Therapeutic Potential of Natural Pharmacological Agents in the Treatment of Human Diseases

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Editorial

Therapeutic Potential of Natural Pharmacological Agents in the Treatment of Human Diseases

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The importance of natural products produced by living organisms from the nature for health and disease treatment has been immense throughout the human evolution. Several natural products derived from plants for thousands of years have been traditionally used to treat various types of human illnesses including general injuries, wound healing, and pain. Natural products have been shown to possess significant pharmacological activities that regulate various vital cell signaling pathways that cause mitogenic, cytotoxic, and genotoxic reactions leading to various disease pathologies. Modern synthetic and combinatorial chemistry associated with the new technological tools such as genomics, proteomics, and metabolomics paved wider use of natural products. Nowadays, most of the natural products are processed and developed as potential pharmacological agents with effective antioxidative, antimitotic, anti-infective, anti-inflammatory, antiangiogenic, and anticarcinogenic properties. In fact, some natural products have been employed as lead compounds to obtain highly biologically relevant semisynthetic pharmacological derivatives with increased efficiency and efficacy for the therapeutic use. The prosperity of knowledge we are assembling from the past several decades will significantly inspire us to develop these natural products as novel potential drugs for future therapeutic strategies.

This special issue compiles 38 excellent manuscripts, including clinical studies, research articles, and reviews, which provides comprehensive evidence demonstrating the significance of natural plant and microbial-derived products in human health and disease.
death are regulated by the essential oils and their constituents via modulating the antioxidant enzymes, oxidative stress, redox sensitive transcription factors, and protein kinases. A brief review article by J. Li et al. describes anti-inflammatory, antiviral, and antiangiogenic actions of glycyrrhizic acid, a triterpene glycoside found in the roots of licorice (Glycyrrhiza glabra) plants. Glycyrrhizic acid has been shown to exhibit anti-inflammatory effects by inhibiting NF-kB mediated production of inflammatory cytokines and chemokines. In particular, this review discusses the pharmacological actions of glycyrrhizic acid in hepatic diseases and its mechanisms of actions in cellular and animal studies. Another review by S. Sreekumar et al. discussed the pharmacological properties of pomegranate fruit. In particular, they have discussed a protective role of methanol extract of pericarp of pomegranate on cardiac and skeletal tissues and as an anticancer agent(s) against various cancer types. These review articles thus provided widespread information on the use of natural plant products in various human diseases.

The research article by M. H. Boskabady and L. G. Mhtaj investigates the effects of Zataria multiflora on animal models of chronic obstructive pulmonary disease (COPD). In this study, hydroethanolic extract of Z. multiflora was examined for its anti-inflammatory role in preventing COPD in guinea pigs. These studies indicate that Z. multiflora could prevent symptoms of COPD and its effects are comparable to the effects of dexamethasone at the concentrations tested. Studies by F. L. Westphal et al. reported the induction of pleuritis, a method that causes the membranes around the lungs to stick together and prevent the fluid buildup, by Copaiba oil in rats. In this study, the effect of induction of pleuritis by Copaiba oil was compared with the silver nitrate. Their results indicate that both Copaiba oil and silver nitrate promoted pleuritis in rats. However, overall better results were seen in the Copaiba group as compared to the silver nitrate group. Silver nitrate group demonstrated greater aggression to the pulmonary parenchyma as compared to Copaiba oil. In another research article, A. Bharathi et al. examined in silico molecular docking and in vitro antiangiotic activity of novel 10-chloro-4-(2-chlorophenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amines (3a–3f). These studies indicate that compound 3e shows selective inhibition of α-amylase and α-glucosidase.

A research article by S. Pal et al. examined the protective effect of ethanol extract of Alocasia indica tuber on hepatoxicity in alcohol treated rat model. These studies report that A. indica tuber extract prevents alcohol-induced liver biomarkers such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), and total bilirubin levels. Further, this plant extract also significantly restored the alcohol-induced levels of malondialdehyde, nitric oxide, glutathione, catalase, and superoxide dismutase suggesting that supplementation of A. indica tuber extract could be protective towards alcohol-induced liver injury. In another research article, A. Sudha and P. Srinivasan identified the DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical scavenging constituents from methanol extract of L. nodiflora using bioassay-guided fractionation. They found that bioactive compound 2-(3,4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (5-hydroxy-3′,4′,7-trimethoxyflavone) isolated from ethyl acetate fraction of plant leaves extract possesses strong antioxidant activity. Similarly, M. Khanapur et al. also reported the antioxidant and antimitogenic activities of flower extracts of Nytcanthes arboritris.

A research article by M. E. Mahomoodally and D. P. Sreekeesoon reported interesting data on the use of natural therapies for the pediatric health care in Mauritius. Based on the interview of parents, they tabulated use of various natural products in pediatric patients of different illnesses. This is one of the first studies to gather primary folk knowledge on the use of plant- and animal-based therapies as natural pharmacological agents for child care. Anticancer properties of the leaves of the medicinal plant Sesbania grandiflora were reported by S. Pajaniradje et al. in their research article. Out of five different solvent fractions from the leaves of S. grandiflora tested for anticancer activities, methanolic fraction was found to be potent growth suppressor of the human A549 non-small cell lung cancer cell line. They found that methanolic fraction of S. grandiflora activates caspase-3 and decreases mitochondrial membrane potential, cyclin D1, and NF-kB in A549 cells. These studies thus indicate that S. grandiflora plant products could be used to prevent lung cancer. Research article by M. P. De Luca et al. demonstrated the antimicrobial properties of propolis varnish against cariogenic bacteria. The paper reports that propolis varnish formulations have satisfactory antimicrobial activity against cariogenic bacteria with low cytotoxicity.

An interesting research study by L. Mei et al. reported antidepressive actions of Pycnogenol (PYC), a natural plant extract from the bark of Pinus pinaster Aiton. These studies indicate that oral administration of PYC prevents depression-like behavior in a chronic corticosterone-treated mice model of depression. Another research study by P. H. M. Nunes et al. reported antioxidative and pharmacological actions of various bark extracts of plant Terminalia fafgofila Mart. & Zucc using rat models of ethanol-induced ulcers and toxicity. In another study, M. A. Hossain et al. investigated the antimicrobial activity of Nymphaea tetragona alone or in combination with antibiotics against different strains of Salmonella bacteria. Their results indicate that combination of ethyl acetate fraction of N. tetragona extract along with antibiotics could be useful to combat drug-resistance Salmonella infections. Z. Li et al., in their research article, demonstrate antitumor activity of icaritin (ICT), a hydrolytic product of icarin from Epimedium genus. By using Burkitt lymphoma cell lines such as Raji and P3HR-1 they found that icaritin prevents cell proliferation and induces apoptosis via activation of caspase-8, caspase-9 and cleavage of PARP and shifting the ratio of Bcl-2/Bax. Further, these studies indicate that icaritin induces cell cycle arrest by increasing the percentage of cells in S-phase and reducing the population of cells in G0/G1 phase. Thus, these studies provide preliminary evidence for the use of icaritin in lymphoma treatment.

Another research article by R. Sudan et al. demonstrated antioxidant and immunomodulatory actions of Arisaema jacquemontii plant extracts in mice. In particular, these
studies indicate that A. jacquemontii leaves have considerable antioxidant and immunomodulating potential as compared to tuber and fruits of the plant. Similarly, another research study by D. Iqbal et al. reported antioxidant, genoprotective, antilipoperoxidative, and HMG-CoA reductase inhibitory properties of traditional medicinal plant, Ficus palmata Forsk. In particular, they report that both F. palmata bark aqueous extract and F. palmata leaves methanolic extract exhibit significant radical scavenging and antioxidant capacity. Further, they report that F. palmata bark aqueous extracts inhibits lipid peroxidation and HMG-CoA reductase activity. In another study, D. Natarajan et al. reported the antimicrobial potential of solvent leaf extracts of P. wightianus. By using 11 human bacterial pathogens and 4 fungal pathogens they showed that leaf extracts of P. wightianus possess antimicrobial activity.

A. Mushtaq et al. examined ethanol, methanol, and aqueous extracts of Eremurus himalaicus for their hypoglycaemic effects in normal Wister Albino rats. They report that as compared to alcohol extracts aqueous extract exhibits significant hypoglycaemic activity in normoglycaemic rats. Similarly, P. Kumar et al. reported that aqueous extract of Trigonella foenum-graecum seeds (AqE-TFG) prevents fat accumulation and dyslipidemia in high fat diet-induced obese rats. Based on the results from this study, they propose that reduction of impaired lipid digestion and absorption, improved glucose and lipid metabolism, enhanced insulin sensitivity, increased antioxidant defense, and decreased lipogenic enzymes could be responsible for preventive effects of AqE-TFG on fat accumulation and dyslipidemia. In the same lines, N. Anupama et al. in their research study examined the anti-inflammatory effects of dried fruit methanol extract of plant Carissa carandas on carrageenan-induced hind paw edema in rats. Another report by A. Bharathi et al. reported in vitro larvicidal and antioxidant activities of dihydrophenanthroline-3-carbonitrile derivatives. A decent research study by N. D. Mahmood et al. investigates hepatoprotective activity of methanol extract of M. calabura leaves (MEMC) in rat models of liver injury. Their results indicate that as compared to N-acetylcysteine MEMC exerts potential hepatoprotective activity as determined by ALT and AST enzymes levels and histological and radical scavenging assays in paracetamol-induced hepatotoxic rats.

In continuation with several reports on antimicrobial effects of natural products in this issue, G. Simonetti et al. also reported their research findings that grape seed extracts (GSEs) obtained from wine and table cultivars of Vitis vinifera L. possess anti-Candida activities. In particular, they indicate that there is a significant correlation between the contents of the flavan-3-ols in GSEs extracts, with a polymerization degree ≥4, and anti-Candida activity. Their conclusion is supported by the studies using an experimental murine model of vaginal candidiasis. In another study, A. Upadhy et al. reported the wound healing property of medicinal plant Cleome viscosa methanol extract (CvME) and its related mechanisms using a Wistar rat cutaneous excision wound model. In this study, the effect of CvME on wound contraction rate, hydroxyproline quantification, and wound granulation were examined. The results indicate that CvME significantly increased wound contraction rate, hydroxyproline content and improved wound granulation. Their data also indicates that C. viscosa promotes a wound repair process by inhibiting Smad-mediated collagen production in wound granulation tissues. In another interesting study, O. Carvajal-Zarrabal et al. reported the effect of avocado oil and olive oil supplementation on the hepatic function in sucrose-fed rats. Their results indicate that avocado oil extracted by centrifugation or using solvent exhibited similar hepatic function effects as olive oil and this suggests that consumption of avocado oil is equally beneficial as olive oil for the liver health.

Curcumin has been well established as an antioxidant, anticarcinogenic, and anti-inflammatory agent. Y. H. Siddique et al. reported the effect of curcumin on oxidative stress and apoptosis in the brains of transgenic Drosophila model of Parkinson’s disease. The exposure of flies expressing human alpha synuclein to curcumin showed a significant dose dependent delay in the loss of activity pattern, reduction in the oxidative stress, apoptosis, and increase in the life span. Thus, these studies suggest potential use of natural plant product curcumin for the treatment of Parkinson’s disease. Similarly, anticervical cancer and antiangiogenic properties of curcumin were also reported by P. Yoyungsnoen-Chintana et al. in their research article. Their studies using nu/nu nude mice xenografts indicate that high dose of curcumin prevents cervical cancer growth and angiogenesis via downregulating VEGF, Cox-2, and EGFR. In an open-label uncontrolled phase-1 pilot study, U. Klickovic et al. examined inducibility of heme oxygenase-1 (HO-1) by orally administered curcumin in healthy male subjects. In addition, these studies also identified a correlation of GT length polymorphism with HO-1. These studies suggest that oral curcumin supplementation has low bioavailability and does not induce HO-1 in human peripheral blood mononuclear cells.

Q. Xu et al. in their article investigated the anticarcinogenic mechanisms of α-mangostin, a natural product isolated from the pericarp of the mangosteen fruit. In particular, they report that α-mangostin prevents pancreatic cancer cells growth in culture and BxPC-3 xenografts in mice via enhancing the apoptosis, modulating the epithelial-mesenchymal transition, and inhibiting the activation of PI3K/AKT signaling pathways. They have also shown that α-mangostin decreases the expression of MMP-2, MMP-9, N-cadherin, and vimentin and increased the expression of E-cadherin in pancreatic cancer cells. Thus, these studies indicate that α-mangostin could be used as a novel adjuvant therapy or complementary alternative medicine for the treatment of pancreatic cancer. Another research study by X. Xu et al. reported that as compared to naringenin alone the combination of naringenin and β-cyclodextrin is more protective in reducing choroidal neovascularization (CNV) in a laser-induced rat model of CNV. The results indicate that treatment of naringenin/β-CD complex demonstrated increased water solubility and improved biological activity that leads to inhibition of CNV formation in rats. Their studies also indicate that naringenin/β-cyclodextrin complex significantly prevents the expression of inflammatory
markers such as VEGF, COX-2, PI3K, p38MAPK, MMP-2, and MMP-9 in retina and choroid tissues and this suggests that the beneficial effects of the combination towards CNV could be linked to the anti-inflammatory activities of narigenin.

Another research paper by H. Zhang et al. investigated the molecular mechanisms by which phytoestrogen, zearalanol could prevent plasma homocysteine levels in a diet-induced hyperhomocysteinemia rat model. They found that zearalanol elevates cystathionine-synthase, an enzyme responsible for homocysteine metabolism, and reduces nitrative stress. In another research study, C. Xun et al. reported the efficacy of salvianolic acid B on motor function recovery in rats with spinal cord injury. These studies indicate that by preventing the activation of IκB-α and NF-κB salvianolic acid B could recover motor function after spinal cord injury. The final research article by H. M. Barreto et al. reported antimicrobial activity of various extracts of *Lippia origanoides* against drug resistant *Staphylococcus aureus* strain. Their results suggest that *Lippia origanoides* could be a source of secondary metabolites for use in association with neomycin and amikacin during antimicrobial treatment.

It is obvious from the ancient medical practices, past literature, and current special issue papers that natural products play a major role in human health and prevention or treatment of human diseases. Although, over the past several years, substantial research has shown that natural products have a crucial physiological role in maintaining the human health, the exact nature of their significance and mechanisms by which these natural products regulate cellular homeostasis that maintain cell survival, differentiation, and death leading to pathological consequences required further detailed investigations. Newly developed technologies such as lipid fingerprinting/lipidomics, metabolomics, and microRNA arrays are important tools that will help to define how the natural plant products provide protection against various pathological conditions. Therefore, the identification and development of important natural products as novel pharmacological agents could help to combat or neutralize the deleterious effects observed during various disease conditions and could be the next important targets for future drug discovery studies.

**Acknowledgments**

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*Kota V. Ramana*

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Review Article


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*Mallotus philippinensis* Muell. Arg (Euphorbiaceae) are widely distributed perennial shrub or small tree in tropical and subtropical region in outer Himalayas regions with an altitude below 1,000 m and are reported to have wide range of pharmacological activities. *Mallotus philippinensis* species are known to contain different natural compounds, mainly phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarins, isocoumarins, and many more especially phenols; that is, bergenin, mallotophilippinens, rottlerin, and isorottlerin have been isolated, identified, and reported interesting biological activities such as antimicrobial, antioxidant, antiviral, cytotoxicity, antioxidant, anti-inflammatory, immunoregulatory activity protein inhibition against cancer cell. We have selected all the pharmacological aspects and toxicological and all its biological related studies. The present review reveals that *Mallotus philippinensis* is a valuable source of medicinally important natural molecules and provides convincing support for its future use in modern medicine. However, the existing knowledge is very limited about *Mallotus* philippinensis and its different parts like steam, leaf, and fruit. Further, more detailed safety data pertaining to the acute and subacute toxicity and cardio- and immunotoxicity also needs to be generated for crude extracts or its pure isolated compounds. This review underlines the interest to continue the study of this genus of the Euphorbiaceae.

1. Introduction

*Mallotus* (family: Euphorbiaceae) is a large genus of trees and shrubs distributed chiefly in the tropical and subtropical regions of the Old World with around 20 species in India [1]. *Mallotus philippinensis* Muell. (commonly called Kamala, Kampillaka, and Kapila, and locally known as Shendri) is a very common perennial shrub or small tree found in outer Himalayas ascending to 1500 meters. Mature fruits have glandular hairs collected as reddish brown powder which is collected in cloth by shaking and rubbing the fruits by hand. The collected material is fine, granular powder, dull red, or madder red-colored and floats on water. This plant is traditionally used for antifilarial [2], antibacterial, anti-inflammatory, and immune-regulatory activity [3] and also used as purgative, anthelmintic, vulnerary, detergent, maturant, carminative, alexiteric and is useful in treatment of bronchitis, abdominal diseases, spleen enlargement, antimicrobial, antiparasitic, and so forth (Figure 18).

Some other medicinal plants reported similar anticestodal activity shown in Table 1. Some medicinal plants exported from India are *Aconitum* species (root), *Acorus calamus* (rhizome), *Adhatoda vasica* (whole plant), *Berberis aristata* (root), *Cassia angustifolia* (leaf and pod), *Colchicum luteum* (rhizome and seed), *Hedychium spicatum* (rhizome), and *Heradeum candicans* (rhizome) [4].

1.1. Scientific Classification. Consider the following:

- kingdom: Plantae,
- subkingdom: Tracheobionta,
- superdivision: Spermatophyta,
- division: Magnoliophyta,
Table 1: Some medicinal plants reported for the antihelmintic activity.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Sources</th>
<th>Part used</th>
<th>Family</th>
<th>Reference</th>
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<td><em>Strobilanthes discolor</em></td>
<td>Leaves</td>
<td>Acanthaceae</td>
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<td>Aerial shoot</td>
<td>Fabaceae</td>
<td>[57]</td>
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<tr>
<td>3</td>
<td><em>Houttuynia cordata Thumb</em></td>
<td>Leaves</td>
<td>Piperaceae</td>
<td>[58]</td>
</tr>
<tr>
<td>4</td>
<td><em>Lasia spinosa Linn</em></td>
<td>Leaves, stalk, stem</td>
<td>Araceae</td>
<td>[59]</td>
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<td><em>Centella asiatica Linn</em></td>
<td>Leaves</td>
<td>Apiaceae</td>
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<td>6</td>
<td><em>Clerodendrum colebrookianum Walp</em></td>
<td>Leaves</td>
<td>Verbenaceae</td>
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<tr>
<td>7</td>
<td><em>Gynura angulosa DC</em></td>
<td>Leaves</td>
<td>Asteraceae</td>
<td>[61]</td>
</tr>
<tr>
<td>8</td>
<td><em>Aloe vera</em> Linn</td>
<td>Leaves</td>
<td>Liliaceae</td>
<td>[61]</td>
</tr>
<tr>
<td>9</td>
<td><em>Psidium guajava Linn</em></td>
<td>Leaves</td>
<td>Myrtaceae</td>
<td>[61]</td>
</tr>
<tr>
<td>10</td>
<td><em>Curcuma longa Linn</em></td>
<td>Rhizomes</td>
<td>Zingiberaceae</td>
<td>[63]</td>
</tr>
<tr>
<td>11</td>
<td><em>Ocimum sanctum Linn</em></td>
<td>Oil/eugenol</td>
<td>Lamiaceae</td>
<td>[64]</td>
</tr>
<tr>
<td>12</td>
<td><em>Albizia anthelmintica</em></td>
<td>Steam bark</td>
<td>Mimosaceae</td>
<td>[65]</td>
</tr>
<tr>
<td>13</td>
<td><em>Berlinia grandiflora</em></td>
<td>Steam bark</td>
<td>Leguminosae</td>
<td>[66]</td>
</tr>
<tr>
<td>14</td>
<td><em>Nicotiana tabacum L.</em></td>
<td>leaves</td>
<td>Solanaceae</td>
<td>[67]</td>
</tr>
<tr>
<td>15</td>
<td><em>Calotropis procera (Ait.) Ait.</em></td>
<td>Flowers</td>
<td>Asclepiadaceae</td>
<td>[68]</td>
</tr>
</tbody>
</table>

class: Magnoliopsida,
subclass: Rosidae,
order: Euphorbiales,
family: Euphorbiaceae,
genus: *Mallotus*,
species: *Mallotus philippinensis*.

1.2. Botanic Description. Trees are small to medium-sized monoecious in nature, up to 25 m tall and with a bole up to 50 cm in diameter, but usually much less in number (Figure 19(a)). Slash turning deep red. Branchlets are reddish-brown glandular. Leaves are alternate and simple, more or less leathery, ovate to lanceolate, cuneate to rounded with two glands at base. Leaves are mostly acute or acuminate at apex, conspicuously 3-nerved, hairy and reddish glandular beneath, petiole size 1–4 cm long, puberulous and reddish-brown in color (Figure 19(b)). Male flowers in terminal and axillary position, 2–10 cm long, solitary or fascicled paniculates spikes, each flowers are with numerous stamens, small; female flowers have spikes or slender racemes, each flower with a stellate hairy, 3 celled ovary with 3 papillose stigmas. Fruit is a depressed-globose; 3-lobed capsule; 5,7 mm, and 10 mm; stellate; puberulous; with abundant orange or reddish glandular granules; 3-seeded (Figure 19(d)). Seeds are subglobose and black in color and 4 mm across (Figure 19(e)) [5].

1.3. Biology. In this genus, *Mallotus philippinensis* (*M. philippinensis*) flowers mature from March to April and fruits mature in July-August. *M. philippinensis* has extra floral nectaries attracting ants.

1.4. Ecology. *M. philippinensis* has a widespread natural distribution, from the western Himalayas, through India, Sri Lanka, to southern China, and throughout Malesia to Australia. Sometimes it is gregarious but more usually mixed with other species, both in forests and open scrubland. Kamala tree is common in evergreen forest, especially in secondary forest, and sometimes even dominant in the undergrowth. Kamala tree withstands considerable shade; it is frost-hardy and resistant to drought.

1.5. Biophysical Limits. Kamala tree is mostly grown at an altitude of 0–1600 m at a mean annual temperature of 16–28°C with mean annual rainfall of 800–2000 mm. Plants will grow mostly in a wide range of soil types, including infertile soils, limestone, acid, and rocky land.

2. Vernacular Names and Traditional Uses


2.2. Traditional Uses. According to Ayurveda, leaves are bitter, cooling, and appetizer. All parts of plant like glands and hairs from the capsules or fruits are used as heating, purgative, anthelmintic, vulnerary, detergent, maturant, carminative, and alexiteric and are useful in treatment of bronchitis, abdominal diseases, and spleen enlargement, and if taken with milk or curd (yoghurt), it can be quite useful for expelling tapeworms [6]. Kamala or Kampilakah is also used as an oral contraceptive. The powder and a few other parts of the
Table 2: Cardenolide and its derivatives.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>R1</th>
<th>R2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coroglaucigenin</td>
<td>CH₂OH</td>
<td>H</td>
<td>[8]</td>
</tr>
<tr>
<td>2</td>
<td>Coroglaucigenin L-rhamnoside</td>
<td>CH₂OH</td>
<td>L-rham</td>
<td>[8]</td>
</tr>
<tr>
<td>3</td>
<td>Corotoxigenin</td>
<td>CHO</td>
<td>H</td>
<td>[8]</td>
</tr>
<tr>
<td>4</td>
<td>Corotoxigenin L-rhamnoside</td>
<td>CHO</td>
<td>L-rham</td>
<td>[8]</td>
</tr>
</tbody>
</table>

Kamala are also used in external applications to promote the healing of ulcers and wounds. They are used to treat parasitic affections of the skin like scabies, ringworm, and herpes.

2.3. Common Adulterants. Glandular hair powder of *M. philippinensis* is commonly adulterated with Annato dye (*Bixa orellana* Linn.), ferric oxide, brick dust, and ferruginous sand. *Casearia tomentosa* (stem bark powder), *Carthamus tinctorius* (flower powder), *Ficus benghalensis* (fruit powder), and *Flemingia macrophylla* (hairs of fruits) are also reported to be used as adulterant or substitute of Kampillaka [7].

3. Chemical Constituents

Major phytochemicals present in this genus contain different natural compounds, mainly phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarin, isocoumarins, and many more to discover. Present knowledge about this endangered species of medicinal plant is still limited with respect to its phytochemistry and biological activity. However, some researchers have contributed towards isolation of some novel constituents and their activity. One of the major chemical constituent, that is, rottlerin of *M. philippinensis*, is listed below with its chemical structure and its major biological activities along with other phytochemicals (Figure 18).

3.1. Cardenolides. *M. philippinensis* seeds contain cardenolides. The seeds of *M. philippinensis* were found to contain after fermentation four cardenolides (Table 2), of which two were newly reported: corotoxigenin L-rhamnoside and coroglaucigenin L-rhamnoside [8].

3.2. Triterpenoids. Some *Mallotus* species were found to have a characteristic feature of pentacyclic triterpenoids with a 6/6/6/6/5 ring system. The petroleum ether extract of the heartwood of *M. philippinensis* yielded triterpenoids: betulin-3-acetate (Figure 1) as a major compound, lupeol acetate (Table 3), and lupeol (Table 3) [9]. Friedelane-type triterpenoids are very common in *Mallotus* species. Friedelin (Figure 2) was obtained from the stem bark of *M. philippinensis* [10]. Most of the genera of family Euphorbiaceae, such as *Drypetes* [11] or *Celaenodendron* [12] also contain friedelin in rich amount. Friedelin is common and is also found in plants from other orders. Other known pentacyclic terpenoids, that is, acetyalleuritolic acid (Figure 3) found to be reported in the petroleum and ether extracts from bark of *M. philippinensis* [9]. The first olean-18-ene triterpene oxidized at C-22 (Figure 4) was isolated from the stem bark.
of *M. Philippinensis* [10]. Apart from above triterpenoids, ursane-type triterpenoid: α-amyrin (Figure 5) reported from the petroleum ether and ethereal extracts of *M. philippinensis* bark [9].

### 3.3. Steroids

Common steroid, β-sitosterol (Figure 6), was isolated from petroleum ether extracts of the heartwood and bark of *M. philippinensis* [9]. Daucosterol was obtained from ether extract from bark of *M. philippinensis* [9].

### 3.4. Phenolic Compounds

Isocoumarins, Bergenin (Figure 7), an isocoumarin, was isolated in 1972 from the heartwood of *M. philippinensis*. This compound was also obtained from the bark and the leaves of *M. philippinensis* [9].

Two new chalcone derivatives (flavonoids), kamalachalcones A and B (Figures 8-9) with a unique ring system caused by dimerization between a dimethylchromene ring and a phenoxyl group, were isolated from kamala (*M. philippinensis*) [13]. Three other novel chalcone derivatives, mallotophillipens C, D, and E (Figures 10, 11, 12, 13 and 14), were isolated from the fruits of *M. philippinensis* [14], lignans, chalcones, and dimeric chalcone derivatives [15].

Four phloroglucinol derivatives (kamalins), rotterlin, and isorallorrotterlin (Figures 15 and 16) were isolated from *M. philippinensis* [16]. Isorotterlin (Figure 17) was also mentioned in *M. philippinensis* [17].

Fruit and bark of plant contain condensed tannins responsible for antioxidant activity. Methanolic bark extract
of *M. philippinensis* subjected to characterization through column chromatography on a Sephadex LH-20 column using ethanol and acetone-water as the mobile phases, tannins and phenols were quantified. Bark extract contains 541 mg/g of total phenolics and fractions ranging from 54 mg/g (fraction I) to 927 mg/g (fraction VI) and condensed tannins were detected in fractions II–VI [18, 19]. In 1989, leaves of *M. philippinensis* were reported for tannins and other related compounds by Saijo et al. [20].

3.5. Other Compounds. Unsaturated fatty acids, that is, triply-unsatured hydroxy acid kamoleneic acid, different fatty acids, and glyceride [21] have been reported from Kamala (*M. philippinensis*) seed oil.

Resinous coloured material contains active parts of rotterin and isorottlerin. It also contains homorottlerine, red role 50%, yellow role 5%, manure 2%, volatile oils, tannin, gum, citric acid, and oxalic acid.

4. Pharmacological Activities

4.1. Antifilarial Activity. The effect of aqueous and alcoholic leave extracts of *M. philippinensis* (Lam.) Muell. was studied on the spontaneous movements of the whole worm and nerve-muscle (n.m.) preparation of *Setaria cervi* and on the survival of microfilariae *in vitro*. Both the extracts result in inhibition of spontaneous motility of whole worm and the n.m. preparation of *S. cervi* characterized by initial stimulation followed by depression in amplitude. The tone and rate of contractions remained visibly unaffected. Aqueous extract at higher concentration showed immediate reduction in tone. The concentration required to inhibit the movements of n.m. preparation was 1/5th for aqueous and 1/11th for alcoholic extract compared to that for the whole worm, suggesting a cuticular permeability barrier. The stimulatory response of acetylcholine was blocked by aqueous extract on whole worm.
movements. On the microfilariae the LC$_{50}$ and LC$_{90}$ were 18 and 20 ng/mL for aqueous and 12 and 15 ng/mL for alcoholic extracts, respectively [2]. Further study will be required to evaluate the same activity with its phytochemicals.

4.2. Antifertility Activity. Seeds extract of *M. philippinensis* exhibits adverse effects on different reproductive parameters of female rats. According to the study, extract reduces serum FSH and LH levels, probably by affecting hypothalamic/pituitary axis in experimental animals. This reduced level may affect follicular development, quality of ovulated eggs, corpus luteum formation, estrus cycle, and maintenance of pregnancy in rats [22]. This antifertility effect of plant extract is supposed to be caused by rottlerin (Figure 15), a phloroglucinol derivative. Acetyl rottlerin may be active, but isorottlerin (Figure 17) is either inactive or slightly active [23]. Effect of pure rottlerin can be further studied so as to clarify the potential of phloroglucinol derivatives.

4.3. Antibacterial and Antifungal Activity. A series of 61 Indian medicinal plants belonging to 33 different families used in various infectious disorders were screened for their antimicrobial properties. Screening was carried out at 1000 and 500 $\mu$g/mL concentrations by agar dilution method against *Bacillus cereus var mycoides, Bacillus pumilus, Bacillus subtilis, Bordetella bronchiseptica, Micrococcus Luteus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumonia, Candida albicans, and Saccharomyces cerevisiae*. Twenty-eight plant extracts showed activity against at least one of the test organisms used in the screening. On the basis of the results obtained, study concludes that the crude extracts of *M. philippinensis* exhibited significant antimicrobial activity [3] and properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents. Steam bark of plant and its chloroform fractions and the methanolic extract significantly inhibit the pathogenic bacteria with significant zones of inhibition comparable to the standard drug used. However, the hexanic extract did not show any significant activity [24]. Glandular hair of fruits of *Mallotus* exhibits significant antibacterial activity against human pathogenic bacteria with MIC ranging 15–20 mg/mL. This extract does not show any inhibition against different species of *candida*. This shows that fruit extract possesses antibacterial activity without any antifungal potential. The results of the study may justify the use of the plant against bacterial pathogens. This probably explains the use of these plants by the indigenous people against a number of infections [25].

However, ethanolic extract shows potent anti-*Helicobacter pylori* activity at the concentration of 15.6–31.2 mg/L against eight *H. pylori* strains. Further purification of extract revealed that rottlerin exhibits potent bactericidal effect with minimal bactericidal concentration (MBC) of 3.12–6.25 mg/L against different resistant strains of clarithromycin.
4.4. Anti-Inflammatory and Immunoregulatory Activity. Chalcones derivatives from the fruits of *M. philippinensis* and *mallotophilippens* C, D, and E (Figures 12, 13, and 14) inhibit nitric oxide (NO) production and inducible NO synthase (iNOS) gene expression by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon-gamma (IFN-gamma). Further investigations suggest the downregulation of cyclooxygenase-2 gene, interleukin-6 gene, and interleukin-1β gene expression. The above results show that these chalcones have good anti-inflammatory and immunoregulatory effects [26].

4.5. Antioxidant Activity and Antiradical Activity. Different fractions of bark and fruit of *Mallotus* were studied for its total antioxidant activity (TAA) and antiradical activity against DPPH on a Sephadex LH-20 column using ethanol and acetone-water as mobile phase. Among different extracts, bark fraction showed the strongest antioxidant activity (TAA value—5.27 mmol Trolox equiv./g) and reducing power. Another extract, that is, phenolic fraction, shows TAA ranging from 0.58 mmol Trolox/g (fraction I) to 6.82 mmol Trolox/g (fraction IV); this is the strongest fraction showing antiradical activity against DPPH and reducing power. TAA of other extracts ranged from 0.05 to 1.79 mmol Trolox equiv./g [18, 19].

4.6. Protein Inhibition Implicated in Cancer Processes. Protein kinase is inhibited with some specificity for PKC by rottlerin, a compound isolated from *Mallotus*. Inhibition of PKC appears due to a strong competition between rottlerin and ATP. CaM-kinase III is suppressed by rottlerin as effectively as PKC δ, among different protein kinases tested. Novel inhibition property and improved selectivity for a distinct PKC isozyme of rottlerin are suggestive from its chemical structure [27, 28]. Rottlerin is also very potent in blocking other kinases including Akt/PKB and p38 MAPK [29–31]. It also inhibits human T cell responses [32], reduces MUC5AC expression in human epithelial cells [33], abrogates reactive oxygen species production in hepatic stellate cells [34], and prevents histamine-induced H1-receptor gene expression in HeLa cells [35]. However, still very limited information is available of rottlerin towards cancer disease and its mechanism of action.

4.7. Hepatoprotective Activity. Methanolic extract of *M. philippinensis* leaves decreases the CCl₄-induced elevation in biochemical parameters (SGOT, SGPT, SALP, direct bilirubin, total bilirubin, and MDA) on pretreatment at doses 100–200 mg/kg and also reversed the functional and antioxidant parameters. This study suggests that leave extract was effective in functional improvement of hepatocytes. Histopathological studies also suggest the hepatoprotective activity of plant [36].

4.8. In Vitro Cytotoxicity against Human Cancel Cell. Glandular hair extract of *Mallotus* fruit powder was assayed
against 14 human cancer cell lines among different fractions; 95% ethanolic extract showed the highest cytotoxic effect as compared to 50% ethanolic and aqueous portion. Further, the chromatographic analysis of the said fraction afforded a polyphenolic molecule rotterin in *Mallotus* plant [37].

4.9. Anticestodal Activity/Veterinary Applications. *M. philippinensis* fruit was found to be very effective against gastrointestinal cestodes in Beetal goats and other ruminants. Comparative anticestodal efficacies of single oral dose treatments with the powdered fruit of *M. philippinensis* (125, 250, and 375 mg/kg), its water or methanol extracts (equivalent to 375 mg/kg), and the total glycosides (25, 50, and 100 mg/kg) were determined in naturally cestode-infected Beetal goats [38]. An ethnobotanical survey has been conducted for anthelmintics in ruminants, so as to document the plants used to treat and control helminthes. *Mallotus* has been frequently used to treat helminthosis in ruminants [39]. *M. philippinensis* fruit extract of 800 mg/kg twice daily for 3 days was observed to have curative efficacy against mature adult worms of *Hymenolepis diminuta*. The total follow-up period of 90 days did not show any further excretion of eggs in the faeces of treated rats. Praziquantel at the dosage of 5 mg/kg also produced a similar effect [40]. *In vitro* scolicidal activity of *M. philippinensis* (Lam.) Muell Arg. fruit glandular hair extract against hydatid cyst *Echinococcus granulosus* protoscoleces at concentrations 10 and 20 mg/mL shows the mortality rate 97% to 99%, respectively, for 60 min treatment, while up to 93% mortality was observed with 20 mg/mL for only 10 min treatment. This proves that the extract has significant scolicidal activity with almost no associated side effects [41]. *In vivo* animal model experiment will be further required to prove its effect against Hydatid cyst.

4.10. Purgative Activity and Anthelmintic Activity. A significant purgative effect after an oral dose (120 mg/kg) in rats was
assessed from resins isolated from plant. Its effect was evaluated from the weight of faeces as well as from surface area of blotting paper soaked by liquid faeces. The anthelmintic effect on tape worm was evaluated in albino rats, from the resin of the plant showed lethal effect of 35.69% and 78.21% respectively in small intestine in concentrations 60 and 120 mg/kg respectively [42, 43].

4.11. Antituberculosis Activity. Organic extract of plant after bioassay-directed fractionation yields five compounds, the most active of which against Mycobacterium tuberculosis was a new compound, 8-cinnamoyl-5,7-dihydroxy-2,2-dimethyl-6-geranylchromone for which the name mallotophilippen F is suggested. The second compound 8-cinnamoyl-2,2-dimethyl-7-hydroxy-5-methoxychromone was isolated from a natural source for the first time, while the remaining three compounds, rottlerin, isorottlerin, or isorottlerin and the so-called "red compound," 8-cinnamoyl-5,7-dihydroxy-2,2,6-trimethylchromene, had been already isolated from this plant. Isolated compounds were identified by 2D-NMR and C-13 NMR [44]. Ethanolic extract of plant was assayed for antimycobacterial activity against M. smegmatis by disc diffusion assay. Further antituberculosis potential of leaves extract was identified by radiometric BACTEC assay; result revealed that ethanolic extract of M. philippinensis showed antituberculosis activity against virulent and avirulent strains of M. tuberculosis H37Rv and M. tuberculosis H37Ra with minimum inhibitory concentrations of 0.25 and 0.125 mg mL⁻¹, respectively. The inhibition in growth index values of M. tuberculosis was observed in the presence of ethyl acetate fraction at a minimum concentration of 0.05 mg mL⁻¹. It suggests that ethanolic and ethyl acetate fraction of plant possesses significant antimycobacterial activity [45]. Steam bark of M. philippinensis has also been reported for its antitumor promoting effect, which was due to the presence of 3α-Hydroxy-D:A-friedooleanan-2-one [46].

4.12. Antiallergic Activity. M. philippinensis fruit contains two new phloroglucinol derivatives, mallotophilippen A and B (Figures 10 and 11) which were identified, using chemical and spectral data, as 1-[5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-isobutyryl-5-methyl-benzyl)-2H-chromen-8-yl]-2-methyl-butan-1-one and 1-[6-(3-Acetyl-2,4,6-trihydroxy-5-methyl-benzyl)-5,7-dihydroxy-2,2-dimethyl-2H-chromen-8-yl]-2-methyl-butan-1-one, respectively. These compounds inhibited the production of nitric oxide (NO) and inducible NO synthase (iNOS) gene expression by a murine macrophage-like cell line (RAW 264.7), which was activated by lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ). Further, phloroglucinol derivatives inhibit histamine release from rat peritoneal mast cells induced by compound 48/80. This study suggests its anti-inflammatory activity [47]. Rottlerin has been tested in animal models of IgE-dependent anaphylaxis and the antiallergic mechanisms of action in mast cells. Antiallergic action of rottlerin has been tested in passive cutaneous anaphylaxis and passive systemic anaphylaxis mouse models and in anaphylactic contraction of bronchial rings isolated from sensitized guinea pigs. This experiments proves antiallergic effect of rottlerin by blocking IgE-induced mast cell degranulation. This report suggests the use of rottlerin in mast cell-mediated allergic disorders including urticaria and allergic asthma [48].

4.13. Anti-Leukaemic Activity. Root extract of M. philippinensis was tested on human promyelocytic leukemia HL-60 cell proliferation, cell cycle regulators, and apoptosis in order to investigate its antileukemic effect. Hexane fraction showed promising toxicity against p53-deficient HL-60 cells (IC₅₀ 1.5 mg dry roots equivalent/mL medium) after 72 h and, interestingly, inhibition of cell proliferation was preceded by the upregulation of the protooncogenes Cdc25A and cyclin D1 within 24 hours suggesting its antileukemic effect in HL-60 cells. After isolation and identification by GC-MS, polyphenols were the main compounds of the hexane extract that inhibited proliferation and induced apoptosis [49].

4.14. Antiproliferative Activity. Antiproliferative effect was evaluated against Thp-1 cell lines from the isolated compounds of M. philippinensis fruit extract, in which 4'-hydroxyrottlerin showed 54% growth inhibition of Thp-1 cell line [50]. Other isolated compounds were also tested against different fungi and were found to be very effective IC₅₀ values.

4.15. Anti-HIV Activity. Four phloroglucinol derivatives, named mallotojaponin (5-methylene-bis-2,6-dihydroxy-3-methyl-4-methoxyacetophenone), mallotochromene (8-acetyl-5,7-dihydroxy-6-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-2,2-dimethylchromone), mallotojaponin (3-(3(dimethylallyl)) S-(acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone), and mallotolerin (3-(3-methyl-2-hydroxybut-3-enyl)-5-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone), were tested for their ability to inhibit the activity of human immunodeficiency virus- (HIV-) reverse transcriptase. The mode of inhibition of mallotojaponin was found to be competitive with respect to the template primer, (rA)n (dT)12–18, and noncompetitive with respect to the triphosphate substrate, dTTP. The Kᵥ value of mallotojaponin for HIV-reverse transcriptase was determined to be 6.1μM [51].

4.16. Antitumor Activity. Four known friedelane-type triterpenoids, friedelin, 3-hydroxy-D:A-friedoolean-3-en-2-one, 2β-hydroxy-D:A-friedooleanan-3-one, and 3α-hydroxy-D:A-friedooleanan-2-one, and two known lupane-type triterpenoids, lupeol and betulin, were isolated from the stem bark of M. philippinensis and were tested for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol 13-acetate (TPA). The inhibitory effect of compounds 2 (IC₅₀ = 292 mol ratio/32 pmol/TPA) and 4 (IC₅₀ = 288) was stronger than those of the other compounds tested and the positive control, curcumin (IC₅₀ = 343). Compound 3α-hydroxy-D:A-friedooleanan-2-one strongly inhibited mouse
skin tumor promotion in an in vivo two-stage carcinogenesis model [46].

4.17. Wound Healing and Mesenchymal Stem Cell (MSC) Proliferation. Bark extract of Mallotus philippinensis has been tested in vitro for wound healing activity by examining the proliferation and migration of MSCs. KUM6 cells proliferation and migration have been enhanced at 0.16–4 μg/mL and unregulated the activity of MSCs by secreting various cytokines to wounded site from bone marrow to systemic circulation and finally remodel wounded tissues [52].

4.18. Toxicities. Seeds of M. philippinensis ethereal extract have adverse effect on various parameters of female rats. Even the extract reduces serum levels of gonadotropins in treated animals at high dose of 100 mg/kg body weight. Reduced weights of ovary and uterus, follicular development, and increased atretic follicular in the ovary are due to subnormal levels of steroid hormones. Thus, pregnancy is very difficult in female rats treated with kamala seed extract [22].

4.19. Colouring Agent-Dye. Glandular hairs of fruit are mostly used as an orange dye for silk [53, 54].

5. Pharmacognostic Evaluation of Mallotus philippinensis

Morphological study shows that fruit depresses globose and is three-lobed capsule, 5–7 mm × 8–10–12 mm, stellate-puberulose, and with abundant orange or reddish glandular granules. Seeds are subglobose and black in color. Organoleptic property of the red fruit shows that it is tasteless and odourless. Microscopic description showed the presence of epicarp, which contained a compactly packed layer of mucilaginous cells, and mesocarp composed of columnar cells which are closely arranged. Its polygonal cells are compactly arranged in 2-3 layers. Presence of lignified vascular arrangement has been observed in the transverse section [55].

6. Conclusion and Future Perspective

Medicinal plants have been clinically used and its interest has been dramatically increased over the past decades throughout the world and its formulations are increasingly cited in media. Daily consumption of the natural products and their formulations by an extensive number of patients lead to serious concern for scientist to study its efficacy and safety. Because of extensive use and its benefits, natural products in many countries are regulated both as medicinal products and as food supplements, often labeled as natural food supplements.

Traditional use and its growing demand for Mallotus philippinensis and its other species lead to compile this review and commented on the current knowledge provided by clinical and preclinical research on the effect of this plant.

Mallotus philippinensis has been widely used as traditional medicine in several parts of countries including India. Every part of this plant possesses its specific medicinal properties and is used mainly in ayurveda to fight against intestinal worms in domestic and grazing animals when administered with jaggery. However, only a few reports are attributed to this plant and its different parts and there is a large scope for investigation. Hence, it is required to explore more of its potential within the field of medicinal and pharmaceutical sciences for novel and fruitful application of this plant in form of natural formulation. Along with this medicinal importance, this plant is used against human pathogens including H. pylori, anti-inflammatory activity, antioxidant, antiradical, protein inhibition, hepatoprotective, antiallergic, anti-HIV activity, and many more. Phytochemical investigation revealed that a large number phenol derivatives and several miscellaneous compounds from different classes have been isolated from this species. The phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarins, and isocoumarins are mostly distributed in all parts of the plant. The other major isolated pure compounds from this species mostly belong to phenolic group exhibiting most of the biological activity. Various types of extracts from different parts and single compounds derived from this species have been found to possess biological activities, including antioxidant, antimicrobial, anti-inflammatory, cytotoxicity, and immune modulatory. Fruit and bark of plant contain condensed tannins responsible for antioxidant activity. Some novel chalcone derivatives, mallotophilipens C, D, and E, were isolated from the fruits of M. philippinensis. Mallotoxin or rottlerin has great anticancerous potential. Among the ever-anticancer agents, rottlerin appears to have great potentiality for being used in chemotherapy. Rottlerin will become a potential molecule for research in future to treat cancerous cell as it will affect cell machineries involved in apoptosis, survival, and autophage. This suggests the view that this species has potential to be a beneficial chemotherapeutic remedy.

Although the data and other reports provided that this medicinal plant is of great biological use in different pharmacological activities including anticancer, further research is needed in different areas regarding the toxicity and efficacy of pure phytochemicals isolated from different parts of this plant. More data will be needed from preclinical and clinical studies on humans to clarify its potency and safety, as lack of knowledge with respect to its adverse effects and methodological accuracy in the literature limits towards its standardized formulation. Furthermore, the mechanism of action of the phytochemicals and extract of Mallotus philippinensis is unclear; more exhaustive studies will be performed to explore its mechanism and structure activity relationship among various constituents.

In conclusion, this review confirms the great potential of Mallotus philippinensis. As very limited information is still known for this species, it leads us to continue the study on different species of Mallotus plant and its interesting pharmacological properties. Further natural product chemistry of isolated moiety and its structural analysis of compounds
responsible for these activities will be an interesting field of research.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


A Review on the Traditional Chinese Medicinal Herbs and Formulae with Hypolipidemic Effect

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Hyperlipidemia, characterized by the abnormal blood lipid profiles, is one of the dominant factors of many chronic diseases such as diabetes, obesity, and cardiovascular diseases (CVD). For the low cost, effectiveness, and fewer side effects, the popularity of using traditional Chinese medicine (TCM) to handle hyperlipidemia is increasing and its role in health care has been recognized by the public at large. Despite the importance of TCM herbs and formulations, there is no comprehensive review summarizing their scientific findings on handling hyperlipidemia. This review summarizes the recent experimental and clinical results of nine representative single Chinese herbs and seven classic TCM formulae that could improve lipid profiles so as to help understand and compare their underlying mechanisms. Most of single herbs and formulae demonstrated the improvement of hyperlipidemic conditions with multiple and diverse mechanisms of actions similar to conventional Western drugs in spite of their mild side effects. Due to increasing popularity of TCM, more extensive, well-designed preclinical and clinical trials on the potential synergistic and adverse side effects of herb-drug interactions as well as their mechanisms are warranted. Hyperlipidemic patients should be warned about the potential risks of herb-drug interactions, particularly those taking anticoagulants and antiplatelet drugs.

1. Introduction

Hyperlipidemia comprises a heterogeneous group of disorders, characterized by high levels in one or more lipids and/or lipoproteins [atherogenic free fatty acids (FA), triglycerides (TG) (hypertriglyceridemia), small dense low-density lipoprotein cholesterol (LDL-C) (hypercholesterolemia), and apolipoprotein (apo) B], and/or low level in antiatherogenic high density lipoprotein cholesterol (HDL-C), in the circulation [1–3]. In 2013, the American Heart Association reported that proportions of American adults aged 20 or above had abnormal blood lipid serum profile (details are shown in Table 1) [4]. These lipid disorders may occur in primary (inherited) [5–7] or secondary form [8]. Secondary hyperlipidemia arises from diet, alcohol intake, estrogen therapy, or diseases such as diabetes mellitus, hypothyroidism, erythematous, and chronic renal diseases [9, 10]. In fact, hyperlipidemia is one of the risk factors of CVD [11], which becomes one of the major killers around the world. It was projected that by 2030 there will be about 23.3 million CVD deaths worldwide [12]. Also, CVD has imposed great medical burden to different societies around the world. The global burden of CVD is beginning to be viewed as high as infectious diseases [13]. Apart from CVD, hyperlipidemia is also closely associated with diabetes, insulin resistance, and obesity [10]. Reduction of total cholesterol (TC) and LDL-C by dietary alterations and medications that affect lipid metabolism [14] is found to reduce the occurrence of atherosclerosis in
animals and clinical cardiovascular events in human [15]. Thus, prevention and treatment of hyperlipidemia are effective approaches to reduce the incident rate of chronic diseases.

Although Western medicines have been the dominant treatment used by hyperlipidemic patients, their adverse effects and part of patients’ intolerance to the pharmacotherapy make traditional Chinese medicine (TCM) one of their alternatives. There is a growing interest in TCM treatment of hyperlipidemia. In the last few decades, hundreds of Chinese herbal medicines in the form of compounds, extracts, single herbs, or formulae have been reported to be effective for the prevention and treatment of hyperlipidemia [16], especially those high-fat diet (HFD) induced cases. In real practice, formulae (at least consist of two herbs) are commonly prescribed to patients. A formula generally is composed of four components: monarch, minister, assistant, and guide. The later three components aid the effects and facilitate the delivery of monarch (the principal component) or lower the toxicity of other components. Based on the patient’s overall body situation, Chinese medicine practitioners always use classical formulae, rather than a single herb, as a foundation to modify the proportion and composition of the ingredients with addition or substitution by other herbs to prepare a specific formulation for individual patients.

Currently there are more than 50 TCM formulae in the form of patent drugs that have been approved by China Food and Drug Administration used for treating hyperlipidemia [16]. This implies that there is a great demand on hyperlipidemic TCM products in the market. This review summarizes TCM herbs and formulae that were proved to have effect in controlling blood lipid profiles in vivo, in vitro, and clinically so as to help people to understand and compare their underlying mechanisms. In this review, nine representative single herbs that have been studied extensively are selected (Table 2). Additionally, seven well-known TCM formulae are also covered here (Table 3).

2. Normal Dietary Lipid Metabolism in the Circulation

Lipid metabolisms involve different lipoproteins in the anabolism and catabolism of these substances (Figures 1 to 2) [3, 9, 14, 17, 18]. TG, phospholipids, and cholesterol esters (CE) are the predominant dietary lipids. These lipids, mainly TG, are hydrolyzed by different pancreatic lipases in the intestine and then absorbed by intestinal mucosal cells and secreted into mesenteric lymphatic vessels in the form of chylomicrons with apoB-48. The newly synthesized TG and CE in the chylomicrons are hydrolyzed by lipoprotein lipase (LPL) to yield chylomicron remnant particles which are cleared by LDL receptors (LDLR) and LDLR-related proteins to the liver. The liver secretes very low-density lipoproteins (VLDL) that contain specific apoB-100, apoC-II, and apoE that bind to enzymes or receptors to facilitate the lipid transfer to the peripheral tissues including vessels for metabolism or storage. ApoB-100 is the main apolipoprotein needed for LDL uptake by the liver. TG is hydrolyzed by LPL in VLDL which is further transformed into TG-reduced intermediate-density lipoprotein (IDL) followed by LDL. LDL is recirculated into the liver or peripheral tissues [19].

HDL plays a critical role in cholesterol homeostasis to induce antiatherogenic effect, which is brought by a process called “reverse cholesterol transport” [2, 20].

The process is the opposing movement of cholesterol from peripheral cells through plasma to the liver. The process involves removal of cholesterol from arterial macrophages and peripheral cells and delivery of the excess cholesterol to the liver for excretion. HDL, containing apoA-1 and other enzymes, is synthesized by the liver in a cholesterol deplete state and takes up excess cellular cholesterol from peripheral cells and arterial macrophages. The excess cholesterol is then delivered to steroidogenic organs for hormone synthesis, or to the liver by binding to scavenger receptor class B, type I (SRBI) for further elimination into the bile as free cholesterol or as biliary acids after metabolism [21]. Liver X receptor (LXR) and farnesoid X receptor (FXR) maintain cholesterol, bile acid, and TG homeostasis [22]. This helps the removal of nonesterified free cholesterol from the blood circulation, thereby preventing the formation of arterial plaques. In indirect reverse cholesterol transport, CE is exchanged 1:1 for TG between apo B-containing lipoproteins (chylomicrons, VLDL, IDL and LDL) and HDL₂ in a process regulated by the cholesterol ester transfer protein (CETP). In addition to the cholesterol removal, HDL also exerts other antiatherogenic and vascular protective functions [2] such as antioxidantive [23, 24], anti-thrombotic [25], and anti-inflammatory actions [26].

Hence, the HDL-C level correlates inversely with the risk of CVD and atherosclerosis while high level of LDL-C highly increases their risk.

3. Lipid Abnormality in Hyperlipidemia Resulting in Atherosclerosis

Lipid abnormality in hyperlipidemia is an increase in nonesterified free FAs in the circulation, an inadequate esterification, and/or a reduced free FA metabolism [27]. The reduced retention of FAs by adipose tissue due to excessive visceral adiposity leads to an increased flux of free FAs

### Table 1: Average percentage of abnormal blood lipid levels among Americans at age 20 or above [4].

<table>
<thead>
<tr>
<th>Blood lipid serum</th>
<th>Total blood cholesterol level</th>
<th>LDL-C level</th>
<th>HDL-C level</th>
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<tbody>
<tr>
<td>Abnormal level (mg/dL)</td>
<td>≥200</td>
<td>≥240</td>
<td>≥130</td>
</tr>
<tr>
<td>Average percentage of Americans</td>
<td>~42.4% men</td>
<td>~12.8% men</td>
<td>~34.4% men</td>
</tr>
<tr>
<td></td>
<td>~12.8% women</td>
<td>~13.6% women</td>
<td>~30.3% women</td>
</tr>
</tbody>
</table>

footnote: Americans include non-Hispanic whites, non-Hispanic blacks, and Mexican-Americans at age 20 or above.
<table>
<thead>
<tr>
<th>Chinese herbal medicines</th>
<th>Sources</th>
<th>Possible bioactive compounds</th>
<th>Effects mentioned in TCMs [54, 94]</th>
<th>Possible hypolipidemic mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alismatis Rhizoma</strong> (Zexie)</td>
<td>Dried rhizome of <em>Alisma orientale</em> (Sam.) Juzep.</td>
<td>Alisol-triterpenes [59]</td>
<td>(i) Promoting urination to drain dampness (ii) Discharging heat (iii) Resolving turbidity and lowering lipid</td>
<td>(i) Decreasing the liver synthesis of cholesterol [57] (ii) Lessening lipid peroxidation and activating antioxidant enzymes [58]</td>
</tr>
<tr>
<td><strong>Coptidis Rhizoma</strong> (Huanglian/Goldthread)</td>
<td>Dried rhizome of <em>Coptis chinensis</em> Franch. or <em>C. deltoidea</em> C. Y. Cheng et Hsiao.</td>
<td>Alkaloids [64], esp. berberine [66, 67]</td>
<td>(i) Clearing heat and drying dampness (ii) Discharging fire and removing toxin (i) Reducing lipid peroxidation [63, 64] (ii) Upregulating PPARx (iii) Negative modulation of FXR to upregulate the gene expression of CYP7A1 for cholesterol conversion into bile acids [64] (iv) Decreasing degradation of dietary polysaccharides [65] (v) Upregulation of LDLR in vitro and in vivo [66]</td>
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<tr>
<td><strong>Crataegi Fructus</strong> (Shanzha/Hawthorn)</td>
<td>Dried ripe fruit of <em>Crataegus pinnatifida</em> Bge. and <em>C. pinnatifida</em> Bge. var. major N. E. Br.</td>
<td>Polyphenols [70] and triterpenic acids for example oleanolic acid and ursolic acid [71]</td>
<td>(i) Promoting digestion and invigorating the stomach (ii) Resolving turgidity and lowering lipid (iii) Moving Qi and dissipation blood stasis (i) Activating PPARx [69] (ii) Inhibiting intestinal ACAT activity in human colon carcinoma cell line Caco-2 [71]</td>
<td></td>
</tr>
<tr>
<td><strong>Ginseng Radix et Rhizoma</strong> (Renshen/Asian ginseng)</td>
<td>Dried root of <em>Panax ginseng</em> C. A. Mey.</td>
<td>(i) Saponins [78, 79] (ii) Acidic polysaccharides [81] (iii) Phenolic extract [83]</td>
<td>(i) Greatly tonifying the original Qi (ii) Resuming pulse and securing collapse (iii) Replenishing “lung” and “spleen” (iv) Engendering fluid and nourishing blood (v) Calming the mental state and enhancing intelligence (i) Improving lipid peroxidation in liver by reduction in serum MDA [76, 83, 87] (ii) Activating LPL activity [81] (iii) Inhibiting pancreatic lipase [82] (iv) Inhibiting food appetite via modifying the serum content and mRNA expression of neuropeptide Y, Y2 receptor, and peptide YY [85]</td>
<td></td>
</tr>
<tr>
<td><strong>Notoginseng Radix et Rhizoma</strong> (Sanqi)</td>
<td>Rhizome and root of <em>Panax notoginseng</em> (Burk.) F. H. Chen</td>
<td>Sanqi saponins [95]</td>
<td>(i) Dissipating stasis and stanching bleeding (ii) Dispelling swelling and relieving pain (i) Reducing HMG-CoA reductase (ii) Reducing lipid peroxidation by increase in the activity of antioxidant hepatic SOD and glutathione peroxidase [95] (iii) Inducing the biosynthesis of bile acids from cholesterol and promoting the β-oxidation of FA in the liver [96] (iv) Acting as a dual FXR/LXR α agonist [124]</td>
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<tr>
<td><strong>Oryzae cum Monasco Semen</strong> (Red yeast rice/Hongqu)</td>
<td>Washed and cooked rice fermented with <em>Monascus purpureus</em> Went</td>
<td>Monacolins, esp. monacolin K [104, 110]</td>
<td>(i) Strengthening “spleen” to improve food digestion (ii) Promoting blood circulation to dissipate blood stasis (i) Resolving the flesh and reducing fever (ii) Engendering fluid to quench thirst (iii) Promoting eruption (iv) Uprasing the middle Qi to relieve diarrhea (v) Unblocking meridian and activating collaterals (vi) Removing wine toxin (i) Promoting cholesterol and bile acids excretion in liver [113] (ii) Estrogen-like effect on lipid metabolism in liver and adipose tissues [116] (iii) Hepatoprotective effect in OVX-induced hepatic steatosis [114]</td>
<td></td>
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<tr>
<td><strong>Puerariae Lobatae Radix</strong> (Gegen/Kudzu root)</td>
<td>Dried root of <em>Pueraria lobata</em> (Willd.) Ohwi</td>
<td>Puerarin [113]</td>
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through the hydrolysis of adipocyte TG, returning to the liver, which stimulates hepatic TG synthesis at hepatocytes, promoting the production of apolipoprotein B and the assembly and secretion of TG containing VLDL. When plasma TG concentration subsequently increases, TG-rich HDL-apoA-I particles are formed and undergo catabolism. Elevated VLDL particles due to hepatic overproduction are lysed and hence fail to bind efficiently to LDLR, while the exchange of CE with these classical hypolipidemic agents is generally well tolerated, most of them have adverse effects [49]; they only target one class of lipoproteins or may not be affordable, deterring most patients from receiving treatments [50]. For instance, statin is a group of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors that inhibit the mevalonate pathway so as to suppress cholesterol absorption with statin, fibrates [41], ezetimibe [42], bile acid sequestrants [43], niacin [44, 45], and intake of omega-3 FA [46–48]. Statins and fibrates are the most commonly used lipid-lowering medications in primary and secondary preventions of atherosclerotic disease. Although these classical hypolipidemic agents are generally well tolerated, most of them have adverse effects [49]; they only target one class of lipoproteins or may not be affordable, deterring most patients from receiving treatments [50].

5. Studies of Single Herbs

5.1. Alismatis Rhizoma. Alismatis Rhizoma, also known as Zexie in Chinese, is the dried rhizome of Alisma orientale (Sam.) Juzep. It is to promote urination to drain dampness, discharge heat, resolve turbidity, and lower lipid recorded in Pharmacopoeia of the People's Republic of China (CP) [54]. A number of Chinese published reports have demonstrated that AR is clinically effective in the treatment of hyperlipidemia. The oral administration of aqueous and alcoholic Zexie extracts (6 g/kg/day, 2 weeks) resulted in significant decreases in serum TG and TC levels in mice fed with HFD [55, 56]. However, the mechanism had not been clearly investigated. Dan. et al. found out that Zexie treatment (2.26 g/kg/day) resulted in an obvious decrease in serum and liver cholesterol, TG level along with elevated serum HDL-C in
Table 3: A summary of antihyperlipidemic effects of different TCM formulae.

<table>
<thead>
<tr>
<th>TCM formulae</th>
<th>Herbs (weight ratio in dose if applicable)</th>
<th>Effects mentioned in TCMs</th>
<th>Effects on blood lipid profile</th>
<th>Possible hypolipidemic mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danggui-Buxue decoction</td>
<td>Astragali Radix and Angelicae Sinensis Radix (1:5) [153]</td>
<td>(i) Raising the Qi&lt;br&gt;(ii) Nourishing the blood [17]</td>
<td>(i) Lowering serum TC and LDL-C levels&lt;br&gt;(ii) Increasing HDL-C level&lt;br&gt;(iii) No significant difference in TG level as compared with diabetic atherosclerosis model group [49, 154]</td>
<td>Downregulating the mRNA expression of MCP-1, ICAM-1, and CD36 [49, 154]</td>
</tr>
<tr>
<td>Danshen-Gegen formula</td>
<td>Salviae Miltiorrhizae Radix and Puerariae Lobatae Radix (7:3) [53]</td>
<td>(i) Promoting blood circulation&lt;br&gt;(ii) Removing blood stasis [200]</td>
<td>(i) A significant dose-dependent decrease in free and esterified TC in the human monocyte derived macrophages in vitro [147]&lt;br&gt;(ii) Lowering TC and LDL-C levels mildly in patients suffering from coronary artery disease compared with placebo [56]&lt;br&gt;(iii) Lowering TC and LDL levels in postmenopausal women with hypercholesterolemia [52]</td>
<td>Dose-related suppression of acetylated LDL uptake by human macrophages [53]</td>
</tr>
<tr>
<td>Erxian decoction</td>
<td>Curculiginis Rhizoma, Epimedii Folium, Angelicae Sinensis Radix, Morindae Officinalis Radix, Anemarrhenae Rhizoma, and Phellodendri Chinensis Cortex (no fixed ratio) [60]</td>
<td>(i) Warming “kidney” Yang&lt;br&gt;(ii) Nourishing “kidney” Yin&lt;br&gt;(iii) Clearing ministerial fire&lt;br&gt;(iv) Harmonizing thoroughfare and conception vessels&lt;br&gt;(v) Balancing Yin-Yang [60]</td>
<td>(i) Suppressing serum TC and LDL-C levels&lt;br&gt;(ii) No significant effect on HDL-C and TG levels in a menopausal rat model [54]</td>
<td>(i) Downregulating HMG-CoA&lt;br&gt;(ii) Upregulating the LDL receptor [54]</td>
</tr>
<tr>
<td>Ling-Gui-Zhu-Gan decoction</td>
<td>Poria, Cinnamomi Ramulus, Atractylodis Macrocephalae Rhizoma, and Glycyrrhizae Radix (4:3:3:2) [63]</td>
<td>(i) Warming Yang for resolving fluid retention&lt;br&gt;(ii) Strengthening the &quot;spleen&quot; to resolve dampness [63]</td>
<td>(i) Reducing TG and TC levels in HFD induced rat models of NAFLD [57]&lt;br&gt;(ii) Lowering ghrelin level in HFD rat groups fasting intermittently supplemented with LGZD [63]</td>
<td>(i) Increasing serum thyroid hormone levels [57]&lt;br&gt;(ii) Improving β-oxidation via modulation of TRβ1 and CPT1A expression in liver [57]&lt;br&gt;(iii) Enhancing metabolism and transport of FA through modulation of SREBP-1c, ACSL and ApoB100 expression [57]</td>
</tr>
<tr>
<td>Shengmai Yin</td>
<td>Ginseng Radix et Rhizoma, Ophiopogonis Radix, and Schisandra Chinensis Fructus (2:1:2) [58]</td>
<td>(i) Replenishing both Qi and Yin energies&lt;br&gt;(ii) Generating body fluids&lt;br&gt;(iii) Restoring the pulses [17]</td>
<td>(i) No significant effect on lipids of Wistar rats fed with HCD&lt;br&gt;(ii) Lowering hepatic cholesterol and TG contents&lt;br&gt;(iii) No effect on fecal cholesterol excretion but higher fecal bile acid content [59]</td>
<td>(i) Stimulating the biosynthesis of bile acid using cholesterol as the precursor&lt;br&gt;(ii) Increasing hepatic cholesterol catabolism [59]</td>
</tr>
<tr>
<td>Turtle jelly</td>
<td>Testudinis Plastrum, plus various Chinese medicinal herbs such as Smilacis Glabrae Rhizoma, Millettiae Speciosae Radix, Mesonaee Chinensis Herba, and Lonicerae Japonicae Flos [173]</td>
<td>(i) Clearing heat&lt;br&gt;(ii) Removing toxin&lt;br&gt;(iii) Promoting urination [51]</td>
<td>(i) Lowering serum TC and LDL levels&lt;br&gt;(ii) Increasing HDL level diet-induced hypercholesterolemic SD rats [50]</td>
<td>(i) Blocking the downregulation of LDLR and PEPCK mRNA and protein expressions&lt;br&gt;(ii) Suppressing the upregulation of PPARα mRNA and protein expressions in the liver [173]</td>
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hyperlipidemic Kunming mice [57]. These results confirmed the efficacy of Zexie in the treatment of hyperlipidemia. They suggested that the herb might act by decreasing the liver synthesis of cholesterol, rather than by increasing the cholesterol catabolism. Zexie methanolic extract (150, 300, and 600 mg/kg/day, 6 weeks) also demonstrated the prevention of the oxidative stress by lessening lipid peroxidation and activating antioxidant enzymes and markedly decreased the serum and liver lipids in nonalcoholic fatty liver disease (NAFLD) rat induced by HFD [58]. Experiments showed that the active lipid-lowering compounds are triterpenoids. Supplement of the atherogenic diet with natural alisol A-24-monoacetate isolated from Zexie in five concentrations (12.2–196.7 mg/kg/day, 10 days) lowered cholesterol levels in the plasma and liver of hypercholesterolemic rats in a dose-dependent manner [59]. Other effective alisol-triterpenes included alisol A-23, 24-diaceitate (derived chemically from alisol A), natural alisol C-23-monoacetate, alisol A, and alisol B-23-monoacetate isolated from Zexie [59]. Also, no adverse effects of triterpenoid-enriched extract of Zexie (360, 720, and 1440 mg/kg/day) were observed in both genders of Sprague-Dawley (SD) rats after feeding for 90 days [60]. The adverse effects of Zexie are correlated with hepatotoxicity in chronic hepatitis B patients and nephrotoxicity following overdosage [61]. Alisol C, 16,23-oxido-alisol B, and alisol O in Zexie may cause nephrotoxicity [62].

### Table 3: Continued.

<table>
<thead>
<tr>
<th>TCM formulae</th>
<th>Herbs (weight ratio if applicable)</th>
<th>Effects mentioned in TCMs</th>
<th>Effects on blood lipid profile</th>
<th>Possible hypolipidemic mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xuefu-Zhuyu decoction</td>
<td>Bupleuri Chinensis Radix, Angelicae Sinensis Radix, Rehmanniae Radix, Paoniae Rubra Radix, Carthami Flos, Persicae Semen, Auranitii Fructus Immaturus, Glycyrrhizae Radix, Platycodi Radix, Chuanxiong Rhizoma, and Achyranthis Bidentatae Radix</td>
<td>(i) Promoting blood circulation to remove blood stasis (ii) Moving Qi to relieve pain [17]</td>
<td>(i) Lowering serum TC and LDL-C levels (ii) Increasing HDL-C level (iii) No significant difference in TG level in HFD fed SD rats as compared with the model group [62] (iv) Lowering TG level and TC/HDL-C ratio in HFD fed Wistar rats [182]</td>
<td>(i) Reversing energy and lipid metabolism disturbance (ii) Decreasing the accumulation acetyl-glycoproteins (iii) Enhancing glutathione biosynthesis [62] (iv) Inhibiting proinflammatory interleukin 8 production [182]</td>
</tr>
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</table>

5.2. *Coptidis Rhizoma*. Coptidis Rhizoma, commonly known as Huanglian in Chinese or Chinese Goldthread in English, is the dried rhizome of *Coptis chinensis* Franch. or *C. deltoidea* C. Y. Cheng et Hsiao. It is to clear heat and dry dampness, discharge fire, and remove toxin recorded in CP [54]. Different clinical, in vivo, and in vitro studies suggested multiple hypolipidemic mechanisms. Yokozawa et al. found that the herb water extract (50 and 100 mg/kg/day, 30 days) significantly reduced the levels of serum TC, LDL and ox-LDL, thiobarbituric acid-reactive substance, and liver cholesterol in a dose-dependent manner in Wistar rats but it did not reduce that of fecal cholesterol, suggesting that it reduced cholesterol synthesis by reducing lipid peroxidation [63]. Cao et al. got a consistent result as Yokozawa’s but used the alkaloid extracts in HFD-fed SD rats instead [64]. The extracts (50, 100, and 200 mg/kg/day) markedly increased the level of liver total bile acid in a dose-dependent manner compared with simvastatin, which was attributed to the positive regulation of PPARα and the negative modulation of FXR to upregulate the gene expression of CYP7A1 to increase its activity in the liver for cholesterol conversion into bile acids [64]. In addition, a new finding suggested that antimicrobial activities of the herb ethanol extract and berberine in HFD-fed C57BL/6 mice might also result in decreasing degradation of dietary polysaccharides, lowering potential calorie intake, and then systemically activating Fiaf protein and related gene expressions of mitochondrial energy metabolism in visceral adipose tissues [65]. Berberine is the main bioactive alkaloid of the herb. Oral administration of berberine (0.5 g twice/day, 3 months) for the hypercholesterolemic Chinese patients resulted in significant decline in serum levels of cholesterol, TG, and LDL-C but no change in HDL-C compared to placebo groups [66]. This was also replicated by another study in human subjects with a mild weight loss (average 5 lb/subject) and in SD rats [67]. The mechanism was explained by the upregulation of LDLR by berberine in the test of human hepatoma cells and the treatment of hyperlipidemic hamsters [66]. Combination of berberine with simvastatin increased the LDLR gene expression to a level significantly higher than that in monotherapies in rats and hypercholesterolemic patients [68]. Human subjects had no side effects observed and were well tolerated with the dosage of berberine [66, 67] or combined with simvastatin [68] so reduction of statin dosage in clinic by adding berberine may be possible.

5.3. *Crataegi Fructus*. Crataegi Fructus (the dried ripe fruit of *Crataegus pinnatifida* Bge. and *C. pinnatifida* Bge. var. major N. E. Br., known as Shanzha in Chinese or hawthorn in English) is a representative herb that is effective in promoting digestion and invigorating the stomach, resolving turbidity and lowering lipid, moving Qi and dissipating blood stasis [54]. Studies have shown that the sugar-free water extract of Shanzha was effective against hyperlipidemia by activating PPARα to lower lipid levels [69]. Apart from
**Figure 1:** The simplified mevalonate pathway of cholesterol production. Potential therapeutic interventions in the pathway using conventional medications and TCMs are indicated. Dotted arrows: skipped pathway.

**Figure 2:** The primary pathways for the normal and abnormal metabolism of human plasma lipoproteins leading to endothelial dysfunction are summarized. Potential therapeutic interventions in the pathway using conventional medications and TCMs are indicated. Dotted arrows: skipped pathway.
Figure 3: The endothelial dysfunction development is summarized and potential therapeutic interventions in the pathway using TCMs are indicated.

This, the extract also inhibited atherosclerosis progression by reducing inflammatory cytokine responses and regulating endothelial function in HFD-fed rats [70]. Shanzha is rich in triterpenic acids (e.g., oleanolic acid and ursolic acid) and polyphenols (such as epicatechin, procyanidins, hyperoside, isoquercitrin, and chlorogenic acid). Oleanolic acid and ursolic acid of Shanzha were found to be particularly responsible for lowering plasma VLDL and LDL cholesterol concentrations by inhibiting intestinal acyl CoA-cholesterol acyl-transferase (ACAT, the enzyme in the mevalonate pathway to synthesize cholesterol) activity in human colon carcinoma cell line Caco-2 [71]. The blunted acetylcholine-induced, endothelium-dependent relaxation of isolated aortas of HCD-fed rats was improved by dried powder of Shanzha crude drug (20 g/kg/day, 4 weeks) [72].

The most frequent mild adverse events were dizziness, nausea, fall, gastrointestinal haemorrhage, circulatory failure, and erythematous rash [73, 74] but many cases had insufficient data supplied to prove the association between the herb and specific adverse effects [75]. The herb was well tolerated and there were no reports of drug interactions [75].

5.4. Ginseng Radix et Rhizoma and Ginseng Radix et Rhizoma Rubra. Ginseng Radix et Rhizoma (Renshen in Chinese and Asian ginseng in English) is the dried root of Panax ginseng C. A. Mey. As recorded in CP, it is used for greatly tonifying the original Qi resuming pulse and securing collapse, replenishing "lung" and "spleen," engendering fluid and nourishing blood, calming the mental state, and enhancing intelligence [54]. Ginseng Radix et Rhizoma Rubra, also called red ginseng, refers to the steamed and dried form of P. ginseng [54]. The lipid-lowering effect has been well studied in the last two decades in clinical trials [76, 77], in vivo [78–85], and in vitro [86].

For the improvement of lipid profile, the 50% alcohol herb extract (2 g three times/day, 8 weeks) decreased TC, TG, and LDL levels and increased HDL in eight normal humans compared to the blood before the trial, which might be induced by antioxidant potential of the extract as one of action mechanisms [76]. The TC and LDL-lowering result was agreed on by a research with 20 hypercholesterolemic patients at a lower dose (100 mg twice/day, 8 weeks) [77]. Yet, the patients had no change of HDL, TG, or VLDL compared to control [77]. Red ginseng saponins (0.01 g/kg for 4 weeks) sustained LPL activity at a normal level or protected LPL activity, resulting in reduction of the rise in serum TG and TC in a cyclophosphamide-induced hyperlipidemia fasted rabbit model [78]. In addition to the saponins, acidic polysaccharides (100 to 1000 mg/kg) isolated from Korean red ginseng using hyperlipidemic rats were also reported to have dose-dependently reduction in TG in serum and liver via the activation of LPL activity [81]. The hypercholesterolemia-enhanced platelet aggregation was also attenuated by Korean red ginseng, rich in glycosylated saponins (200 mg/kg/day, 8 weeks) via the suppression of diacylglycerol liberation in rabbits fed on a diet high in cholesterol, but such effect was weaker than lovastatin [79].

The antioxidant properties of enzyme induced by the herb were also not consistent in humans [76, 87] and rabbit studies [83]. The improvement of lipid peroxidation in liver by reduction in serum malondialdehyde (MDA) was generally observed in most hypolipidemic studies [76, 83, 87]. Apart from that, morphological changes of the aorta in hypercholesterolemic New Zealand white rabbits by the phenolic extract showed positive effects [83].

Weight gain reduction in 3% herb extracted saponins-containing HFD-fed male Balb/c mice may be mediated by pancreatic lipase inhibition [82]. Lin et al. suggested that intraperitoneal injections of ginsenoside Rb1 daily to C57BL/6 mice inhibited the desire for food intake via modifying the serum content and mRNA expression of neuropeptide Y, Y2 receptor, and peptide YY [85]. In the contrast, there were no significant body weight differences in hypercholesterolemic rabbits by the phenolic extract [83] and in HFD-fed C57BL/6 mice by fermented red ginseng powder (150 mg/kg/day, 11 weeks) [88]. However, the combination
of levan (100 mg/kg/day) and the fermented red ginseng powder (150 mg/kg/day) significantly lowered body weight, serum TC and leptin levels, and fat mass with decreasing food efficiency ratio. This suggested improvement of leptin resistance associated with the obese mice. The disagreement in the hypolipidemic effect of the herb may be attributed to the composition of extract used in the experiments, administration method, dosages, and duration [83].

The safety profile of ginseng is generally good [89]. Excessive and uncontrolled intake of ginseng products may cause hypertension, nervousness, irritability, diarrhea, skin eruptions, and insomnia (collectively called ginseng abuse syndrome) [90]. More concerns about interactions between ginseng with anticoagulants, phenelzine [91], or warfarin [92] may increase the risk of bleeding episodes [93].

5.5. Notoginseng Radix et Rhizoma. Notoginseng Radix et Rhizoma is the rhizome and root of Panax notoginseng (Burk.) F. H. Chen, commonly called Sanqi in Chinese, is a herb that invigorates blood circulation and has been widely used in TCM to treat cardiovascular diseases [54, 94]. In CP, the action of Sanqi is to dissipate stasis, stanch bleeding, dispel swelling, and relieve pain [54]. Sanqi crude drug powder supplement incorporated in the HFD for SD rats (10 g/kg, 4 weeks) was found to have improvement in lipid profiles, reduction of HMG-CoA reductase level, and inhibitory effect of lipid peroxidation by increase in the activity of antioxidant enzymes [hepatic superoxide dismutase (SOD) and glutathione peroxidase] [95]. Sanqi saponins including different ginsenosides and notoginsenosides were found to be the main active compounds. A systematic study [96] reported that diet induced hypercholesterolemic SD rats fed with total saponins of Sanqi (30 or 100 mg/kg/day, 4 weeks) could significantly reduce elevated serum TC, TG, LDL-C, and atherogenic index and increase HDL-C. The findings suggested that Sanqi could prevent the development of hypercholesterolemia and atherosclerosis by inducing the biosynthesis of bile acids from cholesterol and promoting the β-oxidation of FA in the liver. Increasing the gene expression of endothelial NO synthase in endothelial cells and Sanqi antioxidative activity might bring about its vasoprotection. Another research also proved that the saponins (100 mg/kg/day, 9 weeks) markedly reduced serum TC, TG, and blood viscosity in HFD-fed SD rats and exerted antiatherosclerosis through an anti-inflammatory action and regulation of the blood lipid profile, involving nuclear factor-κB signaling pathway [97]. Another experiment using the n-butanol extract (30, 60, and 100 mg/kg/day, 4 weeks) showed similar results and revealed that the extract acted as a dual FXR/LXR agonist to prevent the accumulation of abnormal lipid in the hyperlipidemic rats [98]. One study [99] concerned that high fibrinogen is an emerging independent risk factor for cardiovascular diseases but statins could only slightly reduce blood fibrinogen level. In particular, intake of atorvastatin, a very potent lipid lowering agent, might have association with increase in fibrinogenemia in some treated patients [100, 101]. Compared to fluvastatin (3 mg/kg/day), Sanqi powder (43 and 86 mg/kg/day) showed a similar activity in decreasing plasma TC but more marked reduction in fibrinogenemia in HFD-fed rats [99].

More awareness using the intravenous injection of Sanqi saponins should be taken since some adverse reactions such as epistaxis, allergy, and even anaphylactic shock might be caused [102]. Abdominal complaints, nausea, and dyspepsia were observed clinically in a pellet consisting of extracts from Danshen, Sanqi, and Borneol [103].

5.6. Oryzae cum Monasco Semen. Oryzae cum Monasco Semen is commonly called as Hongqu in Chinese and red yeast rice (RYR) in English. It has long been recognized as a TCM to strengthen “spleen” to improve food digestion and promote blood circulation to dissipate blood stasis [94]. It is produced by solid-state fermentation of washed and cooked rice using red yeast (Monascus purpureus Went). RYR is well known for its blood cholesterol lowering effect. The fermentation of RYR produces a family of monacolins that resemble HMG-CoA reductase inhibitors [104]. Among these products, monacolin K is the same substance that the USA Food and Drug Administration approved as lovastatin [105]. Lots of evidences showed that RYR lowers cholesterol levels moderately compared to other statin drugs, but with less adverse effects [106–109]. One recent study demonstrated that 22 patients receiving RYR capsules that contained 7.2 mg lovastatin and a total of 2.4 mg of other monacolins experienced a significant reduction in LDL-C (23.0%) and TC (15.5%) compared to the placebo after 16 weeks of treatment [110]. Another randomized controlled trial showed that RYR preparations had lowering lipid effect, compared with placebo, statins, or other active lipid-lowering agents, and with no treatment received for 12 weeks [111]. In this test, the lipid modification effects of RYR were observed better than nicotinate and fish oils, similar to pravastatin, simvastatin, lovastatin, atorvastatin, or fluvastatin, but equal to or less effective than fenofibrate and gemfibrozil.

Dizziness and gastrointestinal discomfort were also reported in 1.3–36% of participants after RYR intakes [111]. In spite of its therapeutic effect, the yeasts from the same genus of RYR (Monascus strains) could produce citrinin (a nephrotoxic mycotoxin) as a secondary toxic metabolite. A survey indicated that 69.0% of red yeast rice, 35.1% of dietary supplements, and 5.7% of red yeast rice processed products contained citrinin in Taiwan from 2009 to 2012 [112]. The safety control of this kind of products should be cautious.

5.7. Puerariae Lobatae Radix. Puerariae Lobatae Radix (Gegen in Chinese and Kudzu root in English) is the dried root of Pueraria lobata (Willdl.) Ohwi for resolving the flesh and reducing fever, engendering fluid to quench thirst, promoting eruption, uprasing the middle Qi to relieve diarrhea,
unblocking meridian and activating collaterals, and removing wine toxin in CP [54]. For the hypolipidemic effect, puerarin, the major isoflavonoid compound of Gegen, (300 mg/kg/day, 4 weeks), attenuated the increased TC induced by HCD in both serum and liver of SD rats. The cholesterol lowering action might be caused by the promotion of cholesterol and bile acids excretion in liver [113]. Additionally, this herb exhibited beneficial effect in lipid metabolism in ovariectomized (OVX) rats which imitated the postmenopausal situation of the disorders of lipid metabolism. The total isoflavones inhibited the increase in body weight and lipoprotein levels in OVX rats, which exhibited most of the characteristics of human menopausal symptoms compared to the OVX-control rats and exhibited a hepatoprotective effect in OVX-induced hepatic steatosis [114]. Long-term dietary Gegen extract supplementation containing ~25.3% puerarin, 71% daidzin, and 0.8% daidzein (a polyphenol-free diet, with 0.2% kudzu root extract, 2 months) could improve blood glucose, lipid, and pressure control in intact and OVX stroke-prone spontaneously hypertensive rats [115]. The Gegen flavones (100 mg/kg/day, 5 weeks) demonstrated estrogen-like effect on lipid metabolism in liver and adipose tissue compared to estrogen-treated OVX Wistar rats [116].

However, the phytoestrogens of Gegen (coumestrol, genistein, and daidzein) may have potential interactions with the endocrine system, and therefore special attention should be drawn to the herbal formulae containing Gegen, especially the prescriptions for female patients at reproductive age suffering from respiratory diseases [117]. Also, a herb-drug interaction study pointed out that isoflavones and their glycosides and other polyphenols of Gegen might be transformed into conjugated metabolites in SD rats to compete with methotrexate (a drug for cancer treatment and may cause very serious, life-threatening side effects) to delay the elimination of methotrexate, increasing its life-threatening toxicity [118].

5.8. Rhei Radix et Rhizoma. Rhei Radix et Rhizoma (Dahuang in Chinese and rhubarb in English) comes from the root and rhizome of Rheum palmatum L., R. tanguticum Maxim, or R. officinale Baill. Dahuang has been widely used for the treatment of constipation, dysentery, and jaundice [54, 119–122]. Rhein is an anthraquinone and is one of the major components of R. palmatum L. Rhein (150 mg/kg/day) purified from R. tanguticum decreased the plasma levels of cholesterol, TG, LDL-C, and Apo-E, similar to the simvastatin group (20 mg/kg/day) in db/db diabetic mice, but it had a weaker inhibitory effect on HMG-CoA reductase than simvastatin in vitro [123]. Rhein (150 mg/kg/day) was also found to decrease body and fat weight, lower hepatic lipid levels, improve insulin resistance, normalize alanine aminotransferase levels, and reverse hepatic steatosis on NAFLD in HFD-fed mice [124] and might protect against obesity and related metabolic disorders through LXR antagonism and regulation of uncoupling protein-1 expression in brown adipose tissues in HFD-fed mice [125]. Water extract capsules of root of R. officinalis (50 mg/kg/day, oral ingestion with 200 mL of water for six months) was found to significantly lower TC and LDL-C levels but had no change in TG and HDL-C levels in the clinical trial group of patients with atherosclerosis compared to the control group [126]. The researchers suggested that the change aided the improvement in the endothelial function. The same type of water extract was administrated in New Zealand white rabbits and gained similar results [127]. The extract had antiatherosclerotic and plaque stabilizing effects which might occur due to a reduction of some inflammatory cytokines activated by toll-like receptors and nuclear factor-κB signaling [127].

No serious adverse effects were observed throughout the clinical trial [126]. Only five patients in the trial group reported diarrhea, which was mild and resolved without symptomatic therapy [126]. Since Rhubarb has purgative effect due to its anthraquinones [128], the major symptoms of overdose are griping and severe diarrhoea with consequent losses of fluid and electrolytes [129].

5.9. Salviae Miltiorrhizae Radix. Salviae Miltiorrhizae Radix (also called Danshen in Chinese), the dried root of Salvia miltiorrhiza Bunge, is a TCM herb commonly used for the prevention and treatment of CVD. In CP [54], it is used for promoting blood circulation and dispelling stasis, nourishing the blood and calming mental state, regulating menstruation and suppressing pains, cooling blood, and eliminating carbuncle. It has been used for treatment of CVD for long time. The cardiovascular protective effect mainly contributed by Danshen’s antioxidants has been observed in many animal models. Treatment with aqueous extract of Danshen (600 mg/kg/day, 12 weeks) reduced TC and LDL-C with no change in TG and HDL-C and prevented formation of HFD induced fatty liver in an OVX hyperlipidemic rat model [130]. Oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of atherosclerosis. Increased oxidative stress, resulting from increased ROS production, appears to play an important role in hypercholesterolemic atherogenesis [131]. The high antioxidative capacity of the Danshen aqueous extract may facilitate the removal of ROS in the circulation. Another study suggested that this antioxidant capacity and cholesterol lowering effect observed in the hypercholesterolemic restenosis New Zealand white rabbit model fed with 80% ethanol extract of Danshen treatment (4.8 mg/kg/day, 6 weeks) enhanced smooth muscle apoptosis and attenuates neointimal hyperplasia [132]. Same types of model fed with a HCD containing 5% water-soluble Danshen extract for 12 weeks reduced the atherosclerotic area in the abdominal aorta by 56% and cholesterol deposition in the thoracic aorta by 50%, although it was weaker than probucol treated group. It concluded that Danshen’s cholesterol-lowering effect and more importantly, the antioxidant, salvianolic acid B, that accounted for about 75% of antioxidant activity in Danshen contributed to the prevention of endothelial damage and inhibition of LDL oxidation [133]. In another study, hyperlipidemic SD rats were treated with purified aqueous extracts of Danshen that contained Danshensu, rosmarinic acid, and salvianolic...
acid A and salvianolic acid B (50, 100, and 150 mg/kg/day, 4 weeks). They also decreased TC and TG levels and increased HDL-C serum levels, but this improvement likely contributed to the extract acting as a FXR/LXRX co-agonist [134].

There was low or nontoxic acute (32 g/kg, twice/day) or subchronic administrations (5.76 g/kg/day, 13 weeks) of Danshen injection in rats but a significant decrease in TG and increase in total bilirubin [135]. Coadministration of Danshen may exaggerate the anticoagulant response to warfarin since Danshen might increase the bioavailability of warfarin [136, 137]. Therefore, Danshen or its products such as Danshen-Gegen formula or Danshen dropping pills should be used under close medical supervision by people taking similar anticoagulants.

6. Studies of TCM Formulae

6.1. Danggui-Buxue Decoction. Danggui-Buxue decoction (DGBX) is a very common TCM prescription consisting of Astragali Radix and Angelicae Sinensis Radix at a dose of (DGBX) is a very common TCM prescription consisting of Astragali Radix and Angelicae Sinensis Radix at a dose of 1:5 [138]. In CP, its product “Danggui Buxue Koufuye” (a DGBX oral solution) is recorded to raise the Qi and nourish the blood [54]. A study used DGBX (3 or 6 g/kg/day) for 4 weeks to be orally administered to the diabetic atherosclerosis rats, which were induced by NO inhibition plus HFD [139]. Although there was no significant change in TG level, serum TC and LDL-C were significantly lowered, and the HDL-C was higher in the DGBX-treated group than that in diabetic atherosclerosis model groups. Treatment with DGBX in early diabetic atherosclerosis rats resulted in significant inhibition in mRNAs expression of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and CD36 (a FA translocase that mediates oxidized LDL uptake) in aorta so it helped the prevention of diabetic atherosclerosis. Another study showed the similar effect of DGBX (1.68, 8.4 and 16.8 g/kg/day) for 5 weeks but had obvious decrease in TG in all dosages [140]. DGBX (equivalent to 6 g/kg/day, 6 weeks) also demonstrated improvement in hepatic lipid peroxidation by increase in SOD activities and decreased MDA levels in fibrotic livers of HFD-fed rats [141].

The clinical treatment of DGBX preparations at 1.5, 3.0, or 6.0 g/day, 12 weeks was well tolerated, with no serious adverse events noted in postmenopausal women but no significant lipoprotein changes were observed [142].

6.2. Danshen-Gegen Formula. Danshen-Gegen formula (DG), composed of Salviae Miltiorrhizae Radix and Puerariae Lobatae Radix, are traditionally paired clinically to treat atherosclerosis, myocardial infarction, and other cardiac symptoms and have been widely studied in the aspect of cardiovascular effects [143–146]. Various concentrations (0.1–1.0 mg/mL) of the water extract in the ratio of 7:3 were tested in the human monocYTE derive macrophages loaded with acetylated LDL [147]. Compared to the control, the herbal mixture induced a significant dose-dependent decrease in the (free and esterified) cholesterol in the macrophages. However, the herb pair also induced an increase in ICAM-1 expression and monocyte adhesion at higher concentrations [147].

In a clinical trial, DG treatment (3 g/day, 24 weeks) in double-blind parallel fashion in patients suffering from coronary artery disease showed mild decrease in TC and LDL-C [148]. Another clinical research with postmenopausal women treated by the herb pair (two capsules, containing 1 g water extract/day) demonstrated that the herb-pair treated group has a significant improvement in intima-media thickness and a remarkable decrease in TC and LDL as compared to placebo-treated group after 12-month treatment [149]. For the genomic study [149] the herbal-treated group has a higher number of differential gene expressions identified as compared to the placebo-treated groups. It was suggested that the herb pair could diminish the process of cardiovascular deterioration in postmenopausal women.

For the safety of DG, its treatment in patients suffering from coronary artery disease was well tolerated [148]. Yet, coadministration of DG with anticoagulant warfarin or aspirin, respectively, can decrease the prothrombin time of the drugs and cause other significant pharmacokinetic and pharmacodynamic herb-drug interactions in SD rats [150].

6.3. Erxian Decoction. Erxian decoction (EX), designed by Zhang Bo-Na in early 1950s, consists of Curculiginis Rhizoma, Epimedii Folium (monarch), Angelicae Sinensis Radix, Morindae Officinalis Radix (minister), Anemarrhenae Rhizoma, and Phellodendri Chinensis Cortex (assistant) without fixed ratio [151]. It has long been used in TCM to treat Yang and Yin deficiency of “kidney,” menopausal symptoms, and osteoporosis [152, 153]. A study demonstrated that water extract of EX (equivalent to 4.10 g crude drug/kg/day, 8 weeks) had hypolipidemic effects in a menopausal rat model but this was not observed in ethyl acetate (0.11 g/kg), n-butanol (0.470 g/kg), and the aqueous remaining fractions (2.34 g/kg) of the water extracts [154]. Premarin, which is a conjugated estrogen for the replacement of hormone of postmenopausal women, did not show such effect. The serum levels of TC and LDL-C of EX groups were suppressed, possibly through the downregulation of HMG-CoA and upregulation of the LDLR while HDL-C and TG levels had insignificant change. Compared to the premarin, EX containing hypolipidemic components may have the advantage to hyperlipidemic postmenopausal women.

In a clinical trial, the water extract of EX (equivalent to 62 g of crude herbal materials/day, 12 weeks) is well tolerated, with no serious adverse events noted in Hong Kong perimenopausal women [153].

6.4. Ling-Gui-Zhu-Gan Decoction. Ling-Gui-Zhu-Gan decoction (LGZG) is an ancient Chinese herbal formula from “Jin-Gui-Yao-Lue” for warming Yang for resolving fluid retention and strengthening “spleen” to resolve dampness [155]. It consists of four herbs only: Poria, Cinnamomi Ramulus, Atractylodis Macrocephalae Rhizoma, and Glycyrrhizae Radix at the weight ratio of 4 : 3 : 3 : 2 [155]. Caloric restriction
therapy has been studied with metabolic disorders such as type 2 diabetes [156, 157] and obesity [158] to normalize metabolism. Recently, scientists have studied caloric restriction therapy supplemented with LGZD in Wistar rats [155]. Although there were no statistical differences in blood lipid level between fasting and those fed with LGZD groups, the blood level of ghrelin, a starvation hormone [159], was lower in HFD rat groups fasting 24 hours intermittently supplemented with LGZD than those without LGZD. Thus, it was suggested that the decoction may help regulate appetite of the fasting rats and so the same may happen in human patients for easier completion of the caloric restriction therapy when they feel less hunger [155]. Another research group provided evidence on the traditional treatment principle of LGZD using HFD induced rat models of NAFLD [160]. The possible mechanisms of LGZG to regulate lipid metabolism are to increase serum thyroid hormone levels and improve FA synthesis via modulation of thyroid hormone receptor β1 (TRβ1) and carnitine palmitoyltransferase-1A (CPT1A) expression in liver and enhance metabolism and transport of FA through modulation of sterol regulatory element-binding protein 1c (SREBP-1c), long-chain acyl-CoA synthetase (ACSL), and ApoB-100 expression. The combination of Poria and Ramulus Cinnamomi might be crucial in LGZG to warm Yang to activate Qi by increasing serum thyroid hormone levels and hepatic TRβ1 and CPT1A expression and enhancing β-oxidation of FA, so as to relieve water retention while Atractylodis Macrocephalae Rhizoma and Glycyrrhizae Radix assist such effect of warming Yang.

6.5. Shengmai Yin. Shengmai Yin (SMY) which is also called "Shengmai San" or "Pulse-activating decoction" is comprised of Ginseng Radix et Rhizoma, Ophiopogonis Radix, and Schisandra Chinensis Fructus (2:1:2) [161], for treating cardiovascular diseases such as coronary heart diseases [162] and brain impairment [163]. It was first documented in "Nei-Wai-Shang-Bian-Huo-Lun" in the 13th century. Its patent drug "Shenmai Capsule" and SMY are officially recorded in the Food and Drug Administration of China. Shengmai Yin (SMY) which is also called "Shengmai San" or "Pulse-activating decoction" is comprised of Ginseng Radix et Rhizoma, Ophiopogonis Radix, and Schisandra Chinensis Fructus (2:1:2) [161], for treating cardiovascular diseases such as coronary heart diseases [162] and brain impairment [163]. It was first documented in "Nei-Wai-Shang-Bian-Huo-Lun" in the 13th century. Its patent drug "Shenmai Capsule" and SMY are officially recorded in the Food and Drug Administration of China.

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6.6. Turtle Jelly. Turtle (tortoise) jelly, also called Gui-ling-gao in Chinese, is a popular traditional functional food in Southern China to clear heat, remove toxin, and promote urination [172]. The main ingredient is Testudinis Plastrum (tortoise shell), plus various Chinese medicinal herbs such as Smilacis Glabrae Rhizoma, Millettiae Speciosae Radix, Mesonae Chinensis Herba, and Lonicerae Japonicae Flos [173]. The plasma of diet-induced hypercholesterolemic rats fed with turtle jelly (3.3 or 10 mL/kg/day) for 30 days showed hypercholesterolemic effect in terms of lowering the level of the serum TC and LDL, but increasing HDL level with a dose-dependent improvement on the atherogenic index [173]. These also brought the protection of endothelial dysfunction and livers. The hypercholesterolemic effect was suggested to be related to the blockage of downregulation of LDLR and phosphoenolpyruvate carboxykinase (PEPCK) mRNA and protein expressions as well as suppression of the upregulation of PPARα mRNA and protein expressions in the livers in rats fed with HCD.

There are no studies reporting its safety issue. Since the content of turtle jelly is very complicated, its long-term efficacy and safety still need further investigations.

6.7. Xuefu-Zhuyu Decoction. Xuefu-Zhuyu decoction (XFZY) was described in an ancient book called "Yi-Lin-Gai-Cuo" in early 18th century [174] and is composed of two classic formulae (Taohong-Siwu decoction and Sini decoction): Bupleuri Chinensis Radix, Angelicae Sinensis Radix, Rehmanniae Radix, Paeoniae Rubra Radix, Carthami Flos, Persicae Semen, Aurantii Fructus Immaturus, Glycyrhizae Radix, Platycodi Radix, Chuanxiong Rhizoma, and Achyranthis Bidentatae Radix [175]. It is a usual TCM formula to treat blood stasis in the chest region [176]. CP records its proprietary drug "Xuefu ZhuYu liangnang" (a XFZY oral capsule) for promoting blood circulation to remove blood stasis, moving Qi to relieve pain [54]. Its blood lipid lowering effect and improvement of Qi stagnation and no effect on fecal cholesterol excretion but higher fecal bile acid content was observed after the SMY treatment. Increased fecal bile acid excretion (the major degradation of endogenous cholesterol process) might stimulate the biosynthesis of bile acid using cholesterol as the precursor, resulting in an increase in hepatic cholesterol catabolism and so reducing hepatic cholesterol and thereafter TG accumulation.

No adverse reactions were found in clinical trials of SMY capsule [167] or injection to patients with coronary heart disease complicated with diabetes mellitus [168] which is consistent with the review [169]. Yet, a case of a 71-year-old man indicated that an adverse interaction between warfarin and SMY resulted in cerebral bleeding [170]. Our previous mentioned ginseng, one of the main ingredients in SMY, probably contributed to the interaction. Another ingredient Schisandra Chinensis Fructus might also increase the metabolism of the coadministered warfarin [171].
blood stasis syndrome have been proved clinically and in animal researches in China [177–181].

Liu et al. [182] studied the effect of aqueous extract (8 g/kg/day) to HCD-fed Wistar rats. The aqueous fraction significantly lowered TG concentration and decreased TC/HDL-C and thromboxane/prostacyclin ratio (related to platelet coagulation). The 2.5% medium-polar and nonpolar fraction mixture from aqueous fraction (0.2 g/kg/day, 2 weeks), which exhibited comparable effect on serum lipid level and stronger potency to increase the prostacyclin secretion and significantly inhibitory effect on proinflammatory interleukin 8 production, was the antiatherogenic principle of the formula. In 2013, a NMR-based metabolomics approach was conducted to elucidate the mechanisms of XFZY on HFD induced hyperlipidemia [181]. 2.5, 5, and 10 g/kg (equivalent to crude drug mixture) of extract water of six XFZY ingredients (Bupleuri Chinesis Radix, Paeoniae Rubra Radix, Carthami Flos, Persicae Semen, Aurantii Submaturus Fructus, and Chuanxiong Rhizoma at the weight ratio of 3:3:3:2:2:1) were fed to HFD-induced SD rats for seven weeks. Statistical analysis of NMR spectra from blood plasma revealed that XFZY could attenuate hyperlipidemia, by partially reversing energy and lipid metabolism disturbance, decreasing the accumulation of β-hydroxybutyrate (ketone body) and acetyl-glycoproteins (inflammatory mediators), and enhancing glutathione biosynthesis probably due to antihyperlipidemia, antioxidative, and anti-inflammatory effects of its components.

No obvious adverse reaction was found during and after the treatment of XFZY capsules except one case reporting stomach discomfort [167, 176].

7. Discussion

TCMs have been used for over 2000 years in China. In the past few decades, they have drawn more and more attention worldwide to explore their new indications and test if they have potential to treat or prevent the disorders or dysfunctions that Western drugs or single compounds cannot easily regulate. One of the examples is hyperlipidemia. The hypolipidemic effects of TCMs have been extensively investigated and manifested in vitro and in vivo and clinically summarized in Tables 2 and 3.

In ancient TCM theory, the term “hyperlipidemia” does not exist but some scholars suggested that hyperlipidemia have many resemblances to the syndromes of dampness, turbid-phlegm, and blood stasis [183]. Taking turbid-phlegm as an example, excessive inner accumulation of phlegm in the body could block the circulation of blood and Qi, further leading to blood stasis. Patients generally have the symptoms of chest impediment, palpitations, dizziness, or even stroke in serious cases [184, 185]. Most TCM practitioners consider the deficiency of “spleen,” “kidney,” or poor movement of “liver” Qi as the roots (the primary aspect of a disease) of hyperlipidemia while phlegm and stasis are the tips (the secondary aspect of a disease) [53, 185].

Three of the most frequently used strategies by TCM practitioners for the prescription of TCMs [16] are (1) to relieve food retention (e.g., Oryzae cum Monasco Semen, Crataegi Fructus), enhance purgative effect (e.g., Rhei Radix et Rhizoma), and eliminate dampness and water (e.g., Alismatis Rhizoma and Ling-Gui-Zhu-Gan decoction) by targeting the gastrointestinal tract, urinary, and biliary system (e.g., Turtle jelly); (2) to promote blood circulation and relieve blood stasis by improving cardiovascular system (e.g., Salviae Mil-tiorrhize Radix, Notoginseng Radix et Rhizoma, and Xuefu-Zhuuy decoction); (3) to reinforce tonic effects by adjusting entire body functions (e.g., Ginseng Radix et Rhizoma, Danggui-Buxue decoction, and Shengmai Yin). Most of them may provide their effects via multiple approaches, such as red yeast rice that promotes blood circulation and strengthens “spleen” to improve digestions. Hence, patients with hyperlipidemia are prescribed appropriate combination of herbs according to their diagnosed syndromes so as to treat the roots well, rather than only target the tips (symptoms) only.

In addition to the long history of medical uses, in this review, preclinical researches and clinical trials provide supportive evidence to recommend the use of TCM therapy in treating hyperlipidemia.

In the Western medications, some single Western drugs clinically used alone or in coadministration with others in hyperlipidemic patients have their own adverse effects [10, 186, 187] that may not be entirely tolerated by all patients. For example, statins use may induce incidence of myopathy in high dose or in some groups of patients [188–190]. The US National Institutes of Health suggested that many patients under statin treatment alone do not achieve the LDL-C goal [186]. As for patients who want alternative medicines for the replacement of Western medications, TCM may be their choice despite reported mild adverse side effects observed in minor cases clinically. We cannot deny that there may be similar side effects of TCM sharing similar mechanisms as conventional drugs (Figures 1-2). Red yeast rice, for instance, containing monacolins that resemble HMG-CoA reductase inhibitors may have a high potential risk. However, we do not find a report about the intake of the herb causing similar adverse effect. This is greatly attributed to the variation in the drug preparation, dosage, administration period, and population differences. In addition, Chinese herbs are seldom used alone so that their synergistic effect may enhance the efficacy whereas the amount of toxic chemicals is reduced by other coexisting phytochemicals [191] in the herbal mixtures. The combination of herbs that have different pharmacological activities may have better lipid-lowering effects than their individual ingredients or even comparable to single drugs probably contributed to their actions of multiple targets. Better quality control and more efficient drug preparation may enhance TCM oral bioavailability [192] with a lower dosage used. For instance, the oral bioavailability of the Sanqi saponins in crude drug may not be satisfying whereas some adverse reactions might be caused by using the intravenous injection [102]. Using chitosan as a bioadhesive material to prepare a modified Sanqi saponins tablet improved the bioavailability after oral administration for beagle dogs [193].

More importantly, herb–drug interaction has raised growing concern to scientists despite the fact that there is growing popularity of TCM as complementary medicines with
Western drugs in various diseases, including hyperlipidemia, CVD, and diabetes [194, 195]. The literature also warns that there are potentially serious adverse effects and interactions between conventional therapies and TCMs [195, 196]. We found that adverse interactions exist in the conventional drugs with TCMs in the hyperlipidemic treatment while the synergistic effect of hypolipidemic activities using these two types of medicines simultaneously is also possible. For examples, the common hypolipidemic red rice yeast is likely to have drug interactions with niacin that has long been used to increase blood HDL levels. This is because the herb contains monacolins that resemble the pharmacokinetics of lovastatin which has synergistic effect with niacin. Possible adverse effects include flushing, pruritus, rash, or gastrointestinal adverse events [197]. In particular, hyperlipidemic patients who are generally suffering from CVD and taking conventional anticoagulants and antiplatelet drugs should not ignore the potential risks with TCMs [93]. Warfarin, an anticoagulant, is often found to interact with TCMs such as Salviae Miltiorrhizae Radix [136, 137] and Ginseng Radix et Rhizoma [92], increasing the bleeding risk of the patients. The TCM formulation composed of these herbs such as Shengmai Yin [170] and Danshen-Gegen formula [150] should also avoid coadministration of the anticoagulant.

In the contrast, the combination of TCM bioactive compounds and Western drugs may give better hypolipidemic activities than using them solely, such as the fermented red ginseng powder plus levan [88] and berberine from Coptidis Rhizoma which has synergistic effect with niacin. Possible adverse effects include flushing, pruritus, rash, or gastrointestinal adverse events [197]. In particular, hyperlipidemic patients who are generally suffering from CVD and taking conventional anticoagulants and antiplatelet drugs should not ignore the potential risks with TCMs [93]. Warfarin, an anticoagulant, is often found to interact with TCMs such as Salviae Miltiorrhizae Radix and Ginseng Radix et Rhizoma [92], increasing the bleeding risk of the patients. The TCM formulation composed of these herbs such as Shengmai Yin [170] and Danshen-Gegen formula [150] should also avoid coadministration of the anticoagulant.

Since there are insufficient information about the effects of interactions of TCMs with the conventional hypolipidemic medications provided to the clinics and the general public, patients may underestimate or even may not be aware of the potential danger of herb-drug interactions which are similar to drug-drug interactions in terms of their effects on ADME properties [194, 198]. More investigation of herb-drug interactions in vivo and clinically with the main phytochemicals from the single Chinese herbs and more complicated TCM formulae is of high importance to compensate our limited knowledge of TCMs. Since little is known, Chinese medicine practitioners and clinicians should warn hyperlipidemic patients about the potential risks of herb-drug interactions of TCMs with Western drugs, particularly those taking anticoagulants and antiplatelet drugs.

Inconsistencies in the hypolipidemic effect of the herb observed in different studies of this review. It may be attributed to the composition of TCM extract used in the experiments, administration method, dosages, animal models, and duration. Also, due to the limitation of present scientific technology, biological mechanisms of many other TCM formulae have not been completely elucidated [199]. More systematic, well-designed animal and randomized clinical studies in chronic administration with sufficient sample sizes are essential to investigate their exact mechanisms of the hypolipidemic effects, safety, and pharmacokinetics so as to give a more effective alternative to the hyperlipidemic patients.

8. Conclusion

Most of single herbs and formulæ demonstrated the improvement of hyperlipidemic conditions with multiple and diverse mechanisms of actions in spite of their mild side effects. As more and more people tend to use TCM as an alternative medicine, more extensive, well-designed preclinical and clinical trials on the potential synergistic and adverse side effects of herb-drug interactions, as well as their mechanisms, are warranted. Hyperlipidemic patients should be warned about the potential risks of herb-drug interactions, particularly those taking anticoagulants and antiplatelet drugs.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACAT</td>
<td>Acyl CoA-cholesterol acyl-transferase</td>
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<tr>
<td>ACSL</td>
<td>Long-chain acyl-CoA synthetase</td>
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<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
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<tr>
<td>CE</td>
<td>Cholesterol esters</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>CP</td>
<td>Pharmacopoeia of the People's Republic of China</td>
</tr>
<tr>
<td>CPTIA</td>
<td>Carnitine palmitoyltransferase-1A</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acids</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>HCD</td>
<td>High-cholesterol diet</td>
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<tr>
<td>HDL-C</td>
<td>High density lipoprotein cholesterol</td>
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<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-methyl-glutaryl-coenzyme A</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
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<td>LDLR</td>
<td>LDL receptors</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemo-attractant protein-1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element-binding protein 1c</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterols</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese medicine</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TRβ1</td>
<td>Thyroid hormone receptor β1</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoproteins</td>
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</table>
Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Review Article

*Carum copticum* L.: A Herbal Medicine with Various Pharmacological Effects

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*Carum copticum* L. commonly known as "Ajwain" is cultivated in many regions of the world including Iran and India, states of Gujarat and Rajasthan. Traditionally, *C. copticum* has been used in the past for various therapeutic effects including bloating, fatigue, diarrhea, abdominal tumors, abdominal pain, respiratory distress, and loss of appetite. It has other health benefits such as antifungal, antioxidant, antibacterial, antiparasitic, and hypolipidemic effects. This plant contains different important components such as carbohydrates, glucosides, saponins and phenolic compounds (carvacrol), volatile oils (thymol), terpiene, paracymene and beta-pinene, protein, fat, fiber, and minerals including calcium, phosphorus, iron, and nicotinic acid (niacin). In the previous studies, several pharmacological effects were shown for *C. copticum*. Therefore, in this paper, the pharmacological effects of the plant were reviewed.

1. Introduction

*C. copticum* or Ajwain belongs to the Apiaceae plants family and its seeds are used extensively as a food additive in India and mainly therapeutically effective, with hot nature. *C. copticum* is an Egyptian aborigine plant. This plant grows in arid and semiarid fields in different regions of central Europe, Asia, India (most crops are in the states of Rajasthan, Gujarat, and West Bengal), Iran (especially eastern regions of Baluchistan), Iraq, Afghanistan, and Pakistan [1, 2].

In traditional medicine, different therapeutic applications for *C. copticum* have been described and in Persian traditional medicine it is used for thousands of years [3]. The bronchodilatory, antitussive, and antidyspnea effects were demonstrated for *C. copticum* [3]. The therapeutic effects of this plant in gastrointestinal disorders, such as reflux, cramps, abdominal tumors, abdominal pain, and *Helicobacter pylori*, as well as in eye infection disorders, have been demonstrated [3].

Therapeutic uses of *C. copticum* seeds also include carminative, antiseptic, amoebiasis expectorant, antimicrobial, antiparasitic, antiplatelet-aggregatory, and antilithiasis as well as treating common cold and acute pharyngitis [3]. Abortifacient, galactogogic, and diuretic activities have been observed for this plant [4, 5]. There is also anticarcinogenic potential evidence for *C. copticum* [6]. It has been shown that this plant has also foetotoxicity, abortion potential, and galactogogue properties [7].

In previous studies, different pharmacological effects were shown for *C. copticum*. In addition, the plant has been used widely in traditional medicine. Therefore, different pharmacological effects of *C. copticum* and its constituents were reviewed in the present paper.

2. Methods

The following databases and electronic journals were searched from September, 2012, to December, 2013, including Google Scholar, Pubmed, Wiley, Science Direct,
and Springer link. Key search terms were C. copticum, Ajwain, and Trachyspermum ammi and other names of the plant as well as different constituents of the plant and different pharmacological effects. Previously published studies between 1994 and 2014 in the field of different pharmacological effects of C. copticum and its different constituents were reviewed in this paper.

3. Phytology and Morphology

C. copticum is identified in different regions of the world by different names as follows.

Scientific name: Trachyspermum ammi and Sprague, it is synonym of Carum copticum Benth and in some documents Aromaticum has been named by different herbalists.

Different names of the plant in various languages (vernacular name) are Sanskrit: Yamini, Assamese language: Jain, English: Bishop's weed, Hindi, Baluchi: Ajowan and Spica, Gujarati Language: Ajmo, Canada: Oma, Malaysia: Oman, Arabic: Khella or khellin, Persian: nankhah, zenian, khordaneh, and South Khorasan: aigho [8].

C. copticum is a perennial plant; its height is a little more than black cumin and about a meter, but the leaf shape and color of the flowers of the plant are similar to black cumin. Its stem is ramose; its leaves are slurred and filiform with small white flowers. The plant's fruit which is called C. copticum is small, oval, and dark yellow and the fruit surface has five long thin lines of light yellow. Fruits and roots are highly regarded in traditional medicine.

4. Chemical Components

The constituents of the seed of C. copticum included carbohydrate (38.6%), fat (18.1%), protein (15.4%), fiber (11.9%), tannins, glycosides, moisture (8.9%), saponins, flavone, and mineral matter (71%) containing calcium, phosphorous, iron, cobalt, copper, iodine, manganese, thiamine, riboflavin, and nicotinic acid [3, 9]. C. copticum grows in different areas of the world containing different compounds. Main components of the oil of Iranian and African C. copticum oil are carvacrol, γ-terpinene, and p-cymene while thymol (97.9%) is the main component of south Indian plant oil. It was also reported that thymol (45.9%), γ-terpinene (20.6%), and o-cymene (19%) are the major components of the oil of C. copticum but ethylene methacrylate (6.9%), β-pinene (1.9%), and hexadecane (1.1%) were the other constituents of the plant [10]. Thymol (72.3%), terpinolene (13.12%), and o-cymene (11.97%) were also identified as constituents of C. copticum [11]. Chemical composition of C. copticum in two areas in Iran was assessed and results showed that the plant in Kamfiz contains γ-terpinene (48.07%), p-cymene (33.73%), and thymol (17.41%) compared to the composition of plant in Eghlid area which included γ-terpinene (50.22%), p-cymene (31.90%), and nerolidol (4.26%) as main components [12].

Chemical constituents of the essential oil of C. copticum and its acetone extract were also examined by GC and GC-MS analysis. Results showed that 96.3% of the total amount of the essential oil contains 26 components including thymol (39.1%), p-cymene (30.8%), γ-terpinene (23.2%), β-pinene (1.7%), and terpinene-4-ol (0.8%) while 68.8% of the total amount of its acetone extract has thymol (39.1%), oleic acid (10.4%), linoleic acid (9.6%), γ-terpinene (2.6%), p-cymene (1.6%), palmitic acid (1.6%), and xylene (0.1%) [13]. Hydrodistillation and supercritical fluid (CO2) extraction (SFE) methods of the plant were also performed. In hydrodistilled oil, there were 8 components including thymol (49.0%), γ-terpinene (30.8%), p-cymene (15.7%), b-pinene (2.1%), myrcene (0.8%), and limonene (0.7%), but in SFE method with the best condition of temperature, pressure, and dynamic extraction time there were 3 components including γ-terpinene (14.2%), p-cymene (23.1%), and thymol (62.0%) [14].

According to the results of study of Srivastava et al., the main constituents of fruit oil of C. copticum were p-cymene (41.98%), carvacrol (45.20%), and thymol (0.48%) [15]. The content of chromone, an isomer of the coumarin which is a drug with anticoagulant performance, in various stages of growth of C. copticum was determined by high performance liquid chromatography (HPLC) and the results showed that the amount of chromone was higher in unripe than dried [16].

Chemical compounds of C. copticum seeds, cultivated in different studies using gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS), are listed in Table 1.

5. Pharmacological Effects

C. copticum has aromatic odor and spicy taste and is widely used as a spice in the curry powder (curry). The odor of the plant is due to thymol and its aromatic compounds are mainly obtained from methanol extract [19]. Several therapeutic effects were shown for C. copticum and its main constituents which were reviewed in the rest of this paper.

5.1. Respiratory Effects. One of the therapeutic effects of C. copticum is its effect on respiratory system. This plant is used as antiasthma and antidysspnea in traditional medicine. In this context, multiple studies have been carried out including relaxant and inhibitory effects on histamine receptors, stimulatory effect on adrenoreceptors of guinea pigs' tracheal smooth muscles, antitussive effect in guinea pigs, and its bronchodilatory effect on airways of asthmatic patients.

C. copticum showed potent relaxant effect on tracheal smooth muscles which was not due to its content of thymol or competitive antagonistic effect on cholinergic receptors. The existence of α-pinene in essential oil of this plant showed anticholinergic activity (functional antagonism) [20]. Relaxant effects of different fractions from C. copticum including fractions 1, 2, 3, and 4 in guinea pigs' tracheal smooth muscle were shown. For preparation of four fractions, the essential oil was freeze dried at 0°C overnight. The white crystals were collected by filtration, air dried, and subjected to NMR analysis. The filtrate (1 mL) was chromatographed on a silica gel (70–230 mesh). The column was eluted with solvent mixtures comprising petroleum ether (40–60°C) and chloroform with varying concentrations. Fractions (25 mL) were collected and
Table 1: Chemical composition of *C. copticum* based on geolocation or type of extraction.

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<tr>
<td>Thymol</td>
<td>45.9</td>
<td>72.3%</td>
<td>17.41%, 50.22%</td>
<td>39.1%, 39.1%</td>
<td>54.50%</td>
<td>57.18</td>
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<tr>
<td>p-Cymene</td>
<td>—</td>
<td>—</td>
<td>33.73%, 31.90%</td>
<td>30.8%, 1.6%</td>
<td>19.38%</td>
<td>22.55</td>
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<tr>
<td>γ-Terpinene</td>
<td>20.6</td>
<td>—</td>
<td>48.07%</td>
<td>23.2%, 2.6%</td>
<td>22.96%</td>
<td>13.07</td>
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<tr>
<td>o-Cymene</td>
<td>19.0</td>
<td>11.97%</td>
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<tr>
<td>Ethylene methacrylate</td>
<td>6.9</td>
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<td>Hexadecane</td>
<td>1.1</td>
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<td>β-Pinene</td>
<td>1.9</td>
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<td>cis-Limonene oxide</td>
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<td>β-Myrcene</td>
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<td>Myrcene</td>
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<td>α-Thujene</td>
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<td>Carvacrol</td>
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<td>β-Phellandrene</td>
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<td>4-Terpineol</td>
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<td>0.8%</td>
<td>0.11</td>
<td>0.155</td>
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<tr>
<td>β-Fenchyl alcohol</td>
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<td>Terpinolene</td>
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<td>13.12%</td>
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<tr>
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The effect of thymol on tracheal and ileum smooth muscles and ciliary motion of respiratory system in rat showed that thymol has a dose-dependent antispasmodic property and increases mucosa transfer due to ciliary motion [28]. Additionally, the antispasmodic effect of thyme extract was...
Volatile oil compounds such as thymol [29] demonstrated which is suggested to be due to phenolic volatile oil compounds such as thymol [29].

The relaxant effect of carvacrol, one of the main constituents of *C. copticum*, on tracheal smooth muscle of guinea pigs has been shown which was greater than the effect of theophylline [30].

Other plants containing carvacrol such as *Carum carvi* [31] also showed relaxant effects on tracheal smooth muscle. Fraction 2 of *C. copticum*, which is suggested to be carvacrol, also revealed relaxant effect on tracheal smooth muscle [21]. Therefore, the main constituent of *C. copticum*, carvacrol, may have relaxant effects on the tracheal smooth muscle.

To examine the possible mechanism(s) responsible for the relaxant effect of carvacrol on tracheal smooth muscle, its effect on histamine receptors was evaluated in tracheal smooth muscle of guinea pigs by measuring EC<sub>50</sub> histamine (effective concentration of histamine causing 50% of maximum response) in the presence of carvacrol and chlorpheniramine. The results of this study showed a competitive antagonistic effect of carvacrol at histamine H1 receptors. In addition, the results suggested its stimulatory effect on β-adrenergic receptors and also a blocking effect at muscarinic receptors [32] for carvacrol. In fact, stimulatory effect of carvacrol on β2-adrenoceptors was proved by performing isoprenaline concentration response curve and measurement of EC<sub>50</sub> in the presence of the carvacrol, propranolol, and saline on tracheal smooth muscle of guinea pigs in nonincubated and incubated with chlorpheniramine (to block histamine H1 receptors) conditions. The results showed parallel leftward shift of isoprenaline concentration response curve and lower EC<sub>50</sub> in the presence of carvacrol and higher EC<sub>50</sub> in the presence of propranolol compared to the results of saline [33]. These results showed a clear β2-adrenoceptors stimulatory effect for carvacrol. In addition, the inhibitory effect of carvacrol on muscarinic receptors which is the other possible mechanism for its relaxant effect on the tracheal smooth muscle was also studied. The rightward shift in methacholine-response curves and the increased EC<sub>50</sub> in the presence of different concentrations of carvacrol compared with saline were seen which showed possible competitive antagonistic effects of carvacrol at muscarinic receptors [34]. These results suggest that the mechanism of relaxant effect of carvacrol similar to plant extract could have inhibitory effects on muscarinic and histamine receptors and stimulatory effect on β2-adrenoceptors or combinations of the three mechanisms.

However, carvacrol with a potent relaxant effect on tracheal smooth muscle shows no antitussive effect [26].

With regard to the lung inflammation in different respiratory diseases, mainly asthma, the anti-inflammatory and immunomodulatory effects of carvacrol were also examined in several studies. The effect of carvacrol on cell culture supernatants of macrophages in porcine induced alveolar inflammatory showed inhibitory effect of carvacrol on TNF-α, IL-1β, and TGF-β [35]. Carvacrol also inhibited secretion of TNF-α and IL-1β in porcine alveolar macrophage [36]. Anti-inflammatory effect of carvacrol was also evaluated by measurement of exudates volume and leukocyte migration in plural cavity due to carrageenan injection to this cavity which showed a preventive effect of carvacrol on exudates volume and leukocyte migration (*in vivo* and *in vitro*) and suggested an inhibitory effect on COX-1 and COX-2 and 5-lipoxygenase [37]. In addition carvacrol also depicted a preventive effect on serum levels of endothelin, total protein, histamine, NO, and total white blood cells, differential white blood cells (WBC) count and tracheal responsiveness in ovalbumin sensitized guinea pigs [38, 39]. Table 2 summarizes respiratory effects of *C. copticum* and its constituents thymol and carvacrol.

### Table 2: Respiratory effects of *C. copticum* and its constituents thymol and carvacrol.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxant effect on tracheal smooth muscles</td>
<td>[20, 40]</td>
</tr>
<tr>
<td>Inhibitory effect of <em>C. copticum</em> on histamine (H1) receptors</td>
<td>[22]</td>
</tr>
<tr>
<td>Stimulatory effect on beta 2 adrenoceptors</td>
<td>[23]</td>
</tr>
<tr>
<td>Antitussive effects</td>
<td>[26]</td>
</tr>
<tr>
<td>Bronchodilatory effect</td>
<td>[20, 27]</td>
</tr>
<tr>
<td>Thymol</td>
<td></td>
</tr>
<tr>
<td>Increase of mucosa transfer due to ciliary motion</td>
<td>[28]</td>
</tr>
<tr>
<td>Antispasmodic effect</td>
<td>[29]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td></td>
</tr>
<tr>
<td>Relaxant effect</td>
<td>[30, 31, 40]</td>
</tr>
<tr>
<td>Competitive antagonistic effect at histamine H1 receptors</td>
<td>[32]</td>
</tr>
<tr>
<td>Stimulatory effect on β-adrenergic receptor</td>
<td>[33]</td>
</tr>
<tr>
<td>Blocking effect at muscarinic receptors</td>
<td>[34]</td>
</tr>
<tr>
<td>Inhibitory effect on secretion of TNF-α and IL-1β in porcine alveolar macrophage</td>
<td>[35, 37]</td>
</tr>
<tr>
<td>Inhibitory effect on COX-1 and COX-2 and 5-lipoxygenase (anti-inflammatory effect)</td>
<td>[37]</td>
</tr>
</tbody>
</table>

5.2. Cardiovascular Effect. Due to calcium channel blocking effect, *C. copticum* has remarkable role in heart rate and blood pressure. Thymol also made fall in blood pressure and heart rate [41]. Several cardiovascular effects of *C. copticum* and its constituents were shown. Negative inotropic and chronotropic effects due to administration of 1–10 mg/kg thymol in mice were shown which lead to decrease in blood pressure. It was suggested that this effect of thymol could be due to calcium channel blocking property [25].

Kumar et al. examined the effect of juice of *C. copticum* leaves on isolated frog heart. It had positive ionotropic effect
COA reductase [45, 46]. In addition, it was shown that removal or catabolism of lipoproteins and inhibition of HMG antilipidemic effect of the plant is possibly due to enhanced effect of simvastatin (0.6 mg/kg). It was also suggested that and increased HDL up to 60% which was comparable to the triglycerides, and LDL-cholesterol (71%, 53%, and 63%, resp.) which showed that this plant can cause vasodilation of coronary arteries and decreased systemic blood pressure [44].

Lipid-lowering effect of *C. copticum* seeds has been studied in rabbit. In these studies, methanolic extract of the plant (2 g/kg) significantly decreased total cholesterol, triglycerides, and LDL-cholesterol (71%, 53%, and 63%, resp.) and increased HDL up to 60% which was comparable to the effect of simvastatin (0.6 mg/kg). It was also suggested that antilipidemic effect of the plant is possibly due to enhanced removal or catabolism of lipoproteins and inhibition of HMG COA reductase [45, 46]. In addition, it was shown that *C. copticum* seed powder was also effective in increasing secretion of lipase and amylase from pancreas gland in rat [47].

Rajput et al. administered extract of Ajwain with dose of 50 mg/kg and warfarin (0.54 mg/kg) orally to rats and measured coagulation parameters (PT and aPTT). On the 14th day, extract significantly increased PT time compared with warfarin but did not have effect on aPTT. They demonstrated its possible effects on the extrinsic pathway [48].

Administration of thymol orally twice daily (14 mg/kg) to high fat diet rats caused decremented effect on body weight gain and serum lipid peroxidation and increased antioxidant levels [49].

5.3. Urogenital Effects. In an *in vivo* study, the effect of the extract of *C. copticum* seeds on urinary stone of 350 patients was investigated. According to data of this study, Ca oxalate, Ca oxalate/uric acid, and Ca-oxalate/hydroxyapatite stones were treated by 100%, 53%, and 31.25%, respectively, with the extract [50]. Recently in India an anticalcifying protein from the seeds of *C. copticum* has been extracted and was administered in urolithiatic rat model. This protein inhibited calcium oxalate deposition by adhesion to calcium oxalate and prevented growth of stones *in vitro* and also *in vivo* [51]. However, other observations did not show any effect of this plant on the production of urea in 24 hours. The results showed that traditional use of *C. copticum* in the treatment of kidney stones was not statistically significant in laboratory setting [52].

*C. copticum* was tested for abortion in some states of India in 1987. The result of the study showed that *C. copticum* leads to abortion in 50 cases of 75 pregnant women and possibly has fetotoxicity feature. However, the possibility of congenital defect in this region of India increased during the study period. *C. copticum* dry seed has phytoestrogen content with 473 ppm value that can increase milk production [9].

5.4. Gastrointestinal Effects. Traditional use of the *C. copticum* seeds in many gastrointestinal diseases, including intestinal disorders, abdominal pain (colic), or diarrhea, is reported [56]. The alcoholic extract of the plant fruit showed significant reduction effect in ulcer index in an animal model of gastric ulcer [57]. In addition, the extract of crushed fruit from *C. copticum* was effective in relieving stomach pain but increased stomach acid secretion.

Aqueous extract of *C. copticum* (125, 250, and 500 mg/kg) treatment for two weeks improved peptic ulcer induced by ibuprofen in rats which was comparable with the effect of omeprazole. It was also suggested that antiulcer effect of this plant is possibly due to its antioxidant effect [58].

*C. copticum* is able to increase the gastric acid secretion time and the amount of gastric acid. In addition, it was shown that the plant can reduce the transit time of food in the digestive system of mice [59]. Inhibitory effect of *C. copticum* on the contractions of the digestive tract smooth muscle, especially the intestines, increased activities of digestive enzymes and bile secretion was reported [60], which support its effect on gastrointestinal tract.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of calcium oxalate deposition</td>
<td>[51]</td>
</tr>
<tr>
<td>Fetotoxicity feature</td>
<td>[9]</td>
</tr>
<tr>
<td><em>C. copticum</em></td>
<td></td>
</tr>
<tr>
<td>Increase of milk production</td>
<td>[9]</td>
</tr>
<tr>
<td>Reduction of sperm activity and pregnancy</td>
<td>[54]</td>
</tr>
<tr>
<td>Reduction of testes weight</td>
<td>[55]</td>
</tr>
</tbody>
</table>

Table 3: Urogenital effects of *C. copticum*.

Mineraloherbal preparation containing seeds of *C. copticum*, leaves of *Cassia angustifolia* (Senna), fruits of *Terminalia chebula* (Himej) and *Embelia ribes* (Vidang), and roots of *Glycyrrhiza glabra* (Jethimadh) was administered to Sprague-Dawley rats (male and female) by oral route. This preparation reduced number of implantations in females who mated with male rats. However, it did not have significant effect on weight of testis, epididymis, and accessory glands, spermatogenesis, and mating rate in male rats [53].

Effect of *C. copticum* oil on ejaculated human spermatozoa also showed that this plant leads to reduction of sperm activity and pregnancy. Ethanolic extract of *C. copticum* fruit with doses of 100, 200, and 400 mg/kg also was given to male Wistar rats for 60 days. This drug decreased testes weight, number of sperms, and sperm motility dose dependently. In addition, increased level of abnormal sperms was also observed [54]. The viability and membrane integrity of human spermatozoa were evaluated in presence of essential oil of *C. copticum*. This oil reduced viability, antioxidant enzyme, catalase, and mitochondrial function. Cholesterol/phospholipid ratio was increased and therefore the ability of spermatozoa for zygote fertilization is decreased [55]. According to the previous studies *C. copticum* could be a suitable male contraceptive. Table 3 summarizes the urogenital effects of *C. copticum*.
In several studies, hepatic effects of *C. copticum* have been observed. The effect of 125, 250, and 500 mg/kg from *C. copticum* was assessed on peptic ulcer induced by ibuprofen in rats. In addition, the effect of the extract on liver enzymes including aspartate transferase (AST) and alanine transferase (ALT) in rat. In addition, the effect of the extract on liver enzymes of *C. copticum* copticum formulations (containing several plants such as *C. copticum*). In a study, the hepatoprotective effects of polyherbal formulations (containing several plants such as *C. copticum*) administered twice daily for one week after paracetamol (500 mg/kg) administration were evaluated on day 8. Paracetamol increases liver enzymes but treatment with polyherbal formulations improved the liver enzyme which was suggested to be due to cell membrane stabilization and recovery of hepatic tissue.

The effect of *C. copticum* on liver injury induced by CCL4 and lethal dose of paracetamol (1g/kg) in mice was also examined. Oral administration of *C. copticum* reduced liver enzymes (ALT, ALP, and AST) and improved paracetamol-and CCl4-induced hepatic injuries [25]. On the other hand, carvacrol caused apoptosis and antiproliferation on HepG2 cells of human hepatocellular carcinoma. Carvacrol selectively decreases phosphorylation of ERK1/2 and activated phosphorylation of p38 but did not affect JNK MAPK phosphorylation. A significant reduction effect on Bcl-2 gene expression was also shown 24 h after carvacrol treatment. In addition, carvacrol inhibited DNA synthesis and decreased the number of cancer cells and total protein content [62].

The effect of *C. copticum* on isolated guinea pig ileum showed antispasmodic activity of extract of the plant and suggested that this effect may be due to cholinergic receptors inhibition by *C. copticum* [63]. Table 4 summarizes The effects of *C. copticum* on gastrointestinal tract.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antulcer effect</td>
<td>[58]</td>
</tr>
<tr>
<td>Increase of liver enzymes</td>
<td>[58]</td>
</tr>
<tr>
<td><em>C. copticum</em></td>
<td></td>
</tr>
<tr>
<td>Increasing of time and amount of gastric acid secretion</td>
<td>[64]</td>
</tr>
<tr>
<td>Inhibitory effect on the gastrointestinal contractions</td>
<td>[59, 60]</td>
</tr>
<tr>
<td>Hepatoprotective effects</td>
<td>[25]</td>
</tr>
<tr>
<td>Antispasmodic effects</td>
<td>[63]</td>
</tr>
</tbody>
</table>

5.5. Antiparasitic Effects. Infection with filarial nematodes makes lymphatic filariasis and synthetic drug not adequately effective in killing these parasites. Therefore, antifilarial effects of medicinal plant, namely, fruit extract of *C. copticum*, were shown in *vitro* and *in vivo*. *C. copticum*, thymol, and carvacrol have macrofilaricidal properties against adult bovine filarial worm *S. digitata in vitro*. In addition, the plant increased mortality and infertility of female worm of human filarial worm *Brugia malayi in vivo* [65]. The effect of *C. copticum* seeds on treatment of leishmaniasis parasitic was also reported. Hydroalcoholic extract of *C. copticum* showed antileishmanial activity with IC50 15.625 μM which was less than IC50 for macrophage cell line (43.76 μM) [66].

Anthelmintic effect of *C. copticum* in comparison with levamisole (an anthelmintic and immunomodulator drug) on sheep infected with mixed nematode was also evaluated. *C. copticum* powder dose dependently caused reduction in eggs per gram of feces which was more potent compared with levamisole [67].

*Plasmodium falciparum* is genus of parasitic protozoa. Infection with this genus is known as malaria. Ethyl acetate extract of *C. copticum* seed with values of 25 μg/mL also showed in *vitro* antimalarial activity [68].

Pinewood nematode (PWN) makes pine wilt disease. Nematicidal activity of *C. copticum* oil against *B. xylophilus* was evaluated in *vitro* and mortality of nematodes after 24 h was studied. *C. copticum* and its components killed nematodes and likely are suitable as natural nematicides. It was also shown that thymol and carvacrol have a significant effect on nematodes [69, 70]. Considering that one of the most important worldwide parasitic diseases (especially in dirty and unsanitary areas) is hydatid cysts, it was shown that *C. copticum* play a significant role in the removal of hydatid cysts *in vitro*. In a study, *Protoscoleces* were exposed to essential oil of *C. copticum* (3, 5, and 10 mg/mL) for 10, 20, 30, and 60 min. The results showed that the higher concentration in the least time period of the study killed 100% of hydatid cyst protoscolices which was suggested to be due to its phenol compounds [71].

*Coccidian protozoa* such as *Eimeria tenella* live in intestinal tract of animal and cause coccidiosis which in severe cases lead to death. Herbal complex (containing *C. copticum*) with three concentrations (2, 4, and 6 g) was added to water of broiler chickens infected with *Eimeria tenella* and symptoms were compared with amprolium group. This herbal complex in a concentration–dependently manner improved broiler chickens with *Eimeria tenella* [72].

In addition, there are several studies regarding the disinfecting and insecticide effects of *C. copticum* extracts, such as its effects on adult male and female German cockroaches by inhibition of acetylcholine esterase (AChE). In addition, *C. copticum* oil, 0.1 mg/mL, caused 100% larval mortality against *A. aegypti* mosquito larvae. Thus *C. copticum* can be used as botanical insecticides [73, 74]. The effect of thymol vapor on eggs laying of malaria mosquito (*Anopheles stephensi*) was more effective with LD50 1.6-fold than *C. copticum* oil (80.77
versus 48.88 μg/mL) [75]. Table 5 summarizes antiparasitic effects of C. copticum.

5.6. The Antimicrobial Effects. Essential oil from Iranian C. copticum including 72.3% thymol inhibited gram-positive and gram-negative bacteria and viruses in which inhibition rate is associated with thymol content. High dose of thymol inhibits gram-positive more than gram-negative bacteria. It was shown that phenolic compounds interfere with cell membrane, change pH and ions homeostasis, and perhaps in this way act as antimicrobial agents. At all these studies the antimicrobial activity was examined by broth microdilution method [10, 12, 76, 77].

The effect of aqueous extract of C. copticum on several strains of bacteria showed antibacterial effect on Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, P. aeruginosa, S. typhimurium, and Shigella flexneri [78]. The effect of C. copticum on fifty-five bacterial strains showed antimicrobial activity with minimum inhibitory concentration <2% (v/v) except Pseudomonas aeruginosa [79]. It was also shown that ether fraction of C. copticum had better antibacterial and antifungal activity against multidrug resistant (MDR) strains of Candida albicans, Candida krusei, Candida tropicalis, Candida glabrata, Escherichia coli, and reference strains of Streptococcus mutans and Streptococcus bovis than other fractions [80].

Dysbiosis disease occurs due to microbial imbalance in intestinal flora as lactobacilli, bifidobacteria, and coliform bacteria which are lower in fecal counts. In this disease, useful bacteria decreased and harmful bacteria increased in intestinal flora which leads to reduction in energy and body weight. It was shown that C. copticum can lead to reduction in pathogenic microorganisms such as Candida albicans, Clostridium spp., and Bacteroides fragilis while having little effect on microflora and therefore could be effective in dysbiosis treatment [81].

The effect of C. copticum with thymol chemotype (when main component is thymol in contrast carvacrol chemotype) on bacterial strains (S. aureus, B. cereus, L. monocytogenes, E. coli O157:H7, and S. enteritidis) was also evaluated. Bacteria were cultured overnight at 37°C, and the essential oil of the plant and antimicrobial standards (chloramphenicol and ascorbic acid) were added. After incubation at 37°C for 22–24 h, the MIC (mg/mL) was calculated and the microorganism growth inhibition was assayed using an ELISA reader. The results of this study showed that the antimicrobial of C. copticum was more potent than B. persicum and C. cyminum [82].

The antimicrobial effects of C. copticum as MIC and MBC were shown in Table 6.

The overall results of these studies showed that C. copticum essential oil is rich in monoterpenic compounds and could be used as a natural antimicrobial agent in the food and pharmaceutical industries.

Regarding ophthalmic disorders and cataract, it was claimed that the herbal ophthalmic drops (Ophthacare), which is a C. copticum extract product, treat infection, inflammation, and cataract in an experimental study [83].

C. copticum is also able to protect food against microbial invasion in vitro. These antimicrobial properties of C. copticum are due to its two ingredients, thymol and carvacrol [84]. Thymol has microbial killing property against common resistant microbial pathogens to multiple antibiotics drugs from the third generation. Therefore, it can be named as the fourth generation plant antibiotic [85].

Gilani et al. studied antibacterial effect of C. copticum by applying cream containing 5% essential oil of C. copticum to healing wound in rabbits in comparison with iodine tincture. Wound contraction on the 15th day in C. copticum group was 99.68%, compared with the healing effect of iodine tincture group, 100%, and nontreatment group, 96.57%, which indicates a wound healing effect of C. copticum [86].

In a study, bactericidal properties of C. copticum were shown on gram-negative Erwinia carotovora in vitro which is suggested to be due to its phenolic compounds such as thymol and carvacrol [87].

5.7. Antifungal Effects. Antifungal activity of essential oil of C. copticum seeds is also documented against toxigenic Aspergillus species. The oil of this plant also is able to inhibit the growth of this parasite [88]. In another study, C. copticum (900 ppm concentration) showed fungitoxicity activity

Table 5: Antiparasitic effects of C. copticum.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrofilaricidal properties (e.g., S. digitata), increase of mortality, and infertility of female worm (Brugia malayi)</td>
<td>[65]</td>
</tr>
<tr>
<td>Antileishmanial activity</td>
<td>[66]</td>
</tr>
<tr>
<td>Dysbiosis treatment</td>
<td>[81]</td>
</tr>
<tr>
<td>Anthelmintic effect</td>
<td>[61]</td>
</tr>
<tr>
<td>Antimalarial activity</td>
<td>[68]</td>
</tr>
<tr>
<td>Nematicidal activity</td>
<td>[69, 70]</td>
</tr>
<tr>
<td>Killing of hydatid cyst protoscolices</td>
<td>[71]</td>
</tr>
<tr>
<td>Insecticidal activity</td>
<td>[73, 74]</td>
</tr>
<tr>
<td>Thymol</td>
<td></td>
</tr>
<tr>
<td>Nematicidal activity</td>
<td>[69, 70]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td></td>
</tr>
<tr>
<td>Nematicidal activity</td>
<td>[69, 70]</td>
</tr>
</tbody>
</table>

Table 5: Antiparasitic effects of C. copticum.
receptors and nitric oxide pathway [96].

It was suggested that this effect was modulated via potentiation of GABA neurotransmission and suppression of glutamate receptors and nitric oxide pathway [96].

The effect of plant was not due to opioid receptors because analgesic effect of ethanolic extract of Carum copticum was comparable with morphine and this effect is suggested to be due to increase in GABAergic neurotransmission in the brain which reduces neural activity [97].

5.11. Conclusion. C. copticum or Ajwain belongs to the Apiaceae plants family and its most important constituents are thymol and carvacrol. C. copticum seeds have various important medicinal properties such as antipyretic, antitussive, antispasmodic and cardiovasodilator, respiratory, liver protection, urogenital, gastrointestinal, antiparasitic, antimicrobial, and lipid lowering effects. Therefore this plant could be of therapeutic value in treating various disorders. Therefore, further clinical studies regarding various effects of C. copticum and its main constituents are recommended. If significant clinical results were found, proper industrial drug products need to be prepared for clinical use.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Effect of the Zataria multiflora on Systemic Inflammation of Experimental Animals Model of COPD

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The effects of Zataria multiflora (Z. multiflora) on systemic inflammation in guinea pigs model of COPD were examined. Control animals, COPD (induced by exposing animals to cigarette smoke), COPD + drinking water containing three concentrations of the extract of Z. multiflora, and COPD + dexamethasone were studied (n = 6 for each group). Serum levels of IL-8 and malondialdehyde (MDA), total blood WBC (P < 0.01 for all cases), and eosinophil counts (P < 0.05) were higher and weight changes (P < 0.05) were lower in the COPD group compared to controls. IL-8 level (P < 0.001) and weight changes (P < 0.01 to P < 0.001) in all treated groups with Z. multiflora and total WBC number and MDA level in treated groups with two higher concentrations of the extract and lymphocytes percentage (P < 0.05) in the highest concentration of Z. multiflora and dexamethasone (P < 0.05 to P < 0.001) were significantly improved compared to the COPD group. Results showed a preventive effect of hydroethanolic extract from Z. multiflora on all measured parameters in animals model of COPD which was comparable or even higher (in the highest concentration) compared to the effect of dexamethasone at the concentration used.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an epidemic disease in the world [1] and smoking is an important risk factor for development of COPD [2]. COPD is characterized by two pathological features including bronchitis and emphysema. Inflammatory processes, oxidative damage, endothelial dysfunction, endothelial cell apoptosis, proteolysis, and vascular remodeling are lung tissue changes of COPD patients [2–4]. Some cells such as CD8+ cytotoxic, CD68+ macrophages, and neutrophils penetrate the airways and alveoli of COPD patients. These inflammatory cells release some proinflammatory mediators, including TNFα, interleukin-8, and macrophage inflammatory protein-α (MIP-α) which absorb more inflammatory cells and produce a positive inflammatory chain that preserves the inflammatory response [5, 6]. Another inflammatory response in COPD patients is an increase in number of CD68+ monocytes or macrophages in the bronchial mucosa [7, 8]. In severe COPD patients, an increase in the number of neutrophils is also observed [9, 10]. Therefore, antioxidants may have a role in the treatment of COPD [11].

Z. multiflora is a perennial plant with a woody, small leaves, fibrous root, and height of 40–80 cm, with highly narrow branches [12]. This plant has a limited distribution in the world and only grows in Iran, Pakistan, and Afghanistan [13]. Z. multiflora contains various compounds of which some are bioactive chemicals, particularly terpenes such as thymol and carvacrol. Te plant also contains apigenin, luteolin, and 6-hydroxyluteolin glycosides, as well as di-, tri-, and tetramethoxylated. These compounds could be responsible for the therapeutic effects of Z. multiflora [12]. In Iran, Z. multiflora is used in traditional medicine for its antiseptic, analgesic, and carminative properties [14]. The effects of Z. multiflora essential oil on Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus have been described [15]. Antibacterial
effects of this plant on the B. cereus [16], Staphylococcus aureus [17, 18], and Escherichia coli [18], antifungal [19], analgesic [20, 21], antioxidant [22], and immunoregulatory effects [23], its effect on gastrointestinal disorders [24, 25], and anti-inflammatory effects [26] have also been reported. The antispasmodic effect of the plant on different types of smooth muscle was also demonstrated [27–29]. Our previous study also showed relaxant effect of the other plant of this family (Thymus vulgaris) on tracheal smooth muscle [30]. The inhibitory effect of the extract of Z. multiflora and carvacrol on histamine (H1) [31] and muscarinic receptors [32–34] and its stimulatory effect on β-adrenoceptors [35] were demonstrated in our previous studies.

Therefore, in the present study, the effects of Z. multiflora on total and differential WBC in the blood, serum levels of IL-8 and MDA, and weight changes of guinea pig model of COPD have been examined.

2. Materials and Methods

2.1. Plant and Extract. Z. multiflora was collected from mountains in the fluorine mine in a region between Tabas and Yazd, Iran, and was identified by Mr. Joharchi and a sample was kept in the Herbarium of the Faculty of Sciences, Ferdowsi University of Mashhad (herbarium number: 35314). For the preparation of hydroethanolic extract from Z. multiflora, 125 g of dried shoots and powdered plant was mixed with 875 mL of 50% ethanol. The mixture was shaken for 72 hours at room temperature. The extract was then passed through the filter paper and the solvent was removed under reduced pressure. The dried extract was collected and kept at refrigerator temperature. Three concentrations (0.4, 0.8, and 1.6 mg/mL) of the extract were then used for treatment purpose. Extract concentrations were chosen according to the previous study [36–41].

2.2. Characterization of the Extract of Zataria multiflora. In our previous study [35] the characteristic of the extract of this plant was identified, using HPLC (Waters 474, Waters Corporation, MA, USA) fingerprint. The extract was dissolved in the mobile phase and filtered through 0.22 μm membrane filter. An aliquot (20 μL) of sample (50 μg/mL) was injected into the reverse phase HPLC column (C18; 250 × 4.6 mm). The mobile phase consisted of methanol:water (60:40) with an isocratic elution at the flow rate of 1 mL/minute. The peaks were monitored at 254 nm (Figure 1(a)). Figure 1(b) illustrates the chromatographic profile of pure carvacrol (5/1000) with retention time of about 9 min. All used solvents were HPLC graded and supplied by Caledon Laboratories, Georgetown Ltd., Canada. Using the calibration curve, the quantification of carvacrol in a sample of the extract of Z. multiflora was achieved which was about 0.16% w/w (Figure 1).

2.3. Exposure of Animals to Cigarette Smoke. Exposure of guinea pigs to cigarette smoke was performed according to the method described previously [42, 43]. To create a model of chronic obstructive pulmonary disease (COPD) in guinea pigs, animals were placed in special boxes which included two parts: smaller part for the head to which cigarette smoke was administered (the animal’s head) and larger part for the body of guinea pigs. Twenty-milliliter puffs of cigarette smoke were drawn out of the cigarettes using a syringe and then exhausted at a rate of two puffs per minute into the animals’ head (every 30 seconds, one puff of cigarette smoke was transferred into the head part). Animals were exposed for 8–9 minutes to the smoke of one cigarette, and there was a 10-minute break between the two cigarettes. The animals were exposed initially to one cigarette per day and gradually increasing to a maximum of 5 cigarettes per day. The animals were exposed to cigarette smoke (Magna: Nicotine = 5, tar = 6) for 3 consecutive months, 5 days per week, and 5 cigarettes per day (the cigarettes’ filters were not removed).
2.4. Animals and Groups. Thirty-six guinea pigs of both sexes (600–800 g) were used in this study. Animals were kept in a temperature-controlled room. The animals were divided into six groups in random order as follows (for each group, \( n = 6 \)).

1. Control group: the animals were exposed to ambient air and received drinking water alone.
2. COPD group: the animals were exposed to cigarette smoke for 3 months and received drinking water alone.
3. COPD + dexamethasone: the animals were exposed to cigarette smoke and received drinking water containing dexamethasone (50 \( \mu \)g/mL).
4. COPD + Z. multiflora dose 1: the animals were exposed to cigarette smoke and received drinking water containing the extract of Z. multiflora (0.4 mg/mL).
5. COPD + Z. multiflora dose 2: the animals were exposed to cigarette smoke and received drinking water containing the extract of Z. multiflora (0.8 mg/mL).
6. COPD + Z. multiflora dose 3: the animals were exposed to cigarette smoke and received drinking water containing the extract of Z. multiflora (1.6 mg/mL).

The extract or dexamethasone was added to the drinking water daily which was freely available for animals. The volume of uptake of drinking water was checked which was about 100 mL/day for each guinea pig in all groups.

2.5. Biochemical Parameters

2.5.1. Measurement of Malondialdehyde (MDA). After exposing the animal’s chest, a 4 mL blood sample was taken from the heart and collected in a citrate tube. Then blood was centrifuged and the serum was separated and stored in \(-70^\circ\)C until the end of the study to measure the concentrations of IL-8 and MDA in all groups. MDA level, as an index of lipid peroxidation, was measured. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has a peak absorbance at 535 nm. Two mL from reagent of TBA/trichloroacetic acid (TCA)/hydrochloric acid (HCL) was added to 1 mL of serum, and the solution was heated in a water bath for 40 minutes. After cooling, the whole solutions were centrifuged at 1000 g for 10 minutes. The absorbance was measured at 535 nm \[44\]. The MDA concentration calculations were performed using the following formula: 

\[
C(m) = \text{Absorbance}/(1.56 \times 105)
\]

2.5.2. Measurement of IL-8. To measure the concentration of IL-8, a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit was used (Hangzhou Eastbiopharm Co., Ltd., Hangzhou) according to the manufacturer’s protocol.

2.6. Total WBC and Differential WBC Measurement. After opening the animal’s chest, a 4 mL blood sample was taken from the animal’s chest, and was collected in the test tube containing anticoagulant EDTA. Total WBC was counted in duplicate in a hemocytometer (in a Burker chamber) in blood stained with a Turk solution (1:10 dilution consisted of 1 mL of glacial acetic acid, 1 mL of gentian violet solution 1%, and 100 mL distilled). Differential cell counts were done on thin slide, prepared with smearing the blood sample, using Wright-Giemsa stain. According to staining and morphological criteria, differential cell analysis was carried out under a light microscope by counting 100 cells, and the percentage of each cell type was calculated.

2.7. Animals Weight Measurement. Animals were weighted at the beginning of the study (before the first smoke inhalation) and after 3 months (at the end of exposure period); then the difference between the two weights was calculated.

2.8. Statistical Analysis. All data were expressed as mean ± SEM. Comparison between the results of COPD and control groups was performed using unpaired \( t \)-test. The data of treated groups were also compared with COPD group using unpaired \( t \)-test. The comparison between the data of animals treated with three concentrations of Z. multiflora was performed using ANOVA with Tukey-Cramer posttest. Significance was accepted at \( P < 0.05 \). All statistical analyses were made using GraphPad Instat version 3.00 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Serum MDA Level. Serum level of MDA in COPD group was significantly higher than in control group (\( P < 0.01 \)). However, there was a significant reduction in serum levels of MDA in COPD groups treated by dexamethasone (\( P < 0.05 \)) and two higher concentrations of Z. multiflora (\( P < 0.01 \) for both cases) compared to COPD group (Figure 2).

3.2. Serum Levels of Interleukin-8. Serum levels of IL-8 were significantly increased in COPD group compared to control group (\( P < 0.01 \)). However, in treated groups with dexamethasone and three concentrations of Z. multiflora, the level of IL-8 was significantly lower than in COPD group (\( P < 0.001 \), for all cases, Figure 3).

3.3. Total and Differential WBC Counts. Total WBC (\( P < 0.01 \)) and eosinophil counts (\( P < 0.05 \)) were significantly higher in COPD compared to control group (Figure 4). Total WBC number in treated groups with dexamethasone and two higher concentrations of Z. multiflora, eosinophil, and neutrophil percentage in the treated groups with dexamethasone and the highest concentration of the extract were significantly improved compared to COPD group (\( P < 0.05 \) to \( P < 0.01 \)). In addition, lymphocyte percentage in treated groups with dexamethasone and the highest concentration of Z. multiflora was significantly increased compared to COPD group (\( P < 0.05 \), Figure 4).
concentrations of the extract on eosinophil, neutrophil, and the effect of low concentration and the effects of the highest on MDA, total WBC, and weight change were higher than the effect of the two higher concentrations of dexamethasone (50 μg/mL).

The effects of the two higher concentrations of Z. multiflora with dexamethasone, and three concentrations of Z. multiflora (0.4, 0.8, and 1.6 mg/mL) and dexamethasone (50 μg/mL).

Table 1: Weight changes (g) and serum levels of MDA (μg/mL) and IL-8 (pg/mL) in an animal model of COPD treated with three concentrations of Z. multiflora (0.4, 0.8, and 1.6 mg/mL) and dexamethasone (50 μg/mL).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Zataria (0.4 mg/mL)</th>
<th>Zataria (0.8 mg/mL)</th>
<th>Zataria (1.6 mg/mL)</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight changes</td>
<td>19.5 ± 7.13**</td>
<td>82.66 ± 4.97**,###</td>
<td>87.16 ± 2.21**,###</td>
<td>47.16 ± 9.43</td>
</tr>
<tr>
<td>MDA</td>
<td>34.337 ± 2.035†††</td>
<td>21.848 ± 1.395†††</td>
<td>20.491 ± 0.535†††</td>
<td>26.276 ± 1.362</td>
</tr>
<tr>
<td>IL-8</td>
<td>309.93 ± 23.22</td>
<td>243.91 ± 49.19</td>
<td>194.91 ± 35.91</td>
<td>241.51 ± 30.19</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Statistical comparisons between COPD groups treated with Z. multiflora and dexamethasone: † P < 0.05, ‡ P < 0.01. Statistical comparison was done using unpaired t-test. Statistical comparison between the effect of two higher concentrations of the extract (0.8 and 1.6 mg/mL) with its low concentration (0.4 mg/mL): ††† P < 0.001. The statistical comparison was done using ANOVA with Tukey-Kramer multiple posttest.

3.4. Weight Changes. Weight changes at the end of three months of experimental period, in the COPD group, were significantly (P < 0.05) lower than in control group. However, weight changes in the treated groups with dexamethasone and all concentrations of Z. multiflora were significantly increased compared to COPD group (P < 0.01 to P < 0.001, Figure 5).

3.5. Comparison of the Effect of Three Concentrations of Z. multiflora with Dexamethasone. Total WBC number in treated group with the highest concentration of Z. multiflora was significantly lower, and weight changes in the two higher concentrations of the plant were significantly higher (P < 0.05 to 0.01) than in the dexamethasone group. In treated group with low concentration of the extract, total WBC, eosinophils, and neutrophils percentage as well as serum levels of MDA were higher but lymphocyte percentage and weight change were lower than in the treated group with dexamethasone (P < 0.05 to 0.01; Tables 1 and 2).

3.6. Difference between Three Concentrations of Z. multiflora. The effects of the two higher concentrations of Z. multiflora on MDA, total WBC, and weight change were higher than the effect of low concentration and the effects of the highest concentrations of the extract on eosinophil, neutrophil, and lymphocyte were significantly higher than the effect of its low and medium concentration (P < 0.05 to 0.01; Tables 1 and 2).

4. Discussion

In the present study, the preventive effect of Z. multiflora, in a guinea pigs model of COPD by their exposure to cigarette smoke, was studied. Increased interalveolar septum, increased lymphatic tissue in the lung parenchyma, the destruction of alveolar wall, and existence of emphysema in the lung and intra-alveolar bleeding in almost all animals exposed to cigarette smoke were observed, using similar method of exposure to cigarette smoke, which clearly suggest an animal model of COPD [42, 43]. According to previous studies, IL-8 was also used as an inflammation factor in COPD patients [45–48]. Total and differential WBC counts as well as serum level of IL-8 were measured in control, nontreated, and treated COPD groups to evaluate systemic inflammation. Serum level of MDA was also measured to evaluate oxidant condition. The weight change of animals of different groups during the study period was also examined. The results showed increased total WBC and eosinophils count in COPD compared to control group. Serum levels of IL-8 were also increased in COPD compared to control.
Increased total and eosinophil counts as well as serum level of IL-8 indicated a systemic inflammation in animals exposed to cigarette smoke (an animal model of COPD). Systemic inflammation is a well-known phenomenon in COPD patients [46] and animal model of COPD [47]. Previous studies have also shown that inflammatory cells including macrophages, neutrophils, and T lymphocytes play a key role in COPD. Studies have also shown that the eosinophils, as a source of cytokines IL-3, -4, -5, -6, and -8, eosinophil-derived neurotoxin, eosinophil peroxidase, matrix metalloproteinase, and reactive oxygen species have an important role in the pathogenesis of COPD [48]. An increase in serum IL-8 level in smokers with COPD compared to healthy people was also observed previously [49]. The results of the present study also showed increased serum level of MDA which indicated oxidative stress in COPD group. In a previous study, the MDA level was also higher in COPD patients than in normal subjects which supports the results of the present study [50, 51]. The results showed that weight gain in the exposed animals to cigarette smoke during three months was significantly lower compared to control group. Mozzaffarian also showed the effect of cigarette smoking on body weight loss similar to findings of the present study [52]. Increased total and differential WBC counts, IL-8, MDA, and weight change in exposed animals to cigarette smoke showed the induction of an animal model of COPD in the present study and indicated a systemic inflammation in animal model of COPD.

According to traditional medicine, *Z. multiflora* has a beneficial effect on the cough due to colds and bronchial inflammation [53]. Therefore, in the present study, the therapeutic effects of *Z. multiflora* in an animal’s model of COPD were evaluated. Treated COPD animals with all the three concentrations of the extract prevented increased serum level of IL-8 and weight change but total WBC and neutrophils counts as well as MDA level decrease in COPD animals treated with the two higher concentrations of the extract. In addition, eosinophil count was also decreased in only treated animals with the highest concentration of the extract. Therefore, the results suggest that extract of *Z. multiflora* has a preventive effect on systemic inflammation in animal.
model of COPD. In this study, serum levels of MDA were also decreased by the extract of *Z. multiflora*. The effect of the extract on MDA seen in the present study confirms the results of previous studies which have also demonstrated antioxidant effect of *Z. multiflora* [22].

The effect of *Z. multiflora* on all measured parameters was concentration dependent and the effects were increased with increasing extract concentration. The effects of medium and especially high concentrations of the extract were greater than the effect of its low concentration. In addition, the effects of high concentration of the extract on some parameters were also greater than its medium concentration. Concentration-dependent effects of the extract also confirm anti-inflammatory effects of *Z. multiflora*. The effects of the three concentrations of the extract were studied according to the previous studies [36–41] and, using these concentrations, a plateau in the effect of the extract was not achieved.

Comparable effects of the extract with those of dexamethasone are other important reasons for the anti-inflammatory mechanism of *Z. multiflora*. Although the effects of low concentration of the extract were less than dexamethasone, the effects of its high concentration on almost all parameters and the effects of the medium concentration on some parameters were significantly greater than the effects of dexamethasone at used concentrations. In the present study, dexamethasone was used as positive control treatment in COPD according to several previous studies [54–56].

In fact, the previous studies have shown the anti-inflammatory effects of *Z. multiflora*, which support the results of the present study [26]. Antitussive effect [53] of plant also could be due to its anti-inflammatory mechanism and confirm the results of the present study. Our previous studies also showed the effect of the extract on Th1/Th2 balance (IFN-γ/IL4 ratio) toward increasing Th1 subtype activity on both sensitized animals and human mononuclear cells [36] as well as its effect on total and differential WBC count and endothelin level in blood of ovalbumin sensitized guinea pigs [37] which may confirm the immunoregulatory and anti-inflammatory effects of the plant. The effect of carvacrol, the main constituent of the plant, on tracheal responsiveness, inflammatory mediators, total and differential WBC count in blood [38], and serum cytokines and endothelin levels [39] in sensitized guinea pigs was also shown which can support the anti-inflammatory effect of *Z. multiflora*. Therefore, these results suggested an anti-inflammatory and antioxidant activity which lead to a preventive effect for *Z. multiflora* on systemic inflammation in an animal model of COPD (exposed animals to cigarette smoke).

In addition, the protective effect of the plant on inflammatory bowel disease [25], the effect of aequous and ethanolic extracts from the aerial parts of *Z. multiflora* on acute and chronic inflammation [26], and preventive effect of the plant, including total extract, flavonoid fraction, and the essential oil, on carrageenan (CAR) induced rat paw edema [55], have been demonstrated. Antioxidant [22] and immunoregulatory [23] effects of the plant have also been shown. All these studies indicate anti-inflammatory and antioxidant activity of the plant and support the findings of the present study. However, the effect of different constituents of the plant on animal model of COPD as well as the effect of the extract and its constituents on COPD patients should be examined in further studies.

As it is obvious, all aspects of inflammation could not be examined in a single study. Therefore, the effect of the extract on other cytokines, inflammatory mediators, and biomarkers such as ED-1 should be examined in further studies. In the present study, the effects of only three concentrations of the extract were examined which were chosen based on the previous studies [36–41] which were insufficient for determination of IC50 for extracts and its effective dose. Therefore, IC50 for extracts and its effective dose should be examined in further studies.

The preferred route of administration of drugs for treatment of respiratory disorders is inhalation. However, inhaler drugs should be disinfected. In addition, their particle size and flow rate should be known and their proper use should be standardized to ensure the penetration of drugs to the lung. Achievement of these characteristics for a plant extract is very difficult specially the stability, particle size, and proper use in animals. Therefore, in the present study, the extract was dissolved in drinking water of animals and administered orally similar to our previous studies [36–39, 57, 58]. However, in further studies, the effect of standardized inhaled extract should be examined. In addition, we are going to examine the effect of the extract of this plant on asthmatic and COPD patients which will try to use inhaled extract.

### Table 2: Total number and differential percentage of WBC count in an animal model of COPD treated by three concentrations of *Z. multiflora* (0.4, 0.8, and 1.6 mg/mL) and dexamethasone (50 μg/mL).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Zataria (0.4 mg/mL)</th>
<th>Zataria (0.8 mg/mL)</th>
<th>Zataria (1.6 mg/mL)</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC</td>
<td>158.86 ± 2808.33**</td>
<td>90.75 ± 1741.66**</td>
<td>62.8 ± 866.66**</td>
<td>216.86 ± 1630</td>
</tr>
<tr>
<td>Monocyte</td>
<td>13.33 ± 1.05</td>
<td>10.33 ± 1.83</td>
<td>9.1 ± 0.87</td>
<td>12.5 ± 1.86</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>36 ± 1.77</td>
<td>33.83 ± 1.28</td>
<td>33.16 ± 1.13**</td>
<td>28.66 ± 1.22</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>48.33 ± 2.18**</td>
<td>54 ± 1.39</td>
<td>67.16 ± 1.94**</td>
<td>58.5 ± 1.77</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>2.33 ± 0.33**</td>
<td>1.83 ± 0.4</td>
<td>0.5 ± 0.22**</td>
<td>0.66 ± 0.33</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. The data of total WBC is their count in one mL of blood and those of each type are the percentage of total WBC. Statistical comparisons between COPD groups treated with *Z. multiflora* and dexamethasone: **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical comparison was done using unpaired t-test. Statistical comparison between the effect of two higher concentrations of the extract (0.8 and 1.6 mg/mL) with its low concentration (0.4 mg/mL): ¶P < 0.05, ‡P < 0.01, §§P < 0.001. Statistical comparison between the effect of high concentration of the extract (1.6 mg/mL) with its medium concentration (0.8 mg/mL): †P < 0.05, ††P < 0.01, †††P < 0.001. The statistical comparison was done using ANOVA with Tukey-Kramer multiple posttest.
Due to the existence of various constituents, it is very difficult to study the pharmacokinetic of the extract globally. However, the pharmacokinetic of orally administered thymol and carvacrol showed half-lives in total digestive tract that ranged between 1.84 and 2.05 h. Both of them were almost completely absorbed in the stomach and the proximal small intestine. Plasma concentrations (sum of free and conjugated compounds) peaked at 1.39 and 1.35 for carvacrol and thymol, respectively. Both of them were almost completely absorbed in the stomach and the proximal small intestine. Plasma concentrations (sum of free and conjugated compounds) peaked at 1.39 and 1.35 for carvacrol and thymol, which was accompanied by high concentrations in urine. In addition, carvacrol and thymol were not degraded in the intestine. Plasma concentrations (sum of free and conjugated compounds) peaked at 1.39 and 1.35 for carvacrol and thymol, which was accompanied by high concentrations in urine. In addition, carvacrol and thymol were not degraded in the intestine.

In conclusion, the results of this study indicated a preventive effect of Z. multiflora on total and differential WBC, serum levels of MDA and IL-8, and weight change in an animal model of COPD which was comparable or even more potent than the effect of dexamethasone at used concentrations. Therefore, the results suggest a preventive therapeutic effect for Z. multiflora on systemic inflammation in COPD.

5. Conclusion

In conclusion, the results of this study indicated a preventive effect of Z. multiflora on total and differential WBC, serum levels of MDA and IL-8, and weight change in an animal model of COPD which was comparable or even more potent than the effect of dexamethasone at used concentrations. Therefore, the results suggest a preventive therapeutic effect for Z. multiflora on systemic inflammation in COPD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Leila Gholami Mahtaj was responsible for the performance of experiment and helped in the statistical analysis and paper preparation. Mohammad Hossein Boskabady was responsible for study design and supervision of experiments and also helped in the statistical analysis and preparation of paper.

Acknowledgments

This study was financially supported by Research Department of Mashhad University of Medical Sciences (IRAN). The results described in this paper were part of a M.S. student thesis.

References


Exploring natural plant products as an option to find new chemical entities as anticancer agents is one of the fastest growing areas of research. Recently, in the last decade, essential oils (EOs) have been under study for their use in cancer therapy and the present review is an attempt to collect and document the available studies indicating EOs and their constituents as anticancer agents. This review enlists nearly 130 studies of EOs from various plant species and their constituents that have been studied so far for their anticancer potential and these studies have been classified as \textit{in vitro} and \textit{in vivo} studies for EOs and their constituents. This review also highlights in-depth various mechanisms of action of different EOs and their constituents reported in the treatment strategies for different types of cancer. The current review indicates that EOs and their constituents act by multiple pathways and mechanisms involving apoptosis, cell cycle arrest, antimetastatic and antiangiogenic, increased levels of reactive oxygen and nitrogen species (ROS/RNS), DNA repair modulation, and others to demonstrate their antiproliferative activity in the cancer cell. The effect of EOs and their constituents on tumour suppressor proteins (p53 and Akt), transcription factors (NF-κB and AP-1), MAPK-pathway, and detoxification enzymes like SOD, catalase, glutathione peroxidase, and glutathione reductase has also been discussed.

1. Introduction

Cancer has emerged as one of the most alarming diseases in the last few decades throughout the world. It is a multifactorial disease contributing towards uncontrolled growth and invasion of the abnormal cells leading to the formation of tumour. The steep rise in the number of cancer cases may be attributed to the change in food habits, use of tobacco and alcohol, chronic infections, exposure to harmful radiations and chemicals, or more widely due to change in lifestyle and environmental pollution [1]. International Agency for Research on Cancer (IARC) reported that there are approximately 12 million cancer cases and these have accounted for 7.6 million deaths (around 13% of all deaths) in the year 2008 [2]. The recent estimates reveal that the number of new cancer cases and cancer-related deaths has increased by 11% and 79, respectively, in the year 2012 as compared to 2008 [2]. Further, the developing countries have half the number of cancer incidence cases compared to the developed countries [3]. In India, 0.979 million cancer cases were reported in the year 2010 which is expected to increase to 1.148 million by 2020 [4]. The mortality rate among cancer patients is very high. The problem is more serious in economically less developed countries due to the lack of diagnostic techniques, standard methods of treatment, and higher cost of the treatment [5]. People in scientific field are currently overcoming these problems with the use of synthetic drugs. These drugs are designed to specifically target rapidly growing and dividing cells of various tumours. But, these synthetic drugs also affect rapidly dividing normal cells in our body leading to certain other major irreversible side effects. Chemotherapy used in cancer treatment has been reported to induce multidrug resistance
tics [9]. The plant-derived products are expected to induce which are found to have wide applications in cancer therapy. Paclitaxel are classic examples of plant-derived compounds podophyllotoxin, camptothecin, irinotecan, etoposide, and reported to possess significant anticancer properties [8]. Vin-

There are more than one thousand plants which have been reported to possess anticancer properties [10, 11]. Less side effects compared to synthetic drugs. Among plant products classified as alkaloids, saponins, triterpenes, glycosides, and polyphenols among others have shown very promising anticancer properties in both in vitro and in vivo. There are more than one thousand plants which have been reported to possess significant anticancer properties [8]. Vin-
cristine, vinblastine, colchicine, ellipticine, lepachol, taxol, podophyllotoxin, camptothecin, irinotecan, etoposide, and paclitaxel are classic examples of plant-derived compounds which are found to have wide applications in cancer therapeutics [9]. The plant-derived products are expected to induce lesser side effects compared to synthetic drugs. Among plant derived compounds, essential oils (EOs) from aromatic plants have been reported to possess anticancer properties [10, 11].

EOs have also been reported to improve the quality of life of the cancer patients by lowering the level of their agony [12]. EOs-mediated therapy cannot be a substitute to the standard chemotherapy and radiotherapy but can be used in combination with cancer therapy to decrease the side effects of the drugs. Hence, EOs can be used for improving the health of the cancer patients and as a source of novel anticancer compounds. In the last two decades, a number of researches are exploring anticancer potential of EOs and their components in vitro and in vivo models. Recently, Bhalla et al. reviewed EOs as anticancer agents limiting to the recent literature and a short mechanism(s) of action [13]. However, the current review is a comprehensive one, enlisting nearly 130 studies of EOs from various plant species and their constituents that have been studied so far for their anticancer potential. The studies have been classified as in vitro and in vivo for EOs and their constituents. The current review also highlights in-depth various mechanisms of action of different EOs reported in the treatment strategies for different cancers.

2. Chemical Classification, Uses, and Therapeutic Potential of EOs and Their Constituents

EOs are the concentrated hydrophobic liquids with specific aroma produced by aromatic plants [14]. These are also called volatile oils or ethereal oils and are the secondary metabolites present in lower amounts in various plant parts. The composition and other biological properties of the EOs depend on their constituents. The constituents may be terpenes, aromatic compounds and some other compounds of various origins. The constituents of the EOs have been classified on the basis of their chemical structures. EOs are considered more potent than their constituents [15] due to their synergistic and more selective effect. In addition, EOs from plants growing in varied environments differ in their composition and hence have different uses. A general classification based on chemical structures along with examples is enlisted in Table 1.

EOs and their components are used for their specific aromas in perfumery and as flavouring agents in food products since ancient times. EOs have also been used in aromatherapy for improving the health due to sedative and stimulant properties. EOs are used for massage, bath, and inhalation as relaxants and treatment options as aromatherapies for various diseases with active ingredients that are being exploited in medicine [16]. The lipophilic nature of these EOs enables them to easily cross the membranes of the cells and reach inside the cell. EOs are described as strong antioxidants [10, 17] and antimicrobial [18] and are in use for the management of severe diseases like cardiovascular [19], diabetes [20], Alzheimer’s [21], cancer [22], and others. However, the present review focuses only on the anticancer potential of EOs and their constituents.

3. EO and Constituents as Anticancer Agents

EO is one among the most valuable plant products used in the medicine and complementary treatment strategies. Exploration of EOs and their constituents toward their beneficial role in different cancers is currently under lens. A search of PubMed (http://www.pubmed.gov/), the National Institute of Health’s online research shows 543 results for the search “cancer-essential oils” as of February 2014. Further screening of these research papers, nearly 135 correspond to anticancer properties of EO. Out of these 135 research papers, 117 have been published after the year 2005 indicating the sharp increase in number of publications in this field. EOs from different plants have been reported to have anticancer potential against mouth, breast, lung, prostate, liver cancer, colon cancer, and brain cancer and even in leukemia [23–28]. Not only EOs but their constituents like Carvacrol [29], d-limonene [30], Geraniols [31–33], Myrcene [34, 35], perillyl alcohol (POH) [36], α-humulene [37], β-caryophyllene [38], Thymol [39, 40], Citral [41], and others have also been reported to possess cytotoxic effect on the cancer cell lines and in vivo studies. Some of these like POH have gone through phase I [42] and phase II [43] clinical trials in cancer patients. Terpene analogues like Terpinen-4-ol have also been reported to have anticancer properties and induce apoptosis [44].

The current review has extensively collected and documented the available studies indicating EOs from many plants and their constituents as anticancer agents. The overall literature has been divided into different tables. Tables 2 and 3 document the in vivo and in vitro studies of EOs extracted from different plants against different cell lines along with the mechanism reported. Similarly, Table 4 documents in vivo and in vitro studies of constituents of EOs.

4. Mechanism of Action of EOs

Drugs used in cancer treatment target the cancer cell by inducing apoptosis or cell cycle arrest. Hence, natural products causing apoptosis in the cancer cells are valuable resources in cancer suppression. EOs with therapeutic potential can act by two ways—chemoprevention and cancer suppression. Various mechanisms involved in cancer treatment are
Table 1: Chemical classification, general formula, and structure of EO constituents with examples.

<table>
<thead>
<tr>
<th>Component</th>
<th>General formula</th>
<th>General structure</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terpene hydrocarbons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoterpene</td>
<td>C_{10}H_{16}</td>
<td><img src="image" alt="Monoterpene" /></td>
<td>Limonene, α-Pinene, β-Myrcene</td>
</tr>
<tr>
<td>Sesquiterpenes</td>
<td>C_{15}H_{24}</td>
<td><img src="image" alt="Sesquiterpenes" /></td>
<td>Caryophyllene, Humulene, α-Farnesene</td>
</tr>
<tr>
<td>Diterpene</td>
<td>C_{20}H_{32}</td>
<td><img src="image" alt="Diterpene" /></td>
<td>Cembrene C, Kaurene, Camphorene</td>
</tr>
<tr>
<td><strong>Oxygenated terpenes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygenated monoterpene</td>
<td>C_{10}H_{16}O</td>
<td><img src="image" alt="Oxygenated Monoterpenes" /></td>
<td>Camphor, Carveol, Limonene oxide</td>
</tr>
<tr>
<td>Oxygenated Sesquiterpenes</td>
<td>C_{15}H_{25}O</td>
<td><img src="image" alt="Oxygenated Sesquiterpenes" /></td>
<td>Caryophyllene oxide, Humulene epoxide, α-Bisabolene oxide</td>
</tr>
<tr>
<td><strong>Other oxygenated compounds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenols</td>
<td>RC_{6}H_{5}OH</td>
<td><img src="image" alt="Phenols" /></td>
<td>Catechol, Eugenol</td>
</tr>
<tr>
<td>Simple alcohols</td>
<td>R-OH</td>
<td><img src="image" alt="Simple Alcohols" /></td>
<td>Isopropyl alcohol, Butyl alcohol</td>
</tr>
<tr>
<td>Monoterpene alcohols</td>
<td>C_{10}H_{17}OH</td>
<td><img src="image" alt="Monoterpene Alcohols" /></td>
<td>Geraniol, Nerol, Eucalyptol</td>
</tr>
<tr>
<td>Sesquiterpenes alcohols</td>
<td>C_{15}H_{25}OH</td>
<td><img src="image" alt="Sesquiterpenes Alcohols" /></td>
<td>Farnesol, Nerolidol</td>
</tr>
<tr>
<td>Ketones</td>
<td>RC(=O)R'</td>
<td><img src="image" alt="Ketones" /></td>
<td>Acetophenone, Benzophenone</td>
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<tr>
<td>Esters</td>
<td>RCO_{2}R'</td>
<td><img src="image" alt="Esters" /></td>
<td>Bornyl acetate, Ethyl acetate</td>
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<tr>
<td>Lactones and coumarins</td>
<td>C_{3}H_{6}O_{3} (Lactones)</td>
<td><img src="image" alt="Lactones and Coumarins" /></td>
<td>Vernolide, Helenin</td>
</tr>
<tr>
<td></td>
<td>C_{9}H_{6}O_{2} (Coumarins)</td>
<td><img src="image" alt="Lactones and Coumarins" /></td>
<td>Fumarin, Benzofuran</td>
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</table>

activation of detoxification enzymes, modulation of DNA repair signaling, antimetastasis, and antiangiogenesis. Multiple pathways are involved in the antiproliferative activity demonstrated by the EOs in the cancer cells and EOs are even effective in reduction of tumours in animal models. Various targets of EOs for cancer prevention are represented in Figure 1. This makes EOs suitable anticancer agents with no large apparent effects being displayed on the normal cells. Attempts have been made to study various modes of inhibition of cancer cell growth by the EOs in this section.

4.1. Induction of Apoptosis. Apoptosis can occur due to effect on various signaling pathways, genetic material, and other cellular events like changes in the proteins at the intracellular
## Table 2: List of EO bearing plants studied for anticancer potential in *in vitro* models and major observations reported.

<table>
<thead>
<tr>
<th>EO bearing plants</th>
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<tr>
<td><em>Citrus limettioides</em></td>
<td>Colon cancer (SW480) cells</td>
<td>Apoptosis via caspase-3 activation and inhibition of Cox-2 and IL-6, inflammatory proteins</td>
<td>[24]</td>
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<tr>
<td><em>Pulicaria jaubertii</em></td>
<td>Human breast (MCF-7) and liver (HepG2) cancer cell lines</td>
<td>Cytotoxicity</td>
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</tr>
<tr>
<td><em>Drimys angustifolia</em></td>
<td>Human bladder carcinoma (T24) and glioblastoma (U-138 MG) cell lines</td>
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<tr>
<td><em>Boswellia carterii</em> and <em>Commiphora pyraanthoides</em></td>
<td>Human breast (MCF-7) and hepatocellular (HepG2) and cervical (HeLa), skin (HS-1) and small cell lung (A549) cancers cell lines</td>
<td>Cytotoxicity</td>
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<tr>
<td><em>Cymbopogon citratus</em> and <em>C. nardus</em></td>
<td>Human breast cancer (MCF-7) and non-tumorigenic (Vero) cell lines</td>
<td>Cytotoxicity</td>
<td>[114]</td>
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<tr>
<td><em>Tarchonanthus camphoratus</em></td>
<td>Human embryonic kidney and hepatocellular carcinoma cells</td>
<td>Cytotoxicity</td>
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<td><em>Salvia officinalis</em> L.</td>
<td>Human melanoma (A375, M14, and A2058) cell lines</td>
<td>Antiproliferative activity</td>
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<tr>
<td><em>Thymus linearis</em> and <em>T. serpyllum</em></td>
<td>Human breast (MCF-7), hormone dependent prostate carcinoma (LNCaP) and fibroblast (NIH-3T3) cell lines</td>
<td>Antiproliferative activity</td>
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<tr>
<td><em>Porcelia macrocarpa</em></td>
<td>Murine melanoma (B16F10-Nex2), human glioblastoma (U87), cervical carcinoma (HeLa), leukemia (HL-60), colon carcinoma (HCT), breast adenocarcinoma (SKBr), and melanoma (A2058); and non-tumorigenic (HFF) cell lines</td>
<td>Cytotoxicity</td>
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<tr>
<td><em>Thymus fallax</em></td>
<td>Human colorectal cancer (DLD-1) and mouse fibroblast (L929) cell lines</td>
<td>Cytotoxic to cancer but not to normal fibroblast cells</td>
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</tr>
<tr>
<td><em>Achillea wilhelmsii C. Koch</em></td>
<td>Human chronic myelogenous leukemia (K562), umbilical vein endothelial (HUVEC) and cervix carcinoma (HeLa) cell lines</td>
<td>Cytotoxic to cancer but not to normal HUVEC cells</td>
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<td><em>Ducrosia anethifolia</em> and <em>D. flabellifolia</em></td>
<td>Human cancer (K562, LS180 and MCF-7) cell lines</td>
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<tr>
<td><em>Xylopia frutescens</em></td>
<td>Ovarian adenocarcinoma (OVCAR-8), bronchoalveolar lung (NCI-H358M) and metastatic prostate carcinoma (PC-3M) cell lines</td>
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<tr>
<td><em>Annona muricata</em></td>
<td>Human breast cancer (MCF-7) cell lines</td>
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<td><em>Cedrelopsis grevei</em></td>
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<td><em>Libanotis transcaucasica</em></td>
<td>Human cervical adenocarcinoma (HeLa), colon adenocarcinoma (LS180), breast adenocarcinoma (MCF-7) and Raji (human B lymphoma) cell lines</td>
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<tr>
<td><em>Melissa officinalis</em></td>
<td>Human breast cancer (MCF-7) cell lines</td>
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<td><em>Satureja intermedia</em></td>
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<td><em>Origanum majorana</em></td>
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<td>Cytotoxic and induces DNA damage</td>
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Table 2: Continued.

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<td><strong>Guatteria pogonopus</strong></td>
<td>Ovarian adenocarcinoma (OVCAR-8), bronchoalveolar lung carcinoma (NCIH358M), and metastatic prostate carcinoma (PC-3M) cell lines</td>
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<td><strong>Pyrola herba</strong></td>
<td>Human chondrosarcoma (SW1353) cells</td>
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<td><strong>Thymus revolutus</strong></td>
<td>Liver cancer (HepG2) cells</td>
<td>Prooxidant and protective effects</td>
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<tr>
<td><strong>Origanum onites</strong> L.</td>
<td>5RP7 (c-H-ras transformed rat embryonic fibroblasts) cell lines</td>
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<tr>
<td><strong>Capparis spinosa</strong> L.</td>
<td>Human colon carcinoma (HT-29) cell line</td>
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<td><strong>Artemisia campestris</strong> and <strong>Thymelaea hirsuta</strong></td>
<td>Colon cancer (HT-29) cells</td>
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<tr>
<td><strong>Lycopus lucidus</strong> Turcz. var. hirtus Regel</td>
<td>Human liver (Bel-7402 and HepG2), breast (MDA-MB-435S and ZR-75-30), cervix (HeLa) and human renal adenocarcinoma (ACHN) cell lines</td>
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<tr>
<td><strong>Nigella sativa</strong></td>
<td>Human epithelial (Hep-2) cell lines</td>
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<tr>
<td><strong>Thymus vulgaris</strong></td>
<td>Oral cavity squamous cell carcinoma (OCSCC) cells</td>
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<tr>
<td><strong>Aniba rosaeodora</strong></td>
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<td>Apoptosis induction in selective manner</td>
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<td><strong>Boswellia sacra</strong></td>
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<td>Cytotoxic to cancer cells but not to normal cells</td>
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<td><strong>Syzygium aromaticum</strong> L.</td>
<td>Breast cancer (MCF-7 and MDA-MB-231), prostate cancer (DU-145), cervical cancer (HeLa), and Esophageal cancer (TE-13) cell lines</td>
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<td>MCF-7, P 388, and HeLa cell lines</td>
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<td><strong>Mentha spicata</strong> L., Zingiber officinale, Citrus limon Burm. f., C. paradise Macf., Jasminum grandiflorum, lavender, Matricaria chamomilla, Thymus vulgaris, Rosa damascena, and cinnamon**</td>
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<td>[28]</td>
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<tr>
<td><strong>Cinnamomum zeylanicum</strong></td>
<td>Normal rat embryonic fibroblasts (F2408) and c-H-ras transformed rat embryonic fibroblasts (5RP7) cell lines</td>
<td>Cytotoxicity and apoptosis</td>
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<td><strong>Citrus reticulate, C. aurantium, C. limon, and C. aurantium</strong></td>
<td>Ehrlich ascites carcinoma resistant to Endoxan cells</td>
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<td>EO bearing plants</td>
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<td>Reference</td>
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<tr>
<td><em>Morus rotundifolia</em> Koidz</td>
<td>African green monkey kidney (Vero) and human larynx epidermoid carcinoma (Hep2) and colon adenocarcinoma (SW620) cell lines</td>
<td>Cytotoxicity</td>
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<tr>
<td><em>Amomum tsaoko</em></td>
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<td>Cytotoxicity to cancer cells but lesser effect on normal cell line</td>
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<tr>
<td><em>Salvia pisdica</em></td>
<td>Hepatoma G2 (HepG2) and H1299 cell lines</td>
<td>Protective effect against H₂O₂ induced toxicity</td>
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<td><em>Citrus limon</em></td>
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<td><em>Rosmarinus officinalis</em></td>
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<td><em>Hibiscus cannabinus</em></td>
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<td><em>Salvia rubifolia</em> and <em>S. bracteata</em></td>
<td>Human melanoma (M14) cells</td>
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<td><em>Croton regelianus</em></td>
<td>Colon adenocarcinoma (NIH3T3 and SW-480) cells</td>
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<tr>
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<tr>
<td><em>Stachys cretica</em> ssp. vacillans</td>
<td>Breast cancer (MCF-7), melanoma (A375), and liver cancer (HepG2) cell lines</td>
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<tr>
<td><em>Cnidium officinale</em> and <em>Ligusticum chuanxion</em></td>
<td>Human cervix epithelioid carcinoma cells (HeLa) and African green monkey kidney (Vero) cell lines</td>
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<td>Chinese hamster lung fibroblast (V79 cells) cells</td>
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<td><em>Citrus reticulate</em> and <em>Pelargonium graveolens</em></td>
<td>Human promyelocytic leukemia (HL-60 and NB4) cell lines</td>
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<td><em>Boswellia</em> sp.</td>
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<td>[154]</td>
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<td><em>Casearia sylvestris</em></td>
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<td><em>Dictamnus dasycarpus</em></td>
<td>Human breast cancer (MCF-7, ZR-75-30 and MDA-MB-435S), liver carcinoma (Bel-7402 and HepG2), and renal adenocarcinoma (ACHN) cell lines</td>
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<td><em>Juniperus phoenicea</em></td>
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<td><em>Zanthoxylum rhoifolium</em> Lam</td>
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<tr>
<td><em>Thymus broussonetii</em></td>
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<td><em>Thymus sp.</em></td>
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<tr>
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</tbody>
</table>
level. Yu et al., using Bel-7402 cell line, had reported that the glutathione level in the body regulates cell proliferation [10]. A study on human melanoma cells reported that treatment of EOs induces DNA damage in cancer cells which is an indicator of apoptosis [45]. Apart from DNA damage, modification of various genes by the action of EOs is also responsible for apoptosis. Frank et al. studied the action of *Boswellia carteri* EO (frankincense oil) in bladder cancer cells and observed modulation of *CDKN1A, DEDD2, IER3, IL6, SGK, TNFAIP3, GAD45B, and NUDT2* genes involved in apoptosis [46].

EOs were also demonstrated to change expression levels of Bcl-2 and Bax genes leading to release of cytochrome C into cytosol in KB human oral epidermoid carcinoma cells [23]. This happens via activation of caspase-9 leading to caspase-3 formation which in turn cleaves target that causes apoptosis and increased phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 MAPK [23]. EO-induced apoptosis has been also suggested to be involving mitochondrial and MAPKs pathways [23]. Anti-apoptotic Bcl-2 protein is downregulated by the action of EOs on the cancer cells [47]. In mouth cancer KB cells, *Artemisia lavandulaefolia* EO has been shown to decrease Bcl-2 protein level in dose dependent manner [23], which leads to apoptosis in cancer cells that is an important strategy to control cancer development and progression.

EO constituents lead to poly(ADP-ribose) polymerase-1 (PARP) cleavage [48] which is an indicator of apoptosis [49].

### Table 2: Continued.

<table>
<thead>
<tr>
<th>EO bearing plants</th>
<th>Model system</th>
<th>Major findings/mechanism(s) reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrica gale</td>
<td>Human lung carcinoma (A549) and colon adenocarcinoma (DLD-1)</td>
<td>Cytotoxicity</td>
<td>[166]</td>
</tr>
<tr>
<td>Abies balsamea</td>
<td>MCF-7, PC-3, A549, DLD-1, M4BEU, and CT-26</td>
<td>Antitumour activity induced by ROS</td>
<td>[167]</td>
</tr>
<tr>
<td><em>Lavandula stoechas</em> ssp. <em>stoechas</em></td>
<td>Human epidermoid carcinoma (KB), human breast cancer (BCI), lung cancer (LU1), colon cancer (COL-2), drug-resistant KB (KB-V), mouse leukemia (P-388), hormone-dependent human prostate cancer (LNCaP), and rat glioma (ASK) cell lines</td>
<td>Variable cytotoxicity to all except ASK cell line</td>
<td>[168]</td>
</tr>
</tbody>
</table>

### Table 3: List of EO bearing plants studied for anticancer potential in *in vivo* models and major observations reported.

<table>
<thead>
<tr>
<th>EO bearing plants</th>
<th>In vivo models studied</th>
<th>Major findings/mechanism(s) reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pomegranate</td>
<td>Skin tumour in CD1 Mice</td>
<td>Chemopreventive effect</td>
<td>[84]</td>
</tr>
<tr>
<td><em>Cymbopogon citrates</em> STAPF</td>
<td>Female Balb/C mice</td>
<td>Anticarcinogenic activity</td>
<td>[169]</td>
</tr>
<tr>
<td><em>Croton regelianus</em></td>
<td>Sarcoma 180 murine model</td>
<td>Antitumour activity</td>
<td>[145]</td>
</tr>
<tr>
<td><em>Salvia libanotica</em></td>
<td>Mice</td>
<td>Chemoprevention against skin papillomas</td>
<td>[83]</td>
</tr>
<tr>
<td><em>Xylopia frutescens</em></td>
<td>Sarcoma 180 ascites tumour cells injected in mice</td>
<td>Tumour growth inhibition</td>
<td>[120]</td>
</tr>
<tr>
<td><em>Thymus broussonetii</em></td>
<td>DBA-2/PBl5 (H2d) mouse model</td>
<td>Tumour reduction by injection of the EO</td>
<td>[96]</td>
</tr>
<tr>
<td><em>Plectranthus amboinicus</em></td>
<td>B16F-10 melanoma cell line injected C57BL/6 mice</td>
<td>Prevention of lung metastasis</td>
<td>[170]</td>
</tr>
<tr>
<td><em>Lippia gracilis</em></td>
<td>Sarcoma 180 bearing mice</td>
<td>Tumour growth inhibition</td>
<td>[22]</td>
</tr>
<tr>
<td><em>Guatteria pogonopus</em></td>
<td>Sarcoma 180 tumour bearing Swiss mice</td>
<td>Tumour inhibition</td>
<td>[126]</td>
</tr>
<tr>
<td>Neem</td>
<td>RIII/Sa female mice</td>
<td>Tumour reduction</td>
<td>[160]</td>
</tr>
<tr>
<td><em>Curcuma zedoaria</em></td>
<td>Mice</td>
<td>Angiogenesis inhibition</td>
<td>[171]</td>
</tr>
<tr>
<td>α-Pinene from <em>Schinus terebinthifolius</em> Raddi</td>
<td>C57Bl/6 mice with B16Fi0-Nex2 induced melanoma</td>
<td>Antimetastasis</td>
<td>[77]</td>
</tr>
</tbody>
</table>
Table 4: List of EO constituents studied for anticancer potential in both *in vitro* and *in vivo* models, and major observations reported.

<table>
<thead>
<tr>
<th>Constituents used</th>
<th>Model systems used</th>
<th>Major findings/mechanism(s) reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azadirachtin and nimbolide</td>
<td>Hamster buccal pouch (HBP) carcinogenesis model</td>
<td>Cell cycle arrest and apoptosis by intrinsic and extrinsic pathway</td>
<td>[172]</td>
</tr>
<tr>
<td>Azadirachtin and nimbolide</td>
<td>Hepatocarcinoma (HepG2)</td>
<td>G0/G1 phase cell cycle and apoptosis via ROS induction and cytochrome C release in mitochondria</td>
<td>[173]</td>
</tr>
<tr>
<td>Azadirachtin and nimbolide</td>
<td>Hamster buccal pouch (HBP) carcinogenesis model</td>
<td>Chemoprevention of 7,12-dimethylbenz[a]anthracene (DMBA)-induced cancer, prevention of procarcinogen activation and oxidative DNA damage, upregulation of antioxidant and carcinogendetoxification enzymes, inhibition of tumour invasion and angiogenesis</td>
<td>[174]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>Male wistar albino rats with liver cancer induced by diethylnitrosamine (DEN)</td>
<td>Chemoprevention</td>
<td>[29]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>Human cervical cancer cell lines (HeLa and SiHa)</td>
<td>Apoptosis</td>
<td>[175]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>K-562, P-815, CEM, MCF-7 and MCF-7 gem (gemcitabine resistant)</td>
<td>Arrest in S-phase progression</td>
<td>[39]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>Liver cancer (HepG2) cell line</td>
<td>Apoptosis via activation of caspases and mitogen-activated protein kinase (MAPK) pathway</td>
<td>[176]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>Lung cancer (A549) cell line</td>
<td>Growth inhibition</td>
<td>[177]</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>Human metastatic breast cancer (MDA-MB 231) cell line</td>
<td>Apoptosis</td>
<td>[178]</td>
</tr>
<tr>
<td>Carvone</td>
<td>Primary rat neuron and neuroblastoma (N2a) cells</td>
<td>Increase in antioxidant level in primary cells with little potential in treatment of brain tumour</td>
<td>[179]</td>
</tr>
<tr>
<td>Citral</td>
<td>Breast cancer (MCF-7) cell line</td>
<td>G2/M phase arrest and apoptosis</td>
<td>[41]</td>
</tr>
<tr>
<td>Citronellal and synthetic analog C37A (N-citronellylamine)</td>
<td>Human breast cancer (MCF-7) and a non-tumorigenic (Vero) cell line</td>
<td>Cytotoxicity</td>
<td>[114]</td>
</tr>
<tr>
<td>D-limonene</td>
<td>Colon cancer (LS174T) cells</td>
<td>Apoptosis by inactivation of akt pathway</td>
<td>[180]</td>
</tr>
<tr>
<td>Elemene</td>
<td>Laryngeal cancer (Hep-2) cells</td>
<td>Growth inhibition via decrease in eIF4E, eIF4G, bFGF and VEGF</td>
<td>[181]</td>
</tr>
<tr>
<td>Elemene</td>
<td>Colon cancer (LoVo) cells</td>
<td>Inhibition of telomerase activity, cell cycle arrest, and apoptosis</td>
<td>[182]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Primary melanoma (Shc12), radial growth phase (WM3211), primary RGP, radial and vertical growth phase (WM98-1), primary RGP and VGP, Lu-metastatic melanoma (WM1205) and Female B6D2F1 mice with B16 melanomas</td>
<td>Suppresses melanoma via deregulation of the E2F1 transcription factors</td>
<td>[183]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>N-methyl-N’-nitro-N-nitrosoguanidine (MNNNG) induced gastric cancer in rat</td>
<td>Tumour reduction by suppression of NF-κB activation</td>
<td>[184]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Breast cancer (MCF-7) cells</td>
<td>Growth inhibition and apoptosis induction with decrease in levels of intracellular antioxidants</td>
<td>[184]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Androgen-insensitive prostate cancer cells (DU-145) and oral squamous carcinoma (KB) cells</td>
<td>Eugenol and its synthetic analogues inhibited growth without losing membrane integrity</td>
<td>[185]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Human colon cancer (HT-29) cells</td>
<td>Suppression of cyclooxygenase-2 activity and growth</td>
<td>[186]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Colon cancer (HCT-15 and HT-29) cells</td>
<td>Induction of ROS leading to apoptosis</td>
<td>[187]</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Human promyelocytic leukemia (HL-60) cells</td>
<td>Induction of ROS, mitochondrial permeability transition (MPT), reduction of bcl-2 level, cytochrome c release leading to apoptosis</td>
<td>[165]</td>
</tr>
<tr>
<td>Constituents used</td>
<td>Model systems used</td>
<td>Major findings/mechanism(s) reported</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>--------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Human Melanoma (G361) cell line</td>
<td>S-phase cell cycle arrest and apoptosis</td>
<td>[188]</td>
</tr>
<tr>
<td>Furanodiene</td>
<td>Liver cancer (HepG2) cell line</td>
<td>G2/M phase arrest and apoptosis via inhibition of MAPK signalling pathway</td>
<td>[189]</td>
</tr>
<tr>
<td>Geraniol</td>
<td>Human colon cancer (Caco) cell line</td>
<td>Enhances sensitivity to 5-fluorouracil treatment</td>
<td>[32]</td>
</tr>
<tr>
<td>Geraniol</td>
<td>Human colon cancer (Caco) cell line</td>
<td>Inhibits growth and Polyamine biosynthesis-mechanism of inhibition of proliferation</td>
<td>[31]</td>
</tr>
<tr>
<td>Geraniol</td>
<td>Human colon cancer (Caco) cell line</td>
<td>Membrane depolarisation, decreased activity of protein kinase C activity and p44/p42 extracellular signal-regulated protein kinases (ERK)</td>
<td>[190]</td>
</tr>
<tr>
<td>Geraniol</td>
<td>Human tumours (TC-118) induced in Swiss nu/nu mice</td>
<td>Reduction in thymidylate synthase and thymidine kinase expression, synergistic effect of geraniol with 5-fluorouracil</td>
<td>[33]</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>Murine melanoma (B16F10-Nex2), human glioblastoma (U-87 MG), cervical carcinoma (HeLa), leukemia (HL-60), colon carcinoma (HCT), breast adenocarcinoma (SKBr), and melanoma (A2058); and non-tumorigenic (HFF) cell lines</td>
<td>Cytotoxicity</td>
<td>[116]</td>
</tr>
<tr>
<td>Neem oil limonoids</td>
<td>Colon cancer (HCT116 p53−/-, HCT116 p21−/-) LNCaP, PPC1 and MDA-MB231 cell lines</td>
<td>p53 independent apoptosis autophagy</td>
<td>[191]</td>
</tr>
<tr>
<td>Nimbolide</td>
<td>Colorectal cancer (CRC) cell lines and CRC xenografts in nude mouse model</td>
<td>Apoptosis by caspase activation and PARP cleavage and decrease in tumour size in xenograft models</td>
<td>[192]</td>
</tr>
<tr>
<td>Nimbolide</td>
<td>Human hepatocarcinoma (HepG2)</td>
<td>Bcl-2, Bax, cytochrome-c, Smac/DIABLO, caspase-3, and caspase-9 activation leading to intrinsic pathway for apoptosis NF-𝜅B signaling</td>
<td>[193]</td>
</tr>
<tr>
<td>Nimbolide</td>
<td>Colon cancer (WiDr) cells</td>
<td>S-phase cell cycle arrest and caspase-mediated apoptosis</td>
<td>[194]</td>
</tr>
<tr>
<td>Patchouli alcohol</td>
<td>Human colorectal cancer (HCT 116, SW480) cells</td>
<td>NF-𝜅B, p21 activation and suppression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) resulting in apoptosis and decreased growth</td>
<td>[65]</td>
</tr>
<tr>
<td>Perillyl alcohol</td>
<td>Human colon carcinoma (HCT 116) cell line</td>
<td>Dose dependent inhibition attributed to G1 arrest</td>
<td>[70]</td>
</tr>
<tr>
<td>Perillyl alcohol</td>
<td>Female BALB/c mice</td>
<td>Tumour inhibition</td>
<td>[195]</td>
</tr>
<tr>
<td>Perillyl alcohol</td>
<td>BALB/c mice</td>
<td>UV-B induced AP-1 trans-activation inhibition and reduction of the tumours</td>
<td>[60]</td>
</tr>
<tr>
<td>Perillyl alcohol</td>
<td>Lung cancer (A549 and H520) cell lines</td>
<td>Cell cycle arrest and apoptosis</td>
<td>[196]</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>Murine mesothelioma (AE17), melanoma cells (B16-F10), and fibroblasts (L929)</td>
<td>Necrotic cell death and apoptosis to lower extent in cancer cells</td>
<td>[197]</td>
</tr>
<tr>
<td>Thymol</td>
<td>Human promyelocytic leukemia (HL-60) cells</td>
<td>Caspase-dependent and independent apoptosis</td>
<td>[40]</td>
</tr>
<tr>
<td>Thymol</td>
<td>Human liver cancer (Bel-7402) cells</td>
<td>Antiproliferative activity</td>
<td>[198]</td>
</tr>
<tr>
<td>Thymol</td>
<td>K-562, P-815, CEM, MCF-7, and MCF-7 gem</td>
<td>Prevention of G0/G1 phase transition</td>
<td>[39]</td>
</tr>
<tr>
<td>Thymoquinone</td>
<td>Human colon cancer (LoVo, HCT 116, Caco-2, HT-29 and DLD-1) and human intestinal (FHS24Int) cells</td>
<td>ROS generation and mitogen-activated protein kinases (MAPK) JNK and ERK activation in cancer cells leading to apoptosis</td>
<td>[199]</td>
</tr>
<tr>
<td>Trans-caryophyllene</td>
<td>Breast cancer (MCF-7), colon cancer (HCT-116) and murine macrophage (RAW264.7) cell lines</td>
<td>Cytotoxicity</td>
<td>[134]</td>
</tr>
</tbody>
</table>
Table 4: Continued.

<table>
<thead>
<tr>
<th>Constituents used</th>
<th>Model systems used</th>
<th>Major findings/mechanism(s) reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-humulene</td>
<td>Breast cancer (MCF-7), colon cancer (HCT-116) and murine macrophage (RAW264.7) cell lines</td>
<td>Cytotoxicity</td>
<td>[134]</td>
</tr>
<tr>
<td>α-santalol</td>
<td>Breast cancer (MCF-7 and MDA-MB-231) cells</td>
<td>G2/M phase cell cycle arrest and apoptosis with little effect on normal breast cells</td>
<td>[200]</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>Human tumour (MCF-7, DLD-1 and L-929) cell lines</td>
<td>Enhanced activity of α-humulene, isocaryophyllene, and paclitaxel</td>
<td>[38]</td>
</tr>
<tr>
<td>β-Caryophyllene oxide</td>
<td>Human prostate (PC-3) and breast cancer (MCF-7) cells</td>
<td>ROS generation and PI3K/AKT/mTOR/S6K1 signaling activation leading to apoptosis</td>
<td>[201]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>Lung cancer (H460 and A549) cells</td>
<td>G2-M phase cell cycle arrest</td>
<td>[71]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>Melanoma (B16F10) cells</td>
<td>Inhibition of angiogenesis via VEGF factor, antiproliferative and antimetastatic activity</td>
<td>[73]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>Human breast cancer (MCF-7/ADM) cell line</td>
<td>Enhancement of adriamycin effect at its nontoxic concentration</td>
<td>[202]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>G-422 tumour cells in mice</td>
<td>Cross blood brain barrier and inhibit brain carcinomas</td>
<td>[203]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>Lung cancer (A549) cells</td>
<td>PI3K/Akt/mTOR/p70S6K1 signaling pathway inhibition and induces autophagy</td>
<td>[204]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>Human ovarian cancer (A2780 and A2780/CP) cell lines</td>
<td>G2-M cell cycle arrests, cyclin B1 and Cdc2 downregulation and elevation of p53, p21waf1/cip1, p27kip1 and Gadd45 levels</td>
<td>[205]</td>
</tr>
<tr>
<td>β-elemene</td>
<td>Liver cancer (HepG2) cells</td>
<td>G2-M phase cell cycle arrest and apoptosis</td>
<td>[206]</td>
</tr>
<tr>
<td>γ-humulene</td>
<td>Colorectal cancer (HT29) cells</td>
<td>Apoptosis via upregulation of the CD95 receptor and CD95L on cell surface</td>
<td>[207]</td>
</tr>
</tbody>
</table>

Cancer cell (elevated ROS/RNS)

EOs

EOs

Cancer cell proliferation

DNA repair mechanisms

Angiogenesis

MDR

Possible route for EOs-mediated cancer cell death

Figure 1: Multitargeted role of Essential oils (EOs) towards cancer prevention. The EOs-mediated anticancer strategies identified so far include cell cycle arrest, apoptosis, and DNA repair mechanisms. EO reduces cancer cell proliferation, metastasis, and MDR which make them potential candidates toward adjuvant anticancer therapeutic agents.

Major compounds of Salvia libanotica EO like linalyl acetate, terpineol, and camphor have been reported to be very effective against cancer. Synergistic activity of these compounds resulted in the antiproliferative effect on the isogenic colon cancer cell lines HCT-116 (p53+/+ and p53−/−) while no such effect was observed on normal intestinal cell line under similar conditions [48]. Further, Itani et al. also concluded that, in p53+/+ cells, cancer cell death occurs via mitochondrial-mediated caspase dependent pathway while in the other cells, it occurs via caspase-independent way [48]. PARP-1 protein has been shown to be modulated by the EOs and their constituents [23]. Inactivation of PARP results due to the activity of caspases leading to cancer cell death in response to treatment with EOs and their constituents. In a study, Artemisia lavandulaefolia EO and its major compound 1,8-cineole have been shown to adopt the above route for mitochondrial and MAPKs pathways resulting in apoptosis in the mouth cancer, KB cells [23]. EO of Boswellia sacra has also been reported to induce PARP cleavage in MDA-MB-231 cells [50]. Some of the mechanisms leading to apoptosis are summarised below.

4.1.1 Increase in the ROS Levels. ROS are generated inside the cells in response to external stimuli or stress under normal conditions. Enhanced ROS levels in the abnormal cells instigate the cells to undergo apoptosis. Such response in the cancer cell on treatment with EO has been observed as an effective treatment method. EOs from Aniba rosaeodora (rosewood) were reported to induce apoptosis by increasing ROS production [51]. Similar effect has been observed by the EO of Zanthoxylum schinifolium in liver (HepG2) cancer cells which leads to apoptosis [52]. Decreased levels of cellular antioxidants like glutathione [53] and increased ROS production
are the most commonly encountered phenomenon in cancer cells in response to the treatment with EOs that lead to cell death.

4.1.2. Effect on Akt. Akt is an important protein which also regulates p53, a tumour suppressor protein. Boswellia sacra oil influences the Akt protein expression [50]. Vapor of Litsea cubeba seed oil suppressed mTOR and pPDK1 leading to dephosphorylation of Akt protein at serum (Ser<sup>473</sup>) and threonine (Thr<sup>308</sup>), respectively, activating various caspases (caspase 3 and caspase 9) which caused programmed cell death in lung cancer cells [54]. They also reported that the cell cycle gets arrested in the lung cancer cells due to overexpression of p21 resulting from the deactivation of mdm2 due to dephosphorylated Akt protein. Further increased binding of the p21 to cyclins inhibited G<sub>1</sub> - S phase transition [54].

4.1.3. Effect on NF-κB. Nuclear factor, NF-κB, is a transcription factor (TF) that gets activated in growth of the tumour cells [55]. Thus, it serves as a potential target for developing anticancer drugs and blocking of this TF advocates towards anticancer activity of the natural compounds. α-terpineol have been reported to target NF-κB and downregulates its related genes such as IL-1β, IL1RI, IFNG, ITK, and EGFR [56]. Linalyl acetate and α-terpineol monoterpenes act synergistically and inhibit the expression of NF-κB leading to cell death of colon cancer cells [57]. Human leukaemia cell line (HL-60) treated with EO of Cymbopogon flexuosus and its major constituent isointermedeol has been reported to lower NF-κB which is one of the contributing multiple pathways resulting in apoptosis [58]. EO of Artemisia capillaries leads to NF-κB-DNA binding activation at the concentration above 0.5 μL/mL, leading to apoptosis in the mouth cancer KB cells [47].

4.1.4. Effect on AP-1. Activator protein-1 (AP-1) is another TF which plays vital role in different processes like differentiation, proliferation, transformation, and apoptosis of the cells. Its activity is regulated by MAPK proteins which are also affected by EO treatment in cancer cells [47]. Dietary intake of POH results in decreasing the tumours induced by Azoxymethane- (AOM-) induced colon cancer [59]. It prevents the skin cancer induced by UV-B radiations [60] by activation of AP-1. DNA binding activity of AP-1 increases up on effective treatment of Artemisia capillaries EO resulting in apoptosis in mouth cancer cells [47]. AP-1 thus is affected by the EO treatment and its activation mediate apoptosis in the cancer cells.

4.1.5. MAPK-Pathway. MAP kinases get activated in response to oxidative stress in the cells [61, 62]. Various MAPKs like JNK, ERK, and p38 kinase are the signaling molecules of MAPK pathway involved in the apoptosis in cancer cell. EOs mediated apoptosis involves phosphorylated MAPK forms in the cells [62]. These forms increase with time of exposure to the EO of Artemisia capillaris in mouth cancer cells [47].

4.2. Cell Cycle Arrest. Mammalian cells have different cell cycle phases (G<sub>1</sub>, S, G<sub>2</sub>, and metaphase) to complete their life cycle. Fidelity of the cell cycle is lost due to the lack of response to the negative regulators of cell cycle progression in the cancer cells leading to uncontrolled cell division [63]. Regulation of the genes involved in this process is also hampered. Thus, halting any cell cycle event in the cancer cell leads to prevention of their growth and division, a widely employed therapeutic strategy [64]. Various cell cycle checkpoints act as potential targets for cancer treatment. Patchouli alcohol which is an important component of Pogostemon cablin EO has been reported to upregulate p21 expression and suppress cyclin D1 and cyclin-dependent kinase 4 (CDK4) expression in colorectal cancer cells with increase in dose [65]. As p21 is negative regulator of G<sub>1</sub> phase transition, increased expression of this protein by the action of patchouli alcohol is indicative of cell cycle inhibition [65]. Similar arresting of the G<sub>1</sub> transition has also been reported in different types of cancer in response to various other EOs [66, 67]. EOs of Curcuma wenyujin inhibit S/G<sub>2</sub> phase transition leading to cancer cell death [68]. G<sub>2</sub>/M phase transition has been reported on the treatment of liver tumour (J-5) cells with diallyl trisulfide, garlic EO constituents [69]. Various constituents like geraniol, thymol, and carvacrol of EOs inhibit different phases of cell cycle [39, 70–72]. Monoterpenes act by altering the expression of cell cycle. Genes like DDIT3, IL8, and CDKNIA causing cell cycle arrest have been reported to be upregulated by frankincense oil [46]. Therefore, EOs and their constituents serve as effective anticancer substances by targeting cell cycle progression in cancer cells.

4.3. Antimetastatic and Antiangiogenic. Angiogenesis is a process that occurs in the tumours, which helps them to survive and proliferate. Inhibition of this process stops the supply of required nutrients to the cancer cell and is an efficient way to control cancer. Certain anticancer drugs target cancer cell by this way. EO of Curcuma zedoaria has been tested in vitro and in vivo for antiangiogenic effect and it was reported to exhibit antiproliferative activity against various cancer cell lines and also suppressed melanoma growth and lung metastasis in mice [73]. This action was reported to be attributed towards downregulation of matrix metalloproteinases (MMP) [73]. POH which is one of the components of many EOs has been reported as the angiogenesis inhibitor molecule [74]. EO from Citrus sinensis has been reported to inhibit angiogenesis and metastasis in colon cancer cells [75]. Inhibition of vascular endothelial growth factor (VEGF) which plays an important role in angiogenesis is the key indicator of antiangiogenic behaviour displayed by the EOs [75]. In addition, downregulation of matrix metalloproteinase-6 (MMP-6) by the Citrus sinensis EO in a dose dependent manner and blockage of vascular endothelial growth factor receptor 1 (VEGFR1) also confirmed the role of EO in inhibition of metastasis in colon cancer [75]. Limonene and perillic acid are the antimetastatic molecules which are well studied in mice [76]. α-Pinene isolated from the EO of Schinus terebinthifolius also had antimetastatic activity in the C57Bl/6 mice with melanomas [77]. As both these processes are the most harmful and unique properties of the cancer cells, targeting these can prevent spreading of cancer to the other parts and inhibit proliferation of the localised tumours. Efficacy of
the EOs in inhibiting these processes will enable potential treatment strategies for cancer therapy.

4.4. Effect on Detoxification Enzymes. Genotoxins lead to alteration of the internal antioxidants and antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) along with alteration of various important body functions resulting in damage to tissues and membranes. Phase I and phase II detoxification enzymes are responsible for the degradation of the harmful compounds. Certain compounds of the EOs act as inducer of these detoxification enzymes and thus prevent the induced-toxicity and even cancer in the cell line models. Citral is the example of one such compound which increases the activity of a key phase II detoxification enzyme—glutathione-S-transferase [78]. Dietary intake of (POH) also plays a role in the prevention of carcinogenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [79].

EOs have been reported to have preventive effect on cancer treatment [80]. Varying concentrations of Allium sativum (garlic) EO were administered to mouse having diethylnitrosamine induced gastric cancer with basic diet and this EO affected phase I enzymes, SOD, CAT, and GPx activities. EO of Allium sativum has also been reported to be efficient in gastric cancer in mouse model [81]. EOs induce phase I and phase II enzymes which prevent the interaction of carcinogens with DNA. This results in chemopreventive effect of the EOs [15, 81].

EO of holy basil prevents fibrosarcoma tumours induced by 20-methylcholanthrene in Swiss albino mice thighs by increasing the level of endogenous antioxidants [82]. Ocimum sanctum EO affects enzymes, namely SOD, CAT, and GST, and increases the levels of reduced glutathione, a nonenzymatic antioxidant which is responsible for decrease in the size of tumour and its incidence in the mice with the induced toxicity [82]. Salvia libanotica EO has potential to prevent the proliferation of skin papillomas induced by 7,12 dimethylbenz[a]anthracene (DMBA) and 12-0-tetradecanoylphorbol-13-acetate (TPA) in mice [83]. Pomegranate seed oil has ability to inhibit TPA induced skin cancer in the mice [84].

Antioxidant activity of EOs has been reported to be helpful in the scavenging of free radicals generated in the diseased state, leading to the cancer prevention. Wedelia chinensis EO has high antioxidant potential which was evaluated in lung cancer cell line implanted in C57BL/6 mice [85]. Increase in the activity of antioxidant enzymes like CAT, SOD, and GPx along with increased level of glutathione was observed in the mice model, showing the preventive effect of these EOs even in the in vivo models [85]. A proposed overall mechanism by which EOs display anticancer activity is presented in Figure 2.

4.5. Modulation of DNA Damage and Repair Signaling by EOs. Increased ROS production (as discussed above) results in DNA damage and can lead to the cell death. EOs have potential to induce damages at the DNA level that drives the cancer cells towards cell death. This activity is especially harmful in cancer cells, while no such damage is encountered in the normal cells; this provides added advantage of using these EOs. Targeting DNA repair pathways is an effective treatment method currently in use in the cancer to encounter the high proliferation rate in the cancer cells [86, 87].

One of the peculiar properties of the EOs is that though being cytotoxic to cancer cells, these induce proliferation of the normal cells [88]. DNA repair potential is present in various EOs and their constituents. Cells pretreated with the compounds like linalool, myrcene, and eucalyptol were studied for repair activity by their recovery on the normal media and it was found that these can reduce the damage caused by hydrogen peroxide (H2O2), a potential genotoxin, but their coadministration is not that beneficial [34]. Effect of the monoterpenes was dependent on the concentrations used and these had themselves induced breaks in DNA at higher concentrations [34]. Therefore, their dose response studies are important from therapeutic point of view. Camphor and thujone [89] are other monoterpenes reported to mediate via DNA repair process in the cells with induced toxicity and also known as antimutagenic in mammalian cells [89]. Thymus species EO was comparatively nontoxic to the normal fibroblast cells than MCF-7 and LNCAp human cancer cell lines [90]. IC50 values of Tetraclinis articulata EO on blood lymphocytes were reported almost double than for different cancer cells [91].

On the other hand, targeting the DNA repair pathways is helpful in cancer therapy as cells become reluctant to chemotherapy. Downregulation of the repair genes by the EOs can prove to be effective treatment strategy towards targeting DNA repair processes. Genes like H2AFX and HDAC4 are responsible for DNA repair and cell cycle progression and were found to be suppressed by frankincense oil in human bladder cancer (J82) cells using microarray analysis [46]. Therefore, EOs inhibit the cancer cell progression and thereby showing anticancer properties.

More specifically, the DNA polymerases are the enzymes involved in DNA repair and replication (DNA polymerases α, δ, and ε). These have been reported to be very effective targets in the development of drugs for cancer treatment. EOs inhibit the activity of the DNA polymerases [II] and therefore can be used as chemotherapeutic agents in cancer treatment. Chamomile EO was found to be very strong mammalian polymerase (λ and α) inhibitor among many other EOs tested which account for their increased therapeutic potential against cancer [II]. As polymerase α is a DNA replicative polymerase and polymerase λ is a DNA repair/recombination polymerase, hence inhibition of both these polymerases will be helpful in cancer therapeutics [II].

The important DNA damage signaling protein, namely, PARP-1, is most abundantly found nuclear protein almost in all eukaryotes other than yeast. It is the first protein to act on the damaged DNA (single strand DNA and double strand DNA breaks) and initiates the DNA repair by the process of PARsylation and recruiting other DNA repair proteins associated with Base Excision Repair (BER) [86, 92] and non-homologous end joining (NHEJ) [93]. Many EOs and their constituents lead to PARP cleavage [68]. Proteolytic cleavage of PARP-1 by the action of EOs might be indicative of modification of the DNA repair process in the cancer cells.
Figure 2: EOs and their constituents target multiple pathways in cancer cells. EOs by virtue have cell membrane permeability and act on different cellular targets involved in various pathways. EOs increase intracellular ROS/RNS levels which results in apoptosis in cancer cells. Inhibition of Akt, mTOR, and MAPK pathways at different steps by EOs leads to corresponding up-/downregulation of various key biomolecules (and corresponding genes which are not shown in the figure). Alteration in expression of NF-κB by EOs and further binding of NF-κB to DNA result in apoptosis in cancer cells. Dephosphorylation of Akt by the action of EOs results in overexpression of p21, which either induces apoptosis by increasing caspases level or results in cell cycle arrest by binding to cyclins. In addition, EOs-induced mitochondrial stress leads to activation of Bcl-2 and membrane depolarisation resulting in enhanced release of cytochrome-C to the cytoplasm which induces apoptotic cell death in cancer cells. EOs also modulate DNA repair mechanisms by acting as DNA polymerase inhibitors and lead to PARP cleavage which also results in apoptosis in cancer cells.

More elaborative studies are still required in the determination of the role of EOs in modulation of different repair pathways like BER and NHEJ in cancer prevention.

5. Multidrug Resistance (MDR) in Cancer: A Potential Set Back

Multidrug resistance (MDR) is the most frequently encountered problem in the cancer patients, which makes most of the routinely used anticancer drugs ineffective [7, 94]. Lots of research are oriented on circumventing this problem. This arises due to different mechanisms like induction of repair of the damaged DNA in response to drug, change in drug uptake capability, and change in the level and response of the targeted enzymes. Adenosine triphosphate cassette (ABC)-transporter family proteins confer MDR due to their increased activity [95]. EOs can circumvent the reluctance of tumours to respond to the cytotoxic drugs [96]. EOs of thyme are effective against widely used drugs like Adriamycin, Vincristine, and Cisplatin resistant ovarian cancer cell lines and, in addition, tumour size reduction was also observed in vivo which indicates the efficacy of the EO in mammalian system [96, 97]. Juniperus excels EO was effective against MDR P-glycoprotein-expressing CEM/ADR5000 leukemia cells and reversed their resistance indicating the use of EO in MDR treatment in cancer [98]. Melaleuca alternifolia,
tea tree oil, can ameliorate Adriamycin resistance in human melanoma cells and terpinen-1-ol is responsible for this activity [99]. Various EO constituents are reported as the anti-MDR molecules and are summarized below.

Linalool, monoterpenic alcohol, is a constituent of many EOs and is reported to increase the therapeutic potential of Doxorubicin in breast cancer cells MCF-7 (adriamycin resistant) by increasing its accumulation in these cells for effective response [100]. It has also been reported to cause membrane damage in the Epirubicin-resistant lung cancer, H1299 cells [101]. Emergence of Dox resistance is the other most widely encountered chemotherapeutic hurdle [102] in the treatment of cancer patients. Thymoquinone (TQ) is the constituent of Nigella sativa and various other EOs have been found to prevent Dox induced resistance in breast cancer (MCF-7/Dox) cells. It inhibits their growth, induces apoptosis by up-regulation of phosphatase and tensin homolog (PTEN), leading to downregulation of Akt cell survival protein, and causes cell cycle arrest at G1/M phase [103]. Use of the EOs as dietary supplements and coadministration with drugs can enhance the response to the treatment. However, limited studies are available in this aspect but EOs and their active constituents are the promising avenues for combating MDR in cancer patients. Hence, some EOs can be used as combinational therapy in cancer patients due to their beneficial effects after in-depth research on the capability to overcome MDR.

6. Prevention of Side Effects of Cancer Treatment

Cancer patients suffer from different side effects which can be preferentially reduced by alternative methods. EOs are used in the aromatherapy for reducing the agony of brain cancer patients [104]. EO is efficient in depression and reduction of anxiety in cancer patients [105]. Cancer patients undergoing chemotherapy, one of the most frequently used treatment method in cancer, are prone to various side effects [106]. These are nausea and vomiting. Mentha spicata and M. piperita have been found to be effective in overcoming these emetic conditions (chemotherapy-induced nausea and vomiting, CINV) along with the reduction of expenditure on treatment in the cancer patients undergoing chemotherapy [107]. EOs of Leptospermum scoparium and Kunzea ericoides were reported to prevent mucositis in the head and neck cancer patients undergoing radiotherapy when used in the preparation of mouthwash [108]. Some cancer patients having metastatic tumorigenic ulcers of skin develop necrosis and malodour [109]. Patients suffering from such malodour were reported to have improvement in their state on treatment of these ulcers with the mixture of EOs having eucalyptus, melaleuca, lemongrass, lemon, clove leaf, and thyme on a 40% ethanol base [110]. Lavender EO is widely used in aromatherapy and is found to be beneficial in reducing the distress in cancer patients [111]. Hence, EOs serve as the valuable preparations in amelioration of the side effects and sufferings of the cancer patients.

7. Conclusions and Future Perspectives

EOs have been used in medicine from the ancient times and the present review is an attempt to highlight their therapeutic and chemopreventive value with major emphasis on the mechanistic approaches. Main aim of summarizing the research in this area is to provide better understanding of various pathways and mode of action of different EOs. EO constituents are potent in cancer prevention and treatment. Novel potent anticancer molecules can be found in EOs which can further be exploited in therapeutics. EOs can efficiently be exploited in pharmaceutical preparations with more research and some of them are already in the different phases of clinical trials. EOs are more effective in the preliminary studies than the individual constituents. Further, EOs and their constituents can be evaluated as therapeutic agents and can be used in complementation to standard therapies. Research on EOs as anticancer therapeutic agents is still in growing stage and immense potential of the EOs needs to be explored due to the lack of target specific release. Further, studies including clinical trials are required along with the use of advanced techniques for the targeted organ-specific release of the EOs for making the treatment more effective.

Abbreviations

NCBI: National Center for Biotechnology Information
IARC: International Agency for Research on Cancer
WHO: World Health Organisation
EOs: Essential oils
DNA: Deoxyribonucleic acid
PubMed: Publisher Medline
POH: Perillyl alcohol
ERK: Extracellular signal-regulated kinase
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
AP-1: Activator protein-1
CDK4: Cyclin-dependent kinase 4
VEGFR: Vascular endothelial growth factor receptor
VEGF: Vascular endothelial growth factor
MMP: Matrix Metalloproteases
MAPK: Mitogen-activated protein kinases
PARP: Poly(ADP-Ribose) polymerase
ROS: Reactive oxygen species
SOD: Superoxide dismutase
CAT: Catalase
GPx: Glutathione peroxidase
GR: Glutathione reductase
GST: Glutathione-S-transferase
DMBA: Dimethylbenz[A]anthracene
mTOR: Mammalian target of rapamycin
Mdm: Mouse double minute 2 homolog
p21: CDK-interacting protein 1
TPA: 12-0-Tetradecanoylphorbol-13-acetate
TPA: 12-0-Tetradecanoylphorbol-13-acetate
Hydrogen peroxide

Base excision repair

Nonhomologous end joining

Multidrug resistance

Adenosine Triphosphate Cassette (ABC)-Transporter

Phosphatase and tensin homolog

Thymoquinone.

**Conflict of Interests**

The authors have declared that no conflict of interests exists.

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This study aims to assess and compare copaiba oleoresin of *Copaifera multijuga* and 0.5% silver nitrate for the induction of pleurodesis in an experimental model. Ninety-six male Wistar rats were divided into three groups: control (0.9% saline solution), copaiba (copaiba oil), and silver nitrate (0.5% silver nitrate). The substances were injected into the right pleural cavity and the alterations were observed macroscopically and microscopically at 24, 48, 72, and 504 h. The value of macroscopic alterations grade and acute inflammatory reaction grade means was higher in the 24 h copaiba group in relation to silver nitrate. Fibrosis and neovascularization mean in the visceral pleura were higher in 504 h copaiba group in relation to the silver nitrate group. The grade of the alveolar edema mean was higher in the silver nitrate group in relation to the copaiba group, in which this alteration was not observed. The presence of bronchopneumonia was higher in the 24 h silver nitrate group (n = 4) in relation to the copaiba group (n = 0). In conclusion, both groups promoted pleurodesis, with better results in copaiba group and the silver nitrate group presented greater aggression to the pulmonary parenchyma.

1. Introduction

Pleurodesis is a well-established treatment for patients with spontaneous pneumothorax or malignant pleural effusion of multiple etiologies. The objective of pleurodesis is to promote the symphysis of the parietal and visceral pleura by means of induction of fibrosis with the consequent obliteration of the pleural space to prevent the accumulation of either air or fluid in the pleural cavity [1–3].

The ideal agent for pleurodesis has not yet been found. Several substances have been studied both clinically and experimentally, such as ethanolamine oleate, diazepam, erythromycin, macrolides, quinolones, povidone-iodine, and quinacrine, among others [4–7]. All of these studies intend to find an agent with the following characteristics: (1) efficient, (2) widely available, (3) of low cost, and (4) of few side effects. Today, the most commonly used agents are talc, instilled in the pleural cavity by either aspersion or dilution in saline solution, tetracycline derivatives (minocycline and doxycycline), and bleomycin [8]. In the 1980s, tetracycline was the preferred agent for chemical pleurodesis. However, due to cessation of manufacturing, it is no longer available for such an application. Bleomycin has a very high cost and is less efficient in pleurodesis when compared to other medications [9].
Silver nitrate is a substance with irritating, caustic, astrin- gent, and disinfectant properties; it is of low cost and is easy to handle and sterilize and instill in the pleural cavity. Its use for pleurodesis has been increasing, especially after its utilization at smaller concentrations, which has decreased the painful side effects [10]. However, these side effects, in cases that required larger amounts, boost new research in the search for new therapeutic alternatives for inducing pleurodesis.

The Amazon is a main reserve of natural products on the planet, and its population makes wide empirical use of the medicinal properties of these substances due to distrust and the high cost of industrialized medications. Copaiba oil, one of the most popularly used natural products, is known for its antimicrobial, anticancer, antiseptic, and healing effects, among others [11–23]. This oil is composed exclusively of diterpenes and sesquiterpenes, which are naturally produced substances with several biological activities already established in the literature [24, 25].

The effect of copaiba oil injected into the peritoneal and pleural cavities of rats, contrary to what was expected, produced an intense, universal inflammatory reaction with the formation of firm adherences associated with the thickening of the serosas [26, 27]. Based on these findings, we intend to use copaiba oil as an irritating substance, with a possible effect for the induction of pleurodesis.

This study aims to assess and compare copaiba oleoresin of Copaifera multijuga and 0.5% silver nitrate for the induction of pleurodesis in an experimental model.

2. Material and Methods

In an experimental, randomized study, 96 adult, male Wistar rats (Rattus norvegicus) weighing on average 200 g, of the same lineage, were used, from the Instituto Nacional de Pesquisas da Amazônia (INPA) Bioterium. The study was approved by the Ethics Committee for Animal Experimentation of Federal University of Amazonas in September 16, 2008.

The rats were divided into three groups: control (0.9% saline solution), copaiba (pure copaiba oil), and 0.5% silver nitrate, in which each group was composed of 32 animals. After the instillation of the substances in the pleural cavity, each group was further divided into four subgroups of eight rats according to the time of sacrifice, at 24, 48, 72, and 504 h (21 days).

The copaiba oil was extracted from the trunk of a tree from the Copaifera multijuga species located at Forest Reserve Ducke, Manaus, AM, Brazil. The main constituents of the oil were as previously reported in oil C21-1 in Barbosa, Medeiros [28]. Sesquiterpenes were identified by relative retention index, mass spectrum, and comparison with standards. Diterpenes were isolated and NMR data was compared with the literature [28]. The 0.5% silver nitrate and 0.9% sterile saline solution with an electrolyte content of sodium and chloride of 154 mEq/l were obtained commercially.

A volume of 0.4 mL of 0.5% silver nitrate or 0.2 mL of copaiba oil or 1 mL of saline solution was injected into the pleural cavity of the animals. After the rats were weighed, trichotomy was performed at the incision site and, under subcutaneous anesthesia with chloral hydrate (0.2 to 0.3 mL), a 5 mm incision was made, preceded by local asepsis with topical povidone-iodine, in the subxiphoid region (Figure 1(a)). The substances were injected with an epidural anesthesia needle using the transdiaphragmatic approach, in the right pleural cavity (Figure 1(b)). It was determined that rats, with weight less than 200 g, would receive injection of 0.35 mL of substances and over 200 g rats would receive injection of 0.4 mL. This dosage was based on the weight proportion of an average male adult human of 70 kg and an average male of 198.9 g, with this result multiplied by two, due to double pleural cavity of this animal.

At the preestablished time periods (24, 48, 72, and 504 h), the animals were euthanized through the administration of a toxic dose of chloral hydrate (0.8 mL/100 g) in order to induce cardiorespiratory arrest.

Immediately after the sacrifice, a median incision of the thorax and abdomen was performed to observe the macroscopic alterations that were classified as described by Tonietto et al. [29], according to the modification of the pleural cavity and pulmonary parenchyma, in the following grades:

- grade 0: no macroscopic alteration;
- grade 1: presence of exudate, no fibrin reaction or adherences;
- grade 2: presence of exudate, with the presence of mild adherences;
- grade 3: presence of exudate, with the presence of firm adherences;
- grade 4: absence of exudate, incarcerated lung.

After macroscopic analysis, the right lung and the parietal pleura of the right anterolateral chest wall were removed and placed in recipients containing an adequate volume of 10% buffered formalin and randomly numbered from “1” to “96.” After fixation, a longitudinal cut of the right lung was performed with the removal of a longitudinal segment of the anterior chest wall, approximately 1.5 cm long and 0.4 cm wide, containing soft parts, cartilaginous costal segments, and parietal pleura. These samples were submitted to automated histological processing and put in paraffin blocks, at the Pathology laboratory at Incor. In the histological analysis, 4 μm thick sections were stained using the hematoxylin-eosin method and analyzed by a pathologist who was blinded to the medication previously injected. The histological alterations observed were considered separately in relation to the visceral pleura, parietal pleura, and pulmonary parenchyma and according to the components of the inflammatory process they were classified as acute and chronic. Thus, acute inflammation was described by predominance of edema, fibrin deposition, associated or not with inflammatory infiltrate, composed predominantly of neutrophils. In chronic inflammation, there was a predominance of fibroblastic proliferation, vascular neoformation, and presence of infiltrate...
composed predominantly of mononuclear cells and fibrosis, indicated by collagen matrix deposition. These factors were semiquantified both together and separately, and scores were then attributed to them as follows: “0” for the absence of the referred alteration, “1” for alterations of low intensity, “2” for alterations of moderate intensity, and “3” for intense alterations, according to the methodology established by Hurewitz et al. [30].

Qualitative data was obtained by calculating the simple and relative absolute frequencies. Regarding the quantitative data, the mean and the standard deviation (SD) were calculated when there was normal distribution of data, and median, first ($Q_1$) and third quartile ($Q_3$) when the distribution of data was not normal. Fisher’s exact test was used in the association analysis, whereas Student’s $t$-test was used to compare the means of the grades of the macroscopic and microscopic alterations, since there was a normal distribution of data. Kruskal-Wallis’ nonparametric test was used to compare the weight difference. The software used in the analysis was Epi-Info 3.4.3 for Windows developed and distributed by CDC (http://wwwn.cdc.gov/epiinfo/), and the level of significance used in the tests was 5%.

3. Results

The injection of substances in the animals did not produce symptoms and did not require analgesia, and there were no deaths during the experiment. The variation in weight measured before and after the experiment showed no relation to the substance injected. The mean of weight variations in the animals after intrapleural instillation was $-11.48 \pm 15.00$ mg, $-18.05 \pm 17.85$ mg, $-31.38 \pm 25.79$ mg, and $61.82 \pm 56.15$ mg after 24, 48, 72, and 504 h, respectively.

Under macroscopic and microscopic evaluations, all animals of the control group were of grade 0. No significant changes in macroscopic or microscopic evaluations were found in the control group. Greater macroscopic inflammatory reactions were observed in the rats that received copaiba oil, in all the times of sacrifice, when compared to the silver nitrate group, with statistical significance in the 24-hour group ($P < 0.05$) (Figure 2).

Macroscopic and microscopic changes related to the process of inflammation and acute and chronic fibrosis, both in the parietal and visceral pleura, in different times, are detailed in Figure 3. It demonstrates that copaiba oil presents more obvious macroscopic changes every time, with microscopic correspondence at 72 h and 504 h. It is observed that at times 24 h and 48 h there was no development of fibrosis in both groups, which was more evident at times 72 h and 504 h (Figure 3).

Regarding the analysis of the acute inflammatory reaction of the parietal pleura, there was a more intense reaction in the copaiba group at 24 and 72 h ($P = 0.01$ and 0.008, resp.), when compared to the silver nitrate group, as presented in Table 1. In visceral pleura, regarding the same reaction, we observed a greater inflammation effect in the copaiba group at 24 and 72 h, however, without statistical significance (Table 1).

Regarding chronic parietal inflammation, a greater grade of fibrosis was observed in the 72 h silver nitrate group.
Figure 3: Distribution of means of scores presented in macroscopic and microscopic analysis after treatment with the substances, in different times. C: copaiba oil; N: silver nitrate; Macro: macroscopic; API: acute parietal inflammation; AVI: acute visceral inflammation; CPI: chronic parietal inflammation; CVI: chronic visceral inflammation; PF: parietal fibrosis; VF: visceral fibrosis; T1: 24 h; T2: 48 h; T3: 72 h; T4: 504 h; $P < 0.05$ was considered as significant, presenting difference between the treatments.

with statistical significance ($P = 0.019$) when compared to copaiba. There was no difference between the two substances in relation to the visceral pleura (Table 1).

Fibrosis of the visceral and parietal pleura was only observed at 72 and 504 h with increased reaction in the copaiba group to visceral fibrosis at 504 h ($P = 0.017$), as presented in Table 2.

Neovascularization, one of the main indicators of inflammation, was found at a greater grade in the 504 h copaiba group (1.50 ± 1.07), when compared to the silver nitrate group (0.34 ± 0.52; $P = 0.018$) (Table 2).

Edema of the parietal pleura was observed in all cases with the two substances, without statistical significance. In the visceral pleura, edema was more intensely present in the 24 h silver nitrate group when compared to the copaiba group ($P = 0.041$). The alveolar edema difference was more evident in the 24 h 0.5% silver nitrate group, with statistical significance ($P = 0.003$), when compared to the copaiba oil group. In the 48 and 72 h groups, the presence of edema was more frequent in the silver nitrate group (Table 2).

Bronchopneumonia was observed in four rats of the 24 h silver nitrate group, considered statistically significant ($P = 0.038$) when compared to copaiba oil. In the copaiba oil group, only two rats presented this characteristic, in the 48 and 504 h groups, respectively.

4. Discussion

Despite the indication of copaiba oil as anti-inflammatory by the literature [24], a previous work identified an intense inflammatory reaction in the pleural surface when the copaiba oil was applied topically for the treatment of empyema (data not published). Thus, based on this finding, the possibility of testing this phytotherapeutic as a sclerosing agent was identified. Copoaiba oil was used previously, experimentally, in the pleural cavity of rats with the development of great pleural reactions and multiple adherences [26]. The authors described adherences and early lung incarceration at 48 and 72 hours, however, with a higher dose of copaiba oil, which probably caused higher mortality when compared to the current study [26]. In another experimental study, in which copaiba oil was instilled in the peritoneal cavity, the macroscopic alterations observed were similar to those found in the pleura, thus showing that copaiba oleoresin triggers
aggression in the serosa with a corresponding formation of adherences in the process to repair the injury [27].

In this study, the macroscopic alterations were observed in both groups; however, they were more evident in the copaiba group for all of the times analyzed, showing that oleoresin when in contact with the pleural surface causes a greater pleural reaction than that induced by silver nitrate. The most frequent findings were adherences and pleural thickenings. The ventral region contained more alterations, and it is the area of greater contact with the substance in the pleura of rats. Similarly, Kennedy et al. [31], who used talc to induce pleurodesis in rabbits, also observed a greater number of alterations in the same region. Despite talc being the most used substance to pleurodesis induction, in this study, we opted to use silver nitrate because it is easier in the experimental procedure and is equally effective.

The efficacy of the 0.5% silver nitrate solution was tested in other experiments using rabbit models, with a similar effect of pleurodesis when compared to 35 mg/kg tetracycline [32] and superior effect when compared to talc diluted in saline solution at 400 mg/kg [10]. In addition to these studies, silver nitrate already has a well-defined clinical application, as described by Marcheix et al. [33], who showed a 98.9% success rate in pleurodesis when instilled by video-assisted thoracoscopy, however, at a 1% concentration.

The mechanism of the development of pleurodesis after the injection of the substance on the pleural surface has not been fully understood. The first alterations are the descaling of mesothelial cells and the development of pleural effusion with characteristics of exudate. After this, the effusion evolved to pleural adhesions and, in the reparative phase, we observed inflammatory reaction to injury and the regeneration of damaged cells, the migration of conjunctive tissue, the synthesis of extracellular protein matrix, and collagenization, determining a greater consistency of the pleural symphysis. Tissue healing evolution is complex and involves several interrelated processes, including acute inflammatory reaction to the injury on the first day and regeneration of cellular damage with cell migration of the connective tissue to the damaged area between the third and fifth days. Afterwards, protein synthesis of the extracellular matrix begins, a process that can last days or weeks making the healing tissue firmer [10, 31].

The action of copaiba oleoresin in the pleural space can be similar to that observed in most of the substances used for pleurodesis, but the mechanism that leads to the induction of pleurodesis has not been defined. According to Westphal et al. [26], the microscopic analysis of the pleura showed four times greater pleural thickening in the copaiba oil group with statistical significance than the substances compared in that study, crajiru and povidone-iodine, which were mild irritants. In this study, there was pleural thickening, however, at lower intensity, probably due to the reduced dose of copaiba oil injected in the pleural cavity.

The alterations in pulmonary parenchyma due to the use of silver nitrate were described by Vargas et al. [34], who witnessed alveolar collapse and signs of hemorrhage and discrete edema in the first month of follow-up. After the second month, minimal alterations similar to those in the talc group were observed. In this study, we observed a greater reaction in the animals of the silver nitrate group, evidenced by alveolar and visceral pleura edema, in addition to the development of bronchopneumonia in this group when compared to the copaiba group.

Neovascularization associated with the formation of pleural adherences does not differ from its diverse origins since they are similar in the cases of inflammatory and neoplastic processes. The formation of this new tissue is conditioned to the presence of new vascularization as observed in rabbits, in which the presence of neovascularization was observed both in the parietal and the visceral pleura [30]. The importance of neovascularization in the formation of pleurodesis was demonstrated by Guo et al. [35], who reduced it significantly when using the endothelial growth inhibition antibody in rabbits, a substance that inhibits the action of the transforming growth factor (TGF) and is known as a sclerosing agent. In this study, more evident neovascularization was observed in the 504 h time period in the copaiba oil group, when

<table>
<thead>
<tr>
<th>T</th>
<th>Acute inflammation</th>
<th>Chronic inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copaiba</td>
<td>Silver nitrate</td>
</tr>
<tr>
<td>24 h</td>
<td>1.63 ± 1.06</td>
<td>0.38 ± 0.52</td>
</tr>
<tr>
<td>48 h</td>
<td>0.50 ± 0.78</td>
<td>0.50 ± 0.78</td>
</tr>
<tr>
<td>72 h</td>
<td>1.38 ± 0.92</td>
<td>0.25 ± 0.46</td>
</tr>
<tr>
<td>504 h</td>
<td>0.00 ± 0.00</td>
<td>0.25 ± 0.46</td>
</tr>
</tbody>
</table>

* Student’s t-test. Value of P in bold italic indicates statistical difference at 5%. T: time of euthanasia.
Table 2: Distribution according to the mean of the scores of the variables analyzed.

<table>
<thead>
<tr>
<th>Variable</th>
<th>24 h Mean ± SD</th>
<th>48 h Mean ± SD</th>
<th>72 h Mean ± SD</th>
<th>504 h Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parietal fibrosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>—</td>
<td>—</td>
<td>0.63 ± 0.52</td>
<td>0.50 ± 0.78</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>—</td>
<td>—</td>
<td>0.38 ± 0.52</td>
<td>0.75 ± 0.71</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>—</td>
<td>—</td>
<td>0.350</td>
<td>0.506</td>
</tr>
<tr>
<td><strong>Visceral fibrosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>—</td>
<td>—</td>
<td>0.00 ± 0.00</td>
<td>1.38 ± 0.74</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>—</td>
<td>—</td>
<td>0.25 ± 0.71</td>
<td>0.50 ± 0.54</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>—</td>
<td>—</td>
<td></td>
<td>0.017</td>
</tr>
<tr>
<td><strong>Parietal neovascularization</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>—</td>
<td>0.25 ± 0.46</td>
<td>0.63 ± 0.52</td>
<td>0.50 ± 0.78</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>—</td>
<td>0.50 ± 0.53</td>
<td>0.63 ± 0.52</td>
<td>0.63 ± 0.74</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>—</td>
<td>0.334</td>
<td>0.999</td>
<td>0.705</td>
</tr>
<tr>
<td><strong>Visceral neovascularization</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>0.00 ± 0.00</td>
<td>—</td>
<td>0.25 ± 0.46</td>
<td>1.50 ± 1.07</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>0.13 ± 0.35</td>
<td>—</td>
<td>0.62 ± 0.92</td>
<td>0.34 ± 0.52</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>0.334</td>
<td>—</td>
<td>0.319</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>Parietal edema</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>0.50 ± 0.53</td>
<td>0.75 ± 0.46</td>
<td>1.25 ± 0.71</td>
<td>0.63 ± 0.74</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>0.38 ± 0.52</td>
<td>0.88 ± 0.35</td>
<td>1.00 ± 0.53</td>
<td>0.63 ± 0.74</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>0.642</td>
<td>0.554</td>
<td>0.438</td>
<td>0.999</td>
</tr>
<tr>
<td><strong>Visceral edema</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>0.38 ± 0.52</td>
<td>0.87 ± 0.64</td>
<td>1.62 ± 0.92</td>
<td>1.50 ± 0.76</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>0.88 ± 0.35</td>
<td>1.25 ± 0.46</td>
<td>1.38 ± 0.52</td>
<td>1.38 ± 0.52</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>0.041</td>
<td>0.201</td>
<td>0.513</td>
<td>0.705</td>
</tr>
<tr>
<td><strong>Fibroblastic proliferation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parietal pleura</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>—</td>
<td>0.50 ± 0.76</td>
<td>0.62 ± 0.52</td>
<td>0.75 ± 1.03</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>—</td>
<td>0.63 ± 0.52</td>
<td>1.25 ± 0.46</td>
<td>0.63 ± 0.52</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>—</td>
<td>0.705</td>
<td></td>
<td>0.023</td>
</tr>
<tr>
<td><strong>Fibroblastic proliferation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visceral pleura</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>—</td>
<td>0.13 ± 0.35</td>
<td>1.12 ± 0.64</td>
<td>1.12 ± 0.83</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>—</td>
<td>0.25 ± 0.46</td>
<td>0.75 ± 0.89</td>
<td>0.75 ± 0.46</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>—</td>
<td>0.554</td>
<td>0.349</td>
<td>0.285</td>
</tr>
<tr>
<td><strong>Alveolar edema</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copaiba oil</td>
<td>0.00 ± 0.00</td>
<td>0.25 ± 0.71</td>
<td>0.00 ± 0.00</td>
<td>—</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>1.50 ± 1.20</td>
<td>0.50 ± 0.53</td>
<td>0.50 ± 0.76</td>
<td>—</td>
</tr>
<tr>
<td>( P^\ast )</td>
<td>0.003</td>
<td>0.438</td>
<td>0.082</td>
<td>—</td>
</tr>
</tbody>
</table>

* Student’s \( t \)-test. Value of \( P \) in bold italic indicates statistical difference at 5%.

compared to the silver nitrate group, which shows greater effect of copaiba oil in the production of pleurodesis.

The use of rats in experiments to check the effects of pleurodesis can be achieved successfully as observed by Marchi et al. [36]. Several advantages were found in comparison to other animals, among them the easy handling and low cost of maintenance of these animals. In our experiments, we observed that pleurodesis occurred in both groups, thus showing that the use of rats as an animal model in the induction of pleurodesis can be carried out experimentally, allowing the study of pleuropulmonary alterations of pleurodesis.

With respect to chemical constitution, the major constituents in the oil of \( C. \) multitijuga were copalic acid, caryophyllene oxide, and caryophyllene [28], which are common in oils of various species of copaiba trees [25]. Based on this, it is suggested that the action presented in this study may be common in many oils that have the same chemical profile, taken from many other species of \( C. \) paleifera. However, the pharmacological effect of the oleoresin cannot be attributed...
to just one constituent, because the constituents present in oleoresin may interact synergistically in the promotion of the activity observed [13, 25].

5. Conclusions

In conclusion, this study has shown that intrapleural injection of copaiba oil and 0.5% silver nitrate produced pleurodesis; however, the macroscopic alterations and acute inflammation were more evident in the copaiba oil group. During the work period, greater pulmonary alterations occurred in the 0.5% silver nitrate group when compared to copaiba oil. Thus, both groups promote pleurodesis; however, the silver nitrate group presented greater aggression to the pulmonary parenchyma, introducing copaiba oil as a potential treatment for the induction of pleurodesis with milder side effects. However, additional studies are needed to confirm the activity of copaiba oil, identify its potential active compounds in inducing pleurodesis, and evaluate the possible side effects of its use to thereby define its usefulness in patients who have malignant diseases.

Conflict of Interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References


Research Article

In Silico Molecular Docking and In Vitro Antidiabetic Studies of Dihydropyrimido[4,5-a]acridin-2-amines

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An in vitro antidiabetic activity on α-amylase and α–glucosidase activity of novel 10-chloro-4-(2-chlorophenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amines (3a–3f) were evaluated. Structures of the synthesized molecules were studied by FT-IR, 1H NMR, 13C NMR, EI-MS, and single crystal X-ray structural analysis data. An in silico molecular docking was performed on synthesized molecules (3a–3f). Overall studies indicate that compound 3e is a promising compound leading to the development of selective inhibition of α-amylase and α–glucosidase.

1. Introduction

Pyrimidine is a well-known biologically active nitrogen containing heterocyclic compound. In recent years researchers are much interested in the synthesis of pyrimidine analogues. Pyrimidine derivatives posse fungicidal [1], herbicidal [2], antidepressant [3], and antitumor properties [4, 5]. Synthetically prepared amino pyrimidine derivatives display a wide range of biological activities such as antibacterial [6], antitumor [7], and antiviral [8, 9]. Therefore, the substituted amino pyrimidine structure can be found in diverse clinically approved drugs. Interestingly, a substituted amino pyrimidine moiety was also suggested to account for the antioxidant activity [10]. Among those amino pyrimidine heterocycle, 2-aminoypyrimidines have been widely used as pharmacophores for drug discovery. 2-Aminopyrimidines constitute a part of the DNA base pair molecules [11]. Compounds having potent anticancer activity, CDK inhibitory activity, I [12–14], antiproliferative activity [15], kinase inhibitor, II [16], antibacterial agents [17], antitumor, III, antidiabetic activity [18], antimalarial, IV, antiplasmodial agents [19], antimicrobial activity, V [20], and anti-inflammatory activity, VI [21] (Figure 1), contain amino pyrimidine moiety in the structure. The tricyclic, planar acridine moiety is responsible for intercalation between base pairs of double-stranded DNA through π–π interactions and, therefore, causes alteration in the cellular machinery [22]. Intercalative interaction of structurally related well-known intercalators, 9-aminoacridine (9AA), and proflavine (PF) was determined by means of fluorescence quenching study [23].

Due to numerous biological applications of 2-aminopyrimidine and acridine amine and its analogues, we have focused our research on synthesis of 2-aminopyrimidine by using pharmacologically important structural scaffold acridine pharmacophore.

Protein-ligand interaction is comparable to the lock-and-key principle, in which the lock encodes the protein and the key is grouped with the ligand. The major driving force for binding appears to be hydrophobic interaction [24]. In silico techniques help identifying drug target via bioinformatics
tools. They can also be used to explore the target structures for possible active sites, generate candidate molecules, dock these molecules with the target, rank them according to their binding affinities, and further optimize the molecules to improve binding characteristics [25]. Diabetes mellitus (DM) is a leading noncommunicable disease which affects more than 100 million people worldwide and is considered as one of the fine leading diseases which causes death in the world [26]. Type-2 diabetes mellitus is a chronic metabolic disorder that results from defects in both insulin secretion and insulin action. Management of type-2 diabetes by conventional therapy involves the inhibition of degradation of dietary starch by glucosidases such as \(\alpha\)-amylase and \(\alpha\)-glucosidase [27]. The pathogenesis of type-2 diabetes involves progressive development in insulin resistance associated with a defect in insulin secretion, leading to overt hyperglycemia. However, compounds which improve insulin sensitivity and glucose intolerance are somewhat limited warranting the discovery and characterization of novel molecules targeting various pathways involved in the pathogenesis of type-2 diabetes [28]. Currently, there are few drugs that are able to counteract the development of the associated pathologies. Therefore, the need to search for new drug candidates in this field appears to be critical. Aromatic amines and thiazolidinones nuclei would produce new compounds with significant antidiabetic properties [29].

In our research group, we have already reported larvicidal activity of 7-chloro-3,4-dihydro-9-phenylacridin-1(2H)-one and (E)-7-chloro-3,4-dihydro-phenyl-2-[(pyridin-2-y1)methylene]acridin-1(2H)-one [30]. In continuation of our research work on acridine moiety, presently we focus on the synthesis of 10-chloro-4-(2-chlorophenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine, 3a–3f derivatives. All the synthesized dihydropyrimido[4,5-a]acridin-2-amines analogues, 3a–3f, were evaluated for docking and \textit{in vitro} antidiabetic activity.

2. Materials and Methods

2.1. Chemistry. Melting points were determined by open capillary method and are corrected with standard benzoic acid. All solvents were distilled and dried prior to use. TLC was performed on silica gel G and the spots were exposed to iodine vapour for visualization. A mixture of petroleum ether and ethyl acetate was used as an eluent at different ratio. Column chromatography was performed by using silica gel (60–120 mesh). \(^1\)H NMR and \(^{13}\)C NMR spectra were recorded in CDCl\(_3\) on a Bruker advance 400 MHz instrument. Chemical shifts are reported in ppm using TMS as the internal standard. IR spectra were obtained on a Perkin-Elmer spectrum RXI FT-IR spectrometer (400–4000 cm\(^{-1}\)).
resolution: 1 cm⁻¹) using KBr pellets. Molecular mass was determined using ESI-MS THERMO FLEET spectrometer.

2.2. Biological Assays. The dialysis membrane, 1,4-α-D glucan-glucanohydrolase, α-amylase, α-glucosidase, P-nitrophenyl-α-D-glucopyranoside, and acarbose were purchased from Himedia Laboratories, Mumbai, India. All other chemicals and reagents were AR grade purchased locally.

Spectral data of the synthesized compounds are described below.

10-Chloro-4,12-diphenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine (3a). Yellow solid. Yield 70%. mp. 194–196°C. FT-IR (KBr) νmax (cm⁻¹): 3450.30 (–NH₂), 1H NMR (400 MHz, CDCl₃): δ (ppm), 2.99 (s, 2H, –CH₂), 3.18 (s, 2H, –CH₂), 4.43 (s, 2H, –NH₂), 7.25 (m, 6H), 7.46 (d, 2H), 7.53 (m, 2H), 7.60–7.65 (m, 2H), 7.99–8.02 (d, 1H). 13C NMR (400 MHz, CDCl₃): δ (ppm), 23.0, 33.6, 55.8, 56.0, 112.1, 115.8, 120.5, 124.9, 126.2, 127.1, 127.8, 130.2, 131.1, 132.1, 135.0, 138.5, 140.3, 140.7, 144.9, 148.6, 149.3, 157.8, 158.4. EI-MS m/z 495.52 [M+1].

2.3. General Synthesis of 10-Chloro-4,12-diphenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine, 3a–3f. 10-Chloro-4,12-diphenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine, 3a–3f, were synthesized via, (E)-2-benzylidine-7-chloro-3,4-dihydro-9-phenylacridin-1(2H)-ones, 1a–If, (3,9511g, 0.01 mol) were mixed with guanidine carbonate 2 (0.9008 g, 0.01 mol) and 10 mL of 10% alc. NaOH (1g in 10 mL ethanol) and then heated under reflux condition for 5 h. After the completion of the reaction, reaction mixture was cooled and poured into crushed ice. The crude product was separated by column chromatography using 10–15% of ethyl acetate and pet ether solvent to get the target compounds, 3a–3f. The synthetic scheme was presented in Scheme 1 and physical data of all synthesized derivatives were summarized in Table 1.

Spectral data of the synthesized compounds are described below.

10-Chloro-4,12-diphenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine (3a). Yellow solid. Yield 70%. mp. 194–196°C. FT-IR (KBr) νmax (cm⁻¹): 3450.30 (–NH₂), 1H NMR (400 MHz, CDCl₃): δ (ppm), 2.99 (s, 2H, –CH₂), 3.18 (s, 2H, –CH₂), 4.43 (s, 2H, –NH₂), 7.25 (m, 6H), 7.46 (d, 2H), 7.53 (m, 2H), 7.60–7.65 (m, 2H), 7.99–8.02 (d, 1H). 13C NMR (400 MHz, CDCl₃): δ (ppm), 23.0, 33.6, 55.8, 56.0, 112.1, 115.8, 120.5, 124.9, 126.2, 127.1, 127.8, 130.2, 131.1, 132.1, 135.0, 138.5, 140.3, 140.7, 144.9, 148.6, 149.3, 157.8, 158.4. EI-MS m/z 495.52 [M+1].

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10-Chloro-4-(3-methoxyphenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine (3d). Yellow solid. Yield 69%. mp. 168-170°C. FT-IR (KBr) \(\nu_{\text{max}}\) (cm\(^{-1}\)): 3475.73 (–NH\(_2\)), 2924.73-2960.23 (–OCH\(_3\)). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) (ppm), 2.39-2.40 (m, 2H, –CH\(_2\)), 2.71-2.83 (m, 2H, –CH\(_2\)), 3.82 (s, 3H, –OCH\(_3\)), 4.83 (s, 2H, –NH\(_2\)), 6.91 (d, \(J = 1.2\) Hz, 1H), 6.97-6.99 (d, \(J = 7.2\) Hz, 1H), 7.02-7.04 (d, \(J = 8.4\) Hz, 1H), 7.12-7.14 (m, 1H), 7.15 (m, 1H), 7.20-7.22 (m, 2H), 7.24 (m, 2H), 7.31-7.33 (d, \(J = 8.4\) Hz, 1H), 7.34-7.36 (d, \(J = 7.2\) Hz, 1H), \(^13\)C NMR (400 MHz, CDCl\(_3\)): \(\delta\) (ppm), 23.7, 33.8, 55.3, 118.4, 124.8, 126.2, 127.3, 127.9, 128.6, 128.7, 131.1, 132.0, 137.8, 139.3, 146.1, 146.8, 159.5, 160.4, 160.6, 160.9, 163.4. EI-MS \(m/z\) 495.35 [M+1].

2.4. Single Crystal X-Ray Diffraction (XRD) Analysis. Crystals suitable for X-ray analysis were obtained by slow evaporation of a solution of the 10-chloro-4-(2-chlorophenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine, 3f, in ethyl acetate. The measurements were made on Enraf Nonius CAD-4-MV31 single crystal X-ray diffractometer. The diagrams and calculations have been performed using SAINT (APEX II) for frame integration, SHELXTL for structure solution and refinement software programs. The structure was refined using the full-matrix least squares procedures on \(\bar{F}\) with anisotropic thermal parameters for all nonhydrogen atoms. The crystal data and details concerning data collection and structure refinement of compound, 3f, single crystal are summarized in Table 2. As we can see compound 3f crystallizes in triclinic space group, P-1. The single crystal structure and atomic numbering chosen for compound 3f are demonstrated in Figure 2. Selected bond lengths and bond angles are compiled in Table 3. It can be observed that the bond lengths of all kind atoms on the whole molecule are absorbed. The typical C–C single (1.55˚A) and C=C double (1.38˚A) bonds, C–N typical single (1.35˚A) and C=N double (1.33˚A) bonds. This means that the carbon–carbon bond and the carbon-nitrogen bond have double bond character and contribute to form conjugated system. Furthermore, carbon attached with strong electronegative atom chlorine bond length is C–Cl (1.68˚A), in case all C–H (0.97˚A) and C=H (0.93˚A) are absorbed. The primary amine hydrogen bond length is N–H (0.86˚A), respectively (Table 3). Concerning inspection of the torsion angles, the 3f molecule has nearly planar core. Figure 3 illustrates a representative view of compound 3f crystal packing structure. The two molecules of compound 3f are packed in face-to-face arrangement due to the intermolecular hydrogen bonds. The crystal packing structure demonstrates the existence of two intermolecular hydrogen bonds. Interesting result were absorbed from crystal packing structure is the intermolecular hydrogen bonding occurs at C(23)–H(23) and C(24)–H(24) of one molecule to another neighbouring molecule of C(23)–H(23) and C(24)–H(24), not in the case of primary amine containing two hydrogen N(4)–H(A) and N(4)–H(B) Figure 3.
Table 2: The crystallographic data and structure refinement parameters of compound 3f.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C_{27}H_{18}Cl_{2}N_{4}</td>
</tr>
<tr>
<td>Formula weight</td>
<td>469.35</td>
</tr>
<tr>
<td>Temperature</td>
<td>293(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal system, space group</td>
<td>Triclinic, P-1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>(a = 9.9421(5) ) Å, (\alpha = 67.248(2) )°</td>
</tr>
<tr>
<td></td>
<td>(b = 10.9553(5) ) Å, (\beta = 78.530(2) )°</td>
</tr>
<tr>
<td></td>
<td>(c = 11.5126(5) ) Å, (\gamma = 89.875(2) )°</td>
</tr>
<tr>
<td>Volume</td>
<td>1129.39(9) Å³</td>
</tr>
<tr>
<td>(Z)</td>
<td>2</td>
</tr>
<tr>
<td>Calculated density</td>
<td>1.380 Mg/m³</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>0.311 mm⁻¹</td>
</tr>
<tr>
<td>(F(000))</td>
<td>484</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.35 (\times) 0.30 (\times) 0.25 mm</td>
</tr>
<tr>
<td>Theta range for data collection</td>
<td>1.96 to 25.00°</td>
</tr>
<tr>
<td>Limiting indices</td>
<td>(-11 \leq h \leq 11,-12 \leq k \leq 12,-13 \leq l \leq 13)</td>
</tr>
<tr>
<td>Reflections collected/unique</td>
<td>19503/3929 [(R(\text{int}) = 0.0276)]</td>
</tr>
<tr>
<td>Completeness to theta = 25.00</td>
<td>98.8%</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>Semiempirical from equivalents</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.9635 and 0.8623</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least squares on (F^2)</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>3929/6/308</td>
</tr>
<tr>
<td>Goodness-of-fit on (F^2)</td>
<td>1.200</td>
</tr>
<tr>
<td>Final (R) indices [(I &gt; 2 \text{sigma}(I))]</td>
<td>(R_1 = 0.0569, wR_2 = 0.1944)</td>
</tr>
<tr>
<td>(R) indices (all data)</td>
<td>(R_1 = 0.0712, wR_2 = 0.2074)</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.383 and (-0.385) e Å⁻³</td>
</tr>
</tbody>
</table>

2.5. Glucose Diffusion Inhibitory Test

Sample Preparation. Four different concentrations (100, 200, 300, and 400 \(\mu\)g/mL) of samples were prepared. 1 mL of the sample was placed in a dialysis membrane (12000 MW, Himedia laboratories, Mumbai) along with a glucose solution (0.22 mM in 0.15 M NaCl). Then it was tied at both ends and immersed in a beaker containing 40 mL of 0.15 M NaCl and 10 mL of distilled water. The control contained 1 mL of 0.15 M NaCl containing 0.22 mM glucose solution and 1 mL of distilled water. The beaker was then placed in an orbital shaker. The external solution was monitored every half an hour. Three replications of this test were done for 3 hrs [31, 32].

2.6. Inhibition Assay for \(\alpha\)-Amylase Activity. Four different concentrations (100, 200, 300, and 400 \(\mu\)g/mL) of samples and standard drug acarbose were prepared and made up to 1 mL with DMSO. A total of 500 \(\mu\)L of sample and 500 \(\mu\)L of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing \(\alpha\)-amylase solution (0.5 mg/mL) were incubated for 10 minutes, at 25°C. After preincubation, 500 \(\mu\)L of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. This reaction mixture was then incubated for 10 minutes at 25°C. 1 mL of DNSA colour reagent was added to stop the reaction. These test tubes were then incubated in a boiling water bath for 5 minutes and cooled to room temperature. Finally this reaction mixture was again diluted by adding 10 mL of distilled water. % of inhibition by \(\alpha\)-amylase can be calculated by using the following formula. Absorbance was measured at 540 nm [32, 33]:

\[
\text{% inhibition} = \frac{A_{540 \text{ control}} - A_{540 \text{ sample}}}{A_{540 \text{ control}}} \times 100. \quad (1)
\]

Triplicates were done for each sample at different concentrations.

2.7. Inhibition Assay for \(\alpha\)-Glucosidase Activity. Various concentrations of samples and standard drug acarbose were prepared. \(\alpha\)-Glucosidase (0.075 units) was premixed with...
Table 3: Some important bond lengths (Å) and bond angles (°) of compound 3f.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Bond length</th>
<th>Bond</th>
<th>Bond length</th>
<th>Bond angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(1)–C(6)</td>
<td>1.371(6)</td>
<td>N(1)–C(4)–C(5)</td>
<td>123.2(3)</td>
<td></td>
</tr>
<tr>
<td>C(2)–H(2)</td>
<td>0.9300</td>
<td>N(1)–C(4)–C(3)</td>
<td>117.1(4)</td>
<td></td>
</tr>
<tr>
<td>C(11)–H(11A)</td>
<td>0.9700</td>
<td>N(1)–C(9)–C(8)</td>
<td>124.1(4)</td>
<td></td>
</tr>
<tr>
<td>C(11)–H(11B)</td>
<td>0.9700</td>
<td>N(1)–C(9)–C(10)</td>
<td>116.4(3)</td>
<td></td>
</tr>
<tr>
<td>C(11)–H(11B)</td>
<td>0.9700</td>
<td>C(8)–C(9)–C(10)</td>
<td>119.4(3)</td>
<td></td>
</tr>
<tr>
<td>C(13)–N(2)</td>
<td>1.330(4)</td>
<td>C(9)–C(10)–H(10A)</td>
<td>109.5</td>
<td></td>
</tr>
<tr>
<td>C(14)–N(2)</td>
<td>1.332(4)</td>
<td>C(12)–C(11)–C(10)</td>
<td>109.0(3)</td>
<td></td>
</tr>
<tr>
<td>C(14)–N(3)</td>
<td>1.344(4)</td>
<td>H(11A)–C(11)–H(11B)</td>
<td>108.3</td>
<td></td>
</tr>
<tr>
<td>C(14)–N(4)</td>
<td>1.354(4)</td>
<td>C(13)–C(12)–C(15)</td>
<td>115.8(3)</td>
<td></td>
</tr>
<tr>
<td>N(4)–H(4A)</td>
<td>0.8600</td>
<td>C(15)–C(12)–C(11)</td>
<td>124.3(3)</td>
<td></td>
</tr>
<tr>
<td>N(4)–H(4B)</td>
<td>0.8600</td>
<td>N(2)–C(13)–C(8)</td>
<td>117.3(3)</td>
<td></td>
</tr>
<tr>
<td>C(4)–N(1)</td>
<td>1.361(6)</td>
<td>C(12)–C(13)–C(8)</td>
<td>119.1(3)</td>
<td></td>
</tr>
<tr>
<td>C(9)–N(1)</td>
<td>1.317(5)</td>
<td>N(2)–C(14)–N(3)</td>
<td>126.1(3)</td>
<td></td>
</tr>
<tr>
<td>C(9)–C(10)</td>
<td>1.496(6)</td>
<td>N(2)–C(14)–N(4)</td>
<td>117.0(3)</td>
<td></td>
</tr>
<tr>
<td>C(10)–C(11)</td>
<td>1.525(6)</td>
<td>N(3)–C(14)–N(4)</td>
<td>116.9(3)</td>
<td></td>
</tr>
<tr>
<td>N(4)–H(4A)</td>
<td>0.8600</td>
<td>N(3)–C(15)–C(12)</td>
<td>122.4(3)</td>
<td></td>
</tr>
<tr>
<td>N(4)–H(4B)</td>
<td>0.8600</td>
<td>N(3)–C(15)–C(16)</td>
<td>116.7(3)</td>
<td></td>
</tr>
<tr>
<td>C(26)–H(26)</td>
<td>0.9300</td>
<td>C(13)–N(2)–C(14)</td>
<td>116.4(3)</td>
<td></td>
</tr>
<tr>
<td>C(27)–H(27)</td>
<td>0.9300</td>
<td>C(15)–N(3)–C(14)</td>
<td>116.2(3)</td>
<td></td>
</tr>
<tr>
<td>C(1)–Cl(1)</td>
<td>1.681(1)</td>
<td>C(14)–N(4)–H(4B)</td>
<td>120.0</td>
<td></td>
</tr>
<tr>
<td>C(17)–Cl(2)</td>
<td>1.732(4)</td>
<td>H(4A)–N(4)–H(4B)</td>
<td>120.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Release of glucosethrough dialysis membrane to externalsolution (mg/dL).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1.833 ± 0.00</td>
<td>1.515 ± 0.01</td>
<td>1.661 ± 0.00</td>
<td>1.830 ± 0.00</td>
<td>1.5 ± 0.00</td>
<td>1.830 ± 0.00</td>
<td>2 ± 0.00</td>
</tr>
<tr>
<td>60</td>
<td>2.670 ± 0.03</td>
<td>1.831 ± 0.00</td>
<td>1.831 ± 0.00</td>
<td>2 ± 0.01</td>
<td>1.83 ± 0.01</td>
<td>2 ± 0.01</td>
<td>2.16 ± 0.00</td>
</tr>
<tr>
<td>90</td>
<td>2.500 ± 0.00</td>
<td>2.161 ± 0.01</td>
<td>1.831 ± 0.01</td>
<td>2.160 ± 0.00</td>
<td>2.16 ± 0.01</td>
<td>1.831 ± 0.01</td>
<td>2.332 ± 0.07</td>
</tr>
<tr>
<td>120</td>
<td>2.667 ± 0.01</td>
<td>2.5 ± 0.00</td>
<td>2.162 ± 0.03</td>
<td>2.515 ± 0.00</td>
<td>2.5 ± 0.00</td>
<td>2 ± 0.00</td>
<td>2.5 ± 0.01</td>
</tr>
<tr>
<td>150</td>
<td>2.833 ± 0.07</td>
<td>2.331 ± 0.00</td>
<td>2.511 ± 0.04</td>
<td>2.831 ± 0.08</td>
<td>2.66 ± 0.00</td>
<td>1.662 ± 0.00</td>
<td>2.671 ± 0.07</td>
</tr>
<tr>
<td>180</td>
<td>2.833 ± 0.07</td>
<td>3 ± 0.00</td>
<td>2.830 ± 0.00</td>
<td>2.671 ± 0.00</td>
<td>2.831 ± 0.06</td>
<td>1.331 ± 0.00</td>
<td>2.83 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for groups of 3 observations.

Sample. 3 mM p-nitrophenyl glucopyranoside was used as a substrate was added to the reaction mixture to start the reaction [34]. The reaction was incubated at 37°C for 30 min and stopped by adding 2 mL of Na₂CO₃. The α-glucosidase activity was measured by p-nitrophenol release from PNPG at 400nm. % of inhibition can be calculated by using (1). Triplicates are done for each sample at different concentrations [31–33].

2.8. Statistical Analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA). Results are expressed as mean ± SD and n = 3.

3. Results and Discussion

3.1. Glucose Diffusion Inhibitory Test. The results are summarized in Tables 4 and 5. The diffused glucose concentration is given in Table 4 and % of relative movement is given in Table 5. The movement of glucose from inside of membrane to external solution monitored and compared in Figures 4 and 5. Among the six samples only the 3e retains the glucose and shows minimum % of relative movement 41.06% over 180 minutes. All other samples show the highest % of relative movement in glucose diffusion from 30 to 180 minutes.

3.1.1. α-Amylase Inhibitory Assay. The results are given in Table 6. All samples show gradual increase in inhibition, where the sample concentration increased from 100 to 400 µg/mL. Sample 3e shows maximum inhibition of 57% and sample 3d shows 23.55% of inhibition. All other samples show varying less significant inhibition.

3.1.2. α-Glucosidase Inhibition Assay. The results are shown in Table 7. Among the six samples, 3e shows maximum inhibitory activity of 60.25% at higher concentration.
Table 5: % of relative movement in glucose diffusion inhibitory assay.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>81.83 ± 0.01</td>
<td>90.56 ± 0.03</td>
<td>99.83 ± 0.00</td>
<td>81.33 ± 0.00</td>
<td>74.90 ± 0.00</td>
<td>109.11 ± 0.01</td>
</tr>
<tr>
<td>60</td>
<td>68.66 ± 0.07</td>
<td>68.53 ± 0.00</td>
<td>74.91 ± 0.07</td>
<td>68.66 ± 0.01</td>
<td>73.20 ± 0.02</td>
<td>80.90 ± 0.02</td>
</tr>
<tr>
<td>90</td>
<td>86.66 ± 0.05</td>
<td>73.20 ± 0.03</td>
<td>86.40 ± 0.06</td>
<td>86.67 ± 0.02</td>
<td>74.99 ± 0.03</td>
<td>93.33 ± 0.03</td>
</tr>
<tr>
<td>120</td>
<td>93.73 ± 0.04</td>
<td>80.99 ± 0.05</td>
<td>93.74 ± 0.05</td>
<td>93.74 ± 0.05</td>
<td>58.83 ± 0.05</td>
<td>93.74 ± 0.07</td>
</tr>
<tr>
<td>150</td>
<td>82.36 ± 0.01</td>
<td>88.24 ± 0.06</td>
<td>99.89 ± 0.03</td>
<td>94.13 ± 0.06</td>
<td>47.18 ± 0.07</td>
<td>94.13 ± 0.08</td>
</tr>
<tr>
<td>180</td>
<td>105.89 ± 0.02</td>
<td>99.84 ± 0.07</td>
<td>94.24 ± 0.02</td>
<td>100 ± 0.07</td>
<td>41.06 ± 0.06</td>
<td>100 ± 0.06</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for groups of 3 observations.

Table 6: % inhibition of α-amylase assay.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Acarbose</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>32.01 ± 0.09</td>
<td>7.97 ± 0.01</td>
<td>5.43 ± 0.06</td>
<td>3.28 ± 0.00</td>
<td>18.83 ± 0.02</td>
<td>19.20 ± 0.01</td>
<td>12.23 ± 0.00</td>
</tr>
<tr>
<td>200</td>
<td>48.15 ± 0.11</td>
<td>9.41 ± 0.02</td>
<td>5.43 ± 0.02</td>
<td>4.34 ± 0.01</td>
<td>20.65 ± 0.03</td>
<td>21.37 ± 0.00</td>
<td>13.77 ± 0.01</td>
</tr>
<tr>
<td>300</td>
<td>70.03 ± 0.25</td>
<td>11.22 ± 0.07</td>
<td>6.52 ± 0.04</td>
<td>5.070 ± 0.08</td>
<td>21.73 ± 0.01</td>
<td>53.62 ± 0.03</td>
<td>14.85 ± 0.02</td>
</tr>
<tr>
<td>400</td>
<td>80.02 ± 0.71</td>
<td>12.31 ± 0.06</td>
<td>6.52 ± 0.04</td>
<td>6.16 ± 0.06</td>
<td>23.55 ± 0.01</td>
<td>57.96 ± 0.04</td>
<td>16.66 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for groups of 3 observations. **P < 0.05.

Figure 4: Concentration of glucose (mg/dL).

Figure 5: % of relative movement in glucose diffusion inhibitory assay.

3.2. Molecular Docking Studies. The synthesized molecules 3a–3f were constructed using VEGA ZZ molecular modeling package [35]. The obtained structures were then geometrically optimized using AM1 Hamiltonian in MOPAC software package [36]. The X-ray structure of pig α-pancreatic α-amylase (PDB 3L2M) and N-terminal human maltase-glucoamylase with Casuarina (PDB 3CTT) [37], obtained from Brookhaven Protein Data Bank, were used for docking calculations. Docking calculations were performed with AutoDock 4.0 [38].

The crystal structures were refined by removing water molecules and repeating coordinates. Hydrogen atoms were added and charges were assigned to the protein atoms using Kollman united atoms force field by using AutoDockTools-1.5.6. For docking calculations, Gasteiger partial atomic charges were added to the synthesized structures and all possible flexible torsion angles of the ligand were defined by using AUTOTORS. The structures were saved in a PDBQT format for AutoDock calculations.
Autodock requires precalculated grid maps, one for each atom type present in the structure being docked. The auxiliary program Autogrid generated the grid maps. Lennard-Jones parameters 12-10 and 12-6, implemented with the program, were used for modeling H-bonds and van der Waals interactions, respectively. The Lamarckian genetic algorithm method was applied for docking calculations using default parameters. AutoDock uses a semiempirical free energy force field to evaluate conformations during docking simulations. The optimized orientations represent possible binding modes of the ligand within the site:

\[ \Delta G = \Delta G_{vdw} + \Delta G_{hbond} + \Delta G_{elec} + \Delta G_{tor} + \Delta G_{desolv}. \]  

The first three terms are van der Waals, hydrogen bonding, and electrostatics, respectively. The term \( \Delta G_{tor} \) is for rotation and translation and \( \Delta G_{desolv} \) is for desolvation upon binding and the hydrophobic effect.

After docking, the 50 solutions were clustered into groups with RMS deviations lower than 1.0 Å. The clusters were ranked by the lowest energy representative of each cluster.

### 3.2.1. Molecular Docking Study on \( \alpha \)-Amylase

The binding site of the structures was not identified because of the absence of the crystal structure of the ligand, and a blind docking was performed for all the structures 3a–3f with the protein structure 3L2M [39]. Two interacting binding sites were identified [37], one near the entrance of the central beta-barrel of the enzyme and the other near the N-terminal of the protein [39]. The docked structures showed binding energy in the range of \(-4.2\) to \(-4.8\) Kcal/mol. It was observed that the structure 3e exhibits high binding energy of \(-4.8\) Kcal/mol and the structure 3d exhibits binding energy of \(-4.5\) Kcal/mol, respectively. The binding energies (\( \Delta G_{BE} \)) and intermolecular energies (\( \Delta G_{intermol} \)) of the structures 3d and 3e obtained for \( \alpha \)-amy-lase are given in Table 8. When comparing 3d and 3e structures, 3e exhibits high binding energy. The structure 3e shows interaction with the residues Trp58, Trp59, Tyr62, Asp197, and Asp300 present in the binding site of \( \alpha \)-amylase. The amino acids Trp58 and Trp59 were the key residues interacting with all the structures. The docking studies revealed that the van der Waals, electrostatic, and desolvation energies play a key role in binding. It was observed that the Pyrimido ring was fitted well with binding site, via hydrogen bonds through the hydrogen atom of the amino group of 3e with carboxylate group of Asp300. The hydrophobic interactions were formed by Trp58, Trp59, Gln63, Val163, Asp300, His305, and Asp356 in 3e.

### 3.2.2. Molecular Docking Study on \( \alpha \)-Glucosidase

All the binding modes of the structures 3a–3f were explored using the docking calculation in AutoDock. The binding site of the structures was identified using the crystal structure of Casuarina. The docked structures showed binding energy in the range of \(-6.3\) to \(-6.6\) Kcal/mol. The binding energies (\( \Delta G_{BE} \)) and intermolecular energies (\( \Delta G_{intermol} \)) of the structures 3d and 3e obtained for \( \alpha \)-glucosidase are given in Table 8. The structure 3e showed high binding energy when compared to 3d. Based on docking calculations, the synthesized compounds 3d and 3e show better inhibition of \( \alpha \)-glucosidase compared to \( \alpha \)-amylase which is in a good agreement with in vitro studies. The following key residues Asp203, Tyr299, Trp406, Asp443, Met444, Phe450, Lys480, Asp542, Phe575, and Gln603 present in the binding site of \( \alpha \)-glucosidase show noncovalent interactions with 3e. It was observed that the Pyrimido ring of 3e formed hydrogen bonds through the hydrogen atom of the amino group with carboxylate group of Asp203 at a distance of 1.9 Å. The residues Tyr299,

### Table 7: % inhibition of \( \alpha \)-glucosidase assay.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Acarbose</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>30.08 ± 0.05</td>
<td>6.29 ± 0.00</td>
<td>4.29 ± 0.01</td>
<td>20.91 ± 0.00</td>
<td>21.45 ± 0.01</td>
<td>14.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>45.02 ± 0.12</td>
<td>8.32 ± 0.01</td>
<td>5.02 ± 0.01</td>
<td>21.65 ± 0.01</td>
<td>24.62 ± 0.09</td>
<td>10.77 ± 0.03</td>
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<tr>
<td>300</td>
<td>68.25 ± 0.21</td>
<td>10.12 ± 0.01</td>
<td>6.09 ± 0.02</td>
<td>22.71 ± 0.02</td>
<td>56.85 ± 0.01</td>
<td>15.85 ± 0.04</td>
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<tr>
<td>400</td>
<td>78.01 ± 0.07</td>
<td>11.45 ± 0.02</td>
<td>9.19 ± 0.02</td>
<td>23.01 ± 0.04</td>
<td>60.25 ± 0.01</td>
<td>17.71 ± 0.03</td>
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</tr>
</tbody>
</table>

Values are mean ± SEM for groups of 3 observations.

\*P < 0.01

\**P < 0.05.

### Table 8: The binding energy (\( \Delta G_{BE} \)) and intermolecular energy (\( \Delta G_{intermol} \)) of the structures 3d and 3e are given. The docking energy and binding energy are reported in Kcal/mol.

<table>
<thead>
<tr>
<th>Structures</th>
<th>( \Delta G_{BE} )</th>
<th>( \Delta G_{vdw, hb, desolv} )</th>
<th>( \Delta G_{elec} )</th>
<th>( \Delta G_{inter} )</th>
<th>( \Delta G_{tor} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-Amylase</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3d</td>
<td>-4.52</td>
<td>-5.31</td>
<td>-0.40</td>
<td>-1.57</td>
<td>1.19</td>
</tr>
<tr>
<td>3e</td>
<td>-4.83</td>
<td>-5.35</td>
<td>-0.37</td>
<td>-1.54</td>
<td>0.89</td>
</tr>
<tr>
<td>( \alpha )-Glucosidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3d</td>
<td>-6.51</td>
<td>-6.34</td>
<td>-1.37</td>
<td>-1.44</td>
<td>1.19</td>
</tr>
<tr>
<td>3e</td>
<td>-6.61</td>
<td>-6.95</td>
<td>-0.56</td>
<td>-1.41</td>
<td>0.89</td>
</tr>
</tbody>
</table>
nonspecific and produce serious side effects such as GI tract hyperglycemia. The inhibition by natural products is more safe than synthetic drugs. α-Amylase and α-glucosidase are significant enzymes which cleave carbohydrates responsible for absorption of glucose in the blood stream. For this reason, synthetic drug such as acarbose, miglitol, and voglibose are some inhibitors which inhibit α-amylase and α-glucosidase [40]. However, these agents have their limitations, because they are nonspecific and produce serious side effects such as GI tract inflammation and to elevate diabetic complications [41].

4. Conclusion

The present study revealed that we have successfully achieved our target title compound 10-chloro-4,12-diphenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine derivatives. All compounds were confirmed by suitable experimental and spectroscopic techniques. Synthesized derivatives (3a–3f) were evaluated in vitro α-amylase and α-glucosidase inhibitory activity and glucose diffusion test of samples. Among all other derivatives, compounds 10-chloro-4-(4-chlorophenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine, 3e, and 10-chloro-4-(3-methoxyphenyl)-12-phenyl-5,6-dihydropyrimido[4,5-a]acridin-2-amine, 3d, show good inhibitory activity for α-amylase and α-glucosidase with the values of 57.96, 60.27, 23.55, and 23.01% for compounds 3e and 3d, respectively. The docking studies are in good agreement with the in vitro studies. The docking calculations showed that van der Waals, electrostatic, and desolvation energies play a key role in binding. These factors are considered for designing new inhibitors for α-amylase and α-glucosidase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References


Effect of *Alocasia indica* Tuber Extract on Reducing Hepatotoxicity and Liver Apoptosis in Alcohol Intoxicated Rats

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The possible protective role of ethanolic extract of *A. indica* tuber (EEAIT) in hepatotoxicity and apoptosis of liver caused by alcohol in rats was investigated. Treatment of rats with alcohol (3 g ethanol per kg body weight per day for 15 days intraperitoneally) produced marked elevation of liver biomarkers such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), y-glutamyl transpeptidase (y-GT), and total bilirubin levels which were reduced by EEAIT in a dose-dependent manner. Furthermore, EEAIT improved antioxidant status (MDA, NO, and GSH) and preserved hepatic cell architecture. Simultaneous supplementation with EEAIT significantly restored hepatic catalase (CAT) and superoxide dismutase (SOD) activity levels towards normal. The studies with biochemical markers were strongly supported by the histopathological evaluation of the liver tissue. EEAIT also attenuated apoptosis and necrosis features of liver cell found in immunohistochemical evaluation. HPLC analysis of the extract showed the presence of three major peaks of which peak 2 (RT: 33.33 min) contains the highest area (%) and UV spectrum analysis identified it as flavonoids. It is therefore suggested that EEAIT can provide a definite protective effect against chronic hepatic injury caused by alcohol in rats, which may mainly be associated with its antioxidative effect.

1. Introduction

Alcoholic liver disease (ALD) is the most frequent cause of sustained excessive alcohol consumption [1]. ALD may take the forms of acute involvement (alcoholic hepatitis) or chronic liver disease (steatosis, steatohepatitis, fibrosis, and cirrhosis). Furthermore, sustained excessive alcohol intake favours the progression of other liver diseases, such as hepatotoxicity, which refers to liver dysfunction or liver damage [2].

Toxic substances generated during the metabolism of alcohol in the liver may contribute to the development of ALD. These substances include ROS that can destroy vital cell components through a process called oxidation. Recent studies have demonstrated that ROS and the resulting oxidative stress play a pivotal role in apoptosis. Antioxidants and overexpression of superoxide dismutase (MnSOD) can block or delay apoptosis of the vital cells [3]. Oxidative stress may also play a key role in apoptosis [4].

From the ancient past in India, several medicinal plants have been extensively used for the management of liver disorder. *Alocasia indica* is an indigenous herb belonging to family Araceae, traditionally used in inflammation and in diseases of abdomen and spleen [5]. This plant contains flavonoids, cyanogenic glycosides, ascorbic acid, gallic acid, malic acid, oxalic acid, aloacin, amino acids, succinic acid, and β-lectins [6]. The juice of leaves of the plant is used as digestive, laxative, diuretic, and astringent and for the treatment of rheumatic arthritis [7]. The leaves of *A. indica* showed antioxidant, antinociceptive, and anti-inflammatory
activities and hepatoprotective activity against CCl₄ induced liver damage model [8, 9]. It was reported that the leaves of the plant showed anthelmintic [10], antimicrobial [11], antidiarrheal, and in vitro antiprotozoal activities [12]. The rootstocks of the plant demonstrate free radical scavenging activity [13]. Alcoholic extracts of A. indica Schott. leaves (Araceae) showed anti-diabetic and hypolipidemic effect in streptozotocin induced diabetic rats [14]. In a separate report, starch obtained from A. indica Linn. tubers (Araceae) was evaluated as a disintegrating agent [15]. In our previous study, the in vitro antioxidant activity and GCMS analysis of the ethanolic extract of A. indica tuber showed potential antioxidant activity with presence of bioactive phytosterol in the extract [16]. To the best of our knowledge, no other report was available using the tuber of the plant as hepatoprotectant against alcohol-induced liver damage. The objective of this study was to assess the hepatoprotective effects of ethanol extracted A. indica tuber extracts on the alcohol-induced liver damage rat model. This study also aimed to establish the correlation between antioxidant activity and antiapoptotic activity of the extract.

2. Methodology

2.1. Plant Material. The tuber vegetable (Alocasia indica) was collected from the local market of Kolkata, West Bengal, India, and was authenticated by the Botany Department of Serampore College, Hooghly, India. The tuber was chopped (2 x 2 inches) and dried under sun (40 ± 5°C) for a week. The dried tuber was finely powdered in grinder and sieved through the 40-micron sieve and stored in air tight containers.

2.2. Preparation of Ethanolic Extract of A. indica Tuber. 100 g of the dried and powdered tuber of A. indica was extracted in 500 mL of 80% (v/v) ethanol for 72 h in Soxhlet apparatus, and the extract was centrifuged for 15 min at 4000 rpm. Supernatant was taken as ethanolic extract of A. indica tuber (EEAIT), concentrated using rotary evaporator at 40°C, dried in lyophilizer, and kept at −20°C for further use.

2.3. Induction of Experimental Hepatotoxicity by Alcohol. Female Wistar rats weighing 110 ± 4.5 g were kept in at the Central Animal House (ICCB, Kolkata) at 12 h light/dark cycle and at 25 ± 2°C. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) of Indian Institute of Chemical Biology, Kolkata (ICCB/AEC-APP/June meeting/2013). The animals were allocated into four groups with five rats in each group and provided with a control diet composed of carbohydrate (71%), protein (18%), fat (7%), and salt mixture (4%) [17]. The experimental group of animals received alcohol by intraperitoneal injection (i.p.) at the dose of 3 g ethanol (15%, v/v) per kg body weight per day for 15 days. Absolute ethanol was diluted with 0.9% (w/v) NaCl to get the desired concentration. EEAIT was also injected intraperitoneally by the following manner after performing the routine toxicity tests of the extract [9]. The experiment was designed as follows.

Group 1. (C) Control animals received normal rat diet and water and were injected with normal saline (0.9% NaCl) for the whole experimental period.

Group 2. (E) Ethanol treated group had rats that received intraperitoneal injection of ethanol (3 gm/kg body weight/day) for 15 days.

Group 3. (E + AI400) Low dose treated group had rats that received intraperitoneal injection of ethanol (3 gm/kg body weight/day) with EEAIT(200 mg/kg body weight/day) for 15 days.

Group 4. (E + AI400) High dose treated group had rats that received intraperitoneal injection of ethanol (3 gm/kg body weight/day) with EEAIT(400 mg/kg body weight/day) for 15 days.

Group 5. (S) Standard group had rats that received intraperitoneal injection of ethanol (3 gm/kg body weight/day) with silymarin (100 mg/kg body wt/day) for 15 days.

At the end of the experiment, all the rats were sacrificed by cervical dislocation. Blood serum was collected via cardiac puncture and subjected to serum biochemical analysis. Liver was immediately separated for biochemical, histopathological, and immunohistochemical evaluation. All experiments were performed in triplicate.

2.4. Serum Analysis. Activities of liver marker enzyme in blood serum, including AST, ALT, γGT, and bilirubin, were measured using kits obtained from ACCUREX biomedical Pvt. Ltd.

2.5. Preparation of Liver Homogenate. Liver was cut into small pieces on ice and homogenized in 10% Tris HCl buffer (0.1mole/L, pH 7.4) [17]. The homogenate was centrifuged at 10000 rpm at 4°C and the supernatant was collected for the estimation of NO, MDA, and SOD. For catalase and GSH estimation, isotonic phosphate buffer was used for homogenization of liver at pH 7.4, 0.1 M [17] and pH 8, 0.01 M [17], respectively, and then the tissue extracts were prepared according to the method mentioned above.

2.6. Estimation of Protein. Protein in the homogenate of liver tissue was estimated by the method of Lowry using BSA as standard [18].

2.7. Estimation of NO Production. NO production was estimated by Griess reaction, which was expressed in the form of nitrite accumulation. Liver homogenates (100μL) were loaded into microtitre plate followed by addition of 100 μL Griess reagent (1% sulfanilamide in 5% H₃PO₄ and 0.1% naphthyl ethylene diamine dihydrochloride) and incubated at room temperature for 10 minutes. Later, the absorbance
was taken at 550 nm using ELISA Reader (Thermo Scientific, USA) [17]. The results were expressed as μmole/mg protein.

2.8. Estimation of Lipid Peroxidation. Lipid peroxidation was detected by measuring thiobarbituric acid reactive substance (TBARS). Two mL of liver homogenate was mixed with 1 mL of 20% (v/v) TCA and 1 mL of 0.67% (v/v) TBA and then boiled for 10 minutes. After cooling, the mixture was filtered through Whatman filter paper and the reading of filtrate was taken at 530 nm [17]. The amount (mmol) of MDA/mg protein was quantitated as an index of lipid peroxidation.

2.9. Estimation of GSH. In 200 μL PBS, 20 μL of liver extracts was mixed with 10 μL dTNP (4 mg/mL in methanol) and then incubated at room temperature for 15 minutes. After that, readings were taken at 412 nm [17]. Results were expressed as mmol GSH/mg protein.

2.10. Estimation of Antioxidant Enzymes: SOD and CAT. Assay of SOD activity is based on the inhibition of NBT reduction by SOD. Briefly, 2.5 mL of 0.05 mol sodium carbonate buffer (pH 10) was mixed with 0.1 mL of 3 mmol/L EDTA, 3 mmol/L xanthine, 1.5 mg/mL bovine serum albumin buffer at pH 10, and the homogenates of liver. Reaction was initiated by adding 0.1 mL of 56 mU/mL xanthine oxidase [19]. After 30 min of incubation, the reaction was terminated by mixing 6 mL of CuCl₂ and was centrifuged at 350 g for 10 min. Absorbance of blue formazan was recorded at 560 nm. The relative absorbance was then converted into unit of SOD activity per mg protein.

Catalase activity was determined by the decomposition of H₂O₂ at 240 nm at 25° C [20]. 0.2 mL of H₂O₂ solution (10 mmol/L dissolved in 50 mmol/L potassium phosphate buffer at pH 7.0) was mixed with 0.1 mL of liver homogenates and decrease of absorbance in every 30 s over a period of 3 min was recorded. Changes in the rate of absorbance were converted into unit of catalase/mg protein using a conversion factor (3.45), which corresponds to the decomposition of 3.45 micromoles of hydrogen peroxide in a reaction mixture producing a decrease in the absorbance from 0.45 to 0.40 units.

2.11. Histopathological Evaluation. After 15 days of treatment, livers of all animals were fixed in 10% formalin, embedded in paraffin, cut into 5-6 μm by rotary microtome, and stained with haematoxylin-eosin to assess the histopathological changes.

2.12. TUNEL Assay. Liver sections were deparaffinized and rehydrated in descending alcohol concentrations. Apoptotic cells in liver were detected using Apo-BrdU-IHC in situ DNA fragmentation assay kit obtained from BioVision, USA.

2.13. Immunocytochemistry. Detection of NFkB and caspase-3 was done by the method of Giakoustidis et al., 2008 [21]. Deparaffinized and rehydrated liver sections were prepared for incubation with cleaved caspase-3 (Asp 175) antibody (Cell Signaling Technology Inc., Danvers, MA) at a dilution 1/200 or NF-kB p65 antibody at a dilution 1/1000 (Cell Signaling Technology Inc., Danvers, MA) overnight at 48°C. Sections were then incubated with extrAvidin peroxidase conjugates (Sigma-Aldrich) and finally were stained with DAB tablets (Sigma-Aldrich).

2.14. HPLC and UV Spectrum Analysis. HPLC analysis was conducted with a Shimadzu chromatograph equipped with photodiode array detector and a 4.6 × 250 mm reverse phase C18 column. Dried EEAIT was dissolved in appropriate 20% acetonitrile. The sample analysis of the sample was performed at room temperature, in the wavelength range of 254 at 1600 psi using a flow rate of 1.0 mL/min. The injection volume of samples was 50 μL. Total run time was 60 minutes. The gradient elution of two solvents was used—solvent A (TFA 0.1% in water) and solvent B (TFA 0.1% in 100% acetonitrile). The gradient program was begun with 100% A and was held at this concentration for the first 5 minutes. This was followed by 100% eluent B for the subsequent 35 minutes after which the concentration of B was stable 100% for the next 5 minutes and then reduced to 0% in the next 10 minutes. UV spectrum analysis was done in parallel by scanning the chromatogram in a UV range of 200–400 nm in HPLC.

2.15. Statistics. Data were expressed as mean ± SE. Kruskal-Wallis nonparametric ANOVA test was performed to find whether or not scores of different groups differ significantly. To test intergroup significant difference, Mann-Whitney U multiple comparisons test was performed. Differences were considered significant if P < 0.05.

3. Results

3.1. Effect on Serums ALT, AST, γGT, and Bilirubin Levels. Serums ALT and AST are two biochemical markers normally used for early stage assessment of liver injury. Alcohol-induced elevation of the two marker enzymes’ serum ALT (76.63%, P < 0.01) and AST (52.63%, P < 0.01) levels was compared to normal group indicating the incident of liver injury (Table 1). Treatment with EEAIT at the low dose (200 mg/kg/day) displayed the recovery percentage of serums ALT (46.34%, P < 0.05) and AST (28.57%) followed by high dose (400 mg/kg/day) ALT (73.17%, P < 0.01) and AST (100% P < 0.01), when compared to alcohol treated group. Posttreatment with EEAIT recovered serum γGT level by 42.39%, P < 0.05 and 75.72%, P < 0.01 at the concentration of 200 mg/kg/day and 400 mg/kg/day, respectively, which was increased drastically by 81.31%, P < 0.01 in alcohol intoxicated group (Table 1). The same effect was observed in serum bilirubin (Table 1) in which alcohol intoxication increased bilirubin level about four times (341.17%, P < 0.01) in comparison to the control group. EEAIT at the concentration of 200 mg/kg/day and 400 mg/kg/day could reduce the bilirubin level by 1.5 times (P < 0.01) and 2.8 times (P < 0.01), respectively, compared to alcohol treated group. The results are close to the results obtained by standard silymarin treated group with administration of EEAIT at the concentration of 400 mg/kg/day.
effect of EEAIT on the biochemical parameters in ethanol-induced hepatotoxicity in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>E</th>
<th>E + AI200</th>
<th>E + AI400</th>
<th>E + silymarin</th>
<th>Kruskal-Wallis nonparametric ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>21.4 ± 3.4</td>
<td>37.8 ± 4.5**</td>
<td>30.2 ± 2.6b</td>
<td>25.8 ± 2.4**</td>
<td>24.2 ± 1.7**</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>26.6 ± 3.8</td>
<td>40.6 ± 3.4**</td>
<td>36.4 ± 4.4**</td>
<td>26.7 ± 6.1**</td>
<td>26.6 ± 4.4**</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>yGT (U/L)</td>
<td>7.6 ± 1.0</td>
<td>13.78 ± 2.3**</td>
<td>11.16 ± 2.2b</td>
<td>9.1 ± 0.9**</td>
<td>8.5 ± 1.4**</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Bilirubin (U/L)</td>
<td>0.17 ± 0.04</td>
<td>0.75 ± 0.1**</td>
<td>0.49 ± 0.05b**</td>
<td>0.26 ± 0.06**</td>
<td>0.19 ± 0.02**</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>NO (μM/mg protein)</td>
<td>58.6 ± 7.0</td>
<td>79.4 ± 7.7**</td>
<td>64.6 ± 2.1b**</td>
<td>62.2 ± 3.7**</td>
<td>60.3 ± 2.3**</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>GSH (mM/mg protein)</td>
<td>9.3 ± 0.86</td>
<td>7.13 ± 0.46**</td>
<td>7.5 ± 0.65**</td>
<td>8.05 ± 0.56c**</td>
<td>9.1 ± 0.8**</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>MDA (mM/mg protein)</td>
<td>77.9 ± 2.8</td>
<td>99.3 ± 0.88**</td>
<td>89.4 ± 3.1b**</td>
<td>81.7 ± 1.5c**</td>
<td>78.7 ± 1.3**</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>0.3 ± 0.004</td>
<td>0.31 ± 0.002**</td>
<td>0.34 ± 0.006**</td>
<td>0.35 ± 0.004c**</td>
<td>0.34 ± 0.001**</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>CAT (U/mg protein)</td>
<td>153.8 ± 11.4</td>
<td>50.6 ± 28.1**</td>
<td>69.4 ± 36.4**</td>
<td>125.6 ± 17.8**</td>
<td>149.7 ± 12.3**</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

Significance level based on Kruskal-Wallis nonparametric ANOVA and Mann Whitney U multiple comparisons tests—^control versus ethanol, ^ethanol versus AI200, ^ethanol versus AI400, ethanol versus AI400, ^AI200 versus AI400. *P < 0.05, **P < 0.01, ***P < 0.001, NS: not significant (mean ± SD).

3.2. Effect on MDA, NO, and GSH Content in Liver Homogenate. MDA and NO levels were markedly increased in ethanol-attenuated liver, hallmarks of lipid peroxidation, and inflammatory response. Enhanced MDA (27.32%, P < 0.01) and NO (35.64%, P < 0.01) levels in alcohol treated rats were decreased by 46.19%, P < 0.01 and 70.72% in EEAIT treated rat at the concentration of 200 mg/kg/day (Table 1). The recovery percentage of EEAIT at 400 mg/kg/day was enhanced to 82.51% for MDA and NO, respectively.

In the alcohol treated group, there was a significant decrease in GSH content (23.57%, P < 0.01) as compared to control group. EEAIT at 200 mg/kg/day enhanced the GSH level by 17.27% (P < 0.05) but the result is statistically insignificant. The extract at concentration of 400 mg/kg/day can increase GSH level significantly (41.81%, P < 0.05) (Table 1). The results of liver marker enzymes, NO, MDA, and GSH, in the extract coadministered groups were comparable with that of the silymarin treated group.

3.3. Effect on the Level of SOD and CAT in Liver Homogenate. After being intoxicated with ethanol, a decline in the level of SOD (8.51%, P < 0.01) and catalase (67.12%, P < 0.01) was observed in liver injury groups (ethanol-induced) when compared to the normal group. SOD activity was back to normal by EEAIT at the lower concentration though catalase activity recovered significantly by 72.64% (P < 0.01) at the higher doses (Table 1).

3.4. Effect on Serum Levels of Insulin. There was a significant decrease in the levels of insulin hormone in ethanol administration (P < 0.05). Supplementation with 400 mg/kg/day EEAIT significantly recovered ethanol-induced changes in plasma insulin level by 46.53% (P < 0.05) (Figure 1).

3.5. Histopathological Changes of Liver. Histological analysis (Figure 2) showed that there was no pathological abnormality observed in the liver of control rat (Figure 2(a)). In comparison to the normal liver architecture of the control group Figure 2(b) showed the alcohol-induced severe necrotic changes and other changes in liver section such as microvesicular steatosis, increase in sinusoidal space, inflammatory infiltration of lymphocytes, and dilation of the central vein with an increased number of fat droplets. These findings indicated early phases of liver injury in alcohol treated group. In the experimental groups, EEAIT at the concentration of 200 mg/kg/day (Figure 2(c)) showed the moderate protection.
in liver morphology as observed in the normal pattern of the central vein and radiating pattern of cell plates. On the other hand, EEAIT at the concentration of 400 mg/kg/day exhibited significant protection from liver damage (Figure 2(d)) as evidenced by noticeable recovery from ethanol-induced liver damage with fewer microvesicular steatoses, hepatocytes necrosis features, and absence of fat droplets.

3.6. TUNEL Assay of Treated and Untreated Liver Sections. The TUNEL assays (Figure 3) showed no apoptotic nuclei in the control liver tissue (Figure 3(a)), whereas in ethanol treated liver, large quantities of positive TUNEL cells were observed with numerous condensed and fragmented nuclei (brown in colour indicated by arrow, Figure 3(b)). In EEAIT treated group the quantities of positively stained cells were decreased significantly and simultaneously with increasing concentration of EEAIT from 200 to 400 mg/kg/day (Figures 3(c)-3(d)).

3.7. Immunocytochemistry. Activated NF-κB was assessed by immunocytochemistry by using activation specific monoclonal antibodies to NF-κB p65 subunit, the epitope of which binds only after IκB dissociation. In this experiment, much fewer numbers of positively stained cells were observed in the control (Figure 4(a)) group, which was enhanced markedly in alcohol intoxicated liver sections (Figure 4(b)). Supplementation with EEAIT at the concentration of 200 mg/kg/day (Figure 4(c)) and 400 mg/kg/day (Figure 4(d)) can recover the changes as observed in simultaneous reduction of the positively stained cell number from lower dose to the higher dose. Similar patterns of results were also obtained in case of activated caspase-3 (Figures 5(a)–5(d)).
Figure 3: TUNEL assays of liver sections from rats treated with ethanol (3 gm/kg/day) and supplemented with EEAIT. Liver section from control (a), treated with ethanol (b), treated with ethanol and EEAIT at 200 mg/kg/day (c) and 400 mg/kg/day (d). Black arrow indicates apoptotic cells. Sections were taken at 40x magnifications.

3.8. HPLC and UV Spectrum Analysis of EEAIT. HPLC analysis of the crude EEAIT showed the presence of three major peaks at the retention time of 28.91 min, 33.33 min, and 37.95 min designated as peaks 1, 2, and 3, respectively, at 254 nm (Figure 6(a)). UV spectrum analysis of peak 1 showed maximum absorption at 222 nm and 217 nm, peak 2 at 201 and 239 nm, and peak 3 only at 216 nm.

4. Discussion

The present study showed the effect of the extract in reducing alcohol-induced changes in the liver of the rat model.

The hepatoprotective effects of EEAIT were assessed by measuring the serum markers such as AST, ALT, GGT, and bilirubin in an in vivo study in alcohol intoxicated rat. Excessive alcohol administration can accumulate NADH, which is the obvious fate of alcohol metabolism by alcohol dehydrogenases. Enhanced NADH production can synthesize more fatty acids and triglycerides and the leakage of cellular enzymes into plasma associated with serum ALT and AST [22]. Moreover, excess alcohol consumption has been linked with altered liver metabolism with leakage of cytoplasmic liver enzyme γGT into blood [23]. Markers of severe alcoholic hepatitis or cirrhosis also include elevated levels of bilirubin into the plasma. Besides, results from histological images that showed accumulations of fatty droplets in the hepatocytes also provided clear evidence that the preinduction with alcohol (3 gm/kg/day) produced liver damage, including loss of cell membrane integrity and accumulation of fatty acids, in the rat. Thus, by restoring the level of serums ALT, AST, GGT, and bilirubin back to normal in alcohol intoxicated rat, a high dose (400 mg/kg/day) of EEAIT has certified its hepatoprotective effects against alcohol intoxication at least in part by reducing fat droplets in liver. Phenolic compound present in EEAIT [16] may also give membrane stability and repair liver damage caused by alcohol [24]. Liver is the main target organ of insulin and decrease in insulin level by alcohol can provide the dysfunction features of the pancreas which also indirectly affects liver functions. EEAIT also recovered the hormone level which is drastically decreased by alcohol.

GSH depletion is considered to be the chief factor leading to lipid peroxidation [25]. Earlier report revealed that alcohol was capable of generating by ROS glutathione synthesis inhibitor, producing glutathione loss from the
Figure 4: NF-kB p65 expression in immunohistochemistry of liver sections from rats in different experimental groups (a)–(d). (a) Liver section from control showed normal architecture of the liver section. (b) A huge amount of apoptotic dead cells was found in liver section of alcohol treated rat, which was indicated by the white arrow. (d) Moderate apoptotic bodies were shown by EEAIT administration at the concentration of 200 mg/kg/day. (d) Almost normal architect of the liver section found in EEAIT treatment at the concentration of 400 mg/kg/day. All the sections were taken at 40x magnifications.

tissue, and increasing MDA levels [26]. In our experiment increased amount of MDA in alcohol-induced liver signifies the enhanced degree of lipid peroxidation, which can lead to liver damage. Supplementation of EEAIT (200 and 400 mg/kg/day) decreased GSH level with a concomitant increase in MDA level as expected.

NO is an inflammatory mediator and highly reactive oxidant produced by iNOS, released by kupffer cells upon exposure to hepatotoxins such as alcohol [27]. Overproduction of NO by chronic alcohol exposure may stimulate the synthesis of some cytokines, such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, and TNF-α, which in turn stimulate the synthesis of NO [28]. In our study increased production of NO in the ethanol group was restored to the normal level by EEAIT.

As a consequence of the constant oxidative challenge, cells have used their antioxidant systems (SOD and catalase) to counter the peroxidant fluxes [29]. A decrease in both activities in liver tissue of the alcohol-induced group was largely due to the impairment of antioxidant enzymes, which can protect the cells against ROS. Higher amount of total phenolic content and strong antioxidant activity in EEAIT [16] may enhance both the enzyme activities.

A plausible justification for hepatoprotective and antioxidant effects of EEAIT may be due to the presence of flavonoids and phenolic acids, which were highly detected particularly in ethanolic extraction method [16]. In the present study, histological evaluation was undertaken to support the biochemistry profiles. Elevation of the level of liver function biomarkers ALT, AST, γGT, and bilirubin in blood was detected along with the decrease in antioxidant enzyme activity and severe necrosis in histopathological changes by 15-day administration of alcohol at the dose of 3 gm/kg/day. Liver injury hallmarks such as inflammation, necrosis, and fat droplets were restored back close to normal after administration of high dose (400 mg/kg/day) of EEAIT. The result was supported by the decrease in ALT, AST, γGT,
Figure 5: Expression of caspase-3 in immunohistochemistry. (a) Liver section from control showed normal architecture of the liver section. (b) A huge amount of apoptotic dead cell was found in alcohol treated liver indicated by the black arrow. (c) Moderate apoptotic bodies were shown by EEAIT administration at 200 mg/kg/day. (d) Almost normal architect of the liver section found in EEAIT treatment at the concentration of 400 mg/kg/day. All the sections were taken at 40x magnifications.

Figure 6: HPLC chromatogram of EEAIT and UV analysis of the three peaks RT: 28.91, 33.33, and 37.95 min (indicated by black arrow).
bilirubin, NO, and MDA and increase in GSH level, CAT, and SOD activities. Our observation also proves that EEAIT restores the liver injury in a dose-dependent manner.

Indication of apoptosis by TUNEL assay is morphologically characterized by some cellular changes, including DNA fragmentation and the appearance of cytoplasmic apoptotic bodies [30]. In our study, the cell death was detected by an increase in the apoptotic cell number in chronic alcohol-exposed rat livers by TUNEL assay, which was recovered by administration of EEAIT.

Recent investigations suggest that the induction of NF-κB-dependent gene expression in Kupffer cells contribute to alcohol-induced liver injury [31]. In this study downregulation of NF-κB signalling by EEAIT in ethanol-induced injury was due to suppression of NF-κB-dependent target genes in alcohol treated liver. It was also reported that caspase-3 activation in the ethanol treated liver occurred mostly in the hepatocytes around the central vein [32]. Increased expression of caspase-3 was observed in alcohol-induced rat in TUNEL assay and consequence reduction of the expression in EEAIT treatment supports the antiapoptotic action of the extract. The finding was supported by other reports [33]. So the result suggests that activation of NF-κB and caspase-3 may contribute to hepatocyte death in alcohol treated rats. Supplementation with 200 mg/kg/day and 400 mg/kg/day EEAIT in ethanol treated rats successfully reduced these effects of alcohol in liver.

Qualitative analysis EEAIT in reverse phase HPLC showed three consecutive peaks at different retention time of which peak 1 (RT: 28.91 min) and peak 2 (RT: 33.33 min) showed a typical UV absorption spectrum pattern of flavonoid compounds [34] whereas peak 3 (37.95 min) showed maximum absorption in 217 nm wavelength, which may be another secondary compound and have to be analysed. MS analysis and other studies of the three compounds are in our future project.

5. Conclusions

The results strongly imply the potential use of EEAIT in future application for treatment in reducing oxidative stress, alcoholic liver disease, and apoptotic cell death of vital organs such as liver, which can create a lot of possibilities in therapeutic aspects.

Abbreviations

EEAIT: Ethanolic extract of A. indica tuber
ALD: Alcoholic liver disease
ROS: Reactive oxygen species
SOD: Superoxide dismutase
i.p.: Intraperitoneal injection
NaCl: Sodium chloride
AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
γGT: Glutamyl transpeptidase
HCl: Hydrochloric acid
NO: Nitric oxide
MDA: Malonaldehyde
SOD: Superoxide dismutase
GSH: Glutathione
BSA: Bovine serum albumin
H₃PO₄: Phosphoric acid
TBARS: Thiobarbituric acid reactive substance
TCA: Trichloroacetic acid
TBA: Thiobarbituric acid
PBS: Phosphate buffer saline
dTNP: 2′,2′-Dithiobis 5 nitropyridine
NBT: Nitroblue tetrazolium
EDTA: Ethylenediamine tetraacetic acid
CuCl₂: Cupric chloride
H₂O₂: Hydrogen peroxide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Research Article

Bioassay-Guided Isolation and Antioxidant Evaluation of Flavonoid Compound from Aerial Parts of Lippia nodiflora L.

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The present study was designed to identify the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free-radical scavenging constituents from methanol extract of L. nodiflora using bioassay-guided fractionation. The ethyl acetate fraction (EAF) revealed a strong antioxidant activity, compared to other fractions through in vitro DPPH radical-scavenging assay. The repeated fractionation of active EAF by silica gel column chromatography yielded a compound with strong antioxidant potential. The isolated bioactive compound was determined as 2-(3, 4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (5-hydroxy-3',4',7-trimethoxyflavone), by comparing spectral data (UV, IR, $^1$H NMR, $^{13}$C NMR, and MS) with literature reports. The isolated compound demonstrated an excellent antioxidant activity through all antioxidant assays and also significantly inhibited lipid peroxidation at a concentration of 50 μg/mL. The results obtained suggested that extracts from L. nodiflora or its derived phytocompound can be used potentially as a bioactive source of natural antioxidants by contributing beneficial health effects.

1. Introduction

Reactive oxygen species (ROS) are continuously formed as a by-product of metabolisms in aerobic organisms and are also produced on exposure to tobacco smoke, ozone, radiations, organic solvents, and other environmental pollutants [1]. ROS play an important role in various physiological processes, including energy production, phagocytosis, cellular signal transduction, cell proliferation, differentiation, and apoptosis. On the other hand, increasing evidence highlights that overproduction of ROS can induce oxidative damage to all biomolecules (lipids, carbohydrates, proteins, enzymes, DNA, and RNA) and acts as a mediator of numerous disorders, for example, inflammation, arthritis, diabetes, arteriosclerosis, cancer, genotoxicity, and neurological disorders such as Alzheimer’s disease [2]. Antioxidants are very essential for averting degenerative reactions produced by free radicals and reactive oxygen species, which have been concerned with many diseases and in food deterioration and spoilage [3]. However, the safety of some of the synthetic antioxidants used in the food industry has been questioned, because recent studies recognized that they might be carcinogenic [4]. Hence, there is an emerging interest in natural antioxidants, which might help to prevent oxidative damage [5].

Plants and plant products are magnificent sources of phytochemicals and have been found to hold an array of biological activities including antioxidant potential [6]. Plants synthesize antioxidant compounds, mostly flavonoids and polyphenols, which have been reported to protect the human body from various diseases by neutralizing ROS. Recently, phenolic compounds have received increasing significance among various phytochemicals, due to their wide distribution in the plant kingdom and for their biological activities, namely, anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial, and antioxidant activities [7–9]. Antioxidants can be either used as dietary, food supplement or as a drug by the humans [10]. Several studies have revealed that the increased dietary intake of natural antioxidants, such as flavonoids and other phenolic compounds, almost present in plants, exhibits potential protective effects against many degenerative diseases [11–13].
Lippia nodiflora Linn. (Verbenaceae), commonly called Poduthalai in Tamil, is a creeping perennial herb and locally abundant in wet regions, and several medicinal properties are attributed to this plant in the traditional system of medicine. The infusion of the leaves and tender stalks is given to children suffering from indigestion and to women after delivery. The chutney made from the leaves and fruits are often taken to relieve the irritation of internal piles [14]. Numerous pharmacological properties of L. nodiflora including anti-inflammatory, antipyretic, antitussive, antioxidant, antiabetic, and antimelanogenesis effects have been reported [15–18]. The ethnopharmacological relevance of L. nodiflora for skin diseases and in folk cosmetics, such as pimples, carbuncle, and skin burns, has also been revealed [19]. The phytochemical constituents of L. nodiflora, such as flavonoids [20, 21], flavone glycosides [22], alkaloids, essential oils, resin [23], quinol [24], and steroids [25, 26], have been previously reported. Hence, these phytochemicals are considered to be accountable for the pharmacological properties of this plant. Even though there is an evidence for the antioxidant activity of methanolic extract of L. nodiflora, the major antioxidative constituents present in the aerial parts have not been extensively investigated. Hence, the present study was undertaken to isolate active compounds responsible for the antioxidant property of methanol extract of Lippia nodiflora L. through bioassay-guided fractionation using in vitro DPPH assay.

2. Materials and Methods

2.1. General. Nuclear magnetic resonance spectra were recorded on BRUKER, Avance 400 MHz (Switzerland) NMR instrument, operating at 400 MHz for $^1$H and 100 MHz for $^{13}$C nuclei at room temperature and referenced to the residual solvent signal. The chemical shift and coupling constants (J) values are reported in ppm and Hz, respectively. HPLC analysis was performed using a C-18 column (250 × 4.6 mm, 5 μ) in a Shimadzu LC-8A chromatographic apparatus (Shimadzu, Singapore). The mobile phase consisted of methanol-0.5% phosphoric acid in water (60:40, v/v) and the flow rate was held constant at 1 mL/min. The peaks are detected at 280 nm, using variable wavelength UV detector. Silica gel 60 F$_{254}$ plates (20 × 20 cm, 0.2 mm thick; E-Merck, Germany) were used for thin-layer chromatography (TLC) analysis. The ultraviolet spectra were recorded using Varian Cary 5000 scan spectrophotometer, $\lambda_{\text{max}}$ (log ε) in nm, whereas the FTIR spectrum was obtained using a Nicolet 380 (Thermo Scientific, USA). The functional group was identified using potassium bromide (KBr) and scanned in the range of 4000–4000 cm$^{-1}$. Sample was dissolved in methanol and ESI mass spectra were obtained with a Thermo Scientific Exact Mass Spectrometer (Thermo Fisher Scientific, USA).

The reagents such as 2-deoxy-D-ribose, butylated hydroxyl toluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), sodium nitroprusside, and Griess reagent were obtained from (Sigma Chemicals St. Louis, MO, USA). 2, 4, 6-Tripyridyl-s-triazine (TPTZ), thiobarbituric acid (TBA), trichloroacetic acid (TCA) ethylene diaminetetraacetic acid (EDTA), ferric chloride (FeCl$_3$), hydrogen peroxide (H$_2$O$_2$), and nicotinamide adenine dinucleotide-reduced (NADH) were obtained from M/s (Sisco Research laboratories, Mumbai, India). HPLC grade solvents and reagents used for extraction and silica gel (0.075–0.15 mm) for column chromatography were obtained from M/s (Sisco Research laboratories, Mumbai, India). All other chemicals and reagents used in this study were of analytical grade.

2.2. Plant Materials. The plant materials were freshly collected between August and September 2011, from Karaikudi, Sivagangai district, Tamil Nadu. The plant was taxonomically identified and authenticated by Dr. G. V. S. Murthy, Joint Director, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore (BSI/SRC/5/23/2012-13/Tech-19). A voucher specimen of the plant L. nodiflora (DBI-HP: Specimen-1) was prepared and preserved in the Molecular Biology Lab, Department of Bioinformatics, Alagappa University, Karaikudi, Tamil Nadu.

2.3. Extraction, Fractionation, and Isolation of Antioxidant Compound. The aerial parts (stem, leaves, and flowers) were washed with tap water, shade-dried, and reduced to fine powder. The powdered aerial parts of L. nodiflora (1.5 kg) were extracted with 90% methanol (4.5 L × 2) at room temperature. The mixture was filtered through Whatman number 1 filter paper and the solvents from the combined extract were concentrated using a vacuum rotary evaporator (Superfit, India), at 60°C to afford 64.7 g of crude methanol extract (4.31%). The solvent was selected based on its yield from preliminary extraction and phytochemical screening studies. The extract was dissolved in 500 mL of warm water, and the resulting aqueous portion was partitioned with ethyl acetate (EtOAc) (4 × 200 mL) using a separating funnel to afford ethyl acetate fraction (EAF) and aqueous portion. The aqueous phase was then successively partitioned with n-butanol (3 × 200 mL), thus obtaining n-butanol soluble fraction (BF) and water fraction (WF). All the fractions were collected separately and reduced using a vacuum rotary evaporator to remove the solvent and the resultant aqueous extract was lyophilized in vacuo. The samples were then tested for its antioxidant property using 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^*$) assay.

The DPPH$^*$ active EAF (17.2 g) was loaded as a dried slurry of silica gel to column chromatography (45 × 3.5 cm) and eluted with petroleum ether: EtOAc gradient elution (100:0–0:100), in increasing order of polarity. A total of 92 fractions of 100 mL each were collected and analyzed by TLC (Silica gel F$_{254}$ plates 20 × 20 cm, Merck, Germany). TLC analysis was carried out using ethyl acetate: chloroform: formic acid (5:4:1) as the mobile phase and the separated bands were visualized using iodine vapors and vanillin-sulphuric acid reagent. These fractions were pooled to afford seven major fractions (Fr. A: 1–13, Fr. B: 14–35, Fr. C: 36–48, Fr. D: 49–60, Fr. E: 61–70, Fr. F: 71–80, and Fr. G: 81–92) based on TLC analysis. These fractions were tested for bioactivity using DPPH spectrophotometric assay.
For further purification, the highly active Fr. B (2.8 g) was loaded on a silica gel column, eluted with petroleum ether-EtOAc gradients, and the ethyl acetate content of the mixture was increased in a series of 5% steps. The inactive and less active proved fractions were discarded. Finally, the active Fr. B, eluted with petroleum ether-ethyl acetate (85:15) yielded 117 mg of compound. The purity of isolated compound was established by HPLC and its structure was confirmed through the interpretation of the spectral data (UV, FT-IR, $^1$H, $^{13}$C NMR, and MS) and further tested for its antioxidant effects.

2.4. Antioxidant Activities

2.4.1. DPPH Radical Scavenging Assay. The DPPH radical scavenging activities of methanol extract, EAF, BF, and WF were tested according to Yamaguchi et al. [27]. Briefly, 0.2 mL of the sample solutions of different concentrations was added to 1 mL of 0.1 mM of freshly prepared DPPH solution. The reaction mixtures were shaken vigorously and absorbance at 517 nm was determined after 20 min at room temperature. Control sample was prepared containing the same volume without test compounds or reference antioxidants, while DMSO was used as blank. The reference antioxidant BHT was used as the positive control in all the assays. The radical scavenging activity was measured as a decrease in the absorbance of DPPH$^*$ and calculated as follows:

$$\text{Scavenging effect (\%) = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100,}$$

(1)

where $A_{\text{control}}$ is the absorbance of the control and $A_{\text{sample}}$ is the absorbance of the extract or fractions or standard.

2.4.2. Superoxide Radical-Scavenging Assay. The superoxide radical-scavenging effect was determined by the method of Nishikimi et al. [28]. The reaction mixture with NBT (1 mM) in phosphate buffer (0.1 M, pH 7.4), NADH (1 mM) with or without samples, and PMS (0.1 mM) was incubated at room temperature for 5 min and the absorbance was recorded at 560 nm. The inhibition percentage was calculated against a control without the samples. The scavenging ability was calculated using the equation as described for DPPH assay.

2.4.3. Hydroxyl Radical Scavenging Assay. The capacity of the extract and compound to reduce hydroxyl radical-mediated peroxidation was carried out by the method of Hinneburg et al. [29]. Briefly, 0.5 mL of 5.6 mM 2-deoxy-D-ribose in KH$_2$PO$_4$–NaOH buffer (50 mM, pH 7.4), 0.2 mL of 100 μM FeCl$_3$, and 104 mM EDTA (1:1 v/v) solution were added to 0.1 mL of different concentrations of test samples, followed by 100 μL of 1.0 mM H$_2$O$_2$ and 0.1 mL of 1.0 mM aqueous BHT. The reaction mixtures were shaken vigorously and incubated at 50°C for 30 min. Subsequently, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube containing reaction mixture and samples were mixed well again and boiled in a water bath at 50°C for 30 min. The absorbance of solution was read at 532 nm. The hydroxyl radical scavenging ability was calculated using the formula as described for DPPH assay and the values are presented as means of triplicate analyses.

2.4.4. FRAP (Ferric Reducing Antioxidant Power) Assay. The FRAP assay was determined by the method of Benzie and Strain [30] with minor modifications. It depends on the ability of the sample to reduce the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to ferrous tripyridyltriazine (Fe(II)-TPTZ) at low pH. Fe(II)-TPTZ has an intensive blue color which can be read at 593 nm. The stock solutions consist of 300 mM acetate buffer (pH 3.6), 10 mM, 2, 4, 6 tripyridyl S triazine (TPTZ) in 40 mM of HCl, and 20 mM ferric chloride solution. The fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ, and 2.5 mL of FeCl$_3$-6H$_2$O and the temperature was maintained to 37°C before use. The various concentrations of extract, compound, and BHT (10–50 μg/mL) were allowed to react with 2 mL of the FRAP solution for 30 min in the dark condition. The absorbance was recorded at 593 nm. The results are expressed in μM Fe(II)/g and were estimated using aqueous FeSO$_4$-7H$_2$O (20–100 μM) as standard for calibration.

2.4.5. Nitric Oxide Radical Scavenging Assay. At physiological pH, nitric oxide generated from sodium nitroprusside in aqueous solution interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [31]. Briefly, 3 mL of the reaction mixture containing 10 mM sodium nitroprusside and the test samples (10–50 μg/mL) in phosphate buffer were incubated for 150 min at 25°C. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride (0.1% w/v) was added and the mixture was allowed to stand for 30 min at 25°C. A pink colored chromophore is generated and the absorbance was measured spectrophotometrically at 540 nm against a blank sample. The nitric oxide radical scavenging activity of the extract and compound is reported as % inhibition and was calculated using the formula as described for DPPH assay.

2.4.6. Lipid Peroxidation Assay. Lipid peroxidation (LPO) assay was performed according to the protocol described by Damien et al. [32] to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media. Briefly, 0.5 mL of egg homogenate (10% v/v prepared in 1.15% w/v KCl) was added to 0.1 mL of each test samples (10–50 μg/mL) taken in a test tube and made up to 1 mL with double distilled water. Thereafter, 0.05 mL of FeSO$_4$ (0.07 M) was added to induce lipid peroxidation, and the mixture was incubated for 30 min at room temperature. Then, 1.5 mL of 3.5 M acetic acid (pH adjusted to 3.5) was added, followed by 1.5 mL of TBA (0.06 M) in sodium dodecyl sulphate (0.04 M). The resulting mixture was vortex and heated at 95°C for 1 hr. After cooling, 5 mL of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and the above procedure was
followed for the control by using 0.1 mL of SDS instead of the test sample. The percentage inhibition was calculated according to the following formula:

\[
\text{Percentage inhibition of lipid peroxidation} = \left(1 - \frac{E}{C}\right) \times 100,
\]

where \(E\) is the absorbance of the test sample and \(C\) is the absorbance of the fully oxidized control.

2.5. Statistical Analysis. The experiments were carried out in triplicate and data were expressed as means ± standard deviation (SD). All statistical analyses were performed using graph pad prism (version 5.0; Graph Pad software Inc. San Diego, CA, USA). The IC\(_{50}\) value represented the concentration of the test samples that caused 50% inhibition. \(P\) values <0.05 were considered as significant.

3. Results and Discussion

3.1. Isolation and Structure Determination of Antioxidant Compound. The methanol extract and potential antioxidant fractions of \(L.\) nodiflora were initially screened by spectrophotometric DPPH assay and the results are shown in Table I. The DPPH assay revealed that the methanol extract had significant scavenging effects with increasing concentrations in the range of 10–50 \(\mu\)g/mL. Moreover, the scavenging effect of methanol extract was significantly similar to that of standard BHT. At 50 \(\mu\)g/mL, methanol extract and BHT exhibited 79.35% and 86.42% inhibition and the IC\(_{50}\) values (the concentration with scavenging activity of 50%) were 24 and 19 \(\mu\)g/mL, respectively. The result obtained herein was lower than the reported scavenging activity of methanol extract of \(L.\) nodiflora (12.03 \(\mu\)g/mL) [33]. Among the fractions, the DPPH* was significantly scavenged by ethyl acetate fraction (EAF) in a dose-dependent manner, with an IC\(_{50}\) value of 26 \(\mu\)g/mL, followed by WF and BF, with IC\(_{50}\) values of 66 and 83 \(\mu\)g/mL (Table I). Hence, the EAF was subjected to repeated bioassay-guided fractionation on silica gel column chromatography using petroleum ether: EtOAc gradient elution system. The further purification of active fractions obtained from silica gel column chromatography finally yielded a bioactive antioxidant compound from the petroleum ether-ethyl acetate (85:15 v/v) mixture. The extraction procedure for the isolation of active compound was schematically shown in Figure 1. Figure 2(a) shows the HPLC profile of the chemical constituents present in the 90% methanol extract of \(L.\) nodiflora. The HPLC chromatogram of purified compound exposed the presence of a peak, with a retention time of 7.2 min, eluted isocratically with the mobile phase of methanol-0.5% phosphoric acid in water (60:40, v/v) (Figure 2(b)).

The compound was obtained as pale white amorphous powder (yield: 117 mg, 0.68%). The FTIR peaks determined the bonds relevant to alcoholic O–H stretching (3267 cm\(^{-1}\)), =C–H stretching (3010 cm\(^{-1}\)), –C–H stretching (2975 cm\(^{-1}\)), –C=O stretching (1605 cm\(^{-1}\)), –C=C stretching (1506 cm\(^{-1}\)), –C–H bending (1154 cm\(^{-1}\)), C–O stretching (1315 cm\(^{-1}\)), =C–H bending (1030 cm\(^{-1}\)), and O–H bending (993 cm\(^{-1}\)).

1\(^{1}\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) (ppm) 3.91 (3H, s, 4′-OMe), 3.89 (3H, s, 7-OMe), 3.87 (3H, s, 3′-OMe), 6.49 (s, 1H, OH), 6.64 (\(d, J = 6.6\) Hz, 1H, H6), 6.898 (s, 1H, H8), 6.99 (\(d, J = 6.3\) Hz, 1H, H2\(^2\)), 7.52 (\(d, J = 6.3\) Hz, 1H, H5\(^3\)), 8.178 (s, 1H, H6\(^6\)); 13\(^{13}\)C NMR (CDCl\(_3\), 100 MHz): \(\delta\) (ppm) 171.61 (C-4′), 144.30 (C-4′), 125.69 (C-1′), 162.87 (C-2′), 161.77 (C-3′) 156.82 (C-5), 130.87 (C-6′), 114.28 (C-5′), 137.29 (C-9), 103.92 (C-2′), 157.74 (C-7), 98.92 (C-6), 98.19 (C-8), 111.47 (C-3), 113.58 (C-10), 54.76 (7-OMe), 54.88 (4′-OMe) (Figures S\(_1\) and S\(_2\) of the Supplementary Information available online at http://dx.doi.org/10.1155/2014/549836). The compound exhibits a molecular weight of 329 (ESI-MS, \(m/z\) 351.084 [M + Na\(^+\)]) with an elemental formula of C\(_{18}\)H\(_{15}\)O\(_{8}\) (Figure 3). From these spectral interpretations, the isolated compound has been characterized as 2-(3, 4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (5-hydroxy-3′, 4′, 7-trimethoxylavone) (Figure 4), which agreed with the data reported [34].

3.2. Antioxidant Activities of Methanol Extract and Isolated Compound from Ethyl Acetate Fraction (EAF). Flavonoids, a major group of polyphenols, are considered to be the active principles in diverse medicinal plants and have been reported to possess numerous pharmacological properties. The most essential biological activity of flavonoids is mainly due to their antioxidant property by acting as radical scavengers, hydrogen donors, reducing agents, and peroxidation inhibitors [35]. Previous studies reported that the pharmacological effects of \(L.\) nodiflora, such as antioxidant, diuretic, anti-inflammatory, and antimicrobial activities, were recognized due to the presence of the phenol and flavonoid compounds [33, 36]. In the present study, the isolated compound was identified as flavone and its antioxidant activities were examined by using various \textit{in vitro} antioxidant models.

3.2.1. DPPH Radical Scavenging Effect. The substances are considered to be antioxidants, when they are capable of reducing the stable DPPH radical (purple) to the nonradical form DPPH-H (yellow), and thus they act as radical scavengers due to their hydrogen donating abilities [37]. The results of DPPH scavenging activity of all test samples are presented in Figure 5(a). The scavenging activity of methanolic extract, compound, and BHT increased with an increase in sample concentration (10–50 \(\mu\)g/mL). The
Figure 1: Extraction scheme for the isolation of antioxidant compound from aerial parts of *Lippia nodiflora* L.

Figure 2: (a) HPLC chromatogram of whole methanol extract of *L. nodiflora*. (b) HPLC chromatogram of isolated compound (5-hydroxy-3', 4', 7-trimethoxyflavone) at 280 nm. The HPLC profile shows the purity of the isolated compound from aerial parts of *L. nodiflora*. 
highest DPPH scavenging activity for extract, compound, and BHT was found to be 79.35%, 72.66%, and 86.09%, respectively, at 50 μg/mL. It should be noted that the scavenging activity of compound was found to be close to the extract. The IC₅₀ values of scavenging activity on DPPH radical of extract and BHT are given in Table 1, whereas, for compound, it is found to be 27 μg/mL. From these obtained data, the extract and compound were considered as an effective free-radical inhibitor as well as the primary antioxidants, which may limit free-radical damage that takes place in the body.

3.2.2. Superoxide Radical Scavenging Effect. The formation of reactive oxygen species such as hydroxyl radical, hydrogen peroxide, and singlet oxygen in living systems was mainly due to the participation of superoxide anion radicals, either directly or widely through enzyme or metal catalyzed progression [38]. It was therefore anticipated to evaluate the relative interceptive capacity of the extract and compound to scavenge the superoxide radical. The data presented in Figure 5(b), it was noted that the extract, compound, and BHT showed the highest radical scavenging activities (71.07%–84.64%) at 50 μg/mL and the scavenging activity increased with increasing concentration of the samples. The scavenging ability of compound on superoxide radicals was found to be moderate compared to methanolic extract. However, the scavenging activities of extract (83.09%) were found to be very closer to that of BHT (84.64%), which is considered to be a strong superoxide radical scavenger. The IC₅₀ value of plant extract, compound, and BHT was found to be 32, 38, and 26 μg/mL, respectively.

3.2.3. Hydroxyl Radical Scavenging Effect. Hydroxyl radical, an extremely known reactive oxygen species, was competent to attack and spoil almost every molecule in the living cells [11]. They were also capable of stimulating lipid peroxidation process rapidly by attacking the fatty acid side chains of the membrane phospholipids [38]. The scavenging activities of methanolic extract, compound, and BHT on hydroxyl radical inhibition are shown in Figure 6. All the examined samples showed significant hydroxyl radical scavenging activity at 50 μg/mL concentration and the scavenging activity for extract, compound, and BHT was 68.38%, 60.90%, and 74.16%, respectively. The methanolic extract of L. nodiflora (IC₅₀ = 36 μg/mL) was more powerful than the compound (IC₅₀ = 43 μg/mL). The positive control, BHT, was extremely effectual on hydroxyl radical scavenging, with an IC₅₀ value of 32 μg/mL. The observed capacity of the extract and compounds to scavenge *OH radical pointed out that the tested samples could considerably inhibit lipid peroxidation, since *OH radicals are extremely distressed during peroxidation.

3.2.4. FRAP (Ferric Reducing Antioxidant Power) Assay. The ferric reducing/antioxidant power (FRAP assay) is widely used in the assessment of the antioxidant component in dietary polyphenols [39]. The reducing properties are usually related to the presence of compounds which exert their action by breaking the free-radical chain by donating a hydrogen atom [40]. The results of reductive potential of plant extract and compound relative to BHT, a well-known antioxidant data, are shown in Table 2. The reducing ability of the extract was in the range of 23.46–71.14 μM Fe (II)/g. The FRAP values for the methanol extract were significantly higher than that of compound and BHT, while the compound revealed the lowest FRAP values (11.18–51.70 μM Fe (II)/g). At 50 μg/mL, the FRAP value of L. nodiflora extract was found to be 71.14 compared to compound and BHT with FRAP value of 51.70 and 63.18, respectively (Table 2). This result implies that the isolated compound did not show reliable reducing power, when compared to its DPPH and superoxide radical scavenging abilities.

3.2.5. Nitric Oxide Radical Scavenging Assay. Nitric oxide radicals (NO⁻) play a vital role in inducing inflammatory response and their toxic effects increase only when they react with superoxide radicals that damage biomolecules like proteins, lipids, and nucleic acids [41]. The suppression of NO radicals release may be attributed to a direct (NO⁻) scavenging effect as both extract and compound decreased the amount of nitrite generated from the decomposition of sodium nitroprusside through in vitro studies as shown in
Figure 5: (a) DPPH radical scavenging and (b) superoxide radical scavenging activities of extract, compound, and BHT at different concentrations. Values are mean ± SD (n = 3).

Figure 6: Hydroxyl radical scavenging effect of extract, compound, and BHT at different concentrations. Values are mean ± SD (n = 3).

Figure 7(a). The extract and compound at 50 μg/mL exhibited 48.6% and 40% inhibition which was comparable to the standard BHT, which exhibited 49.8% inhibition at 40 μg/mL. However, both extract (IC50 = 54 μg/mL) and compound (IC50 = 67 μg/mL) can inhibit NO radicals but lesser when compared to standard BHT (IC50 = 41 μg/mL). The NO radical-scavenging activities of the extract and compound also followed a concentration-dependent pattern. The results suggest that the isolated flavone compound of *L. nodiflora* might be an effective and novel therapeutic agent for scavenging and regulating the pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite.

3.2.6. Inhibitory Activity towards Lipid Peroxidation. The disturbances in the membrane assembly lead to changes in fluidity and permeability, modifications of ion transport, and inhibition of metabolic processes, altogether revealed to be the collective consequence of reactive oxygen species formed during lipid peroxidation [42]. The extract and compound were capable of preventing MDA formation in a concentration-dependent manner (Figure 7(b)). At 50 μg/mL, methanolic extract and compound possessed 62.44% and 54.95% inhibition, while BHT exhibited 68.64%
inhibition at the same concentration. The IC$_{50}$ values for extract and compound were found to be 39 and 46 µg/mL. There is no significant difference in the IC$_{50}$ values of extract and compound ($P < 0.05$). However, IC$_{50}$ values of extract were in good agreement with BHT (IC$_{50} = 34$ µg/mL). The presence of hydroxyl and electron-donating methoxy group in the compound (5-hydroxy-3', 4', 7-trimethoxyflavone) may be accountable for the antioxidant activity in all the experimental assays.

Based on in vitro antioxidant results of the present work, the methanol extract and isolated compound from ethyl acetate fraction of *L. nodiflora* were believed to be an electron donor, capable of counteracting free radicals. This is the first study to give an account on the antioxidant and free-radical scavenging activity of 5-hydroxy-3', 4', 7-trimethoxyflavone from *L. nodiflora*. The results of the present work also propose that the numerous pharmacological properties exerted by *L. nodiflora* may be partly due to the presence of antioxidant flavone compound.

### 4. Conclusion

The present study was projected to assess the antioxidant and free-radical scavenging activities of extract and fractions from aerial parts of *L. nodiflora* by using in vitro antioxidant models. The ethyl acetate fraction (EAF) exhibited highest free-radical scavenging activity, among the fractions. A bioassay-guided fractionation and purification of EAF resulted in the identification of the flavone compound, namely, 5-hydroxy-3', 4', 7-trimethoxyflavone. The measurement of antioxidant activity of the flavone compound, by using various in vitro antioxidant models, proved it to be a potent antioxidant compound. These results signify that methanol extract, ethyl acetate fractions, and isolated compound exhibited interesting antioxidant properties and afford an essential basis for the use of *L. nodiflora* in the treatment of oxidative damages. Furthermore, these findings hold great perception in the development of alternative antioxidant agents, and still further work is warranted to sort out and characterize the active principles from other fractions, in order to establish their therapeutic efficacy and mechanism of action.

### Conflict of Interests

The authors declare no conflict of interests.

### Acknowledgments

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### References


Research Article

In Vitro Antioxidant, Antiproliferative, and Phytochemical Study in Different Extracts of Nyctanthes arbortristis Flowers

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Nyctanthes arbortristis L. (Oleaceae) is widely used in the Indian system of traditional medicine and is reported to have various biological activities. The present study was intended to evaluate the antioxidant and antiproliferative activities of flower extracts of Nyctanthes arbortristis. The shade dried flowers were extracted with 95% ethanol under sonication and the antioxidant activities were investigated using in vitro assays along with the determination of phytochemical constituents (total polyphenol and total flavonoid). Arborside C and β-monogentiobioside ester of α-Crocetin were identified in crude active extract through LCMS/MS analysis. The antiproliferative activity was carried out by MTT assay by employing different human cancer cell lines. The lowest IC_{50} value of 24.56 ± 6.63 μg/mL was observed against Colo 205 cell line. The extract exhibited significant antioxidant and antiproliferative properties and the observed biological activities in this study provide scientific validation of ethnomedicinal use of this plant.

1. Introduction

Cancer is responsible for 12% of the world’s mortality and the second-leading cause of death in the world [1]. In spite of much progress in the recent past in the cancer treatments, a key problem in tumor therapy with established cytostatic compounds is the development of drug resistance and acute side effects. Most available drugs suffer from insufficient specificity toward tumor cells [2]. Hence, the identification of better antitumor drugs is the need of the hour. Over the last two decades, number of studies has investigated the diverse health benefits and protective effects of natural substances present in the plants, particularly having antioxidant and antiproliferative properties. The scientific evaluation of medicinal plants used in the preparation of folk remedies has provided modern medicine with effective pharmaceutical drugs for the treatment of many infectious and chronic diseases including cancer [3]. Between 1983 and 1994, more than 60% of the approved anticancer drugs in the United States of America were from natural origin. Most of the anticancer agents have been shown to possess antioxidant potential that can play an important role in the protection of some forms of cancer [4, 5].

Plants as master chemists accumulate a wealth of intricate secondary metabolites, which further deliver effective treatments for a plethora of human diseases. Alternative or complementary traditional therapies are used to cure many diseases potentially that are plant derived or other natural sources. In traditional system of medication the importance to be considered is its broad range of outcomes which often treat the whole person rather than a specific symptom or disease. These therapies also make accessibility to the population living in diverse environmental conditions such as geographical and climatic. Thus make the accessibility to the person(s) in need with less efforts and dependence on other resources which in turn could be economically beneficial as well [6]. The phytochemicals found in plant-based foods also possess biological properties along with their antioxidant property. Consuming of antioxidant-rich foods has several health benefits that help to prevent many diseases [7, 8]. The secondary metabolites such as alkaloids, flavonoids, coumarins, and steroids have been shown to
possess antioxidant and anticancer activities in both in vivo and in vitro models [9–11]. Phytomedicine could be in the form of crude preparations (extracts, tinctures, and essential oils) containing a wide variety of compounds or could be pure molecules with a strong and specific activity. The natural products symbolize safety in contrast to the synthetic drugs, but still there is need to check their efficacy with systematic studies. Therefore, the need for new therapeutic options has prompted many researchers to evaluate the efficacy of compounds found in natural products [12].

Nyctanthes arbortristis (Oleaceae) is a mythological plant and possesses high medicinal values in Ayurveda. The popular medicinal uses of N. arbortristis include antihelminthic and antipyretic; besides it is used in disorders like rheumatism and skin ailments and as a sedative. Phytochemical investigations of N. arbortristis indicated the presence of a large number of phenolic compounds, iridoids, and carotenoids, such as arbortristoside (A, B, C) with many biological activities like anticancer, antileishmaniasis, anti-inflammatory, antiallergic, immunomodulatory, and antiviral [13]. The flowers of this sacred plant have not been explored for antiproliferative activity till date. Therefore, the present study was initiated with the aim of investigating the antioxidant and antiproliferative activities of the flower extracts.

2. Materials and Methods

2.1. Reagents. Doxorubicin, DPPH, quercetin, and gallic acid were purchased from Sigma Aldrich, USA. Organic solvents and HCl, hexamethylenetetramine, sodium nitrite, aluminum chloride, NaOH, NaCO₃, and Folin–Ciocalteu reagent were purchased from Merck, India.

2.2. Plant Material. The flowers from well grown and healthy plants of N. arbortristis were collected in and around the University of Hyderabad, Hyderabad. A voucher specimen (UoH/MDP/NA-00005) has been preserved in our laboratory for future reference.

2.3. Preparation of Extract and Phytochemical Screening. The flowers were shade dried and coarsely powdered using electric blender. The powdered material was then extracted with 95% ethanol under sonication. The ethanol solvent was removed under reduced pressure using a rotary vacuum evaporator (Buchii, USA) and dark reddish gummy ethanolic extract of N. arbortristis flowers (NafE) was obtained. This ethanolic extract was taken in double distilled water (ddH₂O) and partitioned with hexane, ethyl acetate, and n-butanol successively and subsequently the organic solvents were recovered under reduced pressure and concentrated. Insoluble part obtained on partitioning between aqueous and organic layers was also collected and concentrated under reduced pressure. Finally, the remaining aqueous part was also concentrated to polar extract by lyophilisation. Thus, N. arbortristis ethanolic extract (NafE), hexane extract (NafEHx), ethyl acetate extract (NafEEa), n-butanol extract (NafEBu), insoluble part extract (NafEln), and aqueous extract (NafEW), total six extracts, were obtained from extraction process and preserved at −20°C for further analysis. Phytochemical screening of secondary metabolites in all six extracts was carried out as described by Harbone [14].

2.4. Determination of Phytoconstituents

2.4.1. Determination of Total Phenolic Contents. The amount of total soluble phenolic content in all six extracts (NafE, NafEHx, NafEEa, NafEBu, NafEln, and NafEW) was determined according to Folin–Ciocalteu method with minor modifications [15]. Briefly, 10 µL of extracts from the stock solution was mixed with 100 µL of Folin–Ciocalteu reagent. After 10 min of incubation at room temperature, 300 µL of 20% Na₂CO₃ solution was added and the volume was adjusted to 1 mL using dH₂O. The mixture was incubated in the dark for 2 h and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against blank sample. The total phenolic content was measured as gallic acid equivalents (mg GAE)/g of dry weight (dw) and the values were presented as means of triplicate analysis.

2.4.2. Determination of Flavonoid Contents. Total flavonoid content in extracts was estimated by a colorimetric method as described by Veronica et al. [16] with minor modifications by taking 20 µL of each extract mixed with 500 µL distilled water and 30 µL of 5% NaNO₂ solution. After 5 min of incubation at room temperature, 60 µL of 10% AlCl₃ solution was added. Subsequently, 350 µL of 1 M NaOH and 40 µL of distilled water were added to make the final volume of 1 mL. Samples were further incubated for 15 min at room temperature and the absorbance of the samples was measured at 510 nm. The total flavonoids were determined as quercetin equivalents (mg QE)/g of dw and the values were expressed as means of triplicate analysis.

2.5. Antioxidant Assays

2.5.1. Total Antioxidant Capacity. The total antioxidant activity of test extracts was evaluated by green phosphomolybdenum complex according to the method of Prieto et al. [17]. An aliquot of 10 µL of extracts was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) in Eppendorf tubes. Tubes were incubated in a dry thermal bath at 95°C for 90 min. After cooling, the absorbance of the mixture was measured at 695 nm against a blank. Ascorbic acid was used for reference and the reducing capacities of the analyzed extracts were expressed as mg of ascorbic acid equivalents (mg AAE)/g of dw.

2.5.2. DPPH® Radical Scavenging Activity. The hydrogen-donating abilities of extracts were examined according to the method of Cuendet et al. [18] with some modifications using 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) as reagent that offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants. Briefly, 0.004% w/v of DPPH radical solution was prepared in methanol and then 900 µL of this solution was mixed with
100 µL of extract solution containing 20–360 µg/mL of dried extracts. The absorbance was measured at 517 nm after 30 min of incubation. Methanol (95%), DPPH solution, and ascorbic acid were used as blank, control, and reference, respectively. The IC50 value represents the concentration of extracts that inhibits 50% of the radical. Scavenging concentration for 50% of DPPH free radical (IC50) was calculated from logarithmic regression equation obtained from the values of at least five dilutions of the primary concentration.

2.6. Evaluation of Antiproliferative Activity. The six different cell lines that were used in study are colorectal adenocarcinoma (Colo 205); retinoblastoma (Y79); chronic myelogenous leukemia (K562); breast adenocarcinoma (MCF7); breast adenocarcinoma (MDAMB231). The cells lines were obtained from the National Centre for Cell Sciences, Pune, India, and were cultured at a seeding density of 0.2 × 10⁶ in DMEM/RPMI medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, respectively, and maintained in a humidified atmosphere with 5% CO2 at 37°C. The samples were dissolved in dimethylsulfoxide (DMSO; not exceeding the final concentration of 0.01%) and further diluted in cell culture medium. The antiproliferative response of extract was assessed by MTT assay [19]. Cells (~10,000) were plated in 200 µL growth medium in the presence or absence of the extract (25, 50, 100, and 200 µg/mL) in 96-well culture plates for 24 h. Then the culture plates were centrifuged at 2000 rpm for 10 min at room temperature. 100 µL of supernatant was discarded and 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37°C. The viability of the cells was determined using a spectrophotometer at 570 nm. The IC50, that is, the concentration of the extract required to inhibit cell growth by 50%, was determined.

2.7. Chromatography Profile: High Performance Liquid Chromatography and Liquid Mass Spectrometry (HPLC/MS/MS). Agilent 1200 series coupled with DAD-UV detector that was equipped with Agilent Technologies 6520 with Accurate Mass Q-TOF mode was used to perform mass spectrometry and Zorbax SB-C18 column rapid resolution (3.5 µm, 4.6 × 150 mm). The flow rate was 0.45 mL/min, and the injection volume was 3 µL. The analyses were performed using binary gradients of Milli-Q water (with 0.1% formic acid + 10 mM ammonium formate) (solvent A) and HPLC grade acetonitrile (with 0.1% formic acid) (solvent B) with the following elution profile: from 0 min: 35% (B) in (A); 10 min: 55% (B) in (A); 25 min: 95% (B) in (A); 35 min: 35% (B) in (A).

2.8. Statistical Analysis. Data were presented as means standard deviation (SD). Statistical analysis was performed using Student’s t-test analysis and one-way analysis of variance (ANOVA). The results were considered statistically significant when P < 0.05. The Dictionary of Natural Products on DVD software (CRC Press, Taylor and Francis Group, https://netbeans.org/) was used to analyze the chromatography profiling data.

![Figure 1: Phytoconstituents content (total flavonoid and total phenolic) in different extracts of N. arbortritis flower.](image)

### Table 1: Phytochemical screening of flower extracts of N. arbortritis.

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Phytochemical constituents</th>
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<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>NaFE</td>
<td>+</td>
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<tr>
<td>NaFEhX</td>
<td>+</td>
</tr>
<tr>
<td>NaFEea</td>
<td>-</td>
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<tr>
<td>NaFEbu</td>
<td>-</td>
</tr>
<tr>
<td>NaFEin</td>
<td>-</td>
</tr>
<tr>
<td>NaFEw</td>
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</table>

Note: +ve represents presence and −ve represents absence of phytochemical; S: steroids; A: alkaloids; F: flavonoids; P: phenolics; Sp: saponins; G: glycosides; T: terpenoids.

### 3. Results

3.1. Extraction, Preliminary Phytochemical Screening, Phytoconstituents Assay, and LCMS/MS Analysis. In the present study, the extraction was carried out under ultrasonication using 95% ethanol as the solvent, followed by fractionation of same extract with various solvents with increasing polarity and the final extracts were designated as NaFE, NaFEhX, NaFEea, NaFEbu, NaFEin, and NaFEW. These six different extracts were subjected to phytochemical screening to check the presence of different phytoconstituents and results are tabulated in Table 1. The UV profile of NaFE, NaFEa, and NaFEbu chromatograms analysed at all wave lengths demonstrated two λmax in the region of 240–280 nm and 300–380 nm thus suggesting the presence of flavonoids [20]. The results of phytoconstituents in different flower extracts of N. arbortritis are presented in Table 1 and Figure 1. The flavonoid content in different extracts (NaFE, NaFEa, and NaFEbu) was found to be in the order of 640 ± 2.09 mg QE/100 g; 590±1.09 mg QE/100 g; and 235±1.81 mg QE/100 g,
respectively, and by Folin-Ciocalteu method for total phenolic content of NafE, NafEa, and NafBu extracts was shown as $991 \pm 0.5$ mg GAE/100 g; $781 \pm 1.02$ mg GAE/100 g; and $591 \pm 0.07$ mg GAE/100 g, respectively.

The NafE was subjected to LC-DAD-ESI-MS to identify the phytochemical(s) by absorption peaks in UV (Figure 2(a)) and with molecular ion in Q-TOF and by their fragmentation (MS/MS) using the positive ionisation mode and observed many peaks (Figures 2(b), 3, and 4); however only two peaks were identified. Peak 1 (Figure 2(b)) (RT = 5.1 min, $\lambda = 280$ nm, and MW = 510.494) (Figure 3) had [M + H]$^+$ at m/z 511 and was identified as Arborside C [21], namely, 6 $\beta$-hydroxyguanine with O-benzoyl substitution with loss of benzoyl that ion appeared at m/z 105, benzoate m/z 121, and glucoside m/z 165 and 6 $\beta$-hydroxyguanine at m/z 244, 242, and 212. Peak 2 (Figure 2(b)) (RT = 20.509 min, $\lambda = 440$ nm, and MW = 652.27) (Figure 4) had [M + H]$^+$ at m/z 653.1994 and was identified as carotenoid, glucosides, namely, $\beta$-monogentiobioside ester of $\alpha$-Crocetin (or Crocin-3) with loss of 1,5-anhydro-D-glucitol that ion appeared at m/z 165.0651 and m/z 491 carotenoid ester with other fragments at m/z 459, 315, and 147 [22].

The reddish-orange coloured tubular calyx of flower of N. arbortristis is due to carotenoid pigments (Crocetin and its derivatives) which are reported from the flowers of this plant [22, 23].

3.2. Antioxidant and Free Radical Scavenging Ability Assays. Phosphomolybdenum assay is a quantitative method to evaluate the antioxidant capacity indicated by electron donating capacity [17]. The results showed that all the extracts exhibited different degrees of activity as presented in Figure 5(a). The highest antioxidant capacity was observed in NafEEa with 30.11 ± 1.77 of AAE/100 g dw of plant material followed by NafE and NafEBu with 28.76 ± 1.51 AAE/100 g dw and 24.66 ± 2.09 AAE/100 g dw, respectively. NafEHx, NafEln, and NafEW were found to be <5.00 ± 2.12 AAE/100 g dw which were considered to be ineffective. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid. N. arbortristis flower ethyl acetate, butanol, and ethanol extracts range between 30.11 and 24.66 mg AAE/g dw and other extracts were very low in concentration.

DPPH assay is a very sensitive qualitative assay for radical scavenging property and the experiment was carried out on the present study and its results can indicate the presence of antioxidant compounds in plant extracts [24]. Figure 5(b) illustrates a significant ($P < 0.05$) decrease in the concentration of DPPH due to the scavenging activities of the extract samples. The samples showed concentration dependent DPPH radical scavenging activities. The IC$_{50}$ values of NafE, NafEHx, NafEEl, NafEBu, NafEln, and NafEW were found to be at 32.71 ± 1.32 $\mu$g/mL, 328.37 ± 2.25 $\mu$g/mL,
Figure 4: Mass spectrum (TOF MS ES+) of peak number 2 in *N. arbortristis* flowers ethanol extract (identified as Crocin-3).

Figure 5: (a) Total antioxidant content in different extracts. All values are expressed as the means ± SEM. (b) DPPH radical scavenging activity of *N. arbortristis* flower in different extracts. All values are expressed as the means ± SEM.

23.98 ± 1.05 μg/mL, 30.29 ± 1.78 μg/mL, 104.11 ± 1.51 μg/mL, and 401.15 ± 1.29 μg/mL, respectively. Percentage inhibition at 40 μg/mL of NafE, NafEEa, and NafEBu was found to be 52.53 ± 2.86%, 69.25 ± 3.96%, and 67.98 ± 3.54%, respectively. Assessment of free radicals scavenging by DPPH method for antioxidant potential is known to be accurate, convenient, and rapid. *N. arbortristis* flowers ethyl acetate, butanol, and ethanol extracts could scavenge DPPH radical effectively 50–70%, respectively, at the highest concentration of 360 μg/mL. There are reports on antioxidant property of this plant with respect to its leaves, flowers, and stem. Extensive work on leaves has been carried out but has not been much studied on flowers. Earlier report suggests that antioxidant activities from leaves, stem, and flower extracts were significantly higher in the extracts from lower polarity over the extracts from higher polarity solvent [13]. This supports our present data where the lower polarity extracts were more active than the higher polarity solvents.

3.3. Evaluation of Antiproliferative Activity. In order to evaluate *N. arbortristis* as a potential candidate for cancer therapy, the above extracts were assayed against a panel of five different human tumor cells, colorectal adenocarcinoma (Colo 205); retinoblastoma (Y79); chronic myelogenous leukemia (K562); breast adenocarcinoma (MDAMB-231), and the chemotherapeutic drug, Doxorubicin, as a positive control. Out of six extracts tested, only two, ethanolic extract (NafE) and ethyl acetate extract (NafEEa), were found to be active, whereas NafEHx, NafEBu, NafEIn, and NafEW extracts did not inhibit the proliferation of tumor cells, thus indicating their noncytotoxicities against the above cancer cell lines. The MTT assay
Figure 6: Antiproliferative activity of *N. arbortristis* flower extracts against (a) colorectal adenocarcinoma—Colo 205 cell line; (b) retinoblastoma—Y79 cell line; (c) chronic myelogenous leukemia—K562 cell line; (d) breast adenocarcinoma—MCF7 cell line; (e) breast adenocarcinoma—MDAMB231 cell line; and (f) human embryonic kidney cells—HEK cell line. Significant values (**∗∗∗*P* < 0.001, **∗∗*P* < 0.01, and **∗*P* < 0.05) were obtained by Student's *t*-test analysis. Composite treatments were compared using one-way analysis of variances (ANOVA) and probability values were found to be equal to or less than 0.05 for all the six cell lines.
Table 2: Percentage inhibition of cancer cell proliferation and IC50 values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell type</th>
<th>% inhibition</th>
<th>IC50</th>
<th>% inhibition</th>
<th>IC50</th>
<th>% inhibition</th>
<th>IC50</th>
<th>% inhibition</th>
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<th>% inhibition</th>
<th>IC50</th>
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<tbody>
<tr>
<td></td>
<td>Colo 205</td>
<td>Y79</td>
<td>K562</td>
<td>MCF</td>
<td>MDA-MB</td>
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<tr>
<td>NafE (200 µg/mL)</td>
<td>72.01 ± 9.40</td>
<td>81.82 ± 2.11</td>
<td>55.87 ± 7.19**</td>
<td>61.82 ± 9.60</td>
<td>53.63 ± 6.84**</td>
<td>54.24 ± 5.39</td>
<td>184.36 ± 5.39**</td>
<td>67.80 ± 0.96</td>
<td>50.97 ± 3.10**</td>
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<tr>
<td>NafEa (200 µg/mL)</td>
<td>70.67 ± 5.30</td>
<td>68.06 ± 2.02</td>
<td>59.50 ± 3.41**</td>
<td>58.48 ± 5.20</td>
<td>98.02 ± 7.47*</td>
<td>46.57 ± 0.64</td>
<td>214.73 ± 0.64*</td>
<td>52.65 ± 2.02</td>
<td>100.81 ± 0.50**</td>
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<tr>
<td>Doxorubicin (10 µg/mL)</td>
<td>92.33 ± 0.45</td>
<td>0.39 ± 0.03</td>
<td>89.42 ± 2.02</td>
<td>0.32 ± 0.10</td>
<td>89.42 ± 2.02</td>
<td>0.36 ± 0.02</td>
<td>92.33 ± 0.45</td>
<td>0.36 ± 0.09</td>
<td>90.65 ± 2.41</td>
<td>0.45 ± 0.11</td>
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<td>DMSO 2% (Solvent Cntl)</td>
<td>3.71 ± 0.56</td>
<td>—</td>
<td>3.73 ± 1.53</td>
<td>—</td>
<td>3.76 ± 1.00</td>
<td>—</td>
<td>4.44 ± 1.70</td>
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<td>4.02 ± 1.17</td>
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</table>

Values were the means of four replicates ± standard deviation (SD). Significant P values (*** P < 0.001, ** P < 0.01, * P < 0.05) were obtained by Student's t-test analysis. Composite treatments were compared using one-way analysis of variances (ANOVA) and probability values were found to be equal to or less than 0.05 for all the four cell lines.
that measures the formazan product at 570 nm clearly proves the cytotoxicity of the tested extracts. Figures 6(a)–6(e) show the cytotoxicity values of two active extracts in tested cell lines in comparison with normal human embryonic kidney cells (Figure 6(f)); the IC50 values are presented in Table 2. The NafE and NafEEa were found to be cytotoxic to tested cell lines. These extracts significantly inhibited the growth of cancer cells in a concentration dependent manner as they caused significant cell death in both sensitive and resistant human cancer cell lines. NafE extract has shown the most potent cytotoxicity on all five cancer cell lines. The percentage inhibition shown by NafE was found to be in the range of 54.24 ± 3.39% to 81.81 ± 2.11% (P < 0.05) against all five cell lines at the highest concentration of 200 μg/mL. The lowest IC50 value was observed against Colo 205 cell line (24.56 ± 6.63 μg/mL). On the other hand, NafEEa extract at the same concentration exhibited slightly lesser percentage inhibition across the cell lines tested (46.57 ± 0.64 to 70.66 ± 5.30%; P < 0.05) with lowest IC50 values found against Colo 205 cell line (25.79 ± 2.69 μg/mL). The difference in the antiproliferative effects between different extracts may have resulted from the different phytoconstituents and their concentrations contained in the extracts due to the sensitivity to the solvent used and mode of extraction. The cytotoxic effect of NafE and NafEEa was also studied in normal embryonic kidney cell line using the MTT method. The results clearly indicated that these two extracts were nontoxic and had no inhibitory effect on cell proliferation in HEK-293 and there was minimal reduction in cell survivability (Figure 6(f)). The percentage viability was above 95% at the highest concentration of 200 μg/mL. This advocates that NafE and NafEEa extracts did not show any kind of toxic effect on the normal cells. Hence, the cytotoxicity of the active extracts was found to be highly selective against the cancer cell lines used.

Crocetin, carotenoid, is an active component of most ancient expensive spice, saffron (Crocus sativus [25]) that is also reported to possess anticancer properties [26]. In dimethylbenzanthracene (DMBA) induced skin tumorigenesis the hydroalcoholic extract of leaves of this plant at 250 mg/kg was found to be as chemopreventive [27] and 4-hydroxyhexahydrobenzofuran-7-one isolated from leaves at 20 mg/kg which inhibited the cell growth of Ehrlich ascites carcinoma cells by 43.27% and did not have any cytotoxic effect [28]. Arbortristoside A and B and iridoid glycosides are reported from seeds at 2.5 mg/kg in mice which possess anticancer activity against methylcholanthrene induced fibrosarcoma [29]. Iridoids and carotenoids are most frequent compounds identified in N. arbortristis and they have been reported for various biological activities [30, 31].

It was an understandable interest to know how high levels of phenolics exhibited high antioxidant activity and also influence the anticancer activity in different extracts. We expected that the extracts with the high content of the total phenolics possessing antioxidant potential possibility have high anticancer activity. Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work [32, 33]. As discussed earlier, several plant species that are rich in flavonoids are reported to prevent and possess therapeutic properties [32–35]. The flowers of this plant were reported with rich phytochemicals diterpenoids, nycanthin, flavonoids, anthocyanins, essential oil, 6, β-hydroxyguanine, carotenoids, β-monogentiobioside, β-digentiobioside, and various biological activities [13]. With the aid of hyphenated techniques LCMS/MS Arborside C and Crocin and β-monogentiobioside ester of α-Crocetin (8,8′-Diapocarotenedioic acid) were identified. Possibly the antioxidant and anticancer activities of ethanolic and ethyl acetate extracts of N. arbortristis are influenced by the presence of phytoconstituents which is in accordance with the findings of phytochemical evaluation which indicated the presence of flavonoids, phenolics, Crocin-3, and Arborside C in extracts with promising activity.

4. Conclusion

N. arbortristis is known for its varied medicinal properties in traditional ayurvedic medicine and reported for various bioactive phytoconstituents. In this study an attempt was made to investigate antiproliferative effects of different extracts of N. arbortristis in different human cancer cell lines apart from its antioxidant potential. The present study indicated that the ethyl acetate and ethanol extracts of N. arbortristis possessed the significant phenolic content and exhibited potent antioxidant and antiproliferative activities, which were comparable to the commercial antioxidant gallic acid, and the anticancer drug Doxorubicin. This seems that the N. arbortristis flower extracts can be considered as good sources for drug discovery. Further investigation is being carried out to identify and characterize the inherent bioactive compounds responsible for the antioxidant and anticancer activities from the ethyl acetate and ethanol extracts of N. arbortristis.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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References


Research Article

A Quantitative Ethnopharmacological Documentation of Natural Pharmacological Agents Used by Pediatric Patients in Mauritius

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The pediatric population constitutes the most vulnerable patients due to a dearth of approved drugs. Consequently, there is a pressing need to probe novel natural pharmacological agents in an endeavor to develop new drugs to address pediatric illnesses. To date, no studies have explored the use of natural therapies for pediatric healthcare in Mauritius. Parents (n = 325) from different regions of the island were interviewed. Quantitative indexes such as fidelity level (FL), informant consensus factor (FIc), and use-value (UV) were calculated. Thirty-two plants were reported to be used by pediatric patients. Gastrointestinal disorders (FIc = 0.97) encompassing regurgitation, infantile colic, and stomach aches were the most common ailments managed with herbs. Matricaria chamomilla used for infantile colic and its pharmacological properties has previously been documented for pediatric patients. Product from A. mellifera (UV = 0.75) was the most utilized zootherapy for managing cough. Most plants and animal products reported in this study have bioactive constituents supported by existing scientific literature but their use for the pediatric population is scant. The present ethnopharmacological study has opened new perspectives for further research into their pharmacology, which can subsequently support and facilitate timely pediatric medicinal product development.

1. Introduction

Many children suffer from diseases that go on to cause extensive mortalities. The WHO (2012) estimated that close to 7 million children under the age of 5 died in 2011 mostly because they did not have access to simple and affordable therapies. Infectious diseases most commonly pneumonia, diarrhoea, and malaria are the main cause of deaths in children younger than 5 years [1, 2]. Caregivers frequently treat their children using natural therapies at home which are justified for various reasons [3–5]. One, natural remedies are easy to access and are also considered effective and safe to use. Second, some caregivers fear adverse effects from conventional drugs. Three, some health conditions including allergies and skin disorders, respiratory problems, and behavioural disorders are challenging to treat conventionally [6–8]. Lastly, there is a lack of approved formulations and drugs for use in the pediatric population because the development of new pediatric drugs is complex and subjected to technological, financial, and ethical challenges [9–11]. As a result, the trend is towards increased use of natural therapies in the pediatric population [7] as supported by the growing number of publications in this respect [4, 5, 10, 12–15].

Nonetheless, in spite of the many studies on the use of natural therapies for all age groups around the globe, none has documented and explored its use for pediatric healthcare in the tropical island of Mauritius. Mauritius possesses a rich biological and cultural diversity which translates into a wealth of traditional knowledge and practices including the use of natural resources for medicinal purposes by various ethnic groups. The island of Mauritius is a developing country with a rich medicinal flora and fauna and the use of traditional medicine is prevalent among the population. While foreign tourists who visit Mauritius see an idyllic, sun-soaked paradise, poverty still prevails and many Mauritians tend to prefer natural therapies for their primary healthcare [16, 17].
Indeed, traditional medicine is omnipresent in the Mauritian community whereby Mauritians still use traditional medicine for the treatment and/or management of various ailments. Nonetheless, with globalization and access to conventional medicines, Mauritians, particularly the younger people, tend to remember their use in the past as such knowledge has not been documented. Therefore, there is a pressing need to record updated primary scientific information on the different plant and animal-based therapies used by Mauritians [17]. This study therefore sets out to investigate and document primary data on herbal and animal-based therapies used by Mauritians for pediatric healthcare. Interestingly, for the first time, a quantitative survey has been designed to collect primary data for natural therapies used for pediatric care in Mauritius. It is believed that the present documentation will serve as a repertoire to record this vanishing knowledge before it is eroded completely from the island and to the scientific community. It is also anticipated that the present documentation will be fundamental to protect traditional knowledge and for the conservation of the rich biodiversity of Mauritius for future generations and to ensure Mauritius’s sovereign rights over its genetic resources and utilization by first documenting them. Specifically, this paper reports a consensus on medicinal plants and animals commonly used, diversity of such therapies used, and the methods of preparation and application of such natural pharmacological agents for child care.

2. Methods

2.1. Background of Study Area. Mauritius is a small volcanic island, 61 km long and 47 km wide, located in the Indian Ocean, 800 km East of Madagascar [54] (Figure 1). The island has a total surface area of 1,865 km² with 330 kms of coastline almost entirely surrounded by coral reefs and with an estimated population of 1,299,000 [55]. The topography of the island rises to its highest point, the Piton de la Riviere Noire, at 828 m. The geography of the land and rain distribution ensures a diverse microclimatic regime throughout the island and hence had a direct impact on both the endemic and exotic vegetation. Consequently, the flora and fauna of Mauritius are characterized by a significant percentage of endemic and indigenous species given its long geographical isolation and evolution [55].

Mauritius became an independent state in 1968 after a long history of colonialism. It was first colonized by the French from 1715 and was then by the British from 1815. The successive waves of immigration consisting of African slaves, white settlers, Indian indentured laborers, and Muslim and Chinese traders, all from different continents, contributed not only to substantial demographic changes but also to the island’s ethnic composition, turning it into a multicultural society [56]. The main ethnic groups are the Bhojpuri-speaking Hindus, constituting 40.2% of the total population. The Tamils are the second largest ethnic community (13.9%), while Telugus (5.6%) and Marathis (4%) represent smaller minorities within the overall Hindu population. The Hindus have a common language (Bhojpuri), the same regional origin (Uttar Pradesh and Bihar), religious practices, and rituals [56, 57]. Previously the economy of the country was based on agriculture. However, recently the economy has successfully been diversified into textiles, tourism, banking, and business outsourcing. Today, the tourism industry in Mauritius is much more lucrative than the sugar industry, and it is also noted that there is an increase in ecotourism and geotourism in Mauritius [58, 59].

The use of traditional medicine is widespread in Mauritius and is comprised of many different forms that include Ayurveda, homoeopathy, traditional Chinese medicine, folk herbalists, traditional midwives, and many other types [16, 17]. Children are still treated using natural products especially for common ailments such as vomiting and gastrointestinal disorders.

2.2. Data Collection. The project was approved by the Department of Health Sciences, Faculty of Science, University of Mauritius, Mauritius. A parent survey was developed as reported in previous studies [5, 60]. The study population encompassed current natural therapies used by parents whose children fell in the age category <15 years [8, 61]. A target sample of 385 participants was interviewed during 2011–2013. This sample size was large enough at 95% confidence interval and a power of 80% with a detectable relative risk of 2 provided that the determinants of using natural therapies within the study population are not uncommon (less than 20%) or very common (greater than 80%). The sample size assumed that at least a quarter of the respondents use natural therapies. The sample size was determined using statistical of EPIINFO version 6 (http://www.cdc.gov/epiinfo CDC, Georgia) for cross-sectional studies.

Proper data was partly collected using the participatory rural appraisal method, as the key informants also became investigators themselves, participating in interviews, informal meetings, open and group discussions, and overt observations with semistructured questionnaire. The content of the semistructured questionnaire was composed of diverse information, including local names of remedies, plant or animal parts used, ailments, methods of preparation and administration, side-effects, and dosage [17, 62].

The interview was performed in vernacular and native languages (“Hindi,” “Bhojpuri,” and “Creole”). The questionnaire developed was strictly confidential and noncompulsory. An information sheet and a consent form were also included in order to inform the participants of the nature, implications, and objectives of this study.

During field visits, when a remedy was mentioned by the participant, where possible, the participant was encouraged to show a sample of the remedy which was collected and/or photographed. The collected sample was then identified with local botanists and experts. The data obtained during the survey was cross-checked (local names/scientific names) according to locally published books [63, 64]. Scientific names of plant species were identified according to the International Plant Name Index (IPNI: http://www.ipni.org). A local database was constructed whereby plant samples were assigned a collection number for future reference.
The questionnaire comprised of structured open-ended and close-ended questions and family demographics such as age, gender, ethnicity, educational level, and area of residence; monthly household income and number of children were included. Parents were also asked information about their children conventional medicine use and questions concerning management of child health conditions were asked during interviews. Voluntary assistance from a local medical doctor was sought to confirm medical conditions and to establish comparisons between the local/vernacular descriptions and standard medical terms. Parents were approached randomly, particularly when they came for vaccination, dentist appointments, and routine follow-ups for their children. They were assured that the information given during the interview would be treated with utmost confidentiality and their decision to participate in the study or otherwise would not influence the treatment their child would receive.

This documentation will fully recognize the contribution of the local people who have been using the indigenous knowledge, protection of community biodiversity and intellectual property rights, and benefits, if any comes out of the study and prior informed consent for publication of the work has been obtained during the survey. Also informants were assured that this research is not for commercial purpose and aims for documentation and information dissemination on the traditional knowledge. Additionally any benefits emanating from its use must be shared according to the rules of the
2.3. Data Analysis. The informant or respondent consensus factor (\(F_{IC}\)) was calculated to estimate user consensus with regard to medicinal plants. \(F_{IC}\) values range from 0.00 to 1.00. High \(F_{IC}\) values are obtained when only one or a few plant species are reported to be used by a high proportion of informants to treat a particular ailment; conversely low \(F_{IC}\) values indicate that informants disagree over which plant to use. The formula used is \(F_{IC} = N_{ur} - N_{i}/(N_{ur} - 1)\), where \(N_{ur}\) is the number of individual plant use reports for a particular illness category and \(N_{i}\) is the total number of species used by all informants for this illness category [65].

The fidelity level (FL), the percentage of informants claiming the use of a certain plant for the same major purpose, was calculated to determine the most important species for the most frequently reported diseases or ailments as follows: FL (\%) = \(N_{p}/N\times100\), where \(N_{p}\) is the number of informants that claim a use of a plant species to treat a particular disease and \(N\) is the number of informants that use the plants as a medicine to treat any given disease [65]. The variety of use was also assessed which indicate the number of different diseases that are treated with a particular species. Use value (UV) was used to establish the relative importance of species known for plants as well as animals. It was calculated as \(UV = \sum U/N\), where \(U\) is the number of citations per species and \(N\) is the number of informants.

3. Results

3.1. Demographic Characteristics. This study was conducted to document herbal and animal-based therapies used to treat and/or manage childhood ailments and health conditions in the tropical island of Mauritius. The respondents were aged 20 years and above (Table 1). The informants were predominantly women (88%) and distributed into Hindus (40.0%), Christians (27.4%), and Muslims (20.6%) faith denominations. Respondent’s average monthly income ranged between 20 001 and 30 000 Mauritian rupees (1 Mauritian rupees = $30). Most participants had acquired at least secondary level (55%) education. The mean number of children from informants was two. An impressive 98% of users have claimed experiencing no side-effects with natural therapies (data not represented graphically).

The majority of informants (77.3%) stated that the natural therapies they have used are helpful, whereas 20% confirmed that such therapies were very important (data not represented graphically). Among users, the most common source of knowledge was family (80.0%). Interestingly, 31.5% and 44.1% of natural therapies users stated that they had been advised by relative and friends, respectively (Figure 2).

3.2. Medicinal Plants Used for Childhood Ailments and Conditions. Thirty-two medicinal plant species (distributed in 22 different families) have been documented to be in use by Mauritians for pediatric healthcare (Table 2). Medicinal plants were most commonly used to treat vomiting, infantile colic, diarrhoea, cough and flu, constipation, and chickenpox. The common methods for herbal preparation, modes of administration, and the parts of plant used have also been documented and presented in Table 2. Leaves are the most popular plant parts used and decoction is the common method of herbal preparation. Different methods of preparations were used for herbs but infusion is the most preferred method for oral preparations in children.

The categories of the plants with high number of uses mentioned for one purpose based on AbouZid and Mohamed (2011) study [66] were also compared with their fidelity level (FL) and summarized in Table 2. The most important species based on the FL scores were *Ayapana triplinervis*, *Matricaria chamomilla*, *Plectranthus madagascariensis*, *Maranta arundinacea*, *Psidium guajava*, and *Punica granatum*.

Plants with the highest number of uses mentioned for all categories were *Ayapana triplinervis* with 140 mentions for vomiting (FL = 66.9), *Matricaria chamomilla* with 99 citations for infantile colic (FL = 100), and *Plectranthus madagascariensis* with 82 mentions for cough and flu (FL = 84.5). The infusion of leaves of *Ayapana triplinervis* flavoured with
a tinct of sugar is the most preferred method of preparation for children. It was also reported that decoction of *A. triplinervis* was used to prepare milk for feeding the newborns. Decoction of *Bidens pilosa* (FL = 75) flowers, *Maranta arundinacea* (FL = 100) oral preparation, leaves infusion of *Polygonum poiretii* (FL = 100), decoction of *Psidium guajava* (FL = 100) leaves, and bark or fruits and decoction of rind of *Punica granatum* (FL = 100) have been reported as effective remedies against diarrhoea by informants. This was strongly supported by the high FL of these plants which demonstrated that respondents agreed on the use of these particular plants for diarrhoea. *Matricaria chamomilla* was the third most highly used herb for infantile colic (FL = 100) which was prepared from infusion of the plant's flowers and *Mentha piperita* had the highest number of mentions for stomach ache (FL = 71.8) in children. Cough and flu with a high $F_{IC}$ value (0.97) were also prevalent ailments amongst pediatric individuals and *Cymbopogon citratus* was commonly used for cough and flu (FL = 52.3). Informants reported that honey and fresh lemon drops are added to extracts of *Zingiber officinale* to enhance the preparation so that it can be easily consumed by the child. *Plectranthus madagascariensis* (FL = 84.5) and *Piper betle* (FL = 79.6) were commonly used for cough and flu and have also been reported to be effective in alleviating asthmatic symptoms in children. *Azadirachta indica* (FL = 81.7) was reported to be helpful in curing chickenpox. Herbal bath prepared from decoction of the *A. indica* leaves was given to children followed by application of extracts of fresh turmeric (*Curcuma longa*). Moreover, Hindus do a special prayer known as "mata" in the local language to help in curing chickenpox, scabies, and measles. Other herbs reported with imperative implications for pediatric healthcare included *Ocimum tenuiflorum* prepared from infusion of the leaves which was reported for sleep disorders (FL = 71.4) and also to soothe infants particularly before going to sleep. Additionally, *Cardiospermum halicacabum* prepared from decoction of the whole plant was reported to be used against childhood eczema (FL = 76.9). High level of consensus (high $F_{IC}$) for some species was observed, particularly for treating skin problems, vomiting, infantile colic, cough and flu, diarrhoea, and abdominal pain amongst others (Table 3).

The specific methods of use and the therapeutic uses of animal products documented from the present survey are summarized in Table 4. As compared to herbal therapies, the number of animal products in use for the pediatric population is less, with only 6 different remedies inventoried. Cough and flu are the most common ailments managed using animal derived products.

As depicted in Figure 3, the most commonly used herbs are *Ayapana triplinervis* (79.5%), *Cymbopogon citratus* (41.4%), *Matricaria chamomilla* (37.6%), *Plectranthus madagascariensis* (36.9%), and *Piper betle* (35.4%).

### 4. Discussion

In spite of the extensive use of natural products with pharmacological properties from traditional medicines to treat and/or manage children ailments in developing countries like Mauritius, little has been done to document such natural pharmacological agents [67, 68]. It is generally agreed that the materials used in traditional medicines need to be carefully documented as a first step to enhance our understanding of the role of herbal and animal products in use for child healthcare and for future drug development before determining whether they are efficacious and safe to use. Although previous studies around the globe have described the use of natural therapies in pediatric patients, this study is the first to document such therapies in the tropical island of Mauritius [13–15, 60]. The main focus of this study was to document common herbal and animal therapies used to manage common childhood ailments in Mauritius.

From the present work, it was observed that Hindus and Christians were common users of natural therapies which can be explained by cultural beliefs and faith in elders' knowledge which has been bestowed upon users from...
<table>
<thead>
<tr>
<th>Family/plant species/ collection number</th>
<th>VN/CEN</th>
<th>Part used</th>
<th>Method of preparation</th>
<th>Pediatric use/ailment treated (Number of citation, fidelity level)*</th>
<th>Recorded literature uses</th>
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<td><strong>Amaranthaceae</strong></td>
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<tr>
<td>Chenopodium ambrosioides L. (PSMTS09)</td>
<td>Bautrisse</td>
<td>L</td>
<td>Decoction of leaves is taken orally twice daily for 5 days</td>
<td>Intestinal worms (3, 100)</td>
<td>Anthelmintic agent [18]</td>
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<td><strong>Asphodelaceae</strong></td>
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<td>Aloe vera (L.) Burm. f. (PSMTS01)</td>
<td>Aloe vera/Aloe vera</td>
<td>G</td>
<td>Gel is smeared on wounds (twice daily) and itchy spots for at least 5 days</td>
<td>Antiseptic (3, 100)</td>
<td>Antimicrobial, anti-inflammatory [19]</td>
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<td><strong>Asteraceae</strong></td>
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<tr>
<td>Ayapana triplinervis (M.Vahl) R.King and H.Robinson (PSMTS03)</td>
<td>Ayapana/NA</td>
<td>AP</td>
<td>Decoction of aerial roots is taken orally for 1 week (3 times per day)</td>
<td>Vomiting (140, 66.9), diarrhoea (6, 2.9), stomach pain (5, 2.4), colitis (58, 27.8)</td>
<td>Antimicrobial, anti-inflammatory [20]</td>
</tr>
<tr>
<td>Bidens pilosa L. (PSMTS05)</td>
<td>Lavilbag/NA</td>
<td>F/WP</td>
<td>Decoction of flowers is taken orally twice daily for 5–7 days</td>
<td>Diarrhoea (3, 75), skin infections (1, 25)</td>
<td>Pain, fever, angina, diabetes, edema (water retention), infections, and inflammation [21]</td>
</tr>
<tr>
<td>Matricaria chamomilla L. (PSMTS17)</td>
<td>Chamomile</td>
<td>F</td>
<td>Decoction of whole plant for 1 week (twice or thrice daily) can be extended for 2–3 weeks</td>
<td>Infantile colic (99, 100)</td>
<td>Antimicrobial and anti-inflammatory [22]</td>
</tr>
<tr>
<td>Tagetes patula L. (PSMTS30)</td>
<td>Genda</td>
<td>F</td>
<td>Infusion of flowers is taken orally once for 5–7 days</td>
<td>Colic (2, 100)</td>
<td>Antifungal [23]</td>
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<td><strong>Apocynaceae</strong></td>
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<td>Catharanthus roseus (L.) G.Don (PSMTS12)</td>
<td>Saponaire</td>
<td>L</td>
<td>Decoction of leaves is taken orally at night for 5 days</td>
<td>Fever (5, 100)</td>
<td>Anticancer, diabetes mellitus, fever, and arrest of bleeding [24]</td>
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<td><strong>Balsaminaceae</strong></td>
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<td>Impatiens balsamina L. (PSMTS15)</td>
<td>Belzamine</td>
<td>L/F</td>
<td>Crushed leaves applied on wounds Decoction once daily is taken orally for 5 days</td>
<td>Apply on wounds (3, 60), colic (2, 40)</td>
<td>Wounds, abscesses, Anaphylactic, antipruritic, expectorant, hypoglycemic, and odontalgic properties [25]</td>
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<td><strong>Brassicaceae</strong></td>
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<td>Nasturtium officinale WT. Aiton (PSMTS20)</td>
<td>Cresson/Watercress</td>
<td>L</td>
<td>Juice extracted from crushed leaves is taken orally daily for 1 week (twice daily) and Can mix with honey</td>
<td>Cough (2, 100)</td>
<td>Antioxidant, de purative, diuretic, expectorant, hypoglycemic, and odontalgic [27] properties</td>
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<td>Carica papaya L. (PSMTS08)</td>
<td>Papaille/papaya</td>
<td>S</td>
<td>Infusion of seeds is taken orally for 1 week each year</td>
<td>Intestinal worms (3, 100)</td>
<td>Anthelmintic activity [28]</td>
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<td>Claoxylon glandulosum Bolvin ex Baill. (PSMTS11)</td>
<td>Bois d’oiseau/NA</td>
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<td>Decoction of leaves is taken (one daily) orally for 5 days</td>
<td>Allergy (2, 100)</td>
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<td>Aspalathus linearis (Burm. f.) R. Dahlgren (PSMTS02)</td>
<td>Rooibos/rooibos</td>
<td></td>
<td>Infusion taken twice daily for 30 days</td>
<td>Infant colic (2, 100)</td>
<td>Asthma, colic disorders, allergies, and dermatological problems [29]</td>
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<td>Family/plant species/ collection number</td>
<td>VN/CEN</td>
<td>Part used</td>
<td>Method of preparation</td>
<td>Pediatric use/ailment treated (Number of citation, fidelity level)</td>
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<td>L</td>
<td>Decoction of leaves is taken orally for 2-3 days</td>
<td>Stomach ache (56, 71.8), colitis (20, 25.6), constipation (2.6)</td>
<td>Antispasmodic, anti septic, anti-inflammatory, antibacterial, and antifungal activities [30]</td>
</tr>
<tr>
<td><em>Ocimum tenuiflorum</em> L. (PSMTS21)</td>
<td></td>
<td>L</td>
<td>Infusion of leaves is taken orally daily for 1 month</td>
<td>Sleep disorders (20, 71.4), cough (8, 28.6)</td>
<td>Cold, headaches, and stomach disorders [31]</td>
</tr>
<tr>
<td><em>Plectranthus madagascariensis</em> (Pers.) Benth. var. <em>madagascariensis</em> (PSMTS23)</td>
<td></td>
<td>L</td>
<td>Juice extracted from crushed leaves of the plant is warmed with honey and fresh lemon twice daily for 1 week</td>
<td>Cough and flu (82, 84.5), bronchitis (5, 5.2), asthma (10, 10.3)</td>
<td>Anti-bacterial, antifungal, and anti-dermatitic [32]</td>
</tr>
<tr>
<td><em>Rosmarinus officinalis</em> L. (PSMTS28)</td>
<td></td>
<td>L</td>
<td>Infusion of leaves is taken orally for 5 days</td>
<td>Stress (4, 100)</td>
<td>Anti-inflammatory effect [33]</td>
</tr>
<tr>
<td><strong>Lythraceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Punica granatum</em> L. (PSMTS27)</td>
<td></td>
<td>RI</td>
<td>Decoction of rind is taken orally for 5 days</td>
<td>Diarrhoea (4, 100)</td>
<td>Antioxidant activity [34]</td>
</tr>
<tr>
<td><strong>Marantaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Moranta arundinacea</em> L. (PSMTS36)</td>
<td></td>
<td>RP</td>
<td>RP is grilled till a brown colour is obtained and then consumed with water for 5 days</td>
<td>Diarrhoea (13, 100)</td>
<td>Tuberculosis, weakness [35]</td>
</tr>
<tr>
<td><strong>Meliaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Azadirachta indica</em> A. Juss. (PSMTS04)</td>
<td></td>
<td>L</td>
<td>Herbal bath is given to child prepared from decoction of the leaves followed by application of green turmeric</td>
<td>Vomiting (140, 66.9), diarrhoea (6, 2.9), stomach pain (5, 2.4), colitis (58, 27.8)</td>
<td>Antimicrobial, anti-inflammatory [20]</td>
</tr>
<tr>
<td><strong>Myrtaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Psidium guajava</em> L. (PSMTS26)</td>
<td></td>
<td>L/B</td>
<td>Decoction taken orally for 3 days, thrice daily</td>
<td>Diarrhoea (55, 100)</td>
<td>Antispasmodic and antimicrobial properties in the treatment of diarrhoea and dysentery and hypoglycemic agent [36]</td>
</tr>
<tr>
<td><em>Syzygium aromaticum</em> (L.) Merr. &amp; L.M. Perry (PSMTS29)</td>
<td></td>
<td>FB</td>
<td>Decoction is taken orally for 5 days</td>
<td>Cough (1, 33.3), gastrointestinal discomfort (2, 66.7)</td>
<td>Antimicrobial, antifungal and antiviral, anti-inflammatory, cytotoxic, and anesthetic properties [37]</td>
</tr>
<tr>
<td><strong>Piperaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Piper betle</em> L. (PSMTS22)</td>
<td></td>
<td>L</td>
<td>Juice extracted from crushed leaves of the plant is warmed with honey and fresh lemon</td>
<td>Cough and flu (74, 79.6), bronchitis (4, 4.3), asthma (15, 16.1)</td>
<td>Antioxidant, antimicrobial, antifungal, anti-inflammatory, and radio-protective properties [38]</td>
</tr>
<tr>
<td><strong>Poaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cymbopogon citratus</em> (DC. ex Nees) Stapf (PSMTS34)</td>
<td></td>
<td>WP</td>
<td>Decoction of plant is taken orally for 7–10 days. <em>Zingiber officinale</em>, sugar and fresh lemon drops, is often added to enhance the preparation</td>
<td>Cough and flu (57, 52.3), fever (52, 47.7)</td>
<td>Inflammation, digestive disorders, diabetes, nervous disorders, and fever [39]</td>
</tr>
<tr>
<td><strong>Polygonaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Polygonum poiretii</em> (Meisn.) K.L. Wilson (PSMTS24)</td>
<td></td>
<td>L</td>
<td>Infusion of leaves is taken orally for 7 days after meal</td>
<td>Diarrhoea (4, 100)</td>
<td>Antimicrobial, anti-inflammatory [41]</td>
</tr>
<tr>
<td>Family/plant species/collection number</td>
<td>VN/CEN</td>
<td>Part used</td>
<td>Method of preparation</td>
<td>Pediatric use/ailment treated (Number of citation, fidelity level)*</td>
<td>Recorded literature uses</td>
</tr>
<tr>
<td>---------------------------------------</td>
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<tr>
<td><strong>Rosaceae</strong></td>
<td></td>
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</tr>
<tr>
<td><em>Prunus persica</em> (L.) Batsch. (PSMTS25)</td>
<td>Peche/peach</td>
<td>L</td>
<td>Decoction of leaves is taken orally for 5 days twice daily</td>
<td>Intestinal worms (2, 100)</td>
<td>Antioxidant properties [42]</td>
</tr>
<tr>
<td><strong>Rubiaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Morinda citrifolia</em> L. (PSMTS39)</td>
<td>Noni</td>
<td>L</td>
<td>Decoction of leaves is taken orally for 3 days after meal</td>
<td>Pain relief (1, 100)</td>
<td>Antioxidant properties [43]</td>
</tr>
<tr>
<td><strong>Rutaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Citrus × aurantium</em> L. (PSMTS10)</td>
<td>Bigarade/citrus</td>
<td>FR/L</td>
<td>Pressed juice of the fruit is taken orally. It is flavoured with sugar. Decoction of leaves is taken orally for 2 weeks</td>
<td>Flu (3, 100)</td>
<td>Anti-inflammatory properties [44]</td>
</tr>
<tr>
<td><em>Toddalia asiatica</em> (L.) Lam. (PSMTS31)</td>
<td>Patte poule/NA</td>
<td>L</td>
<td>Infusion of leaves is taken orally for 3 days</td>
<td>Fever (2, 100)</td>
<td>Fever and pulmonary infections [45]</td>
</tr>
<tr>
<td><strong>Sapindaceae</strong></td>
<td>Liane pok pok/NA</td>
<td>L</td>
<td>Herbal bath is given to child prepared from decoction of the leaves for 10 days</td>
<td>Eczema (30, 76.9), allergy (9, 23.1)</td>
<td>Antioxidant and anti-inflammatory [46] effects</td>
</tr>
<tr>
<td><strong>Solanaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brunfelsia suaveolens</em> (Humb. and Bonpl. ex Wild.) Bercht. and C. Presl (PSMTS06)</td>
<td>Fleur trompette/NA</td>
<td>F</td>
<td>Infusion of flowers is taken orally for 3 days</td>
<td>Asthma (3, 100)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Zingiberaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Curcuma longa</em> L. (PSMTS13)</td>
<td>Safran/green turmeric</td>
<td>R</td>
<td>Taken with milk orally at night for 10 days before sleep</td>
<td>Cough (4, 100)</td>
<td>Antimicrobial, antifungal, insecticidal, anti-inflammatory, and antioxidant properties [47]</td>
</tr>
<tr>
<td><em>Zingiber officinale</em> Roscoe (PSMTS32)</td>
<td>Gingembre/ginger</td>
<td>R</td>
<td>Decoction used <em>Cymbopogon citrates</em> for 10 days before sleep</td>
<td>Cough and flu (4, 100)</td>
<td>Anti-inflammatory, antiemetic, chemoprotective effects [48]</td>
</tr>
</tbody>
</table>

Plant part used: G: gel; AP: aerial parts; L: leaves; WP: whole plant; F: flowers; S: seeds; RP: root powder; B: bark; RI: rind; FR: fruit; FB: flower buds; VN/CEN: vernacular name/common English name, NA: not available. *The categories of the plants with higher number of uses mentioned (for one purpose) were also compared with their fidelity level.
previous generations (ancestors coming mainly from India and Africa). As mentioned in a previous study, therapeutic effect of natural therapies such as medicinal plants is the result of continued traditional use and this trend shows that Mauritians have left no stone unturned in exploring the biological diversity of the island [17]. It was obvious from this study that medicinal plants are the most commonly used natural product among the pediatric population [4, 5]. In a study by Ben-Arye et al. [15], it has been proposed that the use of these therapies differed by the child’s age (e.g., massage in infants, prayer, or faith healing in adolescents), country of origin, and nature of the diseases. The most frequent reasons for using such therapies are based on their perceived effectiveness, due to cultural values and beliefs and fear of adverse effects from conventional drugs among other reasons [6, 7].

It was observed from the present study that various parts of plants were used for the herbal formulation, with leaves being the most frequently used plant parts. The reason for this is possibly the higher concentration of active agents in this part of the plant. This may arise from the fact that leaves act as reservoirs for photosynthesis or exudates that are thought to contain toxins for plant protection and survival which consequently find medicinal values in human health. Use of leaves for herbal remedies is generally considered to be a sustainable approach in herbal therapies as there is less risk to cause much damage and hence less threat to extinction of potential medicinal plants. In an ethnobotanical survey in Nepal, it was reported that underground plant organs were the preferred part for herbal preparations, which consequently might lead to the slow disappearance of these plants [69]. In Nigeria, herbal preparation was mostly prepared from leaves to treat measles [70]. This shows a complete agreement with data collected in the present survey and also with that of Nadembega et al. [71] where leaves were used in highest quantity. It has also been argued [72] that healers tend to use leaves due to its high availability and accessibility and being easy to identify.

This study has also provided some salient information concerning the therapeutic management of gastrointestinal disorders in children with natural therapies. The common gastrointestinal disorders reportedly managed by natural therapies were regurgitation, defecation problems, infantile colic, and frequent stomach aches.

*Ayapana triplinervis* was reported as the most common herb used with the highest number of citations and most frequently used for vomiting. A wealth of published literature is available pertaining to the pharmacological properties of this species and its use in adult population [20], though scant information is available for its use for pediatric healthcare. Extract of *A. triplinervis* has been reported to harbour panoply of bioactive molecules. For instance, a total of seven coumarins known under the trivial names ayapin (or herniarin), ayapin, daphnetin, daphnetin dimethyl ether, daphnetin-7-methyl ether, hydrangetin, and umbelliferone have been characterized from this plant. Coumarins are considered to be components of the general defense response of plants to abiotic and biotic stresses and it has been confirmed that various substituted coumarins exhibit antimicrobial or anti-inflammatory activity and act as inhibitors of numerous enzyme systems [20]. These biological properties tend to support its use as a herbal remedy in Mauritius.

Another common medicinal plant documented in use in the present study is *Cymbopogon citratus* which was reported to be commonly used against cough and flu. *C. citratus* is known to contain various bioactive phytochemicals such as flavonoids, phenolics, terpenoids, and essential oils, which could account for its antimicrobial, antibacterial, antifilarial, antifungal, and anti-inflammatory properties amongst others [73]. *Zingiber officinale* that is mixed with *C. citrates* to enhance the preparation has recently been reported to constitute mainly sesquiterpene lactones, which are responsible for its anti-inflammatory activity by inhibiting arachidonic acid metabolism and thus prostaglandin synthesis [73].

*Matricaria chamomilla* was the third most highly used herb for infantile colic. Interestingly, *M. chamomilla* is included in the pharmacopoeia of 26 countries and as a drug [74]. *M. chamomilla* finds use in flatulence, colic, and hysteria, and it has also been documented to possess anti-inflammatory, antiseptic, antispasmodic, and mildly sudorific activities [74]. The dry flowers of chamomile are in great demand for use in herbal teas, baby massage oil, promoting gastric secretion, and the management of cough and cold and the use of herbal tea preparations has been reported to eliminate colic in 57% infants [74], which further endorses the plant’s popularity for use against colitis in Mauritius. Indeed, active substances of chamomile have been efficient for infant colic [75]. It contains a large group of therapeutically interesting and active compound classes. Sesquiterpenes, flavonoids, coumarins, and polyacetylenes are considered the most important constituents of the chamomile drug [74, 75].

Another plant recorded in the present that has been studied extensively is *Azadirachta indica*. *A. indica* was reported to be helpful in curing chickenpox. It has been reported [76] that *A. indica* possesses panoply of activities including antidermatophytic and antiviral properties. *Ocimum tenuiflorum* commonly known as holy basil was used for sleep disorders and also to soothe infants particularly before going to sleep [31]. It has been shown that *Ocimum tenuiflorum* contains a high concentration of eugenol (1-hydroxy-2-methoxy-4-allylbenzene) that may be a COX-2 inhibitor, similar to modern day pain-killers and hence could possibly account for its soothing effect in children [31]. *Cardiospermum halicacabum* was used against childhood eczema [77]. This species has been found to contain phytochemical constituents such as sterols, tannins, flavonoids, and triterpenes which can account for its anti-inflammatory properties [77].

Zootherapeutic practices represent an alternative to allopathic medicine in the traditional medicine of the local
Table 4: Animal products and the reported therapeutic uses for pediatric health care.

<table>
<thead>
<tr>
<th>SN</th>
<th>VN/CEN</th>
<th>Part(s) used and method of preparation</th>
<th>Therapeutic uses</th>
<th>UV</th>
<th>Reported literature uses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Bos taurus</strong></td>
<td>Martaige vache/cow's ghee Honey is added to cow's ghee and the mixture is warmed. Extracts of water cress are added to form a paste which is then ingested daily (before sleep) for 1 week.</td>
<td>Cough</td>
<td>0.31</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td><strong>Apis mellifera</strong></td>
<td>Mouche di miel/honey Honey is mixed with pressed orange juice or boiled milk and taken orally daily for 1 week. Honey is mixed with cow's ghee as above.</td>
<td>Cough and flu</td>
<td>0.75</td>
<td>Antimicrobial, anti-inflammatory [49]</td>
</tr>
<tr>
<td></td>
<td><strong>Crassostrea spp.</strong></td>
<td>Zouite/oyster Oyster with the shell is burnt for some minutes and then the inside flesh is consumed daily for 1 week.</td>
<td>Asthma</td>
<td>0.31</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td><strong>Donax trunculus</strong></td>
<td>Tek tek/bivalve A soup is prepared of the bivalves. It is administered especially at night for 1 week.</td>
<td>Asthma</td>
<td>0.05</td>
<td>Coughs and cold for children [50], laxative, abdominal bloating pain [51]</td>
</tr>
<tr>
<td></td>
<td><strong>Helix aspersa</strong></td>
<td>Kourpa/snail Live snails are heated and the oily substance is poured in aching ears of children for 5 days before going to bed.</td>
<td>Ear infection</td>
<td>0.25</td>
<td>Used for weakness [52]</td>
</tr>
<tr>
<td></td>
<td><strong>Coturnix coturnix japonica</strong></td>
<td>Di Zeouf caille/quail eggs Ingestion of 1 crude egg daily for 1 month.</td>
<td>Asthma/respiratory diseases</td>
<td>0.53</td>
<td>Asthma [53]</td>
</tr>
</tbody>
</table>

SN: scientific name, VN: vernacular name, CEN: common English name, and UV: use value.

population [78]. Although this knowledge is gradually disappearing with time due to phenomenon like urbanization and modernization, the easy availability and accessibility and limited side-effects of these animal-based therapies are responsible for their continued popularity in the study area. In the present investigation, 6 animal-based therapies have been documented to be used in child care with honey as the most used product against cough [49]. Indeed, several studies have established the beneficial effects of honey. Its antioxidant capacity has been well studied and attributed to its polyphenolic content, namely, flavonoids and phenolics [49]. Additionally, the use of honey in wound healing in medical setting has been linked to its antimicrobial potential. Other therapeutic properties of honey including its anti-inflammatory, antiulcerous, anticancerous, and antiviral effects have been identified [49].

Nonetheless, special precautions should be taken when animal tissues are used as remedies due to the possibility of transmission of serious and widespread zoonoses such as tuberculosis or rabies [79]. According to R. R. N. Alves and H. N. Alves [78], several species of monkeys have been identified as harboring infectious diseases transmissible to man with lethal consequences. Therefore, there is a pressing need for the implementation of sanitary measures to the trade of animal or their parts for medicinal purposes. Moreover, other issues that need to be addressed when using animal-based therapies are the potential interactions among various ingredients (plants and animals), the potential interactions of folk medicines with allopathic remedies, the effects of overdose, and the possibility of toxic or allergic reactions [80].

5. Conclusions and Recommendations

This study is the first attempt to gather primary folk knowledge on the use of plant and animal-based therapies as natural pharmacological agents for child care in Mauritius. Failure to document such knowledge can result in losses in traditional medicines and in scientific documentation of the cultural traditions of natural therapies used in the treatment of human diseases. Our present investigation revealed that the use of plant and animal-based therapies constitutes the common legacy of all Mauritians and despite the penetration of allopathic medicine, natural therapies continue to play a crucial role in the primary healthcare system of Mauritius. This study has also demonstrated that Mauritians exploits a diversity of natural therapies which is perceived by the majority of parents to be effective. Medicinal plants which form part of the Mauritian traditional medicines have been observed to be the most preferred natural products. This can be explained based on cultural, and religious beliefs and on its availability. Moreover, herbal therapies were perceived as having minimal side-effects by parents in the present study. However, pediatricians should realise the fact that such therapies are in use and the possibility of adverse effects is not to be eliminated or ignored. Therefore, clinicians should be more cooperative with patients to enable the reporting of cases and should routinely ask about such use.
Despite the prevalence and the surge of use of natural therapies amongst pediatric individuals, this remains yet a theme to be fully studied and investigated for this particular group. Further research and investigations are needed to explore the potential of medicinal herbs reported herein which could be the basis of an evidence-based investigation to discover new drugs. Natural therapies with the highest number of citations warrant further clinical studies geared towards pediatric healthcare.

Conflict of Interests

The authors declare no conflict of interests. Neither author has any commercial associations that might create a conflict of interests in connection with this paper.

Acknowledgments

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References


Research Article

Antiproliferative and Apoptotic Effects of *Sesbania grandiflora* Leaves in Human Cancer Cells

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Natural phytochemicals and their derivatives are good drug candidates for anticancer therapeutic approaches against multiple targets. We report here the initial findings from our studies on the anticancer properties of the leaves of the medicinal plant *Sesbania grandiflora*. In the current study, five different solvent fractions from the leaves of *S. grandiflora* were tested on cancer cell lines such as MCF-7, HepG2, Hep-2, HCT-15, and A549. The methanolic fraction of *S. grandiflora* was found to exert potent antiproliferative effects especially in the human lung cancer cell line, A549. Caspase 3 was activated in the methanolic fraction treated A549 cells thereby leading to cell death by apoptosis. DAPI staining, DNA laddering, and decrease in mitochondrial membrane potential further confirmed the apoptotic mode of cell death. The high levels of ROS intermediates evidenced by DCF-DA staining could have played a role in the apoptotic induction. Decrease in levels of cyclin D1 and decrease in the activation of NFkB were observed in A549 cells on treatment with methanolic fraction, giving a hint on the possible mechanism of action. These results prove that the medicinal plant *S. grandiflora* can be explored further for promising candidate molecules to combat cancer, especially lung cancer.

1. Introduction

Natural products have been a continuous source of medicines for a very long time. From ancient days, natural products have been the sole means to treat diseases and injury. In most traditional systems of medicine worldwide, plant-based products serve as an integrated part of treatment. The therapeutic effects of these plants have been subsequently proved by medical practices.

Cancer is a major health hazard worldwide. Cancer treatment relies on chemotherapy using cytotoxic drugs, radiation therapy, and surgery. Today a variety of cytotoxic drugs have been reported to combat cancer. Most of these drugs are inadequate not only because of their therapeutic efficacy but also because they have undesirable side effects. With the aim of searching novel compounds without undesirable side effects, we focused on natural medicines. Plants are reported to have a long history in the treatment of cancer [1]. The use of plants and plant-based products for cancer treatment is rapidly growing in medical practices [2]. This led us to choose the medicinal plant *Sesbania grandiflora* L. This plant has been used in the traditional medicine of the Indian Ayurvedic system. Though already proven to have varied medicinal uses like hepatoprotective and cardioprotective roles [3, 4], *S. grandiflora* is the plant of interest in the last couple of years especially for its chemopreventive effects. Studies have shown that the flowers of this plant have potent anticancer activities in various cancer cell models [5, 6]. In many parts of Southeast Asian countries, the flowers and leaves of *S. grandiflora* are used in medicine as well as in traditional food. It has also been shown that the roots of *S. grandiflora* possess antituberculosis activity [7]. The flower extracts of this plant have also been proved to possess antimicrobial activities [8]. In one of our previous studies we have found that the leaves of this plant possess protective roles against rat kidney during alcohol and polyunsaturated fatty acid induced oxidative stress [3]. The leaves have shown anxiolytic and anticonvulsive activity in experimental rats [9]. The leaf juice also seems to possess antiurolithiatic and antioxidant properties [10]. Though the flowers have now
proven to have chemopreventive effects [11], not much work, including mechanism of action and molecular level studies, has been carried out to prove the chemopreventive effects of the S. grandiflora leaves. The current study is focused on the antiproliferative effects of the leaves of S. grandiflora in human cancer cell models.

2. Materials and Methods

2.1. Materials. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), rhodamine 123, dichlorofluorescein diacetate (DCF-DA) and 4',6-diamidino-2-phenylindole (DAPI), and the antibodies for β-actin and NfixB were purchased from Sigma (St. Louis, MO, USA). Primary antibodies for cyclin D1 (SC-246), caspase 3 (SC-7148), and lamin B (SC-6217) and secondary antibodies, goat anti-rabbit IgG-HRP (SC-2004), goat anti-mouse IgG-HRP (SC-2005), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Sample Preparation for Cell Culture. The dried leaves of S. grandiflora were subjected to solvent extraction using solvents of increasing polarity such as petroleum ether, chloroform, acetone, methanol, and water by using Soxhlet extractor. Each solvent fraction was distilled and concentrated. Water insoluble fractions were concentrated using rotary evaporator and subjected to vacuum under reduced pressure overnight yielding the fractions in powder form. Water soluble fraction was subjected to lyophilization (−80°C, in vacuum under reduced pressure for 24 h) and obtained in a powder form. All the fractions were dissolved in the cell culture medium (DMEM) and filtered through 0.22 μm filter before being subjected to cell culture treatments. Water insoluble fractions were initially mixed with DMSO before dissolving in DMEM with a final concentration of 0.1% DMSO during cell treatment.

2.3. Cell Lines and Culture Conditions. Cancer cell lines MCF-7, HepG2, HCT-15, Hep-2, and A549 and normal cell line MRC-5 were obtained from NCCS, Pune, India. The cells were maintained in DMEM supplemented with 10% FBS at 37°C in CO₂ incubator in an atmosphere of humidified 5% CO₂ and 95% air. The cells were maintained by routine subculturing in tissue culture flasks. The culture medium was changed routinely and the cells were split when they reached confluence. The passage number for MCF-7, HepG2, HCT-15, Hep-2, and A549 was P34, P48, P15, P66, and P42, respectively, while performing the cytotoxicity tests.

2.4. MTT Assay. This test is based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which is reduced to a purple-blue insoluble formazan precipitate by the living cells. Experiments were performed in six replicates in 96-well flat-bottomed culture plates (Corning). MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/mL. After 24 h of incubation of cancer cells with different concentrations of various fractions of S. grandiflora, 20 μL of MTT solution was added and the plate was incubated at 37°C for 4 h. After incubation, 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan and transferred to fresh microplate. The amount of colored formazan metabolite formed was determined by its absorbance at 570 nm in a VersaMax ELISA Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.5. DAPI Staining. Cell nuclear morphology was evaluated by fluorescence microscopy following DAPI staining. A549 cells were treated with the methanolic fraction of S. grandiflora for 24 h. The cells were washed with PBS (pH 7.4), fixed with ice cold 70% ethanol and resuspended in DAPI, and incubated for 15 min at 37°C wrapped in aluminium foil. The cells were then washed with PBS and examined under Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA).

2.6. Acridine Orange/Ethidium Bromide Staining. Acridine orange/ethidium bromide (AO/EB) staining was carried out to detect morphological evidence of apoptosis. A549 cells were treated with the methanolic fraction of S. grandiflora for 24 h. The cells were washed with PBS (pH 7.4) and 10 μL of acridine orange/ethidium bromide solution (60 μg/mL of acridine orange and 100 μg/mL of ethidium bromide in PBS) and made up to 100 μL using PBS and incubated for 5 min. The cells were then washed with PBS and examined under Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA).

2.7. Rhodamine 123 Staining. Rhodamine 123 is a fluorescent dye that binds to metabolically active mitochondria. A549 cells were treated with the methanolic fraction of S. grandiflora for 24 h. The cells were washed with PBS (pH 7.4) and fixed with ice cold 70% ethanol and incubated with 5 μg/mL rhodamine 123 at 37°C for 30 min. The cells were then washed with PBS and examined under Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA).

2.8. DNA Fragmentation Analysis. To confirm the apoptotic mode of cell death, DNA fragmentation assay was performed. A549 cells were treated with the methanolic fraction of S. grandiflora for 24 h. After treatment, cells were trypsinized and collected with PBS in 1.5 mL Eppendorf tubes. 100 μL of lysis buffer was added to the pellet and incubated for 30 min on ice. After incubation, centrifugation was carried out at 12,000 g for 30 minutes at 4°C. The supernatant was collected in a fresh tube and mixed with 25:24:1 mixture of phenol: chloroform: isoamyl alcohol and precipitated with two equivalents of ice cold ethanol and one-tenth equivalent of sodium acetate. This was followed by centrifugation at 12,000 g for 20 minutes. The pellet was resuspended in 30 μL of sterile water-RNase solution (15 μg/mL RNase in sterile water) and 6 μL of 6x loading dye for 30 minutes at 37°C which was electrophoresed and imaged in a Syngene Ingenious gel documentation system (Syngene Bioimaging Pvt. Ltd., Haryana, India).
2.9. Western Blot Analysis. Western blot analysis was carried out using cytosolic as well as nuclear fractions [12] of human lung cancer cells A549 treated with selected concentrations of the methanolic fraction of *S. grandiflora*. Protein concentration was determined using Bradford reagent and lysates were resolved on 15% sodium dodecyl sulphate- (SDS-) polyacrylamide gels. The proteins were then electrotransferred onto nitrocellulose membrane (Sigma, St. Louis, MO, USA). After blocking with 5% nonfat milk in Tris-buffered saline (TBS, 0.1M, pH 7.4), blots were subjected to various primary antibody incubations with mouse monoclonal cyclin D1 antibody, rabbit polyclonal caspase 3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit monoclonal NFκB p65 antibody (Sigma, St. Louis, MO, USA) at 4 °C overnight. Protein abundance of β-actin and lamin B served as a control for protein loading for cytosolic and nuclear fractions, respectively. Membranes were incubated with secondary antibody, HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted at an appropriate dilution in 1% BSA, for 1 h at room temperature. After each step, blots were washed thrice with Tris-buffer saline-Tween 20 (TBST). Protein bands were detected by enhanced chemiluminescence method (ECL, Bio-Rad, Hercules, CA, USA). The spectral density of the bands was analyzed by Bandscan 5.0 image analyze system. The protein expression pattern was obtained by normalizing the density to that of β-actin and lamin B for cytosolic and nuclear fractions, respectively.

2.10. Dichlorofluorescein Diacetate (DCF-DA) Staining. ROS levels can be determined by DCF-DA stain. A549 cells were treated with the methanolic fraction of *S. grandiflora* for 24 h. The cells were washed with PBS (pH 7.4) and stained with 10 μM of DCF-DA for 30 min at 37°C wrapped in aluminium foil. The cells were then washed with PBS and examined under Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA).

2.11. Analysis Using Spectrofluorometer for Quantification of ROS Generation. A549 cells were treated with the methanolic fraction of *S. grandiflora* for 24 h. Following the treatment, the cells were trypsinized and collected in Eppendorf tubes. 25 μM of DCF-DA was added and incubated for 40 min at 37°C wrapped in aluminium foil. The fluorescence intensity was recorded by using Fluorolog-FL3-11 spectrofluorometer (HORIBA Jobin Yvon, NJ, USA) with excitation and emission wavelengths of 485 nm and 529 nm, respectively, and slit widths set to 5.0.

2.12. RNA Isolation and Reverse Transcriptase-PCR. Total RNA isolation was carried out with A549 cells treated with selected concentrations of the methanolic fraction of *S. grandiflora* using TRI reagent (Sigma, St. Louis, MO, USA). The primer sequences used for cyclin D1 and beta actin were previously reported for A549 cells [13] and are as follows: cyclin D1:

- forward 5’-CCGTCATGCGGAAGATC-3’;
- reverse 5’-CCTGTCCACTACCGCCTCA-3’;

β-actin:

- forward 5’-AAATCGTGCGTGACATTAA-3’;
- reverse 5’-CTCGTCAACTCCTGCTTG-3’.

2.13. Statistical Analysis. All the data were analyzed using the SPSS 7.5-Windows Students version software (SPSS Inc., Chicago, IL, USA). For all the measurements, one-way ANOVA followed by Tukey’s test was used to assess the statistical significance between groups. *P* ≤ 0.05 was considered to be statistically significant.

### Table 1: IC50 values for the cytotoxicity of various solvent fractions from the leaves of *S. grandiflora* determined using MTT assay.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MCF-7 (μg/mL)</th>
<th>HepG2 (μg/mL)</th>
<th>A549 (μg/mL)</th>
<th>HCT-15 (μg/mL)</th>
<th>Hep2 (μg/mL)</th>
<th>MRC-5 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether fraction</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td>116.8</td>
<td>93.2</td>
<td>74.1</td>
<td>52.3</td>
<td>92.6</td>
<td>104.3</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>332.7</td>
<td>473.9</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>388.1</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Methanol fraction</td>
<td>94.3</td>
<td>62.7</td>
<td>23.6</td>
<td>41.8</td>
<td>106.6</td>
<td>104.4</td>
</tr>
<tr>
<td>Water fraction</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
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</table>

2.14. Effects of *S. grandiflora* Methanolic Extract on Cell Proliferation. As shown in Table 1, the IC50 of *S. grandiflora* methanolic fraction was found to be 23.6 μg/mL in A549 cells which is highly significant compared to the IC50 values in other cancer cell lines which include 41.8 μg/mL in HCT-15, 62.7 μg/mL in HepG2, 94.3 μg/mL in MCF-7, and 106.6 μg/mL in Hep-2. The methanolic fraction showed promising anticancer activity with significantly lesser cytotoxicity in normal cells MRC-5 (IC50 104.4 μg/mL) compared to that of the cancer cells. Similar result was observed when the cancer cells were treated with the chloroform fraction but high toxicity was observed even in the normal cells which made it highly unsuitable for our study. Cytotoxicity was comparatively much lesser/no cytotoxicity was observed when treated with other solvent fractions such as petroleum ether, acetone, and water. Dose response curve was provided for the effect of various fractions on A549 cells (Figure 1). The regression equation for the effect of methanolic fraction on A549 cells is *y* = –13.53ln(*x*) + 92.768, where *x* is
concentration in μg/mL and \( y \) is the percentage of cell viability. Further analyses were focused on A549 cells with or without the methanolic fraction of \( S. \) grandiflora.

3.2. Effects of \( S. \) grandiflora Methanolic Extract on Cellular Morphological Changes of A549 Cells. We studied the mode of death of A549 cells upon treatment with \( S. \) grandiflora methanolic fraction. Apoptosis can be differentiated from necrosis by their characteristic nuclear changes. DAPI is a nuclear stain which is observed as blue fluorescence when excited under fluorescence microscope. In our present study, DAPI staining revealed the changes associated with apoptosis in A549 cells treated with the methanolic fraction of \( S. \) grandiflora (Figures 2(a) and 2(b)). The morphological changes associated with apoptosis such as chromatin condensation, nuclear fragmentation, and margination of nucleus (marked by arrows in Figure 2(b)) are evident in A549 cells upon treatment. Similarly acridine orange/ethidium bromide staining was performed to evaluate the cellular morphological changes in A549 cells treated with the methanolic fraction (Figures 3(a) and 3(b)). Treatment with the methanolic fraction revealed changes associated with apoptosis as indicated by arrows (Figure 3(b)).

3.3. Effects of \( S. \) grandiflora Methanolic Extract on the Mitochondrial Membrane Potential (ΔΨm) of A549 Cells. Mitochondrial membrane potential can be evaluated by staining with rhodamine 123. Green fluorescence is observed in cells with high membrane potential. We found that, upon treatment with the methanolic fraction, the mitochondrial membrane potential was decreased in A549 cells as evidenced by the decrease in the fluorescence compared to the untreated cells (Figures 4(a) and 4(b)). DAPI was used as a counterstain to locate the cells with decreased membrane potential (Figure 4(c)).

3.4. Apoptosis Confirmation by DNA Fragmentation. To gain further insights into the mode of cell death caused by \( S. \) grandiflora methanolic fraction, we determined its effect on the DNA fragmentation, a widely used technique for the detection of apoptosis. The treatment resulted in a dose-dependent increase in the DNA fragmentation levels in A549 cells (Figure 5). DNA fragmentation was clearly visible in the groups with the dose-dependent treatment of the methanolic fractions (Lanes 2 to 7) compared to the control group (Lane 1).

3.5. Protein Expression of Caspase 3, Cyclin D1, and NFκB p65. To confirm the results of \( S. \) grandiflora induced apoptosis in A549, we further investigated whether increased caspase 3 activity was observed through western blotting. As predicted, a dose-dependent increase in the caspase 3 expression was observed (Figure 6(a)). Densitometry of caspase 3 expression showed that there was a significant increase in the levels of caspase 3 starting at 1/4 IC\(_{50}\) dose treatment of methanolic fraction (Figure 6(e)). Since cell cycle plays a major role in many instances of cancer, we planned to evaluate the expression levels of cyclin D1 which helps in the cell cycle G1/S transition. We observed that there was a dose-dependent decrease in the expression levels of cyclin D1 when A549 cells were treated with \( S. \) grandiflora methanolic fraction (Figure 6(b)). Densitometry of cyclin D1 expression showed that there was a significant decrease in the levels of cyclin D1 (Figure 6(f)). We also found that the cytosolic abundance of NFκB increased with the treatment of the methanolic fraction (Figures 6(c) and 6(g)). Nuclear abundance of NFκB decreased with the treatment of the methanolic fraction (Figures 6(h) and 6(i)). The protein expression levels of β-actin were used as the loading control for cytosolic fraction (Figure 6(d)) and lamin B was used as the loading control for the nuclear fraction (Figure 6(i)).

3.6. Evaluation of Intracellular Reactive Oxygen Species (ROS). ROS generation was usually associated with cell apoptosis. To compare the level of ROS generation in A549 cells with or without the methanolic fraction of \( S. \) grandiflora, we used an oxidation-sensitive fluorescent dye, 2',7'-dichlorofluorescein diacetate. Fluorescent microscopy images revealed the increased generation of ROS in the treatment group (Figure 7(b)) compared to that of control (Figure 7(a)). A significant increase in the levels of dichlorofluorescein fluorescence was detected in the treated cells compared to control as evidenced by spectrofluorometer readings (Figures 7(c), 7(d), and 7(e)).

3.7. Gene Expression of Cyclin D1. In order to evaluate whether the decrease in cyclin D1 expression occurred at the translational level or transcriptional level, reverse transcriptase-PCR was performed. The gene expression of cyclin D1 decreased with the treatment of the methanolic fraction (Figure 8(a)) indicating that the cyclin D1 was decreased at the transcriptional level. The gene expression of beta actin served as the loading control (Figure 8(b)).
4. Discussion

The use of naturally occurring plant-based products has shown promising results in the treatment of cancer. One renowned example is the use of taxol, a plant-based bioactive compound used in cancer chemotherapy [14]. Due to the complexity of cancer, novel bioactive compounds with multitargeting efficacy are the need of the hour. We tried to evaluate the anticancer potential of a traditional plant, used as a medicine in India and most parts of Southeast Asia, called *Sesbania grandiflora*. Our present findings demonstrate that the methanolic fraction of *S. grandiflora* has potent
Figure 4: Mitochondrial staining using rhodamine 123 of human lung cancer cells A549 in the presence or absence of the methanolic fraction of S. grandiflora leaves. (a) Untreated A549 cells. (b) A549 cells treated with IC_{50} dose of the methanolic fraction for 24 h showing decreased membrane potential as indicated by the arrows. (c) DAPI costaining of A549 cells treated with IC_{50} dose of the methanolic fraction. Magnification 200x.

Figure 5: DNA fragmentation analysis for human lung cancer cells A549 in the presence or absence of the methanolic fraction of S. grandiflora leaves. Lane 1 is untreated A549 cells. Lane 2 is 5 μg/mL treated. Lane 3 is 10 μg/mL treated. Lane 4 is 15 μg/mL treated. Lane 5 is 20 μg/mL treated. Lane 6 is 25 μg/mL treated. Lane 7 is 30 μg/mL treated. All the treatment is for 24 h.

Anticancer activity against human lung cancer cells and comparable activity in few other cancer cell models while significant cytotoxicity was not observed in the normal cells. One possible reason could be the plant's modulatory effect on apoptosis, which is usually blocked in cancer cells, thereby promoting cell death faster compared to that of normal cells.

Evaluation of apoptosis is crucial to differentiate it from necrosis. Nuclear fragmentation is one of the characteristic features of apoptotic mode of cell death. We used DAPI, a fluorescent DNA-binding agent, and acridine orange/ethidium bromide staining to observe cell death and cellular morphological changes involved in apoptosis [15]. The treatment group showed fragmented apoptotic bodies, shrunken and margined nuclei in contrast to the normal and large nucleus in the untreated cells, proving the apoptotic potential of the extract. Decrease in mitochondrial membrane potential usually indicates apoptosis and helps to distinguish the mode of cell death from necrosis [16]. Our findings confirmed the decrease in the mitochondrial membrane potential as evidenced from the decreased rhodamine 123 fluorescence intensity when A549 cells were treated with the IC_{50} dose of S. grandiflora.

DNA laddering is one of the hallmark indications that helps in the differentiation of apoptosis from necrosis [17,18]. In the treatment group of our present study, the presence of the laddering pattern of DNA fragments and the absence of the necrotic streak further confirm apoptosis. DNA laddering occurs through caspase-activated DNases (CAD) which is activated by caspases. In order to confirm the involvement of caspases in our study, we evaluated the protein expression levels of caspase 3, the common caspase for most of the death signals. As predicted, there was a dose-dependent increase in the levels of active caspase 3.

Arrest of cell cycle can also trigger apoptosis. Cyclins are the proteins which regulate the cyclin-dependent kinases, whose activity in turn regulates the cell cycle checkpoint transitions. One such cyclin, cyclin D1, is involved in the transition of cells from G1 to S phase of the cell cycle. Overexpression of cyclin D1 has been reported in many cancers [19–21] and it was shown that the inhibition of cyclin D1 expression could help in the cancer treatments [22, 23]. In several studies, it was observed that the decrease in cyclin D1 levels is associated with apoptosis [24, 25]. In our present study, we observed a decrease in the expression levels of cyclin D1 which could have probably mediated the antiproliferative and apoptotic effects of the plant.

In order to determine the upstream signal transduction effectors for the possible mechanism of action for the plant's...
modulatory effect on cell cycle and apoptosis, we focused on the transcription factors that might regulate them. One of the important transcription factors is nuclear factor kappa B (NFκB) which has been implicated in cell proliferation as well as tumor development. NFκB is present in the cytosol in an inactive form tightly bound with cytoplasmic IκB proteins [26]. Activated NFκB by the release from IκB proteins translocates into the nucleus to activate the transcription of the target genes. Activated NFκB has been demonstrated to be involved in the prevention of apoptosis [27] as well as in the progression of cell cycle of G1 to S transition through regulation of cyclin D1 [28]. This gives an overall idea regarding the activation of caspase 3 as well as decrease in the protein abundance of cyclin D1. In our present study, the levels of NFκB p65 in the cytosol increased with the concomitant decrease in the nucleus of A549 cells after treatment with the methanolic fraction, thereby preventing the activation of several target genes including cyclin D1. In order to verify the inhibition of cyclin D1 expression at the transcriptional level, RT-PCR was performed which
Figure 7: DCFDA staining for ROS generation of human lung cancer cells A549 in the presence or absence of the methanolic fraction of *S. grandiflora* leaves. (a) Untreated A549 cells. (b) A549 cells treated with IC50 dose of the methanolic fraction for 24 h. (c) Spectrofluorometer measurement for the levels of ROS intermediates in untreated A549 cells. (d) Spectrofluorometer measurement for the levels of ROS intermediates in treated A549 cells. (e) Densitometry graph comparing the levels of ROS intermediates in untreated and treated A549 cells. Values are mean ± S.D from three independent experiments. ANOVA followed by Tukey's test was used to assess the statistical significance between groups. *P* ≤ 0.05, significance relative to control.
confirmed that the decrease in the cyclin D1 expression occurs at the transcriptional level which correlates with the decreased NFκB p65 activation. Thereby the mechanism of action of the methanolic fraction of *S. grandiflora* could be associated with a pathway which prevented the activation of NFκB.

Apoptotic induction can also be mediated through ROS intermediates [29–31]. It was reported that ROS could play a role in downregulating Bcl-2 [32] as well as in triggering the release of cytochrome C from the mitochondria into the cytoplasm along with Fas associated proteins recruitment and finally leading to the activation of caspase 3 and apoptosis.
In our present study, increased levels of ROS in the treatment group indicated that the apoptotic mode of cell death induced by the methanolic fraction of *S. grandiflora* is through the generation of ROS. Thereby, an outline to the mechanism of action for the anticancer effect of *S. grandiflora* leaves involves the prevention of the transcription of major genes by inhibiting NFκB activity and arresting the cells in G1/S phase as well as the generation of ROS intermediates, all triggering the apoptotic cascade leading to cell death (Figure 9).

These observations clearly suggest that the antiproliferative effect exerted by the leaves of *S. grandiflora* is associated with apoptosis. A novel source for a cytotoxic drug is thus warranted from our current findings. Most secondary molecules with apoptosis. A novel source for a cytotoxic drug is 

5. Conclusion

From the results, it is clear that the alcoholic extract of *S. grandiflora* exerted antiproliferative effects especially on lung cancer cells. The mode of cell death of cancer cells by *S. grandiflora* predominately followed apoptosis. A possible G1/S arrest as confirmed through decreased cyclin D1 expression might have triggered apoptosis. The mechanism of action of the methanolic extract in lung cancer cells could possibly involve a pathway that prevents NFκB activation. Thus, the results of this study have led to a new source of plant exerting potent antiproliferative and apoptotic effects. Our present study also suggests the possibility of a lifesaving drug combating different types of cancers especially lung cancer.

Conflict of Interests

The authors declare no conflict of interests.

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References


Glycyrrhizic Acid in the Treatment of Liver Diseases: Literature Review

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Glycyrrhizic acid (GA) is a triterpenoid glycoside found in the roots of licorice plants (Glycyrrhiza glabra). GA is the most important active ingredient in the licorice root, and possesses a wide range of pharmacological and biological activities. GA coupled with glycyrrhetinic acid and 18-beta-glycyrrhetic acid was developed in China or Japan as an anti-inflammatory, antiviral, and antiallergic drug for liver disease. This review summarizes the current biological activities of GA and its medical applications in liver diseases. The pharmacological actions of GA include inhibition of hepatic apoptosis and necrosis; anti-inflammatory and immune regulatory actions; antiviral effects; and antitumor effects. This paper will be a useful reference for physicians and biologists researching GA and will open the door to novel agents in drug discovery and development from Chinese herbs. With additional research, GA may be more widely used in the treatment of liver diseases or other conditions.

1. Introduction

The application of natural compounds in the treatment of refractory diseases is a new trend in modern clinical medicine. Because of their satisfactory efficacy in clinic and low toxicity, more natural products are being used as alternative treatments for many diseases. Many hepatoprotective monomers are derived from natural herbs, especially those from China. Glycyrrhizic acid (GA) is an example of one of these hepatoprotective compounds.

The traditional Chinese medicine Gancao (licorice root) is the dried roots of Glycyrrhiza uralensis Fisch (licorice), G. inflata Bat., or G. glabra L. Gancao which was first described in the Chinese book “Shen Nong Ben Cao Jing” in 200 A.D. as an antidote to toxic substances, ache, and other diseases. Gancao can complement other drugs to reduce toxicity and increase efficacy. The traditional use of Gancao involves a decoction of dried plant roots and stems. Some of the possible therapeutic properties of Gancao include antiarthritic [1], antiallergic [2], antiviral [3], antihepatotoxic [4], anticholinergic [5], antiestrogenic [6], anti-inflammatory [6], antileukemogenic [7], and anticarcinogenic effects [8]. It is commonly used for the treatment of acute and chronic liver injury, viral hepatitis, hepatic steatosis, liver fibrosis, hepatoma, viral myocarditis [9], and other diseases like psoriasis [10] or prostate cancer [11].

The known chemical components of Gancao include saponins (mainly glycyrrhizin (GA), 3.63–13.06%), flavonoids (1.5%), coumarin, alkaloids, polysaccharides, sitosterol, and amino acids [12]. GA (Figure 1) and glycyrrhetinic acid (Figure 2) are well-characterized components of Gancao. GA has been developed as a hepatoprotective drug in China and Japan. GA can generate glycyrrhetinic acid through metabolic processes in the human body. Therefore, the pharmacological effects of GA are essentially the same as glycyrrhetinic acid [13]. GA, also called glycyrrhizin, is a triterpene glycoside from licorice root (Glycyrrhiza glabra) and consists of one molecule of 18β-glycyrrhetinic acid and two
molecules of glucuronic acid (18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronide) [14, 15]. Glycyrrhizin is considered to be the major active component of Gancao as demonstrated by studies with experimental animal models [16] and clinical studies [17]. GA has been used clinically for more than 20 years in patients with chronic hepatitis in China and Japan [18] and shows a satisfactory therapeutic effect in many other diseases. GA is also widely used as a sweetening and flavoring agent in food.

GA is a main substance of licorice, which is one of the most important substances utilized as traditional medicine for almost 2000 years. Moreover, GA was reported to have antiallergic, antiviral, and anti-inflammatory activities. GA was also found to suppress the rise in fasting blood glucose and insulin levels and improve glucose tolerance. Additionally, GA may act as an antidiabetic substance without inducing side effects, although the mechanism is unclear [19].

GA can form two epimers: α-GA and β-GA (Figure 3). α-GA is derived from β-GA by isomerization, and the α- and β-forms differ only in their C18-H-, trans-, and cis-configuration, respectively. Some scholars examined their distribution characteristics in rat tissue and found that the concentrations of α-GA in the liver and duodenum were significantly higher than those of β-GA after i.v. administration. However, the concentrations of α-GA in the other tissues were lower than or similar to those of β-GA and declined rapidly. This indicates that the protective and anti-inflammatory effects of α-GA on the liver may be better than those of β-GA [20].

Several clinical studies reported that GA was efficacious in the treatment of various types of inflammation (mainly in liver [21–30] (Table 1), but also in lung, kidney, intestine, and spinal cord [31]). The most common use of GA is in the treatment of liver disease [32]. GA can reduce steatosis and necrosis of liver cells significantly [33] to inhibit the inter-interstitial inflammation and liver fibrosis and promote cell regeneration. GA has few side effects and is therefore considered to be a drug worth attention and promotion for liver disease.

2. Mechanisms of GA Effects

2.1. Inhibition of Hepatic Apoptosis and Necrosis. Tumor necrosis factor-alpha (TNF-α) is an important cytokine, which is a key mediator of hepatic apoptosis and necrosis in LPS/D-GaAlN-induced liver failure [34]. Plasma TNF-α level is also elevated in patients with chronic hepatitis caused by hepatitis B viral [35] and acute alcoholic hepatitis [36]. Therefore, TNF-α plays a key role in the pathogenesis of not only endotoxin-induced experimental liver injury but also many human liver diseases. Caspase-3 activation is an indicator of almost all apoptosis systems [37], GA has anti-inflammatory and antiapoptotic effects via suppression of TNF-α and caspase-3 and these are used to explain the hepatoprotective effect of GA (Table 2) [38]. GA also significantly inhibits the release of cytochrome C from mitochondria into the cytoplasm. The anti-inflammatory activity of GA may rely on the inhibition of release of TNF-α, myeloperoxidase activity, and translocation of nuclear factor-kB (NF-κB) into the nuclei. GA also upregulated the expression of proliferating cell nuclear antigen, implying that it might be able to promote regeneration of liver injury [39]. Activated Kupffer cells are involved in ischemia-reperfusion- (I/R-) induced liver injury and high-mobility group box 1 (HMGBl) production. GA was shown to inhibit HMGBl production by Kupffer cells and prevented I/R-induced liver injury [40]. GA could also alleviate I/R-induced [41] and spinal cord [42] injury via this
mechanism. In addition, GA conjugates free radicals, which might explain the protective action of GA [43]. For example, GA can be an effective chemopreventive agent against lead acetate mediated hepatic oxidative stress in rats because it binds lead [44]. In concanavalin A- (ConA-) induced mouse model, GA alleviated Con-A-induced inflammation and fibrosis progression in livers via inhibition of CD4+ T cell proliferation in response to ConA via the Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K)/AKT pathways [45].

2.2. Anti-Inflammation and Immunity Regulation. GA suppressed interleukin-6 (IL-6) and TNF-α production induced by the lipid A moiety of lipopolysaccharides (LPS) in RAW264.7 cells. It inhibited lipid A-induced NF-κB activation in Bax/F3 cells expressing toll-like receptor 4 (TLR4)/myeloid differentiation protein-2 (MD-2), cluster of differentiation 14 (CD14), and bone marrow-derived macrophages (BMMs). GA also inhibited activation of mitogen-activated protein kinase (MAPKs), including JNK, p38 protein, and ERK in BMMs. In addition, GA inhibited NF-κB activation and IL-6 production induced by paclitaxel, a nonbacterial TLR4 ligand. It attenuated the formation of the LPS-TLR4/MD-2 complexes, resulting in inhibition of homodimerization of TLR4. Therefore, GA modulated the TLR4/MD-2 complex at the receptor level, leading to suppression of LPS-induced activation of signaling cascades and cytokine production. This indicates that GA can attenuate inflammatory responses and modulate innate immune responses [46]. Moreover, GA can prevent the activation of signal transducers and activators of transcription-3 (STAT-3), reduce the upregulation of intercellular cell adhesion molecule (ICAM-1) and P-selectin expression, reduce formation of poly(adenosine diphosphate-ribose) (PAR) and nitrotyrosine, and reduce polymorphonuclear neutrophil (PMN) infiltration. Some observations suggest that broad anti-inflammatory activity of GA is mediated by interaction with the lipid bilayer, thereby attenuating receptor mediated signaling [47]. GA inhibited the lytic pathway of the complement system and may prevent tissue injury caused by the membrane attack complex. Therefore, GA could be a potent agent for suppressing complement-dependent tissue injury in autoimmune and inflammatory diseases [48]. GA can suppress systemic inflammatory response syndrome (SIRS) associated anti-inflammatory response manifestation via inhibition of CC chemokine ligand 2 (CCL2) production by PMN. It may also have the potential to inhibit anti-inflammatory response-associated opportunistic infections in critically ill patients with severe SIRS [49]. There are also other studies that indicated the same anti-inflammatory mechanisms of GA [50].

2.3. Antiviral Effects. The antiviral mechanisms of GA mainly include the inhibition of viral replication and immunity regulation. GA affects cellular signaling pathways such as protein kinase C and casein kinase II and transcription factors such as activator protein 1 and NF-κB. Furthermore, nitrous oxide (NO) inhibits replication of several viruses like Japanese encephalitis virus 4 (a member of the Flaviviridae family), which can also be inhibited by GA. The powerful anti-inflammatory capabilities of GA make it effective in the treatment of various types of hepatitis like viral hepatitis and nonalcoholic hepatitis. GA was found to inhibit the replication of the SARS-associated virus [51]. In the treatment of HCV (hepatitis C virus) infection, GA can inhibit HCV full-length viral particles and HCV core gene expression or function in a dose-dependent manner and have a synergistic effect with interferon [52]. GA is also involved in biliary secretion and excretion. GA can increase hepatic glutathione levels by the inhibition of biliary excretion of glutathione partly through the inhibition of MRP2 [53], an efflux transporter located at the canalicular membrane of a hepatocyte. MRP2 translocates glutathione, LTC4, bilirubin, methotrexate (MTX), glucuronide (e.g., estradiol-17β-glucuronide [E217G]), or sulfate conjugates and other organic anions from a hepatocyte into the bile canalicus [54–58].

GA can activate certain immune functions, such as IFN production, augmentation of NK cell activity, and modulation of the growth response of lymphocytes via augmentation of IL-2 production [70]. GA can enhance immune function.
<table>
<thead>
<tr>
<th>Experimental drugs</th>
<th>Dose and course of treatment</th>
<th>Combined medication</th>
<th>Case/control</th>
<th>Disease type</th>
<th>Indications and symptoms</th>
<th>Efficacy</th>
<th>Positive control</th>
<th>Side effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizin</td>
<td>200mg + NaCl 100 mL, i.v., q.d., 4 weeks</td>
<td>Prednisolone (20–60 mg)</td>
<td>31/14</td>
<td>Acute onset autoimmune hepatitis (AIH)</td>
<td>Fever, general malaise, fatigue, nausea, vomiting, and right upper quadrant discomfort</td>
<td>Recovery rate was higher in the SNMC group than in the SNMC + CS group ($P = 0.035$)</td>
<td>Glycyrrhizin and corticosteroids (CS)</td>
<td>None</td>
<td>[21]</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>200 mg, i.v., q.d., 52 weeks</td>
<td>None</td>
<td>374/129</td>
<td>Chronic hepatitis C</td>
<td>Inflammatory effect</td>
<td>The proportion of patients with ALT reduction ≥50% after 12 weeks was significantly higher with 5×/week glycyrrhizin (28.7%, $P &lt; 0.0001$) and 3×/week glycyrrhizin (29.0%, $P &lt; 0.0001$) compared with placebo (7.0%).</td>
<td>Placebo-controlled</td>
<td>None</td>
<td>[22]</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>200mg + NaCl 100 mL, i.v., 6c/week, 4 weeks</td>
<td>None</td>
<td>69/13</td>
<td>Chronic hepatitis C</td>
<td>HCV and HCV-RNA positive; serum ALT at least 1.5 times; liver fibrosis or cirrhosis</td>
<td>The mean percentage ALT decrease from baseline at the end of treatment was 26% and 47% for the three times per week and six times per week treatment group, respectively (both $P &lt; 0.001$ versus placebo)</td>
<td>Placebo-controlled</td>
<td>None</td>
<td>[23]</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>200mg + NaCl 100 mL, i.v., q.d. for 8 weeks, then 2–7c/week for 2–16 years</td>
<td>None</td>
<td>453/109</td>
<td>Hepatocellular carcinoma (HCC) occurs in patients with hepatitis C virus-RNA positive chronic liver disease</td>
<td>Inflammatory effect after HCC</td>
<td>Patients treated with SNMC; the 10-year HCC appearance rates in histologic Stages I, II, and III were 3%, and 13%, respectively</td>
<td>Other herbal medicines</td>
<td>None</td>
<td>[24]</td>
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<tr>
<td>Glycyrrhizin</td>
<td>200mg + NaCl 100 mL, i.v., q.d., 0.1–14.5 years</td>
<td>None</td>
<td>1249/796</td>
<td>Interferon-resistant hepatitis C</td>
<td>Chronic hepatitis effect</td>
<td>Crude carcinogenesis rates in the treated and untreated group were 13.3%, 26.0% at the fifth year and 21.5% and 35.5% at the 10th year, respectively ($P = 0.021$)</td>
<td>Others without glycyrrhizin therapy</td>
<td>Hypertension skin rash without itching</td>
<td>[25]</td>
</tr>
<tr>
<td>Experimental drugs</td>
<td>Dose and course of treatment</td>
<td>Combined medication</td>
<td>Case/control</td>
<td>Disease type</td>
<td>Indications and symptoms</td>
<td>Efficacy</td>
<td>Positive control</td>
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<tr>
<td>Diammonium glycyrrhizinate</td>
<td>150 mg + 5–10% glucose injection liquid 250 mL, i.v., q.d., 1 month, 1-2 cycles</td>
<td>None</td>
<td>629/127</td>
<td>Chronic hepatitis, liver cirrhosis</td>
<td>Fatigue, gastrointestinal symptoms, and discomfort in liver area</td>
<td>After 17-day therapy, there are 93.3% patients with ALT normal level in treating group, but 73.3% in contrast group ($P &lt; 0.05$). At day 10, the patient's recovered normal SB were 86.7% in treatment group, but that was 40% in contrast group ($P &lt; 0.01$)</td>
<td>Compound ammonium glycyrrhizinate injection</td>
<td>Headache, facial edema, and blood pressure increased</td>
<td>[26]</td>
</tr>
<tr>
<td>$\beta$-glycyrrhetinic acid</td>
<td>80 mg + 10% glucose injection liquid 250 mL, i.v., q.d., 4 weeks, 100 mg, p.o. tid, 12 weeks</td>
<td>None</td>
<td>80/40</td>
<td>Chronic Hepatitis B</td>
<td>Chronic hepatitis effect</td>
<td>Compared with control group, the TBil, ALT, AST, HA, and IVC are significantly ameliorated in treatment group ($P &lt; 0.01$)</td>
<td>$\alpha$-glycyrrhizic acid</td>
<td>Edema, blood pressure increased, and serum potassium mildly low</td>
<td>[27]</td>
</tr>
<tr>
<td>Magnesium isoglycyrrhizinate</td>
<td>80 mg + 10% glucose injection liquid 250 mL, i.v., q.d., 4 weeks</td>
<td>Hepatoprotective drugs</td>
<td>80/40</td>
<td>Chronic Hepatitis B</td>
<td>Fatigue, gastrointestinal symptoms, and discomfort in liver area</td>
<td>Compared with control group, the TBil, ALT, AST are significantly ameliorated in treatment group ($P &lt; 0.01$)</td>
<td>Diammonium glycyrrhizinate injection</td>
<td>Headache, and blood pressure increased</td>
<td>[28]</td>
</tr>
<tr>
<td>Magnesium isoglycyrrhizinate</td>
<td>150 mg + 5–10% glucose injection liquid 250 mL, i.v., q.d., 4 weeks</td>
<td>None</td>
<td>60/30</td>
<td>Chronic severe hepatitis</td>
<td>Fatigue, gastrointestinal symptoms, discomfort in liver area, and yellow urine</td>
<td>Compared with control group, the TBil, PTA, ALT, and AST are significantly ameliorated in treatment group ($P &lt; 0.01$)</td>
<td>Hepatocyte generation drugs</td>
<td>None</td>
<td>[29]</td>
</tr>
<tr>
<td>Magnesium isoglycyrrhizinate</td>
<td>150 mg + 5–10% glucose injection liquid 250 mL, i.v., q.d., 2 weeks</td>
<td>None</td>
<td>56/28</td>
<td>Liver lesion induced by chemotherapy in cancer</td>
<td>Liver injury effect</td>
<td>Compared with control group, the TBil, PTA, ALT, and AST are significantly ameliorated in treatment group ($P &lt; 0.01$)</td>
<td>Diammonium glycyrrhizinate injection</td>
<td>None</td>
<td>[30]</td>
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</table>

GA: glycyrrhizic acid; TBil: total bilirubin; IVC: type IV collagen; ALT: alanine aminotransferase; AST: aspartate transaminase; PTA: prothrombin time activity.
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<th>Mechanisms of action</th>
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<td>Glycyrrhizin acid</td>
<td>Anti-inflammatory, antiviral, inhibition of hepatic fibrosis</td>
<td>Regulating the expression of inflammation-related factors; inhibition replication of viral mRNA</td>
<td>[46–49]</td>
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<td></td>
<td></td>
<td>[51, 59]</td>
<td>[45]</td>
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<tr>
<td>Compound glycyrrhizin tablet</td>
<td>Improving the liver dysfunction; augmented the entire cytotoxic function mediated by hepatic lymphocytes; inhibiting the cascade leading to apoptosis</td>
<td>Regulating the expression of inflammation-related factors; promoting the growth of hepatocyte; inhibition replication of viral mRNA</td>
<td>[60] [61] [62]</td>
</tr>
<tr>
<td>Glycyrrhetic acid</td>
<td>Anti-inflammatory, antiviral, antiallergic, antitumor proliferation</td>
<td>Regulating the expression of inflammation-related factors; inhibition replication of viral; inhibition of the expression of sensitizing factors and tumor-associated factor;</td>
<td>[63] [64] [65] [66]</td>
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<tr>
<td>18β-glycyrhetic acid</td>
<td>Antiviral, anti-inflammatory</td>
<td>Regulating the expression of inflammation-related factors; inhibition replication of viral mRNA</td>
<td>[67]</td>
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<tr>
<td>Diammonium glycyrrhizinate</td>
<td>Anti-inflammatory, resistance to biologic oxidation and membranous protection, neuroprotective effect</td>
<td>Regulating the expression of inflammation-related factors; regulating the enzymatic reactions' related oxidation</td>
<td>[68]</td>
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<tr>
<td>Dipotassium glycyrrhizinate</td>
<td>Anti-inflammatory</td>
<td>Regulating the expression of inflammation-related factors</td>
<td>[69]</td>
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<td>Application of glycyrrhetinic acid and glycyrrhizic acid in preparing medicaments for preventing or treating pulmonary fibrosis</td>
<td>CN101998780 B</td>
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<td>Application of glycyrrhizic acid in preparation of sunitinib malate cardiotoxicity reduction drug</td>
<td>CN103285020 A</td>
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<td>Application of glycyrrhizic acid on treating dilated cardiomyopathy cardiac remodeling and cardiac dysfunction</td>
<td>CN102247392 A</td>
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<td>Application of glycyrrhizic acid, glycyrrhetinic acid, or salt thereof as well as gel composition and preparation method for gel composition</td>
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<td>Application of glycyrrhizic acid and its breakdown product glycyrrhetinic acid for the manufacture of a medicament for the treatment of inflammatory bowel disease</td>
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<td>Dispersed compound tablet of glycyrrhizic acid and glycyrrhizinate and its preparing process</td>
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<td>Enteric-coated formulation of glycyrrhizic acid and its salt and its preparing method</td>
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<td>Film-coated tablet of glycyrrhizinic acid monopotassium salt and method for preparing the same</td>
<td>CN100341515 C</td>
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<td>Glycyrrhizic acid compounds as foamer in chemically derived surfactant-free dentifrice</td>
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<td>CN1498623 A</td>
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<td>Glycyrrhizic acid antibody and its preparing method and use</td>
<td>CN1293097 C</td>
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<td>Glycyrrhizic acid aureola dimer meditated targeted medication body as well as preparation method and purpose of glycyrrhizic acid aureola dimer meditated targeted medication body</td>
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<td>Glycyrrhizic acid composition</td>
<td>CN101081227 B</td>
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<td>Glycyrrhizic acid double salt and preparation thereof</td>
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<td>Glycyrrhizic acid matrine salt and glycyrrhizic acid marine salt, its preparing method and use</td>
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<td>CN101433529 A</td>
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<td>Glycyrrhizin high-concentration preparation</td>
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<td>US4163067(A)</td>
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<td>Medicine composition of glycyrrhizic acid or its salt, ginseng and astragalus root</td>
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<td>Medicine composition prepared mainly from glycyrrhizic acid or its salt, ginseng and glossy ganoderma</td>
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<td>Sucrose-ammoniated glycyrrhizin sweetening agent</td>
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</table>

in mice [71]. GA treatment could significantly reduce blood immunoglobulin E (IgE), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), NO, TNF-α levels, and nitrous oxide synthase (NOS) activity dose-dependently. GA could also enhance blood immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin-2 (IL-2), and interleukin-12 (IL-12) levels in AR mice. Gr-1⁺CD11b⁺ cells are responsible for numerous pathological processes such as T cell dysfunction after severe trauma or major surgery, leading to increased susceptibility to infection [72]. These cells exercise an inhibitory effect on MBD-1 production of EKs mediated via the suppressor molecules CCL-2 and IL-10. GA acts as a potent inhibitor of these cells and therefore restores MBD-1 levels. This restoration affects T cell dysfunction [73]. In thermally injured mice, GA regulates the burn-associated type 2 T cell responses to recover IL-12 and make it unresponsive, thus restoring the impaired cells [74]. GA acts as a promoter of the late signal transduction of T lymphocytes for IL-2 production. The balance between augmenting and suppressing effects might be dependent on the level of stimulation and stage of the cell. Therefore, this determines quality and quantity of the immunomodulatory action of GA [75]. In blood and nasal mucosa, GA consumption decreases antioxidant enzyme activity, lipid peroxidation, Glutathione levels, and IL-4 levels and enhances IFN-γ, thus protecting the nasal mucosa from oxidative injury and improving immunity activity [76].

GA interferes with some viruses, such as H5N1 [77]. The replication and virus-induced proinflammatory gene expression include inhibition of the virus-induced formation of reactive oxygen species and reduced activation of NF-κB, JNK, and p38, which are redox-sensitive signaling events known to be relevant for replication.

#### 2.4. Antitumor Effects

CYP enzymes are mainly found in the liver and bowel wall. They are responsible for the bulk of phase I or oxidative metabolism of xenobiotics including dietary toxins, carcinogens, mutagens, and drugs. Administration of GA was able to significantly induce CYP content, which reduces the incidence of cancer [78]. GA can also protect against aflatoxin-induced oxidative stress. The protective effect is likely from its capacity to inhibit the metabolic activation of hepatotoxin, a critical factor in the pathogenesis of chemical-induced carcinogenicity [79]. O-carboxymethyl chitosan nanoparticles (CMCNP) modified by GA with
various substitution degrees can efficiently deliver paclitaxel (PTX) to hepatocellular carcinomas (HCC). CMCNP-GA significantly facilitated the increased accumulation of PTX in hepatic tumor tissues and the targeted delivery of PTX to hepatoma carcinoma cells, which resulted in remarkably enhanced in vitro cytotoxicity and in vivo antitumor efficacy [80]. In a diethylnitrosamine-treated experimental animal study, as a chemopreventive agent of HCC, modulation of cell proliferation and apoptosis by GA may be associated with inhibition of HCC. Therefore, GA treatment may inhibit the occurrence of HCC [81].

2.5. Inductive Effect of Liver Enzyme Activity. Some studies showed that GA has an inductive effect on CYP3A activity. Therefore, clinicians should pay attention to other drugs catalyzed by CYP3A, especially those substrates with a narrow therapeutic range such as cyclosporine A, to avoid possible clinically significant interactions with GA [82]. Some studies revealed that the area under concentration-time curve and the mean retention time of methotrexate (MTX) were significantly increased by GA, which increases the adverse reactions of MTX [83]. MTX is an antifolate agent, anticancer agent, and immunosuppressant and is commonly used for anticancer chemotherapy [84], rheumatoid arthritis [85], and severe psoriasis [86]. The adverse reactions of MTX include nausea, vomiting, diarrhea, and hepatotoxicity [87, 88]. A case report showed that combined administration of GA and cilostazol caused pseudodosteronisim [89]. Therefore, the concurrent use of GA with MTX or cilostazol is not recommended. One report shows a case of hypokalemic rhabdomyolysis secondary to chronic GA intoxication [90]. GA ingestion could therefore potentially aggravate hypokalemia in patients with chronic lactic abuse [91], indicating that the use of GA in hypokalemia should be treated with caution.

3. Other Pharmacological Activities

GA is effective in combating hyperglycemia and associated pathological complications such as hyperlipidemia, abnormal histoarchitectures of different organs, and oxidative stress including hemoglobin-induced iron-mediated free radical reactions. The effects of GA on diabetes-associated changes are almost comparable with those of glibenclamide, a standard antihyperglycemic drug, suggesting a possible use of the herbal agent as a drug to prevent complications of diabetes mellitus [92]. Furthermore, GA regulates renal function through the regulation of water channels [93], and GA administration ameliorates the renal concentrating ability and structural lesions in renal tissues in rats with early-phase of ischemia-acute renal failure [94]. As a reduction inhibitor, GA reduces the therapeutic loss of methylprednisolone produced from methylprednisolone 21-sulfate sodium in the large intestine, thus improving the therapeutic property of the prodrug against inflammatory bowel disease [95]. GA also offers protection from the damage induced by UVB radiation in humans. Therefore, it could be considered as a promising agent for addition to topical formulations for the prevention of skin cancer [96]. GA significantly alleviates asthma symptoms [97], inhibits lung inflammation [98], and relieves acute lung injury [35, 99]. It can directly affect cardiac performance and play a role in myocardial and coronary protection in the presence of cardiovascular diseases [100]. GA may prevent brain tissue damage [101], can be a putative therapeutic drug for neurodegenerative diseases associated with cognitive deficits and neuroinflammation such as Alzheimer's disease [102], and could suppress ocular hypertension with potential therapeutic effects in eye disease [103]. GA improves resistance to C. albicans infection by inducing CD4+ T cells, which suppress type 2 cytokine production by Th2 cells [104]. GA inhibits activated macrophage (M2M) generation stimulated with neutrophils. The regulation of neutrophil-associated M2M generation by GA may provide a new therapeutic strategy, which could influence the outcome of certain severe infections in hosts with M2M generation [105].

4. Drugs That Include GA

Drugs made with GA have been on the market for many years, and most have important therapeutic uses. Magnesium isoglycyrrhizinate injection (TianQing GanMei, Chia Tai Tainqing, JiangSu, China) is one example of a drug with GA. Magnesium isoglycyrrhizinate is an effective and safe treatment for chronic liver diseases [106] and is capable of slowing down the progress of pulmonary fibrosis [107]. Moreover, diammonium glycyrrhizinate enteric-coated capsules (TianQing GanPing, Chia Tai Tainqing, JiangSu, China) and diammonium glycyrrhizinate injection (GanLiXin, Chia Tai Tainqing, JiangSu, China) are used for acute and chronic hepatitis associated with elevated alanine aminotransferase. Stronger neo-minophagen C (SNMC, Minophagen Pharmaceutical, Tokyo, Japan) is often used in the treatment of chronic liver disease and can improve liver dysfunction [60]. SNMC is a compound GA tablet that includes GA (2 mg) with glycine acid (20 mg) and L-cysteine hydrochloride (1 mg). SNMC has anti-inflammatory, anti-allergic, steroid-like, anticomplementary, and immunoregulatory effects.

5. GA Combined with Matrine

GA combined with matrine (Mat) can improve CCL4-induced liver fibrosis effectively. This is evidenced by lower levels of collagen, hyaluronic acid (HA), and laminin (LN), less hepatic stellate cells (HSC) proliferation, collagen I, and HA levels secreted by HSC in vitro with combined therapy compared with GA or Mat alone. GA combination with Mat could protect liver cells and inhibit hepatic fibrosis and may therefore be a safe and effective strategy for improving hepatic fibrosis [108]. In an animal model, GA combined with Mat reduced the mortality of acetaminophen overdosed mice, attenuated acetaminophen-induced hepatotoxicity, and reduced the number and area of y-GT positive foci, thus protecting liver function and preventing HCC from occurring [109]. Additionally, the combination of GA and cyclosporine was an effective treatment for nonsevere aplastic anemia [110].
6. Common Derivatives of Glycyrrhizin

Glycyrrhitinic acid (3β-hydroxy-11-oxooleana-12-en-28-oic acid), the aglycone of GA, stimulates glucose-induced insulin secretion in isolated pancreatic islets. Glycyrrhitinic acid treatment enhances plasma insulin levels and reduces the levels of gluconeogenic enzymes in liver. It is a pentacyclic triterpene acid with numerous biological activities, including anti-inflammatory [63], antiviral [64], antiallergic [65], and antitumor proliferative effects [66].

Glycyrrhitinic acid restrains the proliferation of skin tumors in mice and human breast cancer cells (MCF7) and induces apoptosis of cancer cells. The mechanism of apoptosis might be via increased free Ca\(^{2+}\) level in the cells [111]. Mizushina et al. [112] demonstrated that glycyrrhitinic acid potently inhibited the activity of mammalian polymerases, including pol λ. Glycyrrhitinic acid also reduced TNF-α production and NF-κB activation and suppressed mouse ear inflammation stimulated by tissue plasminogen activator. Therefore, glycyrrhitinic acid could be an anti-inflammatory agent based on pol λ inhibition.

Another licorice acid derivative is 18β-glycyrrhetic acid. The triterpene structure of the HMGB1-binding compound is capable of binding to HMGB1 and altering its proinflammatory properties, inhibiting HMGB1-dependent cyclooxygenase (COX) 2 induction [113]. 18β-glycyrrhetic acid has significant antiviral activity against rotavirus replication in vitro, and studies to determine whether 18β-glycyrrhetic acid attenuates rotavirus replication in vivo are underway, although the exact mechanism is unclear. However, some reports show that 18β-glycyrrhetic acid inhibits NF-κB activation, which has been interpreted as 18β-glycyrrhetic acid-mediated regulation of the inflammatory response [114]. 18β-glycyrrhetic acid can also inhibit the activity of tyrosine and prevent melanin growth and whitening. Some reports show that 18β-glycyrrhetic acid is likely responsible for amelioration of dysfunction of glutamate transport in astrocytes, and the inhibition of protein kinase C activity might be related to its pharmacological efficacy [67].

7. Conclusions and Future Perspectives

This review summarized the efficacy of GA in liver disease from clinical trials and its mechanisms of action in vitro and in vivo. Studies indicate that GA could modulate various molecular pathways in liver disease. There are numerous patents for drugs including GA (Table 3). Studies described here highlight the use of GA as a novel chemopreventive agent for liver injury. It is expected that future studies with GA will help to define various molecular mechanisms and targets for inflammation and steatosis. At present, the number of multicenter, large sample, randomized, double-blind, controlled chemoprevention clinical trials with GA is very limited. Extensive clinical research is warranted to evaluate the safety and chemopreventive efficacy of GA alone or in combination with chemotherapy agents.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References


Varnishes are preparations that differ in the polymeric matrix and therapeutic agents. In dentistry they are used to prevent caries. In this study we developed a propolis varnish, considering propolis properties against cariogenic bacteria. To a chitosan polymeric base (CHV) was added ethanolic propolis extract in different concentrations: PV1 (5%), PV2 (10%), and PV3 (15%). Antimicrobial activity was carried out against *Streptococcus mutans* (SM), *Streptococcus sanguinis* (SG), *Streptococcus salivarius* (SS), and *Lactobacillus casei* (LC) through agar diffusion method. The three propolis concentrations incorporated were effective in inhibiting the growth of all microorganisms, but without significant difference between the zones of inhibition observed. Cytotoxicity assay was done by MTT method. Data were analyzed by one-way ANOVA and Bonferroni test. None of the varnishes were cytotoxic, keeping 80% of viable cells, while CHV allowed cellular proliferation (120%). Sustained-release test was carried out by applying 40 μL of each varnish in the buccal surface of bovine teeth and kept in an ethanol/water solution removed in regular times. According to the “independent model approach,” the release profiles were distinct from each varnish and the most prolonged was PV3 (8 weeks). Varnish formulations had satisfactory antimicrobial activity against cariogenic bacteria and have a low cytotoxicity (<50%).

1. *Introduction*

Dental caries is among the most prevalent chronic human infectious diseases affecting children and adults worldwide [1, 2]. One of the mechanisms that allow the control of caries is the decrease of dental biofilms, which is a complex hole of microorganisms and metabolic products fixed in a polymeric matrix adhered to the tooth surface. In this whole, cariogenic bacteria produce acids responsible for the decrease of pH, starting the process of demineralization [3]. Many products are being developed for caries control. The first was fluoride with remineralizing action, followed by chlorhexidine with antimicrobial activity, among others. The vehicle for these substances varies according to its clinical applications and can be found as rinses, gels, or varnishes used as nonsurgical methods for treating and preventing dental caries [4, 5].

Varnishes are preparations that differ in the polymeric matrix, pharmaceutical additives, and therapeutic agents,
generally fluoride and chlorhexidine [6]. This ability to form a film takes place through a polymer, and among the most widely used are the ethyl cellulose and a mixture of copolymer and vinyl acetate-acrylate copolymers.

Recently, natural products are attracting the attention of several studies, mainly due to the increase in bacterial resistance and side effects of the antibiotics most commonly used [7–9]. Moreover, propolis has low toxicity and has several biological activities that strengthens its employment in healthcare. [10]. Several studies have demonstrated the antimicrobial activity of propolis extracts against cariogenic microorganisms [10, 11].

Chitosan is a derivative of chitin, a natural compound that can be found in arthropod exoskeletons, shells of crustaceans, and insect cuticles. Industrially, it is obtained by alkaline hydrolysis of chitin [12]. Chitosan is a biocompatible and biodegradable polymer. Its positive charge combines to the cell wall of bacteria, promoting a bactericidal and bacteriostatic property to this material [13]. These properties, coupled with the ability to form a film and adhere to the tooth, make chitosan an ideal base for sustained drug release [14]. Thus, the objectives of this study were to develop a dental varnish containing Brazilian green propolis ethanolic extract at three different concentrations and to verify the antimicrobial properties compared with chlorhexidine, cytotoxicity, and sustained-release profile of this new product.

2. Material and Methods

2.1. Propolis Sample and Propolis Extract Preparation. Propolis samples produced by honeybees (Apis mellifera) were collected during the spring and obtained in a beekeeping in Caeté, Minas Gerais, Brazil. Brazilian green propolis extract (EPE) was prepared according to Wojtuczka et al. [15]. Propolis was subjected to 14 days of extraction in order to obtain its ethanolic extract, which was later dissolved in 70% ethanol to obtain a 100 mg/mL working concentration. Briefly, the samples were ground mechanically and bottled in 10 g portions. The 10 g portions were put into flask and 100 g of 70% ethanol (w/v) was added. The flask was placed on a rotary shaker in a dark, closed room for two weeks at room temperature. After this period, the extract was cooled at 4°C for 24 h in order to precipitate all insoluble particles, which were removed from the propolis extract by filtration through filter paper (Whatman number 4). Next, the obtained filtrate evaporated to dryness at 40°C using a rotary vacuum evaporator. In order to prepare a working concentration, the brown colored viscous substance was dissolved in 70% ethanol. The propolis used has the main markers that give great quality to it [16, 17].

2.2. Propolis-Chitosan Varnish Preparation. Propolis varnish were prepared by the addition of acetic acid to the EPE with or without dilution in ethanol. After mixing, chitosan (Sigma-Aldrich, St. Louis, USA) was added and Milli-Q (Millipore, Billerica, USA) water was used to complete the formulation volume. Propolis ethanolic extract was added and the compositions were mixed overnight to obtain the 5% (PV1), 10% (PV2), and 15% (PV3) propolis-chitosan formulations (Table 1). For being an innovative dental material, the request of the patent was registered at the National Institute of Industrial Property (INPI) under number 014100004357.

2.3. Antimicrobial Assay. Bacterial sensitivity or resistance to varnishes was detected by the disk diffusion assay, also known as the Kirby-Bauer method [18]. Aliquots of Streptococcus mutans (ATCC 70069), Streptococcus sanguinis (ATCC 10557), Streptococcus salivarius (INQOS-Oswaldo Cruz Foundation, Rio de Janeiro, Brazil/00457), and Lactobacillus casei (LC) (ATCC 393) containing 1.0 × 10⁶ CFU/mL were subcultured in agar Mueller-Hinton (Difco, Trenton, USA), supplemented with 5% of dextrose for Streptococcus spp. L. casei were subcultured and placed in Rogosa medium. Sterile filter papers soaked with 20 μL of each propolis varnish were placed onto the agar. The controls were blank varnish/chitosan (CHV), PV1, PV2, PV3, and chlorhexidine varnish (VCX) (Fórmula e Ação, São Paulo, Brazil). The diameter of inhibition zone around the filter paper formed after 24 and 48 hours at 37°C in an atmosphere of 5% CO₂ was measured in mm and recorded (M ± SD). Any inhibition zone around the filter paper measuring ≤7 mm was considered a negative result.

Minimal inhibitory concentration (MIC) test was carried out using tissue culture microplates (96 wells) containing 100 μL/well BHI. For being highly viscous, the propolis varnish was diluted in an ethanol/water solution at 20% in a proportion of 1:1 (75 mg/mL). After being transferred to the first well, serial dilutions were performed to obtain concentrations ranging from 75 to 0.1 mg/mL. Chlorhexidine at 0.12% (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control and BHI as negative control. The bacterial inoculum (1 × 10⁶ CFU/mL) was added to all wells, and the plates were incubated at 37°C in 5% CO₂ for 24 hours. MIC was defined as the lowest concentration of the propolis varnish that inhibited microorganism visible growth indicated by resazurin 0.01% (Sigma-Aldrich, St. Louis, MO, USA). To determine minimal bactericidal concentration (MBC), an aliquot of each incubated well with concentrations higher than MIC was subcultured on BHI medium. MBC was defined as the lowest concentration of the propolis varnish that allowed no visible growth on the test medium [19].

2.4. Osteoblast-Like Cell Culture. Osteoblasts cells were donated from the Laboratory of Biomaterials and Molecular Entrapment (LEMB), Chemical Department, UFMG. This experiment was approved by the Ethics Committee on Animal Use of Federal University of Minas Gerais (CEUA number 167/2007). Osteoblasts were isolated by collagenase digestion of 20-day fetal rat calvariae [20]. Calvariae were dissected aseptically, and the frontal and parietal bones were stripped of their periosteum. Only the central portions of the bones, free from suture tissue, were collected. The calvariae were treated twice with phosphate-buffered saline (PBS) containing 4 mM EDTA (pH 7.4) for 15 min at 37°C in a shaking water bath. After being washed once in PBS, the calvariae were treated twice with 3 mL of 1 mg/mL collagenase...
for 7 min at 37°C. After the supernatants from these two digestions were discarded, the calvariae were treated two more times with 3 mL of 2 mg/mL collagenase (30 min, 37°C). The supernatants of the latter two digestions were pooled and centrifuged, and the cells were washed in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), suspended in further DMEM-10% FCS, and placed in 75 cm² flasks. After 48 h, the media were changed to minimal essential medium (MEM) with 10% FCS. Confluence was reached within 5–6 days, at which time the cells were subcultured. After trypsinization with trypsin-EDTA (0.05%/0.53 mM), the cells were rinsed in MEM with 5% FCS, resuspended in fresh medium, and then seeded at 5 × 10⁴ cells/mL in 24-well plates (0.5 mL cell suspension/well, i.e., 2.5 × 10⁵ cells/well). The cells were incubated under 5% CO₂-95% air at 37°C.

2.5. Cytotoxicity Assay. The cytotoxicity assay with osteoblasts was carried out according to ISO 10993-5 for cytotoxicity tests in vitro, using MTT colorimetric assay. The cells were cultured at 37°C in a humidified colorimetric assay of 95% air and 5% CO₂ in DMEM (Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum containing penicillin (10 IU mL⁻¹) and streptomycin (10 mg mL⁻¹). Thereafter, in each well 150 × 10⁵ cells were plated. Six wells were prepared for each varnish formulation and three for the control groups. The controls were lauryl sodium sulfate (LSS) (Sigma-Aldrich, St. Louis, USA) diluted in Milli-Q water in concentrations of 0.10%, 0.075%, 0.05%, and 0.025% and plates containing just cells. Plates were incubated at 37°C in 5% CO₂ and 95% humidity conditions. The colorimetric assay was carried out after 24 hours and submitted to absorbance reading at 570 nm in spectrophotometer (Thermo Scientific Multiskan Spectrum; Thermo Fisher Scientific Inc., Boston, USA).

2.6. Sustained-Release Test. Bovine teeth were obtained from carcasses of animals that would be incinerated. The animals were sacrificed at specific slaughterhouse approved by the city of Belo Horizonte to market beef. The teeth were donated by the slaughterhouse before carcasses incineration. For the sustained-release test, ten incisors crowns of bovine teeth obtained post-mortem were cut into four pieces with diamond bur (KG Sorensen, São Paulo, Brazil). Each varnish formulation was applied to the buccal surface of each fragment, using five of them for each varnish and one fragment for CHV in each group. After applying and drying 40 μL of the varnish, each fragment was placed into a tube with 1 mL of 20% ethanol/water solution and incubated in a shaker (KS4000 iControl, IKA, Staufen, Germany) at 37°C and 30 rpm and after 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 24, 72, 168.0, 336.0, 504.0, 672.0, 840.0, 1008.0, 1176.0, 1344.0, 1512.0, and 1680.0 hours, the solution was removed; a new one was added. Each sample of the solution (240 μL) was placed in plates with 96 wells, adding 10 μL of aluminum chloride solution [21]. The reading was carried out in spectrophotometer plate reader in an absorbance of 425 nm. BPE was used as positive control.

2.7. Statistical Analysis. To determine whether the difference of the measures of inhibition zones was significant, data were statistically analyzed through Kruskal-Wallis nonparametric test, performed using SPSS for Windows v. 17 (IBM Inc., Chicago, USA). In the cytotoxicity assay, absorbance results were converted into cell viability percentages and statistically compared by one-way ANOVA and Bonferroni test (Figure 1), using Graphpad Prism 5 (GraphPad Software Inc., San Diego, USA). P values lower than 0.05 were considered significant. The sustained-release profiles were compared using the difference factor and the similarity factor, according to the “independent model approach” [22]. Triplicates from at least three separated experiments were conducted in each assay.

3. Results

3.1. Antimicrobial Assay. All varnishes containing EPE inhibited S. mutans, S. sanguinis, S. salivarius, and L. casei. Table 3 shows results of mean and standard deviation (M ± SD) for all tested formulations. No significant difference between the inhibition zones of the three tested concentrations of propolis (PV1, PV2, and PV3) and pure propolis extract (EPE) was observed. This demonstrates that propolis maintains its antimicrobial properties even when incorporated into the coating of chitosan. In contrast, chlorhexidine (CHX) showed significantly smaller areas than those observed in PV1, PV2, PV3, and EPE inhibition. L. casei appears to be more resistant to chlorhexidine than other microorganisms. The base coating of chitosan (CHV) showed significantly smaller inhibition areas for all microorganisms when compared with CHX and varnishes containing propolis.

MIC and MBC values for all products tested are shown in Table 3. MIC and MBC values ranged from 0.6 to 1.2 mg/mL for propolis varnish and 0.4 to 0.8 mg/mL for chlorhexidine (Table 3).

<table>
<thead>
<tr>
<th>Components</th>
<th>PV1 (5%)</th>
<th>Product amount</th>
<th>PV2 (10%)</th>
<th>PV3 (15%)</th>
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<tbody>
<tr>
<td>EPE</td>
<td>20.0 mL</td>
<td>40.0 mL</td>
<td>60.0 mL</td>
<td></td>
</tr>
<tr>
<td>Ethanol P.A.</td>
<td>40.0 mL</td>
<td>20.0 mL</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>9.0 mL</td>
<td>9.0 mL</td>
<td>9.0 mL</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>1.0 g</td>
<td>1.0 g</td>
<td>1.0 g</td>
<td></td>
</tr>
<tr>
<td>Milli-Q water q.s.p.</td>
<td>100 mL</td>
<td>100 mL</td>
<td>100 mL</td>
<td></td>
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</tbody>
</table>

EPE (propolis ethanolic extract); PV (propolis varnish).

Table 1: Components of the varnish formulations PV1 (5%), PV2 (10%), and PV3 (15%).
Table 2: Susceptibility test of propolis-based chitosan varnish against cariogenic bacteria; inhibition zones; mean and standard deviation (M ± SD) of three experiments.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>PV1 (5%)</th>
<th>PV2 (10%)</th>
<th>PV3 (15%)</th>
<th>EPE</th>
<th>CHX</th>
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<tr>
<td><em>S. mutans</em> (ATCC 25175)</td>
<td>20.3 ± 0.51</td>
<td>20.2 ± 0.88</td>
<td>21.0 ± 0.00</td>
<td>10.4 ± 0.22 *</td>
<td>22.6 ± 0.66 *</td>
</tr>
<tr>
<td><em>S. sanguinis</em> (ATCC 10557)</td>
<td>21.5 ± 0.25 *</td>
<td>21.5 ± 0.25 *</td>
<td>22.3 ± 1.18 *</td>
<td>10.5 ± 0.25 *</td>
<td>21.3 ± 0.31 *</td>
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<tr>
<td><em>S. salivarius</em> (INCQS 00457)</td>
<td>20.5 ± 0.33</td>
<td>20.5 ± 0.33</td>
<td>21.5 ± 0.50 *</td>
<td>8.30 ± 0.33 *</td>
<td>20.5 ± 0.55 *</td>
</tr>
<tr>
<td><em>L. casei</em> (ATCC 393)</td>
<td>19.3 ± 0.25 *</td>
<td>19.3 ± 0.25 *</td>
<td>21.3 ± 0.71 *</td>
<td>9.50 ± 0.55 *</td>
<td>16.0 ± 0.00</td>
</tr>
</tbody>
</table>

PV: propolis-based chitosan varnish; CHV: chitosan varnish; EPE: propolis ethanolic extract; CHX: chlorhexidine 0.12%; INCQS: Instituto Nacional de Controle de Qualidade (National Institute of Quality Control, FIOCRUZ, Rio de Janeiro, Brazil).

* Are related to the statistical difference between the results (P < 0.05).

Table 3: MIC and MBC values from propolis varnish and positive control.

<table>
<thead>
<tr>
<th>Product</th>
<th>MIC (mg/mL)</th>
<th>MBC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varnish 1:1</td>
<td>0.6–1.2</td>
<td>0.6–1.2</td>
</tr>
<tr>
<td>Chlorhexidine (+control)</td>
<td>0.4–0.8</td>
<td>3.91–7.81</td>
</tr>
</tbody>
</table>

3.2. Cytotoxicity Test. Figure 1 shows the results of the cytotoxicity assay concentrations of propolis incorporated chitosan (VA = PV3 = 15%; VB = PV2 = 10%; VC = PV1 = 5%) compared with the chitosan varnish (BV = CHV = 15%) and sodium lauryl sulfate (SLS) at four different concentrations. Cell viability was greater in tests with CHV (≥100% of cells). Cell viability was similar for the three different concentrations of propolis (≥80%). Cytotoxicity was observed for all concentrations of SLS, which showed cell viability below 60%. This demonstrates that the varnish containing propolis and chitosan is not cytotoxic at the tested concentrations. These results confer low cytotoxicity, according to ISO 10993-5 standards.

3.3. Sustained-Release Test. The extended-release profile considered the release of total flavonoids as quercetin. It was very heterogeneous as the varnishes were more viscous or more fluid depending on the concentration of propolis. Almost 100% of total quercetin from EPE was released in 24 hours. Varnish PV3 (15%) designed a release curve of 20% during the first 8 hours, thereafter stopping and restarting after 24 hours, becoming steady for 8 weeks (Figure 2). In the first two hours of the experiment, there was no release of PV2 (10%), starting just before this period and paralyzing after 7 hours. This inactivity lasted 72 hours and after this time the release started again. In this varnish only 30% of total quercetin was released in three weeks. The release of quercetin in PV1 (5%) started after 8 hours and only 10% of total flavonoids as quercetin were released within 24 hours.

4. Discussion

Propolis has been studied due to various biological activities highlighting its antimicrobial activity [11, 23, 24]. The results of antimicrobial susceptibility testing on *S. mutans* and *S. sanguinis* showed that propolis, even when associated with the varnish, is released in a satisfactory way, keeping its antimicrobial property, which makes the use of the drug feasible for this purpose (Table 2) [25]. All formulations inhibited the growth of all bacteria tested to a greater or lesser extent. This difference in size of inhibition zones between the formulations may be due to the concentration of EPE. This may be related to the molecular profile of propolis that has a variety of chemical compounds with different physicochemical characteristics, especially when comparing the inhibition observed for chlorhexidine, which has a characteristic solubility and molecular pattern that allows a better diffusion in agar.

Our results corroborate other studies of antimicrobial susceptibility of the ethanol extract of green propolis with *S. mutans*, *S. sanguinis*, *S. salivarius*, and *L. casei* in vitro, showing high activity of this product [10, 11, 26].
Chitosan varnish (CHV) showed low antimicrobial activity when compared with the other products. However, synergism between propolis and chitosan is possible to occur. The antimicrobial activity of chitosan was reported by several studies \[27, 28\] and this lack of activity may be due to the medium molecular weight of chitosan used in the varnishes, which is not soluble in water, unlike the low molecular weight of chitosan used in other studies.

Chitosan is a nontoxic, biocompatible, and chemically versatile polysaccharide. These properties enable this material to be used in drug delivery systems and tissue engineering, a promising tool in health care \[29\]. Its toxicity depends on the degree of deacetylation of chitin and its molecular weight. Nevertheless, most derivatives of chitosan have low toxicity \[29\], what might be seen in the cytotoxicity assay performed with osteoblasts in this study. BV, besides having not shown toxicity, allowed cell proliferation, increasing the amount in 20%.

Other studies showed that propolis ethanolic extract is cytotoxic on pulp fibroblasts \[30\] and cancer cells \[31\]. In the concentrations tested in this study, the cytotoxicity of the varnish containing propolis was considered low.

In the slow-release test, the components of propolis varnish were not soluble in artificial saliva, and as we could not quantify total flavonoids in this medium, we used an ethanol/water solution. As it does not simulate the oral environment, this test was just an indicative of the release.

The release of PV3 (15%), which has higher amount of EPE, remained stable in the early hours of the experiment, allowing a more constant release, which would ensure an effective and prolonged antimicrobial activity when applied clinically, relevant characteristics for the control of cariogenic biofilm.

For not having released quercetin in the first two hours of the experiment and not keeping regularity, CHV might allow the proliferation of microorganisms during the period of inactivity, although it presented satisfactory results in the antimicrobial susceptibility testing. For these reasons, their release profile might not represent an antimicrobial activity as effective in clinical practice.

PV1 (5%) released no quercetin during the first 8 hours of testing, what could allow bacterial growth in this period. Furthermore, the release of only 10% of quercetin in the oral environment and in just 24 hours limit the indication for the purpose it was developed. The similarity in the release profiles of formulations was compared by the “independent model approach.” In general, values lower than 15% (0–15%) and \(F_2\) values higher than 50% (50–100%) show the similarity of the sustained-release profiles \[21\]. None of the pair of formulations showed \(F_1\) lower than 15% or \(F_2\) higher than 50%, suggesting that all the release profiles are different from each other.

An in vitro study evaluated the release of chitosan containing dexamethasone and concluded this polymer allowed the slow-release of the drug tested \[32\]. Almost 90% was released within the first eight hours of experiment, result not obtained with any other varnish tested. The different characteristics of therapeutic agents used justify these differences.

Varnish formulations must release propolis right after being applied, remaining for about 24 hours onto the tooth surface, sustaining the release of the active principle on a regular and continuous basis, to achieve antimicrobial activity. However, in vitro tests may not reflect the in vivo responses, considering the environmental factors of the oral cavity and the genetic and social characteristics of each individual. Also, the product proved to be innocuous when tested in osteoblasts.

The concentration of 15% (PV3) has presented the largest inhibition zones and releases of higher profile, deserving further studies to prove its effectiveness.

5. Conclusions

Varnish preparations developed in this study showed satisfactory antimicrobial activity against \textit{Streptococcus mutans}, \textit{Streptococcus sanguinis}, \textit{Streptococcus salivarius}, and \textit{Lactobacillus casei} and demonstrated low cytotoxicity on osteoblasts (<50%). Varnish PV3 had the best results, deserving further studies to confirm its possible clinical efficacy.

Conflict of Interests

The authors report no relationships, financial or otherwise, with any entity that may influence the objectivity of this paper.

Acknowledgments

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References


Research Article

Pycnogenol Ameliorates Depression-Like Behavior in Repeated Corticosterone-Induced Depression Mice Model

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Oxidative stress is considered to be a mechanism of major depression. Pycnogenol (PYC) is a natural plant extract from the bark of Pinus pinaster Aiton and has potent antioxidant activities. We studied the ameliorative effect of PYC on depression-like behavior in chronic corticosterone- (CORT-) treated mice for 20 days. After the end of the CORT treatment period, PYC (0.2mg/mL) was orally administered in normal drinking water. Depression-like behavior was investigated by the forced swimming test. Immobility time was significantly longer by CORT exposure. When the CORT-treated mice were supplemented with PYC, immobility time was significantly shortened. Our results indicate that orally administered PYC may serve to reduce CORT-induced stress by radical scavenging activity.

1. Introduction

Stress is a potent risk factor for depression. Recent studies have reported that plasma reactive oxygen species (ROS) content increased in major depression patients [1], and oxidative stress is contributive to major depression [2]. Chronic high levels of glucocorticoids lead to maladaptive anxiety depressive disorders [3]. Stress-based animal models by chronic corticosterone (CORT) treatment might mimic stress-triggered depression in humans [4], and accumulation of oxidative stress has been noted in the brain of stress-induced animal models [5]. A forced swimming test (FST) was developed as an animal model of depression to investigate the effects of new antidepressant compounds in mice [6]. FST has very good reproducibility and is very commonly used by researchers [6].

Pycnogenol (PYC), a family of flavonoids isolated from French maritime pine bark (Pinus pinaster Aiton, synonym Pinus maritima Mill.), is a mixture of procyanidins with potent antioxidants and ROS scavenging properties [7]. As yet there has been no report about the antidepressant effect of PYC. Thus, the present study was conducted to evaluate the efficacy of PYC in protecting against chronic depression in CORT-induced depression mice model using FST.

2. Materials and Methods

2.1. Animals and Chemicals. Eighteen male C57Bl/6J mice weighing 25.3 ± 0.71g were obtained from Charles River Japan Inc. (Tokyo, Japan). The mice were housed in standard cages and fed a commercial diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. The depression model was induced by repeated administration of CORT (14 days of 6.9mg/kg, 3 days of 3.1mg/kg, and then 3 days with 1.6mg/kg) in the drinking water over a period of 20 days according to the method of Gourley et al. [8]. The control group (n = 6) received normal drinking water. Three days after CORT, the mice were randomly separated into 2 groups: the PYC group (n = 6) received PYC at 0.2mg/mL; the CORT-treated group (n = 6) received normal drinking water.
water (Figure 1(a)). Bottles were weighed daily. All procedures for handling animals were approved by the Animal Experimentation Committee of Gifu Women's University Graduate School of Human Life Science. PYC was provided by DKSH Japan K.K., Tokyo, Japan. CORT (4-pregnen-11\(\beta\)-\(\alpha\)-21-DIOL-3 20-DIONE 21-hemisuccinate) was purchased from Sigma-Aldrich (Cat. number C6766, St. Louis, MO, USA), dissolved in tap water, and neutralized to a pH of 7.4.

2.2. FST. FST was performed with an acrylic cylinder (160 mm diameter, 225 mm height) filled to the height of 150 mm with water maintained at 24°C. Mice were placed in the cylinder for 6 min. Behavior was recorded with a video camera to measure immobility time when the mouse remained floating passively in the water (dog paddling in the water without struggling and only making movements necessary for keeping the head above the water) during the last 5 min, with the first 1 min serving as a habituation. Immobility time (passive behavior) was interpreted as a measure of depressive-like behavior. The mice were then removed from the cylinder, dried with tissue paper, and returned to their home cage.

2.3. Statistics. Results are expressed as the mean ± SEM. All data were analyzed statistically using one-way analysis of variance (ANOVA), followed by a post hoc Dunnett’s test. Statistical significance was set at a value of \(P < 0.05\).

3. Results and Discussion

Body Weights and Food Intake. Body weights and food intake did not differ among the groups throughout the experimental period (Figures 1(b) and 1(c)). These results were the same as those of Gourley et al. [8]. It is considered that CORT-induced
CORT-treated group; compared with CORT-treated group; its antioxidant activity. Results showed that PyC supplementation may reduced depression-like behavior in this study.

**Effects of CORT and PyC on Depression-Like Behavior.** The effect of CORT on depression-like behavior was investigated by the FST. Immobility times in the PyC and CORT-treated groups were longer than in the control mice after the CORT treatment period, by post hoc analyses at day 0 (Figure 2). These times were about 200 sec and were the same as in a previous report [8]. Gourley et al. revealed that CORT increased immobility by selectively reduced pERK1/2 in the dentate gyrus and that the increased immobility time by CORT is not due to locomotor differences [8]. These results suggest that oral CORT exposure produced mild depression-like behavior in this study.

Immobility time of the PyC group was shortened 30 days after the CORT treatment period, but a difference did not appear after 14 days by one-way ANOVA (Figure 2). There is a report that it takes four weeks for the oxidative stress to be significantly lowered by PyC [9]. Post hoc analyses indicated that a significant difference was not recognized between PyC and the control group after 30 days (Figure 2). These results suggest that the depression-like behavior was improved by administration of PyC, almost to the control level. Treatment of glucocorticoid increases ROS in the brain of animals [10]. An excess level of ROS can damage cellular components and induce functional abnormalities in many cell types [11]. PyC scavenges free radicals and promotes cellular health [12] and increases the level of antioxidant enzymes [13]. Our results show that PyC supplements may reduce depression-like behavior in CORT mice model by its antioxidant activity.

In the future, we will examine antioxidant enzyme activities of PyC in depression-like behavior and confirm the effects of PyC with behavioral tests and their antioxidant effects.

**4. Conclusions**

The present results suggest that chronic treatment of CORT induced mild stress-mediated depression-like behaviors as observed by FST and that the potent antioxidant activity of PyC can slightly decrease the progression of stress.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgment**

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Research Article

Gastric Antiulcerogenic and Hypokinetic Activities of Terminalia fagifolia Mart. & Zucc. (Combretaceae)

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The acute toxicity, the antioxidant activity, and the pharmacological activity on the gastrointestinal tract of rodents of the ethanolic extract (TFEE) from the bark of Terminalia fagifolia Mart. & Zucc. (Combretaceae) and of its aqueous (TFAqF), hydroalcoholic (TFHAF), and hexanic (TFHEXF) partition fractions have been evaluated. TFEE presented low acute toxicity, antioxidant, and antiulcerogenic activity against ethanol-induced ulcers, which was partially blocked by pretreatment with L-NAME and indomethacin. It reduced the total acidity and raised the pH of gastric secretion. Additionally, TFEE delayed gastric emptying and slightly inhibited the small intestinal transit and also presented a weakly antidiarrheal activity. The antiulcerogenic and antioxidant activity were also detected in TFAqF and TFHAF but not in TFHEXF. The antisecretory and gastroprotective activity of TFEE partially involve the nitric oxide and prostaglandin participation. Nevertheless, TFEE, TFAqF, and TFHAF drastically reduced the mucus layer adhered to the gastric wall of rats treated with ethanol or indomethacin. Complementary studies are required in order to clarify the paradox of the presence of a gastroprotector activity in this plant that, at the same time, reduces the mucus layer adhered to the gastric wall.

1. Introduction

Medicinal plants are used and marketed worldwide as herbal drugs or as single active ingredients over centuries. Besides their popular consumption to treat and cure human illness, plant derived natural products can play an important role as a source of pharmacologic tools to enable the understanding of the biochemical pathways and mechanisms of disease [1, 2].

In Brazil, medicinal plants are widely used in both rural and urban areas. Most are used according to folk tradition developed by natives or brought to the country by Europeans, Africans, and Asians [3]. Considering the cultural and economic perspectives, a scientific verification of plant use is important for the assessment of their quality, safety, and therapeutic efficacy. Terminalia fagifolia Mart. & Zucc. (Combretaceae), commonly known as “chapadeiro,” is a medicinal plant used in traditional folk medicine for its effective treatment of gastrointestinal disturbances, such as ulcer, gastritis, and diarrhea [4]. Diarylpropanes, flavanones, chalcones, flavan, gallic acid, and sitosterol were isolated from the heartwood and trunk bark of T. fagifolia and were evaluated for their in vitro cytotoxic activity against two human cancer cell lines and antioxidant properties [5]. Ayres et al. [6] reported the isolation of (+)-catechin, sitosterol-3-O-β-D-glucopyranoside, α- and β-tocopherol, a mixture of lupeol,
α- and β-amyrin, sitosterol, and a mixture of glycosidic flavonoids in the ethanolic extract of the leaves of *T. fagifolia* and investigated the antioxidant activity of fractions of this extract.

Literature about the botanical family Combretaceae reports the detection of a variety of biological activities and a ubiquitous antioxidant property in genera *Terminalia* and *Combretum* [7–21]. Investigations with *Terminalia arjuna* (Roxb.) Wight & Arn. [22, 23], *Terminalia avicennioides* [24], *Terminalia bellirica* (Gaertn.) Roxb. [25, 26], *Terminalia brasiliensis* [27], *Terminalia catappa* [28], *Terminalia chebula* Retz. [26, 29–31], *Terminalia pallida* Brandis [32], *Combretum leprosum* Mart. & Eiche [33], *Combretum dolichopetalum* Engl. & Diels [34], and *Guiera senegalensis* J.F. Gmel [35] led to interesting results when they focused on the antiulcerogenic activity. The ethanolic extract of *Combretum dolichopetalum* Engl. & Diels also delayed gastric emptying in rats [34]. The methanolic bark extract of *Terminalia arjuna* showed a significant increase in the adherent mucus of the gastric wall and in the protein bound carbohydrate complexes of the gastric juice in rats treated with diclofenac sodium [22].

Gastric ulcer is one of the most common gastrointestinal diseases and results from an imbalance between the action of aggressive and defensive factors on the gastric mucosa [36]. The defense mechanisms are crucial for the maintenance of an effective barrier and are arranged at different levels, which work together to protect the stomach. The preepithelial level or the first line of defense consists of the mucus layer and bicarbonate secreted into the mucus, creating a pH gradient within the mucus. The epithelial level consists of intercellular tight junctions and proton and bicarbonate transport systems. The postepithelial level consists mainly of an effective blood flow and the gastrointestinal autonomic nervous system [37]. Gastric mucosal damage can be induced by aggressive factors like hydrochloric acid, pepsin, leukotrienes, free radicals, nonsteroidal anti-inflammatory drugs (NSAIDs), ischemia, dysmotility, ethanol, nicotine, and stress [38].

Despite the popular use of *Terminalia fagifolia* as a medicinal plant, there are few data about its pharmacological effect, particularly on the gastrointestinal tract. In order to validate the ethnomedical uses of these natural products, the present study has been conducted to evaluate the acute toxicity and the antioxidant and pharmacological activity of the ethanolic extract from the stem bark of *Terminalia fagifolia* (TFEE) and of its partition fractions on the gastrointestinal tract of rodents. Additionally, the total phenolic and flavonoid content of the extract and partition fractions has been determined.

2. Material and Methods

2.1. Animals. Male (246 ± 5 g) and female (164 ± 6 g) Wistar rats and male (27 ± 1.2 g) and female (24 ± 0.8 g) Swiss mice were used for the study. The animals were provided with a rodent-pellet diet (LABINA 5002, EVIALIS do Brasil Nutrição Animal Ltda., Sao Paulo, Brazil) and water *ad libitum*. They were maintained in proper conditions, temperature of 25 ± 2°C, approximately 60% humidity, and 12 h light/dark cycles. The animals were randomly assigned to different control and treatment groups. The experimental protocols were conducted with 6 to 8 animals/group in accordance with the guidelines of the Brazilian Council of Animal Experimental Control and approved by the Ethics Committee for Animal Research at the Federal University of Piaui (Protocol number 042/09).

2.2. Plant Material and Extracts Preparation. The stem barks of *Terminalia fagifolia* Mart. & Zucc. (Combretaceae) were collected in November 2006 at the “Bambu” community, Timon-MA, Brazil. A voucher specimen (TEPB number 21.691) was deposited in the Graziela Barroso Herbarium at the Federal University of Piaui, Teresina, state of Piaui, Brazil. The plant material was shade-dried at approximately 40°C and the stem bark powder was exhaustively extracted with 99.6% ethanol or 50% hydroalcoholic solution at room temperature. After filtration, the solvents were eliminated in a vacuum at 50°C and the concentrates were lyophilized to obtain the dry *Terminalia fagifolia* ethanolic (TFEE) and hydroalcoholic (TFHAE) extracts which were stored under refrigeration until further use. For the experiments these extracts were freshly prepared as a suspension in distilled water.

2.3. Preparation of the Partition Fractions of TFEE. To obtain the partition fractions of TFEE, it was formerly dissolved in a methanol/distilled water solution (1:2) and extracted with ethyl acetate. In sequence, the ethyl acetate phase was concentrated and dissolved in a methanol/distilled water solution (9:1) and extracted with hexane. The phases obtained were concentrated by elimination of the solvents and resulted in the aqueous fraction (TFAqF), hydroalcoholic fraction (TFHAF), and hexane fraction (TFHEXF) of TFEE.

2.4. Chromatographic Analysis of TFEE and Its Partition Fractions. TFEE and its partition fractions (TFAqF, TFHAF, and TFHEXF) were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

The TLC analysis of TFEE, TFAqF, TFHAF, and TFHEXF was performed using silica gel plates on glass (2.5 cm × 6 cm) developed with three solvent mixtures composed of hexane/acetyl acetate (8:2), chloroform/methanol (9:1), or chloroform/methanol/water (65:30:5). The spots on the TLC plates were revealed with cerium sulfate spray solution followed by heating at 100°C for 5 minutes [39].

For HPLC analysis, aliquots of TFEE, TFAqF, and TFHAF were diluted in ethanol and water (8:2) and filtered with 0.45 μm membranes. The samples were injected in high performance liquid chromatography (SHIMADZU Prominence, AUTOSAMPLER SIL-10AF, CTO-20A, LC-6AD, CBM-20A, and SPD-20A). The column used was RP C18 (4.6 × 250 mm, i.d. 5 μm Phenomenex Luna, USA) and the mobile phase consisted of formic acid 2% (v/v) and acetonitrile doped with 0.1% of trifluoroacetic acid (TFA) (v/v), starting with a linear gradient elution of 0–100% in 40 min. Flow rate was 1 mL/min and sample injection was 40 μL. The effluent was
monitored at 276 nm with a UV-VIS detector. Samples of (+)-catechin and (-)-epicatechin were prepared under the same conditions and were used as standard.

2.5. Determination of the Antioxidant Activity by the DPPH Free Radical Scavenging Assay. The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [19] for the determination of the stoichiometry of the reactions (static version) and for the characterization of the reactivity of each sample (dynamic version). For the static version a stock solution (1 mg/mL) of TFEE, TFAqF, TFHAF, or TFHEXF was diluted to final concentrations of 240, 120, 60, 30, 15, and 5 µg/mL, in methanol. Two hundred µL of the solutions with different concentrations was added to 2 mL of 40 µg/mL DPPH methanol solution and allowed to react at room temperature. After 30 min the absorbance values were measured at 516 nm by a UV-VIS spectrophotometer (Biospectro SP-220, EQUIPAR Ltda., Curitiba, Brazil) and used to calculate the EC50 (the concentration of the antioxidant necessary to decrease the initial DPPH concentration by 50%). The reactivity of each sample was evaluated at 60 µg/mL concentration by the measurement of the decrease in the absorbance at 516 nm for 60 min. The values of the absorbance were used to calculate the percentage of the remaining DPPH at each 20 s of interval and the ET50 (efficient time = the time necessary to decrease the initial DPPH concentration by 50%).

The antioxidant activity (AA%) was calculated using the following formula: 
$$ \text{AA}\% = \left[ \frac{\text{absorbance of the control} - \text{absorbance of the sample}}{\text{absorbance of the control}} \right] \times 100.$$ 

Methanol (2 mL) plus plant extract solution (200 µL) was used as a blank. DPPH solution (2 mL) plus methanol (200 µL) was used as a negative control. Catechin and butylated hydroxytoluene (BHT) were used as the standard solutions. Assays were carried out in triplicate. The EC50 and ET50 values were calculated by nonlinear regression.

2.6. Determination of the Total Phenolic and Flavonoid Content. Total phenolic content (TPC) was determined spectrophotometrically by the Folin-Ciocalteu method [40], with minor adaptation. Briefly, reaction medium contains 2 mL of distilled water, 250 µL of Folin-Ciocalteu reagent, and 250 µL of extract or fraction (200 µg/mL). After 5–8 min in the dark, 100 µL of 10% Na2CO3 solution was added and mixed. The mixture was incubated for one hour at room temperature (24°C) in the dark and the absorbance was measured at 760 nm. Gallic acid (10–160 µg/mL) was used to construct a standard curve ($Y = 0.0045X - 0.0246; r^2 = 0.9994$). The results were expressed as mg of gallic acid equivalent (GAE)/g dry weight. All tests were performed in triplicate.

Total flavonoid content (TFC) was determined according to Woijsky and Salatino [41], with minor modifications. Using stopped glass tubes, 1000 µL of samples (500 µg/mL, in ethanol) was mixed with an equal volume of 2% AlCl3, in ethanol. After one hour at room temperature (24°C) in the dark, the absorbance was measured at 420 nm. A standard curve ($Y = 0.0053X + 0.0043; r^2 = 0.9999$) was constructed with rutin (10–160 µg/mL) and the total flavonoid content was expressed as mg rutin equivalent (RE)/g dry weight. Samples were analyzed in triplicate.

2.7. Evaluation of the Acute Oral Toxicity in Mice. This assay was performed according to the Organization for Economic Cooperation and Development (OECD) revised up-and-down procedure for acute toxicity testing [42] in groups of 5 male and 5 female Swiss mice. The animals were fasted overnight (12 h) with free access to water prior to the oral administration of a single dose of 2000 mg/kg of TFEE, TFAqF, or TFHAF and observed continuously for 4 h, intermittently for 24 h, and then once a day for the next 14 days for general behavioral changes, signs of toxicity, and mortality.

2.8. Acute Gastric Ulcer Induced by Ethanol in Rats. The antiulcerogenic activity of TFEE (60.5, 125, 250, or 500 mg/kg) and TFHAF, TFAqF, TFHAF (as a suspension in distilled water), TFHEXF (dissolved in Tween 80 1%), or carbenoxolone at dose 250 mg/kg (in distilled water) was investigated by using the acute ethanol-induced gastric ulcer in rats, adapted from Robert et al. [43].

Male rats maintained under standard conditions as described above were fasted for 24 h and orally received distilled water (control, 5 mL/kg) or vehicle (Tween 80 1%, 5 mL/kg), the sample to be evaluated (test), or carbenoxolone (standard). One hour later, gastric lesions were induced by oral administration of absolute ethanol (1 mL/animal). Animals were euthanized 30 min after ethanol administration with sodium thiopental overdose (100 mg/kg, i.p.) and the stomachs were removed, opened along the lesser curvature, washed with normal saline, and examined in a blinded manner. The quantification of the ulceration induced by ethanol was performed using the ImageJ-NIH computer program (National Institutes of Health, Washington D.C.) to calculate the ulceration index expressed as the percentage of ulcerated area in relation to the area of the corpus of the stomach.

2.9. Pretreatment with Indomethacin on Ethanol-Induced Gastric Ulcer in Rats. Male rats, maintained under standard conditions as described previously, were fasted for 24 h with free access to water then divided into four groups according to the respective treatment. The animals were administered a subcutaneous injection of indomethacin (30 mg/kg, s.c.), a cyclooxygenase (COX) inhibitor. After 30 min, each group received the respective treatment orally (distilled water, 250 or 500 mg/kg of TFEE, or 250 mg/kg of carbenoxolone). One hour later, 1 mL/animal of absolute ethanol was administered orally. The stomachs were removed 30 min after ethanol administration. The gastric mucosal lesions were evaluated and the ulceration index was calculated as described in Section 2.8.

2.10. Pretreatment with L-NAME on Ethanol-Induced Gastric Ulcer in Rats. Male rats, maintained under standard conditions as described previously, were fasted for 24 h with free
access to water and then divided into five groups according to the respective treatment. The animals were administered an injection of N-nitro-L-arginine methyl ester (L-NAME, 70 mg/kg, i.p.), a nitric oxide synthase (NOS) inhibitor. After 30 min, each group received the respective oral treatment (distilled water, 250 or 500 mg/kg of TFEE, or 100 or 250 mg/kg of carbonoxolone). One hour later, 1 mL/animal of absolute ethanol was administered orally. The stomachs were removed 30 min after ethanol administration and the gastric mucosal lesions were evaluated for the quantification of the ulceration index as described in Section 2.8.

2.11. Determination of the Gastric Juice Volume and Acid Secretion in Pylorus Ligated Rats. Female rats were acclimatized under standard conditions as described above for at least 4 days in individual, metabolic, wire-bottom cages to avoid coprophagy. The food was withdrawn 24 h before the experiment but there was free access to drink a 5% glucose solution to reduce fasting stress. The control and experimental groups consisted of 6–8 animals each. All experiments were done in the morning. Pylorus ligation was performed as described by Shay et al. [44] and was done through a midline abdominal incision under ketamine (50 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) anesthesia. TFEE was administered intraduodenally to the animals in a 500 mg/kg dose suspended in 5 mL/kg of distilled water. Control animals received distilled water (5 mL/kg) and the standard group received ranitidine (60 mg/kg). The abdomen was sutured and the animals were allowed to recover from anesthesia. Rats were euthanized with sodium thiopental (100 mg/kg, i.p.) 4 h after treatment and the abdomen was opened and another ligature was placed around the esophagus close to the diaphragm. The stomachs were removed and gastric juice solution was collected. Distilled water (3 mL) was added and the total solution was centrifuged at 3500 rpm for 30 min. The content (mL), the pH, and the total acidity in gastric secretion were determined in the supernatant volume. The total acidity output was determined by titration to pH 7.4 with 0.1 N NaOH in a pH meter (WTW 330i, Wissenschaftlich-Technische Werkstätten GmbH & Co. KG, Weilheim, Germany) and expressed as μEq/h gastric juice. Additionally, glandular segments from the stomachs were excised for determination of gastric wall mucus and nonprotein sulphydryl (NP-SH) group content, as described in Sections 2.12 and 2.13.

2.12. Determination of the Gastric Wall Mucus Content in Pylorus Ligated Rats. Gastric wall mucus was assessed by the Alcian blue method [45]. Stomachs excised from 4 h pylorus ligated rats were opened along the lesser curvature. Glandular segments from the stomachs were removed and weighed. Each segment was transferred immediately to 7 mL of 0.25% w/v Alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8) and incubated for 2 h at room temperature. The free dye was removed by two successive rinses at 15 and 45 min in 0.25 M aqueous sucrose solution. The gastric wall mucus bound dye was extracted by immersion in 5 mL of 0.5 M MgCl₂ for 2 h with 1 min agitation every 30 min. A 4 mL sample of the blue extract was then shaken vigorously with an equal volume of diethyl ether and the resulting emulsion was centrifuged at 3000 rpm for 10 min. The optical density of Alcian blue in the aqueous layer was read against distilled water at 598 nm by a UV-VIS spectrophotometer (Biospectro SP-220, EQUIPAR Ltda., Curitiba, Brazil). The quantity of mucin was expressed as μg of Alcian blue extracted per weight (g) of wet stomach glandular tissue.

2.13. Determination of the Gastric Wall Nonprotein Sulphydryl Group Content in Pylorus Ligated Rats. Gastric wall nonprotein sulphydryl (NP-SH) groups were determined by the method from Sedlak and Lindsay [46]. Stomachs excised from 4 h pylorus ligated rats were opened along the lesser curvature. Glandular segments from the stomachs were removed and weighed. Each segment was transferred immediately and homogenized in 5 mL of refrigerated 0.02 M sodium EDTA (ethylenediaminetetraacetic acid, disodium salt) solution. Tissue proteins (in 4 mL homogenate) were precipitated with 4 mL of 10 g% trichloroacetic acid and centrifuged out (15 min, 3000 rpm) and an aliquot (2 mL) of the supernatant was added to 4 mL of 0.4 M Tris/0.2 M EDTA pH 8.9 and 100 μL of 0.01 M DTNB (5,5’-dithiobis(2-nitrobenzoic acid)), diluted in methanol. The optical density of the TNB (thionitrobenzoic) ion solution was read against distilled water at 412 nm by a UV-VIS spectrophotometer (Biospectro SP-220, EQUIPAR Ltda., Curitiba, Brazil) and the concentration of sulphydryl group was calculated by comparison to a standard calibration curve prepared with cysteine. The content of NP-SH group was expressed as μM SH per weight (g) of wet stomach glandular tissue.

2.14. Determination of the Gastric Wall Mucus and Nonprotein Sulphydryl Group Contents in Rats Treated with Ethanol. Female rats, maintained under standard conditions as described previously, were fasted for 24 h with free access to water then divided into groups according to the respective treatment. The animals orally received distilled water (5 mL/kg, control groups with and without ethanol aggression), TFEE (500 mg/kg, test groups with and without ethanol aggression), TFAqF (250 mg/kg), THAF (250 mg/kg), carbonoxolone (250 mg/kg), or N-acetylcysteine (500 mg/kg). One hour later, gastric aggressive lesions were induced by oral administration of absolute ethanol (5 mL/kg). Animals were euthanized 30 min after ethanol administration with sodium thiopental overdose (100 mg/kg, i.p.) and the stomachs were removed, opened along the lesser curvature, and softly washed with normal saline. Glandular segments from the stomachs were removed and weighed and the gastric wall mucus and nonprotein sulphydryl group contents were determined as described in Sections 2.12 and 2.13.

2.15. Determination of the Gastric Wall Mucus and Nonprotein Sulphydryl Group Contents in Rats Treated with Indomethacin. Male rats maintained under standard conditions as described previously, were fasted for 24 h with free access to water then divided into groups according to the respective treatment. The animals orally received distilled water (5 mL/kg, control groups with and without indomethacin aggression), TFEE
(500 mg/kg), TFAqF (250 mg/kg), TFHAF (250 mg/kg), or carbenoxolone (250 mg/kg). Thirty minutes later, gastric aggressive lesions were induced by subcutaneous administration of indomethacin (30 mg/kg). Animals were euthanized four hours after indomethacin administration with sodium thiopental overdose (100 mg/kg, i.p.) and the stomachs were removed, opened along the lesser curvature, and softly washed with normal saline. Glandular segments from the stomachs were removed and weighed and the gastric wall mucus and nonprotein sulphydryl group contents were determined as described in Sections 2.13 and 2.14.

2.16. Assessment of the Gastric Emptying and Bowel Transit in Rats. The gastric emptying and small intestinal transit were assessed by the phenol red content assay, modified from the method described by Izbeki et al. [47]. Briefly, groups of 6–8 female rats were fasted for 24 h and orally received water (5 mL/kg), TFAE (250 or 500 mg/kg), TFAqF (250 mg/kg), TFHAF (250 mg/kg), or atropine (3 mg/kg, i.p.). One hour later, they all orally received phenol red 0.5 mg/mL in glucose 5 g% (1.5 mL/animal). After 20 min, the animals were euthanized with an overdose of sodium thiopental (100 mg/kg, i.p.) and the stomach and small intestine were removed. The small intestine was divided into the proximal (40%), medial (30%), and distal (30%) portions and each segment was homogenized in 100 mL of 0.1 N NaOH. Tissue proteins (in 5 mL homogenate) were precipitated with 0.5 mL of 20 g% trichloroacetic acid and centrifuged out (20 min, 3000 rpm). From the supernatant, an aliquot of 3 mL was added to 4 mL of 0.5 N NaOH and the concentration of phenol red was determined by absorbance at 560 nm (Biospectro SP-220UVVIS spectrophotometer, EQUIPAR Ltda., Curitiba, Brazil). The content of the dye in each segment was calculated and the retention of the marker was expressed as the percentage of the total amount of phenol red recovered in the four segments.

2.17. Small Intestinal Transit in Mice. Male and female mice, fasted for 24 h, were orally administered distilled water (10 mL/kg), TFAE (500, 750, or 1000 mg/kg), TFHAE (500, 750, or 1000 mg/kg), or atropine sulfate (3 mg/kg) and 30 min later individually received 0.1 mL of a 10% aqueous suspension of charcoal meal. Half an hour after this treatment, each animal was euthanized with a sodium thiopental overdose (100 mg/kg, i.p.) and the intestinal transit of the meal was evaluated by the measurement of the distance travelled in 30 min by the charcoal from the pylorus to the caecum and expressed as the percentage of the full small intestinal length.

2.18. Castor Oil-Induced Diarrhea in Mice. Male and female mice, fasted for 20 h, were divided into six groups of 8 animals each. The first group was orally administered distilled water while the other groups received castor oil (0.1 mL/animal). Half an hour later, the animals orally received distilled water (first and second groups, 10 mL/kg), TFAE (500, 750, or 1000 mg/kg), or loperamide (3.5 mg/kg) and were placed separately in plastic cages with paper sheets. The paper sheet was changed and the number of compact and diarrheal faeces excreted for each animal was scored every hour for 4 h. The severity of the diarrhea was assessed by the total number of compact and diarrheal faeces excreted by each group of animals in the 4 h interval time of observation.

2.19. Statistical Analysis. The results are presented as the mean ± standard error of the mean (M ± S.E.M). The statistical significance for differences between groups was calculated by one-way analysis of variance (ANOVA) and Dunnett’s or Tukey’s test. The differences between groups were regarded as significant at P < 0.05.

3. Results

3.1. Preparation of the Extracts and Partition Fractions. The extraction of the *Terminalia fagifolia* bark powder (630 g) with ethanol (6 L) rendered 120 g (19% yield) of TFEE. Similar procedure using 60 g of bark powder extracted with 900 mL of a 50% hydroalcoholic solution as solvent resulted in 18 g (30% yield) of TFHAE.

The solvent extraction of TFEE (80 g) with acetyl acetate and hexane produced the partition fractions TFAqF (26.8 g, 33.5% yield), TFHAF (43.45 g, 54.3% yield), and TFHEXF (1.7 g, 2.1% yield).

3.2. Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) of TFEE, TFAqF, and TFHAF. The thin layer chromatograms, developed on silica gel plates with three solvent mixtures, suggested the presence of polar compounds like flavonoids, glycosylated flavonoids (yellow spots), and saponins (rose spots) in the aqueous (TFAqF) and hydroalcoholic (TFHAF) partition fractions and fat substances, including steroids (blue spots which become pink and then gray with the extension of the heating) and pentacyclic triterpenoid (rose and orange spots), in the hexanic (TFHEXF) partition fraction of TFEE.

The chromatograms of the HPLC analysis of TFEE, TFAqF, and TFHAF are depicted in Figure 1. The results allowed the identification of components with retention time similar to (+)-catechin and (−)-epicatechin in TFEE and TFHAF and to (−)-epicatechin in TFAqF.

3.3. Antioxidant Activity by the DPPH Free Radical Scavenging Assay. The results of the study of the DPPH free radical scavenging activity of TFEE, partition fractions of TFEE (TFAqF, TFHAF, and TFHEXF), catechin, and BHT are presented in Table 1. TEE showed an EC50 equivalent to that of catechin and much lower (P < 0.001) than that of BHT and of TFHEXF. There was no statistical difference between the EC50 of TFEE and its aqueous or hydroalcoholic fractions. The hexanic fraction of TFEE (TFHEXF) showed an EC50 higher than that of BHT. Nevertheless, the reactivity (ET50) of TFAqF, and TFHAF was higher (P < 0.001) than that of catechin at 60 μg/mL. The reactivity of BHT and TFHEXF was not quantified because of the higher EC50 of these samples.

3.4. Total Phenolic and Flavonoid Content. Table 1 shows the total phenolic (TPC) and flavonoid (PFC) content of
Table 1: Stoichiometry and reactivity of the DPPH free radical scavenging activity and total phenolic (TPC) and flavonoid (TFC) content for the Terminalia fagifolia ethanolic bark extract (TFEE) and the aqueous (TFAqF), hydroalcoholic (TFHAF), and hexanic (TFHEXF) partition fractions of TFEE, catechin, and butylated hydroxytoluene (BHT).

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH scavenging activity</th>
<th>TPC</th>
<th>TFC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (µg/mL)</td>
<td>ET₅₀ (s)</td>
<td>mg GAE/g*</td>
</tr>
<tr>
<td>TFEE</td>
<td>50.0 ± 3.9</td>
<td>4.5 ± 0.6ᵃ</td>
<td>452.3 ± 18.1</td>
</tr>
<tr>
<td>TFAqF</td>
<td>33.9 ± 3.1</td>
<td>8.0 ± 1.9ᵇ</td>
<td>400.5 ± 9.1ᵇ</td>
</tr>
<tr>
<td>TFHAF</td>
<td>31.6 ± 2.0</td>
<td>4.7 ± 1.4ᵇ</td>
<td>404.1 ± 12.9ᵇ</td>
</tr>
<tr>
<td>TFHEXF</td>
<td>&gt;&gt;240ᵃ</td>
<td>ND</td>
<td>76.6 ± 1.3ᵇ</td>
</tr>
<tr>
<td>Catechin</td>
<td>46.3 ± 2.5</td>
<td>51.7 ± 9.7ᵇ</td>
<td>514.5 ± 6.9ᵇ</td>
</tr>
<tr>
<td>BHT</td>
<td>213.8 ± 5.5ᵃ</td>
<td>ND</td>
<td>356.6 ± 9.3ᵇ</td>
</tr>
</tbody>
</table>

The data represent M ± S.E.M. for a triplicate assay. P < 0.01 compared to ᵃcatechin or toᵇTFEE (ANOVA and Tukey’s test). ND: not detectable. * mg gallic acid equivalent/g, ** mg rutin equivalent/g.

TFEE and its partition fractions, catechin, and butylated hydroxytoluene (BHT). There was no significant difference between the TPC (mg gallic acid equivalent/g) of TFAqF (400.5 ± 9.1) and TFHAF (404.1 ± 12.9), which was about 11% lower than that of TFEE (452.3 ± 18.1). The TFC (mg rutin equivalent/g) of TFEE (218.6 ± 2.0), TFAqF (217.5 ± 1.6), and TFHAF (222.0 ± 1.9) was similar. TFHEXF showed a very low TPC (76.6±1.3) and TFC (7.4±0.8), compared to that of TFEE (17% and 3%, resp.).

Table 2: Mortality of male and female mice treated orally with TFEE, TFAqF, or TFHAF and observed for 14 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality (D/T)*</th>
<th>LD₅₀** (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFEE</td>
<td>0/5</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>TFAqF</td>
<td>—</td>
<td>0/5</td>
</tr>
<tr>
<td>TFHAF</td>
<td>—</td>
<td>1/5</td>
</tr>
</tbody>
</table>

*D/T represents the number of deaths (D) among the total (T) of tested animals. ** OECD, guideline 425.

3.5. Acute Oral Toxicity Evaluation. The result of the toxicity study of TFEE, TFAqF, and TFHAF at a limit oral test dose of 2000 mg/kg in male and female mice is shown in Table 2. According to the OECD revised up-and-down procedure for acute toxicity testing [42], the LD₅₀ of TFEE, TFAqF, and TFHAF is greater than 2000 mg/kg for mice with a gender difference in the acute toxicity of TFEE where the females are more sensitive than the males and could be classified as of low acute toxicity hazard category 5 according to the United Nations Globally Harmonized System of Classification and Labeling of Chemicals [48].
Figure 2: Effect of the oral administration of the ethanolic (TFEE, 0–500 mg/kg) and hydroalcoholic (TFHAE, 250 mg/kg) extracts of the bark of *Terminalia fagifolia* and of the aqueous (TFAgF), hydroalcoholic (TFHAF), and hexanic (TFHEXF) partition fractions of TFEE or carbenoxolone (250 mg/kg) on gastric ulcers induced by absolute ethanol. Ulceration index as percentage of the area of the corpus of the stomach (data are presented as the mean ± S.E.M.). *P < 0.05 compared to the respective control group (ANOVA and Dunnett's test).

Table 3: Effect of the intraduodenal administration of the *Terminalia fagifolia* ethanolic bark extract (TFEE) and ranitidine on pH and total acidity of gastric secretion and gastric juice volume and on the gastric wall mucus or nonprotein sulphydryl group contents in four-hour pylorus ligated rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (5 mL/kg)</th>
<th>TFEE (500 mg/kg)</th>
<th>TFAqF (60 mg/kg)</th>
<th>Ranitidine (60 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (units)</td>
<td>2.22 ± 0.07</td>
<td>3.76 ± 0.39*</td>
<td>5.80 ± 0.66*</td>
<td></td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>3.02 ± 0.17</td>
<td>1.18 ± 0.21†</td>
<td>1.48 ± 0.22†</td>
<td></td>
</tr>
<tr>
<td>Total acidity (µEq/h)</td>
<td>69.77 ± 5.51</td>
<td>13.19 ± 4.33*</td>
<td>5.83 ± 3.13*</td>
<td></td>
</tr>
<tr>
<td>Gastric wall mucus (µg/g)</td>
<td>36.07 ± 4.42</td>
<td>34.47 ± 2.83</td>
<td>38.97 ± 2.32</td>
<td></td>
</tr>
<tr>
<td>Nonprotein SH (µM/g)</td>
<td>48.17 ± 1.80</td>
<td>46.11 ± 2.45</td>
<td>42.44 ± 3.37</td>
<td></td>
</tr>
</tbody>
</table>

The data represent the mean ± S.E.M. of groups of 6–8 animals. *P < 0.05 (ANOVA and Dunnett's test) compared to the respective control group.

3.6. Effect of TFEE on Acute Gastric Ulcer Induced by Ethanol in Rats. In the ethanol-induced gastric ulcer model, TFEE was found to possess remarkable ulcer-protective properties at orally administered doses of 125, 250, and 500 mg/kg, showing 56%, 89%, and 97% protection, respectively. The antiulcerogenic activity of TFEE was dose-related, showing an effective dose (ED50) of 113 mg/kg. Inhibition of the gastric ulcerogenic activity of ethanol was also detected in TFAqF (68%), TFHAF (92%), and TFHAE (81%), but not in TFHEXF, at orally administered doses of 250 mg/kg. The standard drug carbenoxolone (250 mg/kg) showed 97% protection (Figure 2).

3.7. Effects of TFEE on Acute Gastric Ulceration Induced by Ethanol with Pretreatment of L-NAME. Pretreatment with the cyclooxygenase inhibitor indomethacin (30 mg/kg, s.c.) weakened the protection against gastric ulceration induced by ethanol from 89% to 52% for TFEE at the 250 mg/kg dose, from 97% to 70% for TFEE at the 500 mg/kg dose, and from 97% to 72% for carbenoxolone at the 250 mg/kg dose (Figure 3).

3.8. Effects of TFEE on Acute Gastric Ulceration Induced by Ethanol with Pretreatment of L-NAME. The protection against gastric ulceration induced by ethanol was weakened by the pretreatment with the nitric oxide synthase inhibitor L-NAME (70 mg/kg, i.p.) from 97% to 51% for TFEE at 500 mg/kg and from 97% to 73% for carbenoxolone at 250 mg/kg dose and it abolished the gastroprotection shown by TFEE at 250 mg/kg dose (Figure 3).

3.9. Effect of TFEE on Gastric Acid Secretion and on Gastric Wall Mucus and Nonprotein Sulphydryl Group Contents in Pylorus Ligated Rats. Intraduodenal treatment with 500 mg/kg of TFEE in four-hour pylorus ligated female rats provoked a significant \( P < 0.05 \) increase in pH and a reduction in the volume and total acidity of gastric juice produced. The response elicited by 60 mg/kg of ranitidine was similar to that obtained with TFEE. However, ranitidine's effect on the total acidity and pH was significantly greater than those of TFEE (Table 3). The intraduodenal administration of TFEE or ranitidine did not result in any significant change in the mucus or NP-SH group contents of the animals' gastric mucosa.

3.10. Effect of TFEE, TFAqF, and TFHAF on Gastric Wall Mucus and Nonprotein Sulphydryl Group Contents in Rats...
**3.11. Effect of TFEE, TFAqF, and TFHAF on Gastric Wall Mucus and Nonprotein Sulphhydryl Group Contents in Rats Treated with Indomethacin.** The effects of the oral administration of the ethanolic extract (TFEE) of the bark of *Terminalia fagifolia* (500 mg/kg) or its aqueous (TFAqF) and hydroalcoholic (TFHAF) partition fractions (250 mg/kg) or carbonoxolone (100 mg/kg) on the gastric wall mucus and nonprotein sulphhydryl (NP-SH) group contents in male rats treated by subcutaneous route with indomethacin are presented in Figure 5. TFEE, TFAqF, and TFHAF showed an effect similar to that of indomethacin, reducing the gastric wall mucus content of the animals treated with this aggressive agent. The treatment of the animals with indomethacin did not produce a significant depletion of the gastric wall nonprotein sulphhydryl (NP-SH) group content, and TFEE, TFAqF, and TFHAF showed no effect on this gastric wall constituent.

**3.12. Effect of TFEE, TFAqF, and TFHAF on the Gastric Emptying and Bowel Transit in Rats.** The percentage distribution of phenol red recovered from the four gastrointestinal segments of treated female rats is detailed in Figure 6. The percentage of dye recovered from the stomach of rats following administration of TFEE at 250 mg/kg (58.9 ± 2.4) and 500 mg/kg (62.4 ± 4.0) was significantly (*P* < 0.01) greater than that observed in control rats (30.0 ± 2.2), indicating that TFEE delays the gastric emptying. The percentage of recovered phenol red was significantly lower (*P* < 0.01) in the proximal and distal small intestinal segments. The animals treated with TFAqF showed a significant increase (*P* < 0.01) in the dye gastric content (46.6 ± 2.0), but there were no differences with respect to the dye contents in the small intestinal segments. The animals treated with TFHAF showed a significant increase (*P* < 0.01) in the dye gastric content (54.0 ± 2.8) and a significant decrease (*P* < 0.01) in the proximal small intestinal segment (25.0 ± 2.8), but there were no differences with respect to the dye contents in the proximal (10.8 ± 1.1) and distal (10.2 ± 1.3) small intestinal segments, compared to the respective control groups (16.1 ± 1.9 and 11.9 ± 0.4). Atropine (3 mg/kg) provoked a significant increase (*P* < 0.01) in the dye contents in the gastric (42.6 ± 2.1) and proximal small intestinal segment (32.4 ± 0.7) and a significant decrease (*P* < 0.01) in the distal (2.6 ± 0.4) small intestinal segments.

**3.13. Effect of TFEE and TFHAE on the Small Intestinal Transit in Mice.** The advancement of the charcoal meal along the small intestine of mice was decreased 21% and 62% by the treatment with TFEE at doses of 750 and 1000 mg/kg, respectively, 21%, 30%, and 34% by the treatment with
**Figure 5:** Effect of the oral administration of the ethanolic extract (TFEE) of the bark of *Terminalia fagifolia* (500 mg/kg) or its aqueous (TFAqF) and hydroalcoholic (TFHAF) partition fractions (250 mg/kg) or carbenoxolone (Carb, 250 mg/kg) on the gastric wall mucus (a) and on the nonprotein sulphydryl (NP-SH) group content (b) in male rats treated by subcutaneous route with indomethacin. The data represent the mean ± S.E.M. of 8 animals/group. *P* < 0.01 (ANOVA and Dunnett’s test) compared to SHAM (a) or control group (b).

**Figure 6:** Effect of oral administration of *Terminalia fagifolia* ethanolic bark extract (TFEE, 250 or 500 mg/kg) or its aqueous (TFAqF, 250 mg/kg) and hydroalcoholic (TFHAF, 250 mg/kg) partition fractions or atropine (3 mg/kg) on the gastric emptying of female rats (8 animals/group). Data are presented as the mean ± S.E.M. ***P* < 0.01 compared to the respective control group (ANOVA and Dunnett’s test).
Table 4: Effect of the oral treatment of mice with different doses of the *Terminalia fagifolia* ethanolic (TFEE) or hydroalcoholic (TFHAE) bark extract or atropine on the small intestinal transit of an aqueous suspension of charcoal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>Advancement of the charcoal (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>12</td>
<td>67.57 ± 2.02</td>
<td>0</td>
</tr>
<tr>
<td>TFEE</td>
<td>500</td>
<td>8</td>
<td>62.99 ± 2.84</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>7</td>
<td>53.47 ± 2.37*</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>7</td>
<td>25.41 ± 6.42***</td>
<td>62</td>
</tr>
<tr>
<td>TFHAE</td>
<td>500</td>
<td>7</td>
<td>53.56 ± 3.96*</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>8</td>
<td>47.12 ± 4.36**</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>7</td>
<td>44.68 ± 4.50**</td>
<td>34</td>
</tr>
<tr>
<td>Atropine</td>
<td>3</td>
<td>8</td>
<td>27.35 ± 3.49***</td>
<td>60</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E.M. *P < 0.05; **P < 0.01 and ***P < 0.001 compared to the control group (ANOVA and Tukey’s test).

TFHAE at doses of 500, 750, and 1000 mg/kg, respectively, and 60% by the treatment with atropine (3 mg/kg). This result was significantly different (P < 0.05) from what was seen in the control group, indicating that TFEE and TFHAE induced an inhibition of the small intestinal transit but only when the extracts were administered at higher doses (Table 4).

3.14. Effect of TFEE on Castor Oil-Induced Diarrhea in Mice.

Using the castor oil-induced diarrhea model on mice, it was observed that the severity of the diarrhea was significantly reduced (P < 0.05) by the treatment of the animals with TFEE (1000 mg/kg) and loperamide (3.5 mg/kg), indicating a weakly antidiarrheal activity in the ethanolic extract of this plant (Figure 7).

4. Discussion and Conclusion

Peptic ulcer is one of the most common gastrointestinal diseases. This pluricausal illness is a resultant of an interaction and imbalance between aggressive factors—like ethanol, free radicals, hydrochloric acid, ischemia, leukotrienes, NSAIDs, pepsin, and stress—and defensive factors like bicarbonate, mucus, mucosal blood flow, sulfhydril and enzymatic activity of superoxide dismutase, and catalase [38]. Prostaglandins and nitric oxide (NO) are important factors involved in gastric defense mechanisms through the regulation of acid and alkaline secretion, epithelial fluid, mucus secretion, and mucosal blood flow [43, 49].

The results of this investigation showed that the oral administration of *Terminalia fagifolia* ethanolic bark extract (TFEE) had an antulcerogenic activity against ethanol-induced gastric ulcer, which was reduced by pretreatment with L-NAME and indomethacin. By intraduodenal route of administration, TFEE showed an antisecretory property but did not change the gastric wall mucus and nonprotein sulfhydril group content in pylorus ligated rats, indicating a systemic activity after the intestinal absorption of the active constituents of the plant extract. Nevertheless, orally administered TFEE and its aqueous (TFAqF) and hydroalcoholic (TFHAF) partition fractions drastically reduced the mucus layer adhered to the gastric wall of rats treated with ethanol or indomethacin. These results point out that the prostaglandins pathway seems to be only partially involved in the gastroprotective effect of TFEE since the gastric wall mucus was reduced by TFEE but not by carbenoxolone, an
agent that enhances the prostaglandins synthesis. Moreover, TFEE delayed gastric emptying and presented a relatively low toxicity and an antioxidant activity similar to that of catechin, used as a comparative standard. Besides these properties, TFEE slightly inhibited the basal and castor oil stimulated small bowel motility, demonstrating a weakly antidiarrheal activity.

The aqueous (TFAqF) and hydroalcoholic (TFHAF) but not the hexanic (TFHEXF) partition fractions of TFEE also presented significant antiulcerogenic activity and delayed gastric emptying, indicating that those properties could be related to the action of polyphenolic compounds like flavonoids or glycosylated flavonoids and saponins present in the bark of *Terminalia fagifolia*.

Ethanol-induced gastric ulcer has been widely used for the evaluation of antiulcerogenic activity of natural products. Ethanol induces ulcers by reducing gastric mucosal blood flow and mucus production in gastric lumen, decreasing endogenous glutathione and prostaglandin levels and increasing ischemia, gastric vascular permeability, acid “back diffusion,” histamine release, efflux of sodium and potassium, influx of calcium, the generation of free radicals, and the production of leukotrienes [38].

Certainly the gastroprotection elicited by the compounds present in the bark of *Terminalia fagifolia* could reflect an inhibition of the gastric acid secretion and an increase in the release of protective substances by the gastric mucosa such as nitric oxide and prostaglandins, since this protection was decreased by pretreatment with L-NAME and indomethacin, which are nitric oxide synthase and cyclooxygenase inhibitors, respectively.

Plants belonging to the botanical family Combretaceae (*Terminalia arjuna*, *T. bellirica*, *T. chebula*, *Combretum leprosum*, *C. dolichopetalum*, and *Guiera senegalensis*) have been shown to exhibit antiulcerogenic and gastroprotective activity with the involvement of prostaglandins and nitric oxide [22, 23, 25, 26, 32–35], apart from its ubiquitous antioxidant properties.

There is evidence that reactive oxygen species and free radicals are involved in the etiology and physiopathology of several human diseases, such as gastrointestinal inflammation and gastric ulcer. The potential antioxidant protective effect of natural products on affected tissues, therefore, is a topic of high current interest [50]. Thus, the free radical scavenging activity detected in *Terminalia fagifolia* bark extracts could contribute to their gastroprotective activity.

Antiulcerogenic or gastroprotective activity was detected in plants containing (−)-epicatechin [51–54]. Reimann et al. [55] showed that (+)-catechin (25 mg/kg), given intraperitoneally, prevented the formation of gastric lesions induced by immobilization in female rats. Nevertheless, orally administered (−)-catechin has not presented antiulcer activity in ethanol-induced gastric ulcers in rats [56] and could not be responsible for the detected antiulcerogenic activity of *Terminalia fagifolia* bark extracts. Probably, this activity must be associated with the action of the substances detected on the HPLC analysis with retention time similar to (−)-catechin in TFEE and TFHAF or to (−)-epicatechin in TFAqF. Flavonoids, a large group of polyphenolic compounds, have been reported to exhibit a wide range of biological activity, including antiulcerogenic effect against gastric damaging agents [57–59], and were detected in TFEE and its partition fractions.

Yano et al. [60] showed that, for rats submitted to restraint and water immersion stress, the formation of gastric lesions was markedly increased after an increase in gastric motility and that this ulcerogenic effect may be due to the “mechanical rubbing” of the gastric mucosa. According to Cho et al. [61] intermittent vagal electrical stimulation increased the intragastric pressure and induced a 100% incidence of hemorrhagic ulcers in the glandular mucosa of rat stomachs. Hypermotility of the stomach has been considered one of the mechanisms of the ulcerogenic action of indomethacin in rats, probably through microcirculatory disturbances, leading to the increased microvascular permeability and cellular damage [62–65]. The endogenous NO also delays gastric emptying and antral motor activity without affecting gastric myoelectrical activity [66]. Flavonoid-rich fraction of *Maytenus ilicifolia* Mart. ex. Reisseck protects the gastric mucosa of rodents through antisecretory activity and formation of nitric oxide and, at the same time, inhibits the gastric emptying and intestinal motility of mice [67, 68]. These findings substantiate the idea that a delaying in the gastric emptying may be considered a beneficial property of a gastroprotective agent against aggressive factors of the gastric mucosa like ethanol and could also contribute to the antiulcerogenic activity of *Terminalia fagifolia* bark extract.

Our results give partial support to the popular use of the bark of this plant to treat gastrointestinal disorders, such as gastritis and gastric ulcers. Besides its antiulcerogenic and antisecretory activities, the plant extract delayed gastric emptying and presented antioxidant activity. Moreover, the toxicity is relatively low and the intestinal transit is inhibited only with higher doses of the extract. However, the assessment of the quality, safety, and therapeutic efficacy of phytotherapeutic preparations requires more scientific investigation.

The results found do not allow the full understanding of the mechanisms involved in the pharmacological activities detected in this study. In reality, the data found in relation to the effects of *Terminalia fagifolia* on the mucus layer bound to the wall of the stomach of rats require further study in order to clarify the paradox of the presence of a significant gastroprotector activity in this plant that, at the same time, drastically reduces the mucus layer adhered to the gastric wall.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

The authors are grateful to the Federal University of Piauí/Brazil and RENORBio/Brazilian Government for providing them with all the laboratory facilities and equipment to perform this research.
References


Salmonellosis is a common and widely distributed food borne disease where *Salmonella typhimurium* is one of the most important etiologic agents. The purpose of this study was to investigate the antimicrobial activity of *Nymphaea tetragona* alone and in combination with antibiotics against *S. typhimurium*. It also aimed to assess the plant for quorum sensing inhibition (QSI) activity and to identify the bioactive compounds. The antibacterial activities of the extract were assessed using broth microdilution method. Disk agar diffusion method was employed to determine the QSI and bioactive compounds were identified by GC-MS analysis. Ethyl acetate fraction of *N. tetragona* extract (EFNTE) demonstrated good antimicrobial activity (MIC 781 μg/mL) against 4 strains out of 5. FIC index ranged from 0.375 to 1.031 between EFNTE/tylosin and 0.515 to 1.250 between EFNTE/streptomycin against *S. typhimurium*. Among all extracts, EFNTE and butanol fraction more significantly inhibited pigment production of *C. violaceum*. Polyphenols were identified as major compound of EFNTE and butanol fraction. These results indicate that combination among *N. tetragona* extract and antibiotics could be useful to combat drug-resistance *Salmonella* infections and polyphenols are promising new components from *N. tetragona* that warrant further investigation as a candidate anti-*Salmonella* agent and quorum sensing inhibitor.

1. Introduction

*Salmonella* species are the leading cause of bacterial gastroenteritis in humans and animals all over the world [1, 2]. Food animals and water are the most important reservoirs of the bacteria [2] where the outbreaks of *Salmonella* infections have increasingly been associated with processed foods [3, 4]. There are 1300 million cases of gastroenteritis, 16 million cases of typhoid fever, and 3 million cases of deaths worldwide each year due to *Salmonella* infections [5]. *Salmonella typhimurium* is one of the most common serovars associated with clinically reported salmonellosis in humans in most parts of the world, accounting for at least 15% of infections [2, 6].

*S. typhimurium* infects a wide range of animal hosts, including poultry, cattle, and pigs, and is termed ubiquitous which usually causes a self-limited gastroenteritis in humans [7]. The use of antibiotics is a major strategy and they are commonly used therapeutically and prophylactically to treat *S. typhimurium* infections in human and animal. However, increased antimicrobial resistance is exacerbating impact on public health worldwide, which leads to increased morbidity, mortality, and treatment costs [8, 9]. Scientific studies showed that tylosin has low or no inhibitory effects on experimentally inoculated *S. typhimurium* in pigs [10, 11]. It was also reported that *S. typhimurium* illustrated resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline in 1980s, in the UK and later distributed extensively through Europe and North America. Additional resistance had attained to trimethoprim-sulfamethoxazole, ciprofloxacin, and extended-spectrum cephalosporins within the 1990s [12]. Development of alternative antibacterial therapies is necessary to overcome this outbreak. Approximately 80% of the world’s inhabitants rely on traditional medicine for
their primary health care and plants also play an important role in the health care system [13]. The possible therapeutic use of Nymphaeaceae may be a good alternative of traditional antibacterial.

The Nymphaeaceae also called water lilies have a broad range of flower colors and are living on the banks of lakes and rivers, distributed in tropical areas around the world [14–16]. A number of species of Nymphaea in Nepal, India, and China are thought to act as functional drug plants [17]. Many bioactive and pharmacologically important compounds have been obtained from Nymphaea species and used in medicine and pharmacy [18]. Flower extract of N. nouchali which possess compounds with high antibacterial and cytotoxic properties [19, 20] and ethyl acetate extract of N. nouchali leaf extract have antibacterial activity against a wide range of strains [21]. Nymphaea lotus extract was reported to have bioactive compounds such as tannins, flavonoids, alkaloids, anthraquinones, saponins, cardiac glycosides, and phenolics where manticin and vancomycin resistant S. aureus, S. pyogenes, and E. coli were highly susceptible to N. lotus [13, 22, 23]. The pygmy water lily, Nymphaea tetragona (Ait.) Georgi (Nymphaeaceae), is one of the widely distributed plants, ranging globally from Asia-temperate, Asia-tropical, Europe, and northern America [24]. It has ethnomedical uses as the rhizome is used to cure acute diarrhea and dysentery by tribal herbal practitioners in Indian region [24].

Although there are many reports in the ethnomedical values of Nymphaea, information on their antibacterial efficacy is scarce and with low scientific caliber for further commercial use. Hence, it is important to determine the antibacterial activity very clearly and to identify the antibacterial active compounds of this plant. Furthermore, the potentials of water lily in combating antimicrobial resistance alone and in combination with antibiotics and the inhibition of quorum sensing controlled virulent factors of microbial pathogens were not explored previously. Thus, the current study was designated to evaluate the antibacterial activity of Nymphaea tetragona extract alone and in combination with commercial antibiotics against Salmonella typhimurium. Quorum sensing inhibition activity of the extract against biometer strain Chromobacterium violaceum was also aimed at assessing in this study. Finally, a GC-MS analysis was performed to identify and quantify the major compounds of the N. tetragona.

2. Materials and Methods

2.1. Bacterial Strains and Culture Medium. Salmonella typhimurium QC strain KTCC2515 and clinical isolates ST171, ST482, ST688, and ST21A were used for experiments in this study (Table 1) which were collected from different farms in Republic of Korea. Bacterial strains were suspended in Mueller Hinton broth (MHB, Difco, USA) and then incubated at 37°C with 200 rpm for 20 h. Mueller Hinton agar (MHA, Difco) was used for the agar diffusion method.

2.2. Plant Extraction and Fractionation. N. tetragona powder of body and root mixture was purchased from Chamsamgol Lotus Farm (Chungju, Republic of Korea). 100 g of the powdered material was boiled with 1000 mL of 50% methanol in a 2000 mL three-neck round bottom boiling flask (Schott Duran, NY, USA) at 100°C setting temperature on nonasbestos surface for 3 h when the % Brix and absorbance of the extract became the highest. The supernatant of N. tetragona 50% methanol extract (NTME) was collected by filtration (70 mm, Advantec; Toyo Roshi Kaisha Ltd., Tokyo, Japan) and solid particles retained on the filter were discarded. The solvent was then removed under reduced pressure in Buchi Rotavapor R-114 (BUCHI Labortechnik AG in Flawil, Switzerland) at 10 rpm and Eyela CCA-1111 (Tokyo Rikakikai Co. Ltd., made in China) and solidified by freeze-drying prior to use. The yield of extract was 10.71%.

10 g of lyophilized N. tetragona extract was suspended in 50 mL of water and fractionated with equivalent amount of dichloromethane, ethyl acetate, and butanol correspondingly by separating funnel. The solvent fractions were solidified and the distribution of extract was 0.43% in dichloromethane, 4.01% in ethyl acetate, and 46.74% in butanol. 47.36% was obtained as residue in water and 1.46% was process loss.

2.3. Antibacterial Resistance Testing. The disk-agar diffusion method validated by the Clinical and Laboratory Standards Institute [25] was used to verify the resistance pattern of four S. typhimurium clinical isolates against different commercial antibiotics. S. typhimurium KTCC 2515 was used as quality control strain in this experiment. Antibiotic sensitivity was considered according to the zone diameter interpretative standards of CLSI [26].

2.4. Determination of MIC and MBC. MIC was determined by the standard broth microdilution method according to the CLSI guidelines [27] in MHB using ~5 × 10^5 CFU/mL of inoculum concentration. The MHB was supplemented with serial dilutions ranging from 24.4–25000, 4.9–2500, 12.2–6250, and 4.9–2500 µg/mL, respectively, for NTME, DFNTE, EFNTE, and BFNT in 100 µL volumes in 96-well plates. Bacterial suspensions were adjusted to 0.1 OD at 600 nm, diluted 1/100, and dispensed in 100 µL aliquots to all the wells, including drug-free controls. Initial CFU/mL of the bacterial suspensions was determined by plating 10-fold dilutions on MHA plates. After overnight incubation at 37°C, inoculums from each well were diluted 10-fold and plated on MHA plates to determine the CFU/mL of each well. MIC was determined by comparing the final CFU with initial CFU. The lowest concentration of the extract inhibiting the increase of CFU was considered as MIC. 100 µL drug dilutions from wells of 96-well plates were cultured on MHA plates to determine the existing bacterial number. MBC was considered as the lowest concentration which can eradicate 99.9% bacteria [28].

2.5. Killing Rate of Salmonella typhimurium. Killing rate was evaluated as previously described by Tia Dubuisson et al., [28] with some modification. 0.25 × MIC, 0.5 × MIC, 1 × MIC, and 2 × MIC of EFNTE were prepared in 10 mL of MHB. Four-hour old S. typhimurium (KTCC 2515) cultures were adjusted to 0.1 OD in 600 nm, diluted to make suitable
diffusion method [29] was used with slight modification. A compared to the zones of individual test [29]. Further test of antagonism shows a reduced zone Generally, synergism shows an improved zone, indifference interface of inhibition zones was observed after incubation. CLSI guideline [25].

zonediameter of individual antimicrobial was determined by in each test plate. Plates were incubated at 35

u a ld r u gsa n dt h ed r u g - f r e em e d i um c o n t r o lw e r ei n c l u d e d combinationsof 2 antibiotics. Similar dilutionsof individual drugs and the drug-free medium control were included in each test plate. Plates were incubated at 35°C for 16 to 20 hours after the addition of ~5 × 10^5 CFU/mL of inoculums. From the MIC of the drugs alone and in combination, we calculated the fractional inhibitory concentration (FIC) and the FIC index (FICI). FIC is the MIC in combination divided by the MIC of the individual drug and FICI is the sum of the FICs of individual drugs. An FICI of ≤0.5 is considered synergistic, an FICI of 4.0 is considered antagonistic, and an FICI of 0.5–4 is considered to indicate no interaction [31].

2.7. Quorum Sensing Inhibition. Quorum sensing inhibition of NTME and its solvent fractions were verified in accordance with the method described by Álvarez et al. [32]. C. violaceum CV12472 was employed to find out the pigment inhibition of extracts for attaining a qualitative screening. 100 μL of fresh culture diluted as 2.5 × 10^5 CFU/mL was poured on media for the preparation of LB agar plates. 60 μL of each extract with specific concentrations was applied to saturate the sterile paper disks (8 mm). Normal saline (60 μL) was used as negative control and purified furanone (100 μg) was applied as positive control whereas tetracycline (10 μg) was employed to compare antibacterial and antagonism sensing activity. Inhibition of pigment production around the disc was checked after 18–24 h incubation at 30°C. The sensitivity to different agents was classified by the diameter of the inhibition zones as follows: “not sensitive” for diameter less than 8 mm, “sensitive” for diameter between 9 and 14 mm, “very sensitive” for diameter between 15 and 19 mm, and “extremely sensitive” for diameter larger than 20 mm [33, 34].

2.8. Gas Liquid Chromatography Coupled Mass Spectrophotometric (GC-MS) Analysis. GC-MS analysis of DFNTE, EFNTE, and BFNTE was performed by “Center for Scientific Instruments” of Kyungpook National University and carried out using a HP 6890 Plus GC gas chromatograph with a (MSD)—HP 5973 MSD mass selective detector (Hewlett-Packard). Samples were diluted 1:1000 (v:v) with HPLC grade dichloromethane. Aliquots of the sample (1 μL) were injected into an HP-5 column. The GC oven temperature was set at 50°C for 4 min, increased to 280°C at a rate of 4°C/min, and held at the final temperature for 2 min. Velocity of the He carrier gas (99.99%) was 0.7 mL/min. Quantitative analysis was performed using the area normalization method.

2.9. Statistical Analysis. The mean values and the standard deviation were calculated from the data obtained from triplicate trials. Analysis of variance (ANOVA) was used to verify
### Table 2: Antimicrobial activity of NTME and its solvent fractions against five strains of *Salmonella typhimurium*.

<table>
<thead>
<tr>
<th></th>
<th>KCTC 2515</th>
<th>ST171</th>
<th>ST482</th>
<th>ST688</th>
<th>ST21A</th>
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<tbody>
<tr>
<td><strong>MIC (µg/mL)</strong></td>
<td></td>
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<td></td>
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<tr>
<td>NOR*</td>
<td>0.063</td>
<td>0.063</td>
<td>0.125</td>
<td>0.063</td>
<td>0.125</td>
</tr>
<tr>
<td>NTME</td>
<td>&gt;2500</td>
<td>&gt;2500</td>
<td>&gt;2500</td>
<td>&gt;2500</td>
<td>&gt;2500</td>
</tr>
<tr>
<td>DFNTE</td>
<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
</tr>
<tr>
<td>EFNT</td>
<td>&gt;781</td>
<td>&gt;781</td>
<td>&gt;781</td>
<td>&gt;781</td>
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<tr>
<td>BFNTE</td>
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<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
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<tr>
<td><strong>MBC (µg/mL)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NTME</td>
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<td>25000</td>
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<tr>
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<td>&gt;2500</td>
<td>&gt;2500</td>
<td>&gt;2500</td>
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<tr>
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<tr>
<td>BFNTE</td>
<td>25000</td>
<td>25000</td>
<td>25000</td>
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<td>25000</td>
</tr>
</tbody>
</table>

NOR: norfloxacin, NTME: *N. tetragona* 50% methanol extract, EFNTE: ethyl acetate fraction of *N. tetragona* 50% methanol extract, DFNTE: dichloromethane fraction of *N. tetragona* 50% methanol extract, BFNTE: butanol fraction of *N. tetragona* 50% methanol extract. *Positive control.

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**3. Results**

3.1. **MIC and MBC of Nymphaea tetragona 50% Methanol Extract.** The antimicrobial activity of NTME and its solvent fractions were confirmed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against different strains of *S. typhimurium*. The results indicated that the EFNTE possessed the strongest antibacterial activity among all fractions. MBC of EFNTE against all tested *S. typhimurium* strains was ≤1562 µg/mL, whereas the MIC values were within 781 µg/mL for all strains except one clinical isolate (Table 2).

3.2. **Killing Rate of Salmonella typhimurium.** Time-kill curves of *S. typhimurium* after treatment with EFNTE are demonstrated in Figure 1. The EFNTE of both the 1 × MIC and 2 × MIC concentrations showed complete inhibition up to 8 hours and started their log phase from this time point. At 24 hours, the growth level was 2-fold lower in 1 × MIC and 3-fold lower in 2 × MIC of EFNTE than the growth control. At 0.25 × MIC and 0.5 × MIC, bacteria reached log phase after 1 hour and stationary phase after 8 hours whereas the control started stationary phase after the 4th hour. None of the tested concentrations showed complete killing effect within 24 h.

3.3. **In Vitro Synergy with Commercial Antimicrobials.** To investigate whether there is any synergy between EFNTE and commercial antibiotics, we examined six antibiotics with EFNTE by disc diffusion method. EFNTE exhibited synergism only with tylosin and streptomycin and has additive/indifferent effects with other antibiotics. There was no antagonistic effect observed in EFNTE with those antibiotics (Figure 2).

To reconfirm the synergistic activity of EFNTE with tylosin and streptomycin, we further performed checkerboard microdilution assay. The results of the checkerboard analysis are summarized in Tables 3 and 4 and Figure 3. An increased sensitivity against tylosin was observed in combination with EFNTE. The corresponding FICIs were ≤0.5 in tested strain, demonstrating a synergistic effect. However, EFNTE in combination with streptomycin showed additive and indifferent instead of synergistic interaction.

3.4. **Quorum Sensing Inhibition of NTME.** The inhibition diameter of *C. violaceum* pigment in presence of NTME and solvent fractions of NTME is presented in Table 5. *C. violaceum* showed sensitivity to all fractions including the crude extract. Although the crude extract and all fractions of extract significantly prevented the pigment, ethyl acetate and butanol fractions showed highest inhibition among all.
Figure 2: Double disk synergy of EFNTE with commercial antibiotics against *S. typhimurium* KCTC 2515. EAF: ethyl acetate fraction of *N. tetragona* 50% methanol extract; TMR: trimethoprim; NOR: norfloxacin; MAR: marbofloxacin; AMP: ampicillin; STR: streptomycin; and TYL: tylosin. Red arrows indicate synergistic interactions.

<table>
<thead>
<tr>
<th>MIC</th>
<th>IND</th>
<th>ADI</th>
<th>SYN</th>
<th>TYL 4096</th>
<th>TYL 2048</th>
<th>TYL 512</th>
<th>TYL 256</th>
<th>TYL 128</th>
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</tr>
<tr>
<td>EFNTE (µg/mL)</td>
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<td>3125</td>
<td>1563</td>
<td>781</td>
<td>391</td>
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<td>IND</td>
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</table>

Figure 3: Combination interaction of EFNTE with (a) tylosin and (b) streptomycin against *S. typhimurium* KCTC 2515 by checkerboard microdilution method. Ash colors indicate bacterial growth and without color zones are free of bacteria. EFNTE: ethyl acetate fraction of *N. tetragona* 50% methanol extract; TYL: tylosin; STR: streptomycin; SYN: synergistic effect; ADI: additive effect; IND: indifferent effect; MIC: minimum inhibitory concentration.

<table>
<thead>
<tr>
<th>MIC</th>
<th>IND</th>
<th>ADI</th>
<th>SYN</th>
<th>STR 128</th>
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<td></td>
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<td>STR 2</td>
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<td></td>
<td></td>
<td></td>
<td>MIC</td>
<td>0</td>
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</table>

Tetracycline demonstrated bactericidal, furanone confirmed pigment inhibitory effect, and the negative control had no activity.

3.5. Gas Liquid Chromatography Coupled Mass Spectrophotometric (GC-MS) Analysis. The major identified compounds with their biological activities are illustrated in Table 6 according to their elusion order. The major chemical compounds in DFNTE were mainly hydrocarbons (about 46.46%) and EFNTE contains methyl gallate (70.44%), 1, 2, 3-benzenetriol or pyrogallol (20.61%), and 6, 8-dimethylbenzocyclooctene (5.90%). The major compounds found in BFNTE were 2-hydrazinoquinoline (57.61%), pyrogallol (20.09%), and methyl gallate (12.77%). GC-MS chromatogram of EFNTE and chemical structures of methyl gallate and pyrogallol are presented in Figure 4. We have shown only the chromatogram of EFNTE and structures of those compounds as they are expected for desired effects and are abundant in EFNTE.

4. Discussion
There are recent reports indicating the resistance of several bacterial strains against different antibiotics that have been
Figure 4: Gas chromatography coupled with mass spectroscopy (GC-MS) chromatogram of EFNTE and chemical structure of major compounds.

Table 3: Fractional inhibitory concentration (FIC) and FIC index (FICI) of combination between tylosin and EFNTE.

<table>
<thead>
<tr>
<th>Tylosin</th>
<th>EFNTE</th>
<th>FICI</th>
<th>Interpretation</th>
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<tr>
<td>Con(^\circ) ((\mu)g/mL)</td>
<td>FIC of drug A</td>
<td>Con(^\circ) ((\mu)g/mL)</td>
<td>FIC of drug B (FIC of A + FIC of B)</td>
</tr>
<tr>
<td>1024</td>
<td>MIC of A</td>
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<tr>
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<tr>
<td>256</td>
<td>0.25</td>
<td>98</td>
<td>0.125</td>
</tr>
<tr>
<td>256</td>
<td>0.25</td>
<td>195</td>
<td>0.25</td>
</tr>
<tr>
<td>128</td>
<td>0.125</td>
<td>391</td>
<td>0.5</td>
</tr>
<tr>
<td>64</td>
<td>0.0625</td>
<td>391</td>
<td>0.5</td>
</tr>
<tr>
<td>0</td>
<td>MIC of B</td>
<td>781</td>
<td>MIC of B</td>
</tr>
</tbody>
</table>

EFNTE: ethyl acetate fraction of \(N.\) tetragona 50% methanol extract; Con\(^\circ\): concentration; drug A: tylosin; drug B: EFNTE; SYN: synergistic effect; ADI: additive effect; IND: indifferent effect; MIC: minimum inhibitory concentration. The FICI was interpreted as follows: synergistic effect (0 < FICI ≤ 0.5), additive effect (0.5 < FICI ≤ 1), and indifferent effect (1 < FICI ≤ 4).

Table 4: Fractional inhibitory concentration (FIC) and FIC index (FICI) of combination between streptomycin and EFNTE.

<table>
<thead>
<tr>
<th>Streptomycin</th>
<th>EFNTE</th>
<th>FICI</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con(^\circ) ((\mu)g/mL)</td>
<td>FIC of drug A</td>
<td>Con(^\circ) ((\mu)g/mL)</td>
<td>FIC of drug B (FIC of A + FIC of B)</td>
</tr>
<tr>
<td>32</td>
<td>MIC of A</td>
<td>0</td>
<td>MIC of A</td>
</tr>
<tr>
<td>32</td>
<td>1</td>
<td>6</td>
<td>0.0077</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>12</td>
<td>0.0154</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>24</td>
<td>0.0307</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>49</td>
<td>0.063</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>98</td>
<td>0.125</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>195</td>
<td>0.25</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>391</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>0.25</td>
<td>781</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0.125</td>
<td>781</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.0625</td>
<td>781</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>MIC of B</td>
<td>781</td>
<td>MIC of B</td>
</tr>
</tbody>
</table>

EFNTE: ethyl acetate fraction of \(N.\) tetragona 50% methanol extract; Con\(^\circ\): concentration; drug A: streptomycin; drug B: EFNTE; ADI: additive effect; IND: indifferent effect; MIC: minimum inhibitory concentration. The FICI was interpreted as follows: synergistic effect (0 < FICI ≤ 0.5), additive effect (0.5 < FICI ≤ 1), and indifferent effect (1 < FICI ≤ 4).
used in the treatment of infectious diseases of human and animals [45]. Thus, to combat infectious diseases associated with resistant pathogens, development of alternative antimicrobial drugs is urgent [46, 47]. The in vitro activity of EFNTE against resistance strains of *Salmonella typhimurium* (Table 1) reflects that the plant could be a good candidate as a source of active phytoconstituents to minimize the development of bacterial resistance and to ensure clinical cure of bacterial infection.

In the present study, MIC results of EFNTE exhibited antibacterial activity against all strains of *S. typhimurium* tested which have been shown to be resistant in one to six out of eight antibiotics (Table 1). The time-kill assay also exposed that the extract fraction effectively inhibited the growth of *S. typhimurium*. Utilities of EFNTE are again explored by the combination interactions with commercial antibiotics where it possessed synergistic effect with tylosin and additive and/or indifferent effects with other tested antibiotics. Quorum sensing inhibition activity of NTME and solvent fractions of the extract demonstrated varied level of effects statistically wherever EFNTE and BFNTE showed the greatest activity among all fractions and crude extract. Furthermore, the GC-MS analysis was performed to investigate possible components from the NTME for its antibacterial potential. This GC-MS analysis efficiently confirmed the existence of some major phenolic compounds (methyl gallate and pyrogallol) along with several other minor constituents.

MIC and MBC of NTME and its solvent fractions were studied against 5 strains of *S. typhimurium*. The results in Table 2 indicated that the EFNTE possessed the strongest antibacterial activity among all the fractions and crude extract. Considering the antibacterial activity, EFNTE was selected for further investigation. Extract having MIC values less than 8 mg/mL was believed as active crude extract [48]. Again, it was suggested to avoid the MIC greater than 1 mg/mL for crude extract and 0.1 mg/mL for isolated compounds. 0.1 mg/mL and 0.01 mg/mL MICs correspondingly for crude extract and isolated compounds would be very interesting activity [49]. Including all, MIC values less than 1 mg/mL were considered as good activity in the current study.

Time-kill curves have been used to determine whether the effects of EFNTE are either bacteriostatic or bactericidal and are useful for the evaluation of the pharmacodynamic characteristics of new antimicrobial agents [50]. According to our results, EFNTE shows bacteriostatic activity against the tested bacteria, since a reduction ≥99.9% of the inoculums was not observed compared to the growth control (Figure 1). At its 1 × MIC and 2 × MIC, EFNTE was able to inhibit *S. typhimurium* in the first 8 h of incubation. Then, 1 × MIC and 2 × MIC of EFNTE treated groups started their log phase. At the end of the incubation period (24 h), 3-fold reduced inoculums concentration in 2 × MIC of EFNTE and 2-fold lower inoculums concentration in 1 × MIC of EFNTE were achieved in contrast to control, indicating that EFNTE displays a bacteriostatic effect.

It is always suggested to treat bacterial infections with a combination of antimicrobial agents for the prevention of drug resistance development and to improve efficacy. Drug combinations having synergistic interactions are generally considered as more effective and, therefore, preferable [28]. Incidentally, EFNTE is proved to have synergistic effects with tylosin against *S. typhimurium*. The EFNTE also has additive and indifferent effects with streptomycin and other antibiotics tested against the same bacterial strain and has no antagonistic interactions with those antibiotics. Excellent in vitro activity combined with synergistic effects with other antibacterial drugs underscores the potential utility of EFNTE for the treatment of *Salmonella* associated infections.

In this study, NTME and its solvent fractions also possessed quorum sensing inhibition activity. EFNTE and BFNTE are considered as very sensitive or sensitive among them, whereas the crude extract and the rest of the fractions are sensitive according to the previous report [33]. Every fraction of extract may have many major and minor compounds with QS-inhibition activity which is reflected in the result. Furthermore, many compounds having antimicrobial activity were identified by the GC-MS analysis of DFNTE, EFNTE, and BFNTE. Methyl gallate, pyrogallol, and some hydrocarbons (hexacosan, heptacosan, octacosan, etc.) are important in response to desired type of effects and the proportion of those compounds in extracts. Pyrogallol is reported to have quorum sensing inhibition activity [36, 37] and antimicrobial activity [38] that is present in both EFNTE (20.61%) and BFNTE (22.09%). Methyl gallate is identified

Table 5: Quorum sensing inhibition activity (as pigment inhibition zone diameters) of *Nymphaea tetragona* 50% methanol extract and solvent fractions of the crude extract against *C. violaceum*.

<table>
<thead>
<tr>
<th>Solvent fraction/antibiotic</th>
<th>Concentration (µg/disk)</th>
<th>Pigment inhibition diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>16.33 ± 0.58</td>
</tr>
<tr>
<td>Furanone</td>
<td>100</td>
<td>16.67 ± 0.58</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>—</td>
<td>ND</td>
</tr>
<tr>
<td>NTME</td>
<td>600</td>
<td>13.67 ± 1.15 (c)</td>
</tr>
<tr>
<td>DFNTE</td>
<td>600</td>
<td>13.33 ± 0.58 (c)</td>
</tr>
<tr>
<td>EFNTE</td>
<td>600</td>
<td>14.67 ± 1.15 (b)</td>
</tr>
<tr>
<td>BFNTE</td>
<td>600</td>
<td>14.33 ± 1.53 (b)</td>
</tr>
</tbody>
</table>

NTME: *N. tetragona* 50% methanol extract, DFNTE: dichloromethane fraction of *N. tetragona* 50% methanol extract, EFNTE: ethyl acetate fraction of *N. tetragona* 50% methanol extract. Diameter of clear zone. ND: no activity detected. Data shown represent the mean ± SD of three replicates. Different alphabets indicate significant difference.
Table 6: Major compound list of three solvent fractions according to their contribution in respective solvent and compounds were listed followed by their elution order including reported activity.

<table>
<thead>
<tr>
<th>RI</th>
<th>% area</th>
<th>Compound</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.46</td>
<td>3.18</td>
<td>Dichloromethane fraction of <em>Nymphaea tetragona</em> 50% methanol extract</td>
<td>—</td>
<td>[35]</td>
</tr>
<tr>
<td>37.12</td>
<td>5.44</td>
<td>Heneicosan</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>38.71</td>
<td>7.9</td>
<td>Tetracosan</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>40.24</td>
<td>11.18</td>
<td>Hexacosan</td>
<td>Antimicrobial effects</td>
<td>[35]</td>
</tr>
<tr>
<td>41.71</td>
<td>10.57</td>
<td>Heptacosan</td>
<td>Antifungal activity</td>
<td>[35]</td>
</tr>
<tr>
<td>43.14</td>
<td>8.19</td>
<td>Octacosan</td>
<td>Antimicrobial effects</td>
<td>[35]</td>
</tr>
<tr>
<td>14.37</td>
<td>5.9</td>
<td>Ethyl acetate fraction of <em>Nymphaea tetragona</em> 50% methanol extract</td>
<td>- 6,8-Dimethylbenzocyclooctene —</td>
<td></td>
</tr>
<tr>
<td>15.79</td>
<td>20.61</td>
<td>Pyrogallol</td>
<td>Antibacterial, QS inhibition, and potent tyrosinase inhibitor.</td>
<td>[36–39]</td>
</tr>
<tr>
<td>16.79</td>
<td>1.31</td>
<td>1,4-Cyclohexanedicarboxylic acid, 2,5-dioxo-diethyl ester</td>
<td>Anticolon cancer.</td>
<td>[40]</td>
</tr>
<tr>
<td>26.90</td>
<td>1.39</td>
<td>1-[7'-Methylbenzofuran-2'-carbonyl]-3-ethylazulene</td>
<td>Derivatives have antibacterial, antifungal, anti-inflammatory, analgesic, antidepressant, anticonvulsant, antitumor, anti-HIV, antidiabetic, antitubercular activity.</td>
<td>[41, 42]</td>
</tr>
<tr>
<td>27.65</td>
<td>70.44</td>
<td>Methyl gallate</td>
<td>Antimicrobial</td>
<td>[43]</td>
</tr>
<tr>
<td>12.86</td>
<td>57.61</td>
<td>Butanol fraction of <em>Nymphaea tetragona</em> 50% methanol extract</td>
<td>2-Hydrazinoquinoline —</td>
<td></td>
</tr>
<tr>
<td>15.8</td>
<td>22.09</td>
<td>Pyrogallol</td>
<td>Antibacterial, QS inhibition, and potent tyrosinase inhibitor.</td>
<td>[36–39]</td>
</tr>
<tr>
<td>27.39</td>
<td>12.77</td>
<td>Methyl gallate</td>
<td>Antibacterial</td>
<td>[43, 44]</td>
</tr>
</tbody>
</table>
from EFNTE (70.44%) and BFNTE (12.77%) which is an antibacterial agent [43]. The scenario is now clear that methyl gallate and pyrogallol were contributing the vital role of the activity of EFNTE where they occupy about 91% of the total. The similar level of QS inhibition activity of EFNTE and BFNTE may be because of the presence of pyrogallol almost in equivalent amount in both fractions. The combined activity of those hydrocarbons or minor compounds present in DFNTE may possess the anti-QS activity.

This is the first study describing the combination interaction with commercial antibiotics and quorum sensing inhibition activity of *N. tetragona* extract. This study is also reporting the existence of methyl gallate and pyrogallol for the first time from this species. Together with all the promising *in vitro* assay findings, we believe that *N. tetragona* 50% methanol extract is expected to become a novel antimicrobial treatment for *Salmonella* infection of animals and human. Further study is needed to explore the mechanism of quorum sensing inhibition, antibacterial, and synergistic activity of the extract. *In vivo* activity and cytotoxic effects of the extract are also necessary to expose.

**Abbreviations**

NTME: *Nymphaea tetragona* 50% methanol extract  
DFNTE: Dichloromethane fraction of *Nymphaea tetragona* 50% methanol extract  
EFNTE: Ethyl acetate fraction of *Nymphaea tetragona* 50% methanol extract  
BFNTE: Butanol fraction of *Nymphaea tetragona* 50% methanol extract  
GC-MS: Gas chromatography-mass spectrometry.

**Conflict of Interests**

None of the authors have any conflict of interests to declare.

**Acknowledgments**

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**References**

Research Article

Cytotoxic Effect of Icaritin and Its Mechanisms in Inducing Apoptosis in Human Burkitt Lymphoma Cell Line

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Icaritin (ICT), a hydrolytic product of icariin from Epimedium genus, exhibits antitumor activities in several human solid-tumor and myeloid leukemia cells with extensive influence on various cell signal molecules, such as MAPKs being involved in cell proliferation and Bcl-2 participating in cell apoptosis. However, the effect of icaritin on Burkitt Lymphoma has not been elucidated. In the present study, we first screened the potential effect of icaritin on Burkitt lymphoma Raji and P3HR-1 cell lines and found that icaritin showed cytotoxicity in both cell lines. We further found that icaritin could significantly inhibit Raji cells proliferation with S-phase arrest of cell cycle and induced cell apoptosis accompanied by activation of caspase-8 and caspase-9 and cleavage of PARP. We also observed that icaritin was able to decrease Bcl-2 levels, thus shifting the Bcl-2/Bax ratio, and it could obviously reduce c-Myc, a specific molecular target in Burkitt lymphoma. Our findings demonstrated that icaritin showed cytotoxicity, inhibited cell growth, caused S arrest, and induced apoptosis in Burkitt lymphoma cells and provided a rationale for the further evaluation of icaritin for Burkitt lymphoma therapy.

1. Introduction

Burkitt lymphoma (BL) is a highly aggressive B-cell lymphoma with extremely short doubling time that presents usually in extranodal sites or as an acute leukemia. As sporadic BL, the incidence is 1-2% of all Non-Hodgkin’s lymphoma (NHL) and accounts for 30–50% of all childhood lymphoma in Western countries [1]. To the current knowledge, Burkitt lymphoma is a so-called double hit (DH) lymphoma that is defined by a chromosomal breakpoint affecting the MYC/8q24 locus in combination with another recurrent breakpoint, mainly a t(14;18)(q32;q21) involving BCL2. By the current standard regimens consists of cyclical intensive chemotherapy and aggressive intrathecal prophylaxis, Burkitt lymphoma have become curable in most patients. However, in high-risk relapsed patients with BL, the prognosis of BL is still very poor. Thus, further studies are needed to better define the therapy regime in older patients and in some relapsed or refractory cases and to elucidate novel anti-Burkitt lymphoma agents with fewer side effects. Icaritin is a hydrolytic product of icariin that is extracted from Epimedium brevicornum, which is a traditional Chinese medicine (TCM). Icaritin has showed significant antitumor activities with minor side effects in various neoplasms, such as inducing apoptosis in human endometrial cancer Hec1A cells [2], leading to cell growth inhibition, G1 arrest, and apoptosis of prostate cancer PC-3 cells [3], resulting in cell cycle arrest at the G2/M phase, apoptotic cell death [4] and enhancing radio sensitivity [5] in breast cancer cells, inhibiting growth [6], and reversing multidrug resistance [7] of hepatoma HepG2 cells. For hematopoietic neoplasms, Li and his team demonstrated the antitumor effect of icaritin in acute myeloid leukemia cells [8], and we reported that icaritin showed potent antileukemia activity on chronic myeloid leukemia in vitro and in vivo [9]. These results indicate that icaritin may possess broad-spectrum antitumor activities to
various malignancies including hematopoietic malignancies. However, there are still no reports on the effect of icaritin on lymphoid neoplasms. This study was aimed to illustrate the cytotoxic effects of icaritin on human Burkitt lymphoma cell lines, for example, Raji and P3HR-1. We have further explored the changes of apoptosis related proteins such as caspase-9, caspase-8, PARP, and the two critical “double hit” factors that are c-Myc and Bcl-2. Herein, our study demonstrated that icaritin showed cytotoxicity, inhibited the two critical factors, c-Myc and Bcl-2, in Burkitt lymphoma cells, and provided a rational for the further preclinical and clinical evaluation of icaritin for Burkitt lymphoma therapy.

2. Materials and Methods

2.1. Chemicals and Reagents. Icaritin with a purity of up to 99.5% was a gift from Dr. Meng-kun at Shen-ao Gene Company (Beijing, China). Icaritin was dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.22 μm filter (Millicon). A stock solution of 20 mM icaritin was kept at −20°C, and serial dilutions from 2 μM to 8 μM were freshly prepared in DMSO for each experiment. The final concentration of DMSO in the culture media was maintained at less than 0.1%, which had no significant effect on cell growth. The anti-Bcl-2 (sc-7382), anti-Bax (sc-7480), anti-c-Myc (sc-764), and anti-actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-cleaved-PARP (no. 9541), anti-caspase-9 (no. 9502), and anti-caspase-8 (no. 9746) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

2.2. Cell Lines. The two Burkitt lymphoma cell lines, Raji cells (ATCC: CCL-86) and P3HR-1 cells (ATCC: HTB-62), were cultured in RPMI 1640 (Hyclone) supplemented with 10% heat-inactivated newborn calf serum (NCS, Hyclone), 50 IU/mL penicillin, and 50 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. All experiments were performed using logarithmically growing cells (4–6 × 10⁵ cells/mL).

2.3. Cell Viability Assay. The in vitro toxicology assay was performed using the MTT based method. Briefly, cells (10⁴ cells/well) were seeded in 96-well microplates and were exposed to different concentrations of icaritin (0 μM, 2 μM, 4 μM, and 8 μM) for 24 h, 48 h, and 72 h, respectively. The MTT were added (0.5 mg/mL), and the cells were incubated for 4 h. After centrifugation for 10 min, the culture medium was discarded and formazan products were dissolved with 150 μL of DMSO for 5 min with shaking at room temperature. The absorbance was measured at 492 nm using a microplate reader. Three reduplicate wells were used for each concentration point, and experiments were repeated three times. IC50 values were determined by plotting a linear regression curve. The present cell viability was calculated as follows:

\[
\text{Cell viability (\%) = OD of treatment} \times 100/\text{OD of control} \quad (1)
\]

2.4. Cell Cycle and Apoptosis Assay. Raji cells were seeded in a 6-well plate and treated with a range of concentrations of icaritin (0 μM, 2 μM, 4 μM, 8 μM) for 48 h. After treatment, the cells were collected, washed twice with prechilled PBS, and fixed in 75% cold ethanol overnight. Ethanol-fixed cells were washed with PBS and incubated with 100 μg/mL of RNase A at 37°C for 30 min followed by 50 μg/mL of propidium iodide (PI) at room temperature for 30 min. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using the ModFit LT 3.0 software packages (Verity Software House, Topsham).

Cell apoptosis was detected with the Annexin V-FITC/PI Apoptosis Detection Kit (Keygen, Nanjing, China) according to the manufacturer’s instructions. Data acquisition was performed using a FACSCalibur flow cytometer and analyzed with CellQuest software.

2.5. Western Blot Assay. The treated cells were washed twice with cold PBS and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, Nantong, China). The cells lysates were quantified with the BCA Protein Assay Kit (Thermo, Rockford, USA) according to the manufacturer’s instructions. In total, 50 μg of each sample was electrophoresed using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond nitrocellulose membranes (Amersham, USA). After being washed briefly with TBS-T and blocked with 5% nonfat milk for 1 h, the membranes were washed and probed with the appropriate antibody overnight at 4°C with shaking. After washing with TBS-T, the membranes were incubated with 1:5,000 dilutions of the appropriate secondary antibody at room temperature for 1 h. The proteins were visualized using ECL chemiluminescence reagents (Thermo, Beijing, China) and exposed to Kodak X-ray films.

2.6. Statistical Analysis. Statistical analysis was carried out with the paired samples t-test. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. Icaritin Inhibited Proliferation of Burkitt Lymphoma Cell Lines. Previous studies have shown that icaritin inhibited the growth of various malignant cells [2–4, 6–9]. To determine whether icaritin inhibits the growth of Burkitt lymphoma cells, Raji and P3HR-1 cell lines were incubated with various concentrations of icaritin for 24, 48, or 72 h. The MTT assays showed that icaritin significantly inhibited the growth of both cell lines in a dose-dependent manner (Figure 1), which indicated that icaritin has antitumor activity on lymphoid malignancies such as Burkitt lymphoma. The IC50 values of icaritin on Raji cells for 24, 48, and 72 hours were 164.14 ± 112.94, 9.78 ± 1.85, and 3.6 ± 0.81 μM while the IC50 values on P3HR-1 cells for indicated time were 9204.11 ± 897.75,
1.2

0.8

0.6

0.4

0.2

0

24 48 72

Cell viability

Exposure to icaritin (hours)

0 𝜇 M

2 𝜇 M

4 𝜇 M

8 𝜇 M

Figure 1: Icaritin inhibited cell growth of Burkitt lymphoma cell lines. The Raji and P3HR-1 cells were treated with DMSO (0 𝜇 M icaritin) or the indicated concentrations of icaritin for the indicated time. The cells were harvested, and the cell viability was evaluated using the MTT assay. Data was expressed in mean ± SD from three independent experiments ($^* P < 0.05$, $^# P < 0.025$, and $^¥ P < 0.0005$ compared to control cells, 0 𝜇 M).

17.69 ± 0.03, and 9.72 ± 1.00 𝜇 M. The so great IC50 values of icaritin on both cell lines for 24 h demonstrated that icaritin inhibits Burkitt lymphoma cells in a time-dependent manner as well.

3.2. Icaritin Induced S-Phase Arrest in Raji Cells. Previous studies have shown that icaritin induces cell cycle arrest accompanied with decreased cell proliferation [3, 4, 9]. To investigate whether the inhibition of growth by icaritin in Burkitt lymphoma cells correlated with cell cycle arrest, we tested the cell cycle distribution of Raji cells that were treated with icaritin using flow cytometry. As shown in Figure 2, in Raji cells, icaritin increased the percentage of S-phase and reduced the population of G0/G1 phase without a concomitant increase in M phase, which indicated that icaritin mainly arrests cell cycle in S-phase, thus retarding cell growth.

3.3. Icaritin Induced Apoptosis in Raji Cells. In various present studies, icaritin induces cell apoptosis in many solid tumors [2–7, 10, 11] and myeloid original malignancies [8, 9]. To determine whether icaritin induces apoptosis in lymphoid malignant cells, Raji cells were treated with different concentrations of icaritin for 48 h and Annexin V-FITC and PI fluorescence assays were performed to evaluate necrotic cells as well as early stage and late stage apoptotic cells. The results showed a significant accumulation of the early and late stage apoptotic cells in icaritin treated Raji cells with dose-dependent manner (Figure 3). We then investigated whether the apoptosis induced by icaritin is associated with proteolytic activation of caspase-8 and caspase-9, two of the classic markers of mitochondrial apoptotic pathway. As shown in Figure 4, icaritin resulted in a significant accumulation of cleaved caspase-8 and cleaved caspase-9. The activation of caspases and cell apoptosis were further confirmed by assessing the cleavage of PARP, a substrate of activated caspases, in icaritin treated Raji cells. As shown in Figure 4, the 89 KDa cleaved fragment of PARP was identified. These results indicated that icaritin induced apoptosis in this lymphoid malignant cell line may be related to mitochondrial apoptotic pathway activation.

3.4. Icaritin Downregulated Expression of c-Myc and Bcl-2 in Raji Cells. Burkitt lymphoma is a so-called double-hit lymphoma that is defined by chromosomal breakpoint affecting the MYC/8q24 locus in combination with another recurrent transposition, mainly a t(14;18)(q32;q21) involving BCL2 [12]. The overexpression of MYC and BCL2 leads to enhanced proliferation [13] and reduced apoptosis [12] in transformed B cells. Moreover, the latest papers demonstrated the inhibition of c-Myc [8] and Bcl-2 [9] caused by icaritin in both acute and chronic myeloid leukemia. To probe the mechanism underlying icaritin-inhibiting growth of Burkitt lymphoma cells, the expression of c-Myc, Bcl-2, and Bax was examined by western blot. As shown in Figure 5, icaritin treatment resulted in the significant decrease of c-Myc and Bcl-2 protein and the increase of Bax protein in a dose-dependent way. These data indicated that icaritin was able to inhibit cell proliferation and induce apoptosis by influencing the two critical factors, c-myc and bcl-2, in Raji cells.

4. Discussion

In this study, we observed the antitumor effect of icaritin in Burkitt lymphoma cell lines, Raji and P3HR-1. Furthermore, we investigated the potential mechanisms in Raji cells. Our...
results showed that icaritin significantly inhibits the proliferation of the two Burkitt lymphoma cell lines in a dose- and time-dependent manner (Figure 1). Then we used Raji cells as a model and found that icaritin causes the accumulation of S-phase cells (Figure 2) and induces cell apoptosis (Figure 3) with activation of caspase-9 and caspase-8 (Figure 4). Finally, we demonstrate that icaritin decreases the two critical signal factors, c-Myc and Bcl-2 (Figure 5), which promote cell proliferation and survival in Burkitt lymphoma cell [12].

Previous studies have shown that icaritin possesses extensive antitumor activities on human solid and hematologic tumor cells including endometrial cancer Hec1A cells [2], prostate cancer PC-3 cells [3], breast cancer MCF-7 and MDA-MB-453 cells [4], hepatoma HepG2 cells [6], renal carcinoma 786-0 cells [11], chronic and acute myeloid leukemia primary cells, and cell lines [8, 9]. However, the report about antitumor activities of icaritin in lymphoid malignancies is still not available. Hence, we, for the first time, demonstrated that icaritin has the potential against Burkitt lymphoma by inhibiting cell proliferation and inducing cell apoptosis.

It is well established that cell cycle is linked to cell proliferation and apoptosis [14, 15]. As a novel anticancer agent, icaritin induces cell death that is accompanied by cell cycle arrest in different phases in various malignancies: mammary cancer MCF-7 cells in G2/M phase [4], prostate cancer PC-3 cells in G1 phase [3], chronic myeloid leukemia K562 cells in G1 phase [9], and acute myeloid leukemia cell lines in S-phase [8]. In this study, we found that icaritin arrests Raji cells in S-phase in a dose-dependent manner (Figure 2). Apparently, the effect of S-phase arrest of icaritin is contributed to the growth inhibition of Raji cells caused by it. As to why icaritin results in different cell phase arrest in various malignant cell lines with the similar inhibition of cell growth, this may be related to the various cell internal environments in different malignant cells.

Notably, icaritin may potently trigger apoptosis in Raji cells that had been treated for 48 h. The phenomenon that icaritin just caused a significant accumulation of Annexin V positive but PI negative cells at 48 h, which indicated that the cell membrane is still undamaged (Figure 3), could elucidate the absence of sub-G1 phase cells at 48 h (Figure 2). To investigate the way by which icaritin prompts apoptosis in Raji cells, we detected and found that icaritin induces the activation of caspase-8, caspase-9 and the cleavage of

**Figure 2:** Icaritin induced S-phase arrest in Raji cells. (a) The cells were treated with DMSO or the indicated concentrations of icaritin for 48 h. The cells were evaluated using PI staining and flow cytometric analysis. The experiments were repeated, and the data from representative experiments are shown. (b) The distribution of cell cycles with the mean ± SD is shown (*P < 0.025; **P < 0.005 compared to control cells, 0 μM).
PARP, which are similar to the report of Tong et al. [2] and Huang et al. [3]. It has been documented that mitochondria play a pivotal role in the signal transduction of apoptosis [16]. The activation of caspase-9 and caspase-3 and subsequent cleavage of PARP and release of cytochrome c from mitochondria imply the activation of mitochondrial-mediated caspases cascade pathway [17]. So, the activation of these proteins that we detected in the pathway suggests that icaritin induced apoptosis in Raji cells might be related to mitochondrial-mediated caspases pathway.

As one of the double hit lymphoma, Burkitt lymphoma cells obtain two critical uncontrolled genes: BCL2 and MYC [12], which make the malignant cells survive and proliferate out of control. Therefore, agents that target one or both of the two factors are able to induce apoptosis and are considered to be the potential drugs that can be used to treat Burkitt lymphoma [12, 18, 19]. Moreover, icaritin shows the ability to decrease the level of Bcl-2 and c-Myc proteins in several studies [2, 4, 8, 9]. To understand the influence of icaritin on the two crucial factors in Burkitt lymphoma cells, we...
Figure 4: Icaritin activated apoptotic pathways in Raji cells. (a) Icaritin treatment induced the cleavage of caspase-8, caspase-9, and PARP. The cells were treated with DMSO or different concentrations of icaritin for 48 h. The cell lysates were subjected to western blot analysis with antibodies against cleaved PARP, caspase-8, and caspase-9. \( \beta \)-actin was used as a loading control. The experiments were repeated and the data from representative experiments are shown. (b) Expression level of each protein was estimated by densitometry and presented as a ratio to the loading control \( \beta \)-actin.

Figure 5: Icaritin reduced c-Myc and Bcl-2 while it increased Bax in Raji cells. (a) The cells were treated with DMSO or different concentrations of icaritin for 48 h, and the cell lysates were subjected to western blot analysis with antibody against c-Myc, Bcl-2, and Bax. \( \beta \)-actin was used as a loading control. The experiments were repeated and the data from representative experiments are shown. (b) Expression level of each protein was estimated by densitometry and presented as a ratio to the loading control \( \beta \)-actin.
detected them with western blot and found that both of them were downregulated by icaritin in dose-dependent manner (Figure 5). Thus, though the more imperative exploration is needed, these data that we obtained have suggested that Bcl-2 and c-Myc were involved in the inhibition of proliferation and survival caused by icaritin, especially in the DH lymphomas.

5. Conclusions

In conclusion, to our knowledge, we have reported for the first time that icaritin shows antitumor effect in lymphoid malignant cell lines. Our experimental results have shown that icaritin is able to inhibit cell growth and induce apoptosis in Burkitt lymphoma cell lines. The underlying mechanisms of icaritin antilymphoma may be related to inhibition of bcl-2 and c-myc. However, considering that the pan influence of icaritin on MAPK/ERK/JNK and JAD2/STAT3/AKT signals has been reported in various tumors, further researches in more lymphoid malignancies and more in-depth experiments remain needed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

This study explored the antioxidant and immunomodulatory potential of ethnomedicinally valuable species, namely, *Arisaema jacquemontii* of north-western Himalayan region. The tubers, leaves, and fruits of this plant were subjected to extraction using different solvents. *In vitro* antioxidant studies were performed in terms of chelation power on ferrous ions and FRAP assay. The crude methanol extract of leaves was found to harbour better chelating capacity (58% at 100 μg/mL) and reducing power (FRAP value 1085.4 ± 0.11 μMFe³⁺/g dry wt.) than all the other extracts. The crude methanol extract was thus further partitioned with solvents to yield five fractions. Antioxidant study of fractions suggested that the methanol fraction possessed significant chelation capacity (49.7% at 100 μg/mL) and reducing power with FRAP value of 1435.4 μM/g dry wt. The fractions were also studied for immune modulating potential where it was observed that hexane fraction had significant suppressive effect on mitogen induced T-cell and B-cell proliferation and remarkable stimulating effect on humoral response by 141% and on DTH response by 168% in immunesuppressed mice as compared to the controls. Therefore, it can be concluded that *A. jacquemontii* leaves hold considerable antioxidant and immunomodulating potential and they can be explored further for the identification of their chemical composition for a better understanding of their biological activities.

1. Introduction

Natural products from plants have gathered significant attention due to the recent evidences suggesting their capacity to ameliorate oxidative stress and other related diseases. Plant secondary metabolites such as phenolics, flavonoids, and terpenoids play an important role as natural antioxidants and immunomodulators [1]. In biological systems, it is often considered that reactive oxygen species (ROS) originate from the interaction of iron with enzymatically and/or nonenzymatically generated superoxide (O₂⁻, Haber-Weiss reaction) and/or hydrogen peroxide (H₂O₂, Fenton reaction) [2]. The human body harbours enzymatic defence mechanisms to counter oxidative stress induced by free radicals which involve superoxide dismutase, catalase, and glutathione peroxidase. Apart from innate defence system, synthetic drugs are also available which improve the capacity of the body to counter oxidative stress and other diseases. But owing to the harmful side effects of synthetic drugs, research on natural products has taken a leap in recent years.

Phytochemicals have also proved to enhance immunity by modulating the innate and adaptive immune responses. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defence mechanism has to be activated under conditions of impaired immune response or when a selective immunosuppression is desired in situations such as autoimmune disorders. Several types of immunomodulators have been identified, including substances isolated and purified from natural sources such as plants including microorganisms. Polyphenolic compounds such as phenolic acids, flavonoids, anthocyanidins, and tannins, produced as secondary metabolites by plants, possess remarkable antioxidant and immunomodulatory activities [3].
The genus *Arisaema* (commonly known as “Cobra Lilies”) is made up of more than 250 herbaceous species, which are distributed throughout temperate to tropical areas. *Arisaema* sp. are traditionally documented and are known to be used by the indigenous people of India and China as antinematodal, anti-inflammatory, analgesic, and antidote and also for treating rheumatoid arthritis [4–7]. *A. heterophyllum*, *A. penisulae*, *A. robustum*, *A. consanguineum*, and *A. japonicum* were frequently used in Chinese herbal medicine as an anticonvulsant [8]. Rhizome of *A. jacquemontii* ground with edible oil forms a paste which is used for massage to regain muscular strength and in skin problems such as blisters and pimples [9].

*Arisaema jacquemontii* is commonly found in the Himalayan forests at an altitude of 2,300–4,300 m. It also occurs in the Nilgiri Hills in southern India and the Khasi Hills region of north-east India. This plant has not been scientifically evaluated for its antioxidant and immunomodulatory potential. Owing to the lack of availability of substantial reports, the present study was planned to explore the biological potential of this traditionally documented plant.

2. Materials and Methods

2.1. Preparation of Extracts and Fractions. *A. jacquemontii* was collected from high altitude (2500–3500 m) from north-western Himalayan region and was identified by the taxonomist of University of Jammu. The leaves, fruits, and tubers were dried separately and grounded in a blender to make fine powder. A total of 100 g powder of each of the leaves, fruits, and tubers were extracted with 500 mL of six different solvents, that is, methanol (AJL1), water (AJL2), and chloroform (AJL3). Methanol and chloroform extracts were obtained by continuous stirring at room temperature for 6 h whereas water extract was prepared at 60 °C overnight. This treatment was repeated thrice and the extracts were pooled, filtered, and evaporated using rotary vacuum evaporator. Active crude extract was further sequentially partitioned with different solvents like hexane (AJL1-H), chloroform (AJL1-C), ethyl acetate (AJL1-E), acetone (AJL1-A), and methanol (AJL1-M) to obtain fractions (Figure 3). All the fractions were freeze-dried using rotary vacuum evaporator and lyophilized.

2.2. Antioxidant Studies

2.2.1. Chelation Power on Ferrous (Fe^{2+}) Ions. The chelating effect on ferrous ions of the prepared extracts was estimated by the method of Dinis with slight modifications [10]. Briefly, 100 μL of each test sample (1 mg/mL) was taken and raised to 3 mL with methanol. 740 μL of methanol was added to 20 μL of 2 mM FeCl_{2}. The reaction was initiated by the addition of 40 μL of 5 mM ferrozine into the mixture, which was then left at room temperature for 10 min and then the absorbance of the mixture was determined at 562 nm.

(i) Ferric Ion Reducing Antioxidant Power (FRAP Assay). FRAP activity was measured according to the method of Benzie and Strain [11]. Briefly, acetate buffer (300 mM, pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine) 10 mM in 40 mM HCl and FeCl_{3}·6H_{2}O (20 mM) were mixed in the ratio of 10:1:1 to obtain the working FRAP reagent. Test sample (0.5 mL) was mixed with 3 mL of working FRAP reagent and absorbance was measured at 593 nm after vortexing. Methanol solutions of FeSO_{4}·7H_{2}O ranging from 100 to 2000 μM were prepared and used for the preparation of the calibration curve of known Fe^{2+} concentration. The parameter equivalent concentration was defined as the concentration of antioxidant having a Ferric-TPTZ reducing ability equivalent to that of 1 mM FeSO_{4}·7H_{2}O.

2.3. Acute Toxicity Study. Acute toxicity study of fractions was conducted as per OECD guidelines 420 (fixed dose procedure) using Swiss mice. Each animal was administered test samples at a dose of 2000 mg/kg by oral route. The animals were observed for any changes continuously for the first 4 h and up to 24 h for any mortality. The animals were then kept for 14 days to observe daily cage side observations and mortality [12].

2.4. Immunomodulatory Studies

2.4.1. Mitogen Activity Test by MTT Assay. Mitogen activity test was based on the method described by Mosmann [13]. Animals (balb/c mice, 18–22 g) were sacrificed; their spleens were removed in sterile conditions. A single cell suspension was prepared in 5 mL of incomplete RPMI. The cell suspension was centrifuged at 1200 rpm for 10 min and supernatant was discarded. RBCs were lysed by Trisammonium chloride treatment. The cells were centrifuged at 1200 rpm for 10 min, after centrifuging, supernatant was discarded and cell pellet was resuspended in complete RPMI. The viability of cells was checked with trypan blue. 1 × 10^6 cells/mL suspension was prepared and 100 mL of it was poured in each well of 96-well microtiter plate. An aliquot of 50 mL of standard mitogens, that is, Concanavalin A (Con A) (1/4) 10 mg/mL and Lipopolysaccharide (LPS) (1/4) 10 mg/mL, and test materials were added according to the experimental setup. The extracts in different concentrations (10^{-4}, 10^{-5}, and 10^{-6} M) were dissolved in DMSO and added to each well of flat bottom microtiter 96-well plate. Plates were placed on a shaker for 5 min. The plates were incubated for 48 h in CO_{2} incubator (37°C, 5% CO_{2}, and 90% relative humidity). After 48 h of incubation, plates were taken out from the CO_{2} incubator and reading was taken on ELISA plate reader at 540 nm. Thereafter, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well. The contents were placed on a shaker for 5 min and plates were incubated for 4–6 h in CO_{2} incubator (37°C, 5% CO_{2}, and 90% relative humidity) to allow the MTT to be metabolized. After incubation, the plates were inverted on a paper towel to remove the medium. The formazan crystals (MTT byproduct) were resuspended in 100 mL DMSO and reading was measured at a wavelength of 570 nm.

2.4.2. Effect on Humoral and Cellular Response in Immune Suppressed Mice. Swiss albino mice (*Mus musculus*) 10–12
The Delayed Type Hypersensitivity Response (DTH).

Saline alone served as a control. Dilution causing haemagglutination was taken as a titre. BSA by Nelson and Mildenhall [14]. The value of the highest serum determined following the microtitration technique described mary antibody titre. Hemagglutination antibody titres were samples were collected on day +7 (before challenge) for primary challenge at the required time schedule.

(1) Antigen (SRBC). Fresh sheep red blood cells (SRBC) collected aseptically from jugular vein of sheep were stored in cold sterile Alsever’s solution, washed three times with pyrogen free sterile normal saline (0.9% NaCl w/v), and adjusted to a concentration of $5 \times 10^8$ cells/mL for immunization and challenge at the required time schedule.

(2) Humoral Antibody Response (Hab). Groups of six mice each were immunized by injecting 0.2 mL of $5 \times 10^8$ SRBC/mL intraperitoneally (i.p.) on day 0 and challenged 7 days later by injecting an equal volume of SRBC i.p. Blood samples were collected on day +7 (before challenge) for primary antibody titre. Hemagglutination antibody titres were determined following the microtitration technique described by Nelson and Mildenhall [14]. The value of the highest serum dilution causing haemagglutination was taken as a titre. BSA saline alone served as a control.

(3) Delayed Type Hypersensitivity Response (DTH). The method of Doherty was followed to assess SRBC induced DTH response in mice [15]. Mice were immunized by injecting 20 μL of $5 \times 10^8$ SRBC/mL subcutaneously into the right hind foot pad. Seven days later, the thickness of the left hind foot was measured with a spherimicrometer (0.01 mm pitch) and was considered as a control. These mice were then challenged by injecting the same amount of SRBC intradermally into the left hind foot pad. The foot thickness was measured again at 0, 4, and 24 hr after challenge.

2.5. Phytochemical Analysis. The fractions obtained from the crude methanol extract of leaves were also investigated for the presence of secondary metabolites like terpenoids, coumarins, glycosides, quinones, saponins, tannins, anthraquinones, alkaloids, phenols, and flavonoids.

2.6. Statistical Analysis. All experiments were carried out in triplicate. Data values are expressed as mean ± standard deviation.

A. jacquemontii

<table>
<thead>
<tr>
<th>Extracts</th>
<th>A. jacquemontii Chelation power on ferrous ions (% at 100 μg/mL)</th>
<th>FRAP (μM/g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tuber</td>
<td>Leaves</td>
</tr>
<tr>
<td>Methanol (AJL1)</td>
<td>12</td>
<td>58</td>
</tr>
<tr>
<td>Aqueous (AJL2)</td>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>Chloroform (AJL3)</td>
<td>13</td>
<td>32</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation. na: not active.

weeks old, with 20–25 g body weight, and male Charles Foster rats (Rattus norvegicus) 10–12 weeks, with 100–150 g body weight, in groups of six were employed for study. In every experiment, one group of animals was used as a vehicle control while another received a standard drug Azathioprine (Aza). The test sample was freshly prepared as a homogenised suspension in 1% w/v acacia gum administered orally daily once a day for the duration of the experiment.

Diverse species of Arisaema hold ethnomedicinal importance in different areas of Asian subcontinent for treating various medical ailments. The rhizomes or tubers of A. calcarum, A. serratum, A.asperatum, A. heterophyllum, and A. amurense are used as analgesic, antitumor, and pesticide agents in traditional Chinese medicine [16]. Chinese herbal traditional medicine system uses A. cumbilis for treating dementia and neurological symptoms [17]. Later on, studies explained that A. cumbilis inhibits the production of proinflammatory cytokines including interleukin (IL-)1β, IL-6, and tumor necrosis factor (TNF-) α [18]. Antihypertrophic cerebrosides have also been isolated from A. amurense [8]. A. erubescens is a widely distributed species in China and it is used as a medicinal herb against damp phlegm, convulsions, and swelling [19]. Pharmacological study has also proved that this species harbors anticonvulsant and anticancer effects [20]. Paenoll, a phenolic compound which possesses antimutagenic, anticonvulsant, and anti-inflammatory activities, has been isolated from A. erubescens [21].

However, sufficient scientific data regarding the biological potential of A. jacquemontii is still lacking. In view of this fact, our study focussed on unearthing the antioxidant and immunomodulating potential of A. jacquemontii, which is found in abundance in the high altitude forests of Himalayas. Extracts of each of tubers, leaves, and fruits of A. jacquemontii were prepared in chloroform, methanol, and water and tested for antioxidant potential by taking in vitro chemical assays, mainly chelation power on ferrous ions and ferric ion reducing antioxidant power (FRAP).

Most reactive oxygen species (ROS) are generated as by-products during mitochondrial electron transport and other metabolic reactions. In addition, ROS are formed as necessary intermediates of metal catalyzed oxidation reactions. The transition metal ion Fe$^{2+}$ possesses the ability to perpetuate the formation of free radicals by gain or loss of electrons. Therefore, the reduction of the formation of reactive oxygen species can be achieved by the chelation of metal ions with chelating agents. Chelation power assay was carried out to assess the chelation capacity of the crude extracts which illustrated that the crude methanol extract of leaves of A. jacquemontii possessed remarkable chelation power at 100 μg/mL (58%) as compared to tuber (12%) and fruit extracts (34%) (Table 1). However, the aqueous and chloroform extracts of tubers, leaves, and fruits showed
negligible activity. Therefore, active crude methanol extract of leaves was subjected to sequential fractionation yielding five fractions, that is, hexane (AJL1-H), chloroform (AJL1-C), ethyl acetate (AJL1-E), acetone (AJL1-A), and methanol (AJL1-M). Chelation power of fractions was also analysed and it was observed that the methanol fraction exhibited significant capacity to chelate ferrous ions in comparison to other fractions with the value of 49.7% at 100μg/mL (Figure 1). Excess of metal ions can lead to various anomalies in the body. The iron (II) chelating activity of plant extracts is of great significance, because it has been proposed that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders like Alzheimer’s and Parkinson’s diseases [22]. Also, chelation therapy is a common practice of neutralising iron overload in the body especially in cases of treatment of Thalassemia and other anemias [23]. The current scenario suggests that the chelation therapy makes use of synthetic compounds which have certain side effects as well. Therefore, chelation of metal ions by natural phytochemicals from Arisaema sp. can prove to be of therapeutic importance.

Another assay, that is, ferric reducing antioxidant power (FRAP), was conducted on all the extracts and fractions of A. Jacquemontii to confirm its antioxidant potential. In this assay, reduction of ferric tripyridyl triazine (Fe³⁺-TPTZ) complex to ferrous form which has an intense blue colour can be monitored by measuring the change in absorption at 593 nm. The results of this experiment were similar to that of chelation power assay; that is, crude methanol extract of A. jacquemontii leaves showed noteworthy FRAP activity with reducing the value of 1085.4 ± 0.11 μM/g dry wt. However, tuber and fruit methanol extracts possessed very low FRAP activity with values of 37 ± 0.021 μM/g dry wt. and 635.4 ± 0.032μM/g dry wt., respectively (Table 1). Also, further FRAP analysis of fractions showed that methanol fraction (AJL1-M) possessed better reducing power with FRAP value of 1435.4 μM/g dry wt. in comparison to other fractions (Figure 2).

Antioxidant studies suggested that the crude methanol extract of leaves (AJL1) and its subsequent methanol fraction (AJL1-M) possessed promising chelating and reducing antioxidant power as compared to all the other extracts of tubers and fruits. All the fractions were also studied for the effects of acute toxicity and behavioural changes using Swiss mice as per OECD guidelines 420 (Fixed dose procedure). It was observed that there was no mortality and noticeable behavioural changes in treated animals as compared to control animals. All the fractions were found to be safe up to 2000 mg/kg body weight p.o.

Many medicinal plants are a rich source of substances which induce para-immunity, non-specific activation of
granulocytes, macrophages, natural killer cells and the comple-ment system. Confirming the safety levels of all the fractions by acute toxicity study in mice, they were further analysed for immune modulating potential by studying the effect on Con A and LPS induced murine lymphocyte proliferation (MTT assay). Concanavalin A and Lipopolysaccharide (LPS) are the mitogens which activate T-cell and B-cell proliferation, respectively, in the splenocytes. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created which can then be quantified using a simple colorimetric assay [13]. Hexane (AJL1-H) and ethyl acetate fraction (AJL1-E) showed significant suppressive effect on LPS induced B-cell proliferation and moderate effect on Con-A induced T-cell proliferation. However, methanol fraction (AJL1-M) also depicted considerable immune suppression on both B-cell and T-cell proliferation (Table 2). Immune suppression finds application in the treatment of autoimmune disorders such as rheumatoid arthritis and multiple sclerosis [24].

Furthermore, the effect of fractions on humoral and cell mediated immune response in immune suppressed mice was also studied (Table 3). The results after 7 days of oral administration of fractions to the immune suppressed mice showed some interesting results. The hexane fraction (AJL1-H) was observed to enhance the humoral and cell-mediated immune response in immune suppressed mice (Table 3). The humoral antibody titre at the concentration of 100 mg/mL was observed to be 141% which was more than that of standard drug, Levamisole (133%). Also, AJL1-H showed noteworthy positive DTH response at all three concentrations of 100 mg/mL (168%), 50 mg/mL (100%), and 25 mg/mL (128%). Also, acetone fraction (AJL1-A) showed moderate humoral and DTH stimulating responses at concentration of 100 mg/mL and 50 mg/mL and methanol fraction (AJL1-M) demonstrated immune stimulating activity only at concentration of 25 mg/mL (Table 3). The other fractions were not found to be much effective as immune stimulants. Therefore, it can be deduced from the study that hexane fraction of A. jacquemontii leaves (AJL1-H) possesses significant immune stimulating potential as it showed potent abrogative effect on humoral antibody response and delayed type hypersensitivity response in immune suppressed balb/c mice and these observations are suggestive of possible therapeutic usefulness in immune compromised patients.

The phytochemical analysis revealed that all the fractions contain high amount of terpenoids. Coumarins, quinones, and glycosides were present in moderate amount (Table 4). Alkaloids, anthraquinones, and flavonoids were found in low quantity and phenols were detected in moderate amount only in acetone and methanol fractions. This was a preliminary analysis of the phytoconstituents present in the fractions. However, the phytochemicals responsible for the antioxidant and immune modulating potential of A. jacquemontii leaves still need to be identified.

### Table 2: Effect of different concentrations of fractions of crude methanol extract of A. jacquemontii leaves on Con A and LPS induced murine lymphocyte proliferation.

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Samples</th>
<th>Conc. (M)</th>
<th>Con A mean ± S.E.</th>
<th>Con A induced T-cell proliferation rate (%)</th>
<th>LPS mean ± S.E.</th>
<th>LPS induced B-cell proliferation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Control</td>
<td>—</td>
<td>—</td>
<td>0.75 ± 0.50</td>
<td>—</td>
<td>1.92 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td>(2) AJL1-H</td>
<td>10^4</td>
<td>0.34 ± 0.04</td>
<td>−54.66</td>
<td>0.39 ± 0.01</td>
<td>−79.68</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10^5</td>
<td>0.76 ± 0.03</td>
<td>+1.33</td>
<td>0.44 ± 0.01</td>
<td>−77.08</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>0.58 ± 0.02</td>
<td>−22.66</td>
<td>0.60 ± 0.01</td>
<td>−68.75</td>
<td>−</td>
</tr>
<tr>
<td>(3) AJL1-C</td>
<td>10^4</td>
<td>0.69 ± 0.12</td>
<td>−22.47</td>
<td>0.82 ± 0.17</td>
<td>−16.32</td>
<td>−</td>
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<tr>
<td></td>
<td>10^5</td>
<td>0.81 ± 0.08</td>
<td>−8.98</td>
<td>0.85 ± 0.06</td>
<td>−13.26</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>0.82 ± 0.14</td>
<td>−7.86</td>
<td>0.83 ± 0.09</td>
<td>−15.30</td>
<td>−</td>
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<tr>
<td>(4) AJL1-E</td>
<td>10^4</td>
<td>0.60 ± 0.11</td>
<td>−20.00</td>
<td>0.36 ± 0.01</td>
<td>−81.25</td>
<td>−</td>
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<tr>
<td></td>
<td>10^5</td>
<td>0.54 ± 0.03</td>
<td>−28.00</td>
<td>0.48 ± 0.01</td>
<td>−75.00</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>0.71 ± 0.00</td>
<td>−5.33</td>
<td>0.59 ± 0.01</td>
<td>−69.27</td>
<td>−</td>
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<tr>
<td>(5) AJL1-A</td>
<td>10^4</td>
<td>0.43 ± 0.02</td>
<td>−42.66</td>
<td>0.54 ± 0.30</td>
<td>−72.21</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>10^5</td>
<td>0.71 ± 0.05</td>
<td>−5.33</td>
<td>0.62 ± 0.30</td>
<td>−67.75</td>
<td>−</td>
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<tr>
<td></td>
<td>10^6</td>
<td>0.64 ± 0.03</td>
<td>−14.66</td>
<td>0.60 ± 0.17</td>
<td>−68.12</td>
<td>−</td>
</tr>
<tr>
<td>(6) AJL1-M</td>
<td>10^4</td>
<td>0.59 ± 0.14</td>
<td>−33.70</td>
<td>0.48 ± 0.05</td>
<td>−75.00</td>
<td>−</td>
</tr>
<tr>
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<td>10^5</td>
<td>0.70 ± 0.01</td>
<td>−21.34</td>
<td>0.52 ± 0.04</td>
<td>−72.91</td>
<td>−</td>
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<td>0.59 ± 0.14</td>
<td>−33.70</td>
<td>0.55 ± 0.09</td>
<td>−71.35</td>
<td>−</td>
</tr>
</tbody>
</table>

+ indicates immune stimulant agents, while − indicates immunosuppressive agents. Results are mean standard error (SE) of three separate experiments. The bold values are shown for those compounds which have proved to be active and those in normal font represent the least significant.
Table 3: Effect of active methanol fraction of *A. jacquemontii* leaves on humoral and cell mediated immune response.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. mg/kg p.o.</th>
<th>Antibody titre Mean ± S.E.</th>
<th>% Activity</th>
<th>DTH Mean ± S.E.</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>6.5 ± 0.21</td>
<td>—</td>
<td>0.80 ± 0.16</td>
<td>—</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>200</td>
<td>4.5 ± 0.21</td>
<td>−30.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>0.35 ± 0.10</td>
<td>−56.25</td>
</tr>
<tr>
<td>Levamisole</td>
<td>2.5</td>
<td>7.16 ± 0.22</td>
<td>+133</td>
<td>1.11 ± 0.22</td>
<td>+168</td>
</tr>
<tr>
<td><strong>AJL1-H (mg/mL)</strong></td>
<td>100</td>
<td>7.33 ± 0.21</td>
<td>+141</td>
<td>1.11 ± 0.22</td>
<td>+168</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.83 ± 0.16</td>
<td>+67</td>
<td>0.80 ± 0.16</td>
<td>+100</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.83 ± 0.16</td>
<td>+33</td>
<td>0.91 ± 0.16</td>
<td>+124</td>
</tr>
<tr>
<td><strong>AJL1-C (mg/mL)</strong></td>
<td>100</td>
<td>5.83 ± 0.16</td>
<td>+67</td>
<td>0.48 ± 0.21</td>
<td>−29</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.66 ± 0.21</td>
<td>+8</td>
<td>0.45 ± 0.22</td>
<td>+23</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5.16 ± 0.16</td>
<td>+33</td>
<td>0.60 ± 0.20</td>
<td>+56</td>
</tr>
<tr>
<td><strong>AJL1-E (mg/mL)</strong></td>
<td>100</td>
<td>5.66 ± 0.21</td>
<td>+58</td>
<td>0.63 ± 0.21</td>
<td>+63</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.16 ± 0.16</td>
<td>+33</td>
<td>0.41 ± 0.16</td>
<td>+14</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5.33 ± 0.21</td>
<td>+42</td>
<td>0.50 ± 0.22</td>
<td>+34</td>
</tr>
<tr>
<td><strong>AJL1-A (mg/mL)</strong></td>
<td>100</td>
<td>6.33 ± 0.21</td>
<td>+91</td>
<td>0.80 ± 0.16</td>
<td>+100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.16 ± 0.40</td>
<td>+83</td>
<td>0.83 ± 0.16</td>
<td>+106</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5.33 ± 0.21</td>
<td>+42</td>
<td>0.53 ± 0.21</td>
<td>+40</td>
</tr>
<tr>
<td><strong>AJL1-M (mg/mL)</strong></td>
<td>100</td>
<td>5.16 ± 0.16</td>
<td>+33</td>
<td>0.58 ± 0.16</td>
<td>+52</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.16 ± 0.16</td>
<td>−17</td>
<td>0.38 ± 0.22</td>
<td>−7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>6.5 ± 0.22</td>
<td>+100</td>
<td>0.93 ± 0.21</td>
<td>+128</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation.

Table 4: Preliminary phytochemical analysis of fractions of crude methanol extract of *A. jacquemontii* leaves.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Hexane (AJL1-H)</th>
<th>Chloroform (AJL1-C)</th>
<th>Fractions</th>
<th>Ethyl acetate (AJL1-E)</th>
<th>Acetone (AJL1-A)</th>
<th>Methanol (AJL1-M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpenoids</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinones</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Glycosides</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tannins</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Phenols</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

+++ indicates high amount; ++ indicates moderate amount; + indicates low amount; − indicates absence.

4. Conclusion

This work is an attempt to identify the biological potential of *A. jacquemontii* growing in the Himalayan region for the first time. The methanol fraction obtained from crude methanol extract of leaves was observed to harbour considerable antioxidant potential and, in case of immune modulating studies, the hexane fraction was observed to show remarkable immune suppressive as well as immune stimulating potential, which could prove to be of immense value in autoimmune diseases as well as in immune compromised patients. However, the phytoconstituents responsible for their significant activity are still unknown. The results obtained in this work provide the basis for designing future experimentation on this species for better understanding of its antioxidative and immunomodulatory system and discovering the phytochemicals responsible for these properties.
Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments
This work was supported by a research grant from the University Grants Commission, New Delhi, India. The authors extend their regards to Dr. Harish Dutt (Assistant Professor, Department of Botany, University of Jammu) for his guidance. They would also like to acknowledge the School of Biotechnology and Department of Bioinformatics, University of Jammu, for their support.

References
Research Article

In Vitro Screening for β-Hydroxy-β-methylglutaryl-CoA Reductase Inhibitory and Antioxidant Activity of Sequentially Extracted Fractions of Ficus palmata Forsk

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Hypercholesterolemia-induced oxidative stress has been strongly implicated in the pathogenesis of atherosclerosis, which is one of the major causes of mortality worldwide. The current work, for the first time, accounts the antioxidant, genoprotective, antilipoperoxidative, and HMG-CoA reductase (EC 1.1.1.34) inhibitory properties of traditional medicinal plant, Ficus palmata Forsk. Our result showed that among sequentially extracted fractions of Ficus palmata Forsk, FPBA (F. palmata bark aqueous extract) and FPLM (F. palmata leaves methanolic extract) extracts have higher phenolic content and also exhibited significantly more radical scavenging (DPPH and Superoxide) and antioxidant (FRAP) capacity. Moreover, FPBA extract also exhibited significantly higher inhibition of lipid peroxidation assay. Additionally, results showed almost complete and partial protection of oxidatively damaged DNA by these plant extracts when compared to mannitol. Furthermore, our results showed that FPBA extract (IC₅₀ = 9.1 ± 0.61 μg/mL) exhibited noteworthy inhibition of HMG-CoA reductase activity as compared to other extracts, which might suggest its role as cardioprotective agent. In conclusion, results showed that FPBA extract not only possess significant antioxidant and genoprotective property but also is able to attenuate the enzymatic activity of HMG-CoA reductase, which might suggest its role in combating various oxidative stress-related diseases, including atherosclerosis.

1. Introduction

Hypercholesterolemia and its induced oxidative stress are now considered to be one of the major contributors in progression of atherosclerosis [1]. An excessive concentration of lipids in plasma may alter the lipoprotein metabolism and results in low density lipoprotein (LDL) accumulation in subendothelial space of arteries where it undergoes oxidative modifications to form oxidized LDL [2], which is highly atherogenic [3]. Several risk factors like hypercholesterolemia and cholesterol-induced oxidative stress enhance the formation of reactive oxygen species (ROS) which leads to the advancement of atherosclerotic lesions in vascular wall [4, 5]. Studies have reported that elevated lipid level, like total cholesterol (TC), triglyceride (TG), and low density lipoprotein (LDL) cholesterol, and a decrease in high density lipoprotein (HDL) cholesterol are directly associated with hyperlipidemia and atherosclerosis [6]. The cholesterol synthesis is regulated by β-hydroxy-β-methylglutaryl-CoA reductase (HMG-CoA reductase) (EC 1.1.1.34), the rate limiting enzyme of cholesterol pathway [7], and catalyzes the conversion of HMG-CoA to mevalonic acid. Currently prescribed drugs that lower cholesterol level mainly work by inhibiting the HMG-CoA reductase enzymatic activity [8, 9]. Treatment and management of hyperlipidemia include dietary changes, weight loss, and use of hypolipidemic drugs. Nonetheless, these oral medications have certain limitation and side effects [10]. Therefore, naturally derived therapeutic agents are in high demand for the treatment of hyperlipidemia and cholesterol-induced oxidative stress as
well as atherosclerosis. Plant products are less toxic, have no side effects and free radical scavengers, and are now considered to be the best source for new hypolipidemic drugs and atherosclerosis development programme [11–14]. The existence of cholesterol lowering agents and HMG-CoA reductase inhibitors has been demonstrated in different plant species including garlic [15], Ananas comosus [16], kiwifruit [17], and Gynostemma pentaphyllum [18]. Ficus palmata Forsk (Moraceae), commonly known as wild fig, is used traditionally in the treatment of constipation and diseases of the lungs and bladder [19]. Leaves, bark, and heartwood of F. palmata contain β-sitosterol and a new tetracyclic triterpene-glaunol acetate. Besides, ceryl behenate, lupeol, and α-amyrin acetate are reported from the stem bark of F. palmata [20]. Since other Ficus species like F. benghalensis [21], F. carica [22], and F. virens [14] are known to possess antioxidant and other pharmacological properties, the present study was initially projected to illustrate the antioxidant, genoprotective, and antilipoperoxidative as well as HMG-CoA reductase inhibitory properties of various sequentially extracted fractions of F. palmata.

2. Material and Methods

All the chemicals used in this study were of analytical grade and procured from HiMedia Laboratories, Mumbai, India, and Merck India. The HMG-CoA reductase assay kit was procured from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Plant Collection and Preparation of Extracts. The fresh plant materials, namely, leaves and stem bark of F. palmata Forsk, belong to the family Moraceae and were collected from herbal garden of Pharmacy Department, Integral University, India. Plant was authenticated by Dr. Tariq Husain, National Botanical Research Institute, Lucknow, India, and has been deposited in Herbarium with accession number 97960. Plants were shed and dried and powder (20 gram) was sequentially extracted with n-hexane (n-hex), dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH), and aqueous solvents in soxhlet apparatus until it turned colorless. The solvent was removed, filtered, and dried at room temperature and residues were stored at −20°C for future use. The percentage yield of different extracts of F. palmata were as follows: F. palmata leaves n-hex extract (FPLH) −3.05%, F. palmata leaves DCM extract (FPDL) −0.9%, F. palmata leaves EtOAc extract (FPLE) −1.25%, F. palmata leaves MeOH extract (FPLM) −9.15%, F. palmata leaves aqueous extract (FPLA) −3.85%, F. palmata bark n-hex extract (FPBH) −1.9%, F. palmata bark DCM extract (FPBD) −0.45%, F. palmata bark EtOAc extract (FPBE) −0.45%, F. palmata bark MeOH extract (FPBM) −4.9%, and F. palmata bark aqueous extract (FPBA) −4.95%.

2.2. Phytochemical Screening and Determination of Total Phenolic Content (TPC). In order to identify the phytochemicals present in various extracts of F. palmata, qualitative chemical tests were carried out by using standard procedure [23]. Total phenolic content of the extracts was determined by using Folin-Ciocalteu’s reagent (FCR) [24] and expressed as µg gallic acid equivalent (GAE) in mg dry weight of extract.

2.3. In Vitro Antioxidant Assay

2.3.1. DPPH Free Radical Scavenging Assay. The radical scavenging assay was measured according to the method of Brand-Williams et al. [25] by using DPPH as a free radical initiator model. Ascorbic acid was used as standard. The scavenging activity of DPPH radicals was measured by using the following equation:

\[
\text{Percent inhibition} = \left( \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right) \times 100.
\]

Further, final result was expressed as IC\textsubscript{50} value that represented the concentration of the extract producing 50% inhibition of DPPH radicals.

2.3.2. Ferric Reducing Antioxidant Power Assay. Ferric reducing antioxidant power (FRAP) assay of different fractions of F. palmata extract was done by following the procedure of Benzie and Strain [26] with some modification [27]. Briefly, an aliquot (100 µL) of an extract (with appropriate dilution) was added to 3 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine solution, and 1 part of 20 mM FeCl\textsubscript{3}) and the reaction mixture was incubated in a water bath at 37°C. The increase in absorbance at 593 nm was measured after 30 min in Eppendorf spectrometer. The standard curve was plotted using ferrous sulphate solution, and results were expressed as µM Fe (II)/mg dry weight of extract.

2.3.3. Superoxide Radical Scavenging Activity. The superoxide radical scavenging assay was done according to the method of Kuchandy and Rao [28] with some modifications. Briefly, 10 µL of nitro blue tetrazolium (1 mg/mL solution in dimethyl sulphoxide (DMSO)), 30 µL of the extract or standard (ascorbic acid), and 100 µL of alkaline DMSO (1 mL DMSO containing 5 mM sodium hydroxide in 0.1 mL water) were added to give a final volume of 140 µL. The samples were incubated for 5 min at room temperature and the absorbance was measured at 560 nm using 96-well microtitre plate using microplate reader. The inhibition percentage was calculated as follows:

\[
\text{Percent inhibition} = \left( \frac{\text{Absorbance test sample} - \text{Absorbance control sample}}{\text{Absorbance test sample}} \right) \times 100.
\]

2.3.4. Assay for In Vitro Antilipoperoxidative Activity. Lipid peroxidation inhibition activity of F. palmata extracts was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. [29] with some modification.
Table 1: Phytochemical screening of FPL and FPB extracts.

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>Flavonoid</th>
<th>Tannins</th>
<th>Terpenoids</th>
<th>Phenols</th>
<th>Proteins</th>
<th>Carbohydrate</th>
<th>Saponins</th>
<th>Reducing sugar</th>
<th>Glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPLH</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>FPLD</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>FPLE</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FPLM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>FPLA</td>
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<td>−</td>
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<td>−</td>
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</tr>
<tr>
<td>FPBD</td>
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<td>++</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>FPBE</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>FPBA</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

[30]. Quercetin was used as reference standard. The percent inhibition activity and IC_{50} value were calculated as described in Section 2.3.1.

2.3.5. Assay for Oxidative DNA Strand Breaks. DNA scission induced by Fenton’s reagent was demonstrated by using supercoiled pUC18 (2686 bp) plasmid DNA according to the method of Lee et al. [31] with slight modifications. Briefly, plant extracts (FPBA and FPLM) were used to protect the oxidative plasmid DNA (100 ng) damage which was initiated by Fenton’s reagent (H2O2 (30 mM), ascorbic acid (100 μM), and FeCl3 (160 μM)), and the final volume of the mixture was raised up to 20 μL. The mixture was then incubated for 45 min at 37 °C and electrophoresis was carried out in Tris-acetate-EDTA buffer (40 mM Tris base, 16 mM acetic acid, and 1 mM EDTA, pH 8.0) for 1.5 h (40 V/20 mA). Mannitol was used as a positive control.

2.4. In Vitro Modulation of HMG-CoA Reductase Inhibitory Activity by Plant Extract. The HMG-CoA reductase assay kit from Sigma-Aldrich (St. Louis, MO, USA) with the catalytic domain of the human enzyme (recombinant GST fusion protein expressed in E. coli) was used, under conditions recommended by the manufacturer, to identify the most effective fraction of plant extract. The concentration of the purified human enzyme stock solution (Sigma) was 0.52–0.85 mg protein/mL. Reference statin drug pravastatin (from Sigma) was used as positive control. To characterize HMG-CoA reductase inhibition under defined assay conditions, reactions containing 4 μL of NADPH (to obtain a final concentration of 400 μM) and 12 μL of HMG-CoA substrate (to obtain a final concentration of 400 μM) in a final volume of 0.2 mL of 100 mM potassium phosphate buffer, pH 7.4 (containing 120 mM KCl, 1 mM EDTA, and 5 mM DTT), were initiated (time 0) by the addition of 2 μL of the catalytic domain of human recombinant HMG-CoA reductase and incubated in Eppendorf BioSpectrometer (equipped with thermostatically controlled cell holder) at 37°C in the presence or absence (control) of 1 μL aliquots of drugs dissolved in DMSO. The rates of NADPH consumed were monitored every 20 sec for up to 15 min by scanning spectrophotometrically.

IC_{50} value was calculated as described above and the % inhibitory enzymatic activity was calculated by using the following formula [17]:

\[
\% \text{Inhibition} = \left( \frac{\Delta \text{Absorbance control} - \Delta \text{Absorbance test}}{\Delta \text{Absorbance control}} \right) \times 100. \tag{3}
\]

2.5. Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis. The fraction which showed maximum inhibition of HMG-CoA reductase activity, that is, FPBA extract, was subjected to LC-MS analysis according to the protocol of Beer et al., [32] at advanced instrumentation research facility, Jawaharlal Nehru University, New Delhi, India. LC-MS and MS/MS analyses were conducted on a Waters Synapt G2 with 2D nanoACQUITY System equipped with a binary pump, in-line degasser, autosampler, and column oven. The system was coupled to a Synapt G2 Q-TOF system (Waters) equipped with an electrospray ionization (ESI) source. LC-MS and MS/MS spectra were compared to literature to tentatively identify peaks and bioactive compound.

2.6. Statistical Analysis. For the entire assays, samples were analyzed in triplicate and the results were expressed as mean ± S.D. IC_{50} value was calculated by Origin version 6.0 Professional software, and the results were evaluated by using one-way analysis of variance (ANOVA) and two-tailed Student’s t-test. Statistical significance was expressed as \(^{\ast} P < 0.05\) and \(^{\ast\ast} P < 0.01\).

3. Results and Discussion

3.1. Phytochemical Screening and Determination of Total Phenolic Content (TPC). The preliminary phytochemical screening of FPLM and FPBA extract showed the presence of tannins, terpenoids, saponins, phenols, carbohydrate, flavonoids, protein, glycosides, and reducing sugar, while FPLA and FPLE extract also revealed the above constituents except tannins and terpenoids (Table 1). In addition, the other extracts also include the varied amount of the above phytochemicals but to a lesser extent compared to FPLM
and FPBA extract. Since, phenolic compounds are known as powerful chain-breaking antioxidants [33] and contribute directly to antioxidative action [34]; our results illustrate the TPC of various extracts of FPL and FPB extracts which were found to be in the following decreasing order: FPBA > FPLM > FPLE > FPBD > FPLD > FPBE > FPBM > FPBH > FPLH. The result presented in Figure 1 clearly demonstrated that FPBA and FPLM extracts have better phenolic content (255.8 ± 2.72 and 223 ± 2.58 µg GAE/mg of dry plant extract, resp.) than other extracts, whereas FPLH and FPBH have lowest phenolic content (17.2 ± 1.32 and 19.6 ± 1.58 µg GAE/mg dry plant extract, resp.). Furthermore, the existence of considerable amount of TPC in FPBA is in fine agreement with prior phytochemical information on different parts of various Ficus species which have phenolic compounds as major components [35–38].

3.2. In Vitro Antioxidant Assay. It is well known that antioxidants slow down or stop the oxidation of oxidizable material, by scavenging free radical and diminishing oxidative stress (excessive generation of ROS and RNS), which play a major role in the oxidation of varieties of biomacromolecules such as enzymes, proteins, DNA, and lipids that could result in the development of various diseases including atherosclerosis. Antioxidant activity of plant extract is associated with the presence of phenolic compound [39]; it is valuable to estimate the role of these sequentially extracted fractions in scavenging free radicals and determining the total antioxidant capacity. Our results showed that FPBA and FPLM extract exhibited significantly higher radical scavenging activity (IC\textsubscript{50} = 23 ± 1.65 and 11.5 ± 0.51 µg/mL, resp.) than other FPB and FPL extracts (Figures 2(a) and 2(b)). From the results presented in Figure 2, it is evident that there is a concentration dependent increase in radical scavenging activity of extracts, with substantial increase in FPBA extract, which is in agreement with previous reported data on F. palmate, which showed antioxidant activity and also observed no cytotoxicity to the normal peripheral blood mononuclear cells [40]. Moreover, the ability of plant extract to reduce ferric ions to ferrous ions is generally used as an index to determine the antioxidant power of extract [41]. The decrease in the concentration of ferric ion is a measure of the antioxidant activity of FPL and FPB extracts. As shown in Figure 2(c), FPBA extract possesses significantly higher antioxidant capacity (2.29 µM Fe\textsuperscript{3+}/mg) followed by FPLM (2.184 µM Fe\textsuperscript{3+}/mg) extract, while other extracts have not shown any significant activity. The ability of FPL and FPB extracts to scavenge superoxide radical, which is known to be a precursor of the more reactive oxygen species and results in various diseases, was also evaluated. Our data exemplified that superoxide radical scavenging capacity (IC\textsubscript{50}) of different extracts ranged from $3 ± 0.21$ µg/mL to $20 ± 1.15$ µg/mL (Figures 2(d) and 2(e)). Furthermore, FPBA extract exhibited significantly higher superoxide radical scavenging capacity (IC\textsubscript{50} = 3 ± 0.21 µg/mL), which was more than the standard ascorbic acid (IC\textsubscript{50} = 10.8 ± 0.64 µg/mL), followed by FPLM (IC\textsubscript{50} = 11.2 ± 0.55 µg/mL). In fact, several scientific studies reported that certain phytochemicals, such as flavonoids, ascorbic acid, tocopherol, tocotrienols, and polyphenols, have been reported as promising antioxidant compounds that might help in attenuating oxidative stress [11–13].

In order to evaluate the role of these extracts against various diseases, including atherosclerosis, which are mediated through excessive generation of free radicals, specifically by damaging DNA and lipid, inhibition of lipid peroxidation and oxidative DNA damage protective activity were evaluated. As revealed from Figures 2(f) and 2(g), all the extracts inhibited lipid peroxidation activity (IC\textsubscript{50} = 88 ± 2.3 µg/mL to >200 ± 4.25 µg/mL), while significantly higher inhibition was observed by FPBA (IC\textsubscript{50} = 88±2.3 µg/mL) and FPLM (IC\textsubscript{50} = 94±4.3 µg/mL) extract when compared to standard quercetin (IC\textsubscript{50} = 72 ± 1.6 µg/mL). On the basis of the above results, it has been concluded that FPBA and FPLM possess significant antioxidant activity among all the tested extracts and were used for oxidative DNA damage protective activity.

Since hydroxyl radicals are involved in damaging DNA molecules, it is important to evaluate the protective role of these extracts at molecular level. The oxidative DNA damage protective activity of FPBA and FPLM extracts showed almost complete and partial protection against OH\textsuperscript{−}-induced oxidatively damaged pUC18 plasmid DNA (Figure 2(h)). Hydroxyl radical generated during incubation of pUC18 plasmid DNA with Fenton’s reagent indicates that OH\textsuperscript{−} generated from iron-mediated decomposition of H\textsubscript{2}O\textsubscript{2} produced both single-strand and double-strand DNA breaks, which in turn was significantly attenuated by the addition of FPBA and FPLM extracts at different concentrations. It is well known that OH\textsuperscript{−} induced the oxidative damage in biological system and this type of damage, which is basically generated by the reaction between H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2} in the presence of redox active metal, can be significantly reduced in the presence of potent antioxidant like plant extract and mannitol. The combined antioxidant results demonstrated that FPBA extract was the most potent free radical scavenger and also completely attenuates the oxidative DNA damage. This profound antioxidant property might be well correlated with high amount of phenolic content present in FPBA fraction (Table 3).

3.3. In Vitro Anti-HMG-CoA Reductase Activity and LC-MS Analysis. In addition to the physiological regulation of
Figure 2: Continued.
HMG-CoA reductase, the human enzyme has been targeted successfully by drugs in the clinical treatment of high serum cholesterol levels and atherosclerosis [42]. The present work is a part of our drug discovery program, which mainly focuses on the development of new natural therapeutic agents with multiple targets and no toxicity after long-term consumption. Paravastatin is the representative of the statin class of drugs that in their active hydrolysed form are specific inhibitors of HMG-CoA reductase. Statins share an HMG-like moiety, which may be present in the form of an inactive lactone form that acts a prodrug, and in addition they also have a rigid hydrophobic substituted decalin ring covalently bound to the HMG-like moiety. Thus, a dose response in vitro study was done to assess the inhibition of HMG-CoA reductase by sequentially extracted fractions of F. palmata.

In this context, our results initially demonstrated the capability of FPL and FPB extract to inhibit HMG-CoA reductase enzymatic activity in a cell-free assay system. For the standardization of the protocol, preliminary screening of pravastatin was done against HMG-CoA reductase activity and observed 50% inhibition at 70.25 nM, which was in concordance with other studies [43]. From the data presented in Table 2 and Figure 3(a), it is evident that FPBA (IC$_{50}$ = 9.1 ± 0.61 μg/mL), FPLE (IC$_{50}$ = 27 ± 1.21 μg/mL), FPBD (IC$_{50}$ = 30 ± 1.51 μg/mL), FPLB (IC$_{50}$ = 35 ± 1.84 μg/mL), FPLH (IC$_{50}$ = 38 ± 1.86 μg/mL), FPBM (IC$_{50}$ = 45 ± 2.13 μg/mL), FPLD (IC$_{50}$ = 58 ± 3.21 μg/mL), and FPLM (IC$_{50}$ = 65 ± 3.42 μg/mL) extracts exhibited significant inhibition of HMG-CoA reductase activity, while other extracts were found to be nonsignificant. Our data clearly demonstrated that FPBA extract significantly inhibited HMG-CoA reductase activity with lowest IC$_{50}$ value and also showed concentration-dependent increase in inhibiting the enzymatic activity of HMG-CoA reductase. The spectrophotometric time scans demonstrated the ability of FPBA and FPLM at different concentrations (2–80 μg/mL) to increasingly mimic

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>IC$_{50}$ value (μg/mL)</th>
</tr>
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<tbody>
<tr>
<td>FPLH</td>
<td>38 ± 2.15*</td>
</tr>
<tr>
<td>FPLD</td>
<td>58 ± 3.15</td>
</tr>
<tr>
<td>FPLE</td>
<td>27 ± 1.15**</td>
</tr>
<tr>
<td>FPLM</td>
<td>65 ± 3.24*</td>
</tr>
<tr>
<td>FPLA</td>
<td>NS</td>
</tr>
<tr>
<td>FPBH</td>
<td>NS</td>
</tr>
<tr>
<td>FPBD</td>
<td>30 ± 1.44**</td>
</tr>
<tr>
<td>FPBE</td>
<td>35 ± 1.64*</td>
</tr>
<tr>
<td>FPBM</td>
<td>45 ± 2.45*</td>
</tr>
<tr>
<td>FPBA</td>
<td>9.1 ± 0.53**</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>70.25** nM</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SD (n = 3). Nonsignificant (NS), significantly different *P < 0.005, and **P < 0.001 versus without inhibitor.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Correlation coefficient ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC-DPPH</td>
<td>0.842</td>
</tr>
<tr>
<td>TPC-superoxide</td>
<td>0.988</td>
</tr>
<tr>
<td>TPC-lipid peroxidation</td>
<td>0.939</td>
</tr>
<tr>
<td>TPC-HMG-CoA reductase</td>
<td>0.803</td>
</tr>
</tbody>
</table>

activity of HMG-CoA reductase. The spectrophotometric time scans demonstrated the ability of FPBA and FPLM at different concentrations (2–80 μg/mL) to increasingly mimic
the inhibitory activity which may suggest direct interaction of plant extracts with this enzyme (Figures 3(b) and 3(c)). Further, FPBA fraction was subjected to LC-MS and MS/MS analysis, and spectra were compared to literature to tentatively identify peaks and bioactive compound (Figures 4(a) and 4(b)). LC-MS showed that extract does contain five prominent peaks having molecular weights 180.06, 193.03, 274.25, 702.29, and 371.09 au. Apart from these peaks there are several small peaks which may have also contributed to its enhanced activity. Alqasoumi et al. [44] claimed that aerial part of *F. palmata* was having five major constituents, including germanical acetate ($m/z$-491), psoralene ($m/z$-168), bergapten (5-methoxypsoralen, $m/z$-216), vanillic acid ($m/z$-168), and psoralenoside ($m/z$-368), which are responsible for its antioxidant and other medicinal properties, whereas in present study distinct peaks of molecular weights ($m/z$-180.06 and 371.09 au) were very similar to psoralene and psoralenoside. These chemical constituents might be responsible for inhibiting the catalytic activity of purified human HMG-CoA reductase in a cell-free assay through FPBA extract, and this extract might signify a novel class of HMG-CoA reductase inhibitors that can directly interact with this enzyme to obstruct the mevalonate pathway and prevent hypercholesterolemia. Further purification of this fraction is required to know the exact bioactive compound. From the above results, it was concluded that FPBA not only showed marked antioxidant activity but also exhibited significant HMG-CoA reductase inhibitory property.

4. Conclusion

Based on our results, it has been concluded that FPBA extract not only possesses significant antioxidant and genoprotective properties but also is able to ameliorate the enzymatic activity
Figure 4: ((a) and (b)) LC-MS/MS analysis of FPBA extract.
of HMG-CoA reductase, which might suggest its role in combating hypercholesterolemia as well as in various oxidative stress-related diseases including atherosclerosis. Moreover, the LC-MS and MS/MS analysis of FPBA extract revealed five prominent peaks having molecular weights 180.06, 193.03, 274.25, 702.29, and 371.09 au. Further, preclinical and clinical studies are needed to explore the role of this extract and their bioactive compound isolated thereof in the prevention and management of cholesterol-induced oxidative stress and hypercholesterolemia as well as atherosclerosis.

**Abbreviations**

FPL: *Ficus palmata* leaves  
FPB: *Ficus palmata* bark  
DPPH: 2, 2-Diphenyl-1-picrylhydrazyl  
FRAP: Ferric reducing antioxidant power.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**References**


Phyllanthus wightianus Müll. Arg.: A Potential Source for Natural Antimicrobial Agents

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Phyllanthus wightianus belongs to Euphorbiaceae family having ethnobotanical importance. The present study deals with validating the antimicrobial potential of solvent leaf extracts of P. wightianus. 11 human bacterial pathogens (Bacillus subtilis, Streptococcus pneumoniae, Staphylococcus epidermidis, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium, Escherichia coli, Shigella flexneri, Proteus vulgaris, and Serratia marcescens) and 4 fungal pathogens (Candida albicans, Cryptococcus neoformans, Mucor racemosus, and Aspergillus niger) were also challenged with solvent leaf extracts using agar well and disc diffusion methods. Further, identification of the active component present in the bioactive extract was done using GC-MS analysis. Results show that all extracts exhibited broad spectrum (6–29 mm) of antibacterial activity on most of the tested organisms. The results highlight the fact that the well in agar method was more effective than disc diffusion method. Significant antimicrobial activity was detected in methanol extract against S. pneumoniae (29 mm) with MIC and MBC values of 15.62 µg/mL. GC-MS analysis revealed that 29 bioactive constituents were present in methanolic extract of P. wightianus, of which 9,12-octadecaenioic acid (peak area 22.82%; RT-23.97) and N-hexadecanoic acid (peak area 21.55% RT-21.796) are the major compounds. The findings of this study show that P. wightianus extracts may be used as an anti-infective agent in folklore medicine.

1. Introduction

Plants play an important role in human life, primarily, as a source of food and medicine. Man is continuously faced with several lethal infectious diseases caused by pathogenic microorganisms [1]. In recent years, several pathogenic microorganisms have gained resistance to currently available synthetic antimicrobial agents and also caused many health problems [2]. Hence, there is an urgent need to discover an alternative new, broad spectrum, more active, and safer antimicrobial agent. Plant materials remain an important resource to combat serious diseases in the world. Especially, the plants from Pinaceae, Cupressaceae, Apiaceae, Bursarceae, Anacardiaceae, Palmaceae, Euphorbiaceae, Dracennaceae, and Fabaceae families are rich source for antimicrobial agents [3, 4]. Plants have an exceptional ability to synthesize de novo antimicrobial agents, in response to microbial attack for its protection [5]. Plant derived natural compounds (such as flavonoids, terpenoids, and steroids) have received considerable attention due to their diverse pharmacological properties including antibacterial and antifungal activities [6].

Antimicrobial components from plants which are mainly secondary metabolites act as inhibitors of bacterial growth, bacterial adherence, exopolysaccharide synthesis, DNA gyrase, cytoplasmic membrane function, and energy metabolism [7, 8]. Berberine, an isoquinoline alkaloid, which is present in roots and stem-bark of Berberis species, shows antimicrobial potential against bacteria, fungi, protzoa, and viruses [9]. Diterpene alkaloids, commonly isolated from the plants of the Ranunculaceae family, have antimicrobial properties [10]. Several phenolic compounds such as, caffeic acid, catechol, and pyrogallol are effective antimicrobial
agents. The antibacterial activity of some monoterpenes, diterpenoids, sesquiterpenes, triterpenoids, and their derivatives isolated from plants was recently reported [11]. Nowadays, search for plants with antimicrobial activity has evolved [12]. Importance of plants in drug discovery is growing due to vast diversity of the secondary metabolites which possess varied biological activities and act as main source of molecule leading the discovery of new, effective, and safer drugs [13]. Recent attention has been paid to extraction and isolation of biologically active compounds from plant species which are used in herbal medicines [14]. Pharmacognostic investigations of plants or plant extracts were needed to ascertain their biological activities which lead to the discovery of novel drugs or templates for the development of new therapeutic agents [15].

Phyllanthus wightianus (Euphorbiaceae) is a monoeocious glabrous shrub which grows up to 4.5 m high and is found in the hills (750 to 1000 m) of peninsular India, on the floor and border of shoals, low altitudes in sandveld, hot dry deciduous, mopane woodlands, along banks of seasonal streams, and rivers [16]. It exhibits various biological properties, such as antimicrobial [17–19], larvicidal [20, 21], analgesic [22], wound healing [23], and antioxidant properties [24]. On the basis of the above information, the present investigation was focused on antimicrobial properties of different solvent leaf extracts and GC-MS analysis of bioactive extract of P. wightianus Müll. Arg.

2. Materials and Methods

2.1. Plant Material. Fresh, matured, uninfected leaves of P. wightianus were collected from higher altitudes (900–1100 m) of Kolli Hills (latitude 10° 12′–11° 7′ N, longitude 76°–77° 17′ E), Namakkal district, Tamil Nadu, India. The plant material was authenticated by Botanical Survey of India (BSI) (reference number: BSI/SRC/5/23/2013-14/Tech/2081) Coimbatore, Tamil Nadu, India. The voucher specimen (specimen number: PU/BT/NDRL/2010/05) has been deposited in the Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India.

2.2. Preparation of Extracts. Collected leaves were washed and shade-dried for three weeks and then powdered. The powdered plant material (500 g) was extracted in increasing polarity order (successively) with hexane, chloroform, acetone, ethyl acetate, and methanol in a Soxhlet apparatus for 72 hours. The extracts were filtered through Whatman number 1 filter paper and evaporated under vacuum at 40°C to yield crude extracts.

2.3. Used Microorganisms. Three gram positive bacteria, namely, B. subtilis (MTCC 441), S. pneumoniae (MTCC 655), S. epidermidis (MTCC 435), and eight gram negative bacterial strains, such as P. vulgaris (MTCC 426), P. aeruginosa (MTCC 741), K. pneumoniae (MTCC 109), S. typhimurium (MTCC 98), E. coli (MTCC 739), and S. flexneri (MTCC 1457), with two clinical isolates (P. vulgaris and S. marcescens) and 4 fungal pathogens (C. albicans, C. neoformans, M. racemosus, and A. niger) were used in this investigation. The fungal strains were obtained from clinical laboratories of Salem District, Tamil Nadu. Each test organism was prepared by inoculating a loop-full of mother culture in a 5 mL of broth (Muller-Hinton broth for bacteria and Sabourd Dextrose broth for fungal cultures) and incubated at appropriate temperature and time for bacterial pathogens (37°C for 16 hours) and fungal strains (room temperature (28°C) for 72 hours). The culture turbidity was adjusted to 0.5 McFarland equivalence (1.5 × 10^n CUF) prior to use.

2.4. Agar Well Diffusion Method. The agar well diffusion method was employed to determine antibacterial activity of extracts as per the modified method of Natarajan et al. [19]. The standardized test cultures (50 μL) were swabbed on the per-molten Müller Hinton Agar (MHA) for bacteria and Sabourd Dextrose Agar (SDA) for fungus using aseptic cotton swab. Six wells were made in the seeded plates using sterile cork borer (5 mm diameter). Then, each extract (50 μL = 50 μg) was separately introduced into wells and allowed to diffuse at room temperature. Equal volume of DMSO was served as negative control. About 25 μL of standard antibiotics like fluconazole (fungus) and ciprofloxacin (bacteria) was used as positive control (each 1 μg/μL). The bacterial and fungal plates were incubated at 37°C for 24 hours and at room temperature for 72 hours, respectively. After the incubation period, the zone of growth inhibition was measured (in mm).

2.5. Disc Diffusion Method. The disc diffusion test was performed by the method of NCCLS [25] with minor modifications. Test microbial suspension culture (50 μL) was spread on the MHA for bacteria and SDA for fungus by a sterile cotton swab. Sterile discs (5 mm diameter) were loaded with each extract (50 μL) and allowed to dry at room temperature. The dried discs were placed aseptically on the seeded plates. Standard antibiotic discs were used as positive control (gentamicin, vancomycin, and ampicillin for bacteria and fluconazole for fungus (10 mcg/disc)). The plates were incubated as the conditions mentioned in the well diffusion methods and the diameter (in mm) of clear zone of growth inhibition was recorded.

2.6. Minimum Inhibitory Concentration (MIC). The MIC was determined by broth microdilution bioassay method using the modified method of Eloff et al. [26]. MIC was carried out on the basis of antimicrobial results, the extracts which exhibited considerable antimicrobial activity against tested organisms. 100 μL of different concentrations (1–1000 μg/mL) of extracts was introduced into 96-well microplates containing 200 μL of Muller-Hinton broth and 20 μL bacterial cultures were added to each well. The microplate was closed with lid and incubated for 24 h at 37°C. After incubation period, 40 μL of p-iodonitrotetrazolium violet (INT) (0.2 mg/mL) was added to the wells to serve as an indicator of bacterial growth and incubated at 37°C for 1 hour. The minimum inhibitory concentration (MIC) was taken as the lowest concentration of the extract that completely inhibited bacterial growth.
were detected in methanolic extract against selected bacterial species. The lowest MIC and MBC value in which no bacterial growth occurred was taken as the Minimum Bactericidal Concentration.

2.7. Minimum Bactericidal Concentration (MBC). The MBC was determined as per method of Khan et al. [27]. MIC test dilutions (5 μL) which showed no color change was subcultured on freshly prepared Mueller Hinton Agar plates and incubated at 37°C for 24 h. The lowest concentration in which no bacterial growth occurred was taken as the Minimum Bactericidal Concentration.

2.8. GC-MS Analysis. GC-MS analysis of bioactive extract was carried out on GC Clarus 500 Perkin Elmer system comprising AOC-20i auto sampler. The spectra of unknown components present in the bioactive extract were identified by compared with known components spectrum which stored in the NIST and WILEY libraries. The name, molecular weight and structure of the components present in the bioactive extract were ascertained. The GC-MS analysis was carried out at Sophisticated Analytical Instrument Facility (SAIF), Indian Institutes of Technology, Chennai, India.

2.9. Statistical Analysis. All determinations were done at least in triplicate and averaged. Values were expressed as mean ± standard deviations. Statistical analyses were conducted using SPPS software (16.0 Version). Analysis of variance (ANOVA) in a completely randomized design and Tukey's multiple range tests were used to compare any significant differences between samples. The confident limits used in this study were based on 95% (P < 0.05).

3. Results and Discussion

The color and extractive yield of the plant material are presented in Table 1. The results of antimicrobial activity of P. wightianus were given in Table 2. All the extracts of P. wightianus show broad spectrum antibacterial activity in the range between 6 and 29 mm. The results of agar well diffusion method show that methanolic extract has significant activity against S. pneumoniae (29 mm) followed by S. epidermidis (17 mm). The considerable amount of antibacterial activity was observed in acetone extract against S. pneumoniae (28 mm) followed by S. flexneri (12 mm). In disc diffusion method, methanol extract exhibits good antimicrobial activity and the maximum growth inhibition was observed in S. pneumoniae (18 mm) followed by S. epidermidis (17 mm). Acetone extract having well to moderate antimicrobial activity and maximum activity was detected in S. epidermidis (18 mm) followed by S. pneumoniae (14 mm). Most of the tested clinical fungal pathogenic strains exhibit no sensitivity on the tested extracts in both agar well and disc diffusion methods. However, high antifungal activity was recorded in methanol extract against C. neoformans (10 mm) followed by hexane extract against M. racemosus (10 mm) in well diffusion method (Table 2). The overall results highlight that methanol extract exhibits significant activity against most of the tested pathogens compared with other extracts.

The MIC and MBC results (Tables 3 and 4) indicate that the crude extracts of P. wightianus inhibited the growth of selected bacterial species. The lowest MIC and MBC value were detected in methanolic extract against S. pneumoniae (15.62 μg/mL) and S. epidermidis (31.25 μg/mL). The rest of extracts showed moderate to high MIC and MBC values (500–1000 μg/mL) against most of the tested bacterial pathogens. Plant extracts are considered as having a good inhibitory activity, if they present MICs ≤ 100 μg/mL, a moderate inhibitory activity, if they present MICs ranging from 100 to 500 μg/mL, a weak inhibitory activity, if they present MICs ranging from 500 to 1000 μg/mL, and no inhibitory activity, if they present MICs > 1000 μg/mL [28, 29]. While considering these reports, the MIC and MBC values recorded from antibacterial activity of present investigation might be good to moderate.

The results highlights that significant antibacterial activity was observed in methanol extract against S. flexneri, S. pneumoniae, and S. epidermidis. Similar observations were made with P. wightianus methanolic extract [17]. Our previous findings, report that acetone extract expresses significant activity against most of the clinical bacterial pathogens compared to methanol extract [19]. Whereas, the present results show MTCC bacterial strains to be more sensitive to the methanol extract than acetone extract of P. wightianus. This difference in observation may be due to the high concentration (5 mg/well/disc) of extracts being used in the earlier study, whereas, in the present findings, low concentrations of extracts did not inhibit bacterial growth.

Studies have shown that methanolic extracts of many Phyllanthus species, such as P. acidus [30], P. muellerianus [31], P. amarus, P. maderaspatensis [32], P. debilis [33], P. amarus, P. emblica [34], and P. niruri [35], harbor promising antimicrobial activity which strengthens the present findings. Several reports stated that methanol is potent solvent for extracting variety of important phytoconstituents, like alkaloids, phenols, tannins, fatty acids, and flavonoids, which harbor antimicrobial potential which support the findings of present investigation [36, 37].

GC-MS analysis shows the presence of 29 compounds which were identified based on their retention time (RT), molecular formula, molecular weight (MW), and concentration (%) (Figure 1 and Table 5). Methanolic extract of P. wightianus has 9,12-octadecadienoic acid (with the peak area 22.82% and retention time 23.970) and N-hexadecanoic acid (with the peak area 21.55% and retention time 21.796) acting as major components. A variety of compounds, such as aliphatic ether, aliphatic carboxylic acid, aliphatic ester, alkenes, and phenolic compounds were identified. Some of the compounds were present only in low quantities (ranging from 0.6 to 4%).

Table 1: Color and extractive yield of extracts from different solvents of P. wightianus.

<table>
<thead>
<tr>
<th>Name of extract</th>
<th>Color</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>Yellowish green</td>
<td>6.24</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Dark green</td>
<td>0.67</td>
</tr>
<tr>
<td>Acetone</td>
<td>Pale green</td>
<td>1.33</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Pale brown</td>
<td>2.49</td>
</tr>
<tr>
<td>Methanol</td>
<td>Brown</td>
<td>4.61</td>
</tr>
</tbody>
</table>
N-Hexadecanoic acid and 9,12-octadecadienoic acid are common secondary metabolites present in several plants [38–43] and are reported as having many biological properties, like antimicrobial, anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, and antioxidant [44, 45]. 9,12-Octadecadienoic acid was also stated as a model compound of unsaturated fatty acids, which selectively inhibits FabI enzyme in S. aureus and E. coli, catalyzing the final and rate-limiting step of the chain elongation process of the type II fatty acid synthesis (FAS-II) in bacteria [46].

Several fatty acids and phenolic compounds were identified in GC-MS analysis of methanol extract which may be responsible for the antimicrobial activity. The mechanisms of antimicrobial action of fatty acids are nonspecific modes of action [47]. However, antimicrobial effects of fatty acids were observed to form mostly either by a complete inhibition of oxygen uptake or stimulating uptake of amino acids into the cells, which occurs in a dose dependent manner [48]. Fatty acids intercalate in the phospholipid bilayer of microbes due to their lipophilicity, which increases the permeability of the cell membrane, dissipation of the proton-motive force, and leakage of inorganic ions, leading to cell death [49, 50].

Studies have shown that phenolic compounds have bacterial action by interfering with bacterial cell adhesins,
enzymes, cell envelope, and transport proteins [51]. They also increase the free radical concentration within the bacterial protoplasm and irreversibly complex with nucleophilic amino acids in microbial proteins determining loss of their function [52]. As a result, this causes bacterial cell lysis [53]. The antibacterial activity of methanol extract is not only caused by their major compounds, but it could be due to a synergism among their other components present in it. Hence, the presence of these components in higher quantity in methanol extract of *P. wightianus* may be responsible for better bioactivity.

### 4. Conclusions

The findings of present investigation show that agar well diffusion method is ideal for determining the antimicrobial activity of *P. wightianus* extracts. Methanolic extract of *P. wightianus* contributed significant activity against most of the

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**Table 3: MIC (µg/mL) for antibacterial activity of *P. wightianus* against some pathogens.**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Methanol</th>
<th>Acetone</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
<th>Hexane</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> (MTCC 441)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (MTCC 739)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (MTCC 109)</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.81&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> (MTCC 426)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.81&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (MTCC 741)</td>
<td>250&lt;sup&gt;c&lt;/sup&gt;</td>
<td>250&lt;sup&gt;c&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> (MTCC 98)</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Shigella flexneri</em> (MTCC 1457)</td>
<td>125&lt;sup&gt;d&lt;/sup&gt;</td>
<td>250&lt;sup&gt;c&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> (MTCC 655)</td>
<td>15.62&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>250&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> (MTCC 435)</td>
<td>31.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are mean of triplicates. Different superscript letters (a–e) in rows indicate significant differences (at $P < 0.05$) when subject to Tukey’s multiple comparison test.

---

**Table 4: MBC (µg/mL) for antibacterial activity of *P. wightianus* against some pathogens.**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Methanol</th>
<th>Acetone</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
<th>Hexane</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> (MTCC 441)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (MTCC 739)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (MTCC 109)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> (MTCC 426)</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (MTCC 741)</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> (MTCC 98)</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Shigella flexneri</em> (MTCC 1457)</td>
<td>250&lt;sup&gt;c&lt;/sup&gt;</td>
<td>250&lt;sup&gt;c&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> (MTCC 655)</td>
<td>15.62&lt;sup&gt;d&lt;/sup&gt;</td>
<td>62.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> (MTCC 435)</td>
<td>31.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>125&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are mean of triplicates. Different superscript letters (a–e) in rows indicate significant differences (at $P < 0.05$) when subject to Tukey’s multiple comparison test.
Table 5: Compounds detected in methanol leaf extract of *P. wightianus* using GC-MS analysis.

<table>
<thead>
<tr>
<th>Peak</th>
<th>R. time</th>
<th>Peak area (%)</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Compound name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.726</td>
<td>0.94</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>116</td>
<td>2-Pentanone, 4-hydroxy-4-methyl-3-2-hydroxy-2-methyl-4-pentanone 2-</td>
</tr>
<tr>
<td>2</td>
<td>9.358</td>
<td>0.28</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>126</td>
<td>Levoglucosenone 6,8-ioxabicyclooct-2-2-en-4-one</td>
</tr>
<tr>
<td>3</td>
<td>10.907</td>
<td>0.67</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O</td>
<td>120</td>
<td>4-Vinylphenol</td>
</tr>
<tr>
<td>4</td>
<td>11.198</td>
<td>0.26</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>174</td>
<td>T-Butyldimethylsilyl acetate</td>
</tr>
<tr>
<td>5</td>
<td>12.387</td>
<td>0.08</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>204</td>
<td>1-(4-Methoxyphenyl)-5-hexen-1-one</td>
</tr>
<tr>
<td>6</td>
<td>13.436</td>
<td>1.60</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O</td>
<td>126</td>
<td>1,2,3-Benzenetriol (pyrogallol)</td>
</tr>
<tr>
<td>7</td>
<td>15.093</td>
<td>6.07</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>236</td>
<td>Butanoic acid, 3-methyl-, 3,7-dimethyl-2,4,6-octatrieny1 ester</td>
</tr>
<tr>
<td>8</td>
<td>15.241</td>
<td>3.56</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;Br&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>390</td>
<td>3,8-Dioxabicyclononane, 6-bromo-4-(1-bromopropyl)-2-[2-penten-4-ynyl]</td>
</tr>
<tr>
<td>9</td>
<td>15.525</td>
<td>1.04</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O</td>
<td>206</td>
<td>Phenol, 2,4-bis(1,1-dimethyl-)-2,4-ditert-butylphenol 1-hydroxy-2, 4-di-tert-butylbenzene</td>
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<tr>
<td>10</td>
<td>16.037</td>
<td>2.46</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;26&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>180</td>
<td>2-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-(2,6,6-trimethyl-2-hydroxyoctahydrilene)acetic acid lactone 4,5</td>
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<tr>
<td>11</td>
<td>16.586</td>
<td>0.55</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>222</td>
<td>1,2-Benzene dicarboxylic acid, diethyl ester phthalic acid</td>
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<tr>
<td>12</td>
<td>17.658</td>
<td>0.77</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>332</td>
<td>β-Dodecahydro</td>
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<tr>
<td>13</td>
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<td>0.71</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>196</td>
<td>2(4h)-Benzo-furanone, 5,6,7,7a-tetrahydro-6-hydroxy-4,4,7a-trimethyl-, (6s-cis)-(--)—lo-loliolide</td>
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<tr>
<td>14</td>
<td>19.716</td>
<td>3.03</td>
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<td>208</td>
<td>Pluchidiol</td>
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<tr>
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<td>20.264</td>
<td>10.65</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;38&lt;/sub&gt;</td>
<td>278</td>
<td>2,6,10-Trimethyl,14-ethylene-14-pentadecene</td>
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<tr>
<td>16</td>
<td>20.347</td>
<td>1.19</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;O</td>
<td>268</td>
<td>2-Pentadecanone, 6,10,14-trimethyl-hexahydrofarnesyl acetone 6,10,14</td>
</tr>
<tr>
<td>17</td>
<td>20.591</td>
<td>3.16</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;40&lt;/sub&gt;</td>
<td>296</td>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2e)-3,7,11,15-tetramethyl</td>
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<td>18</td>
<td>20.831</td>
<td>5.85</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;40&lt;/sub&gt;</td>
<td>296</td>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2e)-3,7,11,15-tetramethyl</td>
</tr>
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<td>20.925</td>
<td>0.36</td>
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<td>Butanal</td>
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<td>20</td>
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<td>0.58</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;</td>
<td>156</td>
<td>6-Octen-1-ol, 3,7-dimethyl-</td>
</tr>
<tr>
<td>21</td>
<td>21.796</td>
<td>21.55</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>256</td>
<td>N-Hexadecanoic acid, palmitic acid, pentadecanecarboxylic acid</td>
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<tr>
<td>22</td>
<td>23.300</td>
<td>0.53</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;O</td>
<td>270</td>
<td>Bis-(3,5,5-trimethylhexyl) ether</td>
</tr>
<tr>
<td>23</td>
<td>23.808</td>
<td>0.40</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;38&lt;/sub&gt;</td>
<td>278</td>
<td>2,6,10-Trimethyl,14-ethylene-14-pentadecene neophytadiene</td>
</tr>
<tr>
<td>24</td>
<td>23.881</td>
<td>3.81</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>186</td>
<td>Nonanoic acid, 7-methyl ester methyl 7-methylnonanoate</td>
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<td>25</td>
<td>23.970</td>
<td>22.82</td>
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<td>280</td>
<td>9,12-Octadecadienoic acid-linoleic acid grape seed oil linoleic linole</td>
</tr>
<tr>
<td>26</td>
<td>24.199</td>
<td>5.93</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>278</td>
<td>9,12,15-Octadecatrienoic acid, linolenic acids alpha-linolenic acid</td>
</tr>
<tr>
<td>27</td>
<td>24.308</td>
<td>0.68</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>284</td>
<td>Octadecanoic acid (stearic acid)</td>
</tr>
<tr>
<td>28</td>
<td>26.055</td>
<td>0.46</td>
<td>C&lt;sub&gt;8&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O</td>
<td>126</td>
<td>(3z)-6-methyl-3,6-heptatrien-1-ol</td>
</tr>
</tbody>
</table>

tested bacterial pathogens with least MIC and MBC values. Hence, methanol is the best solvent system for extracting the bioactive principles from leaves of *P. wightianus* which possesses promising antimicrobial principles which may be used in the treatment of infectious diseases caused by pathogenic microbes. The antimicrobial principles from the bioactive extracts may need further purifications to have its synthetic analogues which will be carried out in the future.
Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Research Article

Phytochemical Screening, Physicochemical Properties, Acute Toxicity Testing and Screening of Hypoglycaemic Activity of Extracts of *Eremurus himalaicus* Baker in Normoglycaemic Wistar Strain Albino Rats

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In the present study EtOAc, MeOH, and aqueous extracts of *Eremurus himalaicus* were evaluated for hypoglycaemic effect in normal rats using both oral glucose tolerance test and 14-day oral administration study. Phytochemical and physicochemical screening was also done. In oral glucose tolerance test the aqueous and MeOH extracts of *Eremurus himalaicus* at a dose level of 500 mg/kg body weight prior to glucose load resulted in a significant fall in blood glucose level within 150 min. of glucose administration. The aqueous extract at a dose level of 250 mg/kg body weight and 500 mg/kg body weight also showed good hypoglycaemic response ($P < 0.001$); this was followed by MeOH extract at a dose level of 500 mg/kg body weight ($P < 0.05$), while MeOH extract at dose level of 250 mg/kg body weight and ethyl acetate extract at dose level of 250 mg/kg body weight and 500 mg/kg body weight exhibited insignificant effect. Phytochemical screening of extracts revealed the presence of alkaloids, terpenoids, phenolics, tannins, saponins, cardiac glycosides, and flavonoids. The results indicate that aqueous extract possess significant hypoglycaemic activity in normoglycaemic rats which may be attributed to the above-mentioned chemical constituents.

1. Introduction

Diabetes, a global burden, is characterized by fast elevation of blood sugar level. The incidence of diabetes mellitus is rising all over the world, especially in Asia. Many oral hypoglycaemic agents, such as biguanides and sulfonylureas are available along with insulin for the treatment of diabetes mellitus, but they have significant side effects and sometimes they are found to be ineffective in chronic diabetic patients [1, 2]. Thus, there is an increasing demand of natural and synthetic products with high anti diabetic potential and lesser side effects. The research conducted over the last several decades has shown that plant and plant-based therapies have high potential to treat and control diabetes and its complications [3]. Diabetes has been treated orally with several medicinal plants or their extracts, based on folklore medicine. Therefore, search for safe and more effective agents has continued to be an important area of active research.

Looking back upon the last 2000 years of the history of medicine mankind has mainly used plants as the best source of medicine [4]. Over 248,000 species of higher plants have been identified and from these 12,000 plants are known to have medicinal properties [5]. The significance of research on natural medicinal plants is gaining momentum owing to its immense potential for improving the health care sector of the globe. The World Health Organization estimated that about 80% of Earth's inhabitants rely on traditional medicine for their primary health care needs that primarily involves the
use of plant extracts or their active components. A concerted research is currently in vogue for understanding the mechanism of action of these traditional procedures. With the sharp rise in popularity of traditional medicine, the economic importance of these plants has increased enormously. The role of medicinal plants is particularly important in the Himalayan region [6]. These areas are richly endowed with a variety of plant species, many of which have medicinal properties.

Eremurus himalaicus Baker commonly known as Himalayan Desert Candle is a wild ornamental herb of Liliaceae family found on rocky slopes of the drier areas of Himalayas that can be easily identified by its tall stout spike-like cluster of hundreds of white flowers with protruding orange anthers [7]. It is locally known as Hulla, Kaithloon, Dharshaag, Chhil hak, and Bulkutor Yalun. Till date no scientific research work has been done on the medicinal values of this plant and the present study is the first attempt to scientifically assess the hypoglycaemic potential of Eremurus himalaicus.

2. Material and Methods

2.1. Plant Material Collection. The plant Eremurus himalaicus was collected from FakirGoojree area of Dhara, Srinagar, in the month of May and was identified and authenticated from The Centre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir under voucher specimen number 1765 (KASH). The plant material was cleaned and dried under shade at room temperature and ground in a grinding mill.

2.2. Preparation of Plant Extracts. The ground plant material was successively extracted with Petrol, EtOAc, MeOH, and water in a Soxhlet extractor. The recovered extracts were then reduced in a rotary evaporator and finally stored in airtight containers at 4°C for further use.

2.3. Preliminary Phytochemical Analysis of the Extracts. The extracts so obtained were subjected to preliminary phytochemical screening as follows.

2.3.1. Tannins. To 2 mL of aqueous extract, 2 mL of 5% FeCl₃ was added and observed for the formation of yellow brown precipitate [8].

2.3.2. Alkaloids. To the 2 mL MeOH filtrate, 1.5 mL of 1% HCl was added. After heating the solution in water bath, 6 drops of Mayor’s reagents/Wagner’s reagent/Dragendorff reagent were added. Formation of orange precipitate was observed to detect the presence of alkaloids [9].

2.3.3. Saponins. Aqueous extract of 2 g powder was made and the solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with few drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion [10].

2.3.4. Cardiac Glycosides. To 2 mL alcoholic filtrate, 1 mL glacial acetic acid and 1-2 drops of FeCl₃ were added followed by 1 mL of concentrated H₂SO₄. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer [11].

2.3.5. Terpenes. To 2 mL of aqueous extract, 5 mL CHCl₃, 2 mL acetic anhydride, and concentrated H₂SO₄ were added carefully to form layer. Reddish brown coloration of interface was observed to detect the presence of terpenes [12].

2.3.6. Flavonoids. 2 g plant material was extracted in 10 mL alcohol or water. To 2 mL filtrate, few drops of concentrated HCl followed by 0.5 g of zinc or magnesium turnings were added. The solution was observed for the appearance of magenta red or pink colour after 3 min [8].

2.3.7. Phenolics. To 2 mL of alcoholic or aqueous extract, 1 mL of 1% ferric chloride solution was added. Blue or green colour indicated phenols [13].

2.3.8. Anthraquinones. 0.5 g of the extract was boiled with 10 mL of H₂SO₄ and filtered while hot. The filtrate was shaken with 5 mL of CHCl₃. The CHCl₃ layer was pipetted into another test tube and 1 mL of dilute ammonia was added. The resulting solution was observed for colour changes [12].

2.4. Physicochemical Parameters. The various physicochemical parameters that were determined as per The Unani Pharmacopeia of India [14] include the following.

2.4.1. Description. It included evaluation of plant by colour, odour, taste, size, shape, and special feature, like touch, texture, and so forth.

2.4.2. Loss on Drying. 10 g of plant material was placed (without preliminary drying) after accurately weighing it in a tared evaporated dish. This was dried at 105°C for 5 h and weighed. Drying and weighing was continued at 1 h interval until we got the constant weight. Constant weight was reached when two consecutive weights, after drying for 30 min. and cooling for 30 min. in a desiccator, showed not more than 0.1 g difference.

2.4.3. Extractive Values (Successive). A known amount of plant material was taken and all the sugars were leached out with cold water, dried thoroughly in a desiccator till weight was constant, and then extracted successively with petrol, EtOAc, MeOH, and water in a Soxhlet extractor for complete extraction and different extracts were weighed quantitatively and percentage with respect to the weight of the plant material taken was calculated.

2.4.4. Total Ash Value. About 2-3 g of ground plant material was incinerated in a tared platinum/silica crucible at a temperature not exceeding 450°C until free from carbon.
Then it was cooled and weighed. The percentage of ash with reference to the air dried plant material was calculated.

2.4.5. Acid Insoluble Ash Value. To the crucible containing total ash, 25 mL of dilute HCl was added. The insoluble matter was collected on an ashless filter paper (Whatmann number 41) and washed with hot water until the filtrate was neutral. The filter paper containing insoluble matter was transferred to the original crucible and dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a suitable desiccator for 30 min. and weighed without delay. The content of the insoluble ash was calculated with reference to the air dried plant material.

2.4.6. Water Soluble Ash Value. The ash was boiled for 5 min with 25 mL of water; insoluble matter was collected in a Gooch crucible or on an ash less filter paper, washed with hot water, and ignited for 15 min. at a temperature not exceeding 450°C. The difference in the weight of the insoluble matter and the weight of ash represented the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried plant material.

2.4.7. Residue on Ignition/Sulfated Ash Test. A platinum/silica crucible was heated to redness for 10 min, allowed to cool in a desiccator, and weighed. Accurately weighed 1-2 g of the plant material was put into the crucible, gently ignited at first, until the substance was thoroughly charred. The residue was cooled, moistened with 1 mL of H₂SO₄, heated gently until white fumes were no longer evolving, and ignited at 800°C ± 25°C until all black particles disappeared. The ignition was conducted in a place protected from air currents. The crucible was allowed to cool; a few drops of H₂SO₄ were added and the crucible was heated. Then it was ignited as before, allowed to cool, and weighed. The operation was repeated until two successive weighing did not differ by more than 0.5 mg.

2.4.8. pH Value at 10% and 1% Dilution

(1) pH of 10% Solution. An accurately weighed 10 g of drug was dissolved in accurately measured 100 mL of water and filtered and the pH of filtrate was checked with a standardized glass electrode.

(2) pH of 1% Solution. An accurately weighed 1 g of drug was dissolved in accurately measured 100 mL of water and filtered and the pH of filtrate was checked with a standardized glass electrode.

2.5. Experimental Animals. Healthy adult Wistar strain male albino rats weighing 190–220 g were obtained from Regional Research Institute of Unani Medicine (RRIUM), Srinagar. The animals were kept under standard conditions. Animal studies had approval of IAEC, RRIUM, and Srinagar.

2.6. Acute Toxicity Testing. The study was performed as per the Organization for Economic Cooperation and Development (OECD) guidelines number 425.

2.7. Effect of Different Extracts of *Eremurus himalaicus* on Normal Rats. It comprised of two tests.

2.7.1. Oral Glucose Tolerance Test. The oral glucose tolerance test was performed in overnight fasted (18 h) normal rats. Healthy rats were randomly selected and distributed into five groups (n = 6). One of those groups was administered distilled water and the rest four groups were given orally EtOAc, MeOH, and aqueous extracts of *Eremurus himalaicus* (500 mg/kg body weight, resp.) and glibenclamide (10 mg/kg body weight). Glucose (2 g/kg body weight) was fed 1 h after the administration of extracts and glibenclamide. Blood was withdrawn from the tail vein at 0, 60, 90, 120, and 150 min of glucose administration and glucose levels were estimated using Accuchek Go blood glucose monitoring kit.

2.7.2. Effect of Different Extracts on Normoglycaemic Rats. Healthy Wistar strain albino rats were selected and randomly divided into different groups with six animals in each group serving as group "A" = normal control, group "B" = EtOAc, 250 mg/kg body weight; group "C" = EtOAc 500 mg/kg body weight; group "D" = MeOH, 250 mg/kg body weight; group "E" = MeOH, 500 mg/kg body weight; group "F" = aq., 250 mg/kg body weight, "G" = aqueous, 500 mg/kg body weight, and "H" = glibenclamide (10 mg/kg body weight). An identification mark was given to the rats of each group using picric acid. The blood glucose level of the rats was measured after overnight fasting. Group “A” was given simple drinking water which served as normal control and rest of the groups were given their respective extracts, mentioned above, orally for a period of 14 days. Blood was collected again on the 7th day and 14th day of dosing, through the retro-orbital sinus of the rats. The serum from the blood was separated and labeled with the animal number. The estimation of glucose level was done on an autoanalyser.

2.8. Statistical Analysis. All the values were expressed as mean ± standard deviation (S.D.) and analyzed for ANOVA and post hoc Dunnett’s t-test. Differences between groups were considered significant at *P* < 0.001 and *P* < 0.05 levels.

3. Results and Discussion

The physicochemical properties revealed that the plant was tall and erect, with medium green spike-like foliage and white inflorescence. The successive extract value of ethyl acetate, methanol and water extracts were found to be 3.10%, 26.12%, and 14.6%. Total ash value of plant material indicated that the amount of minerals and earthy material attached to the plant material and its value was calculated to be 9.702% w/w. The amount of the acid insoluble siliceous matter present in the plant was 8.826% w/w. The water soluble extractive value indicated the presence of sugar, acids, and inorganic compounds. The alcohol soluble extractive values indicated the presence of polar constituents and its value was found to be 1.427% w/w. The value for residue on ignition was 0.963% w/w. The pH values of 1% and 10% solutions were 6.18 and 6.02, respectively. The value for loss on drying was found
Table 1: General physicochemical parameters of *Eremurus himalaicus*.

<table>
<thead>
<tr>
<th>Test parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Medium green foliage, white inflorescence, slightly bitter in taste</td>
</tr>
<tr>
<td>Loss on drying at 105°C</td>
<td>1.419% w/w</td>
</tr>
<tr>
<td>Successive extract value</td>
<td></td>
</tr>
<tr>
<td>Petrol</td>
<td>2.30%</td>
</tr>
<tr>
<td>EtOAc</td>
<td>3.10%</td>
</tr>
<tr>
<td>MeOH</td>
<td>26.12%</td>
</tr>
<tr>
<td>Aqueous</td>
<td>14.60%</td>
</tr>
<tr>
<td>Total ash</td>
<td>9.702% w/w</td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>8.826% w/w</td>
</tr>
<tr>
<td>Water soluble ash</td>
<td>1.427% w/w</td>
</tr>
<tr>
<td>Residue on ignition/sulphated ash</td>
<td>0.963% w/w</td>
</tr>
<tr>
<td>pH of 1.00% w/v soln.</td>
<td>6.18</td>
</tr>
<tr>
<td>pH of 10.00% w/v soln.</td>
<td>6.02</td>
</tr>
</tbody>
</table>

Table 2: Phytochemical analysis of extracts of *Eremurus himalaicus*.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>−</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Phenolics</td>
<td>+</td>
</tr>
</tbody>
</table>

+ sign indicates secondary metabolite is present; − sign indicates secondary metabolite is not present.

Phytochemical investigation of different extracts of *Eremurus himalaicus* revealed the presence of alkaloids, tannins, saponins, terpenoids, flavonoids, phenolics, and cardiac glycosides as secondary metabolites (Table 2). Many of these compounds have been shown to produce potent hypoglycaemic, anti hyperglycaemic, and glucose suppressive activities [15, 16]. These effects might be achieved by facilitating insulin release from beta pancreatic cells, inhibiting glucose absorption in gut, stimulating glycogenesis in liver and/or increasing glucose utilization by the body [15–18].

Acute toxicity studies revealed that the EtOAc, MeOH, and aqueous extracts of *Eremurus himalaicus* were safe up to 2000 mg/kg of body weight and approximate LD₅₀ is more than 2000 mg/kg.

60 min prior administration of the extracts (500 mg/kg of body weight) followed by the glucose load did not allow the blood glucose level to go higher as compared to the normal ones (Table 3). The effect was comparable to that of the standard antidiabetic drug, glibenclamide. Maximum effect was observed for aqueous and MeOH extracts.

The evaluation of the effect of extracts on blood glucose levels of normoglycaemic rats revealed the results that are in accordance with that of the results obtained for oral glucose tolerance test; that is, the aqueous extract (250 mg/kg and 500 mg/kg; \( P < 0.001 \)) followed by MeOH extract (500 mg/kg; \( P < 0.05 \)) showed significant decrease in the fasting blood glucose levels of the rats; however, the ethyl acetate extract (250 mg/kg and 500 mg/kg) and the MeOH extract (250 mg/kg) did not show significant lowering in the blood glucose levels of the rats (Table 4). The maximum reduction was shown by standard followed by aqueous extract which might be due to the presence of saponin glucosides that are soluble in water and have a glucagon lowering effect, therefore, might enhance glucose utilization [19]. Another reason for the plasma glucose lowering action may be due to the decreased gluconeogenesis, which appears to be related to the antioxidant properties of the plant extract [20]. Interference with the absorption of dietary carbohydrates in the small intestine and facilitation of utilization of glucose by peripheral tissues mediated by an insulin dependent glucose transporter can be another reason for the hypoglycaemic nature of the aqueous extract of *Eremurus himalaicus* plant [21, 22]. The hypoglycemic effect may also be due to the presence of insulin-like substance found in various plants [23].

4. Conclusions

The findings of this study indicate the presence of various phytochemicals in the plant extracts, which may be responsible for the pharmacological activity [24, 25]. The aqueous extract is most potent in decreasing the blood glucose levels in normal rats and it might be producing this effect by a mechanism independent from the insulin secretion, for example, by the inhibition of endogenous glucose production [26] or by the inhibition of intestinal glucose absorption [27].

Extracts of *Eremurus himalaicus* appear to be attractive materials for further studies leading to possible drug development for diabetes which is relatively inexpensive and less time consuming and more suited to our economic conditions than allopathic drug development.
The authors declare that there is no conflict of interests.

Data represented as mean ± S.D values of 6 animals each. *P < 0.001 and **P < 0.05 (Dunnett t-test); diabetic control was compared with the normal; extract and standard treated groups were compared with the diabetic control.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References


Research Article

Fenugreek Seed Extract Inhibit Fat Accumulation and Ameliorates Dyslipidemia in High Fat Diet-Induced Obese Rats

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1. Introduction

Obesity is a chronic disorder of carbohydrate and lipid metabolism and is characterized by an increased fat deposition in adipose tissue and other internal organs [1]. Obesity leads to the development of insulin resistance, type-2 diabetes, coronary heart disease, cancer, respiratory disease, and osteoarthritis [2]. During the past 3–4 decades, changes in the food system seem to be the major drivers of the rise of the global epidemic of obesity [3]. As per the World Health Organization (WHO), in 2008, more than 1.4 billion adults (20 years of age and older) were overweight and amongst these over 200 million men and nearly 300 million women were obese [4].

Over the years, many medications have been used to manage obesity but most of them are now withdrawn due to their serious adverse effects [5]. Currently, orlistat is the only Food and Drug Administration (FDA) approved drug for long term management of obesity but this drug has undesirable gastrointestinal side effect such as steatorrhea [6]. In the face of this unmet medical need, there is requirement of new potential antiobesity drug to combat this syndrome.

Fenugreek (Trigonella foenum-graecum L.; TFG) belongs to the family Fabaceae and is used in many parts of the world for the treatment of diabetes. TFG seeds are used as an active ingredient in weight loss and anticholesterol ayurvedic formulation Ayurslim (The Himalaya Drug Company, Bangalore, India). TFG seeds have been shown to possess hypoglycemic, hypolipidemic, and antioxidant effects [7].

Dietary supplemented protein and amino acids have been shown to reduce plasma lipid levels [8, 9]. The presence of proteins and fiber in TFG seeds offers high nutritive value as it contains approximately 26% protein and 48% fiber and might exert a lipid lowering effect [10]. Dietary fibers (galactomannan) in fenugreek seeds are polysaccharides consisting of a mannose backbone with galactose side chains attached at position C6. Galactomannan consist of linear...
chains of (1–4) diequatorially linked D-mannose residues; some contain single-sugar side chains of D-galactose attached by (1–6) glycosidic bonds [11]. Fenugreek galactomannan form a viscous gel in the intestine and inhibit glucose and lipid absorption [12].

Oxidative stress is a biochemical disequilibrium occurring due to enhanced generation of reactive oxygen species (ROS). Elevated oxidative stress could be responsible for the initiation of metabolic disorders due to alterations in the membrane lipids and proteins [13]. In epidemiological studies, polyphenol and flavonoids-rich extract have been shown to possess the hypcholesterolaemic effect due to their antioxidant defense [14].

As plant proteins, dietary fiber and polyphenols do possess health benefits in metabolic disorders; therefore, in the present study, the inhibitory effect fenugreek extract was evaluated on fat accumulation and dyslipidemia in high fat diet-induced obese rats.

2. Materials and Methods

2.1. Plant Materials and Preparation of Extract. Trigonella foenum-graecum seeds were purchased from Indian Council for Agricultural Research (ICAR), New Delhi, India (ref. NISCAIR/RHMD/Consult/-2011-12/1743/43). The seeds were dried at 40°C and ground to a powder. The aqueous extract of *Trigonella foenum-graecum* seeds (AqE-TFG) was prepared as follows: powdered seeds were soaked in hot distilled water (1:20 w/v) and left overnight. The solution was filtered and lyophilized.

2.2. Electrophoretic Fingerprinting of Proteins in AqE-TFG. AqE-TFG was dissolved in phosphate buffer saline (PBS; pH 7.4; 1:10 w/v) and centrifuged (15,000 × g, 30 min). The clear supernatant was dialyzed against PBS at 4°C (5000 cutoff membrane; with fresh buffer changes every 6 h, 4 times). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out as described by Laemmli [15], using 12% resolving gel and 5% stacking gel. Protein bands were stained with Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (5:1:5 v/v/v) and decolorized in 7% acetic acid. Molecular weight of different bands was calibrated with the standard proteins markers: phosphorylase b (molecular weight, 97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.3. Amino Acid Analysis from AqE-TFG. The precolumn derivatisation of amino acid in AqE-TFG was performed with 6-aminouquinolyl-N-hydroxyl succinimidyl carbamate (AQC) and the concentration of amino acid was analysed by reverse phase high performance liquid chromatography (HPLC) (Waters Co., Milford, MA, USA). The excitation and emission wavelengths for fluorescence detector were 250 nm and 395 nm, respectively. The gain setting of the detector was 1, and the 5 μL of samples was injected onto Inertsil ODS-3V column (250×4.6 mm, 4 μm) with the temperature controlled at 37°C and operated with a flow rate of 1.0 mL/min. The linear gradient elution system was used. Mobile phases A, B, and C were acetate buffer (pH 5.05) solution, acetonitrile, and water, respectively. The linear gradient conditions of solvents were modified as follows: initial, 100% A (acetate buffer); 0.5 min, 99% A, 1% B (HPLC grade acetonitrile); 18 min, 95% A, 5% B; 29.5 min, 83% A, 17% B; 33 min, 60% B, 40% C (Milli-Q water); 36 min, 100% A; 65 min, 60% B, 40% C; and 100 min, 60% B, 40% C.

2.4. Determination of Galactomannan in AqE-TFG. AqE-TFG was solubilized in water (1:20, w/v) at 4°C for 3 hours and centrifuged (16,000 × g, 30 min). The supernatant was mixed with absolute ethanol (1:1, v/v) to precipitate galactomannan [12].

2.5. Determination of Phenolic and Flavonoid Contents in AqE-TFG. Phenolic contents of the extract were determined as follows: 50 μL aliquot of extract was assayed with 250 μL of Folin-Ciocalteu reagent and 500 μL of sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 min at room temperature, the absorbance was read at 765 nm [16].

Flavonoid contents of the extract were determined as follows: 0.5 mL of sample; 0.5 mL of 2% AlCl₃ (w/v) in 95% ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm [17].

2.6. Experimental Design. The guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, were followed and prior permission was sought from the Institutional Animal Ethics Committee (IAEC), Hamdard University (registration number 173/CPCSEA; protocol number 773), New Delhi, India, for conducting the study.

The female Wistar rats (150–200 g) were housed in polypropylene cages under controlled conditions (room temperature 25 ± 2°C, air humidity 50 ± 15%, and photoperiod of 12 h light: dark cycle), and water was provided ad libitum throughout the study. After acclimatization to the environment for one week, the animals were randomly divided into 5 groups (n = 10 rats in each group). Normal control rats (Group 1) were fed with normal pellet diet (NPD), while the others (Groups 2, 3, 4, and 5) were fed with high fat diet (HFD) ad libitum for 28 days. From day 8 to 28, Groups 3 and 4 were treated with AqE-TFG (0.5 and 1.0 g/kg body weight (b.w.), orally) while Group 5 was treated with orlistat (10 mg/kg b.w., orally). The solutions of AqE-TFG and orlistat were freshly prepared using normal saline. The compositions of NPD (Amrut rat feed, Mfd. by Nav Maharashtra Chakan Oil Mills Ltd., Delhi, India) and HFD (National Institute of Nutrition, Hyderabad, India) are shown in Table 1.

2.7. Collection of Serum and Tissues. On day 29, blood was collected from the retroorbital plexus of overnight fasted rats under light ether anaesthesia and serum was separated by centrifugation at 3000 rpm for 15 min. Then, the rats were sacrificed. The organs (heart and liver) and white adipose tissue (WAT) (mesenteric, gonadal, and retroperitoneal) were
excised, rinsed in ice-cold physiological saline, and weighed. The serum and organ samples were stored at −20°C until analysis.

2.8. Determination of Anthropometric Parameters. Body weight gain was calculated as a difference of initial and final weight of animals. Body mass index (BMI) was calculated from the formula BMI = body weight (Kg)/length$^2$ (m$^2$) [18]. Food and water intakes were measured daily for a period of 28 days and the average of food and water consumption was calculated. Blood pressure (systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP)) and heart rate (HR) were measured with a noninvasive blood pressure recorder using the rat tail-cuff method (Kent Scientific Corporation).

2.9. Determination of Serum Biochemistries. The blood glucose, serum triglycerides (TGs), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) concentrations, aspartate amino transferase (AST), and alanine aminotransferase (ALT) activities were assayed by enzymatic methods using commercial assay kits (Span Diagnostics Ltd., Surat, India) according to the manufacturer’s protocol. The serum lactate dehydrogenase (LDH) concentration was determined using an enzymatic kit (Reckon Diagnostics Pvt. Ltd., Baroda, India). Rat insulin immunoassay ELISA kit (Crystal Chem, Inc., IL, USA), rat leptin immunoassay ELISA kit (Ray Biotech Inc., GA, USA), rat adiponectin ELISA kit (Ray Biotech Inc., GA, USA), apo-B immunoturbidimetric immunoassay kit (Randox Laboratories Ltd., UK), and lipase kit (BioAssay Systems, CA, USA) were used to measure the serum insulin, leptin, adiponectin, apo-B, and lipase concentrations.

Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) levels were estimated using Friedewald’s equation: LDL-C=TC−HDL-C−VLDL-C; VLDL-C = TGs/5 [19]. The atherogenic index (AI) and coronary risk index (CRI) were calculated as log (TG/HDL-C) [20] and TC/HDL-C [21], respectively.

Homeostasis model assessment for insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) were calculated as fasting glucose (mg/dL) × fasting insulin (μU/mL)/2430 [22] and 1/[log insulin (μU/mL) + log glucose (mg/dL)] [23], respectively.

2.10. Determination of Tissue Biochemistries. The thiobarbituric acid reactive substances (TBARS) [24] and antioxidant enzymes activities, that is, reduced glutathione [25], superoxide dismutase (SOD) [26], and catalase (CAT) [27], were measured in hepatic and cardiac tissues. The fatty acid synthase (FAS) [28] and glucose-6-phosphate dehydrogenase (G6PD) [29] activities were assayed in hepatic and uterine WAT.

2.11. Histopathological Analysis. For histological examination, the liver tissue was collected, fixed in 10% neutral buffered formalin, and embedded in paraffin. Standard sections of 5μm thickness were cut and stained with hematoxylin and eosin (H & E). The slides were examined by light microscopy.

2.12. Statistical Analysis. The data are expressed as means ± S.E.M. All statistical analyses were performed using GraphPad InStat version 3.06 (Graph Pad Software). All data were analyzed using 1-way analysis of variance (ANOVA) followed by Dunnett’s test. Results were considered statistically significant when P < 0.05.

3. Results

3.1. Chemical Compositions of AqE-TFG. The electrophoretic fingerprinting of AqE-TFG revealed that AqE-TFG contains proteins of molecular weight from 14.4kDa to 97.4kDa wherein thick protein bands lie <66.2kDa (Figure 1). The contents of amino acid, phenolic, flavonoid, and galactomanan in AqE-TFG are summarized in Table 2.

3.2. Effect of AqE-TFG on Anthropometric Parameters. The feeding of HFD for 28 days caused a significant (P < 0.01) increase in body weight gain and BMI of rats, in comparison with the NPD control rats. Treatment with AqE-TFG (0.5 and 1.0g/kg) or orlistat (10 mg/kg) for 21 days significantly (P < 0.05 or P < 0.01) suppressed the increase in the body weight gain and BMI of HFD-fed rats. Despite variation in body weight gain and BMI, there was no significant difference in food intake and water intake amongst all groups (Table 3).

A statistically significant (P < 0.01) increase in SBP, DBP, and MAP was observed in the HFD control group than those in NPD group. AqE-TFG (0.5 and 1.0g/kg) or orlistat (10 mg/kg) treatment significantly (P < 0.01) decreased the elevated SBP, DBP, and MAP in HFD-fed rats (Table 4).

The weights of WAT (mesenteric, gonadal, and retroperitoneal) and organ (heart and liver) were significantly (P < 0.01) higher in HFD control group than those in NPD group. AqE-TFG (0.5 and 1.0g/kg) or orlistat (10 mg/kg) treatment significantly (P < 0.05 or P < 0.01) lowered the WAT as well.

### Table 1: Compositions of the normal pellet diet (NPD) and high fat diet (HFD) fed to rats (g/kg).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>NPD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>212.0</td>
<td>342.0</td>
</tr>
<tr>
<td>L-cystine</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Starch</td>
<td>439.0</td>
<td>172.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>172.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Ground nut oil</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tallow</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>100.0</td>
<td>—</td>
</tr>
<tr>
<td>Soya bean oil</td>
<td>50.0</td>
<td>—</td>
</tr>
<tr>
<td>AIN salt mix</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>AIN vitamin mix</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Total (g)</td>
<td>999.0</td>
<td>999.0</td>
</tr>
</tbody>
</table>
Figure 1: Electrophoretic profile of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) proteins. Lane 1 indicates AqE-TFG proteins of molecular weight from 14.4 kDa to 97.4 kDa wherein thick protein bands lie < 66.2 kDa; Lane M indicates standard molecular weight markers (kDa).

### Table 2: Chemical compositions of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG).

<table>
<thead>
<tr>
<th>Compositions</th>
<th>AqE-TFG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids (mg/g)</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>24.30</td>
</tr>
<tr>
<td>Serine</td>
<td>13.70</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>30.50</td>
</tr>
<tr>
<td>Glycine</td>
<td>04.00</td>
</tr>
<tr>
<td>Histidine</td>
<td>32.40</td>
</tr>
<tr>
<td>Arginine</td>
<td>42.20</td>
</tr>
<tr>
<td>Threonine</td>
<td>16.70</td>
</tr>
<tr>
<td>Alanine</td>
<td>07.70</td>
</tr>
<tr>
<td>Proline</td>
<td>10.70</td>
</tr>
<tr>
<td>Cystine</td>
<td>27.50</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>08.80</td>
</tr>
<tr>
<td>Valine</td>
<td>07.60</td>
</tr>
<tr>
<td>Methionine</td>
<td>02.60</td>
</tr>
<tr>
<td>Lysine</td>
<td>03.10</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>09.50</td>
</tr>
<tr>
<td>Leucine</td>
<td>13.10</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10.40</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>04.90</td>
</tr>
<tr>
<td>Galactomannan (%; w/w)</td>
<td>32.5</td>
</tr>
<tr>
<td>Phenolic contents (mg/g)</td>
<td>23.12 ± 0.58</td>
</tr>
<tr>
<td>Flavonoid contents (mg/g)</td>
<td>2.55 ± 0.12</td>
</tr>
</tbody>
</table>

**3.3. Effect of AqE-TFG on Serum Biochemistries.** Table 6 represents the lipid profile of experimental rats. Rats in HFD control group displayed a significant ($P < 0.01$) increase in the levels of TC, TGs, LDL-C, and VLDL-C and a significant ($P < 0.01$) decrease in HDL-C in comparison with NPD group. Similar with the orlistat-treated HFD-fed group, AqE-TFG (0.5 and 1.0 g/kg) administration showed a significant ($P < 0.01$) decrease in the levels of TC, TGs, LDL-C, and VLDL-C or a significant increase in the level of HDL-C after 21-day treatment when compared with HFD control group. The cardiac risk indexes (AI and CRI) were significantly ($P < 0.01$) increased in HFD control group when compared to NPD group, while treatment with AqE-TFG (0.5 and 1.0 g/kg) or orlistat (10 mg/kg) caused a significant reduction in the AI and CRI levels compared to HFD control group.

The HFD-induced obese rats exhibited a significant ($P < 0.01$) increase in the levels of blood glucose, serum insulin, leptin, apo-B, and HOMA-IR as compared to NPD-fed rats. After AqE-TFG (0.5 and 1.0 g/kg) or orlistat (10 mg/kg) treatment for 21 days, the increases of blood glucose, serum insulin, leptin, apo-B, and HOMA-IR were significantly brought down towards normal in HFD-fed rats (Table 7).

A statistically significant ($P < 0.01$) decrease in adiponectin levels and QUICKI was observed in the HFD control group than those in NPD group. AqE-TFG (0.5 and 1.0 g/kg) or orlistat (10 mg/kg) treatment significantly ($P < 0.01$) elevated the reduced levels of adiponectin and QUICKI compared to HFD control group (Table 7).

The serum lipase, LDH, AST, and ALT levels were significantly ($P < 0.01$) increased in HFD control group, when compared to the NPD group. A significant ($P < 0.01$) reduction in lipase, LDH, AST, and ALT was observed in AqE-TFG (0.5 and 1.0 g/kg) or orlistat (10 mg/kg) treated group in comparison to the HFD control group (Table 8).

**3.4. Determination of Tissue Biochemistries.** Table 9 represents the lipogenic enzyme activities of the hepatic and uterine WAT. The HFD control group showed a significant ($P < 0.01$) elevation in levels of lipogenic enzyme (FAS and G6PD) as compared to the NPD control group. Treatment with AqE-TFG (0.5 and 1.0 g/kg) or orlistat (10 mg/kg) for a period of 21 days caused a significant ($P < 0.01$) reduction in levels of FAS and G6PD, respectively, as compared to the HFD control group.

Table 10 represents the oxidative stress marker in hepatic and cardiac tissue. A marked increase of TBARS production and decrease of antioxidant enzyme status (GSH, SOD, and CAT) were observed in the hepatic and cardiac tissue of rats in HFD control group when compared with NPD group. AqE-TFG or orlistat treatment significantly ($P < 0.01$) decreases the TBARS levels and raises the antioxidant enzyme (GSH, SOD, and CAT) activity.

**3.5. Hepatic Histopathology.** Liver histopathologies are shown in Figure 2. The histopathological examination of the NPD control group showed normal cell architecture, while HFD control group showed significant morphological changes with greater hepatic lipid accumulation and fatty degeneration as compared to NPD control rats. On the other hand, treatment with AqE-TFG (0.5 and 1.0 g/kg) or orlistat (10 mg/kg) for a period of 21 days in HFD-fed rats lowered...
Figure 2: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on hepatic histopathological changes in high fat diet- (HFD-) induced obese rats. Group 1 (a): NPD control, showing normal liver architecture and hepatocytes; Group 2 (b): HFD control, showing fatty degeneration and greater hepatic lipid accumulation; Group 3 (c): HFD + AqE-TFG (0.5 g/kg, b.w.), showing mild congestion, no fatty changes, and less hepatic lipid accumulation; Group 4 (d): HFD + AqE-TFG (1.0 g/kg, b.w.), showing no fatty changes and considerably lower hepatic lipid accumulation; Group 5 (e): HFD + orlistat (10 mg/kg, b.w.), showing no fatty changes with lower hepatic lipid accumulation.
Table 3: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on body weight gain, food intake, water intake, BMI, and adiposity index in high fat diet- (HFD-) induced obese rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight gain (g)</th>
<th>Food intake (g/day)</th>
<th>Water intake (mL/day)</th>
<th>BMI (kg/m²)</th>
<th>Adiposity index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>54.60 ± 3.57</td>
<td>15.64 ± 1.07</td>
<td>37.10 ± 0.68</td>
<td>4.23 ± 0.07</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td>HFD control</td>
<td>122.93 ± 8.03**</td>
<td>17.05 ± 0.92</td>
<td>40.53 ± 1.04</td>
<td>5.92 ± 0.27**</td>
<td>2.24 ± 0.08**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (0.5 g/kg)</td>
<td>103.17 ± 5.72</td>
<td>16.40 ± 0.73</td>
<td>38.42 ± 0.85</td>
<td>5.16 ± 0.15**</td>
<td>1.97 ± 0.03**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (1 g/kg)</td>
<td>90.52 ± 4.05**</td>
<td>14.79 ± 0.60</td>
<td>37.98 ± 1.21</td>
<td>4.89 ± 0.08**</td>
<td>1.80 ± 0.06**</td>
</tr>
<tr>
<td>HFD + orlistat (10 mg/kg)</td>
<td>83.76 ± 4.63**</td>
<td>15.95 ± 0.84</td>
<td>39.00 ± 1.36</td>
<td>4.72 ± 0.23**</td>
<td>1.74 ± 0.04**</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. (*n* = 10); **P < 0.01 compared to normal control; *P < 0.05 and **P < 0.01 compared to HFD control.

Table 4: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on blood pressure in high fat diet- (HFD-) induced obese rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>HR (beats/min)</th>
<th>SBP (mm Hg)</th>
<th>DBP (mm Hg)</th>
<th>MAP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>428.5 ± 14.34</td>
<td>126.4 ± 3.40</td>
<td>92.3 ± 5.04</td>
<td>103.67 ± 3.55</td>
</tr>
<tr>
<td>HFD control</td>
<td>530.7 ± 26.39**</td>
<td>153.3 ± 2.16**</td>
<td>112.8 ± 3.54**</td>
<td>126.3 ± 3.02**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (0.5 g/kg)</td>
<td>492.2 ± 18.95</td>
<td>136.5 ± 4.94*</td>
<td>97.4 ± 3.60*</td>
<td>110.43 ± 3.58**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (1 g/kg)</td>
<td>476.1 ± 15.32</td>
<td>134.9 ± 3.87*</td>
<td>96.2 ± 2.63*</td>
<td>109.10 ± 1.98**</td>
</tr>
<tr>
<td>HFD + orlistat (10 mg/kg)</td>
<td>464.5 ± 21.79</td>
<td>131.1 ± 5.63**</td>
<td>94.6 ± 3.35**</td>
<td>106.77 ± 2.40**</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. (*n* = 10); **P < 0.01 compared to normal control; *P < 0.05 and **P < 0.01 compared to HFD control.

Table 5: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on organ weight on white adipose tissue (WAT) weight in high fat diet- (HFD-) induced obese rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Organ's weight (g)</th>
<th>WAT weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Liver</td>
</tr>
<tr>
<td>Normal control</td>
<td>0.71 ± 0.02</td>
<td>7.13 ± 0.24</td>
</tr>
<tr>
<td>HFD control</td>
<td>0.98 ± 0.06**</td>
<td>10.58 ± 0.46**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (0.5 g/kg)</td>
<td>0.84 ± 0.03*</td>
<td>9.04 ± 0.30**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (1 g/kg)</td>
<td>0.80 ± 0.04*</td>
<td>8.20 ± 0.17**</td>
</tr>
<tr>
<td>HFD + orlistat (10 mg/kg)</td>
<td>0.78 ± 0.02**</td>
<td>7.86 ± 0.22**</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. (*n* = 10); **P < 0.01 compared to normal control; *P < 0.05 and **P < 0.01 compared to HFD control.

Table 6: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on serum lipid levels in high fat diet (HFD)-induced obese rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
<th>VLDL-C (mg/dL)</th>
<th>AI</th>
<th>CRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>99.07 ± 2.84</td>
<td>68.32 ± 3.56</td>
<td>32.65 ± 2.25</td>
<td>52.76 ± 4.26</td>
<td>13.66 ± 0.71</td>
<td>0.32 ± 0.03</td>
<td>3.22 ± 0.30</td>
</tr>
<tr>
<td>HFD control</td>
<td>156.36 ± 4.00**</td>
<td>148.90 ± 5.29**</td>
<td>20.19 ± 1.95**</td>
<td>106.38 ± 5.45**</td>
<td>29.78 ± 1.06**</td>
<td>0.89 ± 0.05**</td>
<td>8.70 ± 0.79**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (0.5 g/kg)</td>
<td>133.77 ± 2.45**</td>
<td>103.47 ± 4.70**</td>
<td>29.84 ± 1.62**</td>
<td>83.24 ± 2.99**</td>
<td>20.69 ± 0.94**</td>
<td>0.54 ± 0.04**</td>
<td>4.68 ± 0.36**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (1 g/kg)</td>
<td>118.54 ± 3.20**</td>
<td>83.15 ± 3.86**</td>
<td>32.91 ± 1.56**</td>
<td>70.20 ± 4.04**</td>
<td>16.43 ± 0.77**</td>
<td>0.43 ± 0.03**</td>
<td>3.85 ± 0.27**</td>
</tr>
<tr>
<td>HFD + Orlistat (10 mg/kg)</td>
<td>112.78 ± 4.32**</td>
<td>76.02 ± 4.93**</td>
<td>33.74 ± 2.18**</td>
<td>63.84 ± 5.42**</td>
<td>15.21 ± 0.98**</td>
<td>0.36 ± 0.05**</td>
<td>3.64 ± 0.45**</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. (*n* = 10); **P < 0.01 compared to normal control; *P < 0.05 and **P < 0.01 compared to HFD control.

hepatic lipid accumulation as well as fatty degeneration as compared to HFD control rats, respectively.

4. Discussion

In the present study, consumption of high caloric intake in form of HFD for a period of 28 days induces obesity in rats. BMI is widely used to measure body fat and highly correlated with body fat stores [30]. In our study, a significant reduction in body weight gain and BMI with AqE-TFG treatment indicates that AqE-TFG suppresses the HFD-mediated increase in body weight gain and WAT weight. Despite a significant difference in body weight between the NPD control and HFD control groups, there was no significant difference in the food intake and water intake.
and adiponectin levels in high-fat diet (HFD-) induced obese rats. Results are expressed as mean ± S.E.M. (n = 10); **P < 0.01 compared to normal control; *P < 0.05 and ***P < 0.01 compared to HFD control.

Table 8: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on serum lipase, lactate dehydrogenase (LDH), aspartate amino transferase (AST), and alanine amino transferase (ALT) levels in high fat diet- (HFD-) induced obese rats.

Table 9: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on lipogenic enzymes levels in hepatic and uterine WAT in high fat diet- (HFD-) induced obese rats.

Leptin is a hormone secreted from adipose tissue and regulates appetite and adiposity. With the increase in adipose tissue weight, serum leptin levels also tend to increase [31]. In the present study, the reduction of leptin levels indicated that AqE-TFG treatment caused significant adipocytes loss. This observation was further supported by the AqE-TFG mediated reductions in WAT weights and adiposity index (sum of the weight of the mesenteric, gonadal and retroperitoneal WAT, divided by the weight of the animal, and expressed as percentage) of HFD-fed rats.

Obesity is an independent risk factor for cardiovascular disease (CVD), through its influence on other known risk factors such as dyslipidemia and hypertension [32]. Apo-B is synthesized in liver and indicates the amount of atherogenic lipoproteins in plasma or hepatic tissue, and it is considered as a better predictor of coronary heart disease [33]. In our study, administration of AqE-TFG caused a significant reduction in serum lipids (TC, TGs, LDL-C, and VLDL-C), cardiac risk indexes (AI and CRI), and apo-B levels which may be considered as a better indicator for improvement in risk of coronary heart disease. Further, it is well established that dyslipidemia and hypertension are the risk factors for coronary heart disease [34]. Therefore, cardiovascular risk can be minimised through regulation of dyslipidemia and hypertension. The reduction in the SBP, DBP, and MAP with AqE-TFG indicates that AqE-TFG may be a potential candidate in management of dyslipidemia-induced cardiac complications.

With the increase in adipose tissue fat deposits, as in obesity, the ability of insulin to stimulate glucose transport and metabolism in adipocytes and skeleton muscle is impaired resulting in insulin resistance [35]. Impairment in insulin sensitivity led to dyslipidemia [36]. Further, small adipocytes are more sensitive to insulin than the large adipocytes [37]. The AqE-TFG mediated reduction in HOMA-IR and elevation in QUICKI indicated the improvement in insulin
Table 10: Effect of aqueous extract of *Trigonella foenum-graecum* (AqE-TFG) on hepatic and cardiac thiobarbituric acid reactive substance (TBARS) and antioxidant enzymes (GSH, SOD, and CAT) levels in high fat diet- (HFD-) induced obese rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (nmol/mg protein)</th>
<th>GSH (µmol of P liberated/min/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (nmol of H₂O₂/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Heart</td>
<td>Liver</td>
<td>Heart</td>
</tr>
<tr>
<td>Normal control</td>
<td>0.20 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>27.15 ± 1.49</td>
<td>22.50 ± 1.07</td>
</tr>
<tr>
<td>HFD control</td>
<td>0.68 ± 0.03**</td>
<td>0.52 ± 0.04**</td>
<td>9.87 ± 0.82**</td>
<td>8.36 ± 1.51**</td>
</tr>
<tr>
<td>HFD + AqE-TFG (0.5 g/kg)</td>
<td>0.36 ± 0.04**</td>
<td>0.29 ± 0.02**</td>
<td>20.46 ± 2.01**</td>
<td>17.05 ± 1.83*</td>
</tr>
<tr>
<td>HFD + AqE-TFG (1 g/kg)</td>
<td>0.31 ± 0.02**</td>
<td>0.25 ± 0.02**</td>
<td>25.30 ± 1.14**</td>
<td>19.85 ± 2.30**</td>
</tr>
<tr>
<td>HFD + orlistat (10 mg/kg)</td>
<td>0.23 ± 0.01**</td>
<td>0.21 ± 0.01**</td>
<td>23.65 ± 2.26**</td>
<td>21.17 ± 2.58**</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.E.M. (n = 10); **P < 0.01 compared to normal control; *P < 0.05 and **P < 0.01 compared to HFD control.
sensitivity, which is due to depletion of the adipose tissue triglycerides stores that ultimately results in reductions of lipid levels.

Adiponectin (an adipose-tissue-derived hormone) plays an important role in the regulation of lipid metabolism and insulin sensitivity and also possesses anti-inflammatory and antiatherogenic properties. Adiponectin exhibits insulin sensitizing effect, in part via AMPK activation in peripheral tissues that led to stimulation of fatty acid oxidation and glucose uptake in skeletal muscle, and suppression of glucose production in liver. Circulating adiponectin levels are negatively correlated with obesity, particularly visceral obesity and insulin resistance [38]. In the present study, higher adiponectin levels with AqE-TFG treatment indicated a protective role in the development of metabolic disorders.

The feeding of high fat diet results in excess hepatic triglycerides accumulation due to increased synthesis and decreased secretion of triglycerides and increased de novo lipogenesis [39]. Dietary lipids cannot absorb from the intestinal linings without undergoing hydrolysis by lipase. Inhibition of lipase activity leads to decrease in intestinal lipid digestion and absorption [12]. In the present study, similar to orlistat treated group, the reduced lipase activity with AqE-TFG treatment indicated an inhibitory effect on intestinal dietary fat absorption that leads to decreased triglycerides accumulation in various tissue including liver and WAT. This finding was further supported by the hepatic histopathological studies that showed reductions in hepatic lipid accumulation, fatty degeneration with AqE-TFG, and orlistat treatment.

It is reported that the levels of hepatic and cardiac markers such as ALT, AST, and LDH tend to increase in obesity [40]. The elevated levels of these enzymes in our study indicated that HFD control rats are prone to hepatic and cardiac complications. Treatment with AqE-TFG for a period of 21 days significantly reduced the increased levels of LDH, AST, and ALT.

Factors like enzymes, nutritional conditions, and hormones control the process of fat synthesis and fat breakdown [41]. Elevated lipogenesis is strongly associated with obesity, fatty liver disease, insulin resistance, and type-2 diabetes [42]. Fatty acid synthase (FAS) is responsible for the synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH [43]. Enzymes of the pentose phosphate pathway including glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) produce NADPH which is essential for the biosynthesis of fatty acid and cholesterol [44]. Therefore, modulation of the activities of liver and epididymal WAT lipogenic enzyme (FAS and G6PD) with AqE-TFG treatment indicated the reduced availability of NADPH required for the biosynthesis of fatty acid and cholesterol, leading to rapid decline in fat stores of HFD-fed rats.

Abnormalities in lipid metabolism decrease the strength of the antioxidative defenses [45]. The correlation found between dyslipidemia and oxidative stress in this study shows that dyslipidemia induced by ingestion of high fat diet is the primary cause of lipid peroxidation. Therefore, the possible reason for improvement in dyslipidemia with AqE-TFG may be due to reduction in oxidative stress in HFD-fed rats. In the present study, HFD-induced dyslipidemic rats showed decreased activities of GSH, SOD, and CAT enzymes; therefore, it may be concluded that HFD causes the induction of oxidative stress in the hepatic and cardiac tissue and may lead to the consequences like fatty liver disease and atherosclerosis. Administration of AqE-TFG for a period of 21 days resulted in significant reduction in lipid peroxides levels and elevation in antioxidant enzymes GSH, SOD, and CAT.

Earlier, proteins, galactomannan, and polyphenols from fenugreek seeds have been reported to regulate dyslipidemia in obese and diabetic rodents [12, 14, 46]. Therefore, in our study, the presence of these phytoconstituents justified the fact that AqE-TFG effectively inhibit fat accumulation and ameliorate dyslipidemia in HFD-obese rats, which is due to prevention of impaired lipid digestion and absorption, in addition to improvement in glucose and lipid metabolism, enhancement of insulin sensitivity, increased antioxidant defense, and downregulation of lipogenic enzymes.

**Abbreviations**

(AqE-TFG): Aqueous extract of *Trigonella foenum-graecum* seeds  
(HFD): High fat diet  
(NPD): Normal pellet diet  
(BMI): Body mass index  
(WAT): White adipose tissue  
(a apo-B): Apolipoprotein-B  
(HOMA-IR): Homeostasis model assessment for insulin resistance  
(CVD): Cardiovascular disease  
(AST): Aspartate amino transferase  
(ALT): Alanine amino transferase  
(LDH): Lactate dehydrogenase  
(FAS): Fatty acid synthetase  
(G6PD): Glucose-6-phosphate dehydrogenase  
(TBARS): Thiobarbituric acid reactive substance  
(GSH): Glutathione  
(SOD): Superoxide dismutase  
(CAT): Catalase  
(FAO): World Health Organization  
(FDA): Food and Drug Administration  
(ICAR): Indian Council for Agricultural Research  
(PBS): Phosphate buffer saline  
(AQC): 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate  
(HPLC): High performance liquid chromatography  
(CPCSEA): Committee for the Purpose of Control and Supervision of Experiments on Animals  
(AIEC): Institutional Animal Ethics Committee  
(HR): Heart rate  
(SBP): Systolic blood pressure

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Research Article

Role of Dried Fruits of Carissa carandas as Anti-Inflammatory Agents and the Analysis of Phytochemical Constituents by GC-MS

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Inflammation plays an important role in various diseases with high prevalence within populations such as rheumatoid arthritis, ulcer, atherosclerosis, and asthma. Many drugs are available in the market for inflammatory diseases. They exhibit several unwanted side effects to humans. Therefore, alternative treatments with safer compounds are needed. Carissa carandas plant is used in traditional medicinal system for its various diseases curing property. In the present study, we examined the anti-inflammatory effects of dried fruit methanol extract on carrageenan-induced hind paw edema in rats. C. carandas was defatted with petroleum ether, followed by methanolextraction. The methanolextractsofthedriedfruitsof Carissa carandas were givenorallytotheexperimental rats caused significant activity (P ≤ 0.05) when compared with the control group. The maximum inhibition of paw edema was found to be in Group V, that is, 76.12% with inhibition of paw volume in a dose-dependent manner. The anti-inflammatory activity of the methanol extract of the dried fruits shows that the presence of potential constituents present in this extract may provide assistance in the drug discovery process. The phytochemical compounds of the extract were screened by GC-MS analysis and it was found that 11 compounds are present in methanol extract of dried fruits of Carissa carandas.

1. Introduction

Inflammation is the body’s response to noxious or injurious stimuli, characterized by warmth, redness of the skin, pain, swelling, and loss of function. The response to only stimuli is quite similar. Inflammation which is a part of host defense mechanism includes several tissue factors that are known to be involved in the inflammation reactions such as release of histamines, bradykinin, and prostaglandins. Essentially, there are two types of inflammation: acute and chronic. Inflammatory diseases are mainly treated with nonsteroidal anti-inflammatory drugs and steroidal drugs [1]. During an inflammatory response, mediators such as various inflammatory mediators are released into the rat paw tissue, such as prostaglandins (PGE2), leukotrienes (LTD4), nitric oxide (NO), and oxygen reactive species. These inflammatory mediators have potential to continue stimulating the inflammatory response and its perpetuation [2]. Acute rat paw inflammation is caused by the migration of inflammatory cells into the microvascular system and fluid entering the interstitial tissue. These events are induced by inflammatory mediators which bind to specific receptors on inflammatory and endothelial cells. The first process is a sudden stiffening of these endothelial cells and inflammatory part, which occurs during the first few minutes, after an inflammatory mediator enters in bloodstream. The second event is a firm adhesion mediated by CD11/CD18 molecules and other adhesion molecules, which are necessary to maintain the inflammatory cells into the capillaries. This firm adhesion is followed by transendothelial migration into the paw tissue, leading to cell-mediated tissue injury. A variety of stimuli induce the polymorph nuclear (PMN) migration into the paw.
tissue. Experimental model of carrageenan-induced acute inflammation produces a range of inflammatory responses, due to the increase in the response to chemoattractants increasing PMN migration to the sites of inflammation [3].

Carissa carandas L. (Apocynaceae), commonly known as karaunda, is a widely used medicinal plant. The fruits, leaves, barks, and roots of C. carandas have been used for ethnomedicine in the treatment of human diseases, such as diarrhea, stomachic, anorexia, intermittent fever, mouth ulcer and sore throat, syphilitic pain, burning sensation, scabies, and epilepsy [4]. Chemical constituents include steroids, terpenes, tannins, flavonoid, benzenoids, phenylpropanoid, lignans, sesquiterpenes, and coumarins [5]. There are many drugs available in the market for inflammatory diseases. They exhibited several unwanted side effects to humans. Therefore, alternative treatments with safer compounds are needed. Here, the methanol extract of Carissa carandas dried fruits was evaluated for anti-inflammatory activity and its various phytochemical compounds were analyzed by GC-MS.

2. Materials and Methods

2.1. Plant Material. Carissa carandas fruits were collected from and around Vellore district, Tamil Nadu, India, during the month of October 2012. The plant specimen was authenticated and a voucher of specimen was deposited in the herbarium of Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2013-14/Tech.119). The fruits were dried in the shade at room temperature and ground to powder.

2.2. Preparation of the Extract. The powdered sample material (500 g) was defatted with petroleum ether (1000 mL). Then the residue was extracted with methanol (1000 mL) by maceration at room temperature for 48 hours, stirring several times throughout the process (Figure 1). After filtering through folded paper, the methanol extract was concentrated in a rotary evaporator to yield a dark brown mass (10 g) called methanol extract (MTE). This methanol extract was subjected to GC-MS analysis and anti-inflammatory studies [6].

2.3. Preliminary Phytochemical Studies. The preliminary phytochemical studies were performed by using petroleum ether and methanol extract of dried fruits of Carissa carandas to screen the presence of various secondary metabolites [7].

2.4. GC-MS Analysis. The GC-MS analysis of the C. carandas was performed using a Clarus 680 Perkin Elmer gas chromatography equipped with an Elite-5 capillary column (5% diphenyl, 95% dimethyl polysiloxane) (30.0 m × 0.25 mm ID × 250 μm) and mass detector turbo mass of the company which was operated in EI mode. Helium was the carries gas used at a flow rate of 1 mL/min. The injector was operated at 200 °C and the oven temperature was programmed as follows: 60°C for 2 min and 10°C/min until 300°C. Interpretation of GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components.

Figure 1: Schematic diagram of extraction and biological activity of the extract.
stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained [8].

2.5. Pharmacological Screening. The animal experiments were performed after the approval from Institutional Animal Ethical Committee (IAEC), VIT University Vellore (VIT/IAEC/8th/33). The experiments were conducted according to the standard guidelines.

2.5.1. Anti-Inflammatory Studies

(1) Carrageenan-Induced Paw Edema. In this experiment, a mark was made on both of the hind paws just below the tibiotalar junction so that every time the paw could be dipped in the mercury column of plethysmograph up to the mark to ensure constant paw volume. After 30 min of the above treatment, an inflammatory edema was induced in the left hind paw by injecting 0.1mL carrageenan (1%) in the planter tissue hind paw of all the animals. The right paw served as a reference to noninflamed paw for comparison. The initial paw volume was measured plethysmographically within 30 sec of the injection. The relative increase in the paw volume was measured in control, standard, and treated groups, 4h after carrageenan injection. The percent increase in paw volume over the initial reading was calculated. This increase in the paw volume in animals treated with standard drug and the different doses of methanol extract of the dried fruits of C. carandas were compared with the increase in paw volume of untreated control animals after 3 h. The percentage inhibition of edema volume was calculated using the formula

\[
\text{\% Inhibition} = \left( \frac{V_c - V_t}{V_c} \right) \times 100, \tag{1}
\]

where \(V_t\) and \(V_c\) are the relative changes in the edema of the test and control, respectively.

The results were expressed as % inhibition of edema over the untreated control group [9, 10].

(2) Statistical Analysis. The results were expressed as mean ± SEM. The total variation present in the data was analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s test

3. Results and Discussion

3.1. Phytochemical Screening. The extracts obtained from solvent extraction with petroleum ether and methanol were then subjected to various qualitative preliminary tests for the identification of secondary metabolites. They showed the presence of alkaloids, glycosides, flavonoid, terpenes, steroids, and tannins in the methanol extract, whereas the petroleum ether extract shows only carbohydrates and steroids (Table 1).

3.2. GC-MS Analysis. Methanol extract of dried fruits of Carissa carandas has been analyzed by GC-MS technique. The results are given in Table 2. The extract was shown to contain a mixture of components. 11 components were identified. The analysis of methanol extract of dried fruits of C. carandas showed myo-inositol, 4-c-methyl (27.8%), 2R-acetoxyethyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1t-cyclohexanol (23.10), dichloroacetic acid, 2-ethylhexyl ester (15.39), 12-oleanen-3-yl acetate, (3-alpha) (10.77), other minor compounds like 1-pentatriacontanol (5.66), β-amyrin (2.39), Z,Z-6,28-heptatriactontadien-2-one (1.00), 1-methoxy-25-methyl heptacosan-1-ol (2.12), and 2,4,4-trimethyl-3 hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene (1.63) shown in Table 2 and Figure 2.

3.3. Anti-Inflammatory Activity

3.3.1. Carrageenan-Induced Paw Edema Method. The rats treated with oral administration of methanol extract of dried fruits of C. carandas reduced acute paw edema volume as compared to control. The rats were treated with dried fruit extract at a dose of 100, 200, and 400 mg/kg body weight and exhibited 33.7, 60.95, and 76.12% inhibition of paw edema volume when compared with control at 2 h, respectively. The % inhibition of paw edema increased with time and gave maximum effect at 2 h when compared with control. Here, the methanol extracts of dried fruits of C. carandas exhibited anti-inflammatory activity in dose-dependent manner. The values obtained from each group were expressed as mean ± SEM. To compare the statistical significant changes between control, indomethacin treated rats and with methanol extract of dried fruits of C. carandas, we have done Dunnett’s t-test. The significant levels between the groups were compared between initial at varying time interval. The experiment showed (Table 3) that the extract exhibited statistically significant (\(P < 0.05\)) inhibition of paw volume in a dose-dependent manner. However, maximum inhibition of paw edema which was found to be in Group V is 76.12% [11].

Inflammation plays an important role in various diseases with high prevalence within populations such as rheumatoid arthritis, ulcer, atherosclerosis, and asthma. Many drugs are available in the market for inflammatory diseases. Percentage inhibition of edema volume of methanol extract and standard drug were calculated every 30 minutes up to 2 h duration. There is a dose-dependent inhibition of paw edema in rats as mentioned in Table 3. During the inflammatory response mediators like prostaglandins and bradykinins

---

**Table 1: Phytochemical screening of petroleum ether and methanol extracts.**

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>PET</th>
<th>MET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Tannins and phenolic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

*: negative indicates the absence of the corresponding constituent, +: positive.
were playing an important role in carrageenan-induced paw edema. Here, in this experiment first we induced edema by injecting 1% carrageenan, thereby caused the release of autacoids, histamine, and 5-hydroxy tryptamine (5-HT). Once the inflammation starts declining from maximum, prostaglandins started to act which results in the migration of leukocytes into the inflamed site. Here, indomethacin was used as standard drug. The presence of the reported chemical constituents like myo-inositol, 4-c-methyl, 2R-acetoxy methyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1t-cyclohexanol, dichloroacetic acid, 2-ethylhexyl ester, and 12-oleanen-3-yl acetate, (3-alpha), by GC-MS analysis provided the fact that they might suppress the formation of bradykinin and prostaglandin in the system. The present
Table 2: GC-MS analysis of methanol extract of dried fruits of Carissa carandas.

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Retention time</th>
<th>Compounds</th>
<th>% relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.93</td>
<td>Dichloroacetic acid, 2-ethylhexyl ester</td>
<td>15.39</td>
</tr>
<tr>
<td>2</td>
<td>16.61</td>
<td>1-Pentatriacontanol</td>
<td>5.66</td>
</tr>
<tr>
<td>3</td>
<td>17.12</td>
<td>Myo-inositol, 4-c-methyl</td>
<td>27.8</td>
</tr>
<tr>
<td>4</td>
<td>17.68</td>
<td>Heptacosanoic acid, 1-methoxy-25-methyl heptacosan-1-ol, methyl ester</td>
<td>2.12</td>
</tr>
<tr>
<td>5</td>
<td>19.76</td>
<td>Methyl 13-octadecenoate</td>
<td>1.25</td>
</tr>
<tr>
<td>6</td>
<td>23.19, 24.76</td>
<td>Z,Z-6,28-Heptatriactontadien-2-one</td>
<td>4.24</td>
</tr>
<tr>
<td>7</td>
<td>29.84</td>
<td>12-Oleanen-3-ylacetate, (3-alpha)</td>
<td>10.77</td>
</tr>
<tr>
<td>8</td>
<td>30.45</td>
<td>2R-acetoxymethyl-1,3,3-trimethyl-4-(3-methyl-2-buten-1-yl)-1t-cyclohexanol</td>
<td>23.10</td>
</tr>
<tr>
<td>9</td>
<td>30.91</td>
<td>2R-Acetoxymethyl-1,3,3-trimethyl-4-(3-methyl-2-buten-1-yl)-1t-cyclohexanol</td>
<td>2.39</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>2R-Acetoxymethyl-1,3,3-trimethyl-4-(3-methyl-2-buten-1-yl)-1t-cyclohexanol</td>
<td>8.10</td>
</tr>
</tbody>
</table>

Table 3: Anti-inflammatory activity of methanol extract of dried fruit of Carissa carandas.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>Amount of sample</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>% decrease in paw volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>1 mL/kg</td>
<td>0.19±0.01</td>
<td>0.35±0.027</td>
<td>0.64±0.021</td>
<td>0.77±0.005</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>Standard</td>
<td>50 mg/kg</td>
<td>0.13±0.021*</td>
<td>0.17±0.016*</td>
<td>0.35±0.021*</td>
<td>0.27±0.016*</td>
<td>80.63</td>
</tr>
<tr>
<td>III</td>
<td>Methanol</td>
<td>100 mg/kg</td>
<td>0.21±0.005**</td>
<td>0.29±0.005**</td>
<td>0.48±0.005**</td>
<td>0.57±0.005**</td>
<td>33.7</td>
</tr>
<tr>
<td>IV</td>
<td>Methanol</td>
<td>200 mg/kg</td>
<td>0.19±0.01**</td>
<td>0.22±0.01**</td>
<td>0.4±0.01**</td>
<td>0.45±0.01**</td>
<td>60.95</td>
</tr>
<tr>
<td>V</td>
<td>Methanol</td>
<td>400 mg/kg</td>
<td>0.17±0.01**</td>
<td>0.2±0.01**</td>
<td>0.34±0.05**</td>
<td>0.37±0.01**</td>
<td>76.12</td>
</tr>
</tbody>
</table>

**The mean difference is significant at the 0.05 level, when compared to the control group.

4. Conclusion

Inflammatory diseases are treated by using steroidal and nonsteroidal.

Inflammatory diseases are treated by using steroidal and non-steroidal anti-inflammatory drugs that act through COX inhibition (cyclooxygenase) enzyme can provide relief from the symptoms of inflammation and pain. Mostly these drugs are used for acute inflammation, but they failed to cure chronic inflammatory diseases, such as rheumatoid arthritis or osteoarthritis. Furthermore, these synthetic compounds exhibit several unwanted side effects to humans. Therefore, alternative treatments with safer compounds are needed. Based on the positive result from the experiment, methanol extract of dried fruits of Carissa carandas could potentially be used as food supplement with the purpose of providing anti-inflammatory effects as we used dried fruit extract here [14].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


In Vitro Larvicidal and Antioxidant Activity of Dihydrophenanthroline-3-carbonitriles

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1. Introduction

The special scopes of natural products are discovery of potential drugs with novel structures and varying biological activity. Compounds containing heterocyclic ring systems are of much importance in medicinal chemistry [1]. Because of their excellent chemotherapeutic characters, natural occurrence in living system, varied structure, and chemical properties researchers focused towards the synthesis of heterocyclic rings [2]. Among these, acridone is one of the scaffolds known to associate with several biological activities [3]. And also these acridine analogues are found in plant sources which possess various biological activities. Many plants, particularly plants pertaining to Rutaceae species, possess maximum number of acridine derivatives [4]. Dihydropyridine is an area of interest owing to the key role played in synthesis of intermediate for natural products [5] and other heterocycles. These hydropyridines are valuable synthetic intermediates and can be elaborated to the pharmacologically interesting polysubstituted piperidines and polycyclic alkaloid [6]. The reactivity of dihydropyridine is mainly involved in selective reductions [7] and electrophilic additions [8, 9] and has allowed the completion of total synthesis of alkaloids [10, 11]. Biological importance of dihydropyridines structures was elaborated in Figure 1 [12].

Vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Nowadays researchers are focusing their research on a synthetic compound that kills the larvae at initial stage itself [13]. Plant extracts are acting as a potential larvicidal and antioxidant activity [14]. One of the present research interests is the synthesis of nanoparticles by biosynthetic methods. These nanoparticles are studied for larvicidal activity against various larvae [15]. All these kinds of applications are regarding the presence of various phytochemical compositions in plants. The growing interest is to synthesize heterocyclic compounds, and studying their potential uses in medicinal applications is well proved by the growing number of publications.
Taking these facts into account, our research group have been involved actively to synthesis a drug against larvae. Due to effect of synthetic compounds on larvicial activity, our research group mainly focused on killing the larvae at initial stage itself. Earlier we reported the 7-chloro-3,4-dihydro-9-phenylacridin-1(2H)-one shows an effective larvicidal activity against the early fourth instar larvae of filariasis vector, *Culex quinquefasciatus*, and Japanese encephalitis vector, *Culex gelidus* (Diptera: Culicidae). The compound exhibited high larvicidal effect at 50 mg/L against both the larva with LC₅₀ values of 25.02 mg/L ($r^2 = 0.998$) and 26.40 mg/L ($r^2 = 0.988$) against *C. quinquefasciatus* and *C. gelidus*, respectively [16].

Nowadays, antioxidants that exhibit DPPH radical scavenging activity are increasingly receiving attention (Figure 3) [17]. The generation of free radicals during the metabolic process is now observed to be responsible for wide range of human diseases such as aging, cancer, atherosclerosis, arthritis, viral infection stroke, myocardial infarction, pulmonary condition, inflammatory bowel disease, and neurogenerative disease and others may be produced by reactive oxygen species [18, 19]. Antioxidants act as a major defence against radical mediated toxicity by protecting the damage caused by free radicals. Antioxidative agents are effective in the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer’s disease, and cancer [20, 21].

In the present study, the efforts have been laid down to synthesis of 2-amino-10-chloro-4,12-diphenyl-1,4,5,6-tetrahydrobenzo[j][1,7]phenanthroline-3-carbonitrile analogues, 4a–f, to evaluate the inherent larvicidal activity which was carried out against larva of *Aedes aegypti* and *Culex quinquefasciatus*. The model of scavenging of the stable DPPH radical is extensively used to evaluate radical scavenging activities in a very short span of time in comparison to other methods. Compound under investigation reacts with DPPH (1,1-diphenyl-picrylhydra-zine) due to its hydrogen donating ability at a very rapid rate [22]. When this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons taken up. Such a change in the absorbance produced in this reaction has been widely applied to test the capacity of numerous molecules to act as free radical scavengers [23]. In this way, the scavenging effects of all the synthesized compounds on the DPPH free radical were evaluated.

2. Materials and Methods

2.1. Chemistry. Melting points were measured on Elchem Microprocessor based DT apparatus using an open capillary tube and are corrected with standard benzoic acid. IR spectrum was recorded on a Perkin–Elmer spectrum RXI FT-IR spectrometer (400–4000 cm⁻¹; resolution: 1 cm⁻¹) using KBr pellets. $^1$H and $^{13}$C NMR spectra were obtained on a Bruker Avance 400 MHz spectrometer. Chemical shifts were reported on the scale relative to TMS. Exact mass measurements of the molecular ions were obtained on ESI-MS Thermo Fleet.

2.1.1. General Synthesis of 2-Amino-10-chloro-4,12-diphenyl-1,4,5,6-tetrahydrobenzo[j][1,7]phenanthroline-3-carbonitrile Analogues (4a–f). (E)-2-(Benzylidene-7-chloro-9-phenyl[1,4,5,6-dihydroacridin-1(2H)-ones, 1a–f, were synthesised by our earlier work [24]. Furthermore, synthesis of 2-amino-10-chloro-4,12-diphenyl-1,4,5,6-tetrahydrobenzo [j][1,7]phenanthroline-3-carbonitriles, 4a–f. The compound,
1a–f and malononitrile (0.66 g, 0.01 mol) was dissolved in absolute ethanol (15 mL). The reaction mixture was refluxed for 5 h. After completion of the reaction, the reaction mixture was cooled and poured into crushed ice. The crude products were separated by column chromatography, ethyl acetate, and petroleum ether as a solvent. All synthetic pathways were elaborated in Scheme 1 and synthesized derivatives and physical data of all synthesized compounds, 4a–f, were summarized in Table 1.

3. Biological Activity

3.1. Larvicidal Activity

3.1.1. Insect Rearing. A. aegypti and C. quinquefasciatus larvae were collected from stagnant water area of Melvisharam (12°56'23" N, 79°14'23" E) and identified in Zonal Entomological Research Centre, Vellore (12°55'48" N, 79°7'48" E), Tamil Nadu, to start the colony, and larvae were kept in plastic and enamel trays containing tap water. They were maintained and reared in the laboratory as per the method [25].

3.1.2. Larvicidal Bioassay. During preliminary screening with the laboratory trial, the larvae of A. aegypti and C. quinquefasciatus were collected from the insect-rearing cage and identified in Zonal Entomological Research Centre, Vellore. 1 mg of synthesized compounds, 4a–f (Table 1), was first dissolved in 100 mL of distilled water (stock solution). From the stock solution, 50 ppm was prepared with dechlorinated tap water. DMSO (Qualigens) was used as an emulsifier at the concentration of 2.0% in the final test solution. The larvicidal activity was assessed by the procedure of WHO 1996 with some modification. For bioassay test, larvae were taken in five batches of 20 in 249 mL of water and 1 mL of the desired synthetic compounds, 4a–f, at different concentrations (3.12 to 50 ppm). The control was set up with DMSO 2.0% and dechlorinated tap water. The numbers of dead larvae were counted after 24 h of exposure, and the percentage of mortality was reported from the average of three replicates. The experimental media in which 100% mortality of larvae occurs alone were selected for dose-response bioassay.

3.1.3. Dose-Response Bioassay. From the stock solution, different concentrations ranging from 3.12 to 50 ppm were prepared for larvicidal activity. Based on the preliminary screening results, synthetic compounds, 4a–f, were subjected to dose-response bioassay for larvicidal activity against the larvae of A. aegypti and C. quinquefasciatus. The numbers of dead larvae were counted after 24 h of exposure, and the percentage of mortality was reported from the average of three replicates. However, at the end of 24 h, the selected test samples turned out to be equal in their toxic potential.

3.1.4. Statistical Analysis. The average larval mortality data were subjected to probit analysis for calculating LC$_{50}$, LC$_{90}$, and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit, and chi-square values were calculated using the software [26]. Results with $P < 0.05$ were considered to be statistically significant.

3.2. Antioxidant Activity. All the synthesized compounds, 4a–f, were to be examined for antioxidant activity.
Table 1: Summary of synthesized dihydropyridine derivatives (4a–f) via Scheme 1.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Compounds R</th>
<th>M. Pt. (°C)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4a</td>
<td>144–146</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>4b</td>
<td>230–232</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>4c</td>
<td>238–240</td>
<td>76</td>
</tr>
<tr>
<td>4</td>
<td>4d</td>
<td>140–142</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>4e</td>
<td>210–212</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>4f</td>
<td>255–257</td>
<td>71</td>
</tr>
</tbody>
</table>

Antioxidant assay [27] is based on the measurements of the scavenging ability of compounds towards the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The disappearance of this commercially available radical is measured spectrophotometrically at 517 nm in an ethanolic solution. The antioxidant activity was expressed as the 50% inhibitory concentration (IC\textsubscript{50}) based on the amount of compound required for a 50% decrease of the initial DPPH radical concentration.

DPPH antiradical scavenging activity was also time dependent. The radical scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the following formula:

\[
\text{IC} (\%) = \left[ \frac{A_0 - A_t}{A_0} \right] \times 100, \tag{1}
\]

where \(A_t\) is the absorbance value of the tested sample and \(A_0\) is the absorbance value of blank sample at a particular time. The data for antioxidation is presented as means ± SD of three determinations. The synthesized compounds used for antioxidant assay are of 1 mM concentration. Absorbance taken after 30, 45, 60, 90, and 120 min was plotted against absorption (Figure 1). Percentage inhibition was plotted against various concentrations 0.001, 0.002, 0.004, 0.006, 0.008, and 0.1 (Figure 2); the linear regression analysis equation was used to obtain the IC\textsubscript{50} value. A lower IC\textsubscript{50} value indicates greater antioxidant activity.
4. Spectral Details of Synthesized Compounds (4a-f)

4.1. 2-Amino-10-chloro-4,12-diphenyl-1,4,5,6-tetrahydrobenzo[j][1,7]phenanthroline-3-carbonitrile (4a). Pale yellow solid; M.F: C₃₁H₂₆Cl₂N₅O₃; Yield 79%; M.P: 144–146°C; FT-IR (KBr) ν max (cm⁻¹): 3444 (–NH₂), 3236 (–NH₃), 2222 (–C=O); ¹H NMR (CDCl₃): δ (ppm): 7.58–7.62 (m, 9H, –CH₃), 7.35–7.38 (m, 3H, –CH₃), 7.28–7.32 (m, 3H, –CH₃), 7.23–7.25 (m, 3H, –CH₃), 3.08–3.14 (m, 1H, –CH₂), 3.33 (s, 2H, –NH₂), 3.74 (s, 3H, –OCH₃), 4.11 (s, 1H, –CH), 4.93 (s, 1H, –NH), 6.77–6.95 (m, 3H), 7.21–7.22 (d, J = 2.4 Hz, 2H), 7.25–7.30 (t, J = 7.8 Hz, 1H), 7.35–7.39 (t, J = 8 Hz, 1H), 7.56–7.58 (m, 3H), 7.70–7.73 (dd, J = 2.4 Hz, J = 2 Hz, 1H), 7.96–7.98 (d, J = 8.8 Hz 1H); ¹³C NMR (DMSO-d₆): δ (ppm): 23.5, 32.0, 42.4, 54.9, 57.0, 112.4, 113.5, 118.0, 119.5, 119.8, 120.5, 124.4, 127.6, 127.8, 128.0, 128.1, 128.5, 128.7, 129.7, 129.8, 130.5, 132.8, 137.4, 139.6, 139.9, 144.2, 144.8, 158.1, 158.8, 159.4; Exact Mass: 490.16; Found ESI-MS m/z: 494.0 [M + 4].

4.5. 2-Amino-10-chloro-4-(2-chlorophenyl)-12-phenyl-1,4,5,6-tetrahydrobenzo[j][1,7]phenanthroline-3-carbonitrile (4e). Orange solid; M.F: C₃₀H₂₈Cl₂N₆; Yield 75%; M.P: 210–212°C; FT-IR (KBr) ν max (cm⁻¹): 3466 (–NH₂), 3322 (–NH₃), 2919 (–C=O); ¹H NMR (DMSO-d₆): δ (ppm): 3.60–3.64 (m, 1H, –CH₂), 3.35–3.38 (m, 1H, –CH₂), 3.48 (s, 1H, –CH), 2.89–2.90 (m, 1H, –CH₂), 4.08 (s, 1H, –CH), 5.00 (s, 1H, –NH), 5.03 (s, 1H, –NH), 6.93–6.95 (d, J = 8 Hz, 2H), 7.22–7.24 (d, J = 4 Hz, 1H), 7.22–7.26 (d, J = 8 Hz, 1H), 7.27–7.29 (d, J = 8 Hz, 1H); ¹³C NMR (DMSO-d₆): δ (ppm): 23.8, 32.6, 42.5, 59.5, 117.1, 119.0, 120.3, 125.4, 127.5, 128.0, 128.1, 128.4, 128.9, 129.0, 129.1, 130.1, 130.4, 132.3, 133.6, 138.5, 140.5, 140.9, 141.0, 144.9, 158.0, 158.2; Exact Mass: 494.11; Found ESI-MS m/z: 496.1 [M + 2].

5. Result and Discussion

Results on the larvicidal activities of 4a-f obtained in this study that were summarized in Table 2 confirm their potential for the control of larval population of vectors. Compounds 4a, 4c, and 4d resulted in moderate mortality against A. aegypti 22.4, 72.6, and 52.6% and against C. quinquefasciatus 36.2, 87.0, and 46.2%; however, the highest larval mortality was observed in 4b, 4e, and 4f against A. aegypti 78.2, 100.0, and 82.0 and against C. Quinquefasciatus 92.8, 100.0, and 76.6 at 50 ppm. LC₅₀ and LC₉₀ values are calculated for active compounds. The fourth instar larvae of A. aegypti had values of LC₅₀ 103.40, 69.94, and 158.98 and
Table 2: Larvicidal activity, mean efficacy (percentage ± S.D) of synthetic compounds against fourth instar larvae of Aedes aegypti and Culex quinquefasciatus.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ppm)</th>
<th>% Mortality ( ^\circ )/ppm ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A. aegypti</td>
</tr>
<tr>
<td>4a</td>
<td>50</td>
<td>22.4 ± 6.69</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>16.2 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>12.0 ± 4.01</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>8.2 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>3.12</td>
<td>4.6 ± 0.62</td>
</tr>
<tr>
<td>4b</td>
<td>50</td>
<td>78.2 ± 5.72</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>64.8 ± 1.79</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>55.4 ± 8.38</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>42.2 ± 1.39</td>
</tr>
<tr>
<td></td>
<td>3.12</td>
<td>22.4 ± 6.69</td>
</tr>
<tr>
<td>4c</td>
<td>50</td>
<td>72.6 ± 6.05</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>68.6 ± 2.15</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>45.2 ± 8.83</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>24.2 ± 4.06</td>
</tr>
<tr>
<td></td>
<td>3.12</td>
<td>14.6 ± 2.84</td>
</tr>
<tr>
<td>4d</td>
<td>50</td>
<td>52.6 ± 2.48</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>48.4 ± 2.68</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>39.0 ± 6.73</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>26.4 ± 10.56</td>
</tr>
<tr>
<td></td>
<td>3.12</td>
<td>18.6 ± 3.60</td>
</tr>
<tr>
<td>4e</td>
<td>50</td>
<td>100.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>84.0 ± 1.79</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>65.4 ± 2.38</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>48.2 ± 4.04</td>
</tr>
<tr>
<td></td>
<td>3.12</td>
<td>26.4 ± 2.06</td>
</tr>
<tr>
<td>4f</td>
<td>50</td>
<td>82.4 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>66.1 ± 1.96</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>52.0 ± 1.49</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>28.2 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>3.12</td>
<td>16.8 ± 1.22</td>
</tr>
</tbody>
</table>

LC\(_{50}\) 424.12, 195.70, and 445.78 for 4b, 4e, and 4f, respectively, and larvae of C. Quinquefasciatus had values of LC\(_{50}\) 184.24, 82.29, and 142.22 and LC\(_{90}\) 643.29, 272.36, and 372.34 for compounds 4b, 4e, and 4f, respectively, in Table 3. The \( \chi^2 \) values are significant at \( P < 0.05 \) level. The 95% confidence limits LC\(_{50}\) (LCL–UCL) and LC\(_{90}\) (LCL–UCL) were also calculated. Larval mortality was observed after 24 h exposure; no mortality was observed in the control group. The results of larvicidal activity clearly indicate that the percentage of mortality is directly proportional to the concentration of the compounds. Synthesized compounds, 4a–f, were used at different concentrations, ranging from 3.12 to 50, respectively.

The entire synthesized compounds scavenged DPPH radical significantly in a concentration-dependent manner. Their comparable scavenging activities were expressed in IC\(_{50}\) (concentration required for 50% inhibition of 1 M DPPH concentration) value. As presented in Table 4, compound 4c with IC\(_{50}\) values in the range of 41 \( \mu \text{M} \) showed good radical scavenging activities in comparison with ascorbic acid which was attributed to the presence of two methoxy aryl group of 4c (that can donate hydrogen atoms) and governs the main factor behind their ability to be scavenged by DPPH. After donating a hydrogen atom, compounds exist in their radical form, and the electron conjugation effect in the structure stabilizes the radical of DPPH.

The difference in activity amongst compounds 4a–f was due to the difference in the substitution of these compounds. Amongst them, compounds 4b and 4c with two methoxy substituents showed higher hydrogen donor ability to DPPH radical. Compounds 4b and 4c IC\(_{50}\) values were 54 and 41, respectively, since the corresponding IC\(_{50}\) values for all synthesized compounds were higher than compounds 4b and 4c. Compound 4c has two methoxy groups which are para to each other. Therefore, radical ion resulting from the abstraction of –H atom by DPPH would stabilize by other hydroxyl groups. While in compound 4b both the methoxy groups are in meta and para positions, the radical ion resulted from the abstraction of H atom not that much stabilized from methoxy group compared with 4c. Thus, compound 4d shows better scavenging effect compared to compounds 4e and 4f due to the presence of single methoxy group. Compounds 4a, 4e, and 4f are less active compared with other derivatives because of the absence of any electron releasing group. The results suggest that our compound possesses less activity when compared to 9-aminoacridine propranolol (IC\(_{50}\) = 13.6) [28].

6. Conclusion

Our study demonstrated the novel 2-amino-10-chloro-4,12-diphenyl-1,4,5,6-tetrahydrobenzo[\( j \],[1,7\]phenanthroline-3-carbonitriles 4a–f were synthesized from (\( E \))-2-(3,4-dimethoxybenzylidene)-7-chloro-3,4-dihydro-9-phenylacridin-1-(2H)-ones 1a–f. The synthesized compounds, 4a–f, were used as vector control agents: Aedes aegypti and Culex quinquefasciatus larvae. These encouraging results of the present study provide useful information for further structural optimization of these compounds and a rapid detection for the activity of the target compounds. Furthermore, DPPH radical scavenging activities are evaluated on all synthesized compounds. From the antioxidant results synthesized compound 4c shows higher scavenging activity among all other derivatives.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
Table 3: LC$_{50}$, LC$_{90}$, and other statistical analysis of synthetic compounds against fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Species</th>
<th>LC$_{50}$ ± SE (ppm)</th>
<th>UCL–LCL</th>
<th>LC$_{90}$ ± SE (ppm)</th>
<th>UCL–LCL</th>
<th>$\chi^2$ (df = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b</td>
<td><em>A. aegypti</em></td>
<td>103.40 ± 7.13</td>
<td>128.48–82.30</td>
<td>424.12 ± 49.96</td>
<td>660.92–464.35</td>
<td>10.64</td>
</tr>
<tr>
<td></td>
<td><em>C. quinquefasciatus</em></td>
<td>184.24 ± 6.04</td>
<td>198.02–124.16</td>
<td>643.29 ± 62.28</td>
<td>840.26–629.06</td>
<td>12.88</td>
</tr>
<tr>
<td>4e</td>
<td><em>A. aegypti</em></td>
<td>69.94 ± 2.56</td>
<td>48.89–35.07</td>
<td>195.70 ± 24.30</td>
<td>214.73–147.66</td>
<td>10.56</td>
</tr>
<tr>
<td></td>
<td><em>C. quinquefasciatus</em></td>
<td>82.29 ± 2.74</td>
<td>64.77–46.81</td>
<td>272.36 ± 38.21</td>
<td>238.03–166.62</td>
<td>12.80</td>
</tr>
<tr>
<td>4f</td>
<td><em>A. aegypti</em></td>
<td>158.98 ± 2.54</td>
<td>124.89–115.07</td>
<td>445.78 ± 24.38</td>
<td>273.78–227.64</td>
<td>10.24</td>
</tr>
<tr>
<td></td>
<td><em>C. quinquefasciatus</em></td>
<td>142.22 ± 2.74</td>
<td>87.73–46.82</td>
<td>372.34 ± 18.21</td>
<td>248.03–186.12</td>
<td>8.82</td>
</tr>
</tbody>
</table>

Control: nil mortality; LC$_{50}$: lethal concentration that kills 50% of the exposed larvae; LC$_{90}$: lethal concentration that kills 90% of the exposed larvae; UCL: upper confidence limit; LCL: lower confidence limit; $\chi^2$: chi-square; df: degree of freedom significant at $P < 0.05$ level.

Table 4: 50% inhibition of scavenging activity for compounds 4a–f.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$IC_{50} \times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>38</td>
</tr>
<tr>
<td>4a</td>
<td>53</td>
</tr>
<tr>
<td>4b</td>
<td>54</td>
</tr>
<tr>
<td>4c</td>
<td>41</td>
</tr>
<tr>
<td>4d</td>
<td>68</td>
</tr>
<tr>
<td>4e</td>
<td>71</td>
</tr>
<tr>
<td>4f</td>
<td>80</td>
</tr>
<tr>
<td>9-Amino-acridine-propranolol</td>
<td>13.6</td>
</tr>
</tbody>
</table>

$IC_{50}$ values were determined by linear regression analysis using different concentrations in triplicate.

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References


Amelioration of Paracetamol-Induced Hepatotoxicity in Rat by the Administration of Methanol Extract of *Muntingia calabura* L. Leaves

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*Muntingia calabura* L. is a tropical plant species that belongs to the Elaeocarpaceae family. The present study is aimed at determining the hepatoprotective activity of methanol extract of *M. calabura* leaves (MEMC) using two models of liver injury in rats. Rats were divided into five groups (*n* = 6) and received 10% DMSO (negative control), 50 mg/kg N-acetylcysteine (NAC; positive control), or MEMC (50, 250, and 500 mg/kg) orally once daily for 7 days and on the 8th day were subjected to the hepatotoxic induction using paracetamol (PCM). The blood and liver tissues were collected and subjected to biochemical and microscopical analysis. The extract was also subjected to antioxidant study using the 2,2-diphenyl-1-picrylhydrazyl-(DPPH) and superoxide anion-radical scavenging assays. At the same time, oxygen radical antioxidant capacity (ORAC) and total phenolic content were also determined. From the histological observation, lymphocyte infiltration and marked necrosis were observed in PCM-treated groups (negative control), whereas maintenance of hepatic structure was observed in group pretreated with N-acetylcysteine and MEMC. Hepatotoxic rats pretreated with NAC or MEMC exhibited significant decrease (*P* < 0.05) in ALT and AST enzymes level. Moreover, the extract also exhibited good antioxidant activity. In conclusion, MEMC exerts potential hepatoprotective activity that could be partly attributed to its antioxidant activity and, thus warrants further investigations.

1. Introduction

Liver is a vital organ that plays a role in controlling critical biochemical and physiological activities including homeostasis, growth, energy and nutrient supply, detoxification of drugs and other xenobiotics, and also combating infections [1, 2]. Therefore, it is very susceptible to being damaged by hepatotoxic agents [3]. Many newly developed drugs (e.g., rimonabant, propylthiouracil, or corticosteroids) have been used for treatment of liver diseases; however, these drugs possess harmful side effects such as insomnia, vomiting, constipation, and depression. For that reason, further research on plants and herbs that could potentially substitute the chemical-based drugs is very crucial as many medicinal plants have been found to possess hepatoprotective properties [4]. One of the plants that are currently being investigated for its potential pharmacological activities in our laboratory is *Muntingia calabura*, or locally known as “ceri kampung.” Traditionally, the Peruvian folklore believed that *M. calabura* leaf can reduce gastric ulcer and swelling of prostate gland and alleviate headache and cold [5]. Scientifically, it has been proven that the leaves possess various pharmacologic activities, including antiulcer [6], antinociceptive, antipyretic, and anti-inflammatory activities [7]. An *in vitro* study had...
demonstrated that *M. calabura* possessed antioxidant and antiproliferative activities [8]. From our literature review, no attempt has been made to study the hepatoprotective potential of *M. calabura* leaves. Various reports had shown that the antioxidant and anti-inflammatory activities played significant role in the mechanisms of hepatoprotective activity [9, 10]. Therefore, in accordance with those reports and the fact that *M. calabura* also exerted antioxidant and anti-inflammatory activities as discussed above, the hypothesis that the extract of *M. calabura* will also demonstrate hepatoprotective potential possibly via the same antioxidant and anti-inflammatory mechanisms is worth justifying. Therefore, the present study was aimed at determining the hepatoprotective activity of methanol extract of *M. calabura* leaves (MEMC) using the paracetamol- (PCM-) induced liver damage in rats as the animal model.

2. Materials and Methods

2.1. Chemicals. Paracetamol (PCM; Sigma-Aldrich, USA) and N-acetylcysteine (NAC; Acros Organics, USA) were used in the study. All other chemicals and reagents used were of analytical grade.

2.2. Collection of Plant Material. The leaves of *M. calabura* were collected around Universiti Putra Malaysia (UPM), Serdang campus, Selangor, Malaysia, which were then identified by comparison with specimens available at the Herbarium of the Laboratory of Natural Products, IBS, UPM, Serdang, Selangor, Malaysia. A voucher specimen (SK 2198/13) has been issued. The leaves were dried under shade for 7 days at room temperature, separated, and pulverized by mechanical grinder to form coarse powder.

2.3. Preparation of Plant Extract. The coarse powder of the air-dried leaves of *M. calabura* was subjected to methanol extraction whereby 1 kg of powder leaves was macerated in 20 L of methanol in the ratio of 1:20 (w/v) for 72 hours. The supernatant was filtered sequentially using cloth filter, cotton wool, and Whatman filter paper number. The solvent was then evaporated under reduced pressure (204 mbar) and controlled temperature (40°C) using a vacuum rotary evaporator (Buchi Rotavapor R210/215, Switzerland). The whole processes were repeated twice for the remaining residue [11].

2.4. Animals. Healthy male Sprague Dawley rats at 8-9 weeks of age weighing 180–220 g were used throughout the study. Animals were obtained from the Animal House Facility, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. They were housed at room temperature of 27–30°C and allowed free access to food and tap water *ad libitum*. The animals were acclimatized to laboratory conditions for 7 days before the commencement of experiments. The study protocol of the present study was approved by the Animal House and Use Committee, Faculty of Medicine and Health Sciences, UPM (ethical approval number: UPM/FPSK/PADS/BR-UUH/00449). The rats were handled in accordance with current UPM guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals. All experiments were conducted between 09.30 and 18.30 h to minimize the effects of environmental changes.

2.5. Pharmacological Studies

2.5.1. Antioxidant Activity of MEMC. In an attempt to measure the antioxidant activity, the DPPH free radical scavenging assay was carried out according to the procedure described by Blois [12] with slight modification. Initially, the sample serial dilution was performed to obtain final concentrations of 200, 100, 50, 25, 12.5, 6.25, and 3.13 μg/mL solutions from 1.0 mg/mL stock sample. Next, in 96-well microtiter plate, 50 μL of the previously prepared solutions was added to 50 μL of DPPH (FG: 384.32) (1 mM in ethanolic solution) and 150 μL of ethanol (absolute) in triplicates. The plate was shaken (15 seconds, 500 rpm) and left to stand at room temperature for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 520 nm. Different concentrations of L-ascorbic acid (3.13–200 μg/mL) were used as the standard antioxidant. The control was prepared by adding 50 μL deionized water to 950 μL 100 μM DPPH reagent and the analysis was followed as described above. The results were expressed as percentage inhibition (%I) using the following equation:

\[
I\% = \left( \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \right) \times 100. \quad (1)
\]

Abscontrol is the absorbance of the control reaction with 50 μL deionized water without the extract or ascorbic acid, and Abs_sample is the absorbance in the presence of the sample. The effective concentration of the sample required to scavenge DPPH radical by 50% (EC50) was obtained by linear regression analysis of dose response curve plotting between I% and concentrations.

2.6. Hepatoprotective Assay. The in vivo hepatoprotective activity of MEMC was determined using the PCM-induced hepatotoxicity test in rats. The animals (n = 6) were randomly divided into 6 experimental groups and administered with test solutions as follows.

(i) Group I serving as normal control received 10% DMSO.
(ii) Group II serving as negative control received 10% DMSO.
(iii) Group III serving as positive control received 50 mg/kg NAC.
(iv) Pretreatment groups:
   (a) group IV received 50 mg/kg MEMC,
   (b) group V received 250 mg/kg MEMC,
   (c) group VI received 500 mg/kg MEMC.

These doses of extract (50, 250, and 500 mg/kg) were used in the present study based on previous report on the acute toxicity study performed using three doses (300, 500, and a
maximum dose of 2000 mg/kg MEMC) administered orally, which showed no signs of toxicity in rats [13].

The animals were fasted for 48 hours prior to the experiment under standard laboratory conditions but allowed free access to distilled water (dH₂O) ad libitum. After 48 hours, each group received the respective dose of test solution orally once daily for 7 consecutive days. The oral administration of PCM was performed 3 hours after the last extract administration on the 7th day except for group I, which received only 10% DMSO. After 48 hours of hepatic injury induction, the animals were lightly anesthetized using diethyl ether and the blood was collected by cardiac puncture in sterilized centrifuged tubes which was then centrifuged at 3000 rpm for 10 minutes to get serum for biochemical parameters study. The animals were then sacrificed by cervical dislocation and the liver was removed for histopathological studies.

2.7. Liver Enzymes Assessment. Serum collected was assayed according to the standard liver enzymes assessment methods. Alanine aminotransferase (ALT), alkaline phosphate (ALP), and aspartate aminotransferase (AST) levels were measured using the Hitachi 902 Automatic Chemical Analyser.

2.8. Histopathology. The liver tissue was dissected out and fixed in the 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin wax. The sections, which were 5-6 mm thick, were then prepared using rotary microtome (Leica RM 2125 RTS, Singapore) and stained with hematoxylin and eosin dye for microscopic observation of histopathological changes in the liver. Next, the liver sections were scored and evaluated according to the severity of the hepatic injury as described by El-Beshbishy et al. [14] with slight modifications.

2.9. Phytochemical Screening and HPLC Analysis of MEMC. The phytochemical screening of dried leaves of MEMC was performed according to the standard screening tests and conventional protocols as adopted by Zakaria et al. [7]. The HPLC analysis of MEMC was performed according to the method of Zakaria et al. [7] with slight modifications. Briefly, 10 mg of MEMC was dissolved in 1 mL MeOH and then filtered through the filterer membrane with the pore size of 0.45 μm. The filtered MEMC was then analyzed using a Waters Delta 600 with 600 Controller and Waters 2996 Photodiode Array (Milford, MA, USA), which was equipped with an autosampler, online degasser and column heater. Data was evaluated and processed using the installed Millenium 32 Software (Waters Product). The filtered MEBP was separated on a minibore Phenomenex Luna 5 mm C₁₈ column (dimensions 250 × 4.60 mm) at 27°C using a one-step linear gradient. The sample was eluted using the solvent system consisting of 0.1% aqueous formic acid (labelled as solvent A) and acetonitrile (labelled as solvent B) and two types of elution systems were used as follows: (i) Initial conditions were 85% A and 15% B with a linear gradient reaching 25% B at t = 12 min. This was maintained for 10 min after which the gradient was reduced to 15% B at t = 22 min and maintained for another 8 min (t = 30 min). The programme was returned to the initial solvent composition at t = 35 min. The flow rate used was 1.0 mL/min and the injection volume was 10 μL. The HPLC was monitored at 254 and 366 nm. Further analysis was also carried out to compare the HPLC chromatogram of MEMC against several pure compounds of flavonoid types (e.g. fisetin, quercetin, rutin, quercitrin, naringenin, genistein, pinostrobin, hesperetin and flavanone).

2.10. Statistical Analysis. Data obtained are presented as mean ± standard error of mean (SEM). The data were analysed using one-way analysis of variance (ANOVA) and the differences between the groups were determined using Dunnet post hoc test with P < 0.05 as the limit of significance.

3. Results

3.1. Antioxidant Studies of MEMC. Scavenging of DPPH represents the free radicals reducing activity of antioxidants based on a one-electron reduction which was determined by the decrease of its absorbance at 520 nm. The MEMC exhibited significant antioxidant activity in the DPPH assay in a concentration-dependent manner, as illustrated in Figure I. The IC₅₀ value obtained was 17.39 ± 0.74 μg/mL which is comparable to the reference standard green tea extract (13.90 μg/mL).

3.2. In Vivo Hepatoprotective Study

3.2.1. Effect of MEMC on the Body Weight, Liver Weight, and Liver Weight/Body Weight (LW/BW Ratio) after Induction with PCM. The administration of PCM following pretreatment with 10% DMSO (negative group) did not significantly (P > 0.05) cause increase in the average body weight when compared to the normal control group. The MEMC, at 250 and 500 mg/kg, and 50 mg/kg NAC treated groups showed a significant (P < 0.05) decrease in the average body weight when compared to the negative control group (Table I).
Table 1: Effect of MEMC on percentage change of body and liver weight in PCM-induced hepatic injury rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Body weight, BW (g)</th>
<th>Liver weight, LW (g)</th>
<th>LW/BW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>207.9 ± 4.741</td>
<td>6.190 ± 0.3565</td>
<td>2.967 ± 0.1097</td>
</tr>
<tr>
<td>10% DMSO + PCM</td>
<td>—</td>
<td>217.5 ± 8.258</td>
<td>8.797 ± 0.7331</td>
<td>4.023 ± 0.2399</td>
</tr>
<tr>
<td>NAC + PCM</td>
<td>50</td>
<td>189.9 ± 2.697</td>
<td>8.232 ± 0.3992</td>
<td>4.326 ± 0.1522</td>
</tr>
<tr>
<td>MEMC + PCM</td>
<td>250</td>
<td>195.9 ± 9.105</td>
<td>8.844 ± 0.1816</td>
<td>4.513 ± 0.0718</td>
</tr>
<tr>
<td>MEMC + PCM</td>
<td>250</td>
<td>195.9 ± 3.130</td>
<td>6.340 ± 0.4192</td>
<td>3.583 ± 0.2096</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM of six replicates.

*Significantly different as compared to normal control group, *P* < 0.05.

Table 2: Histopathological scoring of the tissue of PCM-induced hepatic injury rats after pretreatment with MEMC.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Steatosis</th>
<th>Necrosis</th>
<th>Inflammation</th>
<th>Haemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10% DMSO + PCM</td>
<td>—</td>
<td>—</td>
<td>—+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>NAC + PCM</td>
<td>50</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MEMC + PCM</td>
<td>250</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MEMC + PCM</td>
<td>500</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The severity of various features of hepatic injury was evaluated based on those following scoring schemes: − normal, + mild effect, ++ moderate effect, and +++ severe effect.

On the other hand, PCM administration did cause significant (*P* < 0.05) increase in the average liver weight of group pretreated with 10% DMSO when compared to the normal control group. However, only pretreatment with 500 mg/kg MEMC caused significant (*P* < 0.05) reduction in the average liver weight of rats induced with PCM. The 50 mg/kg NAC failed to reduce the increase in liver weight when compared to the negative control group (Table 1).

The mean relative liver weights (LW/BW ratio) of acute PCM-treated animals (negative control) showed significant increase compared to the control normal group (*P* < 0.05). Only the PCM-treated group that was pretreated with 500 mg/kg MEMC showed a significant (*P* < 0.05) decrease in the value of the mean relative liver weights (Table 1).

3.2.2. Histopathological Study of the PCM-Induced Liver Toxicity with and without Pretreatment of MEMC. Histopathological observations (Table 2) performed in this study demonstrated that the normal control group (non-PCM-intoxicated liver pretreated with 10% DMSO) showed normal lobular architecture and normal hepatic cells with intact cytoplasm and well-defined sinusoids (Figure 2(a)). The section of PCM intoxicated liver, pretreated with 10% DMSO (Figure 2(b)), exhibited massive necrosis, presence of haemorrhage, and inflammation with infiltration of lymphocytes involving mainly centrilobular zone 3. Interestingly, these pathological changes were found to be reduced with the increasing doses of MEMC indicating the extract ability to reverse the PCM-induced intoxication (Figures 2(d)–2(f)). Table 3 shows the histopathological scoring of the liver tissues pretreated with the respective test solution. The presence of marked necrosis, hemorrhage, and inflammation following treatment with PCM (shown by the negative control group) had reduced remarkably when pretreated with MEMC or NAC.

3.2.3. Effects of MEMC on Liver Enzymes. In this study, significant elevations of ALT, AST, and ALP were recorded in negative control group as compared to the normal, non-PCM intoxicated group (Table 3, Figure 3). In addition, the histopathological study of the PCM-intoxicated liver pretreated with the respective test solution exhibited correlation with serum biochemical indices. Interestingly, the oral administration of 500 mg/kg MEMC and 50 mg/kg NAC exhibited significant reduction on the level of these enzymes.

3.3. Phytochemical Constituents and HPLC Profile of MEMC. Phytochemical investigation on the crude extract revealed the presence of various compounds, such as flavonoids, tannins, polyphenols, saponins and steroids and the absence of triterpenes and alkaloids.

The HPLC analysis of MEMC was measured at the wavelength of 254 nm and revealed nine major peaks, which were P1 (RT = 2.846 min), P2 (RT = 3.998 min), P3 (RT = 14.584 min), P4 (RT = 19.008 min), P5 (RT = 21.096 min), P6 (RT = 20.349 min), P7 (RT = 22.546 min), P8 (RT = 23.234 min), and P9 (RT = 27.805 min) (Figure 4(a)). Comparison between chromatogram of the standard compounds with chromatogram of MEMC revealed the presence of rutin, quercetin, and fisetin (Figure 4(b)).

4. Discussion and Conclusion

PCM, an over-the-counter drug, is a commonly used anti-pyretic and analgesic which can lead to liver damage if taken in overdose [15, 16]. In therapeutic dose, PCM is converted by drug metabolizing enzymes to water-soluble metabolites and secreted in the urine [17, 18]. Saturated and excess PCM is oxidatively metabolized by hepatic cytochrome p450.
Figure 2: Microscopic observations of liver tissue pretreated with various concentrations of MEMC followed by treatment against PCM-induced liver injury: (a) normal, (b) section of liver tissue of 3 g/kg PCM-treated group (p.o.) showing massive necrosis, haemorrhage, and inflammation, (c) section of 50 mg/kg of N-acetylcysteine liver tissue pretreated on the liver followed by PCM showing preservation of normal hepatocytes, (d) section of pretreated 50 mg/kg MEMC liver tissue followed by PCM showing tissue necrosis and inflammation, (e) section of pretreated 250 mg/kg MEMC liver tissue followed by PCM showing mild inflammation, and (f) section of pretreated 500 mg/kg MEMC liver tissue followed by PCM showing normal histology with mild inflammation (40x magnification). CV: central vein; N: necrosis; I: inflammation; H: haemorrhage.

(CYP450) system to a toxic metabolite, namely, N-acetyl-p-benzoquinone imine (NAPQI) [19–21]. The NAPQI is normally detoxified by a nonprotein thiol known as glutathione (GSH) with both oxidant scavenger and redox-regulation capacities [20]. GSH is a major antioxidant system and a crucial component of host defense which is responsible for scavenging reactive free radicals produced through the metabolism process within the liver to prevent cell injury [16, 22]. The toxic dose of PCM caused the depletion of GSH resulting in accumulation of NAPQI which then covalently binds to the cysteiny1 sulphydryl groups of cellular proteins forming NAPQI-protein adducts [23, 24]. This event results
Table 3: Effect of MEMC on the ALT, AST, and ALP (U/L) level following its pretreatment against the PCM-induced hepatic injury.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>36.05 ± 10.52</td>
<td>124.3 ± 16.14</td>
<td>193.0 ± 41.44</td>
</tr>
<tr>
<td>10% DMSO + PCM</td>
<td>—</td>
<td>1714 ± 142.2*</td>
<td>2266 ± 340.4*</td>
<td>330.0 ± 42.35*</td>
</tr>
<tr>
<td>NAC + PCM</td>
<td>50</td>
<td>884.2 ± 195.4*</td>
<td>1569 ± 106.4*</td>
<td>284.3 ± 5.536*</td>
</tr>
<tr>
<td>MEMC + PCM</td>
<td>50</td>
<td>2734 ± 495.2*</td>
<td>1292 ± 468.0*</td>
<td>315.1 ± 25.64</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1638 ± 174.4</td>
<td>2565 ± 170.5</td>
<td>359.0 ± 32.73</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>244.9 ± 101.9*</td>
<td>526.1 ± 191.1*</td>
<td>22.1 ± 25.55*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM of six replicates.

*Significantly different as compared to normal group, P < 0.05.

The PCM-induced toxicity model is commonly used to study the potential hepatoprotective activity of extracts/compounds [16, 27]. In the present investigation, the 3 g/kg of PCM which is a toxic dose, has resulted in the increment of body weight, liver weight, and LW/BW ratios of rats and showed significant elevation of serum level of hepatic enzymes ALT, AST, and ALP in comparison to normal control group, as expected. Interestingly, administration of MEMC successfully lowered the level of these enzymes and concurrently showed the capability to reduce the liver weight and LW/BW ratios of rats in a dose-independent manner. The failure of PCM to affect body weight in this acute model of hepatotoxicity was parallel with report made by Saad et al. [28] on the failure of thioacetamide to cause changes in body weight of rats in acute liver injury study. Despite the significant changes in liver weights as well as liver body weight ratios observed in PCM-treated rats compared to rats in control groups, measurement of liver body weight ratio is a more accurate approach to determine the changes in liver size compared to the measurement of liver weight alone as the liver weight largely depends on the size of the rat. The enlargement of livers in PCM-treated rats suggested hepatic lesions and liver injury associated with the toxic effects of PCM. These significant changes in the liver weights may be attributed to the accumulation of extracellular matrix protein and collagen in liver tissue.

FIGURE 3: Effect of various doses of MEMC on the serum ALT, AST, and ALP (U/L) levels assessed against PCM-induced hepatic injury in rats. *Significantly different (P < 0.05) as compared to the ALT level in the normal control group. †Significantly different as compared to the AST level in the normal control group. ‡Significantly different as compared to the ALT level in the 10% DMSO + PCM-treated group. ‡Significantly different as compared to the AST level in the 10% DMSO + PCM-treated group. †Significantly different as compared to the ALP level in the 10% DMSO + PCM-treated group.

in the generation of reactive oxygen species (ROS) including the hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^-$), and hydroxyl (OH$^-$) radical that affect the cellular membrane and induce lipid peroxidation and also cause hepatic necrosis [15, 20]. The hepatic cell injuries cause the leaking of cellular enzymes into the blood stream and thus can be measured in the serum [16]. The ALT is an essential serum biomarker of liver damage [25] along with the AST and ALP that are routinely assessed to monitor the function status of the liver [26].
Figure 4: HPLC profile of MEMC. (a) HPLC chromatogram of MEMC at 254 nm and 366 nm. Approximately eleven major peaks were detected at 254 nm with some of them being further highlighted at 366 nm. Each peak was represented by their respective UV-Vis spectra with $\lambda_{\text{max}}$ value. (b) HPLC chromatogram of MEMC at 254 nm compared against several standard pure flavonoids demonstrated the presence of, namely, rutin (1), fisetin (2), and quercetin (3).
and nitric oxide that can affect liver damage or repair [31]. Therefore, it is possible to propose that the extract/compound exerting an anti-inflammatory activity might also demonstrate hepatoprotective activity. In addition, the leaves of M. calabura have also been previously reported to exhibit anti-inflammatory activity [7, 32]. In the present study, MEMC exerted hepatoprotective activity against PCM-induced liver damage in a dose-dependent manner as suggested by the microscopic analysis. Although MEMC did not show a dose-dependent activity against the serum liver enzymes level, the highest dose of MEMC exhibited a significant decrease in serum liver enzymes level. The finding is further supported by the normalization of histopathological changes to preserve the histostructure of hepatocytes. In addition, the MEMC-induced hepatoprotective effects were almost comparable to the standard hepatoprotective drug, NAC.

Phytochemical screening of MEMC demonstrated the presence of flavonoids, saponins, and tannins, as well as the existence of phenolic compounds as indicated by high total phenolic content (TPC) value [8]. The hepatoprotective potential of MEMC can be explained based on the respective phytoconstituents detected in the extract. For example, flavonoids have been reported to exert antioxidant [33, 34], anti-inflammatory [35], and hepatoprotective [34, 35] activities. Moreover, saponins have been reported to exert hepatoprotective activity via modulation of its antioxidant [36] and anti-inflammatory activities [37], while condensed tannins have been suggested to possess free radical scavenging and antioxidant, anti-inflammatory and hepatoprotective activities [38]. Based on all of the reports, the MEMC-induced hepatoprotective activity is suggested to possibly involve the synergistic actions of flavonoids, saponins, and condensed tannins. The HPLC analysis of MEMC demonstrated the presence of at least eleven major fractions with some of the peaks detected at the UV-Vis spectra with $\lambda_{\text{max}}$ value, which falls within the range that detected flavonoids [39]. Moreover, some of those detected peaks have been demonstrated to represent rutin, fisetin, and quercetin.

Detail studies on the phytochemical constituents of M. calabura leaves, in particular, demonstrated the presence of various types of flavonoid-based compounds [40–44]. Interestingly, the isolation and identification of those flavonoid-based bioactive compounds were carried out on the leaves part extracted using methanol, which is similar to the MEMC used in the present study. The presence of flavonoids in MEMC was also expected based on the HPLC analysis wherein some of the peaks detected in the UV spectra represent flavonoid-based compounds. It is important to highlight that flavonoids can be divided into five major subgroups, namely, flavonols, flavones, dihydroflavonols, flavanols, and flavanones [39]. The UV-Vis spectra of flavonoids consist of two absorbance bands labeled as A and B. Band A falls in the range of 310–350 nm for flavones and 350–385 nm for flavonols while Band B falls in the range of 250–290 nm and is similar for all of the abovementioned subgroups. As for the dihydroflavonols and flavanones, the wavelength of Band A falls within the range of 300–330 nm while Band B lies within the range of 277–295 nm. Other than those facts, flavonols and various polyphenols have been shown to exert maximal absorbance at variable wavelengths between 270 and 290 nm. Flavonoids, in particular, have been reported to possess hepatoprotective properties and could be the one responsible for the observed MEMC activity.

Interestingly, the ability of MEMC to exert hepatoprotective activity possibly via its antioxidant action is in line with our previous report on the hepatoprotective activity of methanol extract of Bauhinia purpurea (MEBP) [16]. Comparison was made between the HPLC chromatogram of MEMC and MEBP (chromatogram not shown) and, interestingly, rutin, quercetin, and fisetin were detected in MEMC while gallic acid and catechin were detected in MEBP. Despite almost similar mechanisms of hepatoprotection, both extracts contained different types of phytoconstituents. These differences will provide advantage in improving the antioxidant activity and, concomitantly, the hepatoprotective activity of those plants/extracts if they are combined and tested together due, possibly, to the synergistic effect of various compounds.

MEMC has successfully reversed the PCM-induced hepatotoxic effect by its ability to reduce the elevated level of ALT, AST, and ALP suggesting that these biochemical restorations could be due to the extract ability to inhibit the cytochrome P450 or/and ability to promote the PCM glucuronidation [45]. Furthermore, the ability to lower the enzymes level can be associated with the ability of MEMC to prevent lipid peroxidation of endoplasmic reticulum that is rich in polyunsaturated fatty acid by disrupting the binding of activated radicals to the macromolecules. This process can possibly be achieved via the antioxidant activity of MEMC [46]. Besides, mechanisms of protection that can take place include activation of liver regeneratory by enhancing the protein and glycoprotein synthesis or accelerated detoxification and excretion [47], prevention of lipid peroxidation process, and stabilization of hepatocellular membrane [46]. However, the results obtained warrant further studies, and more detailed investigations are currently underway to determine the possible hepatoprotective mechanism(s) involved and to isolate and identify the responsible bioactive compounds derived from MEMC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References


Research Article

Evaluation of Anti-Candida Activity of Vitis vinifera L. Seed Extracts Obtained from Wine and Table Cultivars

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For the first time, grape seed extracts (GSEs), obtained from wine and table cultivars of Vitis vinifera L., cultured in experimental fields of Lazio and Puglia regions of Italy and grown in different agronomic conditions, have been tested on 43 Candida species strains. We demonstrated a significant correlation between the content of the flavan-3-ols in GSEs extracts, with a polymerization degree ≥4, and anti-Candida activity. Moreover, we demonstrated that GSEs, obtained from plants cultured with reduced irrigation, showed a content of polymeric flavan-3-ols > 250 mg/g with geometric mean MIC values between 5.7 and 20.2 mg/L against Candida albicans reference strains. GSE, showing 573 mg/g of polymeric flavan-3-ols, has been tested in an experimental murine model of vaginal candidiasis by using noninvasive in vivo imaging technique. The results pointed out a significant inhibition of Candida albicans load 5 days after challenge. These findings indicate that GSEs with high content of polymeric flavan-3-ols can be used in mucosal infection as vaginal candidiasis.

1. Introduction

Candida species are major human opportunistic fungal pathogens that cause both mucosal and deep tissue infections. The frequency of mucosal and cutaneous fungal infections has dramatically increased worldwide. Infection caused by Candida spp. affects 70–75% of women at least once during their life. Recurrent vulvovaginal candidiasis occurs in 5% of women with Candida vaginitis [1, 2]. Most of these infections are caused by Candida albicans (C. albicans) and among non-albicans Candida spp., C. glabrata, C. tropicalis, and C. krusei. Azoles are the most common antifungal agents available to treat topical Candida infections. However, these antifungal drugs have several defects related to clinical usage, such as low efficacy and side effects. Therefore, there is an urgent need of new antifungal agents [3]. Natural anti-infective agents represent a promising approach for the treatment of Candida infections [4]. Phytomedicine, which has historically been an important aspect of traditional medicine in nonindustrialized countries, is now becoming an integral part of healthcare in industrialized countries. Plants are the source of thousands of new phytochemicals, and different strategies can be applied to
improve the yields of bioactive metabolites in the plant and to obtain chemically standardized extracts [5, 6]. *Vitis vinifera* L. is the most important fruit species in the world, cultivated especially in Mediterranean area. As reported by the wide literature [7], grapes are rich source of polyphenols, important secondary metabolites produced by higher plants, which play multiple essential roles in plant physiology and showed healthy properties in human organism, mainly as antioxidant, antiallergic, anti-inflammatory, anticancer, antihypertensive, renoprotective, and antimicrobial agents [8, 9]. GSEs are recognized as a complex mixture of monomeric, oligomeric, and polymeric flavan-3-ols. The principal monomers identified are (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCg). Several fungi, including *C. albicans*, are sensitive to EGCg, the main component of green tea extracts [10]. The content of flavan-3-ols in seed grapes is influenced by several factors mainly cultivar, irrigation, nitrogen fertilization, delayed harvest, and storage conditions [11].

Moreover, the application of an extraction process suitable to efficiently recover the target metabolites and an appropriate analytical method for an accurate qualitative and quantitative determination of extract components are required.

In this work, for the first time, anti-*Candida* activity and chemical analysis of GSEs obtained from wine and table cultivars of *Vitis vinifera* L. grown in different agronomic conditions have been evaluated and compared with respect to their phenolic content. The HPLC method with a Poroshell column has allowed to quantify not only the flavan-3-ols oligomers but also the polymeric forms (polymerization degree >4) difficult to be detected with conventional reverse phase columns. Moreover, the effect of GSE treatment on an experimental murine model of vaginal candidiasis was evaluated for the first time by using noninvasive *in vivo* imaging technique.

2. Methods and Materials

2.1. Plant Material. Mature grapes were collected from different cultivars of *Vitis vinifera* L.: Michele Palieri (M. Palieri), Italia, Red Glove, Negroamaro, Pinot, Abbuoto, and Verdicchio. The cultivars M. Palieri, Italia, Red Glove, and Negroamaro were grown in the experimental farm of CRA-UTV in Turi (BA), during 2010 and 2011 with the “tendone” system, a typical cultivation method in the Puglia region (South Italy) whose climate is characterized by scarce rainfalls [12]. The vines were treated with reduced irrigation volume per hectare (1200 m³) (V1) or (2000 m³) (V2) and with reduced nitrogen fertilization (120 kg ha⁻¹) (N1) or with 180 kg ha⁻¹ (N2) that is the quantity generally used in the growing area. Fertilization was carried out at budding (mid-March) and during the growth of the green grapes (first ten days of July). The cultivars Verdicchio, Abbuoto, and Pinot were grown in the experimental field of Lazio region (Center of Italy), during vintages 2006, 2008, and 2011 in normal Mediterranean conditions. The average amount of rainfall accumulated between April and September in 2006, 2008, and in 2011 has been 223 mm, 423 mm, and 245 mm, respectively. The cultivars had training system to Cordon Spur, with plant density of 2.60 × 1 m. In the examined years the same cultural practices were applied in the vineyard. All the grapes were harvested at technological maturation and frozen at −20°C. The seeds have been isolated immediately before use and subjected to extraction process.

2.2. Sample Preparation. The seeds were separated from the flesh and the skin, weighed, and put in liquid nitrogen in a porcelain mortar and ground to obtain a fine powder. They were extracted three times (24 hrs for each extraction) by the mixture EtOH/H₂O (7 : 3 v/v) acidified with formic acid to pH 3; the ratio matrix/solvent was 1 g fresh weight/10 mL. After the removal of the solid residue, the extracts were dried (t ≤ 30°C), weighed, and redissolved in a suitable volume of the same extraction solution to obtain enriched extracts. The samples were centrifuged (12,000 rpm for 5 min) to obtain a limpid solution for the HPLC/DAD/MS analyses. Only the seeds of Abbuoto and Verdicchio cultivars (vintages 2006 and 2008) were treated with a different method using a buffer at pH 3.2 as extractive solution. This method was applied with the aim to simulate the wine-making process. The seeds were manually separated from the berries and extracted with 125 mL of the buffer solution for 144 hours at 30°C. The buffer composition consisted of tartaric acid 5 g; NaOH 1 N 22 mL; Na₂S₂O₃ 2 g; and EtOH 95% 120 mL.

2.3. HPLC/DAD/MS Analysis. The multistep elution method was applied: it started with 95% H₂O for 5 min, then with 86% H₂O for 25 min, 84% H₂O for 5 min; 82% H₂O for 2 min, 80% H₂O for 3 min and a plateau for 4 min, 70% H₂O for 3 min and a plateau for 3 min, up to 20% H₂O for 4 min and a plateau for 5 min; total time of analysis 59 min, equilibration time of 10 min, and a flow rate of 0.4 mL/min. The column was a Poroshell 120 EC18 (150 × 4.6 mm i.d., 2.7 μm) with a precolumn of the same phase maintained at 27°C; the eluents were H₂O (pH 3.2 by HCOOH) and CH₃CN, both of HPLC grade. The HPLC/ESI/MS analysis was carried out using a liquid chromatographic HP 1100 L equipped with an Electrospray (ESI) HP 1100 MDS mass detector with an API interface. The operative conditions of the mass spectrometer were nitrogen flux 10 L min⁻¹, nebulizer pressure 30 psi, gas temperature 350°C, quadrupole temperature 30°C, and capillary voltage 3000–4000 V. The experiments were carried out in negative and positive ionization modes, applying fragmentors between 60 and 220 V. The following standards were used for the identification: (+)-catechin, (−)-epicatechin, ECg, procyanidin B1, and procyanidin B2, all of high purity grade and purchased from Extrasynthese (France). The quantitative analysis of both phenol oligomers and polymers was carried out at 280 nm using only the procyanidin B2 as external standard in a concentration range 0.1–5.7 μg and a five-point calibration curve with R² of 0.999.

2.4. Organisms. For the *in vitro* antifungal evaluation, strains coming from the American Type Culture Collection (ATCC, Rockville, MD, USA), from the German Collection of
Microorganisms (DSMZ, Braunschweig, Germany), and from the Pharmaceutical Microbiology Culture Collection (PMC, Department of Public Health and Infectious Diseases, Sapienza, Rome, Italy) were tested. The strains coming from ATCC were C. albicans ATCC (90028, 90029, 10261, 10231, 3153, and 24433), C. parapsilosis ATCC 22019. The strains coming from DSMZ were C. parapsilosis DSM 11224, C. krusei DSM 6128, and C. tropicalis DSM 11953. The strains coming from PMC were C. albicans PMC (1011, 1075, 1083, 1088, 1097, 1002, 1004, 1006, 1008, 1010, 1012, 1018, 1031, and 1032), C. parapsilosis PMC (0703, 0711, 0706, 0704, 0705, and 0712), C. tropicalis PMC (0908, 0910, 0912, 0913, and 0914), C. krusei PMC (0613, 0625, 0612, and 0622), and C. glabrata PMC (0805, 0849, 0843, and 0822). For the in vivo experiments C. albicans CA1399 carrying the ACT1p-gLuc59 fusion (gLuc59) and C. albicans CA1399 that did not express gLuc59 (control strain) were used [13]. The gLuc59 luciferase reporter has previously been described [13]. C. albicans gLuc59 and the control strain were cultured in YPD as previously described [14].

2.5. Antifungal Susceptibility Testing. The broth microdilution method to evaluate the susceptibility in vitro on strains of Candida spp. was performed according to standardized method for yeast [15, 16]. Briefly, the extracts were dissolved previously in dimethyl sulfoxide at concentrations 100 times higher than the highest test concentration [16]. The final concentration ranged from 0.25 to 512 for total dry seed extracts and from 0.125 to 128 mg/L for Fluconazole (FLC). Microdilution trays, containing 100 μL of serial twofold dilutions of seed extracts or FLC in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MI, USA), were inoculated with an organism suspension of 1.0 × 10^9–1.5 × 10^9 cells/mL. The panels were incubated at 35°C and the growth observed at 48 h. The minimal inhibitory concentration (MIC) was the lowest concentration that caused a prominent decrease (≥50%) in visible growth. The MIC_{90} was defined as the lowest drug concentration that caused ≥90% growth inhibition compared with the drug-free control. Medians, geometric means (GMs), and ranges were calculated.

2.6. In Vitro Induction of Resistance. C. albicans ATCC 10231 was cultured as previously described [17]. In particular, serial subcultures of C. albicans ATCC 10231 were performed in fresh medium every 48 hours, containing rising concentrations of M. Palieri VINI 2010 GSE (starting from 4 mg/L up to 128 mg/L). At the same time the same procedure was carried out for the control without addition of extract. The MIC was evaluated after 48 h of incubation (extracts concentration range 0.5–512 mg/L) according to the CLSI protocol [15].

2.7. Mice. Female CD1 mice obtained from Harlan Nossan Laboratories (Milan, Italy) were used at 4 to 6 weeks of age. Mice were allowed to rest for 1 week before the experiment; by that time the animals were roughly 5 to 7 weeks old. Mice were used under specific pathogen-free conditions that included testing sentinels for unwanted infections; according to the Federation of European Laboratory Animal Science Association standards, no infections were detected.

2.8. Infection and Treatment. Mice infection was performed as previously described with minor adaptations [18]. Mice were maintained under pseudoestrous condition by subcutaneous injection of 0.2 mg of estradiol valerate in 100 μL of sesame oil (Sigma-Aldrich) 5 days prior to infection and weekly until the completion of the study. Mice anaesthetized with 2.5–3.5 (v/v) isoflurane gas were infected with 10 μL of 2 × 10^9 cell/mL of C. albicans gLuc59 or the control strain. Cell suspensions were administered from a mechanical pipette into the vaginal lumen close to the cervix. To favor vaginal contact and adsorption of fungal cells, mice were held head down for 1 min following inoculation. Mice were then allowed to recover for 24–48 h, during which the Candida infection was established. The intravaginal treatment with FLC (200 mg/L, 10 μL/mouse) or with M. Palieri VINI 2010 GSE (50 mg/mL, 10 μL/mouse) has begun 2 h before the challenge and then it was repeated every two days until day +8.

2.9. Monitoring of Mouse Vaginal Infection. After 2, 5, and 8 days after infection, 10 μL (0.5 g/L in 1:10 methanol: H2O) of coelenterazine (Synchem, OHM) was added to the vaginal lumen. Afterwards, mice were imaged in the IVIS-200TM imaging system (Xenogen Inc.) under anaesthesia with 2.5% isoflurane. Total photon emission from vaginal areas within the images (region of interest (ROI)) of each mouse was quantified with Living ImageR software package [19].

2.10. Statistical Analysis. In order to analyze the data among phenolic constituents and MIC values of dried GSEs obtained from selected cultivars of Vitis vinifera L., Pearson’s correlation coefficient (r) was determined. A P value of <0.01 was considered significant. Differences between FLC and M. Palieri VINI 2010 GSE treated and saline treated mice were evaluated by Mann-Whitney U-test. A value of P < 0.05 was considered significant.

3. Results

3.1. In Vitro Antifungal Activity of GSEs. Seed extracts obtained by Vitis vinifera L., wine cultivars, Verdicchio and Abbuoto, cultured in experimental fields of Lazio region of Italy, during 2006 and 2008 vintages, showed great variability of antifungal activity against C. albicans, with GM MIC range from 44.22 mg/L to 203.19 mg/L and MIC_{90} values from 17.9 mg/L to 29.8 mg/L (Table 1). GSEs from the table cultivars M. Palieri, Red Globe, and Italia grown in the 2010 in the experimental farm of CRA-UTV Puglia region of Italy, subjected to reduction of irrigation volume (V1 and V2) and different nitrogen fertilization (N1 and N2), showed potent and comparable antifungal activity against C. albicans with a range of GM MIC values from 8.2 mg/L to 12.8 mg/L and MIC_{90} values from 17.9 mg/L to 29.8 mg/L (Table 2). Moreover, antifungal activity against non-albicans Candida spp. showed MIC values from 6.5 to 8.6 mg/L and MIC_{90} values from 14 to 18 mg/L (Table 3). The same cultivars harvested in 2011 and grown under the same agronomic conditions showed MIC values from 5.66 to 14.59 mg/L.
Table 1: Antifungal activity against *Candida albicans* reference strains of GSEs from table and wine cultivar of *Vitis vinifera* L. harvested in different years.

<table>
<thead>
<tr>
<th></th>
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</thead>
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<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
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<td>128</td>
<td>128</td>
<td>64</td>
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<td>128</td>
<td>256</td>
<td>512</td>
<td>256</td>
<td>128</td>
</tr>
</tbody>
</table>

GM: geometrical mean of MIC.

The values are expressed as median of minimum inhibitory concentration (MIC) determined using Clinical and Laboratory Standard Institute (CLSI) protocol M27-A3. MIC<sub>90</sub>: lowest drug concentration that prevented 90% of growth with respect to the untreated control. GM: geometrical mean of MIC.

Figure 1: Chromatographic profiles at 280 nm of GSEs from Pinot 2011 (a), M. Palieri V1N1 2010 (b), and M. Palieri V1N1 2011 (c). (+) cat: (+)-catechin; proc B2: procyanidin B2; (−) epicatechin: (−)-epicatechin; ECg: epicatechin gallate; Pol 1 and Pol 2: polymeric flavan-3-ols with galloylated residues and a degree of polymerization ≥ 4, determined by mass spectrometry.

and MIC<sub>90</sub> values from 17.55 to 29.17 mg/L for *C. albicans* reference strains demonstrating that the antifungal activity is reproducible over the years (Table 4). Negroamaro V2N2 and V1N2 (from Puglia region) grown in 2011 revealed MIC values against *C. albicans* reference strains of 10.7 mg/L, 20.2 mg/L, respectively (samples 13 and 14 of Table 5), while Pinot (from Lazio region), not subjected to controlled agronomic conditions of water and nitrogen, showed MIC values of 84.5 mg/L (sample 15 of Table 5).

3.2. Chemical Composition of the Extracts. An example of the GSEs HPLC profiles in terms of monomers, oligomers, and polymers is shown in Figure 1. Optimizing the chromatographic method also with the help of an RP 18 Poroshell column several monomer and oligomer compounds have been separated and quantified in all the fifteen extracts (Table 5). The identification of the phenols listed in Table 5 has been done by mean of their UV and mass spectra, by the use of pure standards ((+)-catechin; (−)-epicatechin; procyanidin B1 and B2; ECg) and according to the literature [20–22]. Moreover, two groups of polymeric procyanidins (Pol 1 and Pol 2) with a polymerization degree ≥ 4 have been separated and determined by their mass spectra in negative-ionization mode (Figure 1 and Table 5).

The chemical composition of the different extracts correlated to their antifungal activity against *C. albicans* reference strains is summarized in Table 5. The identified components in the GSEs typically belong to the flavan-3-ols class; (+) catechin and (−) epicatechin are the main monomers; within the polymeric forms 1 + 2 (from 40 to 96% of the total flavan-3-ols) are included some acylated forms with gallic acid residues. The extracts 1–4 and 15 (Table 5) showed the lowest content of flavan-3-ols and in particular the lowest content of polymeric forms compared with all the other samples (Table 5).

3.3. Antifungal Activity of M. Palieri V1N1 2010 GSE against Vaginal Candidiasis. M. Palieri V1N1 2010 GSE has been chosen for in vivo test for best reproducibility of M. Palieri cultivar, confirmed in different years, in MIC values and in
M. PalieriV2N1
MIC
MIC90
8
8
8
64
16
32
16
64
8
16
16
32
8
32
16
32
8
16
8
16
16
32
1
4
8
32
8
16
16
16
16
64
16
32
8
64
8
32
16
32
8.2
17.9
1–32
2–64

M. PalieriV1N1
MIC
MIC90
8
16
16
32
16
32
32
32
8
16
16
32
16
32
32
32
8
16
16
16
16
32
1
4
8
32
4
8
16
32
16
32
16
32
32
64
16
32
16
32
11.18
20.6
1–32
2–64

Red GlobeV2N1
MIC
MIC90
8
16
8
16
8
32
16
64
8
16
16
16
8
64
32
32
8
32
8
16
32
8
2
4
8
32
8
16
8
32
32
64
16
8
32
32
8
64
16
8
10.43
28.2
2–64
4–128

Red GlobeV1N1
MIC
MIC90
8
32
8
32
8
32
16
32
8
32
16
32
16
32
16
64
8
32
16
32
16
64
4
8
16
32
16
32
16
32
32
64
16
32
8
32
16
32
16
32
12.8
29.8
4–64
4–64

ItaliaV2N2
MIC
MIC90
8
16
16
16
16
32
16
64
8
32
16
32
16
32
16
32
8
32
8
16
8
32
1
2
8
16
8
16
8
16
16
16
8
32
16
32
8
32
16
32
8.77
19.2
1–32
2–64

ItaliaV1N2
MIC
MIC90
8
16
8
16
8
16
16
32
8
32
16
16
32
32
8
32
8
16
16
16
16
32
1
1
16
32
16
16
16
16
32
64
8
16
16
32
8
16
16
32
9.29
18.4
1–32
1–64

Fluconazole
MIC
MIC90
0.5
8
16
32
4
32
0.25
1
2
32
1
16
8
32
8
32
8
32
4
8
0.5
32
0.5
64
0.5
8
0.5
8
8
16
8
32
0.25
32
0.25
8
0.25
32
0.25
8
2.5
21.1
0.125–64
1–128

The values are expressed in mg/L as median of minimum inhibitory concentration (MIC) determined using Clinical and Laboratory Standard Institute (CLSI) protocol M27-A3. MIC90 : lowest drug concentration
that prevented 90% of growth with respect to the untreated control. GM: geometrical mean of MIC.

ATCC90028
ATCC3153
ATCC10261
ATCC90029
ATCC10231
ATCC24433
PMC1012
PMC1010
PMC1006
PMC1008
PMC1083
PMC1097
PMC1088
PMC1075
PMC1018
PMC1004
PMC1011
PMC1002
PMC1031
PMC1032
GM
Range

Candida albicans

Table 2: Antifungal activity against Candida albicans of GSEs obtained from selected cultivars of Vitis vinifera L. harvested in year 2010 and cultured under controlled agronomic conditions
of water and nitrogen.

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Table 3: Antifungal activity against *Candida* spp. of GSEs obtained from selected cultivars of *Vitis vinifera* L. harvested in 2010 and cultured under controlled agronomic conditions of water and nitrogen.

<table>
<thead>
<tr>
<th>Candida spp.</th>
<th>M. PalieriV2N1</th>
<th>M. PalieriV1N1</th>
<th>RedGlobeV2N1</th>
<th>RedGlobeV1N1</th>
<th>ItaliaV2N2</th>
<th>ItaliaV1N2</th>
<th>Fluconazole</th>
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<tr>
<td><em>kru sei</em> PMC0613</td>
<td>8</td>
<td>16</td>
<td>8</td>
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<tr>
<td><em>kru sei</em> PMC0625</td>
<td>8</td>
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<tr>
<td><em>kru sei</em> DSM 6128</td>
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<td>8</td>
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<tr>
<td><em>kru sei</em> PMC0612</td>
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<tr>
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<td>8</td>
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<tr>
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<tr>
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The values are expressed in mg/L as median of minimum inhibitory concentration (MIC) determined using Clinical and Laboratory Standard Institute (CLSI) protocol M27-A3. GM: geometrical mean of MIC. MIC\(_{90}\): lowest drug concentration that prevented 90% of growth with respect to the untreated control.
### Table 4: Antifungal activity against *Candida albicans* reference strains of GSEs obtained from selected cultivars of *Vitis vinifera* L. harvested in 2011 and cultured under controlled agronomic conditions.

<table>
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<tr>
<th><em>Candida albicans</em></th>
<th>M. PalieriV2N1</th>
<th>M. PalieriV1N1</th>
<th>RedGlobeV2N1</th>
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<td>ATCC353</td>
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<td>ATCC10231</td>
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<td>ATCC24433</td>
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<td>8</td>
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<td>16</td>
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<tr>
<td>Range</td>
<td>8–32</td>
<td>32–16</td>
<td>8–32</td>
<td>16–64</td>
<td>4–16</td>
<td>16–64</td>
<td>8–16</td>
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</tbody>
</table>

The values are expressed in mg/L as median of minimum inhibitory concentration (MIC) determined using Clinical and Laboratory Standard Institute (CLSI) protocol M27-A3. GM: geometrical mean of minimum inhibitory concentration. MIC<sub>90</sub>: lowest drug concentration that prevented 90% of growth with respect to the untreated control.
phenolic constituents. Moreover, we demonstrated that M. Palieri V1N1 2010 GSE treatment did not induce in vitro resistance on C. albicans ATCC 10231. The MIC value was of 16 mg/L both for control and for M. Palieri V1N1 2010 GSE-treated strain. The in vitro antifungal activity of MP V1N1 2010 GSE and FLC, against C. albicans gLUC59 used in in vivo test, showed MIC values of 15.6 ± 12.7 and 0.60 ± 0.20 mg/L, respectively.

Antifungal activity of M. Palieri V1N1 2010 GSE against vaginal candidiasis was monitored in an experimental murine model of vaginal candidiasis that allowed the visualization of the temporal and spatial progression of infection. In particular the M. Palieri V1N1 2010 GSE was administered intravaginally (50 g/L, 10 μL/mouse) 2 h before the challenge and then it was repeated every two days until day +8. The results reported in Figure 2 showed a significant inhibition of C. albicans load observed 5 days after challenge. The effect was comparable to that observed with FLC. The infection was completely cleared 8 days after infection (Figure 2).

### 4. Discussion

In this work, for the first time, the in vitro anti-Candida activity of GSE from wine and table cultivars of Vitis vinifera L., grown in different agronomic conditions, collected over several years has been evaluated. The results showed that GSEs obtained from cultivars grown in Puglia under hydric stress possess potent antifungal activity in vitro, in some cases similar to that of FLC itself. All GSEs have been chemically characterized. A significant negative correlation coefficient of total flavan-3-ols contained in the different extracts and MIC values has been demonstrated ($r = -0.648$, $P = 0.00896$). Moreover, we demonstrated for the first time that the antifungal activity (MIC) of GSEs is attributable mostly to the polymeric flavan-3-ols (with a polymerization degree ≥4), with a significative negative correlation coefficient ($r = -0.6974$, $P = 0.0038$) (Table 5). Differently, the content of gallate monomers and oligomers did not seem to be correlated to antifungal activity ($r = -0.4334$, $P = 0.1065$). It is important to emphasize that the typical catechin of green tea, EGCg, known to be responsible of growth-inhibitory effect on clinical isolates of Candida spp. [10], is absent in our samples.

We demonstrated that Verdicchio and Abbuto, not subjected to controlled agronomic conditions of water, collected in 2006 (samples 1-2), had a higher content of polymers 1 + 2 (with the polymer 2 from 3- to 10-fold higher) than the same cultivars collected in 2008 (Table 5). These differences could be partially attributable to the rainfall during 2008, twofold higher than that in 2006.

Hydric stress implies a bigger effort to absorb water from the soil and, as a consequence, a lesser vegetative growth and an increase of bioactive molecules production [23]. In particular, Cavaliere et al. demonstrated that the content of flavan-3-ols in grape seeds is influenced by several agronomic conditions mainly irrigation and nitrogen fertilization [11, 24].

In the present work, the dried GSEs obtained from the cultivars M. Palieri, Italia, Red Globe, and Negroamaro, cultured under hydric stress (V1 and V2) (samples 5–14 Table 5), showed the highest content of polymers 1 + 2 > 250 mg/g with the polymer 2 > 46 mg/g and the best antifungal activity with

### Table 5: Phenolic constituents and MIC values of dried GSEs obtained from fifteen selected cultivars of Vitis vinifera L.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flavan-3-ols mg/g</th>
<th>Pol 1 + 2 mg/g</th>
<th>G Mon + Olig mg/g</th>
<th>Pol 1 + 2/ flavan-3-ols</th>
<th>Pol 1/Pol 2</th>
<th>Pol 2 mg/g</th>
<th>MIC GM mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Verdicchio 2006</td>
<td>198.3</td>
<td>156.5</td>
<td>4.9</td>
<td>0.79</td>
<td>1.6</td>
<td>60.2</td>
<td>53.2</td>
</tr>
<tr>
<td>2 Abbuto 2006</td>
<td>96.9</td>
<td>67.2</td>
<td>3.1</td>
<td>0.69</td>
<td>3.5</td>
<td>14.9</td>
<td>44.2</td>
</tr>
<tr>
<td>3 Verdicchio 2008</td>
<td>69.1</td>
<td>32.8</td>
<td>4.1</td>
<td>0.47</td>
<td>4.2</td>
<td>6.3</td>
<td>203.2</td>
</tr>
<tr>
<td>4 Abbuto 2008</td>
<td>98.1</td>
<td>38.9</td>
<td>10.10</td>
<td>0.40</td>
<td>6.2</td>
<td>5.4</td>
<td>64.0</td>
</tr>
<tr>
<td>5 M. Palieri V2N1 2010</td>
<td>820.0</td>
<td>638.5</td>
<td>25.2</td>
<td>0.78</td>
<td>2.8</td>
<td>168.0</td>
<td>10.6</td>
</tr>
<tr>
<td>6 M. Palieri V1N1 2010</td>
<td>748.1</td>
<td>572.9</td>
<td>23.4</td>
<td>0.77</td>
<td>2.9</td>
<td>146.9</td>
<td>11.6</td>
</tr>
<tr>
<td>7 M. Palieri V2N1 2011</td>
<td>581.1</td>
<td>465.4</td>
<td>18.2</td>
<td>0.80</td>
<td>2.8</td>
<td>122.4</td>
<td>12.1</td>
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<tr>
<td>8 M. Palieri V1N1 2011</td>
<td>617.6</td>
<td>448.8</td>
<td>20.7</td>
<td>0.73</td>
<td>4.0</td>
<td>89.8</td>
<td>14.6</td>
</tr>
<tr>
<td>9 Italia V2N2 2011</td>
<td>460.4</td>
<td>429.0</td>
<td>11.9</td>
<td>0.93</td>
<td>2.7</td>
<td>115.9</td>
<td>6.1</td>
</tr>
<tr>
<td>10 Italia V1N2 2011</td>
<td>534.2</td>
<td>514.5</td>
<td>5.4</td>
<td>0.96</td>
<td>1.7</td>
<td>190.9</td>
<td>5.7</td>
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<tr>
<td>11 Red Globe V2N1 2011</td>
<td>471.7</td>
<td>404.9</td>
<td>28.4</td>
<td>0.86</td>
<td>3.6</td>
<td>88.0</td>
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<tr>
<td>12 Red Globe V1N1 2011</td>
<td>300.5</td>
<td>251.6</td>
<td>23.1</td>
<td>0.84</td>
<td>4.4</td>
<td>46.6</td>
<td>12.1</td>
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<tr>
<td>13 Negroamaro V2N2 2011</td>
<td>375.3</td>
<td>354.4</td>
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<tr>
<td>14 Negroamaro V1N2 2011</td>
<td>401.7</td>
<td>306.7</td>
<td>9.0</td>
<td>0.76</td>
<td>4.1</td>
<td>60.1</td>
<td>20.2</td>
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<tr>
<td>15 Pinot 2011</td>
<td>229.5</td>
<td>143.2</td>
<td>11.4</td>
<td>0.62</td>
<td>3.4</td>
<td>32.5</td>
<td>84.5</td>
</tr>
</tbody>
</table>

*Flavan-3-ols: total sum of monomers, oligomers (degree of polymerization ≤ 3) and polymeric forms; Pol 1 + 2: polymeric forms (degree of polymerization ≥ 4); G (gallate) Mon (monomers) + Olig (oligomers) is the sum of (−) epicatechin gallate, monogalloylated dimer, monogalloylated dimer of type A, and monogalloylated trimer. MIC GM: geometric mean MIC against Candida albicans reference strains (ATCC90028, ATCC3153, ATCC10261, ATCC10231, and ATCC24433) determined using Clinical and Laboratory Standard Institute (CLSI) protocol M27-A3.*
Figure 2: In vivo imaging of mice vaginally infected with C. albicans gLUC59 and treated with Fluconazole (FLC) or M. Palieri V1N1 2010 GSE (V1N1). Mice under pseudoestrus condition were infected intravaginally with 10 μL of a 2 × 10^6 cell/mL suspension of C. albicans gLUC59 and treated with 10 μL of saline, 10 μL of FLC (200 mg/L), or 10 μL of V1N1 (0.5 g/L) 2 h before the challenge and then every two days. After 2, 5, and 8 days after infection mice were treated intravaginally with 10 μL of coelenterazine (0.5 g/L) and imaged in the IVIS-200TM imaging system under anesthesia with 2.5% isofluorane. Total photon emission from vaginal areas within the images (region of interest (ROI)) of each mouse was quantified with Living ImageR software package. The reported data come from one of the three experiments with similar results (a). Quantification of total photon emission from ROI was evaluated and the statistical significance was determined with Mann-Whitney U-test. P = 0.04762 (Day +2 postchallenge FLC-treated versus saline-treated mice); P = 0.01587 (Day +5 postchallenge FLC-treated versus saline-treated mice) and P = 0.00794 (Day +5 postchallenge V1N1-treated versus saline-treated mice) (b).

In conclusion, we demonstrated that GSEs obtained from Vitis vinifera plants, grown under hydric stress, had a high and reproducible content of polymeric flavan-3-ols, with a significant inhibition of C. albicans load 5 days after challenge, evaluated by photon emission.
a polymerization degree ≥4, and high antifungal activity. Further studies are in progress to characterize these polymeric fractions. Moreover, we demonstrated, for the first time, anti-

Candida activity of GSE in an experimental murine model of vaginal candidiasis. These findings, together with lack of toxicity and easy way of preparation of the extracts, suggest that GSEs with high content of polymeric flavan-3-ols could be used in Candida infections.

Ethical Approval

All animal experiments were performed in agreement with the EU Directive 2010/63, the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the National Law 116/92. The protocol was approved by Perugia University Ethics Committee for Animal Care and Use (Permit no. 149/2009-B). All the animals were housed in the animal facility of the University of Perugia (Authorization no. 34/2003A) in microisolator boxes with autoclaved diets and wood chips. Mice were acclimatized for a week before starting the experiments. All efforts were made to minimize suffering during experiments.

Conflict of Interests

All the authors declare that they had no personal relationship with other people and organisations that could inappropriately influence our work. G. Pasqua, G. Simonetti, F. D. D’Auria, A. R. Santamaria, D. Antonacci, and N. Mulinacci are named inventors on a patent pending based on Italian priority IT RM20100636 and related to “Extracts obtained from Vitis vinifera seeds and/or pomace and/or green grapes and/or stalks and uses thereof as antifungal agents” PCT/IT2011/000400, which is owned by Sapienza University (70%), CRA (20%), and University of Florence (10%). No commercial agreements are presently in place.

Acknowledgments

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References


[25] G. Pasqua, G. Simonetti, F. D. D’uria, A. R. Santamaria, D. Antonacci, and N. Mulinacci, Patent Pending Based on Italian Priority IT RM20100636 and Related to “Extracts obtained from *Vitis vinifera* seeds and/or pomace and/or green grapes and/or stalks and uses thereof as antifungal agents” PCT/IT 2011/000400, 2013.
Research Article

Topical Application of *Cleome viscosa* Increases the Expression of Basic Fibroblast Growth Factor and Type III Collagen in Rat Cutaneous Wound

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*Cleome viscosa* L. (Cleomaceae) is an important traditional medicine of the Indian-Ayurvedic and Chinese-medicine system documented for rheumatic arthritis, hypertension, malaria, neurasthenia, and wound healing. The plant is also known as Asian spiderflower and is distributed throughout the greater part of India. The present study explored the wound healing property of *C. viscosa* methanol extract (CvME) and its related mechanism using Wistar rat cutaneous excision wound model. Wound contraction rate, hydroxyproline quantification, and histopathological examination of wound granulation tissue were performed. The healing potential was comparatively assessed with a reference gentamicin sulfate hydrogel (0.01% w/w). Western blot for COL3A1, bFGF, and Smad-2, Smad-3, Smad-4, and Smad-7 was performed with 7-day postoperative granulation tissue. Results revealed that the topical application of CvME (2.5% w/w) significantly accelerated the wound contraction rate (95.14%, 24 postoperative days), increased the hydroxyproline content (3.947 mg/100 mg tissue), and improved histopathology of wound tissue as compared to control groups. Western blot analysis revealed that CvME significantly upregulated the expression of COL3A1 and bFGF and increased the Smad-mediated collagen production in granulation tissue. These findings suggest that *C. viscosa* promoted the wound repair process by attenuating the Smad-mediated collagen production in wound granulation tissue.

1. Introduction

Cutaneous wound is a physical injury that results in structural and functional discontinuity of skin tissue, and healing is a tangle tissue restorative process that involves distinct, overlapping phases of homeostasis, inflammation, proliferation, and tissue remodeling [1, 2]. Healing cascade starts from the moment of injury and involves continuous cell-cell and cell-matrix interactions till tissue remodeling of the scar. Blood coagulation at injury site provides temporary fibrin scaffold matrix, which hosts the infiltrating cells and serves as a reservoir of various growth factors and cytokines. Brief initial vasoconstriction at the injury site favors homeostasis and late vasodilation prompts the extravasations of inflammatory cells (neutrophils and macrophages) to remove the nonfunctional cells and destroy the invading infections [2, 3]. Proliferation phase initiated by macrophages involves the formation of granulation tissue and neovascularization. During the final remodeling phase of wound healing, the newly formed collagen fibers cross-link with other structural protein components and contribute to the strength of the scar tissue.

*Cleome viscosa* L. (Cleomaceae) is a sticky herb with yellow flowers, which resembles to mustard, and is distributed throughout the greater part of India. The plant is an important traditional medicine of the Indian-Ayurvedic and Chinese-medicine system for rheumatic arthritis, hypertension, malaria, neurasthenia, and snakebite [4–6]. Various scientific investigations revealed analgesic-antipyretic, anti-inflammatory, hepatoprotective, hypoglycemic, anthelmintic,
and antidiarrhoeal activity of C. viscosa [4, 7–10]. Panduraju et al. [11] reported the topical application of C. viscosa extract accelerated the wound healing in Wistar rat with significant wound contraction rate. Our previous experimental findings showed that methanol extract of C. viscosa ( CvME) possesses potent antioxidant, antimicrobial, and fibroblast proliferation properties as compared to petroleum ether, chloroform, and water extracts [12]. However, to rationalize the wound healing activity of C. viscosa, some major supportive evidences of efficacy such as histopathological examination, estimation of growth factors, and effect on collagen production in healing tissue are yet to be investigated. Therefore, the present study aims to evaluate the C. viscosa induced alteration in fibroblast growth factor and its effect on Smad-mediated collagen (type III) production in Wistar rat excision wound model.

2. Materials and Methods

2.1. Reagents and Chemicals. Glycine, phenazine methosulfate fluoride (PMSF), Bradford reagent, and Tris-HCl were purchased from Sigma (Sigma Chemical Co., USA). Carbopol 934, propylene glycol, gentamicin sulfate, l-hydroxyproline, and p-dimethyl- amino-benzaldehyde were obtained from Himedia (Himedia Pvt. Labs., India). bFGF, COL3A1, Smad-2, Smad-3, Smad-4, and Smad-7, and β-actin primary and secondary antibodies and BCPIP. (3-bromo-4-chloro-5’-indolylphosphate p-toluidine salt–) NBT reagents were procured from Santa Cruz (Santa Cruz, USA). PVDF membrane was from Millipore (Millipore Corp., USA) and Western Max-HRP-Chromogenic detection kit was purchased from Amresco (Amresco, USA). All other chemicals and reagents not mentioned were of the analytical grade.

2.2. Preparation of Extracts. C. viscosa leaves were collected during August-September (2011) from the herbal garden of the Defence Research Laboratory, Tezpur, Assam, India, authenticated by the Botanical Survey of India, Shillong (Accession number 085249). About 100g of shades dried leaf powder was successively extracted using organic solvents (petroleum ether, chloroform, and methanol) by Accelerated Solvent Extractor (ASE1.5, Dionex, USA) as described earlier [12] and concentrated in a rotary evaporator (Rotavac, Heidelberg, Germany) under reduced pressure. Water extract was freeze dried by lyophilization. Preliminary phytochemical screening was performed as described earlier [13].

2.3. Wound Healing Activity

2.3.1. Animals. Healthy adult Swiss albino mice (20–25 g) and Wistar rats (250–300 g) were housed in the Defence Research Laboratory (DRL), Tezpur, Assam, India, and acclimatized for 3 days. Animals were given free access to feed and water ad libitum. The experiments were performed according to the Institutional Animal Ethical Committee guidelines (IAEC/DRL/05/July/2011).

2.3.2. Acute Skin Irritation and Toxicity Study. The acute skin irritation and toxicity study was performed as per OECD guidelines-402 (OECD guidelines, 1987) to determine the therapeutic dose of CvME. CvME hydrogel (1 and 2.5% w/w) was applied to the shaved portion at the back of the mice and observed for 14 days for an abnormal skin response, including irritation, redness, itching, and other related symptoms [14].

2.3.3. Animal Grouping and Excision Wound Creation. Rats were inflicted with excision wounds as described by Atiba et al. [15]. Animals were anesthetized and a circular 20mm diameter wound was created on the shaved dorsal skin up to the depth of loose subcutaneous tissue. Animals were randomly divided into four groups (n = 20): group I, nontreated; group II, vehicle control (Carbopol 934 containing 5% propylene glycol); group III, CvME hydrogel treated (2.5% w/w); and group IV, gentamicin sulfate (0.01% w/w). Treatment was given once daily until complete epithelialization. For one-third of animals, wound granulation tissues (excluding any underlying muscle and extraneous tissue) were harvested on the 7th postoperative day. A portion of tissue was processed for Western blotting and another portion was fixed in Histochoice tissue fixative (Amresco, USA) for histopathological evaluations. Half of the remaining animals were euthanized on day 15 after injury; the entire remodeling tissue was used for histopathological assessment and the remaining animals were observed until complete epithelialization.

2.3.4. Wound Contraction Rate and Hydroxyproline Content Estimation. The progressive changes of wounded area were photographed (Nikon Coolpix-S3000) and evaluated by using special size analysis software ImageJ (National Institutes of Health, ImageJ software, downloaded from http://rsb.info.nih.gov/ij/index.html). Wound contraction was expressed as a percentage of the original wound size (day 0).

Hydroxyproline content was analyzed on day 7 in postinjury granulation tissue as described by Neuman and Logan [16]. Acidic tissue hydrolysate was neutralized to pH 7.0 and mixed with 10 mM CuSO4 and 2.5 N NaOH followed by 6% H2O2. The solution was mixed and incubated at 80°C for 5 min with frequent vigorous shaking. Upon cooling, 3 N H2SO4 was added with agitation. Finally, 5% p-dimethyl-amino-benzaldehyde was added and incubated at 70°C for 15 min. Absorbance was measured at 500 nm using a UV-VIS spectrophotometer (CE7200, CECIL, USA). The standard calibration curve was plotted for pure hydroxyproline and used for estimation of the test samples.

2.3.5. Histopathological Evaluation. Tissues were sectioned (6μm) and stained with hematoxylin-eosin (HE) and Masson’s trichrome (MT) stain. Tissue sections were examined for epithelialization, inflammatory cell infiltration, fibroblast proliferation, neovascularization, and collagen deposition.

2.3.6. Western Blot Analysis. The harvested wound tissues were homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, and 0.05 M PMSF; pH 7.4) and centrifuged at 10,000 × g
for 10 min at 4°C (3-30K, Sigma, Germany). Protein concentration was estimated by Bradford reagent [17]. Equal amount of protein was electrophoresed onto the 12% SDS-PAGE at 80 V for 45 min (Mini Trans-Blot, BioRad Laboratories Inc., USA). Proteins were transblotted on the PVDF membrane (Millipore Corp., USA) and processed with COL3A1, bFGF, Smad-2, Smad-3, Smad-4, and Smad-7, and β-actin primary antibodies (1 : 1000) and corresponding secondary antibodies (1 : 2000). The desired proteins were detected by Western Max-HRP-Chromogenic detection kit and BCIP-NBT solution (Amresco, USA).

2.4. Statistical Analysis. The results are expressed as means ± standard deviation (S.D.). The treated groups were compared with control groups by analysis of variance following Dunnett’s test. A statistical P value less than 0.05 was considered significant.

3. Results and Discussion

3.1. Extraction of Phytochemical from C. viscosa. The powdered C. viscosa leaf (100 g) yielded 2.82 g n-petroleum ether (CvPE), 1.57 g in chloroform (CvCE), 12.03 g in methanol (CvME), and 9.11 g in water (CvWE). The preliminary phytochemical screening showed the presence of alkaloids, flavonoids, terpenes, and carbohydrates in different extracts. In our earlier study, we have reported about its potent antioxidant, antimicrobial, and human dermal fibroblast proliferation activities of CvME [12] which was similar to the previous reports of Koca et al. [18] and Gouthamchandra et al. [19], where the alcoholic extracts were reported as potent antioxidant and antimicrobial and showed significant wound healing property in comparison to other sequential extracts.

Mondal and Suresh [20] also reported the wound healing activity in other species of Cleome, where the methanol extract has shown faster wound contraction rate over other extracts. Therefore, CvME was selected for in vivo wound healing evaluation using Wistar rat.

3.2. Acute Skin Irritation and Toxicity of CvME. Animals treated with CvME hydrogel (1 and 2.5%, w/w) did not show any symptoms of skin irritation and inflammation. Therefore, the extract was considered safe and 2.5% (w/w) of C. viscosa methanol extract hydrogel was used for in vivo wound healing study.

3.3. Effect of CvME on Wound Contraction Rate. Wound contraction is the centripetal movement of surrounding epithelial tissues that depends on the reparative ability and general health of the tissue [21, 22]. The wound appeared clean and free of exudates and distinct granulation tissues appeared at the wounded edges from day 3 after injury in CvME and gentamicin sulfate treated groups. Wound areas were reduced parallel to postoperative days and CvME treated group showed 60.99, 71.13, 82.34, and 90.87% wound contraction, whereas gentamicin sulfate treated group showed 57.2, 71.58, 79.51, and 90.02% in 12, 15, 18, and 21 postoperative days, respectively (Figures 1(a) and 1(b)). On the other hand, nontreated control group showed 23.85, 28.74, 42.92, and 50.63% wound contraction and vehicle treated groups showed 33.75, 41.16, 48.54, and 57.49%, in 12, 15, 18, and 21 postoperative days, respectively (Figure 1(b)). Although the wound contraction rate by CvME was comparable to gentamicin sulfate treated groups, it showed significantly higher P < 0.05 as compared to nontreated and vehicle treated control groups. These findings coincided with the
Table 1: Histopathological evaluation of wound healing process in different treatment groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>S</th>
<th>U</th>
<th>Ed</th>
<th>PMC</th>
<th>MNC</th>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Nontreated</td>
<td>++</td>
<td>++</td>
<td>++/+</td>
<td>+++</td>
<td>+</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vehicle control</td>
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<td>++/+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+/+</td>
<td>–</td>
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<tr>
<td>CvME (2.5%)</td>
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<td>–</td>
<td>–</td>
<td>++</td>
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<tr>
<td>Gentamicin sulfate (0.01%)</td>
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<td>–</td>
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<td>++</td>
<td>+</td>
<td>++</td>
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<td>–</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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</tr>
<tr>
<td>CvME (2.5%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–/+</td>
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<td>+++</td>
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</tr>
<tr>
<td>Gentamicin sulfate (0.01%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–/+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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</tbody>
</table>

HE and MT staining were scored as mild (+), moderate (++), and severe (+++) for epidermal and/or dermal remodeling. S: scab; U: ulcer; Ed: edema; PMC: polymorphonuclear cells; MNC: mononuclear cells; FP: fibroblast proliferation; CD: collagen deposition; RE: reepithelialization; CvME: C. viscosa methanol extract.

3.4. Effect of CvME on Hydroxyproline Content. Hydroxyproline is a basic constituent of collagen structure and contributes to 14% approximately. Measurement of hydroxyproline can be used as an index for collagen turnover [22]. Increase in the hydroxyproline content indicates increased collagen synthesis, which corresponds to the enhanced wound healing. In the present study, the topical application of CvME hydrogel significantly increased \((P < 0.05)\) the hydroxyproline content as compared to nontreated and vehicle treated control groups (Figure 2). CvME treatment also showed higher hydroxyproline content as compared to gentamicin sulfate group, although the data were statistically insignificant. These results therefore reflect the potent wound healing activities of CvME in Wistar rats.

3.5. Effect of CvME on Histopathology of Wound Granulation Tissue. Table 1 indicated that the histopathology examination of the wound granulation tissue supported the findings of CvME accelerated wound healing as an organized wound repair process (inflammation, proliferation, and remodeling). On the other hand, slow epithelialization rate and less collagen density were observed in vehicle and nontreated groups. Histopathological section of 7-postoperative-day wound tissue showed increased infiltration of macrophages/fibroblasts with less inflammatory cells and ulcer-edematous areas in both CvME and gentamicin sulfate treated groups. On the other hand, mild edema with high polymorphonuclear (inflammatory) cell density and less macrophages/fibroblasts were observed in control groups (Figure 3). Fifteen-day CvME topical application significantly accelerated the cutaneous wound repair processes as evident from the appearance of thick, well organized reepithelialized epidermis and compact dermis with abundant collagen fibers. Mason's trichrome (MT, 400×) staining for 15-day postoperative wound tissue depicted distinct mononuclear cells (macrophage and fibroblast) infiltration and faster keratinization with intraepithelial cornification in CvME (Figure 3), whereas slow reepithelialization and less collagen bundle were noticed in control groups. The results of improved histopathology of wound granulation tissue supported the faster wound contraction rate and elevation of hydroxyproline content in CvME hydrogel treated group, which corroborates the earlier reports on wound healing processes [24–26]. These effects of CvME in wound repair...
**Figure 3:** Microscopic view of healing wound tissue and epidermal/dermal remodeling in (1) nontreated, (2) vehicle control, and (3) CvME and (4) gentamicin sulfate treated animal groups. Section shows the hematoxylin and eosin stained epidermis and dermis in (a) and (c) (100x) and Masson's trichrome in (b) and (d) (400x) of 7- and 15-day postoperative treated animal groups, respectively. Arrow points the events of wound healing. S: scab; U: ulcer; Re: reepithelialization; F: fibroblast; PMC: polymorphonuclear cells; MNC: mononuclear cells; C: collagen; and NV: neovascularization.
3.6. Effect of CvME on Granulation Tissue Protein. Proliferation phase of wound healing involves formation and vascularization of granulation tissue, collagen production, and their subsequent maturation. Collagen type III, present in remodeling wound tissues, is produced by the proliferating young fibroblasts under the influence of various growth factors like transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and so forth. The bFGF is a potent angiogenic molecule, which promotes the neo-vascularization and blood supply to the granulation tissue. Increased oxygen supply facilitates the maturation of collagen fibers in granulation tissue [3]. Western blot analysis revealed that application of CvME significantly increased (P < 0.05) the expression of COL3A1 and bFGF protein in wound granulation tissue (Figure 4), thus further confirming the above findings of faster wound contraction rate and higher hydroxyproline content.

Various growth factors such as TGF-β, bFGF, PDGF (platelet derive growth factor), and VEGF (vascular endothelial growth factor) released by the activated platelets, macrophages, and lymphocytes control the proliferation phase of wound healing [1, 3]. Released TGF-β bound to the fibroblast TGF-β receptors in granulation tissue and initiates the TGF-β-Smad-mediated collagen production cascade [30]. Intracellular Smad family protein (TGF-β type 1 receptor kinases substrate) forms heterocomplex (Smad-2/Smad-3/Smad-4) and transduced the extracellular TGF-β signal to fibroblast nucleus for collagen production. Therefore, Smad family proteins were analyzed by Western blot and signal transducer protein (Smad-2, Smad-3, and Smad-4) and showed significantly (P < 0.05) higher expression in CvME hydrogel treated group, whereas the inhibitory protein (Smad-7) was observed to be equal in all treatment groups. β-Actin was used as an internal control. These findings confirmed that the Smad-mediated mechanism is involved in the increased COL3A1 expression, higher hydroxyproline content, and faster wound contraction rate that contributes to overall improved histopathology during wound repair process.

Therefore, the present study suggested that the topical application of C. viscosa methanol extract enhances the wound repair process by attenuating TGF-β-Smad-mediated collagen production in wound granulation tissue.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

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References


Effect of Dietary Intake of Avocado Oil and Olive Oil on Biochemical Markers of Liver Function in Sucrose-Fed Rats

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Metabolic changes, along with cardiovascular and hepatic factors, are associated with the development of diseases such as diabetes, dyslipidemia, and obesity. We evaluated the effect of avocado oil supplementation (centrifuged and solvent extracted), compared with olive oil, upon the hepatic function in sucrose-fed rats. Twenty-five rats were divided into five groups: control (basal diet), a sucrose-fed group (basal diet plus 30% sucrose solution), and three other groups (S-OO, S-AOC, and S-AOS, indicating basal diet plus 30% sucrose solution plus olive oil OO, avocado oil extracted by centrifugation AOC or using solvent AOS, resp.). Glucose, total cholesterol, triglycerides, total protein, albumin, globulin, direct bilirubin, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, alkaline phosphatase, cholinesterase, and α-amylase concentrations were determined and avocado oil effect on them was studied. In some cases the induced metabolic alteration significantly affected total protein and bilirubin levels and also had a highly significant effect on α-amylase levels. AOC and AOS exhibited effects similar to those of olive oil, according to the nonsignificant difference in fatty acid profile observed by other authors. Avocado oil consumption could be beneficial in the control of altered metabolic profile illnesses as it presents effects on hepatic function biochemical markers similar to olive oil.

1. Introduction

The incidence of metabolic syndrome in Mexico is one of the highest in the world, so much that it has become a public health problem. However, little has been done to prevent the factors responsible for this. Epidemiological studies in our country highlight the need to strengthen strategies for its detection, control, and treatment. It involves a set of three or more alterations such as overweight or obesity and disturbance in glucose metabolism and insulin, along with hypertension, dyslipidemia, and other abnormalities of importance that are related to its development and are grouped in different profiles, such as liver, pancreatic, and cardiovascular functions [1–4]. The incidence is about 25% in the general population and there are no differences between men and women, although it varies according to genetic
factors [5, 6]. Reports in the scientific literature show the benefits that the Mediterranean diet and olive oil have on health [7, 8]. These have aroused interest in studying oils rich in monounsaturated fatty acids, especially avocado oil and its effect on manifestations of health disorders in metabolic abnormality. In nonpharmacological treatment, the consumption of monounsaturated fatty acids such as oleic acid, found in different types of oils like olive and avocado, is recommended. The avocado fruit is a rich potential source of oil, mostly monounsaturated [9], and a good source of linoleic acid [10]. It also contains high levels of antioxidants including polyphenols, proanthocyanidins, tocopherols, and carotenoids, which have shown positive outcomes in health. Studies in humans and animal models have showed that it helps to control weight, reduce the risk of diabetes [11], normalizes blood cholesterol, and is involved in liver metabolism [13]. In addition, the phytochemical components of avocado oil are also related to the disease manifestations associated with an altered metabolic profile, so, overall, it is expected that all the beneficial properties of avocado oil together will add up to positive health effects. The purpose of this study was to evaluate the effect of avocado oil supplementation, as compared to that of olive oil, upon biochemical markers and hepatic function associated with a condition induced in rats through the administration of sucrose in drinking water.

2. Materials and Methods

2.1. Avocado Oil Extraction. There are different technologies for extracting oil from avocados and they can affect its quality. The oil was obtained from Hass avocado purchased from a local market in the Port of Veracruz, Mexico. When edible maturity had been reached, the avocados were washed and peeled and the seed removed. Subsequently, the pulp was homogenized by adding tert-butylhydroquinone (TBHQ) at 0.1% (w/w).

2.1.1. Oil Extraction by Centrifugation. The avocado pulp was mixed with water to achieve a 1:1 w/v and NaCl (7.5% w/w), the pH was adjusted to 5.5 with ascorbic acid and the mixture was homogenized in a blender (Black & Decker Model MX 150) at 1,300 rpm for 1 hour at 35°C. Subsequently, the oil was removed by centrifugation at 27000 rpm in a tubular continuous centrifuge (Cepa-Schnell, GLE Model NBS) fed at 2.8 L/min.

2.1.2. Avocado Oil Extraction by Solvent. A homogenate was made with a portion of the avocado pulp and two parts of a mixture of hexane-isopropanol (2:3 v/v) in separate funnels, and the oil phase was collected. Subsequently, the solvent was removed in a rotary evaporator (Buchi R-215, Labortechnik AG, Switzerland) at 30°C and 500 mmHg pressure. The remaining solvent was removed by entrainment with nitrogen gas and then the oil was exposed to high vacuum in a freeze dryer for 24 h. Thereafter the oil was stored in refrigeration and protected from light until use.

2.2. Animals and Treatments. In this experiment 25 male Sprague-Dawley weaned rats (3 weeks old and weighing 240±16 g) were purchased from Teklad, Co. (Mexico City), and caged individually in stainless steel boxes in a room with controlled temperature (25°C) and a light-dark cycle of 12 hours. The experimental protocol for the management of experimental animals was approved by the animal ethics committee, Biochemical and Nutrition Chemistry Area, University of Veracruz. The basal diet was prepared according to the American Institute of Nutrition [14] as shown in Table 1. A mixture of corn-canola oil (7.5 g/100 g diet) was used as a source of dietary fat (Patrona from the local market). The experimental diet was prepared based on the composition of the basal diet plus oil (7.5% w/w), olive oil (Carbonell), and avocado oil extracted by centrifugation or solvent, respectively. Diets were prepared once a week and kept in powder form 4°C until use. As part of this study, the fatty acid composition of the oils used in preparing diets was analyzed and it was found that all the oils had a rather similar composition, mainly oleic and linoleic acids (Table 2).

The animals were divided into two groups: a control group (CG, n = 5) receiving a basal diet and a sucrose-fed group (S, n = 20) which received the basal diet plus 30% sucrose solution as drinking water. The animals had free access to food and water for 16 weeks and food intake was measured daily. At the end of this period, the diet was withdrawn for at least 4 hours and the manifestation of the metabolic characteristics was checked by first determining

<table>
<thead>
<tr>
<th>Table 1: Composition of basal and experimental diets formulated according to AIN-76G.</th>
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<tr>
<td><strong>Ingredients</strong></td>
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<tr>
<td>Cornstarch</td>
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<tr>
<td>Casein</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>Mineral Mix AING-76 G</td>
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<tr>
<td>Vitamin Mix AING-76 G</td>
</tr>
<tr>
<td>DL-Methionine</td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
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<td>Fat†</td>
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</table>

† Corn-canola, olive, or avocado oil. Experimental diets were formulated with basal diet plus oil: olive or avocado oil extracted either by centrifugation or solvent.

<table>
<thead>
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<th>Table 2: Fatty acid composition of dietary oils (%)</th>
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<tr>
<td><strong>Fatty acid</strong></td>
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<tr>
<td>16:0</td>
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<td>16:1</td>
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<td>18:0</td>
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<tr>
<td>18:1</td>
</tr>
<tr>
<td>18:2</td>
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<td>18:3</td>
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Values are expressed as mean of duplicate analysis. Avocado†: avocado oil extracted by centrifugation, Avocado‡: avocado oil extracted by solvent.
body weight, then serum glucose, triglycerides, and cholesterol levels by cardiac puncture.

Once the sucrose-fed model had been obtained, the S animals were divided into four groups of five rats each. One group was maintained on the basal diet (the sucrose-fed group, S); three groups of rats designated, as S-OO, S-AOC, and S-AOS, respectively, received an experimental diet containing 7.5% w/w oil (olive and avocado extracted by centrifugation or extracted with solvent) as the sole source of dietary fat. These four groups received the experimental diets and water with 30% sucrose solution for 4 weeks. The CG group continued to receive only the diet with corn canola oil and no sucrose in the drinking water. Diets were prepared once a week and kept refrigerated until use. Tert-butylhydroquinone (TBHQ) at 0.02% was used to prevent fatty acid oxidation. At the end of the experiment, the diet was withdrawn and the fasting animals were sacrificed through decapitation. Serum glucose, cholesterol, triglyceride, and phospholipid levels were determined. All animals were sacrificed and the organs were extracted for further analysis.

2.3. Assays. Glucose was determined with the glucose oxidase method. Total cholesterol, triglycerides (TG), total protein, albumin, globulin, direct bilirubin, glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), alkaline phosphatase (ALP), cholinesterase, and α-amylase were determined with an automated analyzer (RA 1000 XT, Bayer Technicon) through enzymatic colorimetric methods using commercial kits obtained from Bayer and BioMerieux. The fatty acid profile of vegetable oils was determined through gas chromatography (Hewlett Packard 5890, Palo Alto, CA.) with pentadecanoic acid as internal standard. All the chemicals used were of analytical grade.

2.4. Statistical Analysis. The data are expressed as the mean ± standard deviation (x ± SD). Statistical significance was determined with analysis of variance procedures, with a post hoc Tukey multiple-range test for comparison of means (P < 0.05).

3. Results

3.1. Metabolic Characteristics of Rats in the Control and Sucrose-Fed Group. Table 3 shows growth variables, food and caloric intake, liquid consumption, and biochemical markers to assess rats in the control group (CG) and sucrose-fed rats (S). At 16 weeks, a significant increase (P < 0.05) in final body weight and body weight gain was observed in the S group as compared to the CG group. The food intake in rats in the CG group was significantly higher (P < 0.01) than in the S group. On the other hand, the S group showed a daily liquid intake significantly higher (P < 0.05) as compared with the CG group. However, when the daily liquid intake per 100 g in weight was compared between CG and S group, this was not significant. The caloric equivalent produced by liquid intake was 10.8 ± 1.7 kcal in the S group; the CG group did not have any energy intake because this group received only purified drinking water. Triglyceride levels in the S group were significantly greater (P < 0.01) than in the CG group; however, no significantly different results were found in any group for either glucose or cholesterol levels.

3.2. Effect of Dietary Oils on Liver Function Biochemical Markers. The effect of olive and avocado oils on liver function indicators is shown in Figure 1. S, S-OO, S-AOC, and S-AOS study groups showed changes in total protein levels, all of them significantly higher (P < 0.05) than control group (CG). Additionally, direct bilirubin levels in S-OO and S-AOC groups decreased (P < 0.05) in relation to CG and S groups, but not for the S-AOS group, where a nonsignificant decrease was observed. Albumin and globulin levels in S-OO, S-AOC, and S-AOS study groups were not found to be significantly different either from S or CG groups.

3.3. Effect of Dietary Oils on Pancreatic Function Biochemical Markers. The effect of dietary olive and avocado oils on pancreatic function indicators is shown in Figure 2. Levels of glutamic oxaloacetic transaminase (GOT) in S-AOC and S-AOS groups were similar and not significantly different than either the S or CG group. Significantly lower levels were observed in the S-OO group (P < 0.05) in comparison with all the groups in the study. In the cases of glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP), no significant results were observed among the study groups. The S-AOS group showed similar, nonsignificant values for cholinesterase in comparison with CG. In contrast, S and S-AOC groups both presented significantly lower results (P < 0.05), but in the case of S-OO group these levels decreased in a highly significant manner (P < 0.01) when compared to CG and S-AOS. Levels of α-amylase were all similar for S, S-OO, S-AOC, and S-AOS groups; however, when compared to control group CG, the results were all very significantly higher (P < 0.01).

<table>
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<tr>
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<tr>
<td>Final body weight (g)</td>
<td>445 ± 53</td>
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<td>Body weight gain (g)</td>
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<td>Food intake (g/d)</td>
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<td>Liquid consumption (mL/d)</td>
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<tr>
<td>Liquid consumption (mL/d/100 g bw)</td>
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<tr>
<td>Equivalent in kcal in drinking water</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>114 ± 18</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>104 ± 12</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>79 ± 12</td>
</tr>
</tbody>
</table>

Values are mean ± SD. CG group, n = 5; S group, n = 20. *P < 0.05; **P < 0.01.
4. Discussion

Diabetes, dyslipidemia, and obesity are risk factors with a great impact on the development of diseases associated with an altered metabolic profile or metabolic syndrome. Liver and heart factors also play an important role.

Within this framework, in the present study, significant differences were found for S group as compared to the CG group in final body weight and weight gain, which were significantly higher (6 and 11% resp.), although food intake was significantly lower (54%). These results are consistent with those reported in other studies where metabolic changes were induced by the administration of a sucrose-rich diet in addition to an experimental diet causing changes in the biochemical indicators measured [15, 16]. In relation to serum biochemical indicators associated with the development of metabolic abnormality, it was found that glucose and cholesterol concentrations in S group rats were similar to those in the CG group and not significant. Reaven and Chang [17] have suggested that this is due to hyperinsulinemia developed in metabolic abnormalities which maintains normal levels of blood glucose. TG levels were significantly higher (56%) in S group rats (a 2.3-fold increase). Other studies have found similar results [18, 19]; Piatti et al. [20] reported the association in healthy patients between sudden TG elevation and insulin resistance and suggested that the increase in blood TG in vivo inhibits glucose utilization and oxidation stimulated by insulin action in the peripheral tissues. One way to explain the blood TG elevation might be to consider a possible increase in the reesterification of fatty acids from
Figure 2: Blood serum profile levels of liver function markers in rats fed with different oil diets: (a) GOT; (b) GPT; (c) Alkaline phosphatase; (d) Cholinesterase; (e) α-amylase. Corn-canolan diet (CG group, n = 5); corn-canolan diet plus 30% sucrose in drinking water (S group, n = 5); olive oil diet plus 30% sucrose in drinking water (S-OO group, n = 5); avocado oil diet extracted by centrifugation plus 30% sucrose in drinking water (S-AOC group, n = 5); avocado oil diet extracted by solvent plus 30% sucrose in drinking water (S-AOS group, n = 5). GOT: glutamic oxaloacetic transaminase; GPT: glutamic pyruvic transaminase. Values are mean ± SD. ∗P < 0.05; ∗∗P < 0.01 versus corresponding data in CG group.
the liver as a result of fructose metabolism as reported by Bezerra et al. [18]; this monosaccharide stems from sucrose hydrolysis and, in the liver, fatty acids are mainly used for the high-density lipoprotein (HDL) and TG synthesis, which in turn raise their serum levels.

Sucrose intake did not yield a significant influence on total protein levels as values encountered in sucrose-fed study group and in those fed with olive oil and avocado oil extracted by centrifugation or by solvents were statistically similar (7.9, 7.7, 7.5, and 7.8 g/dL, resp.) for S, S-OO, S-AOC, and S-AOS groups. Nevertheless, total protein values for the sucrose-fed group (S), as well as for S-OO, S-AOC, and S-AOS groups, increased significantly (16, 14, 12, and 15%, resp.) compared to control (CG). This result suggests that avocado oil exerts an effect similar to that of olive oil on liver synthesis of total proteins which are used as markers for liver damage; a rise in these proteins is associated with the development of a nonalcoholic liver pathology, such as fatty liver, linked to metabolic syndrome [21].

Direct bilirubin indicates a loss in hepatocyte function; these levels decreased significantly in S-OO and S-AOC groups in this study compared to control (CG), sucrose-fed group (S), and the group fed with avocado oil extracted by solvent (70, 63, 63% and 60, 50, 50%, resp.). No significant differences were found for albumin and globulin levels in any group in the study. However, albumin levels in the groups fed with avocado oil extracted by centrifugation or solvent were slightly greater, although not significantly so, compared to control (3.6, 4.0 versus 2.9 g/dL, resp.). This indicates that albumin blood levels (a liver damage marker) are not brought back to normal levels by this oil, possibly suggesting that avocado oil composition as a result of the extraction method could have an influence on the level of regulation of this marker.

On the other hand, globulin levels in S group increased (23%), although not significantly, when compared to control. In other groups, S-OO, S-AOC, and S-AOS, globulin levels exhibited a nonsignificant decrease (15, 19, and 21%, resp.) compared to the sucrose-fed group (S) but were slightly higher, again not significantly so (10, 5, and 3%, resp.) than control (CG). These results indicate a return to normal globulin levels as a result of olive and avocado oil consumption. This could have induced a reversion to the damage caused to the liver parenchyma through steatosis, given that in this pathology blood globulin levels remain high.

It was also found that albumin/globulin ratio values (RAG) of rats fed olive oil and avocado oil extracted by centrifugation or by solvents were higher than the sucrose-fed group (0.9, 0.9, and 1.0 versus 0.7, resp.). The results for this marker could suggest a regeneration of liver tissue, as RAG values of the aforementioned study groups were close to 1, similar to the CG value. S group, on the other hand, had quite a low value for this ratio which suggests liver damage.

Mention should be made that to date no reports have appeared in the scientific literature about the effect of avocado oil on the basic profile of protein synthesized by the liver which are used as liver damage markers, specifically cirrhosis of the liver and fatty liver, thus warranting the determination of these values in the present study.

As for control group (CG), GOT and GPT values decreased in the rest of the groups (S, S-OO, S-AOC and S-AOS). An increase, not a decrease, in these enzymes is indicative of liver failure. The effect of administering a diet rich in carbohydrates (30% sucrose), known to contribute to metabolic syndrome in murine models through oxidative stress, or through the activation of adipocyte-specific genes (lipogenesis), among other effects, and in consequence to hepatic metabolic changes [22–24] was not observed in this case related to an increase in GOT and GPT values.

A significant decrease in GOT was observed in the S-OO group. Both olive and avocado oils are recognized as oils with a high percentage of unsaturated fatty acids and a low percentage of saturated [25, 26], (84, 88 and 17, 12%, resp.) in murine models, different transcriptomic responses between diets based on different long-chain polyunsaturated fatty acids have been observed. Furthermore, stereochemistry influences differential responses as seen with linoleic acid isomers [23].

Sucrose intake significantly decreased cholinesterase levels in the sucrose-fed group (S) by 20% compared to control. Olive oil did not improve this situation at all and in fact the results of the S-OO group were significantly even lower than S. Avocado oil extracted by solvent brought cholinesterase levels back to normal, similar to those of control (3866 versus 3999 mg/dL, resp.), but, for the S-AOC group, results remained at the same level statistically as the sucrose-fed group (S).

A decrease in cholinesterase levels can be indicative of hepatic disease. As far as can be ascertained, the different responses of olive and avocado oil observed here have not been reported before. A possible explanation could be based on the specific chemical composition of each oil type and their capacity to overcome the diet induced metabolic alterations. The unsaponifiable fraction of fatty oils contains more than 100 components. A specific example is the case of olive oil whose fraction contains more than 300 components [27]. It has been demonstrated that these minor components also exert effects on the metabolic pathways and according to Osada, 2013, the term “monounsaturated fatty acid–enriched oil” including oils such as olive and other oils, based on the high percentage of oleic acid, “no longer appears appropriate for describing their biological properties since they have different unsaponifiable composition and this fraction is highly active.”

For S, S-OO, S-AOC, and S-AOS groups in the study, α-amylase levels were found to be significantly higher compared to control (36, 47, 43, and 44%, resp.). This points to pancreas damage which was not reversed by the administration of dietary oils (S-OO, S-AOC, and S-AOS); in these groups, significantly higher α-amylase levels were found (1205, 1119, and 1146 versus 996 U/L, resp.) compared to S. To our knowledge, no data are available in the literature about the effect of avocado oil on the levels of this enzyme, even less where sucrose ingestion is concerned.

Sucrose intake caused no effect in ALP levels in S-OO, S-AOC, and S-AOS groups as these were similar and did not differ from control (CG) or sucrose-fed group (S). However, ALP levels in S-AOC and S-AOS groups were seen
to increase (15 and 11%, resp.) in comparison with control and 24 and 20%, respectively, when compared to S. It has been reported that in cases of nonalcoholic fatty liver disease alkaline phosphatase levels increase and are a favorite marker for metabolic syndrome [28].

5. Conclusion

To sum up, these results indicate that sucrose intake affected total protein and bilirubin levels; the results of other markers did not show any evidence of liver damage, probably because the window for manifestation was very short. However, variations in the aforementioned marker levels indicate that the liver was affected and that the albumin/globulin ratio (RAG) under the influence of avocado oil administration revealed the beginning of a regeneration of liver function. On the other hand, neither the administration of olive oil nor avocado oil extracted by centrifugation or using solvent was able to attain normal α-amylase levels, indicating that anomalies in pancreatic function were not reversed. Avocado oil exhibits effects similar to those of olive oil, and no differences in biochemical markers were found between the two methods of avocado oil extraction. This finding is correlated to the nonsignificant difference observed in fatty acid profile of avocado oils obtained by the two aforementioned extraction methods, as reported by Ariza-Ortega et al. [29].

Conflict of Interests

The authors have declared that no competing interest exists.

References


Research Article

Effect of Curcumin on Lifespan, Activity Pattern, Oxidative Stress, and Apoptosis in the Brains of Transgenic Drosophila Model of Parkinson’s Disease

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Background. A time dependent loss of dopaminergic neurons and the formation of intracellular aggregates of alpha synuclein have been reported in PD model flies. Methods. The progeny (PD flies) expressing human alpha synuclein was exposed to 25, 50, and 100 μM of curcumin mixed in the diet for 24 days. The effect of curcumin was studied on lifespan, activity pattern, oxidative stress, and apoptosis in the brains of PD model flies. The activity of PD model flies was monitored by using Drosophila activity monitors (DAMs). For the estimation of oxidative stress, lipid peroxidation and protein carbonyl content were estimated in the flies brains of each treated groups. The cell death in Drosophila brain was analyzed by isolating brains in Ringer’s solution placing them in 70% ethanol and stained in acridine orange to calculate the grayscale values. Results. The exposure of flies to 25, 50, and 100 μM of curcumin showed a dose dependent significant delay in the loss of activity pattern, reduction in the oxidative stress and apoptosis, and increase in the life span of PD model flies. Conclusion. Curcumin is potent in reducing PD symptoms.

1. Introduction

Parkinson’s disease (PD) has been classified as a movement disorder and is characterized by the loss of dopaminergic neurons in substantia nigra [1]. The abnormal expression of alpha synuclein (αS) results in the formation of Lewy bodies: a pathological hallmark of PD [2]. The availability of various experimental models for PD, based on αS overexpression (mutant or wild form) in flies or mice, has led research scientists to study the effects of various compounds on the progression of PD symptoms [3]. Oxidative stress has been attributed as one of the important factors in progression of PD [4]. An emphasis has been given for the use of flavonoids to reduce the oxidative stress in the neurons [5–7]. Curcumin is the principal curcuminoid of the spice turmeric (Curcuma longa), a member of the ginger family [8]. Besides having a number of pharmacological properties [9], in our earlier study it was reported to delay the loss of climbing ability in the PD model flies [10]. In the present study, the effect of curcumin was studied on the life span, activity pattern, oxidative stress, and apoptosis in the brains of transgenic Drosophila model of PD.

2. Materials and Methods

2.1. Drosophila Stocks. Transgenic fly lines that expresses wild-type human synuclein (h-αS) under UAS control in neurons “w[+]; P[w[+mC]=UAS-Hsap/SNCA.F]”5B and GAL4”w[+];P[w[+mC]=GAL4-elavL]”3 were obtained from Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN). When the males of UAS (Upstream Activation Sequence)-Hsap/SNCA.F strains are crossed with the females of GAL4-elav. L (vice versa) the progeny will express the human alpha synuclein in the neurons [1].
2.2. Drosophila Culture and Crosses. The flies were cultured on standard Drosophila food containing 0.83% agar, 4.72% corn meal, 4.16% sugar, and 1.67% yeast at 25°C (24 ± 1) [11]. Crosses were set up as described in earlier published work [12]. The PD flies were exposed separately to different doses of curcumin (Sigma Aldrich, CAS 458-37-7) and mixed in culture medium at final concentration of 25, 50, and 100 μM. The PD flies were also exposed to 10⁻³ M of L-dopamine. The UAS-Hsap/SNC.F acts as a control. The control flies were also separately exposed to the selected doses of curcumin.

2.3. Activity Pattern. From the 12th day onwards, the activity of flies (males) in all treated groups was analysed by using Drosophila Activity Monitor (TriTek, USA). The activity was recorded every hour for a total of 267 hrs and the data was analysed by Actogram J software. The results were presented as a chi-square periodogram [13, 14].

2.4. Lifespan Determination. For the determination of lifespan the newly enclosed male flies (control and PD) were placed in culture tubes (10 flies per tube) containing 25, 50, and 100 μM of curcumin mixed in diet. The flies were transferred to new diet after every 3rd day and the number of dead flies were recorded at 3-day interval until the last one died [7].

2.5. Lipid Peroxidation Assay. Lipid peroxidation assay in the brain homogenate was performed according to the procedure described by Siddique et al. [15]. Reagent 1 (R1) was prepared by dissolving 0.064 g of 1-methyl-2-phenylindole into 30 mL of acetonitrile to which 10 mL of methanol was added to bring the volume to 40 mL. The preparation of 37% HCl served as the reagent R2. The brains of flies were isolated under stereo zoom microscope in ice cold Tris HCl (20mM) (10 brains/group; five replicates/group). Homogenate was prepared in Tris HCl and centrifuged at 3000 g for 20 min and subsequently the supernatant was collected. In a microcentrifuge tube 1300 μL of R1 was taken. A volume of 1 μL (supernatant) was added along with 300 μL of R2 vortexed and incubated at 45°C for 40 min. After incubation, the tubes were cooled in ice and centrifuged at 15,000 g for 10 min at 4°C and read at 586 nm.

2.6. Estimation of Protein Carbonyl Content. The protein carbonyl content was estimated according to the protocol described by Hawkins et al. [16]. The brain homogenate was diluted to a protein concentration of approx 1mg/mL. About 250 μL of each diluted homogenate was taken in eppendorf centrifuge tubes separately. To it 250 μL of 10 mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added, vortexed, and kept in dark for 20 min. About 125 μL of 50% (w/v) trichloroacetic acid (TCA) was added, mixed thoroughly, and incubated at −20°C for 15 min. The tubes were then centrifuged at 4°C for 10 min at 9000 rpm. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol: ethyl acetate (1:1). Finally, the pellets were redissolved in 1 mL of 6 M guanidine hydrochloride and the absorbance was read at 370 nm.

2.7. Analysis of Cell Death in Drosophila Brain. The cell death in Drosophila brain was analyzed as per the method described by Mitchell and Staveley [17]. Flies (5 flies/treatment; 5 replicates/group) were placed in 70% ethanol in a 2 mL microcentrifuge tube for a minute. The brains were isolated in Ringer’s solution under stereo zoom microscope. After removing the Ringer’s solution about 100 μL of freshly prepared acridine orange (5 μg/mL) was added for 5 minutes.

Figure 1: Effect of curcumin on survival rate measured in transgenic Drosophila melanogaster in various treated groups. (C1 = 25μM curcumin; C2 = 50μM curcumin; C3 = 100 μM curcumin).

Figure 2: Effect of curcumin on lipid peroxidation measured in the brains of transgenic Drosophila melanogaster after 24 days of the exposure in treated groups. (C1 = 25μM curcumin; C2 = 50μM curcumin; C3 = 100 μM curcumin).
2.8. Statistical Analysis. The statistical analysis was done using Statistica Soft Inc. The mean values of various fly groups were statistically compared using Student’s t-test.

3. Results

The data collected for the male flies by Drosophila activity monitor (DAM) was analysed by chi-square periodogram. For control flies the number of peaks (significant) were more (Figures S1 (a) and (b)) compared to PD flies (Figures S2 (a) and (b)). A dose dependent significant delay in the loss of activity pattern was observed in the PD flies exposed to 25, 50, and 100 μM of curcumin (Figures S3–5, (a) and (b)). No change in the activity pattern of control flies exposed to 25, 50, and 100 μM of curcumin was observed (Figures S6–8, (a) and (b)). The PD flies exposed to 10⁻³ M of dopamine also showed a delay in the loss of activity (Figures S9 (a) and (b)) as compared to PD flies. The results obtained for the survival rate are shown in Figure 1. The survival rate was measured only in male flies. As is evident from Figure 1 the PD flies exposed to 25, 50, and 100 μM of curcumin showed a dose dependent significant increase in the life span as compared to unexposed PD flies. The control flies showed a life span of about 60 days. The results obtained for the estimation of lipid peroxidation are shown in Figure 2. The PD flies exposed to 25, 50, and 100 μM of curcumin showed a dose dependent significant decrease in the lipid peroxidation as compared to unexposed PD flies and control flies (Figure 2). The results obtained for protein carbonyl content are shown in Figure 3. A dose dependent significant decrease in the mean absorbance values was obtained in PD flies exposed to 25, 50, and 100 μM of curcumin as compared to unexposed PD and control flies (Figure 3). The unexposed PD flies showed the highest mean absorbance value as compared to control flies (Figure 3). The results obtained for cell death in the brains of PD flies was calculated as mean gray scale values shown in Figure 4. A significant dose dependent decrease in the mean gray scale values was obtained for the PD flies exposed to 25, 50, and 100 μM of curcumin as compared to unexposed PD and control flies (Figure 4). The PD flies exposed to 10⁻³ M of dopamine also showed a significant decrease in the mean gray scale value as compared to the unexposed PD flies and control flies (Figure 4).

4. Discussion

The results of the present study reveal that the exposure of PD flies to 25, 50, and 100 μM of curcumin showed a dose dependent significant delay in the loss of activity pattern, reduction in lipid peroxidation, protein carbonyl content, apoptosis, and increase in the life span. Oxidative stress as a result of the accumulation of alpha synuclein has been reported in neurons of PD model flies [18]. It remains still unclear that the degenerating neuron itself or misfolded proteins directly causes toxicity during the progression of PD [19, 20]. In our earlier studies with the same fly models, various plant extracts and flavonoids have been reported to delay the loss of climbing activity and reduced oxidative stress [21–25]. Flavonoids have been reported to show improvements in cognition function possibly by protecting vulnerable neurons or by stimulating neuronal regeneration [26, 27]. In our present study, treatment of curcumin has shown reduction in lipid peroxidation and protein carbonyl content in the brains of PD model flies. This protection is attributed to an antioxidant nature of curcumin [28, 29]. Recent findings have suggested that flavonoids have a remodelling effect on the brain...
nature of α-synuclein fibrils, converting them into nontoxic, smaller amorphous aggregates, thus preventing the formation of reactive oxygen species [30]. On the other hand, an antioxidant nature of the curcumin is attributed to its unique conjugated structure that includes two methoxylated phenols [31]. It has been reported to inhibit the generation of reactive oxygen species (ROS) responsible for DNA and membrane damage [32]. Although the animals are well acquainted with the self-defense mechanism, an enhancement in stress beyond the capacity of an animal to cope up may result in cellular damage leading to the cell death [33]. The exposure of PD flies to curcumin showed a dose dependent decrease in the mean gray scale values, thus confirming an antiapoptotic activity of curcumin [34]. In earlier studies, curcumin has shown the neuroprotection in the 6-OHD model PD due to its antioxidant potential and its capability to penetrate into the brain [35]. It has been reported to alleviate αS-induced toxicity, reduce ROS level, and protect cell against apoptosis [36]. The aggregation of αS in the brain has been implicated as a crucial step in the formation of Lewy bodies and curcumin has antifibrillogenic and fibril-destabilizing properties, thus inhibiting the formation of alpha synuclein fibrils [37, 38]. In Drosophila curcumin have been reported to extend life span in a gender and genotype specific manner [39, 40]. In our present study, the life span and pattern activity were studied on male PD flies. There are reports on the life span extension of curcumin in mice [41] and C. elegans [42]. This extension is due to the neuroprotective and antiaging properties of curcumin [43]. The current pharmacotherapeutic approaches for PD involve improvement in striatal dopamine. The therapies involving natural antioxidants/plant products may be used as adjunct therapy [44]. The results obtained in our present study and our earlier study, in which the alginate-curcumin nanocomposite was studied using the same PD fly strain, results in neuroprotective effects [45]. Tetrahydrocurcumin has been reported to extend the life span of Drosophila and reduce the oxidative stress by regulating O-type forkhead domain transcription factor (FOXO) [46]. Despite having apoptotic properties in various cancerous cell lines there are reports of having antiapoptotic properties of curcumin that corroborate with the findings of our study [47–51]. Cancer cells can accumulate higher intercellular, cellular concentrations of vitamins/antioxidants than normal cells due to loss of homeostatic controls. The high concentration of the antioxidants can alter cancer cell metabolisms and cell signaling [52]. The present study was carried out using Drosophila as a model of PD expressing human wild type α-synuclein in neuron of fly and consequent locomotor dysfunction [1]. The present fly model mimics the motor impairments associated with PD and can be used to study whether or not a variety of compounds or drugs mixed in the fly culture medium have the neuroprotective potential [53].

Conflict of Interests
The authors declare that they have no conflict of interests concerning this paper.

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References


Research Article

Antitumor and Antiangiogenic Activities of Curcumin in Cervical Cancer Xenografts in Nude Mice

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To evaluate the effects of curcumin (CUR) on tumor progression and angiogenesis in cervical cancer- (CaSki-) implanted nude mice and on the angiogenic biomarkers: vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), and epidermal growth factor receptor (EGFR). CaSki cells were subcutaneously injected in nude mice to establish subcutaneous tumors. One month after injection, mice were orally administered vehicle or 500, 1,000, and 1,500 mg/kg of CUR daily × 30 consecutive days. Tumor volume was measured every 3–4 days. At the end of the study, tumor microvasculature was observed under confocal microscope, and immunohistochemical analyses were performed to detect CD31, VEGF, COX-2, and EGFR. CUR at the doses of 1,000 and 1,500 mg/kg showed significant tumor growth retardation (21.03% and 35.57%) versus CaSki + vehicle group. The microvascular density (MVD) in CaSki + vehicle group was significantly increased versus Control + vehicle group and significantly reduced by CUR (1,000 and 1,500 mg/kg). VEGF, COX-2, and EGFR expressions were upregulated in CaSki + vehicle group and attenuated significantly by CUR (1,000 and 1,500 mg/kg). In conclusion, high dose CUR inhibited tumor growth and angiogenesis in CaSki-implanted mice probably mediated by the downregulation of VEGF, COX-2 and EGFR. CUR may have a role in treating human cervical cancer and should be explored further.

1. Introduction

Cervical cancer is the second most common cancer in women worldwide and is the most frequent cancer in many developing countries [1]. Cervical tumors are often highly vascular and bleed spontaneously. The addition of antiangiogenic agents could be important in the treatment of human cervical cancer. Antiangiogenic treatment strategies offer a number of compelling advantages over conventional cytotoxic cancer therapies because endothelial cells are not transformed and drug resistance is not induced. In this regard, discovery of nontoxic antiangiogenic phytochemicals could have greater practical significance compared to nonselective cytotoxic therapies to control the tumor growth and metastasis by targeting angiogenesis.

A number of biological molecules modulate angiogenesis in cervical cancer such as epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2), and vascular endothelial growth factor (VEGF). EGFR is a member of the ErbB family, the tyrosine kinase receptors with growth promoting effects, including a recognized angiogenic potential [2]. Activation of EGFR results in activation of MEK-extracellular signal-regulated kinasel/2 (ERK1/2) and phosphatidylinositol 3-kinase 3-kinase (PI3K-) Akt pathways [3]. These two pathways regulate VEGF expression through changes in VEGF transcriptional activity which is a major mediator of tumor angiogenesis. Recently, Balan et al. reported that EGFR was overexpressed in cervical biopsies of cervical cancer patients [4]. The number of biopsies with intense immunooexpression of EGFR increased with the severity of
the cytological abnormality. Thus, EGFR seems to have an important role in tumor angiogenesis and the prognostic of advanced cervical cancer.

The VEGF, a major mediator of tumor angiogenesis, promotes mobilization of endothelial progenitor cells, cell proliferation, migration, survival, and vascular permeability [5]. VEGF was found to be overexpressed in cervical cancer and associated with a poor prognosis [6, 7]. The COX-2 has a role in the onset and progression of malignancies, including the cervical carcinoma, and is also considered as a marker of tumor aggressiveness. It can potentially predispose to cervical cancer by several mechanisms. An increased expression of COX-2 has been reported to inhibit apoptosis, suppress immune function, promote angiogenesis, and enhance the invasiveness of malignant cells [8]. Kulkarni et al. found that EGF and TGF-α, ligands of EGFR, markedly induced COX-2 in a cervical carcinoma cell line, suggesting that deregulated signaling through EGFR is likely to account, at least in part, for increased expression of COX-2 [9]. Later, Oh et al. reported that COX-2-prostaglandin E2 (PGE2) pathway is also implicated in VEGF expression by HPV 16 E5 [10]. Therefore, EGFR-COX-2-PGE2 pathway also plays an important role in tumor growth and angiogenesis in cervical cancer.

Curcumin (CUR), a phenolic compound extracted from Curcuma longa L., is well known as a chemopreventive agent. Many recent studies have demonstrated that CUR modulates angiogenesis, proliferation, invasion, and tumor progress in various types of cancer [8, 11–13]. However, the effect of CUR on tumor angiogenesis, especially, using cervical cancer- (CaSki-) implanted nude mice model has not yet been reported. Therefore, the present study was designed to determine the effects of CUR on angiogenesis and tumor progression in cervical cancer- (CaSki-) implanted nude mice and to study the possible mechanisms of CUR on angiogenic biomarkers, VEGF, COX-2, and EGFR.

2. Methods

2.1. Cell Line and Cell Culture. Cervical cancer cells (CaSki) were purchased from the American Type Culture Collection. The cell lines were cultured in MEM medium supplemented with 10% fetal bovine serum. All cultures were maintained in an incubator at 37°C with 5% CO2 in a humidified atmosphere.

2.2. CaSki-Induced Tumor Mice. BALB/c-nude mice weighing about 20–25 g were used. The animal experiments were conducted according to the guidelines on experimental animals of The National Research Council of Thailand (1999). The mice were divided into 6 groups: (1) controls supplemented with corn oil (Control + vehicle; n = 6), (2) controls supplemented with CUR (1,500 mg/kg) (Control + CUR; n = 6), (3) CaSki-implanted mice supplemented with corn oil (CaSki + vehicle; n = 6), (4) CaSki-implanted mice supplemented with CUR (500 mg/kg) (CaSki + CUR500, n = 6), (5) CaSki-implanted mice supplemented with CUR (1,000 mg/kg) (CaSki + CUR1000, n = 6), and (6) CaSki-implanted mice supplemented with CUR (1,500 mg/kg) (CaSki + CUR1500, n = 6).

For the CaSki groups, a suspension of 10 × 10⁶ CaSki cells in 0.2 mL MEM [14] was subcutaneously injected into the dorsa of mice at the proximal midline while control group was injected with MEM. The tumors were measured with Vernier calipers every 3-4 days by using the formula \( a^2 \times b \times 0.52 \) (where \( a \) is the shortest diameter and \( b \) is the longest diameter). When the tumor volume was 100–120 mm³, mice were randomized. Then, the mice were daily supplemented with vehicle or CUR (Cayman Chemical, USA) at the doses of 500, 1,000, or 1,500 mg/kg of body weight for one month.

2.3. Study of Tumor Microvasculature. On study day 30, the mice were anesthetized with sodium pentobarbital (50 mg/kg bw, i.p). Fluorescence tracers (0.1 mL of 0.5% fluorescein isothiocyanate- (FITC-) labeled dextran (MW = 200,000, Sigma Chemical, USA)) were injected in the jugular vein. The tumor microvasculature was visualized under confocal microscope.

2.4. Immunohistochemistry for CD31 Expression and Microvessels Density (MVD) Determination. After the microvascular study, the mice were sacrificed and the tumors were fixed in 10% formalin. Immunohistochemistry was performed using 5μm thick paraffin sections. Paraffin sections were dewaxed and rehydrated through xylene and a graded alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. After washing in water, nonspecific binding sites were blocked with 5% bovine serum in phosphate-buffered saline (PBS) for 30 min at room temperature. The tissue slide samples were incubated with primary monoclonal antibody CD31 (Thermo Fischer Scientific, UK) (1:500) at 4°C overnight. The slide was then gently rinsed with PBS and developed by the Envision system/HRP (DAKO cytomation, USA) for 30 min and substrate-chromogen for 10 min at room temperature. The nuclei were counterstained with Mayer’s hematoxylin.

To quantify angiogenesis, microvessel density (MVD) was assessed by immunostaining with the anti-CD31 antibody as previously described [15]. The sections were observed first under the low power (×40), and then the most dense area of microvessels sections was selected and counted under the high power (×200, the surface area of every vision field being 0.4 mm²).

2.5. Immunohistochemistry for VEGF, COX-2, and EGFR Expression. Paraffin sections from dorsal skin tissue were dewaxed and rehydrated with xylene and a graded alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. After washing in water, nonspecific binding sites were blocked with 5% bovine serum in phosphate-buffered saline (PBS) for 30 min at room temperature. The tissue slide samples were incubated with primary monoclonal antibody VEGF (Thermo Fischer Scientific, UK) (1:100) or COX-2 (Thermo Fischer Scientific, UK) (1:50) or EGFR (VENTANA (ready to use), USA) at 4°C overnight. The slide was then gently rinsed
with PBS and developed by the Envision system/HRP (DAKO cytome, USA) for 30 min and substrate-chromogen for 10 min at room temperature. The nuclei were counterstained with Mayer’s hematoxylin.

2.6. Staining Analysis. The sample image from each slide was subtracted from the corresponding background image. The image threshold was standardized to delineate the labeled structures and then applied to all images of an individual experiment. The staining intensity and the percentage of positive staining for VEGF, COX-2, and EGFR were analyzed. Staining intensity (I) was scored as 0 (none), 1+ (weak), 2+ (moderate), and 3+ (strong). The percentage of positive staining (P) was scored as (0) 0% immunopositive cells; (1) ≤25% positive cells; (2) 26–50% positive cells; (3) ≥51–75% positive cells; (4) ≥76%. The sum of both (I) and (P) scores was evaluated for each case and a final score was assigned 0 (negative), 1–3 (weak expression), 4–5 (moderate expression), and 6–7 (strong expression) [16].

2.7. Statistical Analysis. Data were expressed as means with standard error. SPSS.13 software was used for statistical analysis. Student's unpaired t-test was applied for comparison of the means of two groups (Control and CaSki + vehicle groups), and analysis of variance was used for the means of multiple groups. The correlation between COX-2, VEGF, and CD31 expressions and COX-2, VEGF, and EGFR was assessed with Pearson correlation test. For all of the value differences, P value less than 0.05 was considered significant.

3. Results

3.1. Antitumor Effect of CUR in CaSki-Implanted Mice. Tumor growth is shown in Figure 1(a). Tumors in the CaSki + vehicle group doubled in size approximately every 3 days. On day 12, the group treated with 1500 mg/kg of CUR had significantly smaller tumors (439.5 ± 23.66 mm³). The 1,000 mg/kg CUR group showed a significantly reduced tumor volume beginning on day 18 after treatment (918.06 ± 33.86 mm³). At the end of the experiment, CUR treatments at the dose of 1,000 and 1,500 mg/kg significantly retarded the growth of tumors by 21.03% and 35.57%, respectively (Figure 1(b); P < 0.001). Moreover, the CaSki + CUR1500 treated group (1,266.00 ± 36.41 mm³) showed significantly reduced tumor volume versus CaSki + CUR1000 treated group (1,964.78 ± 40.20 mm³) (P < 0.001). However, the percentage of positive staining of VEGF, which was attenuated by the treatments with CUR at the doses of 500, 1,000, and 1,500 mg/kg but was only significantly different in the high dose groups (P < 0.001). The treatment with CUR at the dose of 1,500 mg/kg has also shown a more significant decrease in VEGF positive staining than the treatment with CUR at the dose of 1,000 mg/kg (P < 0.001).

The percentage of positive staining of COX-2, which was 91.80 ± 3.32%, 52.30 ± 3.20%, and 34.00 ± 1.93%, respectively, was attenuated by the treatments with CUR at the doses of 500, 1,000, and 1,500 mg/kg but was only significantly different in the high dose groups (P < 0.001). The treatment with CUR at the dose of 1,500 mg/kg showed a significant decrease in COX-2 positive staining versus the 1,000 mg/kg dose (P < 0.001).

The intensity scores of VEGF and COX-2 expression are shown in Figures 4(b) and 5(b). The CaSki + vehicle group showed strong intensity scores for VEGF (mean score = 2.9) and COX-2 (mean score = 2.5), whereas the control group showed weak intensity scores (mean score = 0.8 and 0.4 for VEGF and COX-2, resp.). Staining intensity scores for VEGF however, the appearance of neocapillaries induced by CaSki was markedly reduced after treatment with high dose CUR (1,000 and 1,500 mg/kg). In addition, the abnormalities of the neocapillary network pattern were attenuated by high dose CUR.

Figure 3(a) shows representative immunostaining for CD31 in control (A and B), CaSki + vehicle (C), and CaSki + CUR (D–F) groups. In normal skin tissue from the control group, few CD31 expressions were detected adjacent to sweat glands, whereas they were highly expressed in CaSki-implanted tissues. High dose CUR attenuated CD31 expression.

In Figure 3(b), the MVD of both Control + vehicle and Control + CUR1500 was similar (6 ± 1.25 and 6 ± 1.02, resp.). The MVD was significantly higher in the CaSki + vehicle group (39 ± 2.38) than in the control group (P < 0.001). The MVD was not significantly decreased by CUR treatment at the dose of 500 mg/kg (36 ± 2.12). High dose CUR: 1,000 (20 ± 1.54) and 1,500 mg/kg (13 ± 2.85), was significantly decreased in MVD as compared to the CaSki + vehicle group and CaSki + CUR500 group (P < 0.001). Moreover, MVD in the CaSki + CUR1500 group was significantly decreased when compared to CaSki + CUR1000 group (P < 0.05).

3.3. Effects of CUR on Angiogenic Biomarkers. Figures 4(a) and 5(a) show the microscopic images of immunohistochemical stained sections for VEGF and COX-2 expression, respectively. Cytoplasm of the tumor cells stained positively for VEGF and COX-2. Figures 4(b) and 5(b) show the percentage of positive staining of VEGF and COX-2, respectively. The percentage of positive staining of VEGF (92.10 ± 2.17%) and COX-2 (73.00 ± 1.23%) expression was significantly increased in the CaSki + vehicle group as compared to control group (VEGF: 13.40 ± 1.17%; COX-2: 76.0 ± 1.27%) (P < 0.001).
Figure 1: (a) Tumor bearing mice. (b) Tumor volume (mm$^3$). *$P < 0.001$ significant difference compared to CaSki + vehicle group and CaSki + CUR500 group. *$P < 0.01$ versus CaSki + CUR1000 group.
expression were reduced in CaSki + CUR500 group (mean score = 2.6), CaSki + CUR1000 group (mean score = 1.6), and CaSki + CUR1500 group (mean score = 1.2) but were significantly different only in the two high dose groups ($P < 0.001$).

In the same fashion, staining intensity scores for COX-2 expression were reduced in the CaSki + CUR500 group (mean score = 2.4), CaSki + CUR1000 group (mean score = 1.3), and the CaSki + CUR1500 group (mean score = 1.1) but were significantly different only in the two high dose groups ($P < 0.001$).

The total scores for VEGF positive staining and intensity revealed that the CaSki + vehicle group had strong VEGF expression (mean total score = 6.9), whereas the control group had weak VEGF expression (mean total score = 1.8). The CaSki + vehicle group had moderate COX-2 expression (mean total score = 5.6), whereas the control group had weak COX-2 expression (mean total score = 1.4).

In the treated groups, the total scores for VEGF expression revealed strong, moderate, and weak expressions in CaSki + CUR500 group (mean total score = 6.6), CaSki + CUR1000 group (mean total score = 4.1), and CaSki + CUR1500 group (mean total score = 3.3), respectively. The total scores for COX-2 expression showed that moderate, weak, and weak expressions were found in CaSki + CUR500 group (mean total score = 5.4), CaSki + CUR1000 group (mean total score = 3.5), and CaSki + CUR1500 group (mean total score = 3.1), respectively.

### 3.4. Effects of CUR on EGFR

The EGFR staining pattern was predominantly in the membrane with occasional cytoplasmic positivity (Figure 6(a)). The quantitative data showed that the percentage of positive staining of EGFR expression significantly increased in the CaSki + vehicle group (90.90 ± 1.26%) compared to control group [9.70 ± 0.79%, $P < 0.001$ (Figure 6(b))]. The percentage of positive staining of EGFR, which was 79.70 ± 1.26%, 61.10 ± 1.03%, and 53.50 ± 1.31%, respectively, was attenuated by the treatments with CUR at the doses of 500, 1,000, and 1,500 mg/kg. Significant reductions in EGFR positive staining were found only in high dose groups ($P < 0.001$).

The intensity score of EGFR expression in CaSki + vehicle group was high (mean score = 2.8) but was low (mean score = 0.7) in the control group (Figure 6(b)). However, staining intensities for EGFR expression were reduced in CaSki + CUR500 (mean score = 2.7), CaSki + CUR1000 (mean score = 1.7), and CaSki + CUR1500 (mean score = 1.5) groups. Again, significance was only achieved in the two high dose groups ($P < 0.001$).

The total score for EGFR expression showed that the CaSki + vehicle group had strong EGFR expression (mean total score = 6.8), whereas the control group had weak EGFR expression (mean total score = 1.7). The EGFR expression extent and intensity scores in the treated group revealed that strong, moderate, and moderate expressions were found in CaSki + CUR500 (mean total score = 6.5), CaSki + CUR1000 (mean total score = 4.7), and CaSki + CUR1500 groups (mean total score = 4.1), respectively.

### 3.5. The Relationship between the Expression of CD31, VEGF, COX-2, and EGFR

The percentage of stained positive cells...
Figure 3: (a) Immunohistochemical staining for CD31 in Control + vehicle group (A), Control + CUR (B), CaSki + vehicle group (C), CaSki + CUR500 group (D), CaSki + CUR1000 group (E), and CaSki + CUR1500 group (F). Bar = 10 μm, 200x. (b) Microvascular density (numbers/0.4 mm²) (mean ± SEM). *P < 0.001 versus Control + vehicle group, †P < 0.001 versus CaSki + vehicle group and CaSki + CUR500 group, and ‡P < 0.05 versus CaSki + CUR1,000 group.
Figure 4: (a) Immunohistochemical staining for VEGF expression in Control + vehicle group (A), Control + CUR (B), CaSki + vehicle group (C), CaSki + CUR500 group (D), CaSki + CUR1000 group (E), and CaSki + CUR1500 group (F). Bar = 10 μm, 200x. (b) The percentage of positive staining (%) and the intensity of VEGF expression (mean ± SEM). *P < 0.001 versus Control + vehicle group, ♠P < 0.001 versus CaSki + vehicle group and CaSki + CUR500 group, and ♣P < 0.001 versus CaSki + CUR1000 group.
Figure 5: (a) Immunohistochemical staining for COX-2 expression in Control + vehicle group (A), Control + CUR (B), CaSki + vehicle group (C), CaSki + CUR500 group (D), CaSki + CUR1000 group (E), and CaSki + CUR1500 group (F). Bar = 10 μm, 200x. (b) The percentage of positive staining (%) and the intensity of COX-2 expression (mean ± SEM). *P < 0.001 versus Control + vehicle group, †P < 0.001 versus CaSki + vehicle group and CaSki + CUR500 group, and ‡P < 0.001 versus CaSki + CUR1000 group.
Figure 6: (a) Immunohistochemical staining for EGFR expression in Control + vehicle group (A), Control + CUR (B), CaSki + vehicle group (C), CaSki + CUR500 group (D), CaSki + CUR1000 group (E), and CaSki + CUR1500 group (F). Bar = 10 μm, 200x. (b) The percentage of positive staining (%) and the intensity of EGFR expression (mean ± SEM). *P < 0.001 versus Control + vehicle group and **P < 0.001 versus CaSki + vehicle group and CaSki + CUR500 group.
for both VEGF and COX-2 expressions was positively correlated and related to MVD in CaSki-implanted mice ($r = 0.99$ and $r = 0.95$, resp., $P < 0.001$) as were the respective intensity scores ($r = 0.877$ and $r = 0.851$, $P < 0.001$). Furthermore, VEGF expression and intensity scores were positively correlated with both COX-2 and EGFR expressions: (i) $r = 0.921$ and $r = 0.964$, respectively, ($P < 0.001$) and (ii) $r = 0.821$ and $r = 0.866$, respectively, ($P < 0.001$).

4. Discussion

In the present study, the experiments were conducted to investigate the effects of CUR on tumor progression and angiogenesis using CaSki-implemented nude mice model. We demonstrated that CUR exhibits antitumor and antiangiogenesis effects in CaSki-implemented nude mice.

One of the mechanisms by which CUR attenuates tumor progression is mediated by its antiangiogenic activity. In the original hypothesis formulated by Folkman in the early 1970s of angiogenic control of tumor growth [17], it was proposed that tumor growth was limited by diffusion to a size of 1-2 mm unless additional blood vessels were recruited to the tumor site. Moreover, it is important to realize that the rate of cell proliferation in such tumors is virtually the same as in rapidly expanding tumors. This has been shown by the work of Hanahan and Folkman, who have followed tumor development in transgenic mice that develop pancreatic islet tumors. They demonstrated that the initial phase of tumor growth is characterized by avascular tumors that maintain a small diameter for a period of weeks until an angiogenic switch is activated and the tumors become vascular and begin to expand in size [18]. Treatment of such mice with angiogenesis inhibitors blocked formation of these tumor colonies [19]. These results indicate that angiogenesis inhibition can lead to tumor regression and, in some cases, to complete elimination of the tumor growth.

In normal tissues, angiogenesis is strictly controlled but in tumors angiogenesis is uncontrolled and immature [20]. Controlled by angiogenic factors and angiogenic inhibitors, tumor cells, endothelial cells, and other cells can produce and release VEGF protein if the local microenvironment is changed by hypoxia [21]. In cervical cancer, human papillomavirus may directly stimulate VEGF production through the upregulation of the E6 oncoprotein [22, 23]. Studies using transgenic mice as well as human cervical tissue suggest that, apart from hypoxia, E6 and E7 oncoproteins can also stimulate VEGF production [24]. López-Ocejo et al. have demonstrated that E6 positive cervical carcinoma cells expressed VEGF mRNA levels two to three times higher than those expressed by E6 negative cells [23]. On the other hand, expression of the antiangiogenic factors thrombospondin-1 (TSP-1) and (TSP-2) was decreased in cells infected by HPV. Consequently, it would appear that cervical cancer expression of HPV-16 (CaSki cell) integrated molecules is able to contribute to a proangiogenic phenotype that might support tumor growth and angiogenesis via upregulation of VEGF expression.

The VEGF pathway plays a crucial role in normal and pathologic angiogenesis, triggering multiple signaling networks that result in endothelial cell survival, migration, proliferation, differentiation, and vascular permeability [25]. In our study, we found that there was a marked increase in neovascularization with a heterogeneous network, hyperpermeability to macromolecules, tortuosity, and dilatation in the CaSki groups as compared to the control group. Moreover, we demonstrated that a strong correlation was found between VEGF expression and increased tumor microvasculature in CaSki + vehicle group. These results suggest strongly that VEGF and angiogenesis promoted by VEGF play important roles in tumor growth. We also found clear heterogeneity in VEGF expression and new vessel formation in cancer tissue. The VEGF expression and MVD were highly correlated in the cancer tissues again suggesting strongly the link between VGF and MVD and the important roles in tumor biological behavior and progression.

Similarly, COX-2 overexpression in CaSki + vehicle group was highly and positively correlated with MVD, providing strong support for its role in tumor-induced angiogenesis in cervical cancer. COX-2 can potentially predispose to cervical cancer by several direct and indirect mechanisms, for example, inhibiting apoptosis, suppressing immune function, promoting angiogenesis, and enhancing the invasiveness of malignant cells [8]. The direct effect of COX-2 on angiogenesis has been demonstrated by Dormond et al. [26]. They showed that inhibition of endothelial cell COX-2 by nonsteroidal anti-inflammatory drugs suppressed αVβ3-dependent activation of the small GTPases, Cdc-42, and Rac, resulting in inhibition of endothelial cell spreading and migration in vitro [26]. Therefore, COX enzymes appear essential for the maintenance of the migration and attachment of endothelial cells through integrin pathways. The indirect pathway of COX-2 on tumor angiogenesis might be mediated by an upregulation of the expression of angiogenic factors like VEGF. This pathway of COX-2 in angiogenesis is thought to be the induction of the synthesis of prostanoids, which then stimulate the expression of proangiogenic factors [10]. Taken together, these findings suggest that both COX-2 and VEGF appear crucial for tumor angiogenesis in cervical cancer, and therapy targeting COX-2 and VEGF pathways should be explored.

Furthermore, we demonstrated that strong expression of EGFR was found in CaSki-implanted mice and that COX-2, VEGF, and EGFR were strongly positively related. We hypothesize that overexpression of the angiogenic biomarkers COX-2 and VEGF may be mediated by the induction of EGFR signaling pathway. EGFR has recently been identified as a promising target for cervical cancer [27]. This receptor is overexpressed in a variety of solid human cancers, such as non-small-cell lung cancer, colorectal cancer, and head and neck cancer. In patients with squamous cell carcinoma of cervix, EGFR is overexpressed in up to 85% of cases, and EGFR expression has been associated with a later tumor stage and a poorer prognosis [13, 28, 29].

Kulkarni et al. found that EGF and TGF-α ligands of EGFR markedly induced COX-2 in a cervical carcinoma cell line [9]. This suggests that deregulated signaling through
EGFR is likely to account, at least in part, for increased expression of COX-2 in cervical cancer. Later, Oh et al. reported that COX-2-prostaglandin E2 (PGE2) pathway is also implicated in VEGF expression by HPV 16 E5 [10]. Collectively, these data provide evidence for an association between EGFR, COX-2, and VEGF expressions in cervical cancer tumor angiogenesis and tumor growth.

In our study, we have demonstrated that the CUR treatment at the doses of 1,000 and 1,500 mg/kg inhibited tumor growth and angiogenesis and that such tumor-associated pathological features as microvascular dilatation, tortuosity, and hyperpermeability were attenuated. Consistent with data from others [28, 29] and our previous work [27], CUR and hyperpermeability were attenuated. Consistent with data from others [28, 29] and our previous work [27], CUR inhibited the expressions of VEGF, COX-2, and EGFR. Interestingly, these high doses did not result in any deaths or overt toxicity.

5. Conclusion

Our data demonstrated that CUR markedly inhibited tumor progression and angiogenesis in CaSkii-implanted nude male mice models. The antiangiogenic effects of CUR partially through VEGF and COX-2 suppression might be mediated by downregulation of EGFR. Our data suggest a potential clinical role for the treatment of cervical cancer and should be explored further.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pomegranate Fruit as a Rich Source of Biologically Active Compounds

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Pomegranate is a widely used plant having medicinal properties. In this review, we have mainly focused on the already published data from our laboratory pertaining to the effect of methanol extract of pericarp of pomegranate (PME) and have compared it with other relevant literatures on *Punica*. Earlier, we had shown its antiproliferative effect using human breast (MCF-7, MDA MB-231), and endometrial (HEC-1A), cervical (SiHa, HeLa), and ovarian (SKOV3) cancer cell lines, and normal breast fibroblasts (MCF-10A) at concentration of 20–320 μg/mL. The expressions of selected estrogen responsive genes (PR, pS2, and C-Myc) were downregulated by PME. Unlike estradiol, PME did not increase the uterine weight and proliferation in bilaterally ovariectomized Swiss-Albino mice model and its cardioprotective effects were comparable to that of 17β-estradiol. We had further assessed the protective role of PME on skeletal system, using MC3T3-E1 cells. The results indicated that PME (80 μg/mL) significantly increased ALP (Alkaline Phosphatase) activity, supporting its suggested role in modulating osteoblastic cell differentiation. The antiosteoporotic potential of PME was also evaluated in ovariectomized (OVX) rodent model. The results from our studies and from various other studies support the fact that pomegranate fruit is indeed a source of biologically active compounds.

1. Introduction

*Punica granatum* L. (Punicaceae) is a nutrient dense fruit rich in phytochemical compounds [1]. Plants produce low molecular weight compounds which are broadly called phytochemicals, usually as a mechanism of defence. Some plants contain distinct families of phytocompounds, which are structurally similar to steroid hormone, 17β-estradiol (E2) and compete with the endogenous hormone for binding to estrogen receptor (ER), thus reducing the hormonal effect of endogenous estrogens [2–4]. These compounds are termed as phytoestrogens. Most of these phytoestrogens present in the diet are inactive compounds, which, on consumption, go through series of enzymatic changes in the gastrointestinal tract, resulting in the formation of compounds having structure similar to that of estrogens [5]. Phytoestrogens have captured major research and clinical attention due to its effectiveness in the prevention and treatment of perimenopausal and menopausal symptoms, over hormone replacement therapy (HRT) [6]. They may act both as agonists and/or antagonists in a site-specific manner, similar to the hormonal action of selective estrogen receptor modulators (SERMs) [7–9]. It can also function as antioxidants and protect DNA from oxidant-induced damage [10]. Research on pomegranate is gaining momentum due to its tremendous nutritional values and medicinal uses. The current review focuses on the use of pomegranate as a phytoestrogen rich and nutraceutical fruit with emphasis to the work done in our laboratory using methanolic extract of pericarp of pomegranate (PME).

2. Chemical Constituents of Pomegranate Fruit and Tree

The chemical composition of the fruits differs depending on the cultivar, growing region, maturity, cultivation practice,
and tree [20–41].

Table 1 represents the key constituents of pomegranate fruit due to their biological and free radical scavenging activities together with flavonoids, anthocyanins, and tannins, which constitute 70–76% of these oils [16]. Phenolic compounds, which is a conjugated isomer unique to pomegranate oil, include sterols, steroids, and a key component of mammalian which 99% is triacylglycerols. Minor components of the oil of pomegranate seed oil comprises over 95% of the oil, of the conjugated linolenic acids. The fatty acid component comprises seed oil and is self-possessed with more than 70% of the fruit were more effective than a single extract [18]. In a combination of pomegranate extracts from different parts of the fruit and this may be one of the reasons why many of the studies demonstrated that combinations of pomegranate extracts from different parts of the fruit were more effective than a single extract [18]. In a comparative analysis, anthocyanins from pomegranate fruit were found to possess higher antioxidant activity than vitamin-E (α-tocopherol), β-carotene, and ascorbic acid [19]. Table 1 represents the key constituents of pomegranate fruit and tree [20–41].

climate, and storage circumstances [11]. About 50% of the total fruit weight corresponds to the peel, which is an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds. Minerals, mainly potassium, nitrogen, calcium, phosphorus, magnesium, and sodium, and complex polysaccharides. The edible part of the pomegranate fruit (50%) consists of 40% arils and 10% seeds. Arils contain 85% water, 10% total sugars, mainly fructose and glucose, and 1.5% pectin, organic acid, such as ascorbic acid, citric acid, and malic acid, and bioactive compounds such as phenolics and flavonoids, principally anthocyanins [12]. The seed cover of the fruit contains delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, and pelargonidin-3-glucoside with delphinidin-3,5-diglucoside being the main anthocyanin in pomegranate juice [13]. 12–20% of total seed weight of pomegranate comprises seed oil and is self-possessed with more than 70% of the conjugated linolenic acids. The fatty acid component of pomegranate seed oil comprises over 95% of the oil, of which 99% is triacylglycerols. Minor components of the oil include sterols, steroids, and a key component of mammalian myelin sheaths, cerebroside [14, 15]. Interestingly, punlic acid, which is a conjugated isomer unique to pomegranate oil, constitutes 70–76% of the seed oil [16]. Phenolic compounds, together with flavonoids, anthocyanins, and tannins, are the main group of antioxidant phytochemicals that are important due to their biological and free radical scavenging activities [17]. Phenolic acids, flavonoids, and tannins are present in different parts of pomegranate fruit and this may be one of the reasons why many of the studies demonstrated that combinations of pomegranate extracts from different parts of the fruit were more effective than a single extract [18]. In a comparative analysis, anthocyanins from pomegranate fruit were found to possess higher antioxidant activity than vitamin-E (α-tocopherol), β-carotene, and ascorbic acid [19]. Table 1 represents the key constituents of pomegranate fruit and tree [20–41].

Table 1: Principal constituents of different parts of pomegranate tree and fruit. The different parts of pomegranate plant like peel, root, bark, flower, leaves, and so forth exhibit different phytochemicals.

<table>
<thead>
<tr>
<th>Pomegranate peel</th>
<th>Pomegranate juice</th>
<th>Pomegranate root and bark</th>
<th>Pomegranate flower</th>
<th>Pomegranate leaves</th>
<th>Pomegranate seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Gallic acid</td>
<td>(i) Simple sugars</td>
<td>(i) Ellagitannins</td>
<td>(i) Carbohydrates</td>
<td>(i) 3,5'-Di-O-methyl gallagic acid</td>
<td></td>
</tr>
<tr>
<td>(ii) Ellagic acid</td>
<td>(ii) Aliphatic organic acids</td>
<td>(ii) Piperidine alkaloids</td>
<td>(ii) Reducing sugars</td>
<td>(ii)</td>
<td></td>
</tr>
<tr>
<td>(iii) Punicalin</td>
<td>(iii) Gallic acid</td>
<td>(iii) Pyrrolidine alkaloid</td>
<td>(iii) Ursolic acid</td>
<td>(iii) Sterols</td>
<td></td>
</tr>
<tr>
<td>(iv) Punicalagin</td>
<td>(iv) Ellagic acid</td>
<td>(iv) Pelletierine alkaloids</td>
<td>(iv) Triterpenoids</td>
<td>(iv) Saponins</td>
<td></td>
</tr>
<tr>
<td>(v) Caffeic acid</td>
<td>(v) Quinic acid</td>
<td></td>
<td>(v) Flavonoids</td>
<td>(v) Flavonoids</td>
<td></td>
</tr>
<tr>
<td>(vi) Ellagitannins</td>
<td>(vi) Quinic acid</td>
<td></td>
<td>(vi) Tannins</td>
<td>(vi) Oleic acid</td>
<td></td>
</tr>
<tr>
<td>(vii) Pelletierine alkaloids</td>
<td>(vii) Amino acids</td>
<td></td>
<td>(vii) Piperidine alkaloids</td>
<td>(vi) Palmitic acid</td>
<td></td>
</tr>
<tr>
<td>(viii) Luteolin</td>
<td>(viii) Amino acids</td>
<td></td>
<td>(viii) Flavone</td>
<td>(vii) Stearic acid</td>
<td></td>
</tr>
<tr>
<td>(ix) Kaempferol</td>
<td>(ix) EGC3G</td>
<td></td>
<td>(ix) Glycoside</td>
<td>(viii) Linoleic acid</td>
<td></td>
</tr>
<tr>
<td>(x) Quercetin</td>
<td>(x) Ascorbic acid</td>
<td></td>
<td>(x) Ellagitannins</td>
<td>(ix) Sterols</td>
<td></td>
</tr>
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</table>


3. Therapeutic Functions of Pomegranate

Extracts of all parts of the pomegranate fruit exhibit therapeutic properties [15] and target a range of diseases including cancer, cardiovascular disorders, diabetes, male infertility, Alzheimer’s disease [42], aging, and AIDS [43] (Figure 1). Although pomegranate’s extensive therapeutic benefits may be attributed to a number of mechanisms, most researchers have determined its antioxidant, anticarcinogenic, and anti-inflammatory properties. Various therapeutic applications of Punica granatum are discussed here.

3.1. Cancer. Research on breast cancer cell lines demonstrated that pomegranate constituents efficiently inhibited angiogenesis [44], invasiveness [40], growth [45], and induced apoptosis [46]. Its anti-inflammatory, antiproliferative, and antimetastatic effects were attributed to the modulation of Bcl-2 proteins, upregulation of p27 and p21, and down-regulation of cyclin-cdk network [47]. Pomegranate constituents inhibit angiogenesis via downregulation of vascular endothelial growth factor (VEGF) in human umbilical vein endothelial and MCF-7 breast cancer cell lines [44], thereby hampering the tumor growth. Prostate cancer cells, when treated with pomegranate juice, increased adhesion and decreased the migration. Molecular analyses revealed that pomegranate juice increased the expression of cell-adhesion related genes and inhibited the expression of genes involved in cytoskeletal function and cellular migration. It would possibly affect prostate cancer because of its apoptotic, antioxidant, antiproliferative, and anti-inflammatory properties, suggesting that it may be beneficial in slowing down or preventing cancer cell metastasis [48]. The application of pomegranate extract to the skin of mice before they were exposed to a carcinogenic agent was shown to inhibit the appearance of erythemas and hyperplasia and the activity of epithelial ornithine decarboxylase [49]. An in vivo study in TRAMP mice model suggested that oral supplementation of
pomegranate fruit extract inhibited metastasis and increased overall survival [50].

Matrix metalloproteinases (MMPs) are good markers of tumor cell invasion and migration [51]. Phytochemicals have been shown to target the activity and secretion of MMPs in estrogen responsive cancers [52]. Constituents of pomegranate minimize tumor cell invasion into normal tissue and metastasis to distant sites and these actions develop due to the inhibition of selected metalloproteinase activity, decreased focal adhesion kinase activity, and reduced VEGF expression [15]. With semiquantitative RT-PCR, we had found out that PME downregulated the transcription of MMP-9 suggesting its possible role in the inhibition of tumor invasion (Figure 2) whereas E2 (10 nM) did not significantly affect the transcription of MMP-9 [53] which correlated with earlier studies suggesting that estrogen stimulated MMP-9 secretion without increasing its gene transcription [54].

We had assessed the estrogenicity/antiestrogenicity of PME in a panel of in vitro biological assays and the expression of endogenous estrogen sensitive markers (pS2 and PR) in breast carcinoma cell lines were analyzed [53]. When MCF-7 cells pretreated with PME were treated with estrogen, the c-Myc expression was not induced as much as when treated with estrogen alone, demonstrating the effect of PME in estrogen regulated mechanism (Figure 3). ER positive cells treated with PPT (4,4′,4′′-(4-Propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol) (ERα selective agonist) and DPN (Diarylpropionitrile) (ERβ selective agonist) clearly showed that PPT increased the pS2 protein levels, whereas DPN did not produce any significant effect. When given in
combination with PPT, PME reduced the pS2 protein levels indicating the role of ERα in mediating the effects of PME on pS2 expression (Figure 4). Thus the effect of PME on expression of pS2 was mediated by ERα and not by ERβ [53].

Pomegranate fruit extract was revealed to inhibit UV-B-mediated phosphorylation of mitogen-activated protein kinase (MAPK) and nuclear factor NF-κB activity [55]. Pomegranate juice almost downregulated the TNFα induced Akt (protein kinase B) activation required for NF-κB activity [56]. Koyama et al. [57] examined the effects of pomegranate extract (POMx) on the IGF system and found out cell growth inhibition and apoptosis. Their findings suggested that POMx treatment reduced mTOR phosphorylation at Ser2448 and Ser2481, whereas IGFBP-3 increased phosphorylation at those sites. These results suggested that POMx decreased prostate cancer cell survival by inhibiting IGF1 expression. To conclude, pomegranate fruit has anticancer properties that can be attributed to different mechanisms.

3.2. Cardiovascular Disorders. In vitro, in vivo and human trials had examined the effects of a range of pomegranate constituents on the prevention and reduction of atherosclerosis and LDL oxidation [58]. Evidence suggested that polyphenolic antioxidants contained in pomegranate juice can cause reduction of oxidative stress and atherosclerosis through the activation of redox-sensitive genes ELK-1 and p-JUN and increased eNOS expression. Their results indicated that proatherogenic effects induced by disturbed shear stress can be reversed by constant administration of pomegranate juice [59]. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduced common blood pressure, LDL oxidation, and carotid intima-media thickness [60]. Azadzoi et al. demonstrated that 8-week administration of pomegranate juice concentrate daily in a rabbit model of atherosclerotic erectile dysfunction significantly increased intracavernous blood flow and smooth muscle relaxation, probably via its antioxidant effect on enhanced nitric oxide preservation and bioavailability [61]. A pilot study in type 2 diabetic patients with hyperlipidemia found that concentrated pomegranate juice decreased cholesterol absorption, increased faecal excretion of cholesterol, had a favourable effect on enzymes concerned in cholesterol metabolism, drastically reduced LDL cholesterol, and improved LDL/HDL cholesterol and total/HDL ratios [62]. Aviram et al. analyzed atherosclerotic lesion size, antioxidant activity, blood sugar, peritoneal macrophages, oxidative status, and lipid profiles for 3 months after giving 6 different pomegranate preparations with varying amounts of total polyphenols and gallic acid content in atherosclerotic apolipoprotein-E deficient mice and found that pomegranate phenolics and pomegranate unique complexed sugars could mimic the antiatherogenic effects of pomegranate extracts [63]. All these evidences suggest the potential cardioprotective effect of pomegranate fruit.
3.3. Antiosteoporotic Potential. Tissue selective estrogen agonist/antagonists are currently being investigated as alternatives to estrogen in the prevention and treatment of postmenopausal osteoporosis [64–66]. Bone loss after ovariectomy is associated with high bone turnover where bone resorption rate exceeds the bone formation rate [67]. To assess the protective role of *Punica* on skeletal system, we had examined the effect of PME on a well-characterized osteoblastic cell population (osteoblastic MC3T3-E1 cells) and examined its effect on Alkaline Phosphatase (ALP), which is a commonly used bone remodelling marker. The results (Figure 5) indicated that PME significantly increased ALP activity, supporting its suggested role in modulating osteoblastic cell differentiation [68].

Ovariectomized rodent model is a well-established system for estrogen deficiency induced bone loss and used by researchers previously [69, 70]. We had evaluated the antiosteoporotic potential of the extract in estrogen deficiency induced osteoporosis in young adult mice of 6–8 weeks of age by assessing the bone turnover by serum ALP. In
Table 2: Effect of E2, PME, and tamoxifen on chosen markers of bone metabolism of ovariectomized mice. Serum calcium, phosphorus, and Alkaline Phosphatase (ALP) levels of sham control (SS Con) and Ovx mice exposed to 0.1% ethanol (vehicle control), E2 (1 mg/kg bwt), PME (50, 100 mg/kg bwt), and tamoxifen (TAM, 10 mg/kg bwt) for 7 days (bwt = body weight). Data are expressed as mean ± SE (n = 5).

<table>
<thead>
<tr>
<th></th>
<th>Sham control</th>
<th>E2 (1 mg/kg bwt)</th>
<th>PME (50 mg/kg bwt)</th>
<th>PME (100 mg/kg bwt)</th>
<th>TAM (10 mg/kg bwt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.46 ± 0.313</td>
<td>10.94 ± 1.18</td>
<td>8.188 ± 0.704a</td>
<td>9.09 ± 0.194</td>
<td>9.908 ± 0.165</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>7.43 ± 0.63</td>
<td>8.818 ± 0.698</td>
<td>7.516 ± 1.731</td>
<td>8.78 ± 1.980</td>
<td>8.146 ± 0.0680</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>140.6 ± 11.28</td>
<td>181.8 ± 34.07a</td>
<td>115.2 ± 23.61b</td>
<td>120 ± 9.02b</td>
<td>134.6 ± 17.54b</td>
</tr>
</tbody>
</table>

*aP < 0.05 versus sham control, bP < 0.05 versus ovx control.

Figure 6: PME did not alter lactoferrin expression in murine uterus. (a) RT-PCR detection of Lactoferrin m-RNA in sham control (SS CON) and ovariectomized mice exposed to 0.1% ethanol (vehicle control, Ovx CON), E2 (1 mg/kg bwt), PME (50, 100 mg/kg bwt), and tamoxifen (TAM, 10 mg/kg bwt) for 7 days. (b) shows the ratio of density of target gene expression to that of endogenous control beta-actin and it represents mean ± SE of three replicates.

Comparison to the sham surgery (SS) control, ovariectomized (Ovx) control animals showed an increase in ALP activity indicating an increase in bone turn-over rate in these animals. PME in higher concentration was found to be effective in decreasing this bone turnover, though E2 was better in controlling the accelerated bone turnover (Table 2). The experimental model differed from aged Ovx mice wherein the osteoporosis is induced only by estrogen deficiency and not by a combination of natural bone loss due to age and ovarian hormone deficiency. An increase in bone turn-over rate was indicated by higher serum ALP level in the Ovx group compared to the SS control group. Therefore, high rate of bone turnover was well corrected by PME suggesting that it might play a protective role against ovarian hormone insufficiency related bone resorption. But E2 as well as PME was able to significantly decrease ALP levels in Ovx mice (Table 2). Serum calcium and phosphorous levels in Ovx control, PME treated, and tamoxifen treated animals were similar to that of SS control animals. Significant decrease in calcium levels was observed in E2 treated animals in comparison to SS control (Table 2). Our findings clearly in- dicated that the possible bone preserving effect of PME is almost comparable with E2 [53]. Earlier studies had shown that an acute or chronic exposure to xenoestrogens or dietary phytoestrogens alters uterine expression of estrogen sensitive genes in mice [71]. So in order to check whether PME has any effect, a semiquantitative RT-PCR was done to analyze uterine mRNA levels of lactoferrin in ovariectomized mice fed with PME for 7 days. Lactoferrin is a well-known estrogen target gene and a biologically active molecule for bone regeneration [72]. The positive control E2 increased the uterine accumulation of lactoferrin mRNA in Ovx animals compared to the vehicle treated Ovx control (Figure 6). Lactoferrin expression did not differ significantly between the groups that received PME (50, 100 mg/kg bwt) and the vehicle (0.1% ethanol) treated Ovx control group, indicating the lack of estrogenicity of PME on uterine endometrium in the doses tested in our study. Tamoxifen (10 mg/kg bwt) was found to increase the
expression of lactoferrin, though not significantly [68]. As there are promising results from both in vitro and in vivo studies, we suggest evaluating the antosteoporotic potential by clinical trials with pomegranate fruit extract that has no side effects on uterine endometrium alongside a significant decrease in bone turn-over rate.

3.4. Other Clinical Applications. In vitro assay showed that fermented pomegranate juice extract is better than red wine and comparable to green tea [37]. There were also reports that pomegranate juice possessed considerably greater antioxidant capacity at much lower concentrations (1000-fold dilutions) than either grape or blueberry juice [73]. Punica granatum peel extract decreased lipid peroxidation in hepatic, cardiac, and renal tissues and at the same time it had a facilitatory effect on the scavenging capability of superoxide anion and hydrogen peroxide [74]. Formerly, it was shown that pomegranate peel extract supplementation alleviated oxidative damage of the liver and enhanced the hepatic structure and function in rats exposed to bile duct ligation [75]. Pretreatment of carbon tetrachloride-induced liver damage in rats with pomegranate peel extract resulted in the reduction of lipid peroxidation and at the same time, the free-radical scavenging activity of catalase, superoxide dismutase, and peroxidase were considerably enhanced [76]. Many studies had keenly explored the anti-inflammatory properties of pomegranate fruit [15, 77–79]. Studies indicated that pomegranate extract inhibited PMACI-induced proinflammatory cytokine assembly by inhibiting the gene expression. This is achieved by blocking JNK and ERK-MAPK activation and NF-κB activation in human KU812 cells [80]. Larrosa et al. showed that pomegranate extract supplementations led to reduced prostaglandin E2 (PGE2) levels in the colon mucosa by downregulating the overexpressed COX-2 and prostaglandin E synthase (PTGES) levels owing to the action of ellagic acid [78]. Punica granatum extract had been found to be particularly effective for controlling oral inflammation, dental plaque, and bacterial and fungal counts in periodontal disease and Candida-associated denture stomatitis [81, 82]. Another study proposed that inhibition of number of signal transduction pathways and the downstream pathogenic cellular response by pomegranate extract or compounds may be a useful approach for the prevention of the onset and severity of inflammatory arthritis [77]. The dynamism of pomegranate fruit in newer areas of pharmacological effects might be delivered in the future.

4. Pomegranate Extract as a Phytoestrogen

Due to the possible adverse side effects of estrogenic stimulation (such as increase in tumor risk), many women have turned to phytoestrogens as an alternative for HRT [83]. The features that facilitate the chemicals to bind with ER are the steric and hydrophobic properties of a compound, as well as the hydrogen bonding between the phenolic hydroxyl group and the ER binding site [84]. Phytoestrogens bind to both forms of ER and showed a lower binding affinity than E2. Some of them exhibit a higher binding affinity to ERα than to ERβ which may indicate that they have different pathways for their actions and explains tissue specific changeability in phytoestrogenic action [85]. Both genomic and nongenomic mechanisms have been projected to explain phytoestrogenic effects on human health [86]. The best move towards the avoidance and handling of estrogen-dependent breast cancer is to selectively hold estrogen activity in the affected tissues without compromising its beneficial effects [87]. Regrettably, at this time, available antiestrogen such as tamoxifen used in the treatment of ER-positive breast cancer has side effects and agonism in the uterine endometrium, leading to an uncertain connection to endometrial carcinoma [88–90]. A competitive radioactive binding study was done to ascertain whether PME interacts with ER and had shown that PME binds to ER and inhibited the binding of labelled estrogen to ER in a dose-dependent manner [53, 91].

5. Pomegranate as a Potential Nutraceutical

According to De Felice, who coined the term nutraceutical, it can be defined as, “a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease” [92]. It may range from isolated nutrients, herbal products, dietary supplements, and diets to genetically engineered “designer” foods and processed products such as cereals, soups, and beverages [93, 94]. Anthocyanins (delphinidin, cyanidin, and pelargonidin) and hydrolysable tannins (such as punicalagin, pedunculagin, punicalin, gallic, and ellagic acid esters of glucose), account for the major antioxidant activity of whole fruit [22, 95]. The peel, which is also a major part of the fruit, is an imperative source of bioactive compounds such as phenolics, flavonoids, ellagitannins, proanthocyanidin compounds [96], minerals, [97], and complex polysaccharides [98]. Aviram and others reported that systolic blood pressure was reduced, after 1 year of pomegranate juice consumption. This was believed to be related to the potent antioxidant properties of pomegranate polyphenols [60]. Hong et al. confirmed that pomegranate juice and pomegranate extracts were more potent inhibitors of cell growth than isolated individual polyphenols in cell lines, influential synergistic and/or additive effects of several phytochemicals including proanthocyanidins, anthocyanins, and flavonoid glycosides [99]. Pomegranate contains agents, particularly polyphenolic flavonoids, which exert actions that could be well conducive to good oral health, particularly in relation to gingivitis development [100]. Pomegranate juice had the greatest antioxidant potency composite index among beverages like black cherry juice, cranberry juice, grape juice, apple juice, orange juice, red wines, blueberry juice, and iced tea; and the antioxidant activity was at least 20% superior to any of the other beverages tested [101–103]. Each and every part of pomegranate provides health benefits, that is, a nutraceutical food.

6. Summary and Conclusions

The discovery that plants generate hormonally active phytochemicals has altered our understanding of the connection
between diet and human health. It is well established that fruit or plant extracts are a complex mixture of various constituents and, in most of the instances, it is not clear whether a single compound or a mixture of compounds is responsible for the reported effects [104]. The thought of the whole herb or multiherb preparation not only addresses multiple targets, but possibly will alleviate the toxicity and side effects of a single, isolated compound from the plant. Many in vitro and in vivo studies pointed out high nutritional and potential tissue specific action of extract of Punica granatum. Proofs are accumulating that compounds present in a fruit or herb extract augment each other’s biological effect. For example, it has been reported that quercetin and ellagic acid (both are also present in pomegranate) together make use of a more prominent inhibitory effect against cancer cell growth than either compound alone [105]. We had found that PME has antioestrogenic effect in the mammary gland, without compromising the beneficial effects of estrogen in the cardiovascular and skeletal system and had no estrogenicity in the uterus [53]. PME could possibly be considered as an ideal SERM and further studies might demonstrate its suitability and possible application in estrogen dependent breast cancers with beneficial effects in other hormone dependent tissues. Figure 7 describes the biological effects of PME, as observed in our studies. Furthermore, it would be valuable to investigate the long-term effects of PME in the in vivo models of estrogen deprivation to demonstrate its suitability in HRT. To achieve this goal, a better understanding is needed regarding the orchestrated action of SERM, receptor and coregulators that contribute to distinct patterns of gene expression. Although scientific research is being carried out to study the biological activity of a lot of food phytochemicals, the health claims attributed to the final marketed nutraceutical products have normally little or doubtful scientific foundation. This is owing to the fact that a great deal of scientific conclusion is derived from animal testing and in vitro assays, while human clinical trials are limited. Some key issues such as metabolism, bioavailability, toxicity, and dose/response of these food bioactive compounds or nutraceuticals themselves have not been well recognized yet. Currently, numerous clinical trials are in progress exploring the therapeutic potential of pomegranate extracts. Its potential use as a nutraceutical needs to be investigated. We may thus anticipate that many of the open issues about the biological effect of extract of Punica granatum will be answered in the near future.

**Authors’ Contribution**

Sreeja Sreekumar, Hima Sithul, Parvathy Muraleedharan, and Juberiya Mohammed Azeez have equal authorship.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Research Article

α-Mangostin Suppresses the Viability and Epithelial-Mesenchymal Transition of Pancreatic Cancer Cells by Downregulating the PI3K/Akt Pathway

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α-Mangostin, a natural product isolated from the pericarp of the mangosteen fruit, has been shown to inhibit the growth of tumor cells in various types of cancers. However, the underlying molecular mechanisms are largely unclear. Here, we report that α-mangostin suppressed the viability and epithelial-mesenchymal transition (EMT) of pancreatic cancer cells through inhibition of the PI3K/Akt pathway. Treatment of pancreatic cancer BxPc-3 and Panc-1 cells with α-mangostin resulted in loss of cell viability, accompanied by enhanced cell apoptosis, cell cycle arrest at G1 phase, and decrease of cyclin-D1. Moreover, Transwell and Matrigel invasion assays showed that α-mangostin significantly reduced the migration and invasion of pancreatic cancer cells. Consistent with these results, α-mangostin decreased the expression of MMP-2, MMP-9, N-cadherin, and vimentin and increased the expression of E-cadherin. Furthermore, we found that α-mangostin suppressed the activity of the PI3K/Akt pathway in pancreatic cancer cells as demonstrated by the reduction of the Akt phosphorylation by α-mangostin. Finally, α-mangostin significantly inhibited the growth of BxPc-3 tumor mouse xenografts. Our results suggest that α-mangostin may be potentially used as a novel adjuvant therapy or complementary alternative medicine for the management of pancreatic cancers.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant and lethal tumors, with an overall 5-year survival rate less than 5% [1]. The lethality of pancreatic cancer is largely due to the aggressive local invasion, metastases, and resistance to chemotherapy [2]. The poor prognosis in pancreatic cancer patients is closely correlated with the high proliferation and metastasis of tumour cells. Gemcitabine has been the standard first-line drug for patients with advanced pancreatic cancer since 1996, but it provides only a modest benefit to patients owing to the problems such as acquired chemoresistance and multiple adverse effects [3]. It is therefore clear that there is an urgent need to develop drugs that are more effective and have less toxicity compared to gemcitabine for the treatment of pancreatic cancer.

Mangosteen, a well-known tropical fruit, is native to Thailand and other tropical countries [4]. The purple pericarp of Mangosteen contains lots of healthy nutrients and pharmacologically active compounds, including xanthones, terpenes, anthocyanins, tannins, and phenols [5]. Since long time ago, the pericarp of this fruit has been used as a native drug by Southeast Asians to treat diseases, such as skin infections and wounds, amoebic dysentery, diarrhoea, and...
cholera [6]. In recent years, commercial products comprised of mangosteen, multiple vitamins and minerals, green tea, and other natural extracts are being widely recommended to cancer patients as a dietary supplement [7]. Although there is no sufficient clinical evidence that mangosteen could suppress the growth of tumors or reduce the incidence of malignancies, the commercial products are one of the best-selling botanical dietary supplements [8].

A class of compounds known as xanthones isolated from mangosteen possess a wide range of biological activities including anti-inflammatory [9, 10], neuroprotective [11], cardioprotective [12, 13], and antioxidant activity [7, 14, 15]. α-Mangostin is one of the major bioactive and most abundant xanthones derived from mangosteen [16]. α-Mangostin, as a chemopreventive and chemotherapeutic bioactive compound, has been widely investigated [17]. It was shown that α-mangostin has a potential inhibitory effect on several carcinomas. It could induce cycle arrest of cancer cell [18], inhibit cell viability [19], induce apoptosis and differentiation [20], reduce inflammation, and decrease adhesion [21–23], invasion [24], and metastasis of cancer cells [23, 25]. However, the underlying pharmacology of its antitumor effect is largely unknown.

In this study, we have investigated the effects of α-mangostin on the cell viability and epithelial-mesenchymal transition in pancreatic cancer BxPc-3 and Panc-1 cells. We have also explored the underlying potential molecular mechanisms and found that the PI3K/Akt pathway was inhibited by α-mangostin.

2. Materials and Methods

2.1. Reagents. α-Mangostin (purity > 98%) was obtained from Sigma (CA, USA) and dissolved in dimethylsulfoxide (DMSO; St. Louis, MO, USA) at the stock concentration of 100 mM. Recombinant human TGF-β1 was purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). Millicell culture plate inserts and Matrigel were purchased from Millipore (Bedford, MA, USA). Antibodies against Bcl-2 or β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anticleaved caspase-3, cyclin-D3, cyclin-D1, Akt, and phospho-Akt (Ser473) antibodies were purchased from Cell Signaling Technology; anti-MMP-2 and anti-MMP-9 antibodies were from Proteintech (USA); antibodies against E-cadherin, N-cadherin, and vimentin were procured from Bioworld (Minneapolis, MN, USA).

2.2. Cell Culture. BxPc-3 and Panc-1 cells were purchased from Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS); hTERT-HPNE cell was purchased from ATCC (Manassas, VA) and cultured at 37°C and 5% CO2 in RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM/High Glucose) (HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, USA), 100 μg/mL ampicillin, and 100 μg/mL streptomycin.

2.3. Cell Viability Assay. BxPc-3, Panc-1, and hTERT-HPNE cells at 50–60% confluency were cultured in medium supplemented with 1% FBS for 24 h to get synchronized G1 phase cells, plated into 96-well plates at a density of 1 × 104 cells per well, and incubated overnight in medium supplemented with 10% FBS. Cells were then treated with various concentrations of α-mangostin in 0.1% DMSO or with 0.1% DMSO alone as control. Following incubation for 6, 12, 24, and 48 h at 37°C, relative cell viability was quantified by MTT assay as previously reported [26]. 20 μL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) was added into each well after media were removed and incubated at 37°C for 4 h. Then, 150 μL DMSO was added to each well and the optical density (OD) was measured at 492 nm on a Multifunction Microplate Reader (POLARstar OPTIMA; BMG, Offenburg, Germany). The proliferation inhibition rate was calculated according to the equation: proliferation inhibition rate = (1 − OD sample/OD control) × 100%.

2.4. Cell Cycle Analysis. Cells (1 × 105 cells/mL) were seeded in six-well plates coated with 1% gelatin and allowed to grow to 80% confluency. Then, the media were replaced with fresh media containing various different concentrations of α-mangostin. After 24 h of incubation, the cells were fixed in 70% alcohol for 30 min on ice. Cells were treated with RNase A (Sigma) at 37°C and stained with propidium iodide in the dark for 30 min. DNA content was assayed on a FACSCalibur (BD, Franklin Lakes, NJ) and cell cycle analysis was conducted using CellQuest software.

2.5. Apoptosis Assay. Apoptosis was assessed with an Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology, Shanghai, China) according manufacturer’s instructions. Cells were seeded (105/well) in 6-well plates in DMEM supplemented with 1% FBS. Twenty-four hours later, the medium was replaced with fresh medium containing various concentrations of α-mangostin. After additional 24 h, cells were trypsinized, washed with PBS, and stained with Annexin V and propidium iodide in the dark. The percentage of apoptotic cells was quantified by flow cytometry.

2.6. Cell Migration and Invasion Assays. Cell invasion and migration assay were performed using Transwell chambers (Millipore) as previously described [27]. The 8 μm pore inserts were coated with or without Matrigel (Sigma-Aldrich) for invasion or migration assays. Cell suspensions containing 1% FBS (200 μL, 5 × 104 cells) were seeded into the upper chamber and 500 μL DMEM containing 20% FBS was added in the lower chamber. Noninvading cells were removed with a cotton-tipped swab after 24 h incubation, and the invading cells on the bottom surface of membrane were stained with 0.1% crystal violet. The invading cell numbers were quantified by counting 10 random fields at ×200 magnification.

2.7. Western Blotting. Total proteins were extracted by RIPA Lysis Buffer (Beyotime, Guangzhou, China) according to the manufacturer’s instruction. Western blotting was performed as previously described [28]. In brief, proteins were separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Roche). Membranes were blocked with 5% nonfat
dry milk in Tris-Buffered Saline and Tween 20 (TBST) at room temperature for 1 h and incubated overnight at 4°C with the following primary antibodies: anti-Bcl-2, anticleaved caspase-3, anti-cyclin-D3, anti-cyclin-D1, anti-MMP-2 and anti-MMP-9. After incubation with HRP-conjugated secondary antibodies for 2 h at room temperature, immunoreactive bands were developed by enhanced chemiluminescence. β-Actin was stained as loading control.

2.8. Real-Time PCR. Total RNA was extracted by Fastgen200 RNA isolation system (Fastgen, Shanghai, China) as the manufacturer’s protocol. Total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Dalian, China). qRT-PCR was conducted using the iQ5 Multi-color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and SYBR Premix Ex Taq II (Takara, Dalian, China) as previously described to quantify mRNA levels of MMP-2, MMP-9, E-cadherin, N-cadherin, and vimentin [29]. The comparative C(T) method was used to quantitate the expression of each target gene using β-actin as the normalization control. The PCR primer sequences for MMP-2, MMP-9, E-cadherin, N-cadherin, vimentin, and β-actin are listed in Supplemental Information Table S1 (see Supplemental Information Table S1 available online in Supplementary Materials at http://dx.doi.org/10.1155/2014/546353).

2.9. Subcutaneous Xenografts In Vivo. Male BALb/c nude mice were purchased and housed in the Animal Center at Medical College, Xi’an Jiaotong University. Twenty animals were randomly divided into four groups, with five animals in each group. The animals in group I received vehicle (100 μL saline) by oral gavage and served as control. The animals in groups 2 and 3 received α-mangostin suspension (dissolved in 100 μL saline and 50 or 100 mg/kg) by oral gavage five times weekly [18]. Body weights were recorded once weekly throughout the study. According to the previous report [27], 2 × 10^6 cells (100 μL) in a 50% Matrigel mixture were injected subcutaneously in nude mice at four to six weeks old. The dimensions, length (L), and width (W) of the tumors were measured with Vernier calipers every 3 days. Tumor volume was calculated using the equation (L × W^2)/2. The mice were euthanized 30 days after the subcutaneous injection.

2.10. Ethics Statement. All in vivo experimental protocols were evaluated and approved by the Animal Care and Use Committee of College of Medicine, Xi’an Jiaotong University.

2.11. Statistical Analysis. Each experiment was performed at least for three times. Data are presented as means ± standard deviation. Differences were evaluated using a Student’s t-test, with P < 0.05 considered to be statistically significant.

3. Results

3.1. Treatment with α-Mangostin Results in Loss of Viability of Pancreatic Cancer Cells. To investigate the effect of α-mangostin on the viability of pancreatic cancer cells, we treated BxPc-3, Panc-1, and hTERT-HPNE cells (normal human pancreatic ductal epithelial cell) with different concentrations of α-mangostin (0, 2, 4, 6, 8, 16, and 32 μM) for 6, 12, 24, and 48 h and assessed cell viability by the MTT assay. Treatment with α-mangostin at concentrations of more than 8 μM significantly reduced the viability of both cancer cell lines in a time-dependent manner (P < 0.05) (Figures 1(a) and I(b)); however, limited inhibitory effect on hTERT-HPNE cells was observed (Figure 1(c)). Treatment with 32 μM α-mangostin resulted in more than 80% loss of cell viability in both pancreatic cancer cell lines and 50% in hTERT-HPNE cells after 48 h.

3.2. α-Mangostin Induces Apoptosis of Pancreatic Cancer Cells. To address the underlying mechanism of the inhibitory effect of α-mangostin on pancreatic cancer cell viability, we measured α-mangostin-induced apoptosis in BxPc-3 and Panc-1 cells by flow cytometry. As shown in Figures 2(a) and 2(b), treatment with 8 μM α-mangostin resulted in increased early (Annexin V-FITC) and late (PI) apoptotic cells compared to untreated controls. Notably, when compared to the untreated group, more than 30% cells of both of the cell lines underwent apoptosis following treatment of 16 μM α-mangostin (Figures 2(a) and 2(b), P < 0.05). Consistent with these results, immunoblots demonstrated that α-mangostin caused a dose-dependent reduction in the antiapoptosis Bcl-2 protein levels and an increase in the levels of cleaved of caspase-3, a marker of cell apoptosis (Figure 2(c)). These results clearly demonstrate that α-mangostin induces apoptosis of pancreatic cancer cells.

3.3. α-Mangostin Induces Cell Cycle Arrest at the G1/G0 Phase in Pancreatic Cancer Cells. Most drugs inhibit cell growth and promote cell apoptosis through inducing cell cycle arrest. To test whether α-mangostin induces cell cycle arrest of pancreatic cancer cells, we treated BxPc-3 and Panc-1 cells with different concentrations of α-mangostin for 24 h and performed cell cycle analysis by PI/flow cytometry. As shown in Figures 3(a) and 3(b), pancreatic cancer cells treated with 8 μM α-mangostin significantly accumulated in the G1/G0 phase, while there were more cells in G1/G0 phase after treatment with 16 μM α-mangostin. In consistence, α-mangostin treatment led to a reduction of cells in S and G2/M phases (Figures 3(a) and 3(b)). The increase of G1/G0 phase in conjunction with the decrease of S and G2/M phases suggests that the reduction of cell viability may be due at least in part to the induction of cell cycle arrest by α-mangostin.

Cyclin D-dependent kinases 4 and 6 (CDK4/6) are the main drivers for the transition of cells from G1 to S phase. Indeed, in BxPc-3 cells, 8 μM α-mangostin slightly and 16 μM α-mangostin markedly reduced the protein level of cyclin-D1 after 24 h of treatment (Figure 3(c)). In Panc-1 cells, 8 μM and 16 μM α-mangostin dramatically decreased the protein level of cyclin-D1 (Figure 3(c)). In both cell lines, the effects of α-mangostin on the protein levels of cyclin-D3 were marginal. These results indicate that α-mangostin inhibits cell cycle progression by downregulating cyclin-D1.

3.4. α-Mangostin Inhibits the Migration and Invasion of Pancreatic Cancer Cells. To evaluate the effect of α-mangostin...
on pancreatic cancer cell migration, we performed Transwell assays and found that α-mangostin at concentrations of 8 μM and 16 μM significantly inhibited the migration of BxPc-3 and Panc-1 cells (Figure 4(a)). Next, we assessed the effect of α-mangostin on pancreatic cancer cell invasion using a Matrigel invasion assay. α-Mangostin significantly suppressed the ability of these cells to invade through the Matrigel in a dose-dependent manner (Figure 4(b)). Moreover, α-mangostin reduced both the mRNA (Figure 5(a)) and protein levels (Figure 5(b)) of MMP-2 and MMP-9, which play critical roles in migration, invasion, and metastasis of cancer cells. These observations demonstrate that α-mangostin inhibits the migration and invasion of pancreatic cancer cells in vitro.

3.5. α-Mangostin Modulates the Expression of EMT-Related Genes in Pancreatic Cancer Cells. The inhibition of cell migration and invasion of pancreatic cancer cells suggests that α-mangostin may affect EMT of cancer cells. To test this, we measured the expression of EMT related-genes by immunoblotting and qRT-PCR. α-Mangostin treatment of

Figure 1: Treatment of pancreatic cancer cells with α-mangostin results in loss of cell viability. Pancreatic cancer BxPc-3 (a), Panc-1 (b), and hTERT-HPNE (c) cells were treated with α-mangostin at the indicated concentrations for 6, 12, 24, and 48 h. Cell viability relative to control was assessed using the MTT assay.
Figure 2: α-Mangostin induces apoptosis of pancreatic cancer cells. (a) Panc-1 and BxPC-3 cells were treated with α-mangostin (0 μM, 8 μM, or 16 μM) for 24 h. Apoptotic cells were evaluated by Annexin V-FITC/PI staining and flow cytometry. Representative FACS plots are shown. (b) Apoptosis rate of the early and late apoptosis was quantified by flow cytometry. *P < 0.05 compared with controls. (c) Protein levels of Bcl-2 and cleaved caspase-3 were detected by Western blotting with β-actin as loading control.
Figure 3: α-Mangostin induces cell cycle arrest in pancreatic cancer cells. (a) The effect of α-mangostin on cell cycle progression in pancreatic cancer cells was assessed by flow cytometry. Cells were fixed, stained, and analyzed for DNA content. (b) The distribution and percentage of cells in G1/G0, S and G2/M phase of the cell cycle are indicated. (c) Protein levels of cyclin-D3 and cyclin-D1 were assessed by Western blotting with β-actin as loading control.
both BxPc-3 and Panc-1 cells resulted in a concentration-dependent increase in the protein levels of the epithelial marker E-cadherin and a decrease in the protein levels of the mesenchymal markers vimentin and N-cadherin (Figure 5(a)). Similar alteration of the mRNA levels of these corresponding genes by α-mangostin was also observed (Figure 5(b)). These results further support the observation that α-mangostin has an inhibitory effect on the acquisition of mesenchymal-like characteristics by pancreatic cancer cells and consequently suppresses their capacity for dissemination and invasion.

3.6. α-Mangostin Suppresses the Activation of the PI3K/Akt Pathway in Pancreatic Cancer Cells. The PI3K/Akt pathway, one of the predominant cell growth-promoting signaling pathways, enhances cell proliferation mainly by increasing D-type cyclins. The arrest of cell cycle in the G1 phase and down-regulation of cyclin-D1 (Figure 3) suggest that α-mangostin may downregulate the activity of the PI3K/Akt pathway in pancreatic cancer cells. To test this hypothesis, we assessed the phosphorylation of Akt at ser473 by immunoblotting of BxPc-3 and Panc-1 cells treated with α-mangostin. Our results revealed that α-mangostin reduced the phosphorylation of Akt at ser473 (Figure 6(a)). We also observed that Akt phosphorylation was significantly decreased after treatment with the α-mangostin in a dose- and time-dependent manner (Figures 6(a) and 6(b)).

The deregulation of PI3K/Akt pathway is associated with enhanced cell migration, invasion, and metastasis of cancer cells. Moreover, a recent study demonstrated that TGF-β induces epithelial to mesenchymal transition through the activation of PI3K/Akt/mTOR pathway [30]. These facts led us to hypothesize that α-mangostin may suppress the migration, invasion, and EMT in pancreatic cancer cells through dampening the PI3K/Akt pathway. We therefore compared the TGF-β-induced phosphorylation of Akt and upregulation of EMT-related genes [31] in BxPc-3 and Panc-1 cells treated with 5 ng/mL TGF-β [31] with or without 16 μM of α-mangostin. While TGF-β enhanced the phosphorylation of Akt and the expression of vimentin and N-cadherin, these TGF-β-induced effects were significantly abolished by α-mangostin (Figure 6(b)). These results demonstrate that α-mangostin induces multiple biological effects in pancreatic cancer cells through inhibition of the PI3K/Akt pathway.

3.7. α-Mangostin Suppresses the Growth of Human Pancreatic Cancer BxPc-3 Cells In Vivo. To extend our in vitro observations to the in vivo context, we conducted in vivo tumor
Figure 5: α-Mangostin modulates the expression of EMT-related genes in pancreatic cancer cells. (a) Panc-1 and BxPC-3 cells were treated with α-mangostin 16 μM at the indicated concentrations for 24 h. Protein (a) and mRNA (b) levels of MMP-2, MMP-9, E-cadherin, vimentin, and N-cadherin were measured by Western blotting and qRT-PCR, respectively. *P < 0.05 compared with controls.

4. Discussion

In this study, we observed that α-mangostin inhibited cell viability and induced cell apoptosis, which were accompanied by the activation of caspase-3 and decreased levels of Bcl-2 in BxPC-3 and Panc-1 cells. Moreover, α-mangostin promoted cell cycle arrest and inhibited cell invasion, which were associated with the decreased expression of cyclin-D and MMPs. Furthermore, we found that α-mangostin suppressed the EMT progression and activity of the PI3K/Akt pathway in pancreatic cancer cells. The subcutaneous tumor xenograft model showed that the oral administration of α-mangostin resulted in significant suppression of the growth of tumor xenografts in nude mice. Our findings suggest that
α-mangostin is a potential drug for adjuvant therapy or a complementary alternative medicine for the management of pancreatic cancers.

Despite great advances in modern medicine during the past two decades, pancreatic cancer is still associated with an extremely high mortality rate (approaching 100%) [2]. Currently, Gemcitabine is considered as a first-line drug for pancreatic cancer, but its efficacy is still disappointing [32] and the pancreatic cancer survival has not improved substantially over the past 25 years. Recently, the dietary and synthetic agents purported to possess greater efficacy and lower toxicity have attracted great attention for the prevention and treatment of pancreatic cancer [33].

Previous studies showed that α-mangostin could inhibit the growth and induce the apoptosis of human prostate cancer [18] and colon cancer [34] cells. Similarly, we found that α-mangostin inhibited the proliferation and induced apoptosis of pancreatic cancer cells in a time- and dose-dependent manner, but limited inhibitory effect on the normal human pancreatic ductal epithelial cell was observed. The caspase-3 cascade is activated by proapoptotic molecules such as cytochrome c when released from mitochondria and is inhibited by antiapoptotic members of the Bcl-2 proteins. We demonstrated that α-mangostin promoted the activation of caspase-3 but decreased Bcl-2, suggesting that α-mangostin may potentially act on mitochondria and induce apoptosis.
**Figure 7:** α-Mangostin suppresses the *in vivo* growth of human pancreatic cancer cells. (a) Body weight was measured weekly and tumor volumes (mm$^3$) were calculated every 3 days throughout the study. (b) Effects of α-mangostin on the growth of xenograft tumors. Photographs of subcutaneous xenograft tumors derived from BxPc-3 cells in nude mice with (left) or without (right) α-mangostin treatment (50 and 100 mg/kg) on day 30 after tumor cell injection.

α-Mangostin has been previously reported to induce cell cycle arrest in breast cancer cells [35] and colon cancer cells [34]. Consistent with these studies, our results demonstrated that α-mangostin significantly inhibited the ability of cells to transit from G1 to the S phase. The transition from the G1 phase to S phase is an important cell cycle control point. Cyclin-D1 is a critical cell cycle regulatory molecule and is required for the cell cycle progression through G1 to S phase [36]. The growth stimulatory signals received by the cells in the early stage of G1 phase increase the levels of cyclin-D1. It was reported that α-mangostin for 24 h suppressed cyclin-D1 which was followed by apoptosis in prostate cancer cells [18]. In agreement with this report, we observed that α-mangostin promoted cell cycle arrest at G1 phase and downregulated cyclin-D1 in BxPc-3 and Panc-1 cells. These results indicate that the inhibitory effect of α-mangostin on the pancreatic cancer cell might be due to the downregulation of cyclin-D1 expression and a consequent delay in the G1/S transition.

Migratory and invasive abilities are important characteristics of metastatic cancer cells. Epithelial-mesenchymal transition is the process wherein epithelial cells acquire fibroblast-like properties and exhibit reduced cell-cell adhesion and increased motility. During oncogenesis, EMT may endow cancer cells with enhanced motility and invasiveness. Recent studies showed that α-mangostin could inhibit the metastasis and invasion in skin, prostate, breast, and lung [25]. In the present study, α-mangostin was demonstrated to decrease the migration and invasion of pancreatic cancer cells. In addition, we found that α-mangostin modulated the expressions of invasion-related (MMP-2 and MMP-9) and EMT-associated (E-cadherin, vimentin and N-cadherin) genes. Our results suggest that α-mangostin may inhibit migration and invasion of pancreatic cancer cells by downregulating MMPs and inhibiting EMT progression.

Dysregulation of the PI3K/Akt pathway is quite common in PDAC. Up to 60% of PDAC tissues and most PDAC cell lines exhibit increased AKT activity [37]. The serine/threonine kinase Akt, the most studied signaling molecule downstream of PI3K, is involved in the stimulation of cell proliferation, inhibition of apoptosis, alteration of the cell cycle, and promotion of invasiveness as well as induction of EMT [38–40]. These findings prompted us to hypothesize that the biological action of α-mangostin may be mediated via PI3K/Akt pathway. Our results showed that α-mangostin
reduced the basal activity of Akt and abolished the TGF-β-induced Akt phosphorylation.

The anticancer potential of α-mangostin was further supported by our in vivo studies that involve generation of subcutaneous xenograft tumors derived from pancreatic cancer cells in nude mice. A previous study showed that pretreatment of rats with α-mangostin (200 mg/kg body wt.) orally for up to 8 days had no observable adverse effects in solid organ systems [13]. Another report demonstrated that F344 rats fed with custom-blended food pellets that contained 0.02 or 0.05% α-mangostin for 5 weeks also showed no apparent adverse effects [41]. In addition, mangosteen that contains abundant amounts of α-mangostin is widely consumed by humans in the form of juices, dietary supplements, and fruit [18]. Therefore, the anticancer activity of α-mangostin suggests that α-mangostin has the potential to be a novel adjuvant therapy or complementary alternative medicine for the management of pancreatic cancer.

5. Conclusions

The present study demonstrated that α-mangostin suppressed the proliferation, migration, and invasion as well as EMT of pancreatic cancer cells. These multiple biological effects might result from the suppression of the PI3K/Akt signaling pathway by α-mangostin. These results suggest that α-mangostin is a potential anticancer agent for the treatment of pancreatic cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Qinhong Xu and Jiguang Ma contributed equally to this work.

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References


Research Article

Preparation of Naringenin/β-Cyclodextrin Complex and Its More Potent Alleviative Effect on Choroidal Neovascularization in Rats

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Choroidal neovascularization (CNV) is characterized by abnormal blood vessels growing from the choroid. Current remedies for CNV have not shown favorable therapeutic efficacy. It is urgent to identify and develop more safe and potent anti-CNV agents via multiple technologies. We previously showed that the natural product naringenin attenuated CNV. However, naringenin has poor water solubility and low bioavailability. Here, we prepared the β-cyclodextrin (β-CD) complex of naringenin and characterized it using infrared spectra and X-ray diffraction analyses. Determination of content and solubility in the complex showed that naringenin accounted for 20.53% in the complex and its solubility was increased by more than 10-fold. Using a laser-induced CNV model in rats we demonstrated that naringenin/β-CD complex more significantly reduced CNV area than naringenin alone in rats. Furthermore, naringenin and its β-CD complex significantly inhibited the mRNA and protein expression of VEGF, COX-2, PI3K, p38MAPK, MMP-2, and MMP-9 in retina and choroid tissues. Naringenin/β-CD complex showed more significant inhibitory effect on VEGF and COX-2 expression than naringenin. These results collectively indicated that naringenin/β-CD complex could be a promising therapeutic option for CNV and that the beneficial effects could be linked to the anti-inflammatory properties of naringenin.

1. Introduction

Choroidal neovascularization (CNV) is a common cause of blindness worldwide. It is characterized by new, abnormal blood vessels growing from the choroid through breaks in Bruch's membrane or the basement membrane of the retinal pigment epithelium [1]. These vessels can leak blood and fluid and are accompanied by fibrous tissue, which often leads to damage of the retinal tissues and vision loss. CNV can be classified according to its location in relation to the fovea: subfoveal CNV is located under the center of the retina; juxtafoveal and extrafoveal CNV are located at increasing distances away from the center [2]. CNV is typically secondary to age-related macular degeneration (AMD) and these conditions are major and also substantially increase causes of blindness among aged people.

Several therapeutic options are currently available to treat CNV with variable efficacy on disease progress. Among existing treatments, there are laser photocoagulation, photodynamic therapies, and local corticosteroids. More recently, the use of antiangiogenic remedies has been emerged for CNV treatment. Angiogenic and angiostatic factors have been found to be pivotal in the pathogenesis of CNV. The discovery of such factors as vascular endothelial growth factor (VEGF) and their mechanisms of action has led to the development of drugs specifically targeting the molecules or their signal transduction pathways. VEGF appears to be one of the major regulators in CNV [3, 4]. Consequently, pegaptanib sodium, an anti-VEGF aptamer, was approved for neovascular AMD in 2004 [5], and the anti-VEGF antibody bevacizumab has also been used intravenously [6] and intravitreally [7] to treat CNV.
Although, by these treatments, very effective results are obtained and their further improvement is still possible, it is also reasonable and necessary to look for more successful and definitive alternatives. Currently, natural products isolated from medicinal herbs have been found to be effective remedies for ophthalmologic diseases. Of note, naringenin is a flavanone compound widely found in medicinal plants. Studies have shown that naringenin could significantly prevent the development of CNV induced by laser in rats, which might be related to its effect on choroidal blood flow [8]. However, naringenin has poor water solubility and low bioavailability, which greatly limit its potential utility in the treatment of CNV. The research in this area is already very active and it can be expected that applications of the recent molecular technologies will bring important advances for CNV remedies. Cyclodextrins are cyclic oligomers of glucose that can form water-soluble inclusion complexes with small molecules and portions of large compounds [9]. These biocompatible, cyclic oligosaccharides do not elicit immune responses and have low toxicities in animals and humans [10]. Of note is that β-cyclodextrin (β-CD) has been widely used to improve drug delivery and bioavailability [11]. In the current study, we prepared naringenin/β-CD complex and characterized its physical and chemical properties, and we further evaluated its efficacy on laser-induced CNV in rats and explored the preliminary mechanisms.

2. Materials and Methods

2.1. Regents and Antibodies. Naringenin (purity > 98%) and β-CD (purity > 98%) were purchased from Beijing Hailin Wei Scientific Co., Ltd. (Beijing, China). The primary antibodies used in western blot analyses against VEGF, COX-2, and p38MAPK were from Cell Signaling Technology (Danvers, MA, USA). The primary antibody against PI3K was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies against MMP-2, MMP-9, and β-actin were from Sigma (St Louis, MO, USA).

2.2. Preparation of Naringenin/β-CD Complex. Naringenin and β-CD (mole ratio 1:1) were accurately weighted. Naringenin was first dissolved in a certain amount of ethanol (0.1 mL/10 mg naringenin), and then β-CD saturated aqueous solution was slowly added with stirring. The mixture was stirred for 30 min at 60°C and then for additional 5 h without heating. The mixture was cooled naturally and stored overnight at 4°C. The mixture was filtered and the filtrate was dried with freeze dryer for 24 h, and finally the naringenin/β-CD complex was obtained. In addition, we also prepared a physical mixture of naringenin and β-CD by grinding naringenin and β-CD (mole ratio 1:1) together in a mortar.

2.3. Characterization of Naringenin/β-CD Complex. Naringenin/β-CD complex of 1.5 mg and KBr of 200 mg were compressed and subjected to infrared spectra analysis (scanning range 4000 cm\(^{-1}\) to 400 cm\(^{-1}\)). The complex samples were also subjected to X-ray diffraction analysis and the detection conditions were as follows: copper target, temperature 50°C, voltage 40 kV, current 100 mA, scanning rate 5°/min, and scanning range 3°–50°.

2.4. Determination of Naringenin Concentration in the Prepared Complex. Naringenin of 16.3 mg was dissolved in ethanol and volumed in 100 mL volumetric flask. Solutions of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mL were, respectively, volumed with ethanol in 50 mL volumetric flasks. The concentration range was 1.63–11.41 μg/mL. The absorbance (A) of these solutions was determined at 288 nm and the standard curve equation was obtained: \( A = 0.0577 \times [C] + 0.06274 \) (\( r = 0.9994 \)). Next, a certain amount of naringenin/β-CD complex was dissolved in ethanol with the aid of ultrasound and volumed in 100 mL volumetric flask. Solution of 1 mL was volumed with ethanol in 10 mL volumetric flask, and the absorbance was determined at 288 nm. According to the above standard curve equation, the concentration of naringenin in the complex could be calculated.

2.5. Determination of Naringenin Solubility in the Prepared Complex. A certain amount of naringenin/β-CD complex was dissolved in water to be oversaturated. The solution was shaked within constant temperature air bath at 37°C and 100 r/min for 48 h. The supernatant was filtered with 0.8 μm Millipore filter. The filtrate was diluted to be 90% of the original concentration and its absorbance was determined at 288 nm. According to the above standard curve equation, the solubility of naringenin in the complex could be calculated.

2.6. Animal Procedures and Treatments. Male Brown-Norway rats (150–180 g weight) were purchased from Beijing Vital River Experimental Animal Co., Ltd. (Beijing, China). All experimental procedures were approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine (Nanjing, China), and all animals received humane care according to the National Institutes of Health (USA) guidelines. Rat CNV model was established according to described methods [12]. Briefly, rats were anesthetized using intramuscular injection with ketamine (50 mg/kg) and then a mixture of 0.5% tropicamide and phenylephrine was used for mydriasis. Krypton laser was concentrated to eight spots between the large retinal vessels at an optic disk. The parameters of laser radiation were 568 nm wavelength, 100 μm spot diameter, 0.1 s exposure duration, and 150–200 mW power. The spots where tiny bubbles appeared after the Bruch membrane was broken were viewed to be qualified. Hemorrhage of retina, choroid, or vitreum was excluded in CNV model establishment. Twenty-four CNV rats were randomly divided into three groups (eight rats/each group), namely, model group, naringenin group, and naringenin/β-CD complex group. Treatments began following retinal photoagulation and rats in treatment groups were intraperitoneally injected with naringenin (20 mg/kg, dissolved in DMSO) or naringenin/β-CD complex (20 mg/kg) once daily for four weeks. Rats in model group were intraperitoneally injected with DMSO of equal volume. In addition, eight rats were normally raised without any manipulation as control group.

2.7. Measurement of CNV Area. After four-week treatment, rats (3 rats (6 eyes)/group) were subjected to sublingual injection with 10% FITC-D (0.2 mL/rat). One hour later, the
eyeballs were removed and fixed in 40 g/L paraformaldehyde. The eyeballs were opened along the equator of eyeball under a microscope. Anterior segments and retinal nerve fibre layer were removed, and the retinal pigment epithelium-choroid-sclera complexes were obtained. With optic nerve as a center, the complex was cut open radially. The retinal pigment epithelium was inverted on cover glass and the graphs were taken using a fluorescence microscope (magnification ×40). ImageJ Software was used to quantify the graphs.

2.8. Real-Time PCR. Total RNA was isolated from retina and choroid of rats (2 rats (4 eyes)/group) using Trizol reagent (Sigma, St Louis, MO, USA) following the protocol provided by the manufacturer. Real-time PCR was performed as described previously [13]. β-Actin was used as the invariant control. Fold changes in the mRNA levels of target genes related to the invariant control glyceraldehyde phosphate dehydrogenase (GAPDH) were calculated as suggested by Schmittgen et al. [14]. The following primers were used in real-time PCR analyses: COX-2 (forward) 5′-TACGAA-GACCCTGCTACGA-3′, (reverse) 5′-GGTGTGTCGCTCAGAACC-3′; VEGF (forward) 5′-CAGTGACTGACAGCTTC-3′, (reverse) 5′-GGATCTGGTCGTCAC-3′; p38 MAPK (forward) 5′-CAGACCTCTGGTGTGATGGCTCAAT-3′, (reverse) 5′-GTGCTCACCTTTGATCCCCAG-3′; p38MAPK (forward) 5′-GGATCTGGTCCCTCTCCTGTTC-3′, (reverse) 5′-GTACCGCTGCAGACTTGTGCT-3′; MMP-2 (forward) 5′-CCGTTATGAGACCTTGAGCC-3′, (reverse) 5′-CAGACCAATCTGTTGCCTCAT-3′; MMP-9 (forward) 5′-GGATCCTCCCCAACCTTATTAC-3′, (reverse) 5′-AGCCAGCTGAGTTCAATCCC-3′; β-actin (forward) 5′-CCCATCTATGAGGTTACGC-3′, (reverse) 5′-TTAATGTCAGCAGATTTTCC-3′.

2.9. Western Blot Analyses. The retina and choroid tissues of rats (3 rats (6 eyes)/group) were homogenised in RIPA lysis buffer (0.1% SDS, 0.5% deoxycholate, 1% Nonidet, 150 mM NaCl, and 50 mM Tris-HCl) containing protease inhibitors on ice. The protein levels were determined using a BCA assay kit (Pierce, USA). Proteins (50 μg/well) were separated by SDS-polyacrylamide gel, transferred to a PVDF membrane (Millipore, Burlington, MA, USA), blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Target proteins were detected by corresponding primary antibodies and subsequently by horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using chemiluminescence reagent (Millipore, Burlington, MA, USA). Equivalent loading was confirmed using an antibody against β-actin. The levels of target protein bands were densitometrically determined using Quantity One 4.4.1. The variation in the density of bands was expressed as fold changes compared to the control in the blot after normalization to β-actin.

2.10. Statistical Analysis. Data were presented as mean ± SD, and results were analyzed using SPSS16.0 software. The significance of difference was determined by one-way ANOVA with the post hoc Dunnett's test. A value of P < 0.05 was considered to be statistically significant.

3. Results

3.1. Characterization and Properties of Naringenin/β-CD Complex. Infrared spectrum analyses showed that naringenin had strong absorption at the wave number ranges of 1790–1540, 1530–1200, and 960–750 cm⁻¹. In naringenin/β-CD complex, red shift occurred in naringenin absorption due to β-CD inclusion. However, the infrared spectrum of physical mixture of naringenin and β-CD was obviously a superposition of their individual spectra (Figure 1). In X-ray diffraction analysis, naringenin had a series of strong diffraction peaks at the positions of 10.7°, 11.4°, 15.6°, 17.3°, 15.2°, 20.6°, 22.3°, 23.7°, 25.7°, and 27.8°, indicating its crystal property. However, β-CD showed several wide peaks
suggesting its amorphous property. The physical mixture of naringenin and β-CD showed superimposed diffraction peaks of naringenin and β-CD without new peaks, but the peak intensity had some changes. Naringenin/β-CD complex showed two wide diffraction peaks of β-CD and the characteristic peaks of naringenin disappeared (Figure 2). All these spectrum analyses above indicated that naringenin was included in β-CD and the naringenin/β-CD complex was successfully prepared. Subsequently, we examined some physical properties of naringenin/β-CD complex. Our results demonstrated that the inclusion rate was 81.06% and that the content of naringenin in the complex reached 20.53%. In addition, the water solubility of naringenin in the complex was enhanced more than 10-fold compared to that of pure naringenin (Table 1), suggesting that naringenin/β-CD complex could have better bioavailability and bioactivity.

3.2. Naringenin/β-CD Complex Significantly Reduces CNV Area in Rats. Examination of CNV area showed that the rats in model group had considerably larger CNV area compared to the normal rats. However, the CNV area in treatment rats was significantly reduced by naringenin (P < 0.05) or naringenin/β-CD complex (P < 0.01) compared to the control rats. In addition, the CNV area in rats treated with naringenin/β-CD complex was also significantly lower than that of rats treated with naringenin (P < 0.05) (Figure 3). These data indicated that naringenin could effectively attenuate CNV in rats and that the naringenin/β-CD complex had more potent effect.

3.3. Naringenin/β-CD Complex Significantly Inhibits the Expression of Key Mediators Involved in the Pathogenesis of CNV in Rats. A number of mediators have been demonstrated to be involved in the pathogenesis of CNV [1]. We subsequently examined the expression of VEGF, cyclooxygenase-2 (COX-2), phosphatidylinositol-3-kinase (PI3K), p38 mitogen-activated protein kinase (p38MAPK), matrix metalloproteinase (MMP)-2, and MMP-9 in retina and choroid tissues of rats. Real-time PCR analyses showed that the expression of the above molecules was significantly upregulated in the CNV rats without treatment. However, both naringenin and naringenin/β-CD complex considerably reduced their mRNA expression to different extents. Of note, naringenin/β-CD complex produced more potent reducing effects on the gene expression of VEGF and COX-2 (Figure 4). We also examined their protein expression of these molecules using western blot assays, showing that both naringenin and naringenin/β-CD complex significantly diminished the increased expression of these molecules at protein level. Similarly, the protein abundance of VEGF and COX-2 was significantly reduced by naringenin/β-CD complex compared to that by single naringenin treatment (Figure 5). Taken together, these results suggested that naringenin and its β-CD complex could downregulate the expression of some key mediators in retina and choroid tissues in rats.

4. Discussion

Classic CNV are associated with bright, early hyperfluorescence that is often well defined and that leaks in the later phase of the angiogram [15]. CNV is an important cause of vision loss in younger patients. Untreated CNV can cause rapid deterioration of central vision and is associated with a poor prognosis. Until recently, many patients with subfoveal CNV could not be treated because of the risk of central vision loss associated with laser photocoagulation [16]. The knowledge of molecular factors and processes involved in a specific pathology represents now one of the major starting points for the identification of new drugs.

Natural compounds have been usually the source for novel drug screening and discovery. Naringenin can be widely found in grape juice, lemon, orange, and Poncirustrifoliate and has a wide range of bioactivities including anti-inflammatory, antioxidant, antiatherosclerotic, and antancer effects [17]. Previous study showed that naringenin could improve the choroidal blood flow and inhibit the expression of COX-2 and iNOS, leading to downregulation of VEGF expression and inhibition of CNV formation [18]. However, naringenin suffers from poor water solubility and low bioavailability. Current technologies in pharmaceutics material sciences allow for the production of target specific and/or function-specific chemical ligands and drugs [19]. Cyclodextrin structurally contains an electron-rich hydrophobic cavity and hydrophilic outer wall [20]. Cyclodextrin has been commonly used as a carrier for improving the deficiency of drug molecules, especially those who are water-insoluble and unstable. For example, Zhang and Cui prepared P-glycoprotein substrate berberine hydrochloride/HP-β-CD complex and used single-pass intestinal perfusion method to evaluate the effect of cyclodextrin infusion on the intestinal absorption of berberine hydrochloride in rats, and they found that HP-β-CD inclusion significantly enhanced the absorption rate constant and the permeability coefficient of berberine hydrochloride.

![Figure 2: X-ray diffraction analyses of β-CD (a), naringenin (b), physical mixture of naringenin and β-CD (c), and naringenin/β-CD complex (d).](image)

![Table 1: Several properties of naringenin and its β-CD complex.](table)

<table>
<thead>
<tr>
<th></th>
<th>Inclusion rate</th>
<th>Naringenin content</th>
<th>Naringenin solubility (µg/mL)</th>
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<td>Naringenin</td>
<td>N/A</td>
<td>N/A</td>
<td>71.85</td>
</tr>
<tr>
<td>Naringenin/β-CD complex</td>
<td>81.06%</td>
<td>20.53%</td>
<td>846.28</td>
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Note: N/A means not applicable.

![Table 1](image)
Figure 3: Naringenin/β-CD complex significantly reduces CNV area in rats. (a)–(d) are representative fluorescent graphs of choroidal flat mounts with FITC-D in the four groups. (e) is the quantification of CNV area in rats. Group 1: CNV rats without treatment; group 2: CNV rats with naringenin treatment; group 3: CNV rats with naringenin/β-CD complex treatment. Data are expressed as mean ± SD; * P < 0.05 versus group 1, ** P < 0.01 versus group 1.

in small intestine [21]. Shulman and Cohen demonstrated that HP-β-CD inclusion increased the water solubility of naringenin by 400-fold and enhanced the transport capacity crossing Caco-2 cells by 11-fold. Further, their pharmacokinetic data showed that AUC was increased by 7.4-fold and C_max increased by 14.6-fold in rats [22]. In the present study, we prepared the naringenin/β-CD complex and characterized it using X-ray diffraction and FTIR methods. Determination of solubility showed 11.8-fold increases in water solubility of naringenin. Using laser-induced CNV model in rats, we further demonstrated that naringenin/β-CD complex had more potent inhibitory effects on CNV than those of naringenin alone, suggesting the significant improvement of bioavailability and biological activity by β-CD inclusion.

Studies have shown that inflammation plays an important role in the formation of CNV [23]. COX-2 is a critical regulatory enzyme in inflammatory processes and is closely associated with the formation of ocular neovascularization [24]. COX-2 can promote angiogenesis via pathways mediated by prostaglandins, thromboxane A2, and MMPs, of which VEGF-MMP system plays an important role in endothelial cell proliferation and migration and the eventual formation of new blood vessels [25, 26]. The CNV model was induced by laser in the current study, and it was actually manifestation of the inflammatory responses induced by thermal injury. Our data showed that the mRNA and protein expression of VEGF, MMP-2, and MMP-9 were significantly upregulated in CNV rats, confirming the critical role of VEGF-MMP system in the formation of CNV. Meanwhile, the expression of COX-2 was also increased concomitant with upregulation of VEGF and MMPs, suggesting that laser-induced CNV could be associated with COX-2 induction of VEGF and MMPs. We found that naringenin and naringenin/β-CD complex could downregulate the expression of these molecules and inhibit CNV formation, presumably due to the anti-inflammatory effect of naringenin resulting in the downregulated expression of COX-2 as well as VEGF-MMP system. Our results also showed that naringenin/β-CD complex, compared to naringenin alone, had more effective inhibitory effect on the expression of COX-2 and VEGF, although all the tested molecules could be downregulated by naringenin and its β-CD complex. It could be postulated that naringenin could strongly inhibit the production of prostaglandin E2 resulting in potent suppression of COX-2 and VEGF.

The present study also investigated the role of PI3K and MAPK signal pathways in the formation of CNV. These two cascades can be activated by inflammation and injury and regulate cell proliferation, differentiation, apoptosis, and motility, and they are also involved in COX-2 activity [27, 28]. PI3K is a type of kinase specifically catalyzing the 3-hydroxyl phosphorylation of phosphatidylinositol, which generates inositol lipids as second messengers such as p70S6K, protein kinase B and AKT. The PI3K/AKT pathway is a primary
downstream signal transduction of PI3K. COX-2 can activate the serine-threonine kinase, leading to activation of AKT signal pathway, while celecoxib could dose-dependently inhibit AKT phosphorylation in vascular endothelial progenitor cells and cause apoptosis [29]. MAPK pathways are important signal transduction systems within cells, of which p38MAPK is commonly activated by inflammatory factors, stress stimulation, lipopolysaccharide, protein synthesis inhibitor, and bacterial pathogens [30]. Activated p38 translocates to the nucleus and transcriptionally regulates the expression of genes involved in immune modulation, inflammatory responses, and apoptosis under stress [30]. COX-2 is localized in endoplasmic reticulum and nuclear membrane and catalytically produces prostaglandin E2 within cells increasing activities of MAPKs [31]. Studies showed that p38 pathway activation could enhance interleukin-1β-induced COX expression and increase prostaglandin E2 synthesis, but these effects could be completely abrogated by p38 inhibitor SC68376 [32]. Our current study revealed that PI3K and p38MAPK expression was significantly upregulated concomitant with upregulation of COX-2, VEGF, MMP-2, and MMP-9 in CNV animals, indicating that PI3K and p38MAPK signal systems were related to the formation of CNV. Naringenin and its β-CD complex could downregulate the expression of PI3K and p38MAPK, possibly due to the inhibition of COX-2. This could also be supported by the
Figure 5: Naringenin/β-CD complex significantly inhibits the protein expression of key mediators involved in the pathogenesis of CNV in rats. (a)–(f) are western blot analyses for protein levels of VEGF, COX-2, PI3K, p38MAPK, MMP-2, and MMP-9 and their quantification. β-Actin was used as an invariant control for equal loading. Group 1: normal rats; group 2: CNV rats without treatment; group 3: CNV rats with naringenin treatment; group 4: CNV rats with naringenin/β-CD complex treatment. Data are expressed as mean ± SD; *P < 0.05 versus group 1, **P < 0.01 versus group 1, *P < 0.05 versus group 2, and ***P < 0.01 versus group 2.
observation that significant differences only existed in the expression of COX-2 and VEGF, rather than other tested molecules, altered by naringenin and its β-CD complex. Further investigations are needed to explore whether some other pathways mediated naringenin regulation of PI3K and p38MAPK or naringenin directly regulated them.

In summary, the prepared naringenin/β-CD complex showed increased water solubility and improved biological activity leading to more potent inhibitory effect on CNV formation in rats. PI3K and p38MAPK signal pathways were involved in the pathogenesis of CNV. Attenuation of CNV by naringenin and its β-CD complex could be associated with the anti-inflammatory properties especially with the inhibition of COX-2.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Research Article

Alleviation of Plasma Homocysteine Level by Phytoestrogen α-Zearalanol Might Be Related to the Reduction of Cystathionine β-Synthase Nitration

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1. Introduction

Hyperhomocysteinemia (HHcy) is considered as an independent risk factor for cardiovascular diseases [1]. Gene deficiency or poor activity of cystathionine β-synthase (CBS), the key enzyme involved in homocysteine metabolism, is a vital cause of hyperhomocysteinemia. Studies have demonstrated that estrogen could protect cardiovascular system from hyperhomocysteinemia and ameliorate the level of plasma total homocysteine; however, the underlying mechanisms remain to be clarified. The aim of this research is to investigate the possible molecular mechanisms involved in ameliorating the level of plasma homocysteine by α-zearalanol. By the successfully established diet-induced hyperhomocysteinemia rat models, we found that, after α-zearalanol treatment, the activity of cystathionine β-synthase, the key enzyme in homocysteine metabolism, was significantly elevated and level of nitrative stress in liver was significantly reduced. In correlation with this, results also showed a decreased nitration level of cystathionine β-synthase in liver. Together data implied that alleviation of plasma homocysteine level by phytoestrogen α-zearalanol might be related to the reduction of cystathionine β-synthase nitration.
studies have demonstrated that α-zearalanol supplement not only attenuated homocysteine-induced vascular injuries but also alleviated plasma total homocysteine (tHcy) level in hyperhomocysteinemia rats [14]; however, the underlying mechanisms remain to be clarified. Celano et al. have reported that cystathionine β-synthase could be nitrated and consequently inactivated by peroxynitrite (ONOO−). Consequently inactivated by peroxynitrite (ONOO−) reported that cystathionine β-synthase could be inactivated. Celano et al. have reported that cystathionine β-synthase could be nitrated and consequently inactivated by peroxynitrite (ONOO−). Consequently inactivated by peroxynitrite (ONOO−), remethylations remain to be clarified. Celano et al. have reported that cystathionine β-synthase could be nitrated and consequently inactivated by peroxynitrite (ONOO−).

2. Materials and Methods

2.1. Materials. 3-Nitrotyrosine (NT) antibody ab110282 was purchased from Millipore (USA); cystathionine β-synthase (179–199) antibody sc-271886 and Protein A/G Plus-Agarose were purchased from Santa Cruz (USA); GAPDH antibody and HRP conjugate (GAIR) were purchased from THERMO SCIENTIFIC(USA); cystathionine β-synthase enzyme linked chemiluminescent kit was purchased from GENMED (USA); rat homocysteic acid (Hcy) ELISA kit was purchased from RapidBio (USA); α-zearalanol was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals and Treatment Protocols. This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication number 85–23, revised 1996) and was performed with approval of the local institutional animal care and use Committee.

2.3. Group Distribution. Sixteen adult female Wistar rats (SPF grade) after ovariectomy were randomly divided into 2 groups: (1) OVX + Met (HHcy) and (2) OVX + Met + α-ZAL (α-ZAL + HHcy). HHcy rats were fed with 2.5% methionine diet for 16 weeks, and α-ZAL + HHcy rats were fed with 2.5% methionine diet plus α-ZAL for 16 weeks (2.5 mg/kg/day). The blood samples were collected from carotid arteries for tHcy detection. Livers were quickly removed for the NT and cystathionine β-synthase detection.

2.4. Measurement of Plasma tHcy by ELISA. The level of plasma tHcy was determined by commercial ELISA kit assay as in [14].

2.5. Measurement of Cystathionine β-Synthase Activity in Livers. After rats were anesthetized with 10% chloral hydrate, the livers were harvested. The cystathionine β-synthase activity in livers was determined by commercial enzyme linked chemiluminescent kit according to the instructions provided by the manufacturer.

2.6. Measurement of Cystathionine β-Synthase and NT Expression in Livers by Western Blot. Equal amounts of proteins from the liver were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Nonspecific sites were blocked with 5% nonfat dry milk in trisbuffered saline with Tween 20 (TBS-T) for 1 hour at room temperature, following which the membrane was washed three times for 10 min each. The blots were then incubated over night at 4°C with appropriate primary antibody (mouse anti-cystathionine β-synthase 1:500, mouse anti-NT 1:1000, and mouse anti-GAPDH 1:8000) and then HRP-conjugated secondary antibody for 2 hours at room temperature. After washing by TBST three times for 15 minutes each, ECL Plus substrate (Thermo Scientific, Inc.) was applied to the blot, images were captured in a gel documentation system. Relative optical density of protein bands was analyzed using gel software image lab 3.0.

2.7. Measurement of NT Content in Livers by Immunohistochemistry. The livers were fixed in 4% paraformaldehyde for 4°C over night, dehydrated in ethanol, embedded in paraffin, and cross-sectioned (5 μm). Parallel sections were subjected to standard immunohistochemical staining (mouse anti-NT 1:250 and goat anti-mouse IgG:1:500).

2.8. Measurement of Nitrated Cystathionine β-Synthase in Livers. For immunoprecipitation assay, 1 mg of protein extracts from each group was preincubated with 10 μL protein G or A Sepharose for 2 hours and centrifuged at 2500 rpm for 5 min to withdraw nonspecific binding. 4 μg of anti-cystathionine β-synthase or nonspecific mouse IgG antibody was added to the supernatant for 4 hours at 4°C, followed by incubation with 40 μL protein G or A Sepharose over night at 4°C. The beads were washed three times with 500 μL Wash Buffer I (1% Triton-X-100, 0.1% SDS, 50 mM HEPES, 150 mM NaCl, and PH 7.8), Wash Buffer II (1% Triton-X-100, 0.1% SDS, 50 mM

Figure 1: The total homocysteine level of rats’ plasma (36.29 ± 7.09 μmol/L) was obviously above the normal level (15 μmol/L) after they were fed with 2.5% methionine diet for 16 weeks. α-Zearalanol treatment significantly lowered the total homocysteine level (19.20 ± 9.23 μmol/L). Data were expressed as mean ± SD; n = 8. Student t-test; *P < 0.05 versus HHcy. HHcy: hyperhomocysteinemia; tHcy: total homocysteine; α-ZAL: α-zearalanol.
Figure 2: $\alpha$-Zearalanol treatment increased cystathionine $\beta$-synthase activity in the liver of HHcy rats. (a) The cystathionine $\beta$-synthase expression was detected by Western blot and normalized by GAPDH expression. (b) The cystathionine $\beta$-synthase activity was detected by enzyme linked chemiluminescent kit. Data were expressed as mean ± SD; $n = 3$. Student $t$-test; **$P < 0.01$ versus HHcy. CBS: cystathionine $\beta$-synthase; HHcy: hyperhomocysteinemia; $\alpha$-ZAL: $\alpha$-zearalanol.

### 3. Results

3.1. $\alpha$-Zearalanol Treatment Lowered Plasma tHcy Levels in HHcy Rats. Conventionally, hyperhomocysteinemia is defined as plasma tHcy above 15 $\mu$mol/L. To compare the tHcy levels among each group of rats, we detected the level of tHcy by ELISA. Results showed that the tHcy levels of methionine diet-induced HHcy rats (36.29 ± 7.09 $\mu$mol/L) were obviously above the normal level (15 $\mu$mol/L). Meanwhile, $\alpha$-zearalanol treatment significantly lowered the tHcy levels (19.20 ± 9.23 $\mu$mol/L) when compared with HHcy ($P < 0.05$, Figure 1).

3.2. $\alpha$-Zearalanol Treatment Increased Cystathionine $\beta$-Synthase Activity but Not Expression in the Livers of HHcy Rats. To explore the effects of $\alpha$-zearalanol on cystathionine $\beta$-synthase in HHcy rats, we measured the cystathionine $\beta$-synthase expression and bioactivity of each group. Interestingly, the cystathionine $\beta$-synthase expression of each group displayed no significant difference ($P = 0.44$) (Figure 2(a)), whereas $\alpha$-zearalanol treatment increased the activity of cystathionine $\beta$-synthase ($P < 0.01$ versus HHcy, Figure 2(b)).

3.3. $\alpha$-Zearalanol Treatment Decreased 3-Nitrotyrosine Content in the Livers of HHcy Rats. 3-Nitrotyrosine (3-NT) would be generated when protein is modified by peroxynitrite; thus it is commonly used to reflect the level of peroxynitrite in vivo. It was previously reported that cystathionine $\beta$-synthase could be nitrated and consequently inactivated by peroxynitrite ($\text{ONOO}^-$) in vitro. To explore whether $\alpha$-zearalanol could influence the level of peroxynitrite or not, we further observe the effect of $\alpha$-zearalanol treatment on NT content in the livers of HHcy rats. Result showed that with $\alpha$-zearalanol treatment, there was a significant attenuation of NT content in HHcy rats ($P < 0.05$ versus HHcy, Figure 3),
implying that the high level of nitrative stress could be reduced by α-zearalanol.

3.4. α-Zearalanol Treatment Attenuated Cystathionine β-Synthase Nitration in the Livers of HHcy Rats. Based on the above findings, furthermore, we explored the effect of α-zearalanol on cystathionine β-synthase nitrination by immunoprecipitation along with Western blot. As expected, our results showed that α-zearalanol treatment significantly attenuated cystathionine β-synthase nitrination in the livers of HHcy rats \((P < 0.01\) versus HHcy, Figure 4), further implying that α-zearalanol might protect cystathionine β-synthase bioactivity by inhibiting cystathionine β-synthase protein nitrination in HHcy rats and consequently preventing tHcy elevation.

4. Discussion

Homocysteine is a sulfur-containing amino acid derived from the dietary amino acid methionine \([16]\). Methionine can be metabolized into homocysteine in vivo, and high methionine diet can cause overaccumulation of plasma total homocysteine (tHcy). Here we fed rats with 2.5% methionine diet for 16 weeks to duplicate the hyperhomocysteinemia models. To exclude the influence of endogenous estrogen, we intentionally ovariectomized all the rats before duplicating the hyperhomocysteinemia models. And in agreement with our previous study \([14]\), this time our result again showed that α-zearalanol treatment could significantly attenuate tHcy in HHcy rats. Why did this happen? What would be the underlying mechanisms?

In mammals, homocysteine is formed in the methionine metabolism cycle and catalyzed by cystathionine β-synthase in the transsulfuration pathway. Since transsulfuration pathway metabolizes homocysteine via an irreversible pathway which forbids reconstruction, its key enzyme cystathionine β-synthase is more essential than other enzymes in terminal removal of homocysteine. Recent studies have demonstrated that deficiencies in cystathionine β-synthase expression or activity contribute to hyperhomocysteinemia, and vitamin B6, the coenzyme of cystathionine β-synthase,
along with Western blot. Data were expressed as mean ± SD; n = 3. Student t-test; *P < 0.05 versus HHcy. CBS: cystathionine β-synthase; HHcy: hyperhomocysteinemia; α-ZAL: α-zearalanol.

Figure 4: α-Zearalanol treatment attenuated cystathionine β-synthase nitration in the livers of HHcy rats. Nitrated cystathionine β-synthase in liver tissue was detected by immunoprecipitation along with Western blot assay displayed the fact that α-zearalanol treatment significantly alleviated the level of cystathionine β-synthase protein nitration, further implying that α-zearalanol might protect cystathionine β-synthase bioactivity by inhibiting cystathionine β-synthase protein nitration in HHcy rats and consequently preventing elevation of tHcy.

Taken together, in this study, firstly we indicated that α-zearalanol alleviated plasma total homocysteine via protecting cystathionine β-synthase bioactivity in HHcy rats. Furthermore, we found that the improvement of cystathionine β-synthase bioactivity by α-zearalanol might be related to the reduction of cystathionine β-synthase nitration. Although it is possible that nitration of other enzymes involved in homocysteine metabolism may also take roles in hyperhomocysteinemia development, and the detailed molecular mechanisms responsible for α-zearalanol still remain to be explored, this new insight gained from the current study may shed a novel light on better mechanistic understandings of α-zearalanol and provide new ideas for the prevention and treatment of hyperhomocysteinemia.

Conflict of Interests
The authors declare no conflict of interests regarding the publication of this paper.

Authors’ Contribution
Hui Zhang and Qi Sun contribute equally to this work.

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References


Study of Effect of Salvianolic Acid B on Motor Function Recovery in Rats with Spinal Cord Injury

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In this study effect of salvianolic acid B was observed on motor function recovery of rats with spinal cord injury. 50 rats were selected and after inducing SCI their recovery under controlled conditions was studied using Sal B and PBS (as control). Both compounds were introduced intraperitoneally in respective groups of traumatic rats at the same time intervals for 28 days. It was observed that Sal B introduced at 5 mg/kg/day resulted in better motor function recovery. BBB score was recorded which increased significantly along with the reduction in cavity area observed by bright field microscopy of tissues, that is, from 1 to 10 and from 0.20 ± 0.05 mm$^2$ to 0.10 ± 0.03 mm$^2$, in Sal B treated group, respectively, compared to PBS group. Statistical analysis was carried out using SPSS software (SPSS, Chicago, IL, USA), values were expressed as mean ± SEM, and $P$ value < 0.01 was considered significant. Effect of Sal B on expression of NF-kB p65 and IkBa was studied and OD values of densitometry of western blots were taken. MPO activity was also studied. It was observed that treatment of Sal B significantly reduced the expression of both compounds in Sal B treated group as compared to control group after 28 days of treatment.

1. Introduction

Traditionally used in Chinese medicine the bioactive compound extracted from Salvia miltiorrhiza has been responsible for exhibiting characteristics like anti-inflammatory and neuroprotective both in vivo and in vitro [1, 2]. The anti-inflammatory and antioxidant activities of Sal B may be attributed to the presence of phenolic hydroxyl group in the structure of the compound [3]. Liu et al., 2006 [4], showed that Sal B can penetrate the blood brain barrier and can trigger differentiation and proliferation of stem cells of nervous system and can also protect neurons from apoptosis [5]. Other possible therapeutic uses of Sal B identified so far include the protection against amyloid β protein (Aβ) induced cytotoxicity [6] and protection against TNF-α injury in human aortic vascular endothelial cells [7]. That is why this compound is now used for treating cardiovascular diseases as well as for stroke [8]. In the treatment of spinal cord injury (SCI) it has been shown to play an important role by reducing inflammatory responses and affecting the processes that could lead to secondary regeneration; in experimental models of cerebral ischemia and brain injury the neuroprotective effect of Sal B has been demonstrated [9–11].

In traumatic injury of spinal cord a number of cellular and molecular events occur that can be included in primary and secondary injury pathways. The pathology of SCI can be increased significantly by secondary injury in association with the primary injury [12, 13]. A major component contributing to the pathogenesis in case of secondary injury in SCI is the inflammatory responses [14]. These responses are mediated by induced or enhanced gene expression. In this case the major component is nuclear factor-kB (NF-kB) with the family of transcription factors like (cRel, RelA/p65, RelB, p50, and p52) [15, 16]. The inhibition of activation of NF-kB has been demonstrated as a possible strategy for the attenuation of secondary damage in SCI [17, 18]. The direct inhibition of IkB kinase (IKK) has been demonstrated in different studies to regulate the inhibition of NF-kB gene products [19, 20]. Therefore targeting of IKK/NF-kB pathway can result in improving the recovery of locomotor function by the reduction of infiltration of inflammatory cells and
apoptosis after SCI in rats and has been reported in different studies [21, 22].

In this study we have made an effort to explore the use of Sal B as a potential inhibitor of IKK/NF-kB pathway and evaluation of expression of IkBα and NF-kB p65 was carried out by selecting two experimental groups of rats with traumatic SCI where Sal B and PBS (control) groups were tested. The study of functional recovery of locomotor function in rats using Basso-Beattie-Bresnahan (BBB) scale was also performed and MPO activity was studied in both groups to establish the effect that treatment of Sal B might have on reducing inflammation after injury.

2. Materials and Methods

Sal B (molecular formula: C_{36}H_{30}O_{16}, molecular weight: 746, purity: 98.5%, Green-Valley, Shanghai, China) was used in the experiment while phosphate buffered saline (PBS) was used as a control. A total of 40 adult SD female rats (weight: 200–230 g) were obtained and divided into two main groups of Sal B and PBS (20 rats per group). In each group there were four subgroups having 5 rats each (n = 5). Four subgroups were monitored till 28 days where in each week one subgroup was used. Sal B was injected intraperitoneally in all the subgroups of Sal B group from 0, 1 to 28 days and PBS was injected in all the corresponding subgroups of PBS group like Sal B in the same amount after injury at similar time interval for the same duration of 0, 1, 2 to 28 days. In every assigned group the treatment was stopped at the end of the week subsequently while in the other subgroups the treatment continued and potential recovery of SCI was studied. At T9-T10 laminectomy was performed after administering 10% chloral hydrate anesthesia. A 10g NYU impact rod centered above T9 was dropped from 12.5 mm height and a consistent partial, incomplete SCI was induced and then the postinjury care was taken out according to the previously described methods of Sal B treatment on SCI using bright field microscopy of Sal B and PBS groups the rats were given Sal B 5 mg/kg per day intraperitoneally and in PBS group PBS was given only as a control.

Then transcardial perfusion was made using 4% paraformaldehyde after animals were anaesthetized with 10% chloral hydrate. T9-T10 portion of spinal cord was removed and then immersed in the same fixative for 24 hrs. Tissue was taken and sectioned sagitally after embedding in paraffin for 24 hours. For all the subgroups of Sal B and PBS groups sections were collected on microscopic slides and after the removal of paraffin graded ethanol was used to rehydrate the slides and then all sections were stained with hematoxylin eosin (HE) for general purpose histology. Behavior testing of rats was done using Basso-Beattie-Bresnahan (BBB) scale at different points before and after injury (1, 7, 14, 21, 28 d) in both Sal B and PBS control groups. Here a score of 0 represents absence of locomotion while 21 shows normal locomotor function and subsequent points show the improvement in function up till 21 [23]. Statistical analysis was carried out using SPSS software (SPSS, Chicago, IL, USA) and the values were expressed as the mean ± SEM, one way ANOVA followed by Bonferroni post hoc test was used for multiple comparison, and P value of <0.01 was considered significant. NF-kB p65 and phosphorylated IkBα were tested according to the previously described methods with slight modification. Total proteins were extracted from the 10 mm spinal cord segment that contained the injury epicenter using Total Protein Extraction kit (Applygen Technologies Inc., Beijing, China). The concentration of proteins was determined using BCA Protein Assay Kit (Applygen Technologies Inc., Beijing, China), following the manufacturer’s protocol. Samples were diluted in sample buffer and boiled for 5 min and then 50 microgram of protein from each sample was loaded on 4–20% polyacrylamide gel, then separated by electrophoresis, and transferred to polyvinylidene difluoride membrane. After blocking, the membrane was incubated with specific primary antibodies: mouse anti-rat NF-kB p65 monoclonal antibody (1:1000; Santa Cruz Biotechnology Santa Cruz, CA, USA) and monoclonal rabbit anti-rat phosphorylated IkBα (Ser32) antibody (1:500; Cell Signaling Technology, Danvers, MA, USA). The reactive protein bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:2000; Jackson, West Grove, PA, USA) and an ECL western blotting kit (Applygen Technologies Inc, Beijing, China), which was performed according the manufacturer’s instructions. The membranes were exposed to X-ray film for 10 s to 1 min. A polyclonal rabbit anti-actin antibody (1:500; Santa Cruz Co., Santa Cruz, CA, USA) was used to detect actin in the samples as a loading control. The protein bands were scanned and digitized, and the optical density (OD) of each band was determined using the Gel-Pro analyzer 4.0 software.

Studies have shown that in case of spinal cord injury inflammatory responses are triggered because inflammatory cells at the site of injury release neurotoxins and other inflammatory mediators that can result in the generation of reactive oxygen and nitrogen species resulting in cellular damage [25]. In order to investigate the anti-inflammatory effect of Sal B we studied the infiltration of leukocytes within the injured spinal cord. As myeloperoxidase activity can be used as an indicator of polymorphonuclear leukocyte accumulation so we studied the MPO activity in both groups.

3. Results

After carrying out the treatment with Sal B by administering at the rate of 5 mg/kg/day in Sal B group and 5 mg/kg/day PBS in the PBS group intraperitoneally from 0 to 28 days the spinal cord sections were selected to monitor the effects of Sal B treatment on SCI using bright field microscopy (Figures 1(a) and 1(b)). HE stained sagittal sections were used to measure the cavities in spinal cord and from 15 sections in every rat in all subgroups of PBS and Sal B group. The cavity was measured by average area in all subgroups of both groups and it was observed that Sal B significantly reduced the cavity area from 0.20 ± 0.05 mm² to 0.10 ± 0.03 mm² compared with the PBS group (P < 0.01, Figure 2) in the fourth subgroup of both Sal B and PBS groups (i.e., from 21
to 28 days of treatment). A difference in the performance in locomotor function was observed in Sal B treated animals as compared to PBS group where Sal B group rats demonstrated partial weight supported ambulation and after 14 days of Sal B injections a significant statistical difference in BBB score was observed while after 28 days a significant difference of 8 points on BBB scale between Sal B and PBS groups was achieved (Figure 3). The graph shows significant difference in BBB score between Sal B and PBS groups where $P < 0.01$ was significant.

In order to observe the effect of Sal B on IKK/NF-κB pathway, the expression of NF-κB p65 in spinal cord tissues was observed using western blotting where after 24 hours of SCI the increase in NF-κB p65 protein was observed in both groups and then a significant attenuation of NF-κB p65 was observed among the Sal B treated group as compared to PBS group after 28 days of the treatment ($P < 0.01$) (Figures 4 and 5). The expression of phosphorylated IkBα was also observed using western blotting where again after 24 hours an increase in phosphorylated IkBα was found in Sal B and PBS groups; however, a significant attenuation in the expression of IkBα was observed in Sal B group after postinjury treatment ($P < 0.01$) after 28 days of treatment (Figures 6 and 7).

It was observed that infiltration of leukocytes is increased in SCI in spinal cord tissues but it can be controlled and reduced by the help of Sal B. The evaluation of MPO activity in subgroups of both Sal B and PBS control groups was performed and it was observed that the treatment of Sal B...
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Sal B PBS

Figure 5: Graphs showing a difference in OD values of PBS control and Sal B treated animal groups. The OD values are from the subgroup 4 after 28 days of the treatment because a remarkable improvement in locomotor recovery and reduction in the cavity area was observed in this group. Significant decrease in the expression of NF-κB is observed as compared to the control, \( ^c P < 0.01 \).

Sal B and PBS control groups showing the amount of NF-κB

Sal B and PBS groups showing the amount of IkBα

Sal B and PBS control groups bars showing MPO activity comparison

Figure 6: Western blotting showing expression of IkBα in both Sal B and PBS groups after 28 days of Sal B and PBS administration in respective groups.

Figure 7: OD values showing the reduction in expression of IkBα in Sal B treated group with a statistical difference, \( ^c P < 0.01 \), as compared to the PBS control group after 28 days of treatment.

Figure 8: Graphic representation of MPO activity in Sal B treated group and PBS control group. The increase of MPO activity in PBS group was significantly reduced in Sal B group \( ^c P < 0.01 \).

Sal B and PBS control groups bars showing MPO activity comparison

It has been reported that secondary inflammation is regulated by IKK/NF-κB pathway [27] and if this pathway is successfully targeted the pathogenesis in SCI models can be reduced [17]. The selective inhibition of IKKβ, the main catalytic subunit of IKK, can result in reduced inflammatory cells and neuronal apoptosis after SCI in rats [28]. Studies have shown that inflammatory responses are significant contributors to the secondary complications after SCI [29]. NF-κB being the major transcriptional regulator is very important factor and its activation can be done by various pathways, one of which involves IkB kinase complex [30], and IKKβ is the main catalytic subunit of IKK that can activate NF-κB by phosphorylation of inhibitory protein of IkB [31]. It is this reason that NF-κB expression and IkBα level were monitored in both groups where Sal B group showed a significant decrease in the expression of NF-κB p65 and IkBα. NF-kB p65 is one of the different Rel family proteins like RelB, c-Rel, p50, and p52. The results of Sal

4. Discussion

In the present study it can be concluded that the use of Sal B significantly improved the locomotor function recovery in rats with induced spinal cord injury as compared to the PBS group where Sal B was not administered. A dose of 5 mg/kg/day improved BBB score in rats as evident in the results as compared to the PBS control group and moreover the cavity area was also reduced in this group. The most obvious improvement was observed in the group that received Sal B for 28 days after injury. There can be many reasons attributed to this recovery including the protective effect on neural cells that were injured by SCI and recovery of these neurons. A number of particular genes that are called neuroprotective genes can be induced in a variety of conditions like electrical stimulation, cerebral ischemia, and brain injury [26]; therefore, the induction of such genes directly by Sal B can result in the treatment of SCI. It can downregulate the infiltration of spinal cord tissues by neutrophils. A comparison of result of MPO activity in the fourth subgroup of both Sal B and PBS is shown in Figure 8 where \( ^c P < 0.01 \) is found significant as compared to the PBS control group. The use of Sal B therefore can reduce tissue damage by inhibition of neutrophil infiltration.
B group showed downregulation of infiltration of spinal cord tissues by neutrophils providing additional evidence of therapeutic potential of Sal B. Different compounds have been used from Chinese herbal medicine for this purpose and has shown successful results in the treatment of SCI in rats as, for example, Lua et al. [32] recently reported butein a compound obtained from Caragana jubata and Rhus verniciflua to exhibit inhibition of NF-kB pathway and help regulate the expression of these genes inducing secondary inflammation in SCI. However, the use of Sal B alone for treating SCI in rats is the first report of this potential use of the compound to the best of our knowledge. Also the parameters studied are physiological as well as cellular expressions, both providing significant evidence of the possible uses of the Sal B; therefore, further studies in this regard will be helpful in exploring this new possible therapeutic agent. The selective inhibition of stimulatory pathways along with the physiological recovery like cavity reduction, improvement in motor functions provides a possible incite for future investigations that should be explored further.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**References**


Phytochemical Prospection and Modulation of Antibiotic Activity In Vitro by Lippia origanoides H.B.K. in Methicillin Resistant Staphylococcus aureus

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The Lippia origanoides H.B.K. ethanol extract (LOEE) and hexane (LOHEX), dichloromethane (LODCM), and ethyl acetate (LOEA) fractions were tested for their antimicrobial activity alone or in combination with antibiotics against a methicillin resistant Staphylococcus aureus (MRSA) strain. The natural products did not show antimicrobial activity against multidrug resistant strain at the clinically significant concentrations tested. However, a modulatory effect in the antibacterial activity of the neomycin and amikacin was verified when LOEE, LOHEX and LODCM were added to the growth medium at subinhibitory concentrations. A similar modulation was found when the natural products were changed for chlorpromazine, an inhibitor of bacteri al efflux pumps, suggesting the involvement of resistance mediated by efflux system in the MRSA tested. The fractions LOHEX and LODCM showed a modulatory activity bigger than their majority compounds (carvacrol, thymol, and naringenin), indicating that this activity is not due to their majority compounds only, but it is probably due to a synergism between their chemical components. These results indicate that L. origanoides H.B.K. can be a source of phytochemicals able to modify the phenotype of resistance to aminoglycosides in MRSA.

1. Introduction

Methicillin resistant S. aureus (MRSA) remains as an important cause of infectious diseases acquired in hospitals and communities worldwide [1–3]. This pathogen is a common cause of cutaneous and soft tissue infections, as well as invasive illness, such as bacteremia, septic arthritis, osteomyelitis and necrotizing pneumonia [4, 5]. The therapy of these infections is often complicated once many MRSA strains carry multiple genes of antibiotic resistance [6, 7].

The high prevalence of infectious diseases caused by MRSA and other multidrug resistant bacteria has motivated the search for new antimicrobial agents and/or new compounds able to potentiate the antimicrobial activity of
old antibiotics [8–10]. Several studies have evidenced that secondary metabolites derived from plant metabolism may be active against multi-drug resistant bacteria, including MRSA [11, 12].

The family Verbenaceae is made up of 75 genera with about 3000 species and the genus Lippia has about 200 species distributed in countries of Central and South America, as well as countries of tropical Africa [13]. Lippia origanoides H.B.K. (Figure 1) is a medicinal plant known in the Piauí state as alecrim-do-campo. Its leaves have been used as culinary seasoning and in traditional medicine as remedy for gastrointestinal disorders and as a general antiseptic for mouth, throat, and wounds [14, 15]. The essential oil is the most studied product obtained from L. origanoides H.B.K., which exhibits antigenotoxic effect in bacterial cells, antioxidant activity, low toxicity, as well as antimicrobial and antiparasite activities [16–21].

On the other hand, biological activities of extracts and partition fractions obtained from L. origanoides H.B.K need further studies, and as far as we know, the antibiotic-resistance modifying activity of its extracts has not been investigated yet. In the present study, the ethanol extract obtained from L. origanoides H.B.K., and different partition fractions and their majority compounds were tested for their antimicrobial activity alone or in combination with amino-glycoside antibiotics, aiming to evaluate its potential as a natural source of secondary metabolites acting as modulators of antibiotic resistance.

2. Materials and Methods

2.1. Strains and Drugs. All tests were performed with multidrug resistant bacterial strain of Staphylococcus aureus SA10 isolated from rectal swab (resistant to cephalothin, cepalexin, cefadroxil, penicillin G, ampicillin, amoxicillin, oxacillin, erythromycin, clarithromycin, azithromycin, clindamycin, moxifloxacin, ciprofloxacin, levofloxacin, nalidixic acid, tetracycline, neomycin, gentamicin and amikacin), which was kindly provided by Laboratory of Clinical Microbiology of the Universidade Federal da Paraíba. The standard strain Staphylococcus aureus ATCC25923 was used as positive controls. The strains were maintained on Nutrient Agar (Himedia, India) slant at 4°C, and prior to assay the cells were grown overnight at 37°C in Brain Heart Infusion (BHI, Himedia, India). Neomycin, amikacin, naringenin, carvacrol, thymol, and chlorpromazine were obtained from Sigma Chemical Corp., St. Louis, MO, USA. Antibiotics and chlorpromazine were dissolved in sterile water. Naringenin, carvacrol, and thymol were dissolved in dimethylsulfoxide (DMSO-MERCK) and the stock solutions were diluted with sterile water.

2.2. Plant Material. Leaves of Lippia origanoides H.B.K were collected in the county of José de Freitas (latitude 04° 45' 23" south and longitude 42° 34' 32" west), Piauí, Brazil. The plant material was identified and a voucher specimen was deposited with the number TEPB09205 at the Herbarium “Graziela Barroso” of Universidade Federal do Piauí (UFPI).

2.3. Preparation of Extracts and Fractions. The leaves were dried at room temperature and powdered. The powdered material (1,120 g) was extracted by maceration using ethanol as solvent in the ratio of 1:3 (m/v) and the homogenate was allowed to stand for 72 h at room temperature. This procedure was realized in triplicate. The supernatants were then filtered, gathered, and concentrated under vacuum in a rotary evaporator (model Q-214B-Quimis, Brazil) and ultra thermal bath (model Q-214M2-Quimis, Brazil), yielding 309.5 g of ethanol extract (LOEE). A part of this extract was suspended in ethanol/water (1:1, v/v) and partitioned in solvents with increasing polarity (hexane, dichloromethane, and ethyl acetate), obtaining fractions hexane (LOHEX), dichloromethane (LODCM), and ethyl acetate (LOEA), respectively. The fractions were concentrated under vacuum in a rotary evaporator and lyophilized. After extraction with ethanol, the residue was macerated with ethanol/water (1:1, v/v) in the ratio of 1:3 (m/v), and the homogenate was allowed to stand for 72 h at room temperature. This procedure was repeated for three consecutive times. The supernatants were then filtered, gathered and concentrated under vacuum in a rotary evaporator and lyophilized, thereby obtaining the hydro alcoholic extract (LOHA).

2.4. Gas Chromatography/Mass Spectrometry (GC-MS). The fractions’ constituents were converted to silylated derivatives according to Isidorov et al. [22], with modifications. Two milligrams of each fraction were mixed with 100 μL of bis(trimethylsilyl)trifluoroacetamide and trimethylsilyl chloride 1%. The mix was maintained at 85°C under agitation for 1 h. The silylated fractions were analyzed after injection of 1 μL in a gas chromatograph (Shimadzu GC-17A) with a flame ionization detector (FID) model ISQ and coupled to an mass spectrometer model GCMS-QP5050A equipped with a DB-5 HT (Agilent, Palo Alto, CA, USA) 95% methyl-polyoxsiline and 5% phenyl capillary column (internal diameter = 0.25 mm, length 30 m, film thickness = 0.1 μm). Operating conditions were as follows: injector temperature, 260°C; detector temperature, 300°C; carrier gas (Helium), flow rate of 1 mL min⁻¹. Oven temperature was initially 60°C (0.5 min) and was then raised to 260°C (5 min) at a rate of 6°C min⁻¹; then it was heated to 300°C (10 min) at a rate of 12°C min⁻¹.
The mass spectrometry conditions were as follows: scan mode with acquisition time of 52.21 min; ionization voltage, 70 eV; mass range, 40–650 Da; ion source temperature, 200°C. Compounds were preliminarily identified by characteristic fragmentation and by comparison of NIST 2.0 mass spectra libraries.

2.5. Evaluation of the Antibacterial Activity. Stock solutions of LOHEX, LODCM, and LOEA were prepared by dissolving 10 mg of each product in 1 mL of dimethyl sulfide, thus starting with an initial concentration of 10 mg/mL. The resulting solution was then diluted to 1024 μg/mL in sterile water. Minimal inhibitory concentrations (MICs) of the LOHEX, LODCM, LOEA, carvacrol, thymol, naringenin, and chlorpromazine were determined by the microdilution assay in BHI broth 10% with bacterial suspensions of 10^5 CFU/mL. The final concentrations of the fractions and compounds ranged from 512 to 8 μg/mL [23]. MICs of neomycin and amikacin were determined for the same method with antibiotic concentrations ranging from 2,500 to 2.4 μg/mL.

2.6. Modulation of the Antibiotic Activity. For evaluation of extracts and their majority compounds as antibiotic resistance modulators, MICs of the antibiotics were determined in the presence or absence of subinhibitory concentrations (1/8 MIC) of the extracts (128 μg/mL), naringenin (128 μg/mL), carvacrol and thymol (32 μg/mL), and chlorpromazine (8 μg/mL) [24]. The plates were incubated at 37°C for 24 h.

2.7. Statistical Analysis. Each experiment was performed six times and the results were normalized by calculation of geometric mean values. Error deviation and standard deviation of the geometric mean were revealed. Statistical analyses were performed using GraphPad Prism, version 5.02. Differences between treatment with antibiotics in the absence and the presence of the L. origanoides extract and fractions, as well as carvacrol, thymol, naringenin, and chlorpromazine were examined using two-way analysis of variance (ANOVA). The differences mentioned above were analyzed by Bonferroni posttest and they were considered statistically significant when P < 0.05.

3. Results and Discussion

3.1. Evaluation of the Antibacterial Activity. The antibacterial activity of the natural products from L. origanoides H.B.K., as well as the majority compounds of the LOHEX (carvacrol and thymol) and LODCM (naringenin) was tested by microdilution method against S. aureus strains (ATCC 25923 and SA10). Plant extracts are considered as having a good inhibitory activity if they present MICs ≤ 100 μg/mL, a moderate inhibitory activity if they present MICs ranging from 100 to 500 μg/mL, a weak inhibitory activity if they present MICs ranging from 500 to 1000 μg/mL, and no inhibitory activity if they present MICs > 1000 μg/mL [25, 26]. According to these criteria, the extract and fractions, as well as, naringenin did not show antibacterial activity against the S. aureus strains (once they showed MICs ≥ 1024 μg/mL). On the other hand, carvacrol and thymol presented a moderate inhibitory activity (MIC 256 μg/mL) against the S. aureus strains.

3.2. Modulation of Antibiotic Activity. When LOEE or the fractions LOHEX and LODCM were added to the growth medium at subinhibitory concentration, a reduction in the MIC of at least 10-fold for neomycin and at least 5-fold for amikacin was verified (Figures 2–4). To the best of our knowledge, this is the first time that Lippia origanoides H.B.K. extracts are described as enhancers of the aminoglycosides activity against MRSA, and the results were compatible with those found for other medicinal plants [10, 27–29].

After crossing the cell wall and the cell membrane, aminoglycosides bind to the ribosomal subunit 30S, interfering with the binding of mRNA, leading to the synthesis of anomalous proteins, which are inserted into plasma membrane changing its permeability [30]. Resistance to aminoglycosides in S. aureus is frequently related to enzymatic inactivation [31], but it can be mediated by membrane transporter proteins able to pump the antibiotic to the extracellular medium, as LmrS efflux protein [32, 33].

Decrease in the MICs of antibiotics tested was also verified when LOEE, LOHEX, and LODCM were replaced by chlorpromazine at subinhibitory concentration. The role of chlorpromazine as inhibitor of bacterial efflux systems has already been well studied [34, 35]. This result indicates a possible mechanism mediated by efflux pump for resistance to neomycin and amikacin in the SA10 strain, which could be inhibited by phytochemicals present in LOEE and in both
3.3. Phytochemical Prospection. Through phytochemical prospection of the fractions studied, several classes of secondary metabolites were identified. The majority compounds found in the LOHEX were the monoterpenoids carvacrol and thymol, besides other minority components (Table 1 and Figure 6). The antimicrobial activity of phenolic compounds such as carvacrol and thymol has been demonstrated previously [36], including against MRSA [37].

Due to their lipophilicity, these compounds can intercalate in the phospholipid bilayer, increasing the permeability of the cell membrane, dissipation of the proton-motive force, and leakage of inorganic ions, resulting in lysis and cell death [38, 39].

Flavonoids aglycones were the main phytochemicals found in the fraction LODCM (Table 2 and Figure 6), including the flavanones naringenin (in the keto and enol forms), eriodictyol and hesperetin, the isoflavones dihydroglycitein and 3'-hydroxy-irilone, and the flavan-3-ol (+)-catechin. Some lipophilic flavonoids can react with bacterial membrane [40], besides forming complexes with proteins [41]. As already mentioned, these mechanisms may increase the membrane permeability and inhibit efflux systems dependent of proton-motive force, leading to a bigger accumulate of antibiotics into the bacterial cell [34]. In fact, the modulatory effect of flavonoids on resistance to quinolones and tetracycline has already been verified in Staphylococcus aureus [42–44].

With regard to the modulatory effect found in this study, it could be attributed to an interaction of LOHEX and LODCM components in the plasma membrane, leading to an increase in the cell permeability to the aminoglycosides. Furthermore, this interaction could inhibit efflux systems dependent of proton-motive force, as LmrS protein, contributing to the increase in the intracellular levels of antibiotics and leading to an enhancement of the aminoglycoside activity against MRSA.

Thymol and carvacrol did not modulate the antistaphylococcal activity of the neomycin or amikacin in the SA10 strain.
Table 2: Chemical composition of *Lippia origanoides* H.B.K. dichloromethane fraction (LODCM).

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Compound</th>
<th>RTa</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thymol</td>
<td>9.524</td>
<td>1.26</td>
</tr>
<tr>
<td>2</td>
<td>Carvacrol</td>
<td>9.864</td>
<td>1.77</td>
</tr>
<tr>
<td>3</td>
<td>2,3-Dimethylcyclopentenol</td>
<td>12.983</td>
<td>1.41</td>
</tr>
<tr>
<td>4</td>
<td>Theophylline</td>
<td>13.239</td>
<td>0.48</td>
</tr>
<tr>
<td>5</td>
<td>Cycloleucine</td>
<td>13.357</td>
<td>0.34</td>
</tr>
<tr>
<td>6</td>
<td>Not identified</td>
<td>13.547</td>
<td>4.98</td>
</tr>
<tr>
<td>7</td>
<td>7-Methyloxanthine</td>
<td>14.700</td>
<td>0.64</td>
</tr>
<tr>
<td>8</td>
<td>Not identified</td>
<td>15.713</td>
<td>1.37</td>
</tr>
<tr>
<td>9</td>
<td>3'-Hydroxy-irilone</td>
<td>27.474</td>
<td>0.48</td>
</tr>
<tr>
<td>10</td>
<td>Dihydroglycitein</td>
<td>32.615</td>
<td>4.77</td>
</tr>
<tr>
<td>11</td>
<td>Not identified</td>
<td>33.277</td>
<td>4.49</td>
</tr>
<tr>
<td>12</td>
<td>Not identified</td>
<td>33.550</td>
<td>29.49</td>
</tr>
<tr>
<td>13</td>
<td>Naringenin (enol form)</td>
<td>33.593</td>
<td>15.56</td>
</tr>
<tr>
<td>14</td>
<td>Naringenin (keto form)</td>
<td>33.637</td>
<td>10.4</td>
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<tr>
<td>15</td>
<td>Not identified</td>
<td>33.754</td>
<td>6.3</td>
</tr>
<tr>
<td>16</td>
<td>Eriodictyol</td>
<td>33.830</td>
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</tr>
<tr>
<td>17</td>
<td>Hesperetin</td>
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<td>18</td>
<td>Not identified</td>
<td>34.755</td>
<td>7.82</td>
</tr>
<tr>
<td>19</td>
<td>(+)-Catechin</td>
<td>34.899</td>
<td>2.62</td>
</tr>
<tr>
<td>20</td>
<td>2,4,6-Trihydroxybenzoic acid</td>
<td>36.439</td>
<td>0.79</td>
</tr>
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</table>

aRetention time.

Table 3: Chemical composition of *Lippia origanoides* H.B.K. ethyl acetate fraction (LODCM).

<table>
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<tr>
<th>Peaks</th>
<th>Compound</th>
<th>RTa</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethylene glycol</td>
<td>3.625</td>
<td>0.97</td>
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<tr>
<td>2</td>
<td>Glycerol</td>
<td>9.080</td>
<td>8.19</td>
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<tr>
<td>3</td>
<td>Not identified</td>
<td>16.937</td>
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<tr>
<td>4</td>
<td>Protocatechuic acid</td>
<td>19.191</td>
<td>9.68</td>
</tr>
<tr>
<td>5</td>
<td>L-Rhamnose</td>
<td>19.366</td>
<td>5.63</td>
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<tr>
<td>6</td>
<td>2-Keto-D-gluconic</td>
<td>19.492</td>
<td>1.15</td>
</tr>
<tr>
<td>7</td>
<td>D-Xylopyranose</td>
<td>20.740</td>
<td>2.12</td>
</tr>
<tr>
<td>8</td>
<td>Gallic acid</td>
<td>21.575</td>
<td>1.46</td>
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<tr>
<td>9</td>
<td>D-Mannose</td>
<td>22.312</td>
<td>1.27</td>
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<td>10</td>
<td>Not identified</td>
<td>27.956</td>
<td>0.92</td>
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<td>11</td>
<td>Not identified</td>
<td>29.948</td>
<td>1.06</td>
</tr>
<tr>
<td>12</td>
<td>Isomaltulose</td>
<td>31.022</td>
<td>16.37</td>
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<td>13</td>
<td>Isomaltulose</td>
<td>31.377</td>
<td>1.29</td>
</tr>
<tr>
<td>14</td>
<td>D-Turanose</td>
<td>34.476</td>
<td>1.62</td>
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<tr>
<td>15</td>
<td>Catechin</td>
<td>34.838</td>
<td>11.61</td>
</tr>
<tr>
<td>16</td>
<td>3'-Hydroxy-5-furanoside-dihydroirilone</td>
<td>44.546</td>
<td>4.54</td>
</tr>
<tr>
<td>17</td>
<td>3'-Hydroxy-5-inosose-irilone</td>
<td>44.980</td>
<td>2.32</td>
</tr>
<tr>
<td>18</td>
<td>3'-Hydroxy-5-piranoside-dihydroirilone</td>
<td>46.179</td>
<td>3.07</td>
</tr>
<tr>
<td>19</td>
<td>3'-Hydroxy-5-inosose-dihydroirilone</td>
<td>47.991</td>
<td>5.08</td>
</tr>
</tbody>
</table>

aRetention time.

(Figure 3), and the modulatory activity verified for naringenin was lower than that showed by LODCM (Figure 4). These results suggest that the modulatory activity verified for LOHEX and LODCM is not only caused by their majority compounds, but it could be due to a synergism among their components. However, further investigations with different phytochemicals alone and in different combinations between them are needed to test this hypothesis.

LOEA also did not modulate the activity of the aminoglycosides tested (Figure 5). In this fraction was found a high content of irilone O-heterosides, besides the aglycone catechin (Table 3 and Figure 6). It has been demonstrated that addition of hydrophilic groups as glycosides becomes the compound less effective in inhibiting Gram-positive bacteria [45, 46]. It was suggested that the lack of affinity for the phospholipid bilayer or specific receptors on the cell membrane is the motive for this decrease in the antibacterial activity [47]. The lack of modulatory effect in the activity of the aminoglycosides tested showed by LOEA also may be related to the bigger hydrophilicity of the compounds present in this fraction.

### 4. Conclusions

In the present work, it was verified that LOEE and the nonpolar fractions LOHEX and LODCM were able to potentiate the activity of neomycin and amikacin against a MRSA strain in vitro, which may be related to the low polarity of its phytochemicals. Aminoglycosides have been used in the initial empirical treatment of endocarditis caused by Gram-positive bacteria, including *S. aureus*, but the high frequency of resistant strains and adverse reactions have discouraged its clinical use [48]. The results obtained indicated that *Lippia origanoides* H.B.K. could be a source of secondary metabolites for use in association with neomycin and amikacin in the antibiotic chemotherapy of infections caused by MRSA.
Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References


Clinical Study

Human Pharmacokinetics of High Dose Oral Curcumin and Its Effect on Heme Oxygenase-1 Expression in Healthy Male Subjects

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Purpose. Heme oxygenase-1 (HO-1) has been proposed to exert pharmacological benefits by its antioxidative and anti-inflammatory effects. HO-1 expression may be affected by the GT length polymorphism in the promoter region of the HO-1 gene. We investigated the inducibility of HO-1 by orally administered curcumin in healthy male subjects and its correlation with the GT length polymorphism.

Methods. In an open label uncontrolled phase-1 pilot study, ten male subjects received 12 g of oral curcumin. To investigate the effects of the GT length polymorphism on the inducibility of HO-1, five subjects with homozygous short and five with homozygous long GT genotypes were studied. Plasma concentrations of curcumin, bilirubin, HO-1 mRNA, and protein expression in peripheral blood mononuclear cells (PBMCs) were analyzed over 48 hours.

Results. At a detection limit of 1 μg/mL curcumin could not be detected in plasma of any subject. Compared to baseline, HO-1 mRNA and protein levels were not induced in PBMCs at any time point up to 48 hours. There was no correlation between any of the parameters and GT length polymorphism.

Conclusions. Oral curcumin administration has low bioavailability and does not induce HO-1 on mRNA or protein level in PBMCs.

1. Background Information

1.1. Curcumin and Heme Oxygenase. Curcumin, a bioactive component of turmeric, has been proposed to modulate multiple cell signaling pathways and to interact with numerous molecular targets, including cell cycle, apoptosis, proliferation, angiogenesis, and inflammation [1]. Beside its beneficial effect on cancer cells [2], orally ingested curcumin improved glucose metabolism in type 2 diabetes animal models [3]. Curcumin is also suggested to be a stimulant of heme oxygenase-1 (HO-1). This has been shown in vitro in human breast cell lines [4] and in human hepatocytes. The strong therapeutic potential of curcumin analogues for treating diabetic diseases and their ability to induce HO-1 expression has also been discussed in the recent published article by Son et al. [5].

HO-1 is the rate-limiting enzyme that catalyzes the degradation of heme b (Fe-protoporphyrin-IX) into biliverdin (which is rapidly converted to bilirubin), carbon monoxide (CO), and free iron (Fe2+). In humans two genetically distinct isozymes of HO have been characterized: a constitutively expressed form HO-2 and an inducible form HO-1 [6]. HO-1 is a member of the heat-shock protein family (HSP 32) being expressed in endothelial, epithelial, and smooth muscle cells [7]. It serves as a protective enzyme due to its anti-inflammatory, antioxidant, antiapoptotic, and anti-proliferative mechanisms of actions [8]. There is a GT length polymorphism (GT)n dinucleotide repeat polymorphism in the proximal promoter region of the HO-1 gene [9]. This (GT)n repeat is highly polymorphic and modulates gene transcription by means of oxidative challenge [10]. In vitro studies evidenced that a longer (GT)n repeat corresponds to
lower transcriptional activity of the HO-1 promoter region [11, 12] and is associated with a susceptibility to large number of diseases [13], including the coronary artery disease in type 2 diabetic patients [14, 15]. Oral administration of curcumin to patients after cadaveric renal transplantation led to an increase of HO-1 protein levels in urinary epithelial cells and improved renal function [16]. The molecular steps and signal transduction pathways underlying the HO-1 upregulation in general, and by curcumin in particular, remain largely undefined. PI3K and p38MAPK pathways under the control of the transcription factor NF-E2 related factor 2 (Nrf2) and NF-κB might play a substantial role in the HO-1 induction [2, 17, 18].

However, bioavailability of curcumin after oral administration is low [19] due to extensive metabolic breakdown in the gastrointestinal tract [20]. To date, there are no sufficient studies available that have assessed the pharmacokinetics of a single high dose oral curcumin and its association with HO-1 expression. We therefore investigated the pharmacokinetics of a single high dose oral curcumin and its ability to induce HO-1 expression in humans among different human specific (GT)n polymorphisms.

2. Methods

2.1. Study Participants. 132 healthy white European male subjects aged between 18 and 45 years (inclusive) were screened for the (GT)n length polymorphism in the promoter region of the HO-1 gene (Figure 1). Subjects were stratified in two groups according to their HO-1 genotype, which may affect transcription of the HO-1 gene after oral curcumin administration. Five subjects with a homozygous short GT genotype (S/S; age: 29 ± 4; BMI: 23.7 ± 2.0 kg/m²) and five with a homozygous long GT genotype (L/L; age: 27 ± 4; BMI: 23.7 ± 1.8 kg/m²) were included in this study and exposed to oral curcumin (Figure 2). The study protocol was approved by the Ethics Committee of the Medical University of Vienna, and written informed consent was obtained from all participants before study entry. The study is registered at ClinicalTrials.gov (NCT 00895167).

2.2. Study Design. In this open label, uncontrolled phase-1 pilot study, each participant passed a screening examination that included medical history, a physical examination, vital sign measurement, a 12-lead electrocardiogram, laboratory tests, drug screening, and test-strip urinalysis between 2 and 14 days before the first drug administration. 12 g of oral curcumin (12 capsules containing a powder of Curcumin C3 Complex) was administered after an overnight fast. Curcumin and total bilirubin plasma levels, HO-1 mRNA, and protein expression in peripheral blood mononuclear cells (PBMCs) were analyzed before and at 2.5, 5, 7.5, 10, 24, and 48 hours after the study drug administration. Laboratory safety tests including chemistry, hematology, coagulation, and urine chemistry were performed at screening and at the end-of-study visit (EOS). Vital sign measurement and a 12-lead electrocardiogram were recorded during the investigational period at −0.5 and 7.5 hours and at EOS. Adverse events were recorded throughout the study.

2.3. Study Drug. Curcumin C3 Complex (Sabinsa Corporation, NJ, USA) is known to be well tolerated when taken at high doses of 12 g/day [20, 21]. The study drug consists of 73–78% curcumin I (curcumin; molecular formula: C15H10O5; molecular weight: 268.208 g/mol); 18–22% curcumin II (demethoxycurcumin; molecular formula: C16H12O5; molecular weight: 338.347 g/mol); and 2–5% curcumin III (bisdemethoxycurcumin; molecular formula: C19H14O4; molecular weight: 308.328 g/mol). In order to increase bioavailability of this complex and to inhibit rapid drug decomposition, the study drug contains 5 mg Bioperine, which is a standardized extract from the fruits Piper nigrum L and Piper longum L, containing 95% of piperine [22].

3. Laboratory Assessment

3.1. Plasma Curcumin Measurement. Plasma curcumin was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) method using a Hitachi LaChrom Elite HPLC with L-2400 UV detector (Hitachi HTA, Life Sciences Division, CA, USA), and Waters μBondapak C18 column. Detection wave lengths were 428 and 300 nm for curcumin and 4-hydroxybenzophenone (as internal standard, IS), respectively. The internal standard (IS) was determined with 50 ng/mL of curcumin. The lower limit of quantification was 1 ng/mL.

3.2. HO-1 Genotype Assessment. Genomic DNA was isolated from whole blood using standard techniques. Polymerase chain reaction amplifications of the HO-1 (GT)n repeat length polymorphism were performed as described in [9, 10]. We divided allelic repeats into two subclasses following a classification based on transfection studies with low and high GT repeats [6, 9, 10, 14, 15]: short repeats with <27 (GT)n were designated as allele class S (short) and longer repeats with ≥27 (GT)n as allele class L (long).

3.3. HO-1 mRNA Expression in PBMCs. PBMCs were isolated from EDTA blood with Ficoll-Plaque (Amersham Biosciences, UK) prefilled tubes (Leucosep, Greiner Bio-One, Austria). Cell pellets for HO-1 mRNA analysis were treated with lysis buffer (Buffer RLT, Qiagen Sciences, MD, USA). First-strand cDNAs were synthesized from approximately 1 μg of total RNA with the use of MLV reverse transcriptase and random hexamer primers according to the manufacturer instructions (RT-PCR Core Kit; Takara Bio). For quantitative real-time polymerase chain reaction (RT-PCR), sense and antisense primers (Invitrogen, Paisley, Scotland) and fluorogenic probes (Eurogentec, Herstal, Belgium) for HO-1 and 18S were used. Results are expressed as the target/reference ratio. The difference between the HO-1 mRNA levels of curcumin and vehicle-treated cells was considered as the cell expression of HO-1 mRNA and is expressed as ΔHO-1 mRNA.
3.4. HO-1 Protein Levels in PBMCs. HO-1 protein levels in PBMCs were detected using Western blot analysis. The dry cells were lysed with a lysis buffer containing protease inhibitors. After centrifugation the resulting supernatant was used for protein concentration measurements using a bicinchoninic acid protein assay kit (Thermo Scientific). 30 µL of a solution containing SDS loading buffer and mercaptoethanol were combined with 50 µg of sample protein and lysis buffer up to 100 µL. Samples were heated at 93°C and separated by polyacrylamide gel electrophoresis (PAGE) at 120 V. They were transferred to polyvinylidene fluoride (PVDF) blot membrane (20 V for 1.5 hrs) using the semidyry method. The membranes were blocked in 5% milk for 1 hour, washed three times with TBST (tris-buffered saline including 0.05% Tween-20), incubated with the primary HO-1 antibody over night at 4°C, and washed and incubated with the second antibody for 1 hr. The membranes were incubated in substrate solution and imaged using clear X-ray films. The antibody staining and development procedure was repeated again using an anti-β-actin antibody for normalisation of the results.

3.5. Safety Laboratory Parameters. Complete blood counts as well as blood, dialysate, and urine chemistry were performed by standard procedures in an ISO 9001: 2000 certified laboratory.

4. Statistical Methodology and Analysis

For the statistical analyses, SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA) was used. Nonparametric statistics were applied for comparison between groups and within groups. Differences between baseline characteristics were assessed by the Wilcoxon test. The Mann-Whitney-U test was performed to assess a possible interaction (effect modification) between genetics and treatment. All P values are results of two-sided tests, and P values < 0.05 are considered statistically significant. Since the study has an exploratory character, no adjustment for multiple testing was performed.

5. Results

5.1. HO-1 Genotype Characteristics. A total of 132 subjects were screened for the GT length polymorphism in the
HO-1 promoter region. The (GT)n repeats ranged between 21 and 37, with 23 and 30 being the most common alleles (Figure 1). Using the cutoff of 27 repeats, the prevalences of the genotypes for homozygous S/S and L/L and heterozygous S/L were 9.1%, 40.2%, and 50.8%, respectively [23].

5.2. Baseline Characteristics. Five subjects with a homozygous short (S/S) GT genotype and five with a homozygous long (L/L) GT genotype of the HO-1 length polymorphisms were investigated in our pilot study. The demographic data and relevant baseline laboratory values are summarized in Table 1. The demographic data and relevant baseline laboratory values are summarized in Table 1.

5.3. Curcumin Plasma Levels. Curcumin was not detectable before or after oral administration of study drug at any timepoint. RP-HPLC (detection level: 1 ng/mL) did not detect any quantified curcumin plasma levels at any timepoint.

5.4. Bilirubin Plasma Levels. Lower levels of conjugated bilirubin were determined in the S/S group (0.15 mg/dL) compared with the L/L group (0.20 mg/dL; \( P = 0.015 \)) at predose. No difference in \( \Delta \text{AUC}_{48h} \) of mean bilirubin (total fraction and subfractions) could be observed after oral curcumin administration compared with the individual baseline levels of the study participants. Comparing the two predefined genotype groups, no significant difference could be detected for both, total fractions and subfractions of plasma bilirubin.

5.5. HO-1 mRNA. HO-1 mRNA baseline concentrations of both genotype groups are presented in Table 1. No change in the area under curve over 48 h (\( \Delta \text{AUC}_{48h} \)) of mRNA concentrations from the individual baseline level was observed (\( P = 0.878 \), Figure 3(a)).

5.6. HO-1 Protein Levels. HO-1 protein baseline levels are presented in Table 1. There was no significant difference in the maximal concentration (\( C_{\text{max}} \)) between the L/L group (\( t_{\text{max}} = 5.01 \)) compared with the S/S group (\( t_{\text{max}} = 6.52 \)). The area under curve \( \Delta \text{AUC}_{48h} \) of HO-1 protein did not differ between the groups. For the HO-1 protein, no genetic effects could be detected in the area under curve \( \Delta \text{AUC}_{48h} \) (\( P = 0.459 \)) and maximal concentration \( C_{\text{max}} \) (\( P = 0.169 \)) comparing both genotypes.

5.7. Safety Parameter. No clinical relevant safety issue was detected during the investigational period.

6. Discussion

Multiple studies have been already published postulating the beneficial cellular effects of oral curcumin [1–3]. The recently published article by Chuengsamarn et al. reported a benefit of daily oral doses of 1.5 g of curcumin capsules, lowering the number of incidences of type 2 diabetes mellitus in a prediabetes population and improving overall \( \beta \)-cells functioning [24].

The underlying molecular mechanisms of curcumin are largely unknown. The HO-1 expression was thought to be one of the possible pathways of curcumin action, but in the
current study we could not detect any significant increase in HO-1 mRNA and protein concentration after a single high dose of 12 g oral curcumin at any timepoint throughout a reasonable observation period of 48 hours (Figures 3(a) and 3(b)).

Heme oxygenase-1 is identified as particularly important in protection against a large number of diseases resulting from increased production or decreased removal of reactive oxygen species [25]. Relevant, for the present study, HO-1 was observed to be induced by curcumin in vitro in hepatocytes and in vivo in urinary epithelial cells [16, 26].

HO-1 expression was analyzed in PBMCs because these cells are regarded as important mediators of inflammation in a large number of diseases and are also considered to be prime targets of the cytoprotective actions of HO-1 [27]. The human specific (GT)n polymorphism of HO-1 gene has been reported to be associated with various chronic diseases [13–15, 28, 29], where in general a shorter (GT)n repeat polymorphism has protective effects and resulted in vivo in a higher HO-1 baseline activity and increased inducibility [10–12]. However, it has not yet been demonstrated that the (GT)n polymorphism is relevant to a pharmacological induction of HO-1 in humans. Furthermore, we could show that the (GT)n polymorphism does not influence HO-1 induction by heme arginate in healthy males in a clinically relevant way [23]. In the present study, stratifying our cohort according to the (GT)n polymorphism did not display any difference in the parameters observed.

Animal studies have shown that orally administered curcumin has low absorption rates (60–66%) and about approximately 75% of the ingested curcumin is excreted unmetabolized in the feces and only negligible amounts in the urine [30, 31]. Pharmacokinetic studies in humans have generally produced similar data [19, 21, 32]. Plasma levels of curcumin could only be measured in higher orally administered doses (8 to 12 g/day) [19]. Due to curcumin’s poor systemic bioavailability after oral dosing usually piperine, a known inhibitor of hepatic and intestinal glucuronidation was used as an additive to enhance the therapeutic use of curcumin. Shoba et al. have shown that the poor bioavailability is increased by piperine, in rats by 154% and in humans by up to 2000% [22]. Despite the fact that the study drug contained 5 mg of Bioperine, no detectable curcumin plasma levels could be observed.

A recently performed clinical trial generated tolerability and clinical and biomarker efficacy data on Curcumin C3 Complex in persons with Alzheimer’s disease. Due to the low measured plasma levels, no clinical or biochemical evidence of efficacy of Curcumin C3 Complex could be demonstrated [33]. However, the lack of curcumin traces in plasma after oral dosing does not support a direct curcumin-mediated effect and urges for further investigations to present the pharmacological activity and clinical mode of action of this dietary constituent.

To understand the pharmacological action of curcumin, an analysis of plasma concentrations, conjugates, and metabolites (curcumin sulfate, tetrahydrocurcumin, and hexahydrocurcumin) would be imperative for any ongoing clinical investigation. Furthermore, the molecular steps and signal transduction pathways underlying the HO-1 upregulation by curcumin remain largely unknown.

7. Limitation

Regulation of promoter activity by the (GT)n repeat length may differ in blood cell types and in response to different stimuli [10, 11]. We do not exclude the possibility that the proportion of HO-1 expressing cells varied across PBMCs tested in S/S and L/L groups. Further investigations are needed to determine whether different PBMCs subpopulations respond differently to curcumin. Since a single high dose of oral curcumin was investigated in the current study, no observation on altered resorption after multiple dosing was performed.

In this clinical trial we used the HPLC-MS method for the analysis of plasma curcumin concentrations since this assay was successfully applied to the pharmacokinetic studies of curcumin in rats [34]. Additional assays could provide more useful information on the determination of curcumin in human plasma samples.

8. Conclusion

The present study shows that despite a single high oral dose of curcumin, no measurable plasma levels could have been detected nor any change in the HO-1 expression could have been detected. The present work builds on previous demonstrations that curcumin strongly upregulates HO-1 activity, an effect that could explain one of the pharmacological actions of curcumin. It is anticipated that increasing clinical use of curcumin and other inducers of HO-1 activity will be of enormous benefit to healthcare worldwide.

Abbreviations

AUC: Area under the curve
CO: Carbon monoxide
EOS: End of study
(GT)n: Dinucleotide guanosine thymine repeat
HO: Heme oxygenase
L/L: Homozygous for the long GT repeat length polymorphism in the promoter of the HO-1 gene
PBMCs: Peripheral blood mononuclear cells
S/S: Homozygous for the short GT repeat length polymorphism in the promoter of the HO-1 gene

Conflict of Interests

The authors state no conflict of interests.
Authors’ Contribution

Uros Klickovic and Daniel Doberer contributed equally to this study and should be considered cofirst authors.

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