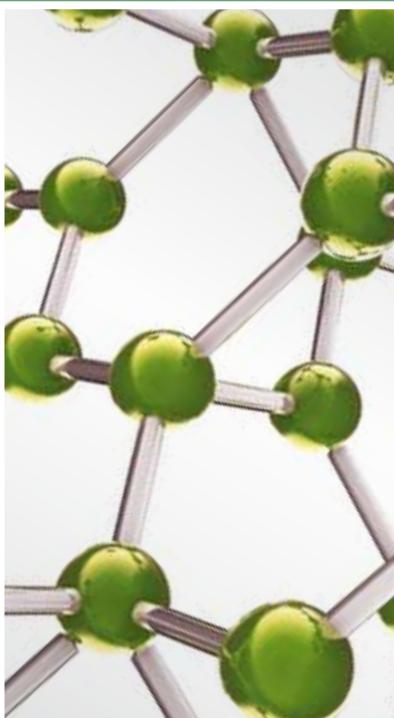


BOTANICALS IN DIETARY SUPPLEMENTS

GUEST Editors: WEENA JIRATCHARIYAKUL, LUDGER BEERHUES, GAIL B. MAHADY,
TANAWAN KUMMALUE, and MOLVIBHA VONGSAKUL



Botanicals in Dietary Supplements

Botanicals in Dietary Supplements

Guest Editors: Weena Jiratchariyakul, Ludger Beerhues,
Gail B. Mahady, Tanawan Kummalue, and Molvibha Vongsakul



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Editorial

Botanicals in Dietary Supplements

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Botanicals are accepted worldwide as medicinal agents and nutraceuticals. Extensive scientific investigations have been performed over the past 200 years which have resulted in the evolution of botanical utilization. This special issue highlights some of this research in 7 review articles and in 24 original research articles.

Review Articles. W. Jiratchariyakul and G. B. Mahady wrote about the botanical status and popular herbs in EU, US, and Thailand. M. Miroddi et al. updated the market and the regulatory of botanical products in EU and US. Traditional Chinese and Indian Medicines (TCM and TIM) play an important role in Asian countries. Z. Wang et al. presented and discussed the role of TCM in the treatment of epidemic type II diabetes mellitus. M. M. Pandey et al. reported the use of TIM as a nutritional supplement in malnutrition. Y. Kamisah et al. presented the chemoprevention and antioxidation of *Parkia speciosa*. A. P. Bartolome et al. reviewed laboratory evidence of *Bidens pilosa*. T.-P. Huynh et al. discussed the promising botanical compounds for prevention and treatment of eye diseases.

Research Articles. New biological and pharmacological activities of botanicals are reported. They included the enhancement of learning, memory and antistress of *Acanthopanax*

trifoliatus (P. Sithisarn et al.), the sedative effect of *Ziziphus mauritiana* (A. M. M. San et al.), the cardioprotective effect of *Phyllanthus emblica* (L. Chularojmontri et al.), the cytoprotection and antioxidative stress of *Citrus maxima* (L. Chularojmontri et al.), anti-influenza viral activity of *Momordica charantia* (V. Pongthanapisith et al.), antioxidation from *Nypa fruticans* (N. Prasad et al.), *Nigella glandulifera* (J. Zhao et al.), TIM (M. M. Pandey et al.), *Herba Cynomorii* (J. Chen et al.). The alleviation of metabolic disorder of *Citrus ichangensis* (X. Ding et al.), and anticancer activity of *Vitex agnus-castus* (S. Li et al.).

The mechanisms of anticancer action are deeply investigated with the botanical compounds, zerumbone from *Zingiber zerumbet* (N. M. Nadzri et al.), phenyl butenoid dimer from *Zingiber cassumunar* (T. Anasamy et al.), and girinimbine from *Murraya koenigii* (S. Mohan et al.). Biochanin A, the major isoflavone from *Trifolium pratense*, prevented the bone loss in the ovariectomized rat (S.-J. Su et al.). Quercetin isolated from *Caesalpinia mimosoides* had neuroprotective effect (N. Tangsaengvit et al.) and inhibited eosinophile (M. K. Asano Sakai-Kashiwahara). C. Li et al. reported new anti-inflammatory triterpenoids from *Illicium difengpi*.

Besides the herbal activities, the cohort study of TCM, Si-Wu-Tang, in postpartum women was performed to evaluate

the health benefits. The effectiveness of the modern herbal drug was carried out using the double-blind randomized controlled clinical trial, as shown under the title “Antiherpetic effects of *Gynura procumbens*” (S. Jarikasem et al.).

The research on the quality assessment of the botanicals was also presented in this issue. It included the HPLC analysis of *Moringa oleifera* (B. Vongsak et al.) and *Pueraria tuberosa* (S. Rastogi et al.). In addition, the arsenic accumulation in *Zingiberaceous rhizomes* was reported (C. Ubonnuch et al.).

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Review Article

Overview of Botanical Status in EU, USA, and Thailand

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The botanical status in EU, USA, and Thailand is different owing to the regulatory status, the progress of science, and the influence of culture and society. In the EU, botanicals are positioned as herbal medicinal products and food supplements, in the US they are regulated as dietary supplements but often used as traditional medicines, and in Thailand, they are regulated and used as traditional medicines. Information for some of the most popular botanicals from each country is included in this review.

1. Introduction

Currently botanicals, herbal medicines, herbal medicinal products, botanical dietary supplements, and traditional medicines are diversely defined according to the regulations of the country in which the product is manufactured and its final destination. In the European Union (EU) and USA, the extracts and extract fractions (special extracts) are primarily used as active compositions in the products, which are regulated as herbal medicinal products (HMPs), and food supplements in EU, and dietary supplements in USA. In Asia, the crude drugs are often used as active composition in the products, which are usually classified as traditional medicines. Herbal medicinal products are intended for use as modern and traditional drugs or as food supplements that are used to maintain health. In Thailand, as a representative of Asian countries, the botanical products are regulated both as traditional medicines and health foods. Complementary and alternative medicine also includes the use of botanicals in prevention and therapy of disease states. The progress in science and technology of botanicals started approximately between 1811 and 1815, when the term “pharmacognosy” was firstly used in EU [1]. The purpose of this review is to give a brief overview of the regulatory status of botanicals and some of the more recent data on the therapeutic effects of some of the most popular botanicals in these countries.

2. Botanicals in EU

The advancement of botanicals in Germany resulted from the improvement of the drug act in 1976, which required the evidence for the registration to support the quality, effectiveness, and safety of the drugs, including herbal medicines. Herbal medicines are classified as special drugs because of the difficulties in providing evidence of quality and effectiveness. In the later 1970s, the German Ministry of Public Health appointed the Commission E, a group that was comprised of experts in herbal drugs and preparations from medicinal plants. In 1978, the Commission E reviewed botanicals and issued the evaluation criteria for the safety and efficacy of botanicals as follows [2].

- (1) The traditional use and the long use of botanicals may indicate the safety and the potential effectiveness of the botanicals.
- (2) The chemical evidence, especially the specific active substance, may indicate the potential activity and/or the toxicity of the active substance. However, it depends on the type and the quantity of the active substance.
- (3) *In vitro* and *in vivo* pharmacology and toxicity studies of the botanical extracts and the isolated active substances provide evidence for the potential use of

- the botanicals. This result is very useful where there are no results from clinical studies.
- (4) The resulting data from the clinical studies confirms the safety and the efficacy of botanicals.
 - (5) The field and epidemiological studies provide relevant information about the duration of the species particular environment, which are useful for the evaluation of botanical safety and effectiveness.
 - (6) The case reports from the physicians are also useful for the evaluation of the botanical effectiveness. However, the evidence is not as strong as the controlled clinical studies.
 - (7) The unpublished information and study from the manufacturers are also useful for the evaluation.

The Commission E issued botanical monographs until the year 1995, when the European Union started to harmonize drug regulations. At that point, the Commission E monographs became historical documentation and a basis for the scientific documentation of botanicals [3].

One of the most popular botanicals in EU is *Ginkgo biloba* L. [4–6]. It is thought that, in the EU, Ginkgo became extinct during the ice era but was reintroduced to EU in the eighteenth century from East Asia, where the Ginkgo leaf had been traditionally used as a remedy for bronchial asthma and topically as a wound-plaster.

Ginkgo biloba, the last plant in the family Ginkgoaceae, is thought to be one of the oldest trees still surviving on earth [4–6]. Some specimens are over 30–40 meters high and several hundred years of age. The leaf form looks like two lobes of a spreading fan or maiden's hair. *Ginkgo biloba* has many common names, one of which is the maidenhair tree. The body of research data for *Ginkgo* is extensive, and research on various aspects of the plant safety, quality, and efficacy is currently ongoing. Nowadays, the plant is cultivated in Japan, South Korea, USA (South California), and France (Bordeaux).

Monographs for Ginkgo leaf (Folium Ginkgo; *Ginkgo* Folium) are present in many pharmacopoeias and official publications worldwide including the Chinese Pharmacopoeia, the European Pharmacopoeia, the USA Pharmacopoeia, and the WHO monographs. In terms of chemistry, there are several groups of compounds present in Ginkgo leaves. They include the following.

- (1) Flavonoid glycosides: mono-, di-, and triglycosides (0.5–1.8%), including quercetin- and isorhamnetin-3-O-glucosides, kaempferol-7-O-glucoside, quercetin-3-O-rhamnoside, 4'-O-methyl-myricetin-3-O-glucoside, kaempferol-3-O-rutinoside, kaempferol-3-O-glucorhamnoside, quercetin-3-O-rutinoside, 4'-O-methyl-myricetin-3-O-rutinoside, syringetin-3-O-rutinoside, kaempferol-3-O-glucorhamnoside, and quercetin-3-O-glucorhamnoside.
- (2) Flavonol-acyl-glucosides (0.06–0.2%): kaempferol- and quercetin-3-O-[6-p-coumaroyl-glucorhamnoside] (Figure 1).

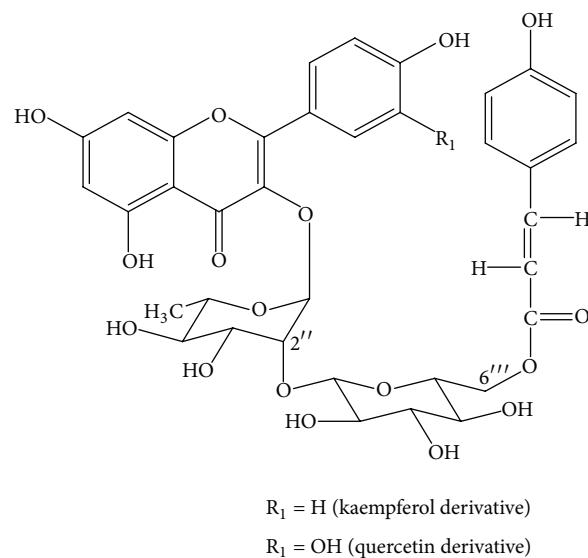
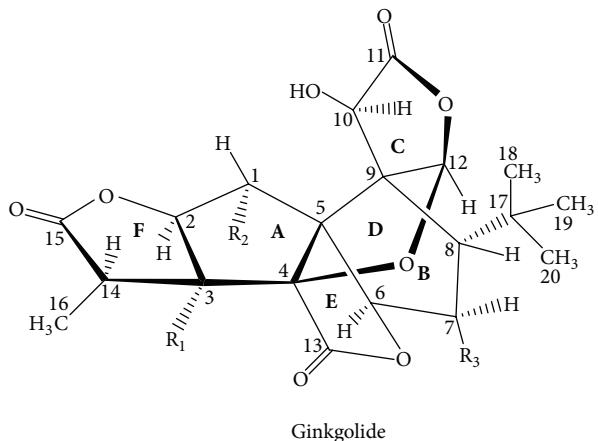


FIGURE 1: Two major flavonol-acyl-glucosides of *Ginkgo biloba* [7].

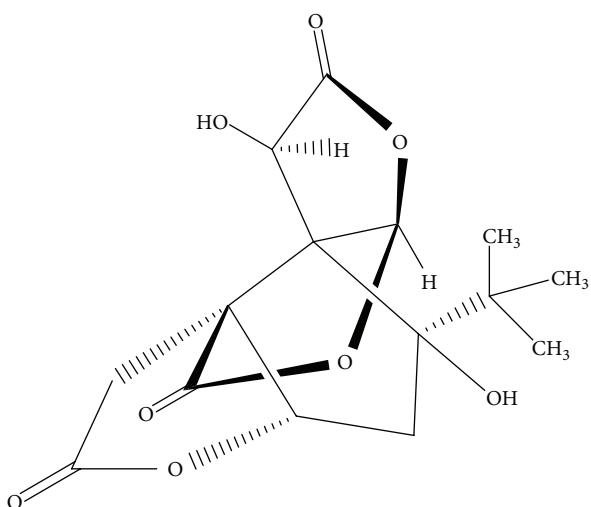
- (3) Biflavonoids (0.4–1.9%): amentoflavone, bilobetin, 5-methoxy-bilobetin, ginkgetin, isoginkgetin and sciadopitysin.
- (4) Catechins (up to 0.04%) and oligomeric proanthocyanidins (8–12%), of which the major skeletons belong to delphinidin and cyanidin. In addition (+)-catechin, (−)-epicatechin, (+)-allocatechin, and (−)-epigallocatechin are also found.
- (5) Terpenoids include diterpenes (ginkgolides A, B, C, J, and M; up to 0.23%) and sesquiterpenes (bilobalide; up to 0.2%) (Figure 2).
- Ginkgo terpenoids containing three groups of lactones and a tertiary butyl group cause the bitterness of the ginkgo extract.
- (6) Steroids include sitosterol, campesterol, 22-dihydro-brassicasterol, and sitosterylglucoside.
- (7) Organic acids include shikimic acid, 3-methoxy-4-hydroxy benzoic acid, 4-hydroxy benzoic acid, and ginkgolic acid.
- (8) Nitrogen containing compounds.

The first nitrogen containing compound, 6-hydroxy-kynurenic acid, was recently found in Ginkgo leaf. The quantity of this compound depends on the plant age. The leaves collected in the fall contain the maximum content (up to 0.24%) of this compound, which is the major metabolite of tryptophan.

The active composition or drug material from *G. biloba* leaf is prepared using 60% aqueous acetone as extraction solvent; the biflavonoids and ginkgolic acid are removed, and the drug-extract ratio is 50:1 (average). The extract fraction or special extract of *G. biloba* leaf contains ginkgo flavones glycosides (22–27%) and terpene lactones (ginkgolides and bilobalide) (5–7%). The level of ginkgolic acid must not exceed 5 ppm.



R ₁	R ₂	R ₃	Ginkgolide (diterpene)
OH	H	H	A
OH	OH	H	B
OH	OH	OH	C
OH	H	OH	J
H	OH	OH	M



Bilobalide (sesquiterpene)

FIGURE 2: Terpene lactones, ginkgolides and bilobalide, of *Ginkgo biloba* [7].

The special extract of Ginkgo leaf possesses the following pharmacological activities: increases the tissue resistance to hypoxia, especially the brain tissue to the oxygen-deficient blood; it inhibits the brain edema; decreases retinal edema and lesions; it inhibits aging and the reduction of muscarinic cholinergic receptor and α_2 -adrenergic receptor and it increases choline uptake in the hippocampus; it increases

cognition by enhancing memory and learning; it increases blood circulation, especially microcirculation; it improves the blood flow; it removes toxic oxygen radicals (by flavonoids); it inhibits the platelet activating factor, PAF (by ginkgolides), and it provides neuroprotection (by ginkgolides A and B; bilobalide).

The special extract from Ginkgo leaf has several mechanisms of action leading to various therapeutic uses. An important use of the extract is indicated for age associated memory impairment, also known as cerebral insufficiency. The symptoms cover the following: lack of concentration, memory loss, sensitivity, being easily tired, lack of interest, being depressed, being anxious, being dizzy, tinnitus, and headache. Some symptoms indicate that a disorder of cerebral blood flow is the beginning of the oxygen-deficiency in brain and degenerative dementia.

Observed adverse events of Ginkgo leaf are rare. They include headache, dizziness, palpitations, gastrointestinal disorders, and skin allergies. Ginkgolic acid is a potent contact allergen; therefore, the ginkgolic acid level in the extract must not exceed 5 ppm. The special Ginkgo extract is not mutagenic or carcinogenic and is nontoxic to the reproductive system.

The patients can drive or work with machinery when they take Ginkgo extracts. The injectable form of Ginkgo extract is not allowed due to potential allergic reactions, which may include serious symptoms such as arrhythmia and anaphylaxis.

The therapeutic indications allowed for Ginkgo leaf extracts by the German Ministry of Health are as follows. The therapeutic indications allowed for Ginkgo leaf extracts by the German Ministry of Health are as follows.

- (1) Relief of the symptoms and uneasy feeling from the cerebral insufficiency and degenerative dementia: the drug administration: 120–240 mg, 2-3 times a day, for at least 8 weeks. After 3 months the physician should consider the continuation of the drug administration.
- (2) For patients with peripheral arterial occlusive diseases, along with walking therapy: the drug administration: 120 mg, 3 times a day, for at least 6 weeks.
- (3) Tinnitus, dizziness: the drug administration: 160 mg, 3-4 times a day; the administration longer than 6–8 weeks is not necessary.

2.1. Botanicals as Food Supplement and Special Food [7, 8]. Besides carbohydrates, fats, and proteins, humans also need secondary metabolites from botanicals. These botanicals make food colorful (carrot, tomato, etc.) and digestible (spices). The citrus extract with bioflavonoids, the extract of *Allium ursinum*, and the extract of *Linum usitatissimum* are the examples of food supplements. Some botanicals are used as special foods in the chronic diseases, such as cardiovascular, hypercholesterolemia, diabetes, rheumatoid arthritis, and osteoporosis. Red wine and its protective phenols are used for preventing the cardiovascular diseases. Soy and its estrogenic isoflavones are used as alternative for steroid hormones in the climacteric period.

3. Botanical Dietary Supplements in the USA

For the past thirty years, the consumption of botanical dietary supplements (herbal preparations) by USA consumers has increased, and approximately half of adults in the USA use at least one dietary supplement [9]. Supplement sales in the USA were estimated at \$11.5 B in 2012, an increase of seven percent [10]. The most common reasons cited for using dietary supplements are to improve overall health as well as maintain health [9]. Other reasons for increased use of dietary supplements included improvements in bone health for women, mental health, prostate health for men, weight loss, and menopause or hot flashes for women [9]. Many botanical dietary supplements (BDS) are also used by the USA population for the treatment or prevention OF a wide array of ailments including the common cold, depression, and other nonlife-threatening medical conditions, although they are not regulated for this purpose [9, 10]. Consumers over the age of 65, baby boomers, and adolescents make up the major of consumers using dietary supplements. Interestingly, condition-specific products are increasingly being used, perhaps due to the older age populations buying these products. Some of the categories of dietary supplements that are very popular in the USA include the digestive supplements (probiotics), omega-3 fatty acids, joint support, and eye health, all which increased in market share in 2012 [9].

The increasing use of condition specific products is interesting in that dietary supplements are regulated according to the Dietary Supplements Health and Education Act of 1994, which states that dietary supplements are not to be used for treating any condition or disease state but only as support for maintaining and promoting good health [11]. Each dietary supplement package must state that the product is a "Dietary Supplement," and "*These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.*" Thus, it appears that regardless of how supplements are regulated in the USA, consumers still use these products to manage symptoms and treat specific conditions. This is problematic as many dietary supplements do not have sufficient data to support efficacy, and in some cases there are contraindications, drug interactions, and adverse effects associated with the use of specific supplements, of which consumers are not fully aware.

According to the National Center for Complementary and Alternative Medicine at the National Institutes of Health, the five most searched-for botanical dietary supplements of 2012 included Aloe vera, Echinacea, evening primrose oil, fenugreek, and St. John's wort [11]. We have included a brief overview of each of these botanicals; for a more detailed review, including pharmacopoeial details, reviews of pharmacology, and chemistry, please refer to the WHO monographs on selected medicinal plants, volumes 1–4 [12–15] (WHO 2009).

3.1. Aloe Vera: *Aloe vera* (L.) Burm. f. (Liliaceae). *Aloe vera* is a succulent perennial that resembles a cactus and is recognized by many common names including aloe, Aloe Vera, aloes,

cape aloe, and curacao aloe [12]. This plant is the source of two botanically distinct drugs, "aloe" which is a bitter yellow latex originating in the cells of the pericycle and adjacent leaf parenchyma, and *Aloe vera* gel, which is a colorless mucilage obtained from the parenchymatous cells in the leaves. The biologically active chemical constituents of the yellow latex are the hydroxyanthracene derivatives, aloin A and aloin B (Figure 3), whereas the gel consists primarily of water and polysaccharides (pectins, hemicellulose, glucomannan, acemannan, and mannose derivatives) [12, 16, 17].

Clinically, aloe (solidified yellow latex) is used for the short-term treatment of occasional constipation [12]. It should not be used continuously for longer than 1–2 weeks and may cause adverse reactions, including abdominal spasms and pain which can occur even after a single dose [12, 16]. Overdose can lead to abdominal spasms and pain, as well as the formation of watery stool. Chronic abuse of this drug as a laxative can lead to hepatitis, electrolyte disturbances (hypokalaemia and hypocalcaemia), metabolic acidosis, malabsorption, weight loss, albuminuria, haematuria, weakness, and orthostatic hypotension [12, 16]. Another adverse effect is secondary aldosteronism that may also occur after chronic use. Aloe, as a laxative, is contraindicated in patients with intestinal obstruction or stenosis, atony, severe dehydration with electrolyte depletion, or chronic constipation. It should not be administered to patients with inflammatory intestinal disease, children under 10 years of age, during pregnancy, or during lactation (unless under the supervision of a physician) [12]. The safety of aloe latex was reported in 2007 [17]. This study showed that the administration of the latex to mice at a dose of 100 mg/kg for three months caused inflammation, genotoxicity, general toxicity, and sperm damage [17].

Aloe vera gel is also used in complementary and alternative medicine (CAM) to treat wounds and burns. In clinical trials, fresh *Aloe vera* gel has been shown to promote healing of burns, including radiation burns [12]. Clinical studies further suggest that fresh *Aloe vera* gel may promote wound healing; however, it should be noted that only fresh gel or preparations containing 10–70% fresh gel are active [18]. Dried or dated preparations are ineffective in either burn or wound healing. A 2012 systematic review that assessed the clinical trials using *Aloe Vera* gel for acute and chronic wounds suggested that the clinical trial data are in general of poor quality, biased, and conflicted [18]. *Aloe vera* gel has few adverse reactions and is only contraindicated in cases of known allergy to the Liliaceae [12, 16]. In terms of safety, recent studies in rodents have shown that *Aloe vera* gel is not genotoxic and has no toxic effects after 13 weeks of feeding [17, 19].

3.2. Echinacea: *Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.), and *Echinacea purpurea* (L.) Moench (Asteraceae). *Echinacea*, a native American plant and traditional medicine, is popular in the USA and Europe for the prevention and treatment of the symptoms of cold, flu, and upper respiratory infections [12, 16]. Clinical research suggests that *Echinacea* may enhance the immune response,

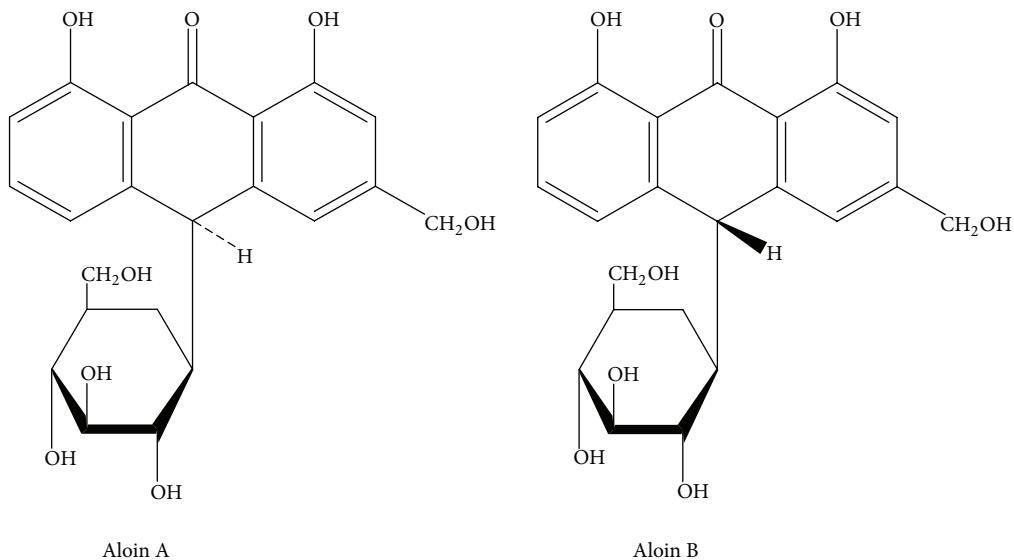


FIGURE 3: The structures of aloin A and B, two of the major constituents of *Aloe vera* latex.

but this effect is likely product specific, as certain preparations of *Echinacea*, including fresh pressed juice or isolated polysaccharides, appear to be more effective than other products [20, 21]. The chemistry of *Echinacea* is complicated but is well documented, and several groups of constituents, including alkamides and caffeic acid derivatives such as cichoric acid, echinacoside, cynarin, and cichoric acid methyl ester, are considered important for activity (Figure 4) [12].

A 2007 meta-analysis of 14 clinical trials evaluated the safety and efficacy of *Echinacea* on the duration and severity of the common cold [22]. The results of this analysis suggested that pretreatment with *Echinacea* reduced the odds of developing a cold by 58 percent and reduced the average duration of the infection by 1.4 days [22]. In the most recent clinical trials, the results assessing the effects of various *Echinacea* products on the severity and duration of the common cold are conflicted [23, 24]. The first clinical trial, using an unknown product for the treatment and prevention of the common cold, resulted in a negative outcome [23], where administration of *Echinacea* had no significant effect on the symptoms or duration of the common cold. The second study was a positive prevention trial [24] that showed a positive preventative effect of a specific product “Echinaforce” (Bioforce, Switzerland) on the common cold [24].

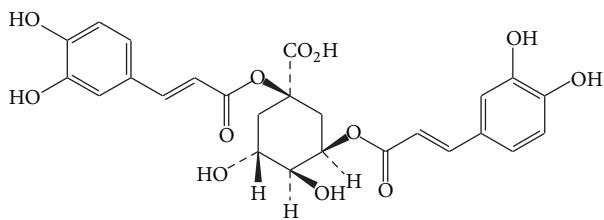
Interestingly, many of the clinical trials involving pediatric patients have negative outcomes as well as a higher incidence of skin rashes reported after treatment as an adverse effect [25]. Thus, at this time, it does not appear that there is any benefit from administering *Echinacea* products to children. In addition, people with allergies to the Asteraceae (ragweed and chamomile) may also have cross-sensitivity to *Echinacea* [12]. Furthermore, *Echinacea* products are not recommended during pregnancy.

3.3. Evening Primrose Oil: *Oenothera biennis* L. (Onagraceae).

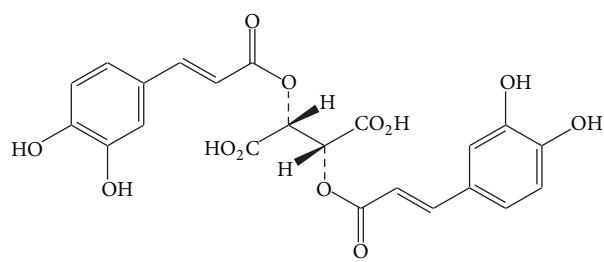
the seeds of *O. biennis*, a native American wild flower introduced into Europe in the early 17th century [13]. EPO has been used internally for the management of atopic eczema, diabetic neuropathy, premenstrual syndrome, and cyclic mastalgia [13]. Mastalgia is a common cyclic breast condition, with swelling and breast pain being severe enough to interfere with usual daily activities. The major chemical constituents of the oil include *cis*-linoleic acid (65–80%), γ -linoleic acid (8–14%) (Figure 5), oleic acid (6–11%), palmitic acid (7–10%), and stearic acid [13], most of which have antiinflammatory effects.

A review of the clinical trials for evening primrose oil up to the year 2001 is available in the WHO Monographs of Selected Medicinal Plants [13]. A more recent randomized, double-blind, placebo-controlled clinical trial involving 85 women with cyclic mastalgia showed that daily administration of EPO at a dose of 3000 mg per day (in divided doses) for 6 months decreased the pain and severity associated with this condition [26]. In terms of safety, EPO is known to inhibit platelet aggregation in animals; thus, patients taking anticoagulants should be closely monitored and checked by their physician prior to using products containing EPO. In terms of adverse effects, headaches, nausea, and minor diarrhea have all been reported [13]. In addition, EPO should be used with caution in patients with a history of epilepsy, as it may precipitate symptoms of undiagnosed temporal lobe epilepsy in schizophrenia patients taking epileptogenic drugs, particularly the phenothiazines [13].

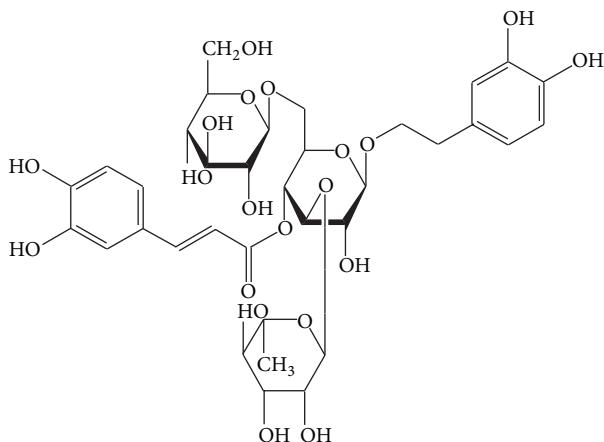
3.4. Fenugreek: *Trigonella foenum-graecum* L. (Fabaceae). *Trigonella foenum-graecum* L., commonly known as fenugreek, is a plant that has been extensively used as a source of antidiabetic compounds from its seeds and leaf extracts, including trigonelline (Figure 6) [27]. Preliminary human trials and animal experiments suggest possible hypoglycaemic and antihyperlipidemic properties of fenugreek seed



Cynarin (1,5-dicaffeoylquinic acid)



Cichoric acid



Echinacoside

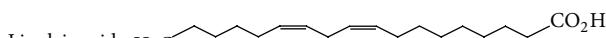
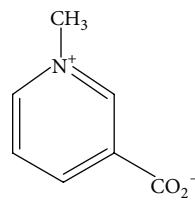
FIGURE 4: Structures of the caffeic acid derivatives that make up some of the major chemical compounds in *Echinacea*.

FIGURE 5: Structures of the fatty acid derivatives that make up some of the major chemical compounds in evening primrose oil.

powder when taken orally. One study showed that the action of fenugreek in lowering blood glucose levels is almost comparable to the effect of insulin [27]. In animal models, the soluble fiber of fenugreek appears to seed improve glucose homeostasis by delaying carbohydrate digestion and absorption and enhancing insulin action [28, 29].

However, the clinical data for fenugreek are currently inadequate to recommend it's use at this time and new clinical data for fenugreek soluble fiber are needed before any clinical



Trigonelline

FIGURE 6: The structure of trigonelline, one of the chemical constituents of fenugreek.

recommendations can be made about its therapeutic use for diabetes [26].

3.5. St. John's Wort: *Hypericum perforatum L.* (Clusiaceae). *Hypericum perforatum L.* is a herbaceous aromatic perennial plant, commonly known as St. John's wort. St. John's wort (SJW) is prepared from the dried aerial parts and flowering tops of *H. perforatum*, and used in both the USA and Europe to manage mild to moderate depression [12, 13, 30]. In the United States, St. John's wort is regulated as a dietary supplement; however, in reality it is used more as a CAM therapy for depression. The major chemical constituents of St. John's wort include the naphthodianthrones hypericin and pseudohypericin and the acylphloroglucinols hyperforin and adhyperforin (Figure 7) [13].

Earlier clinical trials have suggested that standardized extracts of St. John's wort were as effective as low doses of selective serotonin reuptake inhibitors or tricyclic antidepressants for the treatment of mild to moderate depression [13]. Meta-analyses of randomized controlled trials have found that St. John's wort was superior to placebo and similarly effective as standard antidepressants in the acute treatment of mild to moderate depression [31]. In addition, there was a reduced frequency of adverse effects, lower treatment withdrawal rates, low rates of side effects, and good compliance, with St. John's wort as opposed to standard antidepressant drugs [31]. A more recent study demonstrated the cost-effectiveness of using St. John's wort products as a first line of therapy for mild to moderate depression [32]. In general, St. John's wort can interact with many important prescription drugs that are used by patient populations more susceptible to depression such as transplantation patients, HIV/AIDS, women, and cancer patients [33, 34]. The metabolism of drugs such as cyclosporin, protease inhibitors, estrogens, and chemotherapeutic agents is increased when given in combination with therapeutic doses of St. John's wort. Since SJW can interact with any drug metabolized through the cytochrome P450 enzymes, many interactions with prescription medicines are possible, and patients should consult their physician or pharmacist prior to use [31].

4. Botanicals in Thailand

Botanicals are important part in the traditional medicine system that was established in China, India, Mesopotamia,

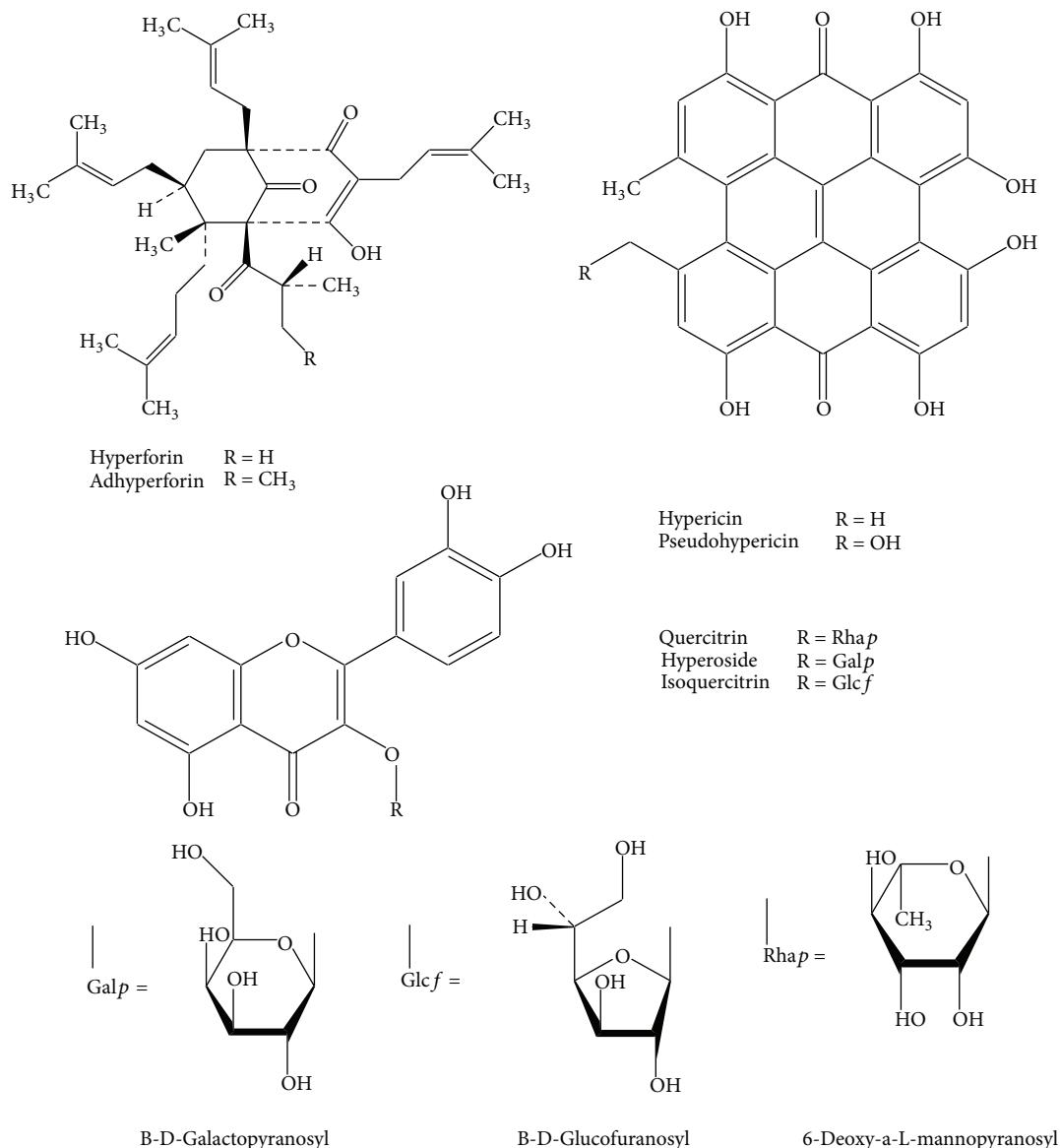


FIGURE 7: Structures of some of the major chemical constituents present in St. John's wort extracts.

and Egypt in 5000 A.C. During 500 A.C.–500 A.D., the traditional medicine systems of Graeco-Roman and Islam evolved and transformed into a modern medicine system in 1800 A.D. [35]. Drugs are defined in modern medicine system as chemicals with well-defined structures, isolated from botanicals or chemically synthesized. For example, vinblastine and vincristine, isolated from *Catharanthus roseus*, are anticancer drugs. Another evolution path of botanicals is the utilization of botanical extracts. Research in botanicals is multidisciplinary as shown by the publications in this current issue. New discoveries from botanicals are going on, while the indigenous and traditional uses of botanicals have been developed and applied in the community. By the beginning of ASEAN Economic Community (AEC) in the year 2015, harmonization of laws and regulations relative to botanicals in ASEAN countries will be established. This encourages each

country in the region to improve the laws and regulations relative to botanicals.

Asian countries have a long historical use of traditional medicines and botanicals. They have their own traditional medicinal systems that are accepted by their people and regulated by the states. However, there are some difficulties in integrating botanicals to the national health system. World Health Organization (WHO) has realized the botanical importance in Asia, in which 80% of the populations still rely on traditional medicine system. WHO has developed the guidelines of herbal medicines in the Southeast Asia region for supporting the integration of traditional and modern medicines [15]. In the guidelines, herbal medicines are divided into four categories based on distinguishing the used form and its origin. They are indigenous, traditional, modified, and export/import herbal medicines. Indigenous

and traditional herbal medicines use the combination of crude drugs as drug materials. The modified herbal medicine mostly comprises single evidence-based herb in the form of standardized extract, and it is available in the modern dosage form.

The traditional medicine system in Thailand has been developed and influenced by Ayurveda and Chinese traditional medicine systems since the Ayutthaya period (1350–1767 A.D.) [36]. During the reign of King Rama V, Siriraj hospital was established in the year 1888, and the system was a combination of modern and traditional medicine systems. In the year 1913, the traditional medicine system was no longer taught to the medical students owing to the different doctrines and the separation from the modern medicine. The regulation on traditional medicine was issued in the year 1967 under the Drug Act 1964, which is divided into two parts covering modern and traditional medicines.

Because of the return of the botanical importance, the Ministry of Public Health, Thailand, established in 2002 the department involving traditional medicine system. The Department of Thai Medicine and Alternative Medicine, which works together with the Department of Medical Science, issued the list of essential medicines (herbal medicine). The first list was approved in 1999. The latest List, in 2012, covers 52 traditional drugs and 21 single herbal drugs, which are classified as modified herbal medicine and is divided into 4 groups covering the modified Thai traditional medicine, the modified traditional medicine (e.g., ayurveda, Chinese), the single herbal drug, and the modern herbal drug [37]. Currently, there is only one modern herbal drug (silymarin as hepatoprotective) that is licensed because of the strong evidence requirement of safety, efficacy, and quality. It is quite difficult or impossible to prove the efficacy of the botanical products, especially those with mild-moderate effect, with the same standard as the modern medicine. Most of the imported herbal products are licensed as food supplements, of which the medical indication cannot be claimed.

The following are the examples of well-known single herbal products included in the list of essential medicine (Herbal Medicines).

Andrographis paniculata, the aerial parts, contained total lactones not less than 6% (calculated as andrographolide, Figure 8). The indications cover the relief of noninfectious diarrhea, with the daily dose ranging from 2 to 8 g and the relief of common cold, with the daily dose ranging from 6 to 12 g. The lactones have weak antimicrobial activity. The therapeutic effect may also be due in part to other activities as well, such as anti-inflammatory and immunostimulatory effects. A review of this botanical is published as a monograph in the WHO Monographs on Selected Medicinal Plants volume 2.

Curcuma longa, the rhizome containing curcuminoids not less than 5% (calculated as curcumin, Figure 9) and volatile oil not less than 6%. It is indicated for the relief of flatulence. The daily dose ranges from 2 to 4 g. The volatile oil is carminative. The curcuminoids (curcumin) in rhizome and cineole in volatile oil stimulate the bile secretion, which aids the digestion and relieves the flatulence. The monograph of

C. longa was published in the WHO Monographs on Selected Medicinal Plants, volume 1.

Both botanicals are used in the form of powdered crude drug. The used form of drug material determines the method of quality control and the daily dose. The quality control of the crude drug is performed according to the monograph in the Thai herbal pharmacopoeia (THP), which is established by the Department of Medical Sciences, Ministry of Public Health. There are three volumes of THP issued in 1998, 2000, 2007 (supplement), and 2009. They cover 37 monographs of crude drugs [41, 42].

One interesting botanical in the list of essential medicine (herbal medicine) is *Momordica charantia* fruit, which is classified as a single herbal drug and indicated as remedies for fever and aphthous ulcer and as a bitter tonic. The daily dose ranges from 3 to 6 g (infusion) and from 1.5 to 3 g (oral). The use of this botanical is limited to the traditional indication in the list of essential medicine. However, the research on *M. charantia* still continues, and new health benefits from this plant are being revealed.

Momordica charantia (bitter gourd, bitter melon) has been used as medicine for thousand years in Africa, Asia, and Latin America. In India, the fruit has been used in ayurvedic medicine as remedies for diabetes, liver disease, gout, and arthritis. In Thai medicine, the leaf has been used as one ingredient in “green recipe,” which relieves fever and the root as remedies for liver disease and blood disorder [43]. Scientific research on *M. charantia* has been continuously performed since 1962, when the antidiabetic substance, charantin, was discovered by Lotlikar and Rao [44]. In 1965 Sucrow identified the structure of charantin as a mixture of sitosteryl- and 5, 25 stigmastadiene-3 β -ol-D-glucoside (in the ratio 1:1, Figure 10). Baldwa et al. isolated the insulin-like compound from the fruit in the year 1977 [45]. Khanna et al. identified the insulin-like compound as a polypeptide with the molecular weight of 11 kD, 166 amino acids, and named it polypeptide-P [39]. The plant also contained cucurbitacins (Figure 11), and they were momordicosides, momordicines, karavilosides, and charantosides [46]. These cucurbitacins have antidiabetic effects [47]. *M. charantia* fruit contained several antidiabetic compounds and lowered the blood sugar via several mechanisms such as the stimulation of insulin secretion from pancreas, the decrease of sugar formation from the liver, the increase of glycolysis, the renewal of pancreatic β cells, the increase of insulin sensitivity, the increase of glucose tolerance, and the inhibition of α -glucosidase [48–52]. *M. charantia* affects carbohydrate and lipid metabolism through the stimulation of thyroxine and AMPK (AMP-activated protein kinase) [53]. It can also suppress the insulin-signaling pathway resulting in the increase of insulin sensitivity [54].

The *in vivo* studies of the fruit juice of *M. charantia* were given to rabbits and mice, showing antidiabetic effect [55–58]. Some reports stated that *M. charantia* could alleviate the complications resulting from the long time uncontrolled blood sugar level, such as kidney damage, cataract, and peripheral neuritis [59–61].

One clinical study in type II diabetic patients showed that *M. charantia* fruit improved glucose tolerance, decreased

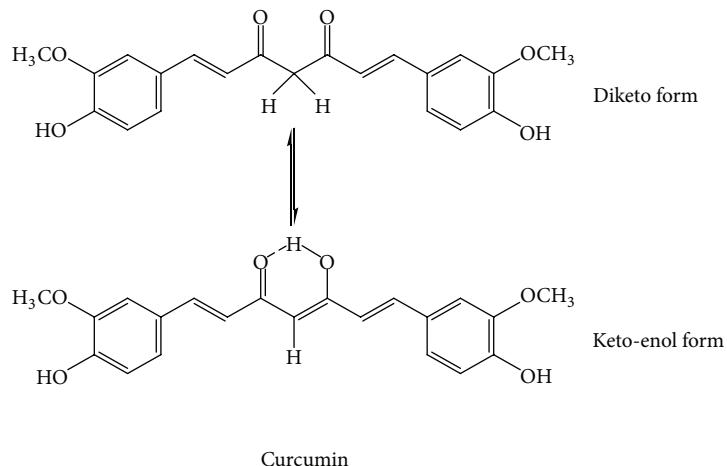


FIGURE 8: Two forms of curcumin of *Curcuma longa*. The keto-enol form is more preferable [7].

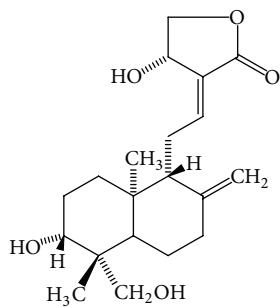


FIGURE 9: Andrographolide from *Andrographis paniculata* [14].

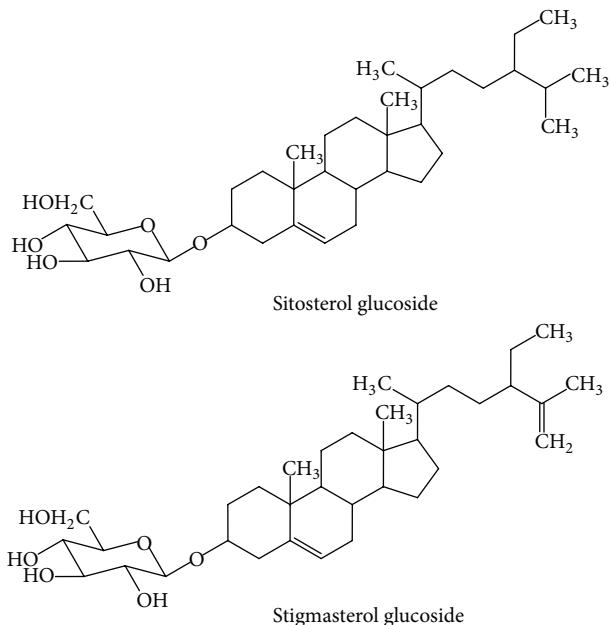


FIGURE 10: Charantin (a mixture of sitosterol and stigmasterol glucosides, 1:1) from *Momordica charantia* [38].

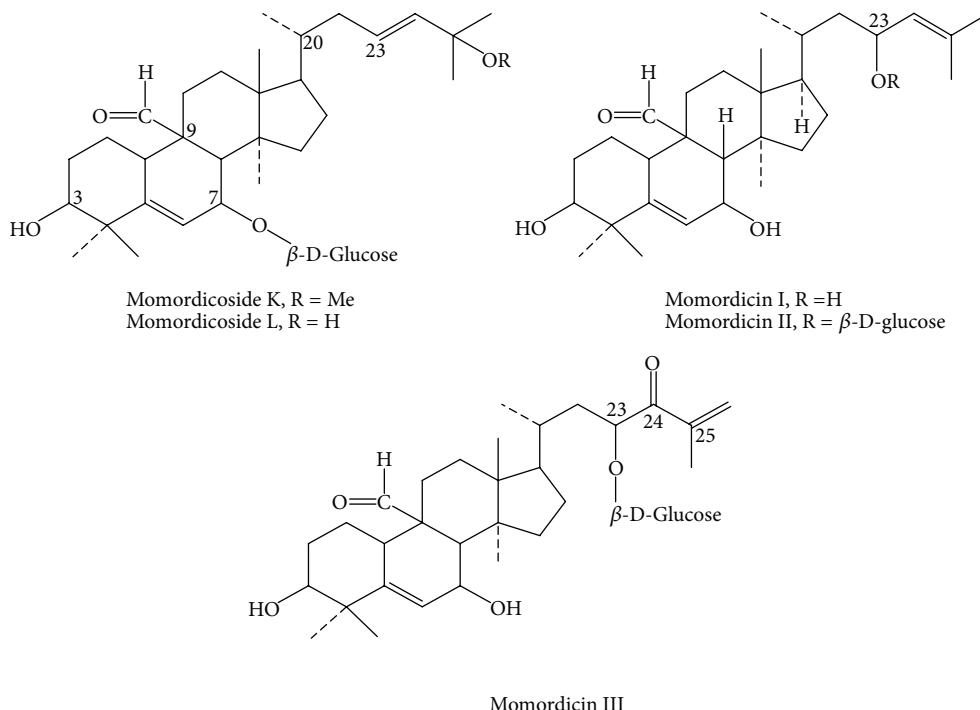
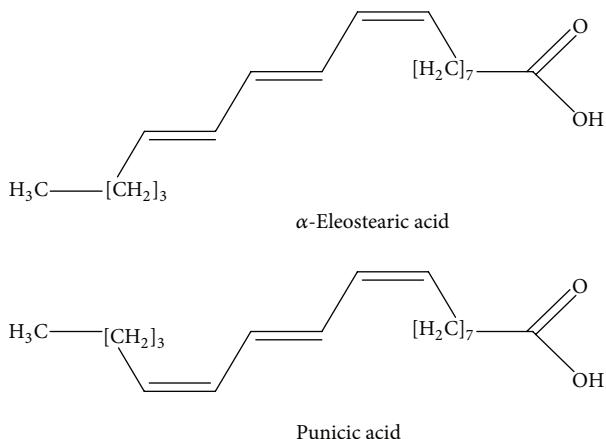
the postprandial hyperglycemia, and lowered the urination frequency [62]. The diabetic patients normally suffer from hypertriglyceridemia, hypertension, and obesity, and thus, *M. charantia* fruit seems to be an appropriate complementary and alternative medicine because of the presence of bioactive compounds, which are responsible for the lowering of blood sugar, blood lipids, blood pressure, and body weight [52, 63–66]. Currently, the clinical evidence of *M. charantia* fruit as an antidiabetic agent is insufficient. Such clinical results usually occur with the food plants that have mild-moderate activity. Well-designed clinical trials and quality control as well as production process control are recommended for further clinical investigation. The increase of prediabetes and diabetes incidences caused a great burden on the health-care expense. The use of nonmodified botanicals for self-medication like the fruit juice of *M. charantia* may be a good choice of complementary and alternative medicines, and it is cost-effective [40, 67].

Other parts of *M. charantia* have also been investigated for the medicinal purposes. The seed oil contained conjugated linolenic acid, namely, α -eleostearic and punicic acids (Figure 12), which are strong antioxidant and anti-inflammatory agents [68].

The seed oil could decrease the blood lipids and prevent cardiovascular disease [69]. The seed proteins, α -momorcharin, Momordica anti-HIV protein 30 (MAP30), and Mara Khee Nok 29 (MRK29), which are type I ribosome inactivating proteins (RIPs I), have antitumor, antiviral, and anti-HIV effects [70–72]. According to the strong cytotoxicity of cucurbitacins, *M. charantia* has been also intensively investigated for its anticancer effect. It could inhibit several cancer cell lines, for example, adrenocortical, breast, nasopharynx, and prostate cancer cell lines [40, 73–76].

5. Summary

Botanicals have been recognized as food and medicines in every country in the world for thousands of years. The progress of science and technology and the vast amount of

FIGURE 11: Cucurbitacins from *Momordica charantia* [39].FIGURE 12: α -Eleostearic acid and punicic acid from *Momordica charantia* [40].

research on botanicals have given rise to the evolution of botanical utilization. Herbal products are regulated under different laws on different continents, and harmonization of some of these laws would be beneficial to everyone. Botanical extracts have been developed and are used as active composition in HMPs (traditional and modern) including food supplements in EU and dietary supplements in USA, which are classified as not drugs or food. In Thailand, the use of a single or combined crude drugs as traditional medicine still exists. However, modern botanical products (HMPs/phytopharmaceuticals, food supplements, and dietary supplements), in which the quality, effectiveness, and

safety are supported by clinical data, and the understanding of the modern practitioners should be integrated into the modern drug system. This would make for a better healthcare system globally and make it cost effective. In addition, research on botanicals will eventually give rise to the discovery of new drugs, providing that there is sufficient funding for such research.

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Research Article

Antiherpetic Effects of *Gynura procumbens*

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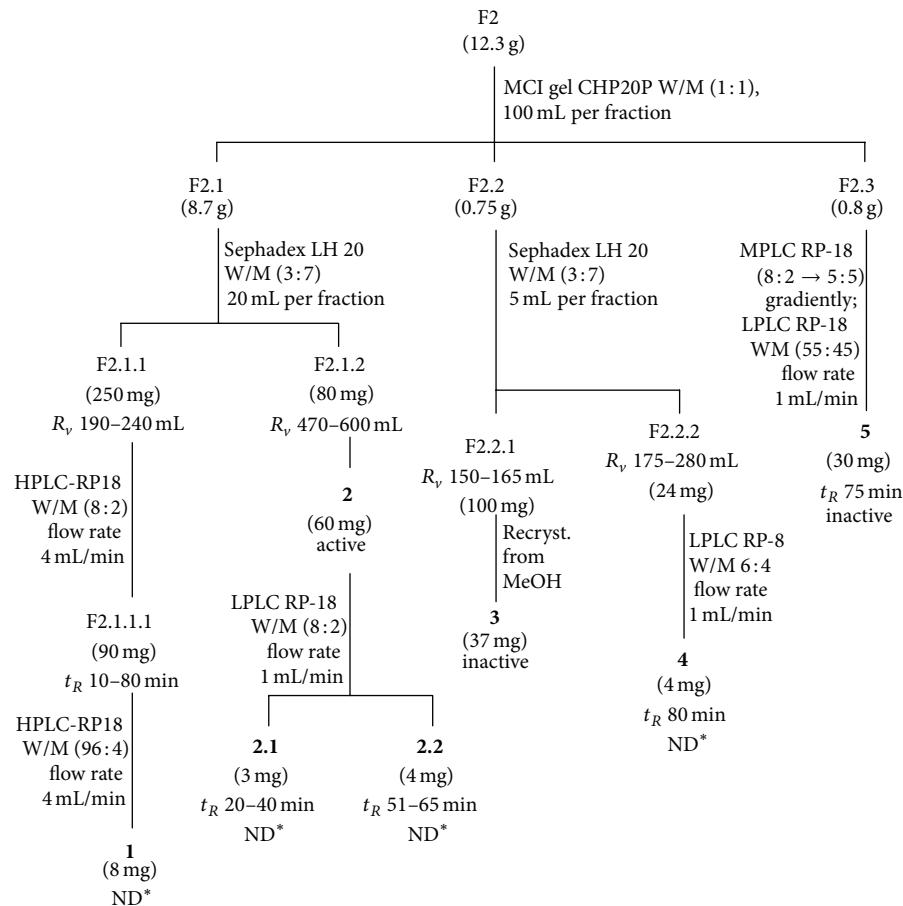
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The ethanol extract of *Gynura procumbens* showed virucidal and antireplicative actions against herpes simplex virus HSV-1 and HSV-2. It was further chromatographed on MCI gel CHP20P column giving the extract fractions F1 (water), F2 (water-methanol) F3 (methanol), and F4 (ethyl acetate). All but F1 had virucidal action against both viral types. We reported here the active compounds from F2 and F3. The antiherpetic compounds of F2 was a mixture of dicafeoylquinic acids with virucidal and antireplicative actions against HSV-2 (IC_{50} 96.0 and 61.0 μ g/mL, resp.) Virucidal compounds of F3 were a mixture of β -sitosterol and stigmasterol (IC_{50} 250.0 μ g/mL against HSV-1), a mixture of β -sitosteryl and stigmasteryl glucosides (IC_{50} 50.0 μ g/mL against HSV-2) and 1, 2-bis-dodecanoyl-3- α -D-glucopyranosyl-sn-glycerol (IC_{50} of 40.0 μ g/mL against HSV-2). Herbal products containing 1 and 2% of standardized ethanol extract were prepared. Double-blind randomized controlled clinical trial of the products was performed in patients with recurrent herpes labialis. Results showed that the number of patients, whose lesions healed within 7 days and the average healing time of both groups differed insignificantly. Viral culture on D7 indicated a decrease of infected patients from 48.7% to 7.69% in treated group whereas in placebo group the infected patients decreased from 31.25% to 20.00%. The viral reduction in treated group indicated the benefit of the product. Insignificant result might arise from a low number of participated patients and insufficient concentration of plant extract in herbal product.

1. Introduction

Gynura procumbens (Lour.) Merr. is mentioned in traditional Chinese medicine as a topical anti-inflammatory remedy [1]. In Southeast Asia, *Gynura* plants are widely distributed. In Thailand, *G. pseudochina* var. *hispida* (Thai name: Wan Mahakaan) is externally used as anti-itching,

anti-inflammatory, and antiherpes virus [2]. In Singapore, Malaysia, and Indonesia, the plant has been traditionally used as remedies for eruptive fever, rash, kidney disease, migraine, constipation, hypertension, diabetes mellitus, and cancer [3]. The ethanol extract from the leaf reduced the mouse ear oedema induced by croton oil [4]. Phytochemical studies of *Gynura* plants resulted in the discovery of



ND*: not determined according to the insufficient amount of the isolated substance

W: water

M: methanol

FIGURE 1: Isolation of components 1–5 from F2.

pyrrolizidine alkaloids [5–7], spirostanol [8], coumarins [7–9], and anthocyanins [10]. Since *G. procumbens* so far has not been explored towards antiherpes viral activity, on the other hand, according to traditional Thai medicine, the utilization of the plant may be associated with herpes viral infection; the investigation of the plant components for the antiherpetic activity is performed.

2. Materials and Methods

Plant Material. *Gynura procumbens* was collected from Chanthaburi province, Thailand, in October 1994. The plant was identified by Ms. Leena Phupatpong, an expert botanist at the Forest Herbarium (BKF), Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. A voucher specimen (BKF no. 127362) was deposited at the same place.

Extraction and Isolation. The aerial parts of the plant (25 kg, fresh) were washed, cut into pieces, dried in a hot-air oven (60°C), and ground, yielding 1.7 kg of the coarse-powdered drug. It was successively extracted in a Soxhlet apparatus using petroleum ether (40–60°C), dichloromethane,

and ethanol. The solvents were removed under reduced pressure. The dry ethanol extract (114 g) was further separated using chromatographic columns with different packing materials. MCI gel CHP20P column fractionated the extract into the fractions F1 (water), F2 (water-methanol 1:1), F3 (methanol), and F4 (ethyl acetate). F2, F3, and F4 were active against herpes virus.

F2 (12.3 g) and F3 (6.4 g) were progressively chromatographed on MCI gel CHP20P using aqueous methanol (1:1) for F2 and methanol for F3 as solvent systems, resulting in F2.1 (8.7 g), F2.2 (0.75 g), and F2.3 (0.8 g) and F3.1 (2.0 g), F3.2 (3.0 g), and F3.3 (0.8 g), respectively. Compounds 1, 2, 3, 4, and 5 were isolated from F2 (Figure 1), while compounds 6, 7, 8, and 9 were isolated from F3 (Figure 2). F4 was not further studied.

Cell Line. The Vero cell line (African green monkey kidney cells) was grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics (5×10^4 cells per well).

Virus. HSV-1, strain KOS, and HSV-2, strain Baylor 186, were obtained from the Department of Microbiology, Faculty

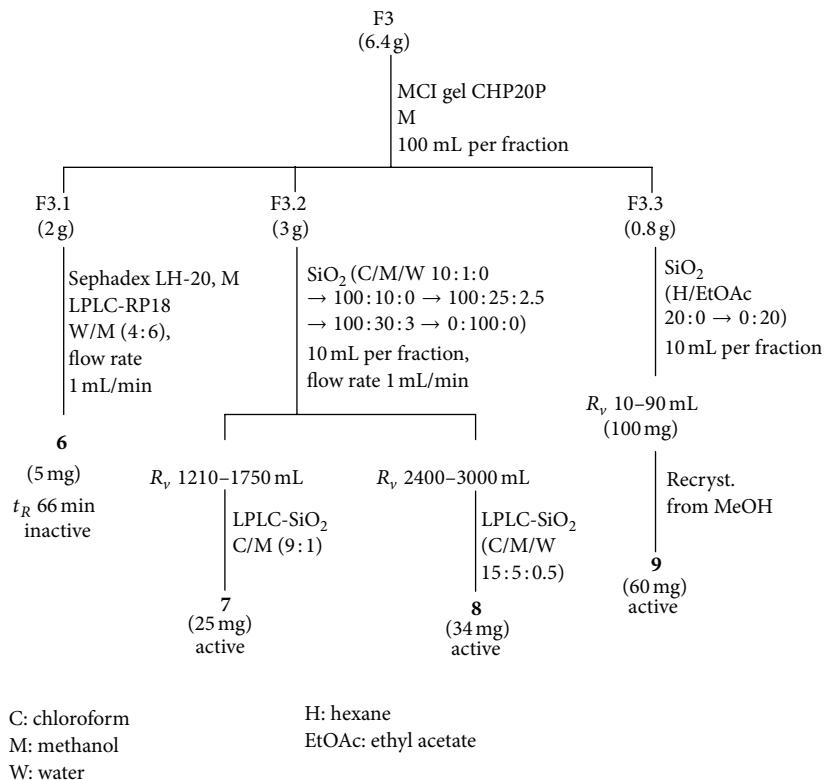


FIGURE 2: Isolation of components 6–9 from F3.

of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The quantity of 100 plaque forming unit (PFU) per mL were used for experiments.

Evaluation of Cytotoxicity. Serial 2-fold dilutions of the test sample in the maintenance medium were added to Vero monolayer. After incubation at 37°C for 5 days, cytotoxicity was determined by vital staining with 1% crystal violet in 10% formalin for 30 min. The highest concentration of the test sample which did not exhibit cytotoxicity represented the maximum nontoxic dose (MNTD). Serial 2-fold dilutions of MNTD were used to perform the antiviral assay.

Antiviral Assay. Antiviral activity was determined by plaque reduction assay on confluent Vero cells growing in 96-well tissue culture plates. The test included three treatments, that is, inactivation and pre- and posttreatments.

Inactivation (To determine the neutralizing activity of the test sample against virus infectivity (virucidal action)): virus (100 μL) was incubated with test sample (100 μL) at 37°C for 1 h. The mixture was added in duplicated wells of monolayer cells and incubated at 37°C for 1 h. After washing the cells, MEM and semisolid media (0.4% gum tragacanth) were added to the cultures, which were then incubated at 37°C for 3 days, and stained with crystal violet.

Pre-treatment (to determine the inhibitory activity of viral adsorption or penetration): test sample (100 μL) was added to duplicated wells of monolayer cells and incubated at 37°C for 24 h. After washing the culture, cells were infected with virus (100 μL) and incubated at 37°C for 1 h. Semisolid media were added to the duplicated wells of monolayer cells

after washing out the non adsorbed virus. The cultures were incubated at 37°C for 3 days and then stained with crystal violet.

Post-treatment (to determine the inhibitory activity of intracellular viral replication): the monolayer cells were infected with virus and incubated at 37°C for 1 h. After washing the cells, the test sample (100 μL) and semisolid media were added in duplicated wells, incubated at 37°C for 3 days and then stained with crystal violet.

Control. Cell control: monolayer cell in MEM and semisolid media. Acyclovir was used as the positive control. Virus control: monolayer cells infected with 100 PFU/mL of virus, MEM, and semisolid media.

Calculation of plaque inhibitory capacity: percent of plaque reduction compared to the culture without treatment (virus control) was calculated. The 50% inhibition concentration (IC_{50}) of the active substance or sample was determined as the lowest concentration which reduced plaque numbers by 50% in treated compared to untreated cultures.

The samples were dissolved in dimethyl sulfoxide (DMSO) and diluted with the maintenance medium. The final concentration of DMSO in the test sample was 0.3%.

Viral Culture. Vero cells were grown in growth medium, MEM (Earle's salt, JR Scientific, Inc., Woodland, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA) and 100 unit/mL penicillin G, and 100 $\mu\text{g}/\text{mL}$ streptomycin (M&H Manufacturing Co., Ltd., Thailand), and 0.01 M HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) (Gibco, Grand Island, USA). Maintenance

media were prepared as growth media except the concentration of fetal bovine serum was reduced to 2%.

Vero cell monolayers in culture flask were dispersed by using 0.25% (1x) trypsin (JR Scientific, Inc., Woodland, CA, USA). It was performed after removing the growth media and washing twice with 5 mL PBS. After discarding PBS, one mL of trypsin was added and cells were incubated at 37°C for 2–5 min; then the culture flask was gently shaken until the cells were detached and 5 mL of the growth media was added. These cells were counted to 10⁵ cells/mL and 1.5 mL of cells were added into the culture tube containing cover glass and then incubated at 37°C in 5% CO₂ overnight. After the incubation, the confluent Vero cells were inoculated with 0.1 multiplicity of infection (MOI) of HSV-1 and HSV-2 viruses (100 μL/culture tube) (Nunclon surface, Nunc Brand product, Denmark) as positive control. The culture was incubated at 37°C in 5% CO₂ for 1 h with shaking at 15 min interval time before adding the maintenance media. The sample from vesicle swab collected in virus transport media (VTM) was processed by refrigerated centrifugation at 10,000 rpm for 10 min. Then, 100 μL of the supernatant was inoculated in 10⁵ cells/mL confluent Vero cells of culture tube containing cover glass and incubated virus at 37°C in 5% CO₂ for 1 h with shaking at 15 min interval time before adding the maintenance media. The confluent Vero cells of 10⁵ cells/mL per culture tube added with maintenance media were used as negative control. Each experiment was carried out in duplicate tubes. All culture tubes of samples, positive and negative control were incubated and examined for cytopathic effect (CPE). The Vero cells on the cover glass were determined for HSV-infected cells and the supernatants were kept at -70°C in order to subpassage culture again. The Vero cells on the cover glass were fixed with chilled acetone for 10 min and air-dried. The detection of HSV in Vero cells using a specific anti-serum was conjugated with fluorescein isothiocyanate (FITC) (Polyclonal antibody, DAKO A/S, Denmark) that was diluted to 1:40 and applied to fixed cells and incubated for 30 min at 37°C. The fixed cells were washed and counterstained with Evans blue for 5 min, then washed again with distilled water, air dried, and mounted with PBS-glycerol buffer. The direct immunofluorescence of the HSV-infected Vero cells was examined under a fluorescent microscope. The negative control was acetone-fixed uninfected cells which processed the same as the test. The infected cells were observed, scored, and recorded for the fluorescent pattern, localization, and intensity of the fluorescent conjugate of antiserum.

Preparation of the Plant Extract, the Herbal, and the Placebo Gels. The plant was macerated with ethanol and evaporated as dry extract, which was standardized with 7, as a specific marker compound or standard. The standardized ethanol extract was used as drug material, which was prepared as 1 and 2% herbal gels. Carbopol, the gelling substance, was dispersed in cool boiled water and neutralized with sodium hydroxide solution. The plant extract was dissolved in propylene glycol, added to the gel, and mixed thoroughly. The placebo gel was prepared similarly, but the coloring agent (caramel) was used instead of the plant extract.

Patients and Method. This study is a double-blind placebo trial in the treatment of recurrent herpes labialis with *G. procumbens* gel compared to placebo. It was approved by the Ethical Clearance Committee on Human Rights Related to Researches Involving Human Subjects. The clinical trial was performed in accordance with the ICH-GCP guidelines and the Declaration of Helsinki. The patients were informed prior to commencement of the trial and provided written consensus of the participation. It was conducted at three hospitals, that is, Ramathibodi, Chulalongkorn, and Phra Mongkutklao hospitals.

The participated patients were more than 18 years of age, diagnosed as recurrent herpes labialis, and the symptom appeared within 48 hours. The exclusion criteria were the patients during pregnancy or lactation and the patients with chronic diseases and HIV infection.

On the first day (D0) of the trial commencement, the history and symptoms of the patients were recorded and the infected lesions were photographed. The Tzanck smear and viral culture were performed to confirm the diagnosis. The baseline laboratory examination included CBC, SGOT, SGPT, BUN creatinin, and urine examination.

The patients were allocated into three groups by block randomization. They were group A receiving 1% herbal gel, group B receiving 2% herbal gel, and group C receiving the placebo gel. All patients were supplied with identical tubes of gel.

They were advised to apply the gel thinly on the infected area every two hours on the first day and four times a day on the following days until the lesion healed. The severity of pain and itch was recorded daily by the patient on a linear visual analogue scale of 0–10 from none to very severe. Dates of full crusting and complete healing were also recorded. The patients were assessed by the investigators on days 2 or 4 and 7 and the day after which lesions completely healed. The assessment on each follow-up visits included the severity of pain and itch and days of full crusting and complete healing of the lesions. The adverse reactions or any patient complaints were also recorded. On D2–D4 and D7, the lesions were photographed and viral cultures were performed. The blood and the urine were collected and examined again on the last visit.

Statistical Methods. We calculated that if success rate occurred in 80% of patients with drug treatment and 50% for placebo patients, it would require 45 drug-treated and 45 placebo patients to detect the success rate at 5% significance level with a power of 80%. We inflated our calculated sample size to 50 patients to compensate for 10% of loss follow-up rate. The Fisher exact test was used to compare the success rate between the drug-treated and placebo groups. Descriptive statistics were reported as mean with standard deviation. Statistical analysis was performed on a completed study basis.

3. Results

The isolation of aqueous methanol F2 and the methanol F3 fractions resulted in four antitherapeutic components, that is, 2 from F2 (Figure 1, Table 1) and 7, 8, and 9 from F3 (Figure 2,

TABLE 1: Antiviral activity of the extract, extract fractions, and isolated compounds from *G. procumbens*.

Extract/extract fractions/compounds	MNTD μg/mL	Antiviral activity (IC_{50} , μg/mL)					
		Inactivation (virucidal action)		Pre-treatment inhibition of viral adsorption and penetration		Post-treatment inhibition of intracellular viral replication	
		HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
Ethanol extract	>2000	625	675	—	—	584	568
F2	1000	320	366	—	—	—	—
F3	1000	362	391	—	—	ND	266
F4	1000	347	312	+	+	+	446
1	ND	ND	ND	ND	ND	ND	ND
2	200	+	96	—	—	+	61
3	200	—	—	—	—	—	—
4	ND	ND	ND	ND	ND	ND	ND
5	200	—	—	—	—	—	—
6	10	—	—	—	—	—	—
7	100	+	50	+	+	—	—
8	100	—	40	—	—	—	—
9	500	250	—	ND	ND	—	—

Positive control: 50 μg/mL of Acyclovir completely inhibited the plaque formation in all test methods.

—: inactive at subtoxic concentration (MNTD/2) (inhibition of plaque forming < 50%).

+: active but IC_{50} is not determined.

MNTD: maximum nontoxic dose.

ND: not determined.

Table 1). The flavonoids (a mixture of **3.1** and **3.2** and **5**) which were isolated from F2 and **6** from F3 had no antiviral activity. Compounds **2**, **7**, **8**, and **9** were identified using spectroscopic methods, especially NMR with field gradient technique [11–14].

2: A Mixture of Dicaffeoylquinic Acids(**2.1** and **2.2**)

2.1 (Figure 4): 3,5-Di-O-Caffeoylquinic Acid. Amorphous yellow powder; R_f 0.55, silica gel 60, CHCl₃/MeOH/H₂O/acetic acid/(21:15:3:1); UV (MeOH) λ_{max} 326 nm. ¹H-NMR (CD₃OD, 300 MHz) δ 7.62, 7.58 (d, J = 16 Hz, H-7', H-7'', each) 7.08, 7.06 (d, J = 8.2, H-2', H-2'', each) 6.98, 6.96 (dd, J = 8, 2 Hz, H-6', H-6'', each) 6.78 (d, J = 8.0, H-5', H-5'', each) 6.41, 6.30 (d, J = 16 Hz, H-8', H-8'') 5.55 (ddd, J = 10.2, 9.7, 6.0, H5ax) 5.38 (ddd, J = 3, 3, 3 Hz, H-3eq) 3.90 (dd, J = 7, 3 Hz, H-4ax) 2.28 (dd, J = 15.1, 3.1 Hz, H-2).

2.2 (Figure 5): 4,5-Di-O-Caffeoylquinic Acid. Amorphous yellow powder, R_f 0.50, silica gel 60, CHCl₃/MeOH/H₂O/acetic acid/(21:15:3:1); UV (MeOH) λ_{max} 326 nm. ¹H-NMR (CD₃OD, 300 MHz) δ 7.57, 7.49 (d, J = 16 Hz, H-7', H-7'', each) 7.02, 6.98 (d, J = 2 Hz, H-2', H-2'', each) 6.89, 6.86 (dd, J = 8, 2 Hz, H-6', H-6'', each) 6.73, 6.71 (d, J = 8 Hz, H-5', H-5'', each) 6.26, 6.18 (d, J = 16 Hz, H-8', H-8'', each) 5.69 (ddd, J = 10.3, 9.7, 7.0 Hz, H-5ax) 5.10 (dd, J = 10.0, 3.0 Hz, H-4ax) 4.31 (ddd, J = 3.0, 4.0, 3.0 Hz, H-3eq) 2.0–2.4 (broad m, H₂-2, H₂-6).

7: A Mixture of β-Sitosteryl (**7.1**, Figure 11) and Stigmasteryl (**7.2**, Figure 12) Glucosides. White powder; mp 254–256°C (with decomposition); R_f 0.48, silica gel 60, CHCl₃/MeOH/H₂O/(20:5:0.5); FAB-MS [M-Na]⁺ m/z

599 and 597 (calcd for C₃₅H₆₀O₆ 576 and C₃₅H₅₈O₆ 574 for **7.1** and **7.2**); EI-MS (pos. ion mode) m/z 414 and 412 [M+H]⁺ (corresponding aglycones); m/z 396 and 394 (low intensity) [M+H-H₂O]⁺; ¹H-NMR (C₅D₅N, 300 MHz) δ 5.35 (dd, J = 4.5, 1.5 Hz, H-6) 5.23 (dd, J = 15.5, 9 Hz, H-22, **7.1**) 5.08 (dd, J = 15.5, 9 Hz, H-23, **7.1**) 5.04 (d, J = 7.9 Hz, H-1') 4.55 (dd, J = 11.6, 1.8 Hz, H-6'a) 4.40 (dd, J = 11.6, 4.9 Hz, H-6'b) 4.26 (dd, J = 15.6, 8.5 Hz, H-3', H-4') 4.04 (t, J = 8.2, 7.9 Hz, H-2') 3.96 (m, H-3, H-5') 2.72–2.47 (m, H-4) 2.14–1.74 (m, H-2) 1.93 (m, H-1) 1.7 (m, H-25) 1.4 (m, H-20) 1.0–1.8 (m, H-8, H-9, H-14, H-17) 1.28 (m, H-28) 1.09 (d, J = 6.7 Hz, H₃-21, **7.2**) 1.0 (d, J = 6.4, H₃-21, **7.2**) 0.94, 0.96 (m, H-24) 0.93 (d, J = 6.9 Hz, H₃-27) 0.92 (d, J = 7 Hz, H₃-26) 0.95 (s, H₃-19) 0.90–1.30 (m, H-23, H-23, **7.2**) 0.90 (d, J = 6.1 Hz, H₃-27) 0.89 (d, J = 6.7 Hz, H₃-26) 0.88 (t, J = 7.67, 7.67 Hz, H₃-29) 0.8–2.2 (m, H-7, H-11, H-12, H-15, H-16) 0.69 (s, H₃-18, **7.1**) 0.67 (s, H₃-18, **7.2**); ¹³C-NMR (C₅D₅N, 75 MHz) δ 140.955 (C-5) 138.833 (C-22) 129.521 (C-23) 121.921 (C-6) 102.607 (C-1') 78.605 (C-3, 3') 78.440 (C-5') 75.331 (C-2') 71.761 (C-4') 62.894 (C-6'a, 6'b) 56.971 (C-14, **7.2**) 56.889 (C-14, **7.1**) 56.297 (C-17, **7.1**) 56.132 (C-17, **7.2**) 51.460 (C-24, **7.1**) 50.404 (C-9) 46.097 (C-24, **7.2**) 42.527 (C-13, **7.1**) 42.396 (C-13, **7.2**) 40.783 (C-20, **7.1**) 40.010 (C-12) 39.368 (C-4) 37.526 (C-1) 36.967 (C-10) 36.424 (C-20, **7.2**) 34.269 (C-22, **7.2**) 32.196 (C-7) 32.114 (C-8, 25 **7.1**) 30.290 (C-2) 29.531 (C-25, **7.2**) 29.317 (C-16, **7.1**) 28.560 (C-16, **7.2**) 25.714 (C-28, **7.1**) 24.579 (C-15, **7.2**) 24.546 (C-15, **7.1**) 23.444 (C-28, **7.1**) 21.502 (C-21, **7.1**) 21.305 (C-11) 19.446 (C-19, **7.2**) 19.216 (C-19, **7.1**) 19.051 (C-21, **7.2**) 12.191 (C-18, **7.1**) 12.010 (C-18, **7.2**).

8:1,2-bis-Dodecanoyl-3-α-D-Glucopyranosyl-sn-Glycerol (Monoglucoyl Diglyceride, Figure 13). Colorless waxy mass, R_f

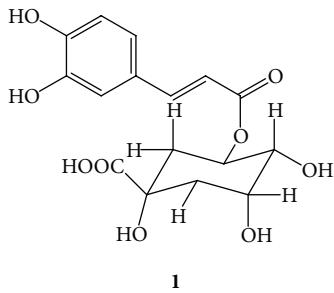
**1**

FIGURE 3

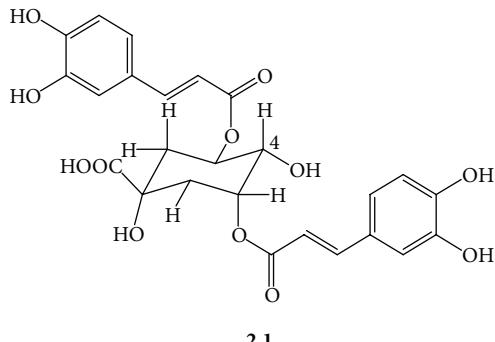
**2.1**

FIGURE 4

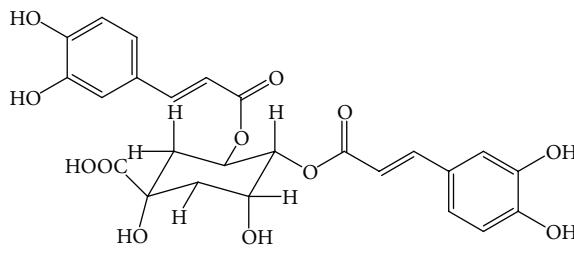
**2.2**

FIGURE 5

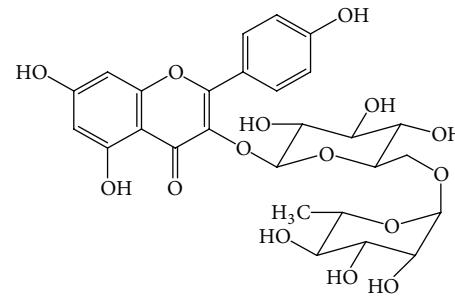
**3.1**

FIGURE 6

0.15, silica gel 60, $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}/(20:5:0.5)$; $^1\text{H-NMR}$ (CD_3OD , 300 MHz) δ 5.28 (dd, $J = 7.0, 6.4, 4.0, 4.1$ Hz, H-2) 4.77 (d, $J = 3.8$ Hz, H-1'') 4.50 (m, H-1a) 4.14 (dd, $J = 12.0, 7.0$ Hz, H-1b) 4.00 (dd, $J = 10.8, 6.4$ Hz, H-3a; ddd, $J = 9.8, 9.0, 3.4$ Hz, H-5'') 3.60 (dd, $J = 9.5, 9.0$ Hz, H-3'') 3.56 (dd, $J = 10.8, 6.4$ Hz, H-3b) 3.41 (dd, $J = 9.7, 3.8$ Hz, H-2'') 3.28 (dd, $J = 14.3, 9.7$ Hz, H-6'') 3.19 (dd, $J = 9.7, 9.0$ Hz, H-4'') 3.00 (dd, $J = 14.3, 3.4$ Hz, H-6'') 2.30 (t, $J = 7.6, 7.6$ Hz, H-2', 2'') 1.57 (m, H-3', 3'') 1.25 (m, H-4'-13', 4''-13'') 0.84 (t, $J = 6.4, 6.0$ Hz, H-14', 14''); $^{13}\text{C-NMR}$ (CD_3OD , 75 MHz): δ 192.31 (C-1', 1'') 99.08 (C-1'') 74.00 (C-3'', 4'') 73.00 (C-2'') 71.50 (C-2) 70.00 (C-5'') 66.00 (C-3a, 3b) 64.00 (C-1a, 1b) 61.00 (C-6'') 34.69 (C-2', 2'') 29.52-30.06 (C-4'-11', 4''-11'') 25.27 (C-3', 3'') 23.02 (C-13', 13'').

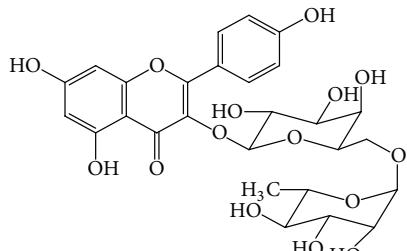
9: A Mixture of β -Sitosterol (**9.1**, Figure 14) and Stigmasterol (**9.2**, Figure 15). White needles, mp 145–148°C; R_f 0.75, silica gel 60, $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}/(20:5:0.5)$; $^1\text{H-NMR}$ (CDCl_3 , 300 MHz) δ 5.34 (broad, d, $J = 5.4$ Hz, H-6) 5.15 (dd, $J = 15, 8.5$ Hz, H-22, **9.1**) 5.01 (dd, $J = 15, 8.5$ Hz, H-23, **9.1**) 3.52 (dd, $J = 11, 10, 5, 4$ Hz, H-3) 2.26 (m, H-12) 2.08–1.78 (m, H-22, **9.2**) 1.3–0.8 (m, H-23, **9.2**) 1.7 (m, H-25) 1.28 (m, H-28) 1.2 (d, $J = 6.4$ Hz, H-21) 1–1.18 (m, H-9, 14, 17, 20) 1.0 (s, H_3 -19) 0.95 (m, H-24) 0.84 (d, $J = 6.4$ Hz, H-26*) 0.82 (d, $J = 6.4$ Hz, H-27*) 0.80 (t, $J = 6.5, 6.5$ Hz, H-29) 0.8–2.2 (m, H-1, 2, 7, 11, 15, 16) 0.68 (s, H_3 -18, **9.2**) 0.70 (s, H_3 -18, **9.1**); $^{13}\text{C-NMR}$ (CDCl_3 , 75 MHz) δ 140.77 (C-5) 138.30 (C-22, **9.1**) 129.30 (C-23, **9.1**) 121.70 (C-6) 71.82 (C-3) 56.88 (C-14, **9.1**) 56.79 (C-14, **9.2**) 56.09 (C-17, **9.2**) 55.99 (C-17, **9.1**) 51.24 (C-24, **9.1**) 50.19 (C-9, **9.1**) 50.16 (C-9, **9.2**) 45.87 (C-24, **9.2**) 42.33 (C-4) 42.23 (C-13) 40.47 (C-20) 39.80 (C-12, **9.2**) 39.70

(C-12, **9.1**) 36.53 (C-10, **9.1**) 36.52 (C-10, **9.2**) 36.15 (C-20, **9.2**) 33.97 (C-22, **9.2**) 32.27 (C-1) 31.92 (C-7, **9.1**) 31.88 (C-7, **9.2**) 31.69 (C-2) 29.19 (C-25, **9.2**) 28.91 (C-16, **9.1**) 28.24 (C-16, **9.2**) 26.13 (C-23, **9.2**) 25.40 (C-28, **9.1**) 24.37 (C-15, **9.1**) 24.31 (C-15, **9.2**) 23.09 (C-28, **9.2**) 21.21 (C-26*, **9.1**) 21.09 (C-21, **9.1**) 19.81 (C-26*, **9.2**) 19.39 (C-19) 19.05 (C-27**, **9.1**) 18.99 (C-27**, **9.2**) 18.79 (C-21, **9.2**) 12.23 (C-29) 12.05 (C-18, **9.1**) 11.86 (C-18, **9.2**). *,** Pairs interchangeable.

The antiherpetic activities of **1** and **4** were not determined because of the minute amount. The compounds **3**, **5**, and **6** were inactive. The chemical structures of **1**, **3**, **4**, **5**, and **6** were identified as follows.

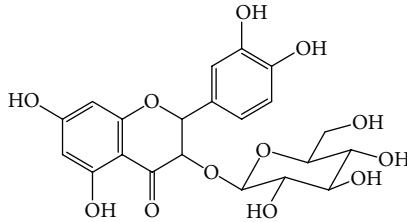
1: 5-O-Caffeoyl-D-Quinic Acid (Chlorogenic Acid, Figure 3). Colorless amorphous powder; UV (MeOH) λ_{\max} 326 nm, shoulder at 299 nm; ESI MS (pos. ion mode) m/z 355 [M+H]⁺; (calcd for $\text{C}_{16}\text{H}_{18}\text{O}_9$, 354); $^1\text{H-NMR}$ ($\text{DMSO}-d_6$, 300 MHz) δ 7.40 (d, $J = 16.0$ Hz, H-7') 7.00 (d, $J = 2.0$ Hz, H-2'), 6.94 (dd, $J = 8.0, 2.0$ Hz, H-6') 6.73 (d, $J = 8.0$ Hz, H-5') 6.14 (d, $J = 16.0$ Hz, H-8') 5.10 (ddd, $J = 8.0, 7.0, 6.0$ Hz, H-5ax), 3.90 (broad, H-3eq), 3.48 (dd, $J = 7.0, 4.0$ Hz, H-4ax), 1.97 (m, H-2a), 1.86 (m, H-6), 1.71 (m, H-2b); $^{13}\text{C-NMR}$ ($\text{DMSO}-d_6$, 75 MHz): δ 175.00 (C-7), 165.90 (C-9'), 148.20 (C-3'), 145.47 (C-4'), 144.67 (C-7'), 125.56 (C-1'), 121.18 (C-6'), 115.68 (C-5'), 114.70 (C-8'), 114.45 (C-2'), 73.20 (C-1), 71.50 (C-3), 71.19 (C-5), 69.52 (C-4), 36.92 (C-2).

3: A Mixture of Kaempferyl-3-O- α -L-Rhamnosyl (1→6)- β -D-Glucopyranoside (**3.1**, Figure 6) and Kaempferyl-3-O- α -L-Rhamnosyl (1→6)- β -D-Galactopyranoside (**3.2**, Figure 7). Yellow amorphous powder, mp 188–190°C (Lit. mp 198–200°C) [12]; UV (MeOH) λ_{\max} 265 nm (band II) and



3.2

FIGURE 7



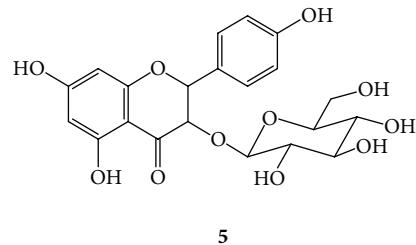
4

FIGURE 8

349 nm (band I); $^1\text{H-NMR}$ ($\text{DMSO}-d_6$, 300 MHz) δ 12.48 (d, $J = 3.7$ Hz, 2 \times 5-OH) 10.10 (OH) 8.04 (d, $J = 8.9$ Hz, H-2', H-6', 3.1) 7.96 (d, $J = 9.0$ Hz, H-2', H-6', 3.2) 6.87 (d, $J = 8.9$ Hz, H-3', H-5', 3.1) 6.85 (d, $J = 8.9$ Hz, H-3', H-5', 3.2) 6.18 (d, $J = 2.0$ Hz, H-6a, H-8a) 6.40 (d, $J = 1.8$ Hz, H-6b) 6.38 (d, $J = 2.0$ Hz, H-8b) 5.31 (d, $J = 7.5$ Hz, H-1'') 5.27 (d, $J = 1.5$ Hz, H-1'') 5.16 (d, $J = 4.4$ Hz, OH) 5.05 (d, $J = 4.7$ Hz, OH) 5.03 (d, $J = 6.3$ Hz, OH) 4.88 (d, $J = 5.0$ Hz, OH) 4.55 (d, $J = 5.3$ Hz, OH) 4.52 (d, $J = 4.1$ Hz, OH) 4.38 (d, $J = 6.0$ Hz, H-1'') 4.37 (d, $J = 6.0$ Hz, H-1'') 3.68 (d, $J = 10.0$ Hz, OH) 3.58 (m, H-2'', H-4'', H-6'') 3.10–3.58 (m, H-3'', H-5'', H-2''', H-3''', H-4''', H-5''') 1.05 (d, $J = 6.1$ Hz, 6'''-CH₃-Rha) 0.97 (d, $J = 6.3$ Hz, 6'''-CH₃-Rha).

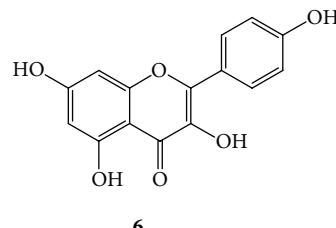
$^{13}\text{C-NMR}$ ($\text{DMSO}-d_6$, 75 MHz) δ 177.00 (C-4) 165.00 (C-7) 161.00 (C-5) 160.00 (C-4') 156.00 (C-2, C-9) 133.00 (C-3) 131.00 (C-2', C-6') 121.00 (C-1') 115.01 (C-3', C-5', 3.2) 115.00 (C-3', C-5', 3.1) 103.00 (C-10) 101.00 (C-1'') 100.00 (C-1'') 98.50 (C-6) 93.50 (C-8) 75.65 (C-3'', 3.1) 75.50 (C-5'', 3.1) 73.93 (C-2'', 3.1) 73.31 (C-5'', 3.2) 72.74 (C-3'', 3.2) 71.50 (C-2'', 3.2) 71.50 (C-2''', C-4''', 3.1) 70.33 (C-2''', C-4''', 3.2) 70.33 (C-3''') 70.02 (C-4'', 3.1) 67.66 (C-4'', 3.2) 67.66 (C-5'') 66.49 (C-6'') 17.41 (C-6''', 3.2) 17.31 (C-6''', 3.1) [13].

4: *Quercetin-3-O-β-D-Glucopyranoside* (Figure 8). Yellow amorphous powder; UV (MeOH) λ_{\max} 256 nm (band II) and 358 nm (band I); $^1\text{H-NMR}$ (CD_3OD , 300 MHz) δ 7.72 (d, $J = 2.1$ Hz, H-2') 7.52 (dd, $J = 8.5, 2.2$ Hz, H-6') 6.89 (d, $J = 8.5$ Hz, H-5') 6.36 (d, $J = 2.0$ Hz, H-8) 6.21 (d, $J = 2.0$ Hz, H-6) 5.15 (d, $J = 7.5$ Hz, H-1'') 3.71 (dd, $J = 11.9, 2.4$ Hz, H-6''a) 3.56 (dd, $J = 11.9, 5.2$ Hz, H-6''b) 3.48 (dd, $J = 8.0, 7.5$ Hz, H-2'') 3.42 (dd, $J = 9.0, 8.0$ Hz, H-3'') 3.34 (dd, $J = 10.0, 9.0$ Hz, H-4'') 3.22 (ddd, $J = 10.5, 5.5, 2.5$ Hz, H-5''); $^{13}\text{C-NMR}$ (CD_3OD ,



5

FIGURE 9



6

FIGURE 10

75 MHz): δ 179.49 (C-4) 166.19 (C-7) 159.04 (C-3) 158.49 (C-2, C-9) 149.86 (C-4') 145.92 (C-3') 135.60 (C-3) 123.19 (C-1') 123.09 (C-6') 117.56 (C-5') 116.03 (C-2') 105.39 (C-10) 104.31 (C-1'') 99.65 (C-6) 94.78 (C-8) 78.38 (C-3'') 78.11 (C-5'') 75.72 (C-2'') 71.24 (C-4'') 62.56 (C-6'').

5: *Kaempferyl Glucopyranoside* (Figure 9). Yellow amorphous powder, mp 165–170°C (with decomposition) [14]; UV (MeOH) λ_{\max} 266 nm (band II) and 349 nm (band I); IR (KBr) ν_{\max} 3350–3450 (O-H), 2933 (aliph. (C-H)), 1633 (C=O), 1611 (C=C), 1604 (C=O) cm⁻¹; ESI MS: M_R 448. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$, 300 MHz) δ 12.50 (broad s, 5-OH), 8.01 (d, $J = 9.0$ Hz, H-2', H-6', each), 6.84 (d, $J = 9.0$ Hz, H-3', H-5', each), 6.38 (d, $J = 2$ Hz, H-8), 6.17 (d, $J = 2$ Hz, H-6), 5.41 (d, $J = 7.1$ Hz, H-1''), 5.24 (2''-OH), 4.96 (5''-OH), 4.86 (3''-OH, 4''-OH), 4.16 (6''-OH), 3.55–3.30 (broad s, H-6''), 3.18 (H-5''), 3.16 (broad s, H-2''), 3.06 (broad s, H-3'', H-4''); $^{13}\text{C-NMR}$ ($\text{DMSO}-d_6$, 75 MHz) 177.13 (C-4), 165.10 (C-7), 161.09 (C-5), 156.43 (C-9), 155.88 (C-2), 133.08 (C-3), 130.75 (C-2', C-6'), 120.88 (C-1'), 115.02 (C-3', C-5'), 103.43 (C-10), 100.97 (D, C-1''), 99.00 (C-6), 93.78 (C-8), 77.41 (C-3''), 76.39 (C-5''), 74.17 (C-2''), 69.84 (C-4''), 60.80 (C-6'').

6: *Kaempferol (3,5,7,4'-tetrahydroxyflavone, Figure 10)*. Yellow amorphous powder; UV (MeOH) λ_{\max} 260 nm (shoulder) and 365 nm (band I); $^1\text{H-NMR}$ (CD_3OD , 300 MHz) δ 8.07 (d, $J = 8.5$ Hz, H-2', H-6'), 6.91 (d, $J = 8.5$ Hz, H-3', H-5'), 6.40 (d, $J = 1.7$ Hz, H-8), 6.19 (d, $J = 1.7$ Hz, H-6); $^{13}\text{C-NMR}$ (CD_3OD , 75 MHz) δ 177.42 (C-4), 165.56 (C-7), 162.46 (C-5), 160.56 (C-4'), 158.24 (C-9), 148.10 (C-2), 137.16 (C-3), 130.68 (C-2', C-6'), 123.76 (C-1'), 116.31 (C-3', C-5'), 104.56 (C-10), 99.29 (C-6), 94.486 (C-8).

To evaluate the antiherpetic effectiveness of *G. procumbens*, we performed the double-blind, randomized, controlled clinical trial in patients with recurrent herpes labialis of the

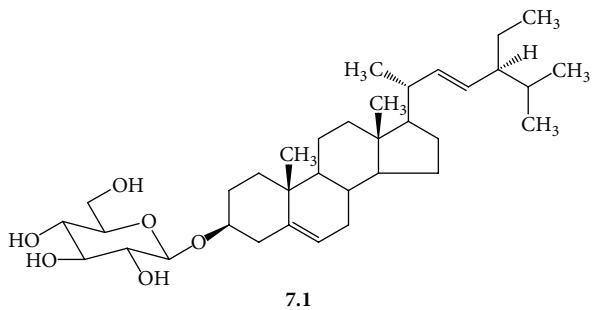


FIGURE 11

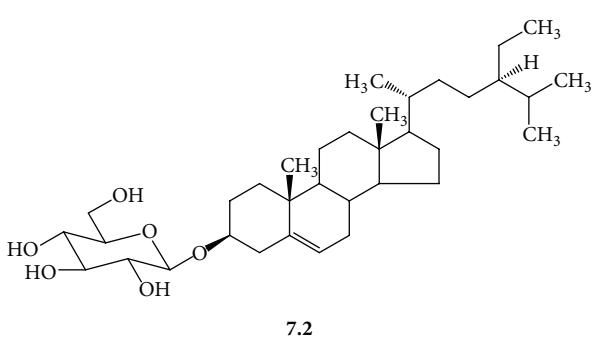


FIGURE 12

herbal products, which contained 1 and 2% of the standardized plant extracts.

A total of 65 patients participated in the study. Four patients (three in placebo group, one in the treated group) did not appear for the followup. One patient in the treated group did not record the date of full crusting and complete healing. One patient in the placebo group could not continue treatment due to allergic contact dermatitis. Therefore, only 59 patients were evaluated. They were divided into the treated group with 1% herbal gel (19 patients), the treated group with 2% herbal gel (22 patients), and the placebo group (18 patients). The antiherpetic results of the treated groups (1 and 2% herbal gels) were not different significantly. The data from both treated groups were thus combined (41 patients, 10 males and 31 females) and compared to the placebo group which comprised 18 patients (3 males, 15 females). Both groups were comparable for age, sex, pain, and itching scores, proportion of patient who are suffering from pain, and percentage of patients who had positive tests for Tzanck smear and viral culture. The only significant difference was the proportion of itching patients. It was greater in the treated group as shown in Table 2.

The positive results of Tzanck smear of the treated and control groups were 70.0 and 72.2%, respectively. The viral culture showed the presence of HSV-1 in the treated and control groups 46.2 and 31.3%, respectively. The patients in the treated and control groups were not significantly different, apart from the itching in the treated group, which was significantly different from the control (Table 2).

The first followup (D2, D4) indicated that the treated group still suffered the pain, but the number decreased from

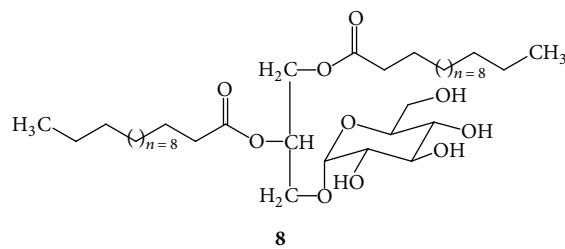


FIGURE 13

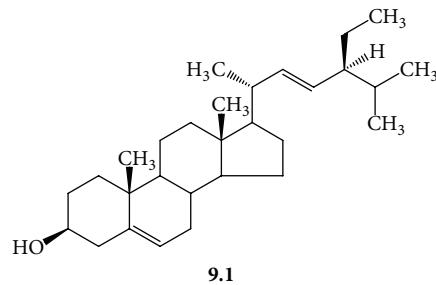


FIGURE 14

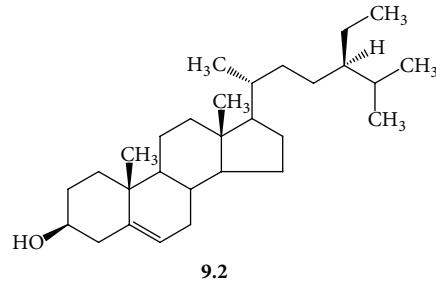


FIGURE 15

27 patients (65.90%) to 20 patients (34.15%), the itching decreasing from 68.29 to 48.78%. The patients who are suffering from pain in the placebo group decreased from 11 in 18 patients (61.10%) to 6 in 18 patients (33.33%), but the proportion of patients with itching remain unchanged (7 in 18 patients, 38.89%). The average pain scores of the treated and placebo groups were 1.11 and 1.41, respectively. The average itching scores of the treated and the placebo groups were 1.60 and 1.44, respectively. The results of both groups were not significantly different (Table 3).

Twenty-one of 41 patients in the treated group (51.22%) had full crusting within 4 days. It was comparable to 9 of 18 patients (50%) in the placebo group. Lesions completely healed within 7 days in twenty-six patients (63.41%) of the treated group whereas it was 8 in 18 patients (44.44%) for the placebo group. In the treated group, the average time for full crusting was 4.9 days and for complete healing was 8.7 days. The same figures for the placebo group were 5.1 and 9.4 days, respectively. The viral culture on D7 showed the number of patients in the treated group who had positive culture to be reduced from 19 to 3 whereas those in the placebo group

TABLE 2: Patients clinical and laboratory data before treatment in the treatment and placebo groups.

Patients	The treated groups	The placebo groups	P value
Sex			
Male	10	3	0.51
Female	31	15	
Total	41	18	
Age (years)			
Average	37.15	41.72	
Range	16–71	20–69	0.20
Standard deviation	11.31	15.25	
Pain on the first day (D0)			
Case/total (%)	27/41 (65.90%)	11/18 (61.10%)	0.72
Pain score (average)	2.85	2.86	0.90
Itching on the first day (D0)			
Case/total (%)	28/41 (68.29%)	7/18 (38.89%)	0.03
Itching score (average)	2.73	2.10	0.46
Tzanck smear before the treatment			
Positive result (%)	70.0	72.2	0.86
Viral culture before the treatment			
Positive result (%)	19/39 (48.7)	5/16 (31.25)	0.23

TABLE 3: The results of the double-blind randomized, controlled clinical trial of *Gynura procumbens* gel product.

Result	The treated groups	The placebo groups	P value
The pain on D2–D4			
Number of patients/total (%)	14/41 (34.15)	6/18 (33.33)	0.95
Average pain score	1.11	1.41	0.65
The itching from D2–D4			
Number of patients/total (%)	20/41 (48.78)	7/18 (38.89)	0.48
Average itching score	1.60	1.44	0.80
The crust day			
Within 4 days (patients/total)	21/41 (51.22%)	9/18 (50.00%)	0.93
Average time (days)	4.9	5.1	0.84
The healing day			
Within 7 days (patients/total)	26/41 (63.41%)	8/18 (44.44%)	0.18
Average time (days)	8.7	9.4	0.56
The viral culture			
Proportion +ve culture (%)			
On D2–D4	8/38 (21.05)	3/15 (20.0)	1.0
On D7	3/39 (7.69)	3/15 (20.0)	0.33

reduced from 5 to 3 patients. The results differed from the placebo insignificantly (Table 3).

Side Effects. The blood chemistry (CBC, UA, SGOT, SGPT, BUN, and Cr) of the participated patients was normal after the treatment. There was one patient in the placebo group suffering from allergic contact dermatitis, so the treatment was discontinued. There were 10 patients (24.4%) in the treated group and 5 patients (27.8%) in the placebo group who had mild itching and irritation, but all patients could tolerate the symptoms without disruption of the treatment. The side effects in the treated and placebo groups were not significantly different.

4. Discussion

The phytochemical work on *G. procumbens* showed that the ethanol extract of the aerial plant parts, the drug material, had the virucidal action against HSV-1 and HSV-2 (IC_{50} 625.0 and 675.0 μ g/mL, resp.) and prevented the viral replication with IC_{50} 584.0 and 568.0 μ g/mL, respectively. Several antiherpetic compounds were isolated from the extract. Some of them such as dicaffeoylquinic acids (2) were known for the antiviral activity [15]. We found that the extract contained other antiherpetic compounds, that is, 7, 8, and 9. The antiherpetic activity against HSV-2 of 8, the glycosphingolipid, was stronger than 2 (dicaffeoylquinic acids).

In addition, several flavonoids were found in the extract. They probably exerted the activity at the concentration above the MNTD. The test concentrations should not exceed the MNTD because the cultured cells would be damaged. However, the presence of the flavonoids possibly imparted anti-inflammatory effect [1, 4, 16], which could alleviate the infectious symptoms.

The laboratory evidence directed us to carry on the clinical study. The double-blind randomized, controlled clinical trial of recurrent herpes labialis with herbal and placebo gels was conducted. The ethanol extract was standardized using a mixture of sitosteryl and stigmasteryl glucosides as a marker compound (standard) because it could be isolated in a sufficient amount without difficulty. The standardized extract was incorporated in gel base as 1 and 2% herbal gels. The placebo gel was prepared similarly, colored like herbal gel but without the extract.

We could not achieve the number of the participated patients as calculated (50 in each group), though the trials were conducted at three hospitals. There were only 65 patients participating in the trial, but only 59 patients completed the study. Thus, the power of this clinical trial reduced from 80% to only 50%. Though the antiherpetic result of herbal gel differed insignificantly from the placebo, it tended to have some benefits. These included the tendency of the decreasing number of patients who suffered from itches in the treated group, the greater number of patients healing within 7 days, and the lesser number of patients infected with HSV-1. From the traditional, laboratory, and clinical pieces of evidence, we could postulate that *G. procumbens* tended to reduce the suffering from HSV-1 infection and might have some antiherpetic action. We performed the stability test of the herbal gel and found that the appearance and the content of the marker compound remained unchanged when storing the gel at 25°C for 24 months and at 40°C for 6 months. To improve the efficacy result of this herbal gel for the treatment of recurrent herpes labialis, we recommend the change of gel base to reduce the irritation and increase the potency of it. For further clinical investigation, we recommend increasing patients number and high concentration of the plant extract in the herbal gel.

5. Conclusion

Gynura procumbens comprised antiherpetic compounds, that is, caffeoylquinic acid derivatives, phytosteryl glucosides, and glycoglycerolipids. The flavonoids in this plant possibly imparted anti-inflammatory effect which was advantageous to the herpetic patients. The laboratory evidence and the reduction of the infection incidence in patients supported the antiherpetic effect of *G. procumbens*. The insignificant result of the clinical study might arise from the low participated patient number and insufficient extract concentration in the herbal product.

Conflict of Interests

All authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Parkia speciosa Hassk.: A Potential Phytomedicine

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Parkia speciosa Hassk., or stink bean, is a plant indigenous to Southeast Asia. It is consumed either raw or cooked. It has been used in folk medicine to treat diabetes, hypertension, and kidney problems. It contains minerals and vitamins. It displays many beneficial properties. Its extracts from the empty pods and seeds have a high content of total polyphenol, phytosterol, and flavonoids. It demonstrates a good antioxidant activity. Its hypoglycemic effect is reported to be attributable to the presence of β -sitosterol, stigmasterol, and stigmast-4-en-3-one. The cyclic polysulfide compounds exhibit antibacterial activity, while thiazolidine-4-carboxylic acid possesses anticancer property. The pharmacological properties of the plant extract are described in this review. With ongoing research conducted on the plant extracts, *Parkia speciosa* has a potential to be developed as a phytomedicine.

1. Introduction

Parkia speciosa Hassk., or stink bean, is a plant that is abundantly found in the tropical regions like Malaysia, Indonesia, Thailand, and Philippines [1, 2]. It is a plant that belongs to the genus *Parkia* and species *speciosa* in the family Fabaceae (also placed in Leguminosae and Mimosaceae). It is known as petai in Malaysia, Singapore, and Indonesia [1, 3], sator or sataw in Thailand [4, 5], u'pang in Philippines [3], and yongchak in India [6]. It grows up to 40 meter high [6]. It bears green long and flat beans which are called pods in stalks. The stalks are 2 to 6 cm wide and 30 to 45 cm long. The light green stink bean seeds with seed coats are encapsulated in these pods (Figure 1). The seeds have a peculiar smell and can be eaten raw as “ulam” (a Malay word for uncooked) or cooked. The seeds are the most consumed “ulam” in Malaysia [7]. Half-ripened seeds are also usually pickled in brine. The plant seeds have been used by the locals to treat various diseases and symptoms like diabetes, kidney disorder, and headache [1, 8, 9].

2. Nutritional Values

P. speciosa seeds contain many nutritional values such as protein, fat, and carbohydrate. They are also a good source for minerals (Table 1). The seeds have a considerable amount of vitamin C [10] and α -tocopherol (vitamin E) [11]. Among fourteen types of vegetable which were commonly consumed by the southern Thais, *P. speciosa* seeds had relatively the highest content of thiamin (vitamin B₁, 2.8 μ g/g) but insignificant antithiamine factor [12]. High concentration of tannin was detected in its seed coats and pods compared to other fruit vegetables [5]. Tannin has been reported to decrease protein and amino acid digestibility [13]. Therefore, it is not advisable for children to consume the seeds in high amounts as good protein absorption is necessary for good body development.

3. Chemical Compounds

Table 2 tabulates the phytochemicals screening in various parts of *P. speciosa*. Almost all major chemical compounds



FIGURE 1: The pods and seeds with (grey, A) or without (green, B) seed coats of *Parkia speciosa*. The plant materials were collected from a plantation at Batang Kali, Selangor, Malaysia, in January 2013.

are present in the seeds. Phenolic compounds are also present in almost all parts of the plant. To date, not many studies have been done to elucidate the chemical properties in the pods. In the seeds, the terpenoids detected using gas chromatography were β -sitosterol, stigmasterol, lupeol, campesterol, and squalene [14, 15]. Interestingly, lupeol was found to possess anticarcinogenic [16], antinociceptive, and anti-inflammatory properties [17]. No flavonoid like quercetin, myricetin, luteolin, kaempferol, or apigenin was detected in the methanolic extract of *P. speciosa* seeds using a reversed-phase high performance liquid chromatography [18], but it was noted to be present in the ethanolic extract when screened using a colorimetric assay [10]. Besides, alkaloids and saponins were also found in the plant. The seeds also contain cyclic polysulfides, namely, hexathionine, tetrathiane, trithiolane, pentathiopane, pentathiocane, and tetrathiepane [19] which are responsible for its strong pungent smell and taste, while the presence of djenkolic acid in the seeds is thought to cause blockage of the ureter [20].

Chromatography analysis of the stink bean seeds had identified presence of fatty acids which were undecanoic, myristic, palmitic, oleic, linoleic, elaidic, stearic, stearoic, lauric, arachidonic, and linoleic acids [14]. In the seeds, formation of thiazolidine-4-carboxylic acid, a thioproline, was remarkably increased after boiling when detected using gas chromatography-thermal energy analyzer [21]. This compound was reported to have an anticarcinogenic property [22]. The chemical structures of lupeol, thiazolidine-4-carboxylic acid, and some cyclic polysulfides (tetrathiane, tetrathiepane, trithiolane, and pentathiocane) are shown in Figure 2.

4. Properties of *P. speciosa*

Studies conducted on *P. speciosa* have revealed many potential properties of the plant. However, it needs to be ventured further to understand and to identify its mechanism of actions for future use in humans. Each property of the plants is described and discussed.

4.1. Antioxidant Activity. Oxidative stress has been implicated to play an important role in many pathological

TABLE 1: Nutritional value of *Parkia speciosa* Hassk. seeds.

Component	Composition (per 100 g edible portion)
Ash (g)	1.2–4.6
Protein (g)	6.0–27.5
Fat (g)	1.6–13.3
Carbohydrate (g)	13.2–52.9
Crude fiber (g)	1.7–2.0
Energy (kcal)	91.0–441.5
Calcium (mg)	108.0–265.1
Iron (mg)	2.2–2.7
Phosphorus (mg)	115.0
Potassium (mg)	341.0
Magnesium (mg)	29.0
Manganese (ppm)	42.0
Copper (ppm)	36.7
Zinc (ppm)	8.2
Vitamin C (mg)	19.3
α -Tocopherol (mg)	4.15
Thiamin (mg)	0.28

References: [10–12, 70].

conditions such as hypertension [23], hyperbilirubinemia [24], stress-induced gastric lesion [25], hyperhomocysteine-mia [26], cancer [27], atherosclerosis [28], and diabetes [29]. Therefore, there is a growing interest to study plants with potential antioxidant property to treat various diseases. *P. speciosa* is not an exception.

Commonly, the simple ways to measure natural antioxidant in plant extracts are the total phenolic content, reducing ferric ion antioxidant potential (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assays. Plants are a major source of phenolic compounds such as cinnamic, p-coumaric, caffeic, ferulic, chlorogenic, protocatechuic, and gallic acids [10]. The total phenolic content assay commonly uses the gallic acid as a standard. The FRAP assay is done to determine the antioxidant activity of a compound by measuring its redox property to reduce the ferric ion by single electron donation, while the DPPH radical-scavenging assay measures the ability of the compound to donate hydrogen to DPPH radical. There is a strong positive correlation between the FRAP and DPPH assays [30, 31] and between both assays and the total phenolic content [31].

Table 3 summarizes screening studies of the antioxidant properties of *P. speciosa*. The antioxidant capacity was relatively very high in the pods and seeds mixture where the methanolic extract had larger capacities than the aqueous extract for all the three assays [32]. Methanolic extract contains hydrophilic and intermediate hydrophilic compounds, whereas the aqueous extract contains hydrophilic constituents only. This difference explains the greater capacity of the methanolic extract. Antioxidant activity was also present in the seeds and leaves of the *P. speciosa* but with lower activities when compared to the activity in the pod and seed mixtures. This suggests that the pods retain greater antioxidant content than the other parts of the plant.

TABLE 2: Phytochemical substances in *P. speciosa*.

Parts	Alkaloid	Saponin	Terpenoids	Phenolic	Flavonoid	Tannin
Seeds	+	-	+	+	+	-
Barks	+	-	-	+	-	ND
Leaves	-	-	+	+	+	ND
Seed coats	+	+	-	-	+	+
Pods	ND	ND	ND	+	ND	+

Abbreviations: +: present; -: absent; ND: not determined. References: [5, 10, 32, 71].

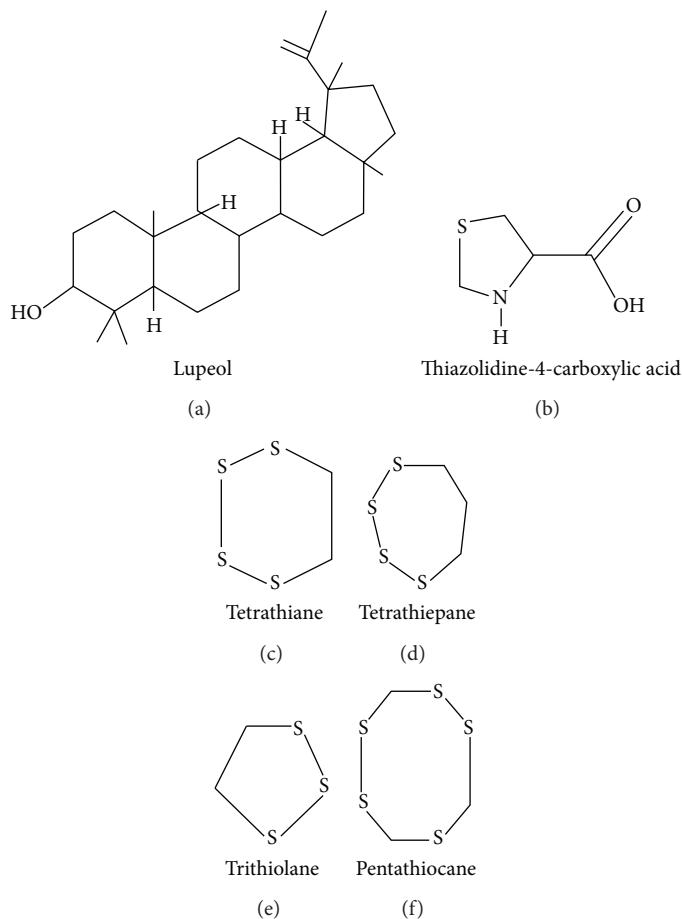


FIGURE 2: Chemical structures of lupeol (a), thiazolidine-4-carboxylic acid (b), and some cyclic polysulfides (c-f) found in *Parkia speciosa*.

Flavonoids which possess antioxidant property were detected in the ethanolic extract of the seeds [10], but none was found in its methanolic extract [18]. The antioxidant content and activity of *P. speciosa* is considered high amongst edible plants [10, 32, 33] especially its total phenolic contents. There was a difference in the total phenolic and flavonoid contents from the seeds extracted in ethanol and methanol. Both compounds were found to be higher in the ethanolic extract than the methanolic extract [10, 18], which could be due to more hydrophobic compounds being retained in the ethanol compared to the methanol extract. This is further shown in two different studies [18, 34] where the total phenolic content

was totally absent in the former study [18] but detected at a larger content in the latter study [34]. There are many factors that may affect the chemical content or composition in plants such as species, method of extraction, storage condition, and season and age of the plant parts at the time of harvest as well as geographical factors.

The DPPH radical-scavenging activity of the plant was also determined based on median inhibition concentration (IC_{50}). The pod ethanolic extract of the plant exhibited mean IC_{50} of $10.03 \mu\text{g/mL}$ compared to those of standard antioxidants used, quercetin ($0.45 \mu\text{g/mL}$), and butylated hydroxytoluene (BHT, $3.47 \mu\text{g/mL}$). The finding indicated

TABLE 3: Antioxidant activity in various *P. speciosa* extracts.

Plant part	Extract	Total phenolic content (mg GAE/g) ^a	DPPH assay (μmol Trolox/g) ^a	FRAP assay (μmol Trolox/g) ^a	Total flavonoids (mg RE/g) ^a	Tannin (mg/g) ^a	Reference
Pod and seed	Aqueous	1557.6 ^{b,c}	7418.3 ^{b,d}	1617.3 ^{b,d}	—	—	Ayub Ali et al. [32]
Pod and seed	Methanol	2464.3 ^{b,c}	5936.9 ^{b,d}	1898.0 ^{b,d}	—	—	Ayub Ali et al. [32]
Pod	Ethanol	—	—	—	—	250	Tunsaringkarn et al. [5]
Seed	Ethanol	51.9 ^a	—	—	20.3 ^a	—	Maisuthisakul et al. [10]
Seed	Methanol	—	—	—	0	—	Miean and Mohamed [18]
Seed	Methanol	120 ^{b,c}	40 ^{b,e}	—	—	—	Tangkanakul et al. [34]
Seed	Aqueous	6.5	67.62	44.67	—	—	Reihani and Azhar [30]
Seed coat	Ethanol	—	—	—	—	350	Tunsaringkarn et al. [5]
Leaf	Ethanol	44.7	89.26 ^f	—	—	—	Tangkanakul et al. [4]
Leaf	Aqueous	22.7	57.4 ^f	—	—	—	Tangkanakul et al. [4]
Leaf	Aqueous	32.73	22.7	49.9	—	—	Wong et al. [33]

^aDry weight basis, ^bfresh weight basis, ^c(mg GAE/100 g), ^d(mg Trolox/100 g), ^e(mg vitamin C equivalent/g), and ^f(mg BHA equivalent/g).

that the extract possessed a relatively high antioxidant property when compared to other plant extracts which gave IC₅₀ in the range of 0.06 to 2016.64 μg/mL [35]. Another similar study showed a lower DPPH radical-scavenging mean IC₅₀ of which was 0.667 μg/mL in the ethanolic extract of the seeds [10], suggesting that this extract also has higher antioxidant activity in the seeds.

The antioxidant activity of the plant was also assessed using a Heinz body induction using an *in vitro* model [5]. In the study, packed red cells that were mixed with acetylphenylhydrazine (a hemolytic agent) and incubated with the extracts from *P. speciosa* seed coat and pericarp (pods) showed lower Heinz body formation than other plant extracts. This indicated that the extracts were able to inhibit oxidative destruction to the erythrocytes. The seed coat extract exhibited the highest inhibitory activity, while the pericarp extract was the third highest amongst the twenty-one plants tested. The IC₅₀ for the former was 3.90 mg/mL and the latter was 46.29 mg/mL. The inhibitory activity was found to be positively correlated to the tannin concentration in the plants ($r = 0.658$, $P < 0.01$). This proved that tannin which was found in the plant extracts had a strong Heinz body inhibitory ability and antioxidant ability [5]. The findings of the study showed that *P. speciosa* has a potential to be used as an agent to reduce hemolytic jaundice.

4.2. Hypoglycemic Activity. Diabetes is a general term that describes a disease with an elevation of blood glucose more than normal or known as hyperglycemia, when the body is unable to metabolize glucose properly. There are two types of diabetes mellitus; insulin-dependent (IDDM, type 1) and noninsulin-dependent (NIDDM, type 2). The former type is manifested by a low insulin release due to destruction of the pancreatic cells and it can be controlled by regular administrations of insulin [36]. In type 2 NIDDM, the body fails to use insulin properly or becomes less responsive to insulin [37].

Many plants have been screened for its hypoglycemic property. Studies regarding the hypoglycemic property of *P. speciosa* had started in early 1990s [38, 39]. The plant showed good hypoglycemic activity in *in vivo* and *in vitro* experiments. Many crude *P. speciosa* extracts from the empty pods (pericarp) and seeds were tested for the activity. Research works done by Jamaluddin and Mohamed [38] and Jamaluddin et al. [40] showed that the antidiabetic activity was only observed in the chloroform extract, either in seeds or empty pods, and none in other extracts such as petroleum ether, dichloromethane, ethyl acetate, ammoniacal chloroform, and methanol. Oral administration of the chloroform extract of the empty pods and seeds significantly decreased glucose level 2 hours after ingestion and the effect lasted for at least 24 hours in alloxan-induced diabetic rats. The activity was higher in the seeds than in the empty pods [38] with a minimum effective dose of 25 mg/kg and 50 mg/kg, respectively [39, 40]. β-Sitosterol and stigmasterol, the two major phytosterols present in the seeds of *P. speciosa* were responsible for the hypoglycemic activity. They acted synergistically but no hypoglycemic effect was observed when they were tested individually [39], while in the empty pods, stigmast-4-en-3-one was identified and elucidated to be the active compound that produced the effect. It reduced blood glucose level by 84% at 100 mg/kg body weight compared to 111% reduction by glibenclamide (5 mg/kg body weight) [40]. However, the possible hypoglycemic mechanism of the pure compounds whether they had any effect on insulin release or glucose absorption from the gut was not elucidated in these studies.

Other than alloxan-induced diabetic rat model, the hypoglycemic property of the plant was also tested in *in vitro* experiments by measuring the activities of α-amylase and α-glucosidase. α-Amylase is an enzyme that is involved in carbohydrates breakdown to produce simpler saccharides, whereas α-glucosidase is the enzyme involved in the carbohydrates intestinal absorption [41]. Therefore, inhibition of both enzymes would be beneficial in NIDDM treatment due to the delayed digestion and uptake of glucose from

the intestinal tract. Decreased postprandial hyperglycemia is one of therapeutic approaches in the management of diabetes. Strong inhibitor of both enzymes like Acarbose [42] produces common side effects such as abdominal distention and flatulence, due to its strong inhibition on pancreatic α -amylase [43], thus results in colonic bacterial fermentation of undigested carbohydrates [44]. Therefore, a drug with a weak α -amylase inhibition but a good inhibitory property against α -glucosidase would be a good alternative.

The hexane and dichloromethane extracts of *P. speciosa* seeds showed no inhibitory effect on α -amylase activity [45]. However, the aqueous extract of the plant demonstrated a good α -glucosidase inhibitory activity with the empty pods having almost 15 times higher activity than the seeds [46, 47]. The α -glucosidase inhibitory activity was also observed in the petroleum ether, dichloromethane, and ethanolic extracts. Similar to the aqueous extract, the empty pods of these extracts possessed better activity than the seeds about twofold [48]. However, the aqueous extract of the seeds was able to increase insulin release [49].

P. speciosa has the potential to be developed as an oral hypoglycemic agent. Nevertheless, it was not shown to have any effect on blood glucose level in normal healthy experimental animals [38, 40]. Several possible mechanistic approaches need to be carried out before its possible use in humans.

4.3. Antitumor and Antimutagenicity. Cancer is one of the leading causes of death worldwide. Much attention has been paid to explore any potential antitumor agents in edible plants, for future use in humans. Amongst many medicinal plants screened, the methanolic extract of *P. speciosa* seeds demonstrated a moderate antimutagenic activity in the Ames test [34], while in inhibition assay of Epstein-Barr virus (EBV), the antitumor promoting activity of the seeds was considered as weakly active [50]. Nevertheless, it had been reported that consumption of the raw seeds reduced the incidence of esophageal cancer in Southern Thailand [51].

The methanolic extract of the seed coats exhibited selective cytotoxicity on breast cancer (MCG-7 and T47D), colon cancer (HCT-116), and hepatocarcinoma (HepG2) cells [52]. The methanolic extract of the seeds on the other hand did not show significant cytotoxic effect on any cancer cell lines. However, the ethyl acetate subextract of the methanolic extract showed selective cytotoxicity on hormone sensitive breast cancer cells, MCF-7 [53]. These findings are not conclusive yet due to the nature of the studies that used crude extract rather than pure compounds.

Development of a tumor is always associated with host immune response. Increased immune system enhances the ability of the host to resist tumor development as well as infectious diseases. Compounds with the ability to increase mitogenesis of lymphocytes may have a potential to be used as antitumor agents [54]. Lectin isolated from the *P. speciosa* seeds had shown mitogenic effect on human lymphocytes and rat thymocytes. The lectin stimulated incorporation of [3 H]-thymidine into cell DNA [55, 56]. Its activity increased with the increasing dose, before declining to an optimum point.

The effect was comparable to other known T-cell mitogens such as concanavalin A, pokeweed mitogen, and phytohemagglutinin [55]. Lectins with mitogenic activity usually exert antiproliferative, immunomodulatory, and antitumor properties [54]. Similar to lectins from other sources, the *P. speciosa* lectin also had a strong hemagglutinating activity for rabbit, goat, rat, and human erythrocytes [47, 56, 57]. These findings indicate that lectin from the seeds may increase DNA synthesis and, therefore, may enhance immune response against infections and tumors. The effect of natural products on tumor cell viability was found to be negatively associated with their mitogenic activity [58].

Angiogenesis is a critical process which is involved in many physiological and pathological conditions such as metastasis of solid tumors. The methanolic extract of the fresh pods of the *P. speciosa* demonstrated antiangiogenic activity. *In vitro*, the extract inhibited microvessel outgrowth in rat aortae more than 50%, which was not observed in the water and hexane extracts. It also inhibited the ability of human umbilical vein endothelial cells (HUVEC) to form capillary-like structures in matrigel matrix [53]. Both extracts of hexane and methanol of the seed coats showed antianangiogenic activity in rat aortic rings with vessels outgrowth inhibition of 74% and 82%, respectively [52]. The effect might be due to the formation of many vacuoles in the endothelial cells observed in light microscopy [53]. The presence of the vacuoles indicates cellular starvation due to nutritional deprivation which is an essential characteristic to maintain the viability of the cells [59]. This property is beneficial in the treatment of cancer due to its ability to prevent neovascularization of the tumors.

Thiazolidine-4-carboxylic acid, a thioproline, might be responsible for the antitumor effect of the seeds. It was shown to possess antiproliferative effects against cancer cells [22]. It was found in cooked seeds of *P. speciosa* but was undetectable in uncooked seeds [21]. The compound is an effective nitrite-trapping agent which can inhibit the endogenous formation of carcinogenic N-nitroso compounds [60]. Its derivatives were previously designed and synthesized as novel influenza neuraminidase inhibitors [61].

4.4. Antimicrobial Activity. The seeds of *P. speciosa* have been used by the Orang Asli in West Malaysia to treat kidney disorder which is believed to be urinary tract infection [1]. Studies regarding the antimicrobial property of *P. speciosa* are still lacking where, so far, only the seeds of the plant have been screened for its antimicrobial activity. The extracts of the seeds in petroleum ether, chloroform, and methanol demonstrated antibacterial activity against *Helicobacter pylori* but none was found in the water extract. The activity was the highest in the chloroform extract followed by methanol and petroleum ether. Comparatively, the chloroform extract showed a moderate inhibition zone diameter to mg extract ratio (25.0), while the ratio of other plant extracts was in the range of 1.5 to 117.5 [62]. A previous study also showed the ability of the seed extract in methanol to inhibit *H. pylori* growth, while the ethyl acetate extract was effective against *Escherichia coli*. These extracts, however, had no inhibitory

effect on *Salmonella typhimurium*, *Salmonella typhi*, and *Shigella sonnei* growth [63]. An aqueous suspension of the seeds displayed an ability to inhibit the growth of *Aeromonas hydrophila*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus anginosus*, and *Vibrio parahaemolyticus*. However, the suspension was ineffective against *Citrobacter freundii*, *Edwardsiella tarda*, *Escherichia coli*, *Vibrio alginolyticus*, and *Vibrio vulnificus*. These bacteria were isolated from moribund fishes and shrimps [64].

Collectively, it may be assumed that the seeds of *P. speciosa* are more effective against Gram-negative bacteria. However, the spectrum of the activity depends on the type of the extract. The antibacterial property is due to the seed content of hexathionine and trithiolane, two cyclic polysulfide compounds [19]. It was also screened for antiplasmoidal activity against *Plasmodium falciparum*, but no activity was found [65].

4.5. Effects on Cardiovascular System. Decoction of the roots of *P. speciosa* has been used in folk medicine in Malaysia to treat hypertension [9, 66]. To date, however, no scientific data regarding the plant effects on hypertension is available. Hypertension increases the risk of atherosclerosis, an artery clogging process which leads to heart attacks and strokes. Angiogenesis plays an important role in atherosclerosis. As mentioned earlier, the methanolic extract of the empty pods also possessed antiangiogenic property. It is suggestive that the plant extract may inhibit or reduce the development of atherosclerosis, thus needs to be explored further. Vascular endothelial growth factor (VEGF) is a factor that is involved in pathological angiogenesis or hypervascularization [67] which also plays a crucial role in atherosclerotic lesions [68]. The methanolic extract was shown to inhibit the expression of VEGF and neovascularization in rat aortic rings [53].

Its possible fibrinolytic activity was screened among other Thai indigenous plants using an *in vitro* experiment by measuring the clear zone area of fibrinogen and thrombin mixture. Relatively, *P. speciosa* had the lowest fibrinolytic activity which was only 1.5 mm^2 (the plant with the highest activity was 50.2 mm^2) [69]. Thus, it can be considered that the plant has no significant fibrinolytic activity.

5. Pharmacokinetics and Toxicity

To date there is no single study conducted on the pharmacokinetics of *P. speciosa*. This could be due to research being carried out on this plant so far only used its crude extracts rather than the pure compounds. A few studies conducted had detected the active compounds responsible for its hypoglycemic effects (β -sitosterol, stigmasterol, and stigmast-4-en-3-one), antibacterial activity (hexathionine and trithiolane), and antitumor (lectin and thiazolidine-4-carboxylic acid) [19, 39, 40, 56], but to date no study had used the pure compounds for the respective effects. Other studies performed were still at the stage of activity screening. Pharmacokinetic data of *P. speciosa* is important and vital in order to better understand its pharmacodynamic effects. The extent of its absorption would affect the amount of dosage

needs to be administered. Its metabolism pathway should also be studied to determine whether the produced metabolites are toxic or otherwise. Its excretion is believed to be through the kidneys due to the odorized urine after its consumption. Other possible routes are not known.

Its toxicity study is also lacking. No *in vivo* toxicity study has been carried out. Only Aisha et al. [53] had performed a cytotoxicity study of the plant using HUVEC. In their study, the methanolic extract of the fresh pods ($100 \mu\text{g/mL}$) did not show any significant cytotoxic effect on the cell lines. Information gathered from the locals, consumption of the seeds up to 30 seeds (two long pods) in a serving almost everyday does not cause any adverse effect.

6. Conclusion

Parkia speciosa Hassk. which is rich in antioxidant content especially total phenolic has many potentials to be developed as a phytomedicine. The properties are attributable to the presence of β -sitosterol, stigmasterol, stigmastenone, thiazolidine-4-carboxylic acid, hexathionine, and trithiolane in the plant. Traditionally, it is used to treat hypertension, diabetes, and headache, but with no scientific evidence so far. Many scientific studies have been performed on its hypoglycemic, antitumor, antimicrobial, and antiangiogenic properties. This still warrants further studies to explore the potential properties of the plant including its antihypertensive, analgesic, or anti-inflammatory (due to its lupeol and flavonoid contents) properties. Further toxicity studies and research in humans should also be conducted.

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Research Article

Antiviral Protein of *Momordica charantia* L. Inhibits Different Subtypes of Influenza A

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The new antiviral activity of the protein extracted from *Momordica charantia* was determined with different subtypes of influenza A. The protein was purified from the seed of *M. charantia* using an anion exchanger and a Fast Protein Liquid Chromatography (FPLC) system. At the concentration of 1.401 mg/mL, the protein did not exhibit cytotoxicity in Madin-Darby canine kidney cells (MDCK) but inhibited 1×10^5 FFU influenza A/PR/8/34 H1N1 virus at 56.50%, 65.72%, and 100% inhibition by the protein treated before the virus (pretreated), the protein treated alongside with the virus (simultaneously treated), and the protein treated after the virus (posttreated) during incubation, respectively. Using 5, 25, and 100 TCID₅₀ of influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2 and A/Thailand/1(KAN-1)/2004 H5N1, the IC₅₀ was calculated to be 100, 150, and 200; 75, 175, and 300; and 40, 75, and 200 $\mu\text{g}/\text{mL}$, respectively. Our present finding indicated that the plant protein inhibited not only H1N1 and H3N2 but also H5N1 subtype. As a result of the broad spectrum of its antiviral activity, this edible plant can be developed as an effective therapeutic agent against various and even new emerging subtypes of influenza A.

1. Introduction

Influenza A viruses have continued to be a significant public health concern with epidemics responsible for serious morbidity and mortality. Epidemics of influenza occur almost every year according to an antigenic drift of the two viral surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA). Currently, sixteen hemagglutinin subtypes (H1–H16) and nine neuraminidase subtypes (N1–N9) have been recognized [1]. In the year 1918, the highly pathogenic strains of influenza A virus emerged in an unpredictable manner, causing the death of 20–40 million people worldwide [2, 3]. Pandemic strains of the virus usually possess antigenically different, novel glycoproteins, causing the limitation of duration and cross strain protection of currently available vaccines. For example, the emerging strains of avian influenza viruses (H5N1) appeared in humans in Hong Kong in

1997 [4]. These viruses had an extremely high virulence in humans, killing 6 out of 18 infected individuals. Recently, the outbreak of influenza A/H5N1 infection has occurred among poultry in Asia and had transmission to 499 people in 15 countries with a high case fatality rate of approximately 60% [5]. According to the report of WHO on April 14, 2013, a new influenza virus H7N9 emerges in eastern China and possibly spreads into Vietnam, causing at least 13 deaths among 60 infections.

Presently, the two inhibitors of the M2 protein, amantadine, and rimantadine and the two neuraminidase inhibitors, zanamivir and oseltamivir are the available anti-influenza A agents [6]. However, up to 30% of individuals who received amantadine or rimantadine excreted viral resistant strains [7, 8]. Additionally, many reports indicated that neuraminidase inhibitor resistant strains arose rapidly in the presence of therapeutic agents [9–13]. Consequently, much attention has

been focused for the development of new antiviral drugs for the effective treatment and overcoming the resistant viruses. Many researchers are attempting to find new anti-influenza agents both from chemically synthesized and naturally active compounds [14–17]. Among the medicinal plants investigated, *Momordica charantia* L. that has been reported to contain many potent antiviral activities that might be a good candidate. For instance, the proteins from this plant strongly inhibit several viruses including hepatitis B virus, dengue virus, herpes simplex virus, and Human Immunodeficiency Virus [18–21]. *M. charantia*, an edible vegetable belonging to the Cucurbitaceae family, is commonly cultivated in Africa and Asia and is known as a bitter melon or bitter ground. It has been traditionally used as folk medicines for several ailments such as antidiabetic, antitumor, anthelmintic, antimarial, and antiviral agents [22]. For this current work, we have determined the new antiviral activity of the protein of *M. charantia* with various influenza A subtypes including influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1(KAN-1)/2004 H5N1. Our discovery has clearly indicated that the protein of *M. charantia* has an effective, broad antiviral activity against different subtypes of influenza A.

2. Materials and Methods

2.1. Plant Materials. The ripe fruits of *M. charantia* were purchased from a local market in Bangkok during January–February 2006. Briefly, the seeds were cleaned and dried by removing red tissue covers. Then, they were added with 10 mM sodium phosphate buffer pH 6.3 (PBS) containing 0.15 M NaCl with a ratio of 2 mL buffer for a gram of seeds and blended with a homogenizer for 5 minutes to form fine emulsion at 4°C. The emulsion was stirred at 4°C for 1 hour, filtrated through double cheesecloth, and centrifuged at 12,000 ×g for 10 minutes at 4°C. The supernatant was called the crude protein fraction.

2.2. Purification of the Protein by Fast Protein Liquid Chromatography. The crude protein was purified using a Hitrap Q anion exchanger equipped with a Fast Protein Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech, USA). Briefly, the crude protein fraction was filtrated with a syringe filter (0.45 μm) and applied to the FPLC at 4–10°C in a cool cabinet. The column was washed with PBS containing 1 N NaCl, and the protein was eluted by varying the pH and ionic strength. The proteins migrated under an electric field with the pH gradient corresponding to their pI. The UV detector was set at 280 nm and the fraction size of the proteins was collected for each 1 mL. The desirable peak of the protein fraction recorded by the UV detector was collected, and the protein sample was divided into small 0.5 mL tubes and kept at 4°C for further investigation.

2.3. Protein Concentration Determination and SDS-PAGE Analysis. The protein concentration was determined by Bradford assay with Coomassie Plus Protein Assay Reagent (PIERCE, USA) using a spectrophotometer (Spectronic 3000 array spectrophotometer, USA). SDS-PAGE was performed

according to the method described by Laemmli using the Bio-Rad Mini Protein apparatus (Bio-Rad, USA). Normally, 15% separating and 5% stacking gel with 100 constant voltage were used.

2.4. Cells and Viruses. MDCK cells was obtained from Virology Department, Microbial Research Institution, Osaka University (Riken cell bank), and maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% MEM nonessential amino acids, 1% penicillin-streptomycin solution, and 1% glutamine (Invitrogen). The influenza virus A/PR/8/34 H1N1 (PR 8) was also from Virology Department, Research Institution Microbial Disease, Osaka University. Influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1(KAN-1)/2004 H5N1 subtypes were provided from Virology Department, Siriraj Medicine Faculty, Mahidol University.

2.5. Cytotoxicity of Proteins by Cell Proliferation Assay. The cell proliferation assay kit (CHEMICON International) was used for fast and sensitivity quantification of cell proliferation and viability. The assay was based on the cleavage of tetrazolium salt WST-1 to formazan by cellular mitochondrial hydrogenases. One hundred μL (1×10^5 cells) of MDCK cells were added in each well of a 96-well plate. After incubation at 37°C, 5% CO₂ for 24 hours, twofold serial dilutions of the protein were added and incubated at 37°C, 5% CO₂ for 24 hours. Ten microliters of WST-1/ECS solution were added in each well and then incubated at 37°C, 5% CO₂ for 1 hour. The number of viable cells was measured using a colorimetric assay system that measured the reduction of tetrazolium salt to formazan at an absorbance of 450 nm.

2.6. Detection of Influenza Virus A/PR/8/34 (H1N1) (PR8) Infection in MDCK Cells by an Immunofluorescence Assay. The infection of influenza virus A/PR/8/34 in MDCK cells was determined by an immunofluorescence assay. A 24-well plate was added with 1000 μL/well (1×10^5 cells) of MDCK cells and incubated at 37°C for 24 hr. The cells were then added with 0.175 to 1.401 mg/mL of the protein sample and incubated at 37°C for 1 hour. The plate was washed with PBS buffer twice. Then, 1×10^5 focus-forming units/mL (FFU/mL) of the influenza virus A/PR/8/34 was inoculated in the wells, and the plates were further incubated at 37°C for 1 hr. After incubation, the cells were fixed with 500 μL of 4% paraformaldehyde. The infected cells were identified with the nucleoprotein antibody (150 μL/well) of influenza A virus (anti-NP : PBS = 1 : 1000) and incubated at room temperature for 30 min. Finally, 150 μL of fluorescein isothiocyanate conjugated goat anti-mouse (anti-FITC : PBS = 1 : 500) were added in each well. Influenza A-infected cells were observed under a fluorescence microscope.

2.7. Detection of Influenza Virus A/PR/8/34 Infection in MDCK Cells by a Peroxidase Antiperoxidase (PAP) Staining Method. After inoculation with influenza virus A/PR/8/34, MDCK cells were incubated at 37°C for 24 hours. The infected cells were fixed with methanol for 10 minutes and dried at

room temperature. Fifty μL of the first antibody (Mouse IgG to influenza NP : PBS = 1:1000) was added in each well, and the plates were incubated at 37°C for 30 minutes. After incubation, the plates were washed with PBS buffer twice. Fifty μL of the second antibody (Rabbit IgG to Mouse IgG : PBS = 1:1000) was added in each well, and the plates were incubated at 37°C for 30 minutes. The plates were washed twice with PBS buffer. Finally, 50 μL of the third antibody (Goat IgG to rabbit IgG : PBS = 1:500) was added in each well, and the plates were incubated at 37°C for 30 minutes and washed twice with PBS buffer. After washing, 50 μL of PAP solution (diluted with PBS to 1:1000) was added in each well, and the plates were incubated at 37°C for 30 minutes. Simultaneously, DAB substrate solution was prepared by adding approximately 2 mg of DAB and 2 μL of H₂O₂ in 10 mL of PBS. After washing, 50 μL DAB substrate solution was added in each well. The plates were incubated at room temperature for 20 minutes until the solution was changed to an umber color. After the plates were washed with tap water and dried at room temperature, the number of infected cells (foci) was counted under a light microscope.

2.8. Determination of the Incubation Time Effect of the Protein on Influenza Virus A/PR/8/34. In each experiment, 1×10^5 MDCK cells were plated in each well of a 96-well plate. For the pretreated assay, the cells were added with twofold serial dilutions of different protein (called the pretreated protein) concentrations ranging from 0.17 to 1.40 mg/mL and further incubated at 37°C 5% CO₂ for 1 hour. After washing with PBS twice, the cells were inoculated with 1×10^5 FFU/mL (1 MOI) of influenza virus A/PR/8/34 and incubated at 37°C 5% CO₂ for 24 hours. After incubation, the number of infected cells was determined by PAP staining assay as mentioned above.

For the simultaneously treated assay, the protein (called the simultaneously treated protein) was added immediately after the cells were inoculated with the virus. For the post-treated assay, the protein (called the posttreated protein) was added after the cells were preinoculated with the virus for one hour. The following steps were the same as described for the pretreated assay.

2.9. Determination of IC₅₀ of the Protein on Influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1(KAN-1)/2004 H5N1. The IC₅₀ values of the protein on different subtypes of influenza A were determined using 100, 25, and 5 tissue culture infectious dose₅₀ (TCID₅₀) of influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1(KAN-1)/2004 H5N1. Briefly, various protein concentrations ranging from 12.5 to 400 $\mu\text{g}/\text{mL}$ were added to each well containing 1×10^5 cells of MDCK cells. After incubation at 37°C, 5% CO₂ for 1 hour, the cells were individually inoculated with 1×10^5 FFU/mL of different subtypes of influenza A and further incubation overnight. The infected cells were individually identified with nucleoprotein antibody against different influenza A viruses using an immunofluorescence assay as mentioned previously. The IC₅₀ was determined by graphically plotting the inhibition of viral growth as a function of the protein concentrations.

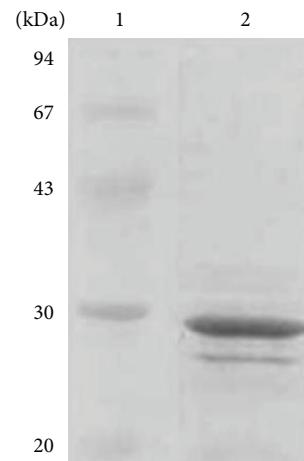


FIGURE 1: SDS-PAGE of the proteins isolated from *M. charantia*. The proteins were analyzed in 12% SDS-PAGE. Lanes 1 and 2 are molecular weight markers and the protein fraction after the FPLC separation, respectively.

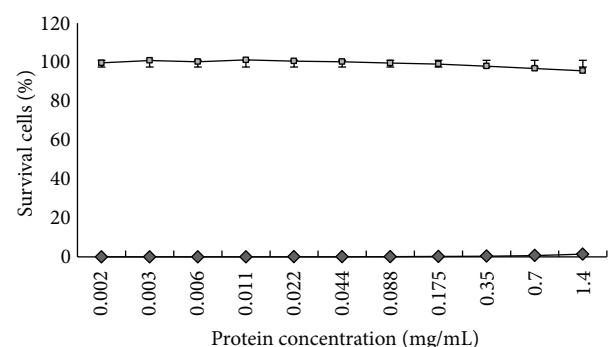


FIGURE 2: The cytotoxicity test of the protein of *M. charantia* on MDCK cells. The twofold serial dilution of the protein concentration ranging from 0.002 to 1.40 mg/mL was used in the experiment.

2.10. Statistical Analysis. Student's *t*-tests or one-way ANOVA were used for statistical analysis. In all cases, *P* values <0.05 were considered statistically significant.

3. Results

3.1. Purification and Characterization of the Protein from *M. charantia*. After being purified with a Hitrap Q anion exchanger and a Fast Protein Liquid Chromatography (FPLC) system, the majority of the protein band appeared on the SDS-PAGE was of 30 kDa (Figure 1). This partially purified protein was used to determine its biological activities.

3.2. Cytotoxicity of the Protein from *M. charantia*. To evaluate the cytotoxicity effect of extracted proteins, MDCK cells were grown in different dilutions of the protein for 24 hours. The protein concentration ranging from 0.002 to 1.40 mg/mL did not exhibit any cytotoxicity in MDCK cells (Figure 2).

3.3. The Protein Effectively Inhibited Influenza A/PR/8/34 H1N1 Demonstrated in MDCK Cells. The twofold serial

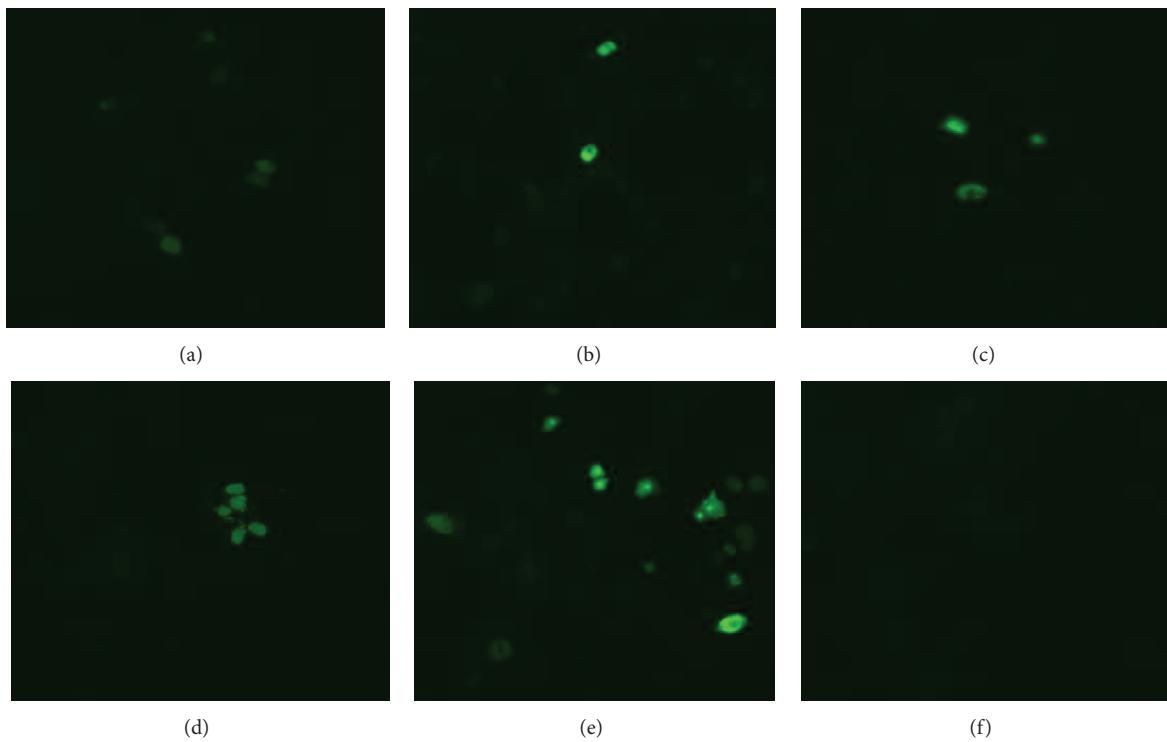


FIGURE 3: Determination of the inhibitory effect of the protein of *M. charantia* against influenza virus A/PR/8/34 (H1N1) infected in MDCK cells. The twofold dilution of the pretreated protein of 1.4 to 0.175 mg/mL ((a)–(d), resp.) was used on the assay. The viral infected cell (e) and noninfected cell (f) were used as the positive and negative controls, respectively.

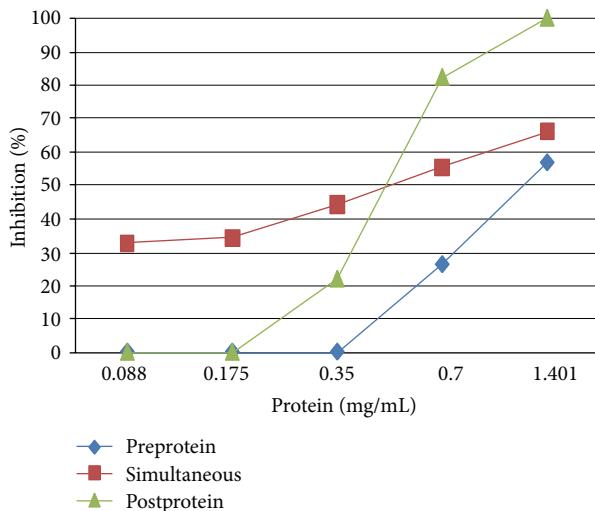


FIGURE 4: Determination of time incubation effect of the protein on influenza A/PR/8/34. The twofold serial dilutions of 0.088 to 1.40 mg/mL of the pretreated, simultaneously treated, and posttreated proteins were individually added in the viral infected cells.

dilutions of the extracted protein ranging from 0.0175 to 1.40 mg/mL were applied to determine the inhibitory effect against influenza virus A/PR/8/34 H1N1 at 0.1 MOI in 1×10^5 MDCK cells (Figure 3). The protein (the pretreated protein) was added in each well for one hour before adding the virus. By using the immunofluorescence assay, the viral infection

in MDCK was clearly reduced, especially at the protein concentration of 1.40 mg/mL (Figure 3(a)).

3.4. Time Incubation Effect of the Protein on Influenza Virus A/PR/8/34. In order to determine the time incubation effect of the protein on influenza virus A/PR/8/34, the twofold serial dilution of 0.088 to 1.40 mg/mL of the pretreated, simultaneously treated, and posttreated proteins were individually added in the viral infected cells. The results indicated that at the concentration of 1.40 mg/mL of the pretreated, simultaneously treated, and posttreated proteins exhibited different antiviral efficacy of 56.50%, 65.72%, and 100% inhibition, respectively (Figure 4).

3.5. IC_{50} of the Posttreated Protein on Influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1 (KAN-1)/2004 H5N1. The IC_{50} values of the posttreated protein against different subtypes of influenza A were determined. The results showed that at 5, 25, and 100 TCID₅₀ of influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1 (KAN-1)/2004 H5N1, the IC_{50} values were calculated to be 100, 150, and 200; 75, 175, and 300; and 40, 75, and 200 μ g/mL, respectively (Figure 5).

4. Discussion

Momordica charantia was reported to possess several antiviral activities including hepatitis B virus, dengue virus, and Human Immunodeficiency Virus. In our laboratory, we

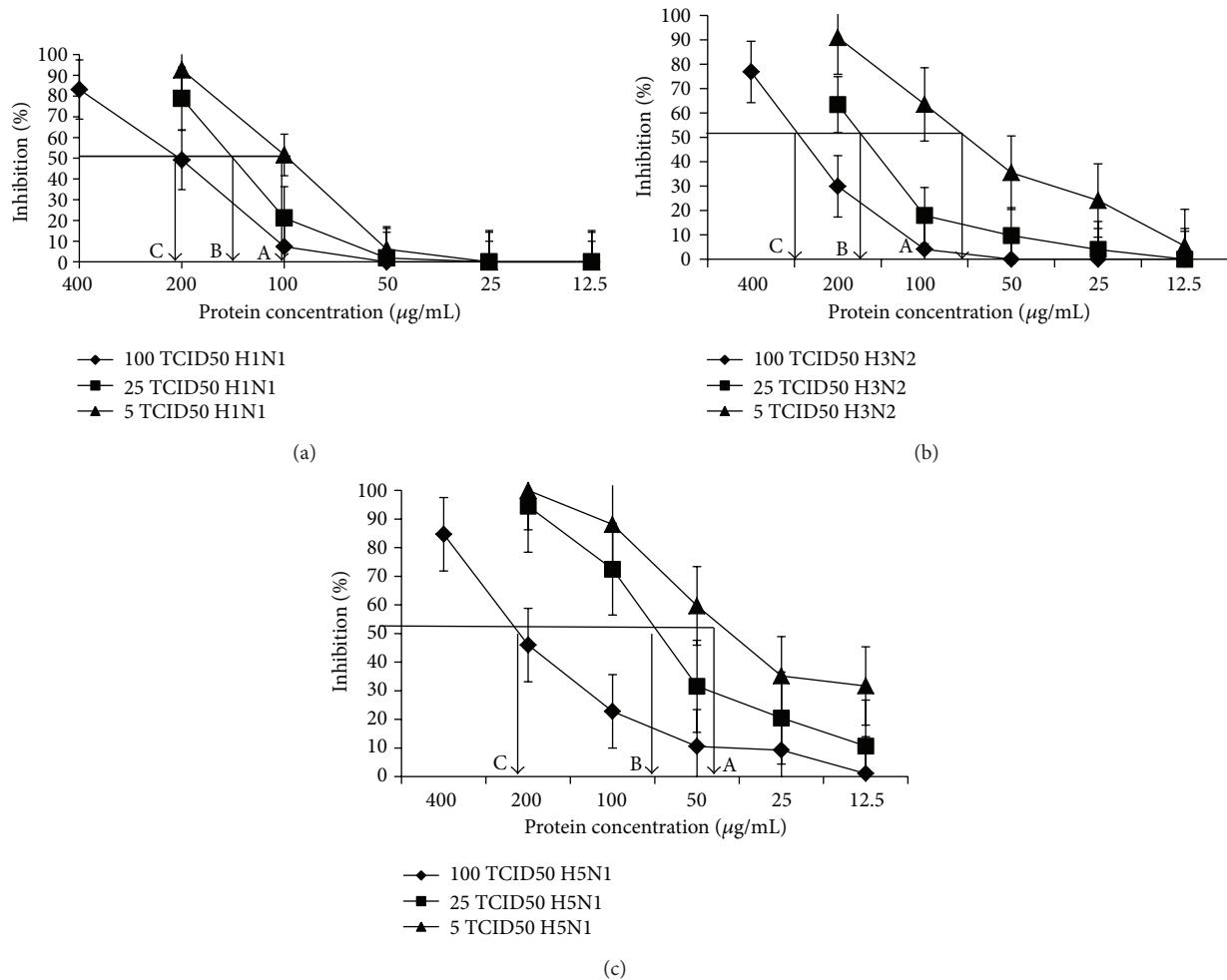


FIGURE 5: The IC_{50} of the posttreated protein on influenza A/New Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1 (KAN-1)/2004 H5N1 ((a), (b), and (c), resp.). Using 5, 25, and 100 TCID₅₀, the IC_{50} was calculated by graphically plotting the % inhibition of viral growth as a function of the protein concentrations.

discovered that the protein purified from the ripe seeds of *M. charantia* contained effectively anti-HIV-1 activity [20]. For the present work, we have determined the novel antiviral property of the protein from this plant. At the concentration of 1.401 mg/mL, the protein did not exhibit any cytotoxicity in MDCK cells but effectively inhibited H1N1 as a dose-dependent manner (Figures 2 and 3). For the incubation time effect of the protein on H1N1, the posttreated protein displayed more potent antiviral action than the pretreated and simultaneously treated protein. This information implies that the protein of *M. charantia* can be effectively used after patients are being exposed to the virus. Interestingly, we found that the posttreated protein of 1.401 mg/mL completely inhibited at least 10^7 FFU/mL of H1N1 (data not shown). This discovery confirmed the high antiflu activity of the protein of *M. charantia*.

To further investigate the antiviral activity of the plant protein, the MDCK cells were prior infected with Caledonia/20/99 H1N1, A/Fujian/411/01 H3N2, and A/Thailand/1 (KAN-1)/2004 H5N1 and later treated individually with the

postprotein. As expected, the protein strongly inhibited not only H1N1 but also H3N2 and H5N1 subtypes. Its inhibitory effect was almost equally potent against the three viral subtypes. At the high dose of 100 TCID₅₀, the protein still exhibited effective anti-influenza activity. Taken together with the former finding of its anti-HIV property, we believe that the protein of *M. charantia* possess the broad antiviral activity against various types of viruses in addition to HIV and influenza. According to previous publications, the antiviral and anticancer activities of the proteins belong to the action of the protein called ribosomal inactivating proteins (RIPs). So far, several RIPs have been identified from the plants in the Cucurbitaceae family such as MAP30 from *M. charantia* and GAP31 from *Gelonium multiflorum* [20, 23]. Additionally, our recent finding indicates that Cochinin B, a novel RIP purified from the seeds of *Momordica cochinchinensis*, contained a potent antitumor activity [24]. Nevertheless, whether the anti-influenza activity of the protein from *M. charantia* is associated with the action of RIP or not, further investigation is needed.

5. Conclusion

We have found that the protein purified from *M. charantia* possessed effective antiviral activity to a broad range of influenza A subtypes including H1N1, H3N2, and H5N1. Thus, this plant protein holds a great promise to be developed as an effective therapeutic agent against various and even new emerging subtypes of influenza A such as H7N9, which is now pandemic in China.

Conflict of Interests

The authors would like to declare that there is no conflict of interests.

Acknowledgment

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Review Article

***Bidens pilosa* L. (Asteraceae): Botanical Properties, Traditional Uses, Phytochemistry, and Pharmacology**

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There are 230 to 240 known *Bidens* species. Among them, *Bidens pilosa* is a representative perennial herb, globally distributed across temperate and tropical regions. *B. pilosa* has been traditionally used in foods and medicines without obvious adverse effects. Despite significant progress in phytochemical and biological analyses of *B. pilosa* over the past few years, comprehensive and critical reviews of this plant are anachronistic or relatively limited in scope. The present review aims to summarize up-to-date information on the phytochemistry, pharmacology, and toxicology of *B. pilosa* from the literature. In addition to botanical studies and records of the traditional use of *B. pilosa* in over 40 diseases, scientific studies investigating the potential medicinal uses of this species and its constituent phytochemicals for a variety of disorders are presented and discussed. The structure, bioactivity, and likely mechanisms of action of *B. pilosa* and its phytochemicals are emphasized. Although some progress has been made, further rigorous efforts are required to investigate the individual compounds isolated from *B. pilosa* to understand and validate its traditional uses and develop clinical applications. The present review provides preliminary information and gives guidance for further basic and clinical research into this plant.

1. Introduction

The United Nations World Health Organization estimates that as many as 5.6 billion people, 80% of the world population, utilize herbal medicine for primary health care [1]. Plants have formed the foundation of complicated traditional medicine systems for thousands of years. Medicinal herbs are applied to treat a wide range of disease categories. The first written documentation of the use of medicinal herbs dates from the 26th century BCE in Mesopotamia, and the first record of the use of medicinal herbs by the Egyptians and Greeks dates from 18th century BCE and the 5th century BCE, respectively. Starting around the 11th century BCE, the Chinese and Indians started to develop herbal medicine systems, Chinese herbal medicine, and Ayurvedic medicine,

respectively, that continue to be widely practiced today [2]. Therefore, since antiquity, medicinal herbs have played a prominent role in human health.

B. pilosa is an easy-to-grow herb that is widely distributed all over the world. It is considered to be a rich source of food and medicine for humans and animals [3, 4]. There is increasing global interest in the use of *B. pilosa* as shown by the many studies conducted on the plant in recent years. The folkloric use of *B. pilosa* has been recorded in America, Africa, Asia, and Oceania [5]. To explore the potential clinical application of *B. pilosa*, it is important to link its traditional use with rigorous evidence-based scientific study. The present review focuses on recent studies on the botany, traditional usage, phytochemistry, pharmacology, and toxicology of *B. pilosa*. The information provided here highlights the possible

TABLE 1: Taxonomy of *B. pilosa* [6].

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	<i>Bidens</i>
Species	<i>Bidens Pilosa L.</i>

usefulness of *B. pilosa* and its isolated compounds and offers insights into possible future research directions. Studies of *B. pilosa* are divided into three groups: (1) the botany, ethnomedical uses, plant chemistry, pharmacology, and biosafety of *B. pilosa*; (2) scientific studies that validate the ethnomedical uses of *B. pilosa*; and (3) the therapeutic and future research potential of *B. pilosa*.

1.1. Botany. *B. pilosa* was first collected and named by Carl Linnaeus in 1753 [3]. Taxonomically, it is assigned to the *Bidens* genus (Asteraceae) as shown in Table 1. This genus is estimated to include 230 to 240 species worldwide [3, 4]. *B. pilosa* has several varieties such as *B. pilosa* var. *radiata*, var. *minor*, var. *pilosa*, and var. *bisetosa*. Alongside examination of morphological traits, authentication of *B. pilosa* can be aided by chemotaxonomy and molecular characterization [7].

B. pilosa is an erect, perennial herb widely distributed across temperate and tropical regions. *B. pilosa* is either glabrous or hairy, with green opposite leaves that are serrate, lobed, or dissected. It has white or yellow flowers, and long narrow ribbed black achenes (seeds). It grows to an average height of 60 cm and a maximum of 150 cm in favorable environments [8] (Figure 1). *B. pilosa* prefers full sun and moderately dry soil. However, it can grow in arid and barren land from low to high elevations. With the advantage of being fast-growing, in the 1970s, the Food and Agricultural Organization actively promoted the cultivation of *B. pilosa* in Africa [9]. *B. pilosa* propagates via seeds. A single plant can produce 3000–6000 seeds. Dry mature seeds from *B. pilosa* can be germinated in 3 to 4 days in moist soil or after being soaking in water. Seeds are viable for at least 3 years [10]. Minimal agricultural techniques are required for *B. pilosa* cultivation. Due to its invasive tendencies, *B. pilosa* is generally considered to be a weed [11].

B. pilosa is thought to have originated in South America and subsequently spread all over the world [12]. *Bidens* species and their varieties bear vernacular names based on their characteristics. For example, *Bidens* species are known by such names as Spanish needles, beggar's ticks, devil's needles, cobbler's pegs, broom stick, pitchforks, and farmers' friends in English and some other languages because of their sticky achenes [8] and are sometimes known as *xian feng cao* ("all bountiful grass") in Chinese because of their prosperous growth.

1.2. Traditional Uses. *B. pilosa* is used as an herb and as an ingredient in teas or herbal medicines. Its shoots and leaves, dried or fresh, are utilized in sauces and teas [13, 14]. In the 1970s, the United Nations Food and Agriculture Organization (FAO) promoted the cultivation of *B. pilosa* in Africa because it is easy to grow, edible, palatable, and safe [15]. The nutritional value of *B. pilosa* is shown in Table 2.

All parts of *B. pilosa* plant, the whole plant, the aerial parts (leaves, flowers, seeds, and stems), and/or the roots, fresh or dried, are used as ingredients in folk medicines. It is frequently prepared as a dry powder, decoction, maceration or tincture [16]. Generally, this plant is applied as dry powder or tincture when used externally, and as a powder, maceration, or decoction when used as an internal remedy [14].

As summarized in Table 3, *B. pilosa*, either as a whole plant or different parts, has been reported to be useful in the treatment of more than 40 disorders such as inflammation, immunological disorders, digestive disorders, infectious diseases, cancers, metabolic syndrome, wounds, and many others [17–20]. *B. pilosa* is usually ingested; however, it can also be utilized externally. For instance, fresh *B. pilosa* is used to treat snake bites and wounds [21], and in Trinidad and Tobago the aqueous solution of the leaves of *B. pilosa* is used to bathe babies and children [22].

B. pilosa is sometimes used alone; it is also used as an ingredient in medicinal mixtures together with other medicinal plants such as *Aloe vera*, *Plectranthus mollis*, *Valeriana officinalis*, and *Cissus sicyoides* among others [23–26]. Whether the commonly used mixtures of *B. pilosa*, or the mixtures of the compounds found in *B. pilosa* afford synergistic effects is not yet clear and needs to be verified by further studies. However, one study has suggested that *B. pilosa* varieties share similar phytochemical compositions and may be substituted for each other [7].

2. Phytochemicals

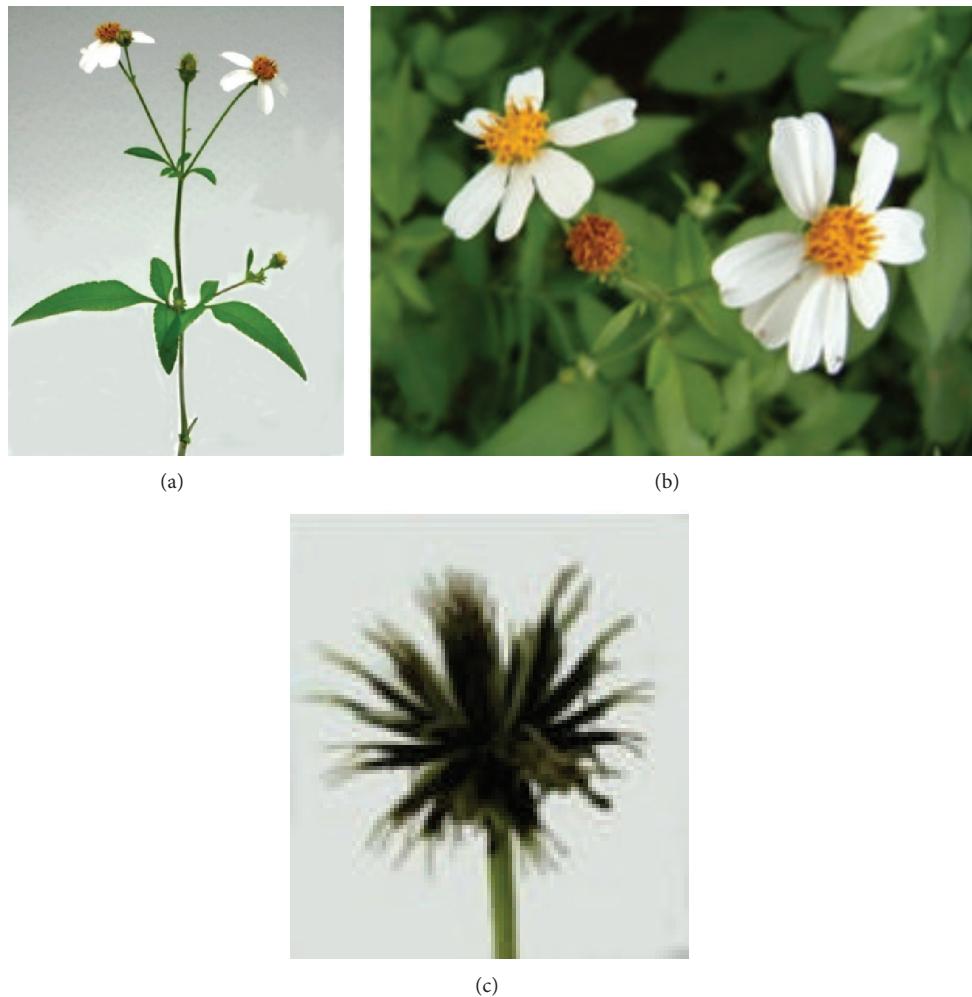
Interest in basic research and application of *B. pilosa* has increased since its first identification in 1753. This is mainly due to its wide application in medicines, foods, and drinks. Around 116 publications have documented the exploitation and medical use of *B. pilosa*. To date, 201 compounds comprising 70 aliphatics, 60 flavonoids, 25 terpenoids, 19 phenylpropanoids, 13 aromatics, 8 porphyrins, and 6 other compounds, have been identified from this plant as compiled previously [27]. The structures of these compounds are presented in Tables 4, 5, 6, 7, 8, 9, and 10, respectively. However, the association between *B. pilosa* phytochemicals and their bioactivities is not yet fully established and should become a future research focus. In the present review, we explore possible associations (Table 11), describe the importance of the known compounds in relation to their biological activity and discuss their likely mechanisms of action (Table 3). Compelling evidence suggests that the various diverse bioactivities reported for *B. pilosa* reflect its phytochemical complexity.

B. pilosa is an extraordinary source of phytochemicals, particularly flavonoids and polyyne. Plant flavonoids are

TABLE 2: Nutritional facts about *B. pilosa*, courtesy of the United Nations Food and Agriculture Organization [15].

Plant (100 g)	Energy (kcal)	Moisture (%)	Protein (g)	Fat (g)	Carbohydrate (g)	Fiber (g)	Ash (g)	Calcium (mg)	Phosphorus (mg)	Iron (μg)	Carotene equivalent (μg)	Thiamine (mg)
Raw	43	85.1	3.8	0.5	8.4	3.9	2.2	340	67	—	1800	—
Dried	33	88.6	2.8	0.6	6	1.3	2	111	39	2.3	—	—

“—” denotes not detectable.

FIGURE 1: *B. pilosa* (a) and its flowers (b) and achenes (c).

commonly reported to possess anticancer, antiinflammatory, antioxidant, and other bioactivities. However, the bioactivities of only seven of the 60 flavonoids present in *B. pilosa* have been studied. The bioactivities of the remaining 53 flavonoids are poorly understood and deserve further investigation. Other classes of compounds found in *B. pilosa* are also in need of further examination, as shown in Table 11.

3. Pharmacological Properties

As outlined in Table 3, *B. pilosa* is traditionally used to treat a wide variety of ailments. Different preparations of its whole

plant and/or parts have been purported to treat over 40 categories of illnesses. Scientific studies, although not extensive, have demonstrated that *B. pilosa* extracts and/or compounds have antitumor [28–36], antiinflammatory [18, 32, 37–42], antidiabetic and antihyperglycemic [7, 43–46], antioxidant [47–49], immunomodulatory [29, 50], antimalarial [30, 51], antibacterial [51–53], antifungal [53, 54], antihypertensive, vasodilatory [19, 55], and antilulcerative [17] activities. In this section, the primary pharmacological properties of *B. pilosa* extracts and phytochemicals are presented and discussed.

3.1. Anticancer Activity. Folkloric reports revealed the possible antitumor efficacy of *B. pilosa*, and several scientific

TABLE 3: Ethnomedical information about *B. pilosa*.

Disorder	Plant part ^b	Mode of use	Region/Country	References
Stomachache	LE	Decoction	India Africa	[23, 25, 56]
Colics	WP	Decoction	Africa China	[25]
Catarrh	WP	Juice or decoction; taken orally	Cuba	[24]
Diarrhea	LE and WP	Fresh leaves or Decoction	Africa Uganda	[25, 57]
Constipation	WP	Decoction	Africa	[25]
Dysentery/Bacillary dysentery	WP	Decoction	Africa China	[25]
Choleretic	WP	Decoction	Middle America	[25]
Anti-inflammatory	WP	Not stated	China Cuba	[22, 24]
Asthma	WP	Decoction or maceration; taken orally	Cuba China	[21, 24]
Antirheumatic	RT and WP	Juice and decoction	Hong Kong Zulu, Africa	[22, 25]
Acute appendicitis	WP	Decoction	Hong Kong	[21]
Enteritis	WP	Decoction	Africa China	[25]
Pruritus	WP	Decoction	Hong Kong	[21]
Conjunctivitis	WP	Decoction	Africa China	[25]
Otitis	WP	Decoction	Africa China	[25]
Pharyngitis	WP	Decoction	Africa China	[25]
Gastritis	WP	Juice; taken orally	Cuba	[24]
Diabetes	WP	Decoction; taken orally	Cuba Taiwan	[24, 43]
Headache	WP	Decoction	Bafia, Cameroon	[26]
Diuretic	WP	Decoction	Middle America	[25]
Hypotensive	WP	Decoction; taken orally	Bafia, Cameroon	[26]
Colds	LE and WP	Fresh or decoction	China Middle America Uganda	[21, 25, 57]
Yellow Fever	LE and WP	Fresh or decoction	Middle America Uganda China	[21, 22, 25, 57]
Influenza	LE and WP	Fresh or decoction	Middle America Uganda	[21, 25, 57]
Acute infectious hepatitis	WP	Decoction	Hong Kong	[21]
Intestinal worms	WP	Decoction	Africa	[25]
Malaria	RT and WP	Juice	Africa China	[21, 25]
Eye Infection	LE and WP	Fresh or juice	Uganda Middle America	[25, 57]

TABLE 3: Continued.

Disorder	Plant part ^b	Mode of use	Region/Country	References
Antimicrobial	AP	Decoction for drinking; bathing/external use	Trinidad and Tobago	[22]
Pulmonary tuberculosis	WP	Decoction or maceration; taken orally	Cuba China	[21, 24]
Bacterial infections in gastrointestinal tracts	WP	Decoction	Trinidad and Tobago	[22]
Renal infection	LE	Decoction; taken orally	Cuba China	[24]
Sore throat	LE and WP	Fresh or decoction	Middle America Uganda	[21, 25, 57]
Cough	WP	Decoction; taken orally	Cuba China	[21, 24]
Coolness of the uterus	WP	Decoction; taken orally	Cuba	[24]
Menstrual irregularities	WP	Decoction; taken orally	Cuba	[24]
Dysmenorrhea	WP	Decoction	Bafia, Cameroon	[26]
<i>Hyperemesis gravidarum</i> (morning sickness)	WP	Decoction	Africa	[25]
Hemorrhoids	WP	Decoction	Hong Kong China	[21]
Nose bleeds	LE and WP	Fresh or decoction	Middle America Uganda	[21, 25, 57]
Stomach ulcers	LE and WP	Maceration or juice; taken orally	Cuba Middle America Uganda	[21, 24, 25, 57]
Cuts, burns, and skin problems	LE and WP	Fresh plant or decoction; topical application/bathing	Trinidad and Tobago Africa China Cameroon Brazil Venezuela China	[17, 22, 25]
Wounds	WP	Crushed herb	Africa Central America Hawaii	[21]
Snake bites	WP	Pulverized herb	China	[21]

^bLE: leaves, ST: stem, FW: flower, WP: whole plant, RT: roots, AP: aerial parts.

in vitro studies have supported the claim that *B. pilosa* extracts and isolated compounds possess anti-cancer activities against a variety of cancer cells. Several studies have used bioassay guided isolation and fractionation methods to discover new compounds from *B. pilosa*. For example, Kwiecinski and colleagues tested hydroalcoholic crude extracts, chloroform, ethyl acetate, and methanol fractions for anti-tumor activity [35]. The cytotoxicity of the extracts was assessed using brine shrimp, hemolytic, MTT, and neutral red uptake (NRU) assays. *In vivo* studies were performed using Ehrlich ascites carcinoma in isogenic BALB/c mice. Among them, the chloroform fraction was the most toxic with a half maximal inhibitory concentration (IC_{50}) of 97 ± 7.2 and $83 \pm$

$5.2 \mu\text{g/mL}$ in NRU and MTT, respectively [35]. Kumari and colleagues also reported the anti-cancer and anti-malarial activities of *B. pilosa* leaves [30]. Based on a cytotoxicity-directed fractionation strategy, they identified phenyl-1,3,5-heptatriene with IC_{50} values of 8 ± 0.01 , 0.49 ± 0.45 , 0.7 ± 0.01 , and $10 \pm 0.01 \mu\text{g/mL}$ against human oral, liver, colon, and breast cancer cell lines, respectively. However, phenyl-1,3,5-heptatriyne showed lower activity against breast cancer cell lines than the chloroform leaf extract which had an IC_{50} value of $6.5 \pm 0.01 \mu\text{g/mL}$. Moreover, the positive control, taxol, showed higher activity than phenyl-1,3,5-heptatriyne [30]. Furthermore, *in vitro* comet assays were performed to evaluate the toxicity of n-hexane, chloroform, and methanol

TABLE 4: Aliphatic natural products isolated from *B. pilosa* [27].

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
1	Heneicosane		$\text{CH}_3(\text{CH}_2)_{19}\text{CH}_3$	Aerial (Tanzania)	[25]
2	Docosane		$\text{CH}_3(\text{CH}_2)_{20}\text{CH}_3$	Aerial (Tanzania)	[25]
3	Tricosane		$\text{CH}_3(\text{CH}_2)_{21}\text{CH}_3$	Aerial (Tanzania)	[25]
4	Tetracosane		$\text{CH}_3(\text{CH}_2)_{22}\text{CH}_3$	Aerial (Tanzania)	[25]
5	Pentacosane		$\text{CH}_3(\text{CH}_2)_{23}\text{CH}_3$	Aerial (Tanzania)	[25]
6	Hexacosane		$\text{CH}_3(\text{CH}_2)_{24}\text{CH}_3$	Aerial (Tanzania)	[25]
7	Heptacosane		$\text{CH}_3(\text{CH}_2)_{25}\text{CH}_3$	Aerial (Tanzania)	[25]
8	Octacosone		$\text{CH}_3(\text{CH}_2)_{26}\text{CH}_3$	Aerial (Tanzania); not found (Taiwan)	[25, 58]
9	Nonacosane		$\text{CH}_3(\text{CH}_2)_{27}\text{CH}_3$	Aerial (Tanzania); not found (Taiwan)	[25, 58]
10	Triacontane		$\text{CH}_3(\text{CH}_2)_{28}\text{CH}_3$	Aerial (Tanzania); not found (Taiwan)	[25, 58]
11	Hentriacontane		$\text{CH}_3(\text{CH}_2)_{29}\text{CH}_3$	Aerial (Tanzania); not found (Taiwan)	[25, 58]
12	Dotriacontane		$\text{CH}_3(\text{CH}_2)_{30}\text{CH}_3$	Aerial (Tanzania); not found (Taiwan)	[25, 58]
13	Tritriacontane		$\text{CH}_3(\text{CH}_2)_{31}\text{CH}_3$	Aerial (Tanzania); not found (Taiwan)	[25, 58]
14	2-Butoxy-ethanol		$\text{CH}_3(\text{CH}_2)_3\text{OCH}_2\text{CH}_2\text{OH}$	Whole (Taiwan)	[59]
15	Tetracosan-1-ol		$\text{CH}_3(\text{CH}_2)_{22}\text{CH}_2\text{OH}$	Aerial (Tanzania)	[25]
16	Hexacosan-1-ol		$\text{CH}_3(\text{CH}_2)_{24}\text{CH}_2\text{OH}$	Aerial (Tanzania)	[25]
17	1-Octacosanol		$\text{CH}_3(\text{CH}_2)_{26}\text{CH}_2\text{OH}$	Aerial (Tanzania)	[25]
18	1-Hentriacontanol		$\text{CH}_3(\text{CH}_2)_{29}\text{CH}_2\text{OH}$	Not found (Taiwan)	[58]
19	Tetradecanoic acid	Myristic acid	$\text{CH}_3(\text{CH}_2)_{12}\text{CO}_2\text{H}$	Aerial (Tanzania)	[25]
20	Hexadecanoic acid	Palmitic acid	$\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H}$	Aerial (Tanzania)	[25]
21	Octadecanoic acid	Stearic acid	$\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H}$	Aerial (Tanzania)	[25]
22	Eicosanoic acid	Arachidic acid	$\text{CH}_3(\text{CH}_2)_{18}\text{CO}_2\text{H}$	Aerial (Tanzania)	[25]
23	Docosanoic acid	Behenic acid	$\text{CH}_3(\text{CH}_2)_{20}\text{CO}_2\text{H}$	Leaves (Philippines)	[60]
24	2-butenedioic acid			Aerial (China)	[61, 62]
25	(Z)-9-Octadecenoic acid	Oleic acid		Aerial (Tanzania)	[25]

TABLE 4: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
26	(E)-9-Octadecenoic acid	Elaidic acid		Leaves (Philippines)	[60]
27	(Z,Z)-9,12-Octadecadienoic acid	Linolic acid or Linoleic acid		Aerial (Tanzania); whole (Taiwan)	[25, 63]
28	(Z,Z,Z)-9,12,15-Octadecatrienoic acid	α -Linoleic acid		Whole (Taiwan)	[59]
29	(Z,Z)-9,12-Octadecadienoic acid, ethyl ester	Ethyl linoleate		Whole (Taiwan)	[59]
30	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, methyl ester	Methyl linolenate		Whole (Taiwan)	[59]
31	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, ethyl ester	Ethyl linolenate		Whole (Taiwan)	[59]
32	(Z)-9-Octadecenoic acid, 2-butoxyethyl ester	2-Butoxyethyl oleate		Whole (Taiwan)	[59]
33	2-Butoxyethyl linoleate			Whole (Taiwan)	[59]
34	(Z,Z,Z)-9,12,15-Octadecatrienoic acid, butoxyethyl ester	2-Butoxyethyl linolenate		Whole (Taiwan)	[59]
35	1,7E,9E,15E-Heptadecatetraene-11,13-diyne	Heptadeca-2E,8E,10E,16-tetraen-4,6-diyne		Not found (China)	[64]

TABLE 4: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
36	1,11-Tridecadiene-3,5,7,9-tetrayne			Roots (not stated)	[65]
37	1-Tridecene-3,5,7,9,11-pentayne	Pentayneene		Leaves (not stated) and not found (Egypt)	[65, 66]
38	5-Tridecene-7,9,11-triyne-3-ol			not found (Egypt)	[66]
39	2,10,12-Tridecatriene-4,6,8-triyn-1-ol			Part not specified (Not stated)	[67]
40	2,12-Tridecadiene-4,6,8,10-tetrayn-1-ol	1,11-Tridecadiene-3,5,7,9-tetrayn-13-ol		Roots (Not stated); not found (Egypt)	[65, 66]
41	2,12-Tridecadiene-4,6,8,10-tetraynal	1,11-Tridecadiene-3,5,7,9-tetrayn-13-al		Roots (Germany)	[68]
42	2,12-Tridecadiene-4,6,8,10-tetrayn-1-ol, 1-acetate	1,11-Tridecadiene-3,5,7,9-tetrayn-13-acetate		Roots (not stated)	[65]
43	(5E)-1,5-Tridecadiene-7,9-diyne-3,4,13-triol			Aerial (China)	[69]
44	(6E,12E)-3-oxo-tetradeca-6,12-dien-8,10-diyne-1-ol			Aerial (China)	[69]
45	(E)-5-Tridecene-7,9,11-triyne-1,2-diol	1,2-Dihydroxy-5(E)-tridecene-7,9,11-triyne		Whole (Taiwan)	[28]
46	(E)-6-Tetradecene-8,10,12-triyne-1,3-diol	1,3-Dihydroxy-6(E)-tetradecene-8,10,12-triyne		Whole (Taiwan)	[28, 70, 71]
47	(2R,3E,11E)-3,11-Tridecadiene-5,7,9-triyne-1,2-diol	Safynol		Not found (Egypt and China)	[64, 66]

TABLE 4: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
48	5,7,9,11-tridecatetrayne-1,2-diol	1,2-Dihydroxytrideca-5,7,9,11-tetrayne		Whole (Taiwan)	[28, 70, 72]
49	(R)-3,5,7,9,11-tridecapentayne-1,2-diol	(R)-1,2-Dihydroxytrideca-3,5,7,9,11-pentayne		Aerial (Japan)	[51]
50	(4E)-1-(Hydroxymethyl)-4-dodecene-6,8,10-triyn-1-yl-β-D-glucopyranoside	2-β-D-Glucopyranosyloxy-1-hydroxy-5(E)-tridecene-7,9,11-triyne		Aerial (USA); whole (Taiwan); leaves (Taiwan)	[7, 44, 45, 50, 71, 73]
51	(4E)-1-(2-Hydroxyethyl)-4-dodecene-6,8,10-triyn-1-yl-β-D-glucopyranoside	3-β-D-Glucopyranosyloxy-1-hydroxy-6(E)-tetradecene-8,10,12-triyne		Aerial (USA and China); whole (Taiwan); leaves (Taiwan)	[7, 44, 45, 50, 62, 69, 71, 73]
52	3-Hydroxy-6-tetradecene-8,10,12-triynyl-β-D-glucopyranoside	β-D-Glucopyranosyloxy-3-hydroxy-6E-tetradecene-8,10,12-triyne		Whole (Mexico)	[74]
53	1-(Hydroxymethyl)-4,6,8,10-dodecatetrayne-1-yl-β-D-glucopyranoside	Cytopiloyne, 2-β-D-Glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne		Whole (Taiwan); leaves (Taiwan)	[7, 46, 50]
54	2-O-D-Glucosyltrideca-11E-en-3,5,7,9-tetrayn-1,2-diol		Leaves (Brazil)	[18]	

TABLE 4: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
55	(R)-1-(Hydroxymethyl)-2,4,6,8,10-dodecapentayn-1-yl- β -D-glucopyranoside	2- β -D-Glucopyranosyloxy-1-hydroxytrideca-3,5,7,9,11-pentayne		Aerial (China and Japan)	[51, 62]
56	1-[[(Carboxyacetyl)oxy]methyl]-4,6,8,10-dodecatetraynyl- β -D-glucopyranoside			Aerial (Japan)	[75]
57	(4E)-1-[(Carboxyacetyl)oxy]-4-methyl-4-dodecene-6,8,10-triynyl- β -D-glucopyranoside			Aerial (Japan)	[75]
58	(4E)-1-[(Carboxyacetyl)oxy]-4-ethyl-4-dodecene-6,8,10-triynyl- β -D-glucopyranoside			Aerial (Japan)	[75]
59	(5E)-5-Heptene-1,3-diyne-1-yl-benzene	1-Phenylhepta-1,3-diyen-5-en		Whole (Taiwan)	[59]
60	7-Phenyl-2(E)-heptene-4,6-diyne-1-ol			Roots (not stated); Aerial (China)	[65, 69]
61	7-Phenyl-2(E)-heptene-4,6-diyne-1-ol-acetate			Roots (not stated; Brazil)	[65, 76, 77]
62	7-Phenyl-4,6-heptadiyn-2-ol	Pilosol A		Whole (Taiwan); aerial (China)	[69, 78]

TABLE 4: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
63	7-Phenylhepta-4,6-diyne-1,2-diol			Aerial (China)	[69]
64	1,3,5-Heptatriyn-1-ylbenzene	1-Phenylhepta-1,3,5-triyne		Leaves (not stated); leaves of tissue culture (not stated); aerial (Tanzania; China); Whole (Taiwan); roots (Brazil)	[25, 61, 65, 69, 77-79]
65	7-Phenyl-2,4,6-heptatriyn-1-ol			Leaves (not stated); aerial (China)	[65, 69]
66	7-Phenyl-2,4,6-heptatriyn-1-ol-acetate			Leaves (not stated)	[65]
67	5-(2-Phenylethyynyl)-2-thiophene methanol			Aerial (China)	[69]
68	5-(2-Phenylethyynyl)-2β-glucosylmethylthiophene			Aerial (China)	[69]
69	3-β-D-Glucopyranosyl-1-hydroxy-6(E)-tetradecene-8,10,12-triyne			Leaves (Cameroon)	[19]
70	1-Phenyl-1,3-diyne-5-en-7-ol-acetate			Leaves (Brazil)	[18]

S.N. denotes serial number.

extracts of *B. pilosa* and its ethyl acetate, acetone, and water fractions on Hela and KB cells. The ethyl acetate fraction from the methanol extract exhibited the highest activity with half maximal cytotoxic concentrations (CTC_{50}) of $965.2 \mu\text{g}/\text{mL}$ and $586.2 \mu\text{g}/\text{mL}$ against Hela and KB cells, respectively. Despite the moderate toxicity, these findings suggest that these *B. pilosa* extracts/fractions could be useful for future studies [36]. Hot water extracts of *B. pilosa* var. *minor* Sheriff were also assessed for its antileukemic effects on leukemic cell lines L1210, U937, K562, Raji, and P3HR1 using XTT-based colorimetric assays. The extract inhibited the five cell

lines with IC_{50} values ranging from $145 \mu\text{g}/\text{mL}$ to $586 \mu\text{g}/\text{mL}$. L1210, K562, Raji, and P3HR1 were more sensitive to *B. pilosa* extract with IC_{50} values below $200 \mu\text{g}/\text{mL}$ [101].

Consistent with the antitumor activities of *B. pilosa* extracts and fractions, some of its phytochemicals also showed anticancer activity as outlined in Table II. Among them, luteolin (103), a well-studied flavonoid with multiple bioactivities, was more effective against tumor cell proliferation than its derivatives with IC_{50} values ranging from $3 \mu\text{M}$ to $50 \mu\text{M}$ in cells, and 5 to $10 \text{ mg}/\text{kg}$ in animals. Luteolin was also found to fight cancer as a food additive at concentrations

TABLE 5: Flavonoids isolated from *B. pilosa* [27].

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
71	2-[{(3,4-Dihydroxyphenyl)-methylene]-6-hydroxy-3(2H)-benzofuranone	Sulfuretin		Aerial (China)	[62]
72	2-[{(3,4-Dihydroxyphenyl)-methylene]-6,7-dihydroxy-3(2H)-benzofuranone	Aurone, (Z)-6,7,3',4'-tetrahydroxy; maritimetin		Aerial (China)	[62]
73	2-[{(3,4-Dihydroxyphenyl)-methylene]-6-(β-D-glucopyranosyloxy)-7-hydroxy-3(2H)-benzofuranone	Aurone, (Z)-6-O-β-D-glucopyranosyl-6,7,3',4'-tetrahydroxy; maritimtein		Leaves (Japan and China); aerial (China)	[62, 80, 81]
74	2-[{(3,4-Dihydroxyphenyl)-methylene]-7-(β-D-glucopyranosyloxy)-6-hydroxy-3(2H)-benzofuranone	Aurone, (Z)-7-O-β-D-glucopyranosyl-6,7,3',4'-tetrahydroxy		Leaves (Japan)	[80]
75	6-[(6-O-Acetyl-β-D-glucopyranosyloxy)-2-[{(3,4-dihydroxyphenyl)-methylene]-7-hydroxy-3(2H)-benzofuranone	Aurone, (Z)-6-O-(6-O-acetyl-β-D-glucopyranosyloxy)-6,7,3',4'-tetrahydroxy		Leaves (Not stated); aerial (China)	[62, 80]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (C, country)	References
76	6-[{(3,6-di-O-Acetyl- β -D-glucopyranosyl)oxy]-2-[(3,4-dihydroxyphenyl)-methylene]-7-hydroxy-3(2H)-benzofuranone	Aurone, (Z)-6-O-(3,6-di-O-acetyl- β -D-glucopyranosyl)-6,7,3',4'-tetrahydroxy-β-bidenoside A		Leaves (China)	[81]
77	6-[(4,6-di-O-Acetyl- β -D-glucopyranosyl)oxy]-2-[(3,4-dihydroxyphenyl)-methylene]-7-hydroxy-3(2H)-benzofuranone	Aurone, (Z)-6-O-(4'',6''-diacetyl- β -D-glucopyranosyl)-6,7,3',4'-tetrahydroxy		Leaves (not stated); aerial (China)	[61, 62, 82]
78	2-[(3,4-Dihydroxyphenyl)-methylene]-7-hydroxy-6-[(2,4,6-tri-O-acetyl- β -D-glucopyranosyl)oxy]-3(2H)-benzofuranone	Aurone, (Z)-6-O-(2'',4'',6''-triacetyl- β -D-glucopyranosyl)-6,7,3',4'-tetrahydroxy		Leaves (not stated); aerial (China)	[61, 82]
79	2-[(3,4-Dihydroxyphenyl)-methylene]-7-hydroxy-6-[(3,4,6-tri-O-acetyl- β -D-glucopyranosyl)-oxy]-3(2H)-benzofuranone	Aurone, (Z)-6-O-(3'',4'',6''-triacetyl- β -D-glucopyranosyl)-6,7,3',4'-tetrahydroxy		Aerial (China)	[61, 62]
80	2-[(3,4-Dihydroxyphenyl)-methylene]-7-hydroxy-6-[[6-O-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]- β -D-glucopyranosyl]oxy]-3(2H)-benzofuranone	Aurone, (Z)-6-O-(6-O-p-coumaroyl- β -D-glucopyranosyl)-6,7,3',4'-tetrahydroxy		Leaves (Japan)	[80]

TABLE 5: Continued.

S.N.	IUPAC names	Common names	Structure	Plant part (Country)	References
81	1-[2-(β -D-Glucopyranosyloxy)-4-hydroxyphenyl]-2-hydroxy-3-(3-hydroxyphenyl)-2-propen-1-one	Chalcone, α ,3,2',4'-tetrahydroxy- β -D-glucopyranosyl-2-O-		Aerial (China)	[62]
82	1-(2,4-Dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-propen-1-one	Butein		Aerial (China)	[62]
83	3-(3,4-Dihydroxyphenyl)-1-(2,3,4-trihydroxyphenyl)-2-propen-1-one	Okanin		Leaves (China)	[81]
84	3-(3,4-Dihydroxyphenyl)-1-[3-(β -D-glucopyranosyloxy)-2,4-dihydroxyphenyl]-2-propen-1-one	3'-O- β -D-glucoside Okanin		Leaves (Germany); flowers (Germany)	[82-84]
85	3-(3,4-Dihydroxyphenyl)-1-[4-(β -D-glucopyranosyloxy)-2,3-dihydroxyphenyl]-2-propen-1-one	4'-O- β -D-glucoside Okanin		Flowers (Germany); leaves (Japan)	[80, 84]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
86	Okanin 4'-O- β -D-(6''-O-acetyl)glucoside			Flowers (Germany)	[84]
87	1-[4-[(4,6-di-O-Acetyl- β -D-glucopyranosyl)-oxy]-2,3-dihydroxyphenyl]-3-(3,4-dihydroxyphenyl)-2-propen-1-one	Okanin 4'-O- β -D-(4'',6''-diacetyl)-glucopyranoside		Aerial (China)	[61]
88		Okanin 4'-(2'',4'',6''-triacetyl)-glucoside		Leaves (Germany)	[85]
89		Okanin 4'-O- β -D-(3'',4'',6''-triacetyl)-glucoside		Aerial (China)	[61]

TABLE 5: Continued.

S.N.	IUPAC names	Common names	Structure	Plant part (Country)	References
90	1-[2,3-Dihydroxy-4-[[6-O-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]- β -D-glucopyranosyl]oxy]-phenyl]-2-propen-1-one	Okanin 4'-O- β -D-(6"-trans- <i>p</i> -coumaroyl)-glucoside		Leaves (Germany)	[85]
91	Okanin 4'-O- β -D-(4"-acetyl-6"-trans- <i>p</i> -coumaroyl)-glucoside			Leaves (Germany)	[86]
92	Okanin 4'-O- β -D-(2'',4''-diacetyl-6"-trans- <i>p</i> -coumaroyl)-glucoside			Leaves (Germany)	[86]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
93	4'-O- β -D-(3'',4''-diacetyl-6''-trans- <i>p</i> -coumaroyl)-glucoside			Leaves (Germany)	[86]
94	Okanin4'-O-[β -D-glucopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside]			Flower (Germany)	[84]
95	Okanin 3',4'-di-O- β -D-glucoside			Flower (Germany)	[84]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
96	1-[3-(β -D-Glucopyranosyloxy)-2,4-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)-2-propen-1-one	Okanin 4-methyl ether- β -D- glucopyranoside		Leaves (Germany); aerial (China)	[62, 83]
97	Okanin 4-methyl ether- β -(4',6',4'',6'')- tetraacetyl)- glucopyranoside			Aerial (China)	[69]
98	Chalcone, 2',4',6'-trimethoxy- 4-O-D-glucopyranosyl- dihydro			Leaves (China)	[81]
99	2-(3,4-Dihydroxyphenyl)-2,3-dihydro-7,8-dihydroxy-4H-1-benzopyran-4-one	Okanin, iso		Leaves (China)	[81]
100	2-(3,4-Dihydroxyphenyl)-2,3-dihydro-8-hydroxy-7[(2,4,6-tri-O-acetyl- β -D-glucopyranosyl)oxy]-4H-1-benzopyran-4-one			Aerial (China)	[61]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
101	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	Apigenin		Aerial (Tanzania; China)	[25, 69]
102	7-(β -D-Glucopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	Apigenin 7-O-glucopyranoside		Aerial (Tanzania)	[25]
103	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one	Luteolin		Aerial (Tanzania; China; Vietnam)	[25, 61, 62, 69, 87]
104	2-(3,4-Dihydroxyphenyl)-7-(β -D-glucopyranosyloxy)-5-hydroxy-4H-1-benzopyran-4-one	Luteolin 7-O- β -D-glucopyranoside		Aerial (Tanzania)	[25]
105	5,7-Dimethoxy-6-(5-methoxy-6-methyl-4-oxo-4H-pyran-3-yl)-2-phenyl-4H-1-benzopyran-4-one	5-O-Methylhosstudin		Aerial (Uganda)	[88]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
106	3-(β -D-Glucopyranosyloxy)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	Astragalin; Kaempferol-3-O- β -D-glucopyranoside		Aerial (China)	[62]
107	Kaempferol 3-(2,3-di-E-p-coumaroyl- α -L-rhamnopyranoside)		Not found	Aerial (Vietnam)	[87]
108	2-(3,4-Dihydroxyphenyl)-7-(β -D-glucopyranosyloxy)-5-hydroxy-3,6-dimethoxy-4H-1-benzopyran-4-one	Axillaroside		Aerial (China)	[69]
109	5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6-dimethoxy-4H-1-benzopyran-4-one	Centaureidin		Whole (Taiwan)	[29]
110	7-(β -D-Glucopyranosyloxy)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6-dimethoxy-4H-1-benzopyran-4-one	Centaurein		Aerial (Japan); whole (Taiwan)	[29, 47, 89]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
111	5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-6,7-dimethoxy-4H-chromen-4-one	Eupatorium, <i>iso</i>		Not found (China)	[64, 90]
112	2-(3,4-Dimethoxypyphenyl)-7-(β -D-glucopyranosyloxy)-3,5-dihydroxy-8-methoxy-4H-1-benzopyran-4-one			Not found (Japan)	[91]
113	7-(β -D-Glucopyranosyloxy)-5hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,8-dimethoxy-4H-1-benzopyran-4-one			Not found (Japan)	[91]
114	Isorhamnetin 3-[O- α -L-rhamnopyranosyl-(1-2)- β -D-glucopyranoside]			Aerial (Vietnam)	[87]
115	7-[(6-deoxy- α -L-Mannopyranosyl)oxy]-3- β -D-glucopyranosyloxy)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-1-benzopyran-4-one	Luteoside		Aerial (China)	[69]

TABLE 5: Continued.

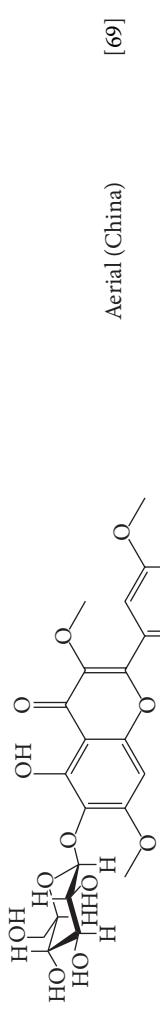
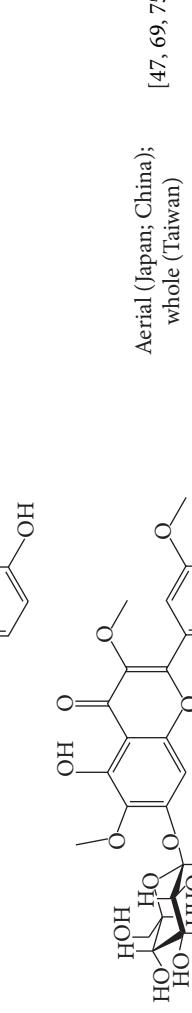
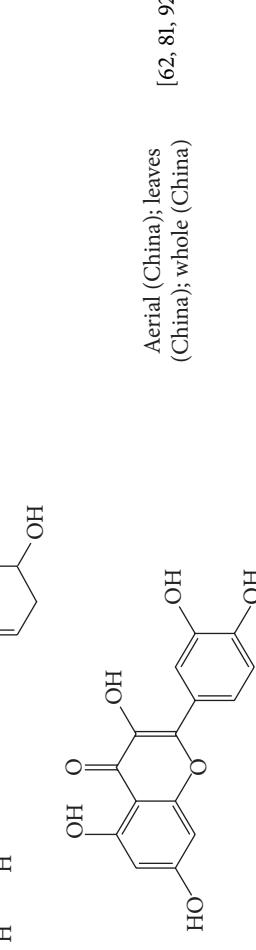
S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
116	Luteolin 3-O- β -D-Glucopyranoside			Aerial (Tanzania)	[25]
117	5,7-Dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,6-dimethoxy-4H-1-benzopyran-4-one	Quercetagelin 3,6,3'-trimethyl ether		Aerial (China)	[69]
118	6-((2S,3S,4S,5S)-Tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-2-yloxy)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one	Quercetagelin 3,7,3'-trimethyl ether-6-O- β -glucoside		Aerial (China)	[69]
119	7-(β -D-Glucopyranosyloxy)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,6-dimethoxy-4H-1-benzopyran-4-one	Jacein; Quercetin 3,6,3'-trimethyl ether-7-O- β -glucoside		Aerial (Japan; China); whole (Taiwan)	[47, 69, 75]
120	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one	Quercetin		Aerial (China); leaves (China); whole (China)	[62, 81, 92]

TABLE 5: Continued.

S.N.	IUPAC names	Common names	Structure	Plant part (Country)	References
121	2-(3,4-Dihydroxyphenyl)-3-(β -D-galactopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one	Quercetin 3-O- β -D-galactoside; hyperin; hyperoside		Aerial (Tanzania; Japan); Leaves (China); Whole (China)	[25, 37, 64, 81, 89, 92]
122	2-(3,4-Dihydroxyphenyl)-3-(β -D-glucopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one	Quercetin 3-O- β -D-glucopyranoside		Aerial (Tanzania; China; Japan); Leaves (Japan)	[25, 37, 62, 80]
123	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-1-benzopyran-3-yl- β -D-glucopyranosiduronic acid	Quercetin 3-O- β -D-glucuronopyranoside		Aerial (Tanzania; Japan)	[25, 89]
124	3-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-galactopyranosyloxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one	Quercetin 3-O-robinobioside		Aerial (Japan); Whole (Taiwan)	[47, 89]

TABLE 5: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
125	7-(β -D-Glucopyranosyloxy)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-methoxy-4H-1-benzopyran-4-one	Quercetin 3,3'-dimethyl ether 7-O- β -D-glucopyranoside		Roots (Brazil)	[5, 77, 93]
126	7-[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyloxy]-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-methoxy-4H-1-benzopyran-4-one	Quercetin 3,3'-dimethyl ether 7-O- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside		Roots (Brazil)	[77, 93]
127	7-[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyloxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3-methoxy-4H-1-benzopyran-4-one	Quercetin 3,4'-dimethyl ether-7-O-rutinoside		Aerial (China)	[61, 62]
128	2-(3,4-Dihydroxyphenyl)-3-(β -D-glucofuranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one	Isoquercitrin		Aerial (Japan; China)	[62, 89]

TABLE 5: Continued.

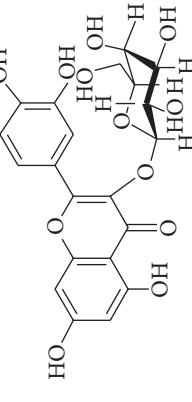
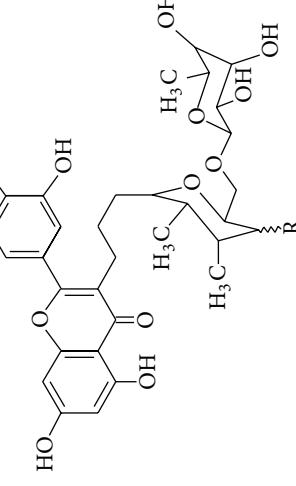
S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
129	Quercetin 3-O- β -D-galactopyranoside			Aerial (not stated)	[25]
130	Quercetin 3-O-rutinoside			Whole (Taiwan)	[47]

TABLE 6: Terpenoids isolated from *B. pilosa* [27].

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
131	3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene	Bicyclogermacrene		Leaves (Brazil)	[94]
132	4,11,11-Trimethyl-8-methylenecyclo[7.2.0]undec-4-ene	E-Caryophyllene		Leaves (Brazil)	[94]
133	1-Methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene	Germacrene D		Leaves (Brazil)	[94]
134	4-(1,5-Dimethyl-4-hexen-1-ylidene)-1-methyl-cyclohexene	Z- γ -Bisabolene		Leaves (Brazil)	[94]
135	Decahydro-1,1,4-trimethyl-7-methylene-1H-cycloprop[e]-azulene	β -Gurjunene		Leaves (Brazil)	[94]
136	2,6,6,9-Tetramethyl-1,4,8-cycloundecatriene	α -Humulene; α -caryophyllene		Leaves (Brazil)	[94]
137	1,2,3,4,4a,5,6,8a-Octahydro-1-isopropyl-7-methyl-4-methylenenaphthalene	δ -Muurolene		Leaves (Brazil)	[94]
138	1,2,3,4,4a,5,6,8a-Octahydro-4a,8-dimethyl-2-(1-methylethylidene)-naphthalene	Selina-3,7(11)-diene		Leaves (Brazil)	[94]
139	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Phytol		Whole (Taiwan)	[78]

TABLE 6: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
140	3,7,11,15-Tetramethyl-2-hexadecenoic acid	Phytanic acid		Whole (Taiwan)	[78]
141	3,7,11,15-Tetramethyl-2-hexadecenyl ester-heptanoic acid	Phythyl heptanoate		Leaves (Not stated)	[60]
142	(3S,10R,13R)-17-((2R,5R)-5-Ethyl-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-10,13-dimethyl-1H-cyclopenta[a]phenanthren-3-ol	Campesterol		Aerial (Tanzania)	[25]
143	Not found	Phytosterin-B	Not found	Not found (Taiwan; Egypt)	[66, 95]
144	Stigmast-5-en-3-ol	β -sitosterol		Aerial (Tanzania); whole (Taiwan)	[25, 58, 78]
145	13,14,15,16,17-Tetradecahydro-10,13-dimethyl-1H-cyclopenta[a]phenanthren-3-yloxy)-tetrahydro-6-(hydroxymethyl)-2H-pyran-3,4,5-triol	β -Sitosterol glucoside		Not found (Egypt)	[66]
146	5 α -Stigmasta-7-en-3 β -ol			Whole (Taiwan)	[78]
147	5 α -Stigmasta-7,22t-dien-3 β -ol			Whole (Taiwan)	[78]
148	Stigmasta-5,22-dien-3-ol	Stigmasterol		Not found (Taiwan); aerial (Tanzania); leaves (not stated); whole (Taiwan)	[25, 58, 60, 78]

TABLE 6: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (Country)	References
149	Lup-20(29)-en-3-ol	Lupeol		Not found (Egypt)	[66]
150	Lup-20(29)-en-3-ol, acetate	Lupeol acetate		Not found (Egypt)	[66]
151	Olean-12-en-3-ol	β -amyrin		Not found (Egypt)	[66]
152	5,9,13-Trimethyl-24,25,26-trinorolean-3-ol	Friedelan-3 β -ol		Aerial (Tanzania)	[25]
153	5,9,13-Trimethyl-24,25,26-trinorolean-3-one	Friedelin; friedelan-3-one		Aerial (Tanzania)	[25]
154	2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene	Squalene		Aerial (Tanzania); Leaves (Not stated); Whole (Taiwan)	[25, 60, 78]
155	β,β -Carotene	β -Carotene		Leaves (Not stated)	[96]

TABLE 7: Phenylpropanoids isolated from *B. pilosa* [27].

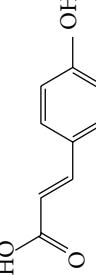
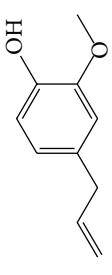
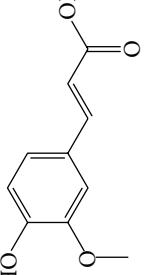
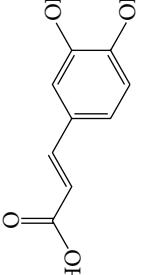
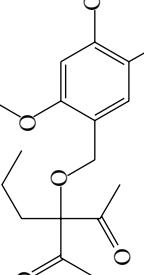
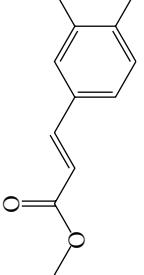
S.N.	IUPAC names	Common names	Structure	Plant part (country)	References
156	3-(4-Hydroxyphenyl)-2-propenoic acid	<i>p</i> -Coumaric acid		Whole (Japan)	[97]
157	2-Methoxy-4-(2-propen-1-yl)-phenol	Eugenol		Leaves and roots (Japan)	[97]
158	3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid	Ferulic acid		Whole (Japan)	[97]
159	3-(3,4-Dihydroxyphenyl)-2-propenoic acid	Caffeic acid		Whole and aerial (Japan)	[37, 97]
160	3-Propyl-3[(2,4,5-trimethoxyphenyl)-methoxy]-2,4-pentanedione	3-Propyl-3-(2,4,5-trimethoxybenzoyl)pentan-2,4-dione		Leaves (India)	[98]
161	3-(3,4-Dihydroxyphenyl)-2-propenoic acid, ethyl ester	Caffeate, ethyl		Not found (Taiwan); whole (Taiwan)	[28, 38, 71]

TABLE 7: Continued.

S.N.	IUPAC names	Common names	Structure	Plant part (country)	References
162	2-[[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-3,4-dihydroxy-2-methylbutanoic acid	<i>d</i> -Erythronic acid, 2-O-caffeoyle-2-C-methyl d-Erythronate, methyl 2-O-caffeoyle-2-C-methyl		Leaves (Japan)	[99]
163	2-[[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-3,4-dihydroxy-2-methylbutanoic acid, methyl ester	<i>d</i> -Erythronate, methyl 2-O-caffeoyle-2-C-methyl d-Erythronate, methyl 3-O-caffeoyle-2-C-methyl		Leaves (Japan)	[99]
164	3-[[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-2,4-dihydroxy-2-methylbutanoic acid, methyl ester	<i>d</i> -Erythronate, methyl 3-O-caffeoyle-2-C-methyl		Leaves (Japan)	[99]
165	4-(Acetyloxy)-3-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-2-hydroxy-2-methylbutanoic acid	Not found (Japan)		Leaves (Japan)	[91]
166	3-(3,4-Dihydroxyphenyl)-tetrahydro-4-hydroxy-4-methyl-5-oxo-3-furanyl ester-2-propenoic acid	3-O-Caffeoyl-2-C-methyl-D-erythro-1,4-lactone		Leaves (Japan)	[99]
167	3-[[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-1,4,5-trihydroxy-cyclohexanecarboxylic acid	Chlorogenic acid		Aerial (Japan); whole (Taiwan)	[37, 47, 89]

TABLE 7: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
168	4-[[(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]oxy]-1,3,5-trihydroxy-cyclohexanecarboxylic acid	4-O-Caffeoylquinic acid		Aerial (Japan)	[89]
169	3,4-bis[[2(<i>E</i>)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-oxy]-1,5-dihydroxy-cyclohexanecarboxylic acid	3,4-Di- <i>O</i> -caffeoylequinic acid		Aerial (Japan); Whole (Taiwan)	[44, 47, 71, 89]
170	3,4-bis[[2(<i>E</i>)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]-oxy]-1,4-dihydroxy-cyclohexanecarboxylic acid	3,5-Di- <i>O</i> -caffeoylequinic acid		Aerial (Japan); whole (Taiwan)	[44, 47, 71, 89]

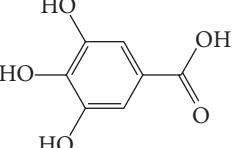
TABLE 7: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
I71	3,4-bis[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propen-1-yl]oxo]-1,5-dihydroxy-cyclohexanecarboxylic acid	4,5-Di-O-caffeoylequinic acid		Whole (Taiwan)	[44, 47, 71]
I72	3-[4-[[6-O-[3-(4-Hydroxyphenyl)-1-oxo-2-propen-1-yl]-β-D-glucopyranosyl]-oxy]phenyl]-2-propenoic acid	β-D-p-Coumaric acid, 4-O-(6-O-p-caoumaroyl-glucopyranosyl)		Leaves (Japan)	[80]
I73	3-[4-[[2-O-Acetyl-6-O-[3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl]-β-D-glucopyranosyl]-oxy]phenyl]2-propenoic acid	β-D-p-Coumaric acid, 4-O-(2-O-acetyl-6-O-p-caoumaroyl-glucopyranosyl)		Leaves (Japan); aerial (China)	[61, 80]
I74	6,7-Dihydroxy-2-chromenone	Esculetin, cichorigenin		Not found (Egypt)	[66]

TABLE 8: Aromatic compounds isolated from *B. pilosa* [27].

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
175	1,2-Benzenediol	Pyrocatechin		Whole (Japan)	[97]
176	4-Ethyl-1,2-benzenediol	Pyrocatechol		Whole (Japan)	[97]
177	Dimethoxyphenol; 2,6-dimethoxyphenol			Roots (Japan)	[97]
178	4-Ethenyl-2-methoxyphenol	<i>p</i> -Vinylguaiacol		Whole (Japan)	[97]
179	2-Hydroxy-6-methylbenzaldehyde	6-Methyl-salicylaldehyde		Whole (Japan)	[97]
180	Benzene-ethanol	2-Phenyl-ethanol		Whole (Japan)	[78]
181	4-Hydroxy-3-methoxybenzaldehyde	Vanillin		Aerial (Japan)	[97]
182	3-Hydroxy-4-methoxybenzaldehyde	Vanillin, <i>iso</i>		Leaves (Japan)	[97]
183	4-Hydroxybenzoic acid	<i>p</i> -Hydroxybenzoic acid		Whole (Japan)	[97]
184	2-Hydroxybenzoic acid	Salicylic acid		Stem and roots (Japan)	[97]
185	3,4-Dihydroxybenzoic acid	Protocatechuic acid		Whole (Japan)	[97]
186	4-Hydroxy-3methoxybenzoic acid	Vanillic acid		Aerial (Uganda); Roots (Japan)	[88, 97]

TABLE 8: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
187	3,4,5-Trihydroxybenzoic acid	Gallic acid		Whole (China)	[100]

of 50 to 200 ppm [31] and prevent skin cancer [31] and cancer invasion [102]. Lee and colleagues reported that luteolin prevents cancer by inhibiting cell adhesion and invasion [102]. Significant inhibition concentration was reported to be 5 μM and complete inhibition concentration was reported to be 40 μM . Moreover, luteolin was reported to inhibit hepatocyte growth factor (HGF)-induced cell scattering as well as cytoskeleton changes such as filopodia and lamellipodia which was determined using phase-contrast and fluorescence microscopy. Furthermore, luteolin also inhibited the HGF-induced phosphorylation of c-Met, ERK 1/2, and Akt as well as the MAPK/ERK and PI3 K-Akt pathways [31, 102]. Other mechanisms underlying the anticancer activities of luteolin are the inhibition of topoisomerase I and II, which inhibits cell replication and DNA repair thus promoting apoptosis, regulation of PI-3-Kinase/Akt/MAPK/ERK/JNK, activation of apoptosis in the mitochondrial pathway by activating caspase 9 and caspase 3, which were found in malignant cells but not in normal human peripheral blood mononuclear cells, death receptor-induced apoptosis, and a cell-cycle arrest mechanism, inhibition of fatty acid synthase which is upregulated in many cancer cells, and sensitization to chemotherapy whereby luteolin increases the susceptibility of cancer cells to chemotherapy [31]. Butein (82) is another flavonoid that showed a cytotoxic effect on human colon adenocarcinoma cell proliferation with a reported IC₅₀ value of 1.75 μM . Butein at 2 μM affected the incorporation of [¹⁴C]-labeled leucine, thymidine, and uridine which can cause the inhibition of DNA, RNA, and protein synthesis of human colon cancer cells. Moreover, butein also exhibited noncompetitive inhibition of 1-chloro-2,4-dinitrobenzene (CDNB) in glutathione S-transferase (GST) activity. Tumor resistance was correlated with high levels of GST, thus, butein inhibited proliferation of cancer cells [33]. Another flavonoid present in *B. pilosa*, centaureidin (109), also showed anti-cancer activity in B lymphoma cells. Centaureidin, isolated from *Polymnia fruticosa*, inhibited tubulin polymerization *in vitro* and induced mitotic figure formation in CA46 Burkitt lymphoma cells. Using turbidimetric assay, the IC₅₀ value of centaureidin in inhibition of mitosis was 3 μM [103]. Cytotoxicity of centaureidin was further analyzed using American National Cancer Institute (NCI) 60 human tumor cell lines. The cytotoxicity potency of centaureidin, expressed as GI₅₀ (50% growth inhibition in the NCI tumor line panel), was 0.24 μM [34]. These data mark centaureidin as a promising antimitotic agent for tumor therapy.

In addition to anti-tumor flavones, polyynes found in *B. pilosa* have also been shown to possess anti-tumor properties.

Based on a bioactivity-directed isolation approach, Wu and colleagues identified two polyyne aglycones from the ethyl acetate fraction of *B. pilosa* [70]. 1,2-Dihydroxytrideca-5,7,9,11-tetrayne (48) and 1,3-Dihydroxy-6(*E*)-tetradecene-8,10,12-triyne (46) exhibited significant anticell proliferation activity in primary human umbilical vein endothelium cells (HUVEC) with IC₅₀ values of 12.5 μM and 1.73 μM , respectively. They also decreased angiogenesis and promoted apoptosis in human endothelial cells. Their anti-angiogenic and cytotoxic effects correlated with activation of the CDK inhibitors and caspase-7 [70]. In addition, 1,2-Dihydroxy-5(*E*)-tridecene-7,9,11-triyne (45) showed antiangiogenic effects in HUVECs with an IC₅₀ value of 12.4 μM as evidenced by a decrease in the tube formation and migration of HUVECs [28]. The IC₅₀ value of compound 45 in the inhibition of basic fibroblast growth factor-induced HUVEC growth was 28.2 μM . However, it had higher IC₅₀ values than those for lung carcinoma cells and keratinocytes. This compound could also inhibit cell proliferation of HUVECs, lung carcinoma A549 cells and HACAT keratinocytes. The mechanism by which compound 45 inhibits HUVEC growth and angiogenesis is complicated and includes decreasing the expression of cell cycle regulators (CDK4, cyclins D1 and A, retinoblastoma (Rb), and vascular endothelial growth factor receptor 1), caspase-mediated activation of CDK inhibitors p21 (Cip1) and p27 (Kip), upregulation of Fas ligand expression, downregulation of Bcl-2 expression, and activation of caspase-7 and poly (ADP-ribose) polymerase [28].

3.2. Anti-Inflammatory Activity. *B. pilosa* is commonly used to treat inflammatory disorders. The anti-inflammatory phytochemicals present in *B. pilosa* are listed in Table II. Cyclooxygenase-2 (COX-2) is a physiologically important enzyme that converts arachidonic acid to prostaglandin (PGE₂). Its expression is induced by a wide variety of external stimuli indicating its involvement in inflammatory diseases, and it is used as an inflammatory marker [42]. Yoshida and colleagues studied the effects of the aqueous extracts of *B. pilosa* aerial parts in the production of COX-2 and PGE₂ as well as on the activation of mitogen activated protein kinases (MAPKs) in normal human dermal fibroblasts (HDFs) in response to inflammatory cytokine, IL-1 β . This work showed that IL-1 β activated MAPKs such as ERK1/2, p38, and JNK to different extents and induced COX-2 expression. The COX-2 expression in HDFs was regulated mainly by p38 following IL-1 β stimulation. Consistently, the p38 inhibitor SB203580 blocked this expression. Using this cell platform, *B. pilosa* extracts were tested for inhibition of inflammation.

TABLE 9: Porphyrins isolated from *B. pilosa* [27].

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
188	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(15S,16S)-10-ethenyl-5-ethyl-1,16,18,20-tetrahydro-6,11,15,22-tetramethyl-18,20-dioxo-15H-9,12-imino-21,2-metheno-4,7:17,14-dinitriopyrano[4,3-b]azacyclononadecine-16-propanoic acid	AristophyllC		Leaves (Taiwan)	[98]
189	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(2S,18S,19S,20bR)-13-ethenyl-8-ethyl-2a,18,19,20b-tetrahydro-20b-(methoxycarbonyl)-9,14,18,24-tetramethyl-4H-12,15-imino-3,5-metheno-7,10:20,17-dinitriolo-1,2-dioxeto-[3',4':3,4]-cyclo-pent[1, 2]azacyclononadecine-19-propanoic acid	Bidenphytin A		Leaves (Taiwan)	[98]
190	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(2S,18S,19S,20bR)-13-ethenyl-8-ethyl-2a,18,19,20b-tetrahydro-2a-hydroxy-20b-(methoxycarbonyl)-9,14,18,24-tetramethyl-4H-12,15-imino-3,5-metheno-7,10:20,17-dinitriolo-1,2-dioxeto[3',4':3,4]-cyclo-pent[1,2-b]-azacyclononadecine-19-propanoic acid	Bidenphytin B		Leaves (Taiwan)	[98]
191	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(3R,4S,21R)-14-ethyl-21-hydroxy-21-(methoxycarbonyl)-4,8,9,13,18-pentamethyl-20-oxo-3-phorbinepropanoic acid	(13 ² R)-13 ² -Hydroxypheophytin A		Leaves (Taiwan)	[98]
192	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(3R,4S,21S)-14-ethyl-21-hydroxy-21-(methoxycarbonyl)-4,8,9,13,18-pentamethyl-20-oxo-3-phorbinepropanoic acid	(13 ² S)-13 ² -Hydroxypheophytin A		Leaves (Taiwan)	[98]

TABLE 9: Continued.

S. N.	IUPAC names	Common names	Structure	Plant part (country)	References
193	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(3R,4S,21R)-14-ethyl-13-formyl-21-hydroxy-21-(methoxycarbonyl)-4,8,9,18-tetramethyl-20-oxo-3-phorbinepropanoic acid	(13 ² R)-13 ² -Hydroxypheophytin B		Leaves (Taiwan)	[98]
194	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(3R,4S,21S)-14-ethyl-13-formyl-21-hydroxy-21-(methoxycarbonyl)-4,8,9,18-tetramethyl-20-oxo-3-phorbinepropanoic acid	(13 ² S)-13 ² -Hydroxypheophytin B		Leaves (Taiwan)	[98]
195	(2E,7R,11R)-3,7,11,15-Tetramethyl-2-hexadecen-1-yl ester-(3S,4S,21R)-9-ethenyl-14-ethyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-3-phorbinepropanoic acid	Pheophytin A		Leaves (Taiwan)	[98]

The extract dose-dependently suppressed the activation of p38 and JNK and moderately suppressed ERK1/2, as well as suppressing COX-2 expression and PGE₂ production [42]. This work supports the use of *B. pilosa* as an anti-inflammatory agent; however, no compounds responsible for the anti-inflammatory activity of *B. pilosa* were identified.

A further study also reported the anti-inflammatory activity as well as the antiallergic activity of *B. pilosa* [37]. In this study, dried powder of the aerial part of *B. pilosa*, which had been pretreated with the enzyme cellulose, was used for further tests. The results showed that oral administration of the cellulose-treated *B. pilosa* lowered the level of serum IgE in mice 10 days after immunization with DNP (2,4-dintrophenyl)-Ascaris as an antigen. This treatment also reduced dye exudation in skin induced by passive cutaneous anaphylaxis and production of inflammatory mediators, histamine, and substance P in rats [37]. Phytochemical analysis showed that cellulose treatment increased the percentage of caffeic acid and flavonoids. This study suggests that *B. pilosa* and its phenolics have anti-inflammatory functions.

Phenolics and polyynes are major anti-inflammatory phytochemicals present in *B. pilosa* (Table 11). Unsurprisingly,

phenolics such as luteolin (103) and ethyl caffeate (161) that are major constituents of *B. pilosa* have also been reported to possess anti-inflammatory activity. Luteolin was reported to exhibit anti-inflammatory activity in macrophages. Xagorari and colleagues showed that luteolin inhibited the release of inflammatory cytokines, TNF- α and interleukin-6, in RAW 264.7 cells following LPS stimulation [41]. It inhibited TNF- α production with an IC₅₀ value of 1 μ M. The underlying anti-inflammatory mechanism of luteolin was reported to be the inactivation of Akt and NF- κ B activation [41]. In addition, luteolin was reported to confer anti-inflammatory activity through inhibition of LPS-stimulated iNOS expression in BV-2 microglial cells. It inhibited LPS-activated microglia in a dose-dependent manner with an IC₅₀ value of 6.9 μ M. Moreover, immunoblot and RT-PCR data proved that luteolin suppressed I κ B- α degradation and iNOS expression in LPS-activated microglia [39]. Kim and colleagues stated that luteolin may have beneficial effects on inflammatory neural diseases through inhibition of iNOS expression [39]. A related study revealed that luteolin decreased the transcriptional activity of NF- κ B RelA via partial inhibition of TNF-mediated NF- κ B DNA binding activity. Luteolin also

TABLE 10: Other compounds isolated from *B. pilosa* [27].

S.N.	IUPAC names	Common names	Structure	Plant part (country)	References
196	3,7-Dihydro-1,3,7-trimethyl-1H-purine-2,6-dione	Caffeine		Aerial (Uganda)	[88]
197	1-((2R,4S,5R)-Tetrahydro-4-hydroxy-5-(hydroxymethyl)furan-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione	Thymidine		Not found (China)	[64]
198	1-(2-Thienyl)-ethanone	2-Acetyl-thiophene		Roots (Germany)	[68]
199	(2R,3S,4S,5S)-2-(Heptan-2-yloxy)-tetrahydro-6-((tetrahydro-3,4-dihydroxy-5-(hydroxymethyl)furan-2-yloxy)methyl)-2H-pyran-3,4,5-triol	Heptyanyl 2-O- β -xylofuranosyl-(1 \rightarrow 6)- β -glucopyranoside		Whole (Taiwan)	[47]
200	2-[(3R,7R,11R)-3-Hydroxy-3,7,11,15-tetramethylhexadecyl]-3,5,6-trimethyl-2,5-cyclohexadiene-1,4-dione	α -Tocopheryl quinone		Whole (Taiwan)	[59]
201	7-O-(4'',6''-Diacetyl)- β -D-glucopyranoside	Not found		Leaves (China)	[81]

inhibited Akt phosphorylation and induced degradation of a transcription factor, interferon regulatory factor (IRF) [40].

Chiang and colleagues showed that ethyl caffeate (**161**) significantly inhibited NO production in mouse macrophages, RAW 264.7 cells [38]. Based on MTT assays, they concluded that this inhibition was not due to the cytotoxicity of ethyl caffeate. The IC₅₀ value of ethyl caffeate in the inhibition of NO production was 5.5 μ g/mL, slightly lower than curcumin (positive control) which has an IC₅₀ value of 6.5 μ g/mL. They demonstrated that ethyl caffeate exerted anti-inflammatory activity via the reduced transcription and translation of iNOS (inducible nitric oxide synthase) in RAW 264.7 cells. In addition, this compound also suppressed COX-2 expression in RAW 264.7 cells

and MCF-7 cells. The *in vivo* anti-inflammatory effect of ethyl caffeate was verified by testing in TPA-treated mouse skin. Like celecoxib, the positive control, ethyl caffeate significantly abolished COX-2 expression in a dose-dependent manner. Ethyl caffeate at 1 mg/200 μ L/site (24 mM) inhibited COX-2 expression at a level comparable to celecoxib at 1 mg/200 μ L/site (13 mM). Remarkably, ethyl caffeate at 48 mM (2 mg/200 μ L/site) was more effective than celecoxib at 131 mM (10 mg/200 μ L/site). Moreover, this compound inhibited the activation of nuclear factor- κ B (NF- κ B) by LPS via the prevention of NF- κ B binding to DNA [38]. In addition, 3 ethyl caffeate analogs (ethyl 3,4-dihydroxyhydrocinnamate, ethyl cinnamate, and catechol) also showed different degrees of NF- κ B binding to DNA as

TABLE 11: Chemical constituents of *B. pilosa* and their biological activities.

S.N.	Name	Classification	Molecular formula	Biological activities
109	Centaureidin [52]	Flavonoid	C ₁₈ H ₁₆ O ₈	Anti-listerial [29, 52] Cytotoxic [9]
110	Centaurein [52]	Flavonoid	C ₂₄ H ₂₆ O ₁₃	Anti-listerial [29, 52] Cytotoxic [9] Anti-viral [104]
103	Luteolin [105]	Flavonoid	C ₁₅ H ₁₀ O ₆	Anti-viral [106, 107] Cytotoxic [30] Anti-inflammatory [108] Anti-allergic [108]
82	Butein [109]	Flavonoid	C ₁₅ H ₁₂ O ₅	Anti-leishmanial [110] Cytotoxic [31]
48	1,2-Dihydroxytrideca-5,7,9,11-tetrayne [111]	Polyyne	C ₁₃ H ₁₂ O ₂	Anti-angiogenic [111]
46	1,3-Dihydroxy-6(<i>E</i>)-tetradecene-8,10,12-triyne [111]	Polyyne	C ₁₄ H ₁₆ O ₂	Anti-angiogenic [111]
45	1,2-Dihydroxy-5(<i>E</i>)-tridecene-7,9,11-triyne [112]	Polyyne	C ₁₃ H ₁₄ O ₂	Anti-angiogenic [112] Anti-proliferative [112] Anti-microbial [114] Anti-malarial [3] Cytotoxic [3] Antifungal [14]
64	1-Phenylhepta-1,3,5-triyne [113]	Polyyne	C ₁₃ H ₈	Anti-viral (100) Cytotoxic [23]
27	Linoleic acid [32]	Fatty acid	C ₁₈ H ₃₂ O ₂	Anti-inflammatory [38]
161	Ethyl caffeoate [38]	Phenylpropanoid	C ₁₁ H ₁₂ O ₄	Immunosuppressive and Anti-inflammatory [17]
54	2-O- β -Glucosyltrideca-11(<i>E</i>)-en-3,5,7,9-tetrayn-1,2-diol [17]	Polyyne	C ₁₉ H ₂₀ O ₇	Anti-diabetic [115] Anti-inflammatory [50]
53	2- β -D-Glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne [50]	Polyyne	C ₁₉ H ₂₂ O ₇	Anti-diabetic [19] Anti-inflammatory [55]
69	3- β -D-Glucopyranosyl-1-hydroxy-6(<i>E</i>)-tetradecene-8,10,12-triyne [19]	Polyyne	C ₂₀ H ₂₆ O ₇	Anti-diabetic [19] Anti-inflammatory [55]
50	2- β -D-Glucopyranosyloxy-1-hydroxy-5(<i>E</i>)-tridecene-7,9,11-triyne [19]	Polyyne	C ₁₉ H ₂₄ O ₇	Anti-inflammatory [55] Anti-malarial and antibacterial [51]
129	Quercetin 3-O- β -D-galactopyranoside [25]	Flavonoid	C ₂₁ H ₂₀ O ₁₂	Anti-inflammatory [116]
170	3,5-Di-O-caffeoylequinic acid [55]	Phenylpropanoid	C ₂₅ H ₂₄ O ₁₂	Anti-viral [78] Antioxidant [47]
171	4,5-Di-O-caffeoylequinic acid [55]	Phenylpropanoid	C ₂₅ H ₂₄ O ₁₂	Anti-viral [78] Antioxidant [47]
169	3,4-Di-O-caffeoylequinic acid [55]	Phenylpropanoid	C ₂₅ H ₂₄ O ₁₂	Anti-viral [78] Antioxidant [47]
126	Quercetin 3,3'-dimethyl ether 7-O- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside [117]	Flavonoid	C ₂₁ H ₂₀ O ₁₁	Anti-malarial [118]
125	Quercetin 3,3'-dimethyl ether-7-O- β -D-glucopyranoside [117]	Flavonoid	C ₂₁ H ₂₀ O ₁₂	Anti-malarial [118]
70	1-Phenyl-1,3-diyn-5-en-7-ol-acetate [18]	Polyyne	C ₁₅ H ₁₂ O ₂	Anti-malarial [18]
199	Heptanyl 2-O- β -xylofuranosyl-(1 \rightarrow 6)- β -glucopyranoside [47]	Miscellaneous	C ₁₈ H ₄₄ O ₁₀	Antioxidant [47]
124	3-O-Rabinobioside [47]	Saccharide	C ₂₇ H ₃₈ O ₁₅	Antioxidant [47]
130	Quercetin 3-O-rutinoside [47]	Flavonoid	C ₂₇ H ₃₈ O ₁₅	Antioxidant [47]
167	Chlorogenic acid [47]	Phenolic	C ₁₆ H ₂₆ O ₉	Antioxidant [47]
119	Jacein [47]	Flavonoid	C ₂₄ H ₂₆ O ₁₃	Antioxidant [47]
49	(<i>R</i>)-1,2-dihydroxytrideca-3,5,7,9,11-pentayne [51]	Polyyne	C ₁₃ H ₈ O ₂	Anti-malarial and Antibacterial [51]

listed in Table 12. Ethyl cinnamate lacks a catechol moiety which results in ineffective inhibition of NF- κ B binding to DNA [38].

Pereira and colleagues assessed the anti-inflammatory and immunomodulatory activities of *B. pilosa* methanol extract as well as one polyyne, 2-O- β -glucosyltrideca-11(*E*)-en-3,5,7,9-tetrayn-1,2-diol (**54**) in T lymphocytes and a zymosan-induced arthritis mouse model [18]. They first examined the *in vitro* effect of the *B. pilosa* extract and compound **54** on cell proliferation of human T cells stimulated with 5 μ g/mL phytohemagglutinin (PHA) or 100 nM 12-O-tetradecanoyl phorbol-13-acetate (TPA) plus 15 μ M ionomycin and on cell proliferation of mouse T cells stimulated with 5 μ g/mL concanavalin A (Con A). The data demonstrated that both methanol extract and compound **54** suppressed T-cell proliferation in a dose-dependent manner. The estimated IC₅₀ values of the *B. pilosa* extract against human T cells stimulated with 5 μ g/mL PHA and 100 nM TPA plus 15 μ M ionomycin were 12.5 and 25 μ g/mL, respectively. In comparison with the methanol extract, compound **54** showed 10-fold more inhibition of human T-cell proliferation with an estimated IC₅₀ value of 1.5 μ g/mL. Accordingly, the *B. pilosa* extract and compound **54** dose-dependently suppressed mouse T-cell proliferation with estimated IC₅₀ values of 30 and 2.5 μ g/mL, respectively. Taken together, the data indicate that the *B. pilosa* extract and compound **54** act on human and mouse T cells. To test the *in vivo* effect of the *B. pilosa* extract and compound **54**, a zymosan-induced arthritis mouse model was used. This model was established from B10.A/SgSnJ mice with an injection of zymosan (0.15 mg). The zymosan-injected mice received an intraperitoneal injection of the *B. pilosa* extract (1, 5, or 10 mg) at one dose a day for 5 days. Popliteal lymph node (PLN) weight was monitored to check the development of arthritis. The results revealed that 10 mg of the methanol extract of *B. pilosa* extract could significantly diminish inflammation as evidenced by PLN weight [18]. This work suggests that *B. pilosa* (and compound **54**) can suppress immune response and inflammation.

3.3. Antidiabetic Activity. Anti-diabetic agents are primarily developed from plants and other natural resources [7, 43–46]. *B. pilosa* is one of 1,200 plant species that have been investigated for antidiabetic activity [119, 120]. *B. pilosa* is used as an anti-diabetic herb in America, Africa, and Asia [45, 119, 121]. Many studies have indicated that *B. pilosa* could treat type 1 diabetes (T1D) and type 2 diabetes (T2D) in animals.

Etiologically speaking, T1D is caused by autoimmune-mediated destruction of pancreatic β cells, leading to insulin deficiency, hyperglycemia, and complications. Currently, there is no cure for T1D. Polarization of Th cell differentiation controls the development of T1D. Suppression of Th1 cell differentiation and promotion of Th2 cell differentiation ameliorate T1D [122]. One study showed that the butanol fraction of *B. pilosa* inhibited T-cell proliferation, decreased Th1 cells and cytokines, and increased Th2 cells and cytokines, leading to prevention of T1D in nonobese diabetic

TABLE 12: Structure and activity relationship studies of ethyl caffeate using *in vitro* NF- κ B/DNA binding assays [38].

Compound	Concentration (μ M)	NF- κ B/DNA binding
Ethyl caffeate	50	100% inhibition
Ethyl 3,4-dihydroxyhydrocinnamate	100	100% inhibition
Catechol	400	100% inhibition
Ethyl cinnamate	400	No inhibition

(NOD) mice [44]. Based on a bioactivity-directed isolation strategy, 3 polyynes, 2- β -D-Glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (**53**), also known as cytopiloyne, 3- β -D-Glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**69**), 2- β -D-Glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**50**) were identified from *B. pilosa* [44, 46]. The IC₅₀ value of the butanol fraction was 200 μ g/mL. This inhibition was reported to be partially attributed to cytotoxicity because the butanol fraction at 180 μ g/mL could cause 50% death of Th1 cells. Moreover, this study suggested that the butanol fraction may prevent diabetes in NOD mice *in vivo* via downregulation of Th1 cells or upregulation Th2 cells that have effects which are antagonistic of those of Th1 cells [44]. This was proven by intraperitoneal injection of the butanol fraction at a dose of 3 mg/kg BW, 3 times a week, to NOD mice from 4 to 27 weeks. This dosage resulted in lower incidence of diabetes (33%). At a dose of 10 mg/kg, the butanol fraction of *B. pilosa* totally eliminated (0%) the initiation of the disease. To further support this result, assessment of IgG2a and IgE production was performed in the serum of NOD mice. As *in vivo* results obtained from intracellular cytokine staining, experiments were not very conclusive levels of IgG2a and IgE were measured since Th1 cytokine IFN γ ; and Th2 cytokine IL-4 favor the production of IgG2a and IgE, respectively. As expected, high levels of IgE and some decline in the levels of IgG2a were observed in the serum. Profiling of the butanol extract revealed five compounds, 3- β -D-Glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**69**), 2- β -D-Glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**50**), 4,5-Di-O-caffeoylequinic acid, 3,5-Di-O-caffeoylequinic acid, and 3,4-Di-O-caffeoylequinic acid. Only the first two compounds showed similar effects on the prevention of diabetes in NOD mice as the *B. pilosa* butanol fraction. Moreover, compound **50** showed greater activity than compound **69** in terms of enhancement (by 34% compared to 8%) of differentiation of Th0 to Th2 at 15 μ g/mL (both compounds) and inhibition (by 40% compared to 10%) of differentiation to Th1 at the same concentration [44].

Among the three polyynes found in *B. pilosa*, cytopiloyne (**53**) had the most potent anti-T1D activity [46]. To test the *in vivo* effect of cytopiloyne, NOD mice received intraperitoneal or intramuscular injection of cytopiloyne at 25 μ g/kg BW, 3 times per week. Twelve-week-old NOD mice started to

develop T1D, and 70% of NOD mice aged 23 weeks and over developed T1D. Remarkably, 12- to 30-week-old NOD mice treated with cytopiloyne showed normal levels of blood glucose (<200 mg/dL) and insulin (1-2 ng/mL). Consistent with T1D incidence, cytopiloyne delayed and reduced the invasion of CD4⁺ T cells into the pancreatic islets [46].

In vitro study showed that cytopiloyne (53) inhibited the differentiation of naïve Th (Th0) cells (i.e., CD4⁺ T cells) into Th1 cells and promoted differentiation of Th0 cells into Th2 cells [50]. The *in vitro* data are consistent with the *in vivo* results indicating that cytopiloyne reduced Th1 differentiation and increased Th2 differentiation as shown by intracellular cytokine staining and FACS analysis [46]. In line with the skewing of Th differentiation, the level of serum IFN- γ and IgG2c decreased while that of serum IL-4 and serum IgE increased compared to the negative controls (PBS-treated mice). Cytopiloyne also enhanced the expression of GATA-3, a master gene for Th2 cell differentiation, but not the expression of T-bet, a master gene for Th1 cell differentiation, further supporting its role in skewing Th differentiation [46].

Also importantly, cytopiloyne partially depleted CD4⁺ rather than CD8⁺ T cells in NOD mice [46]. As shown in Table 13, coculture assays showed that the depletion of CD4⁺ T cells was mediated through the induction of Fas ligand expression on pancreatic islet cells by cytopiloyne, leading to apoptosis of infiltrating CD4⁺ T cells in the pancreas via the Fas and Fas ligand pathways. However, cytopiloyne did not induce the expression of TNF- α in pancreatic islet cells and, thus, had no effect on CD8⁺ T cells [46].

In addition, Chang and colleagues showed that cytopiloyne dose-dependently inhibited T-cell proliferation stimulated by IL-2 plus Con A or anti-CD3 antibody, using [3 H] thymidine incorporation assay [46].

Overall, the mechanism of action of cytopiloyne and, probably, its derivatives in T1D includes inhibition of T-cell proliferation, skewing of Th cell differentiation, and partial depletion of Th cells. Due to the anti-diabetic mechanisms of action, it was hypothesized that cytopiloyne protects NOD mice from diabetes by a generalized suppression of adaptive immunity. To evaluate this hypothesis, ovalbumin (Ova) was used as a T-cell dependent antigen to prime NOD mice, which had already received cytopiloyne or PBS vehicle. Ova priming boosted similar anti-Ova titers in cytopiloyne-treated mice and PBS-treated mice, but a difference in immunoglobulin isotype was observed in the two groups. Thus, it was concluded that cytopiloyne is an immunomodulatory compound rather than an immunosuppressive compound [46, 50].

T2D is a chronic metabolic disease with serious complications resulting from defects in either insulin secretion, insulin action, or both [123]. A study by Ubillas et al. showed that the aqueous ethanol extract of the aerial part of *B. pilosa* at 1 g/kg body weight (BW) lowered blood glucose in db/db mice, a T2D mouse model [45]. Based on a bioactivity-guided identification, compounds **69** and **50** were identified. Further, the mixture of the compounds (**69**:**50**) in a 2:3 ratio significantly decreased blood glucose concentration and reduced food intake on the second day

TABLE 13: Apoptosis in cocultures of T cells and pancreatic β cells [46].

Cell/Medium	% Apoptosis and necrosis
CD4 ⁺ T cells/control medium	<4
CD4 ⁺ T cells/with PBS-treated β cells of NOD-SCID mice	2
CD4 ⁺ T cells/with cytopiloyne-treated β cells	18
CD4 ⁺ T cells/with cytopiloyne-treated β cells in the presence of α -FasL antibody	7
CD8 ⁺ T cells/control medium	4
CD4 ⁺ T cells/with PBS-treated β cells of NOD-SCID mice	4
CD8 ⁺ T cells/with cytopiloyne-treated β cells	4
CD8 ⁺ T cells/with cytopiloyne-treated β cells in the presence of α -FasL antibody	4

of treatment when administered at doses of 250 mg/kg twice a day to C5BL/Ks-db/db mice. When tested at 500 mg/kg, a more substantial drop in blood glucose level as well as the stronger anorexic effect (food intake reduced from 5.8 g/mouse/day to 2.5 g/mouse/day) was observed [45]. In this study, it was suggested that the blood glucose lowering effect of *B. pilosa* was caused, in part, by the hunger suppressing effect of its polyynes [45]. However, the hunger suppressing effect of the ethanol extract of *B. pilosa* was not found in the studies described below. In another study [43], water extracts of *B. pilosa* (BPWE) were used in diabetic db/db mice, aged 6–8 weeks, with postprandial blood glucose levels of 350 to 400 mg/dL. Like oral anti-diabetic glimepiride, which stimulates insulin release, one single dose of BPWE reduced blood glucose levels from 374 to 144 mg/dL. The antihyperglycemic effect of BPWE was inversely correlated to an increase in serum insulin levels, suggesting that BPWE acts to lower blood glucose via increased insulin production. However, BPWE had different insulin secretion kinetics to glimepiride [43]. One flaw in current anti-diabetics is their decreasing efficacy over time. The authors investigated the long term anti-diabetic effect of BPWE in db/db mice. BPWE reduced blood glucose, increased blood insulin, improved glucose tolerance, and reduced the percentage of glycosylated hemoglobin (HbA1c). Both long-term and one-time experiments strongly support the anti-diabetic action of BPWE [43]. In sharp contrast to glimepiride, BPWE protected against islet atrophy in mouse pancreas. The investigators further evaluated anti-diabetic properties of 3 *B. pilosa* varieties, *B. pilosa* L. var. *radiate* (BPR), *B. pilosa* L. var. *pilosa* (BPP), and *B. pilosa* L. var. *minor* (BPM) in db/db mice [7]. One single oral dose (10, 50 and 250 mg/kg body weight) of BPR, BPP, or BPM crude extracts decreased postprandial blood glucose levels in db/db mice for up to four hours, and the reduction of glucose levels in the blood appeared to be dose-dependent. Comparing the three variants, BPR extract resulted in a higher reduction in blood glucose levels when administered at the same dose as the other two varieties. In terms of serum insulin levels, a dose

of 50 mg/kg of each extract was used, and the BPR extract, together with the three polyyne, significantly increased the serum insulin level in db/db mice. Long-term experiments (28-day treatment) were then conducted using diabetic mice with postprandial glucose levels from 370 to 420 mg/dL, and glimepiride was used as positive control. The range of dosages applied was from 10 mg/kg BW to 250 mg/kg BW. Results showed that the positive control as well as the crude extracts of the three varieties lowered the blood glucose levels in db/db mice. However, only BPR extract, containing a higher percentage of cytopiloyne (53), reduced blood glucose levels and augmented blood insulin levels more than BPP and BPM. The percentage of glycosylated hemoglobin A1c (HbA1c) was also measured and found to be $7.9\% \pm 0.5\%$ in mice aged 10–12 weeks, and $6.6\% \pm 0.2\%$, $6.1\% \pm 0.3\%$ and $6.2\% \pm 0.3\%$ in the blood of age-matched mice following treatment with BPR crude extract (50 mg/kg), glimepiride (1 mg/kg), and compound 53 (0.5 mg/kg), respectively [7]. Among the polyyne found in *B. pilosa*, cytopiloyne was the most effective against T2D. Hence, cytopiloyne was used for further study on anti-diabetic action and mechanism [124]. The data confirmed that cytopiloyne reduced postprandial blood glucose levels, increased blood insulin, improved glucose tolerance, suppressed HbA1c level, and protected pancreatic islets in db/db mice. Nevertheless, cytopiloyne failed to decrease blood glucose in streptozotocin (STZ)-treated mice whose b cells were already destroyed. Additionally, cytopiloyne dose-dependently increased insulin secretion and expression in b cells as well as calcium influx, diacylglycerol, and activation of protein kinase C α . Collectively, the mechanistic studies suggest that cytopiloyne treats T2D via regulation of insulin production involving the calcium/DAG/PKC α cascade in b cells.

The studies detailed above point to the conclusion that cytopiloyne and related polyyne (compounds 69 and 49) are anti-diabetics in animal models. The data uncover a new biological action of polyyne. It should be noted that, like all anti-diabetic drugs, cytopiloyne failed to prevent or cure diabetes completely but reduced diabetic complications [124]. Intriguingly, 34 polyyne have been found in *B. pilosa* so far. It remains to be seen whether all the polyyne present in this plant have anti-diabetic activities.

3.4. Antioxidant Activity. Free radicals can damage cellular components via a series of chemical reactions [48] leading to development and progression of cardiovascular disease, cancer, neurodegenerative diseases and ageing [47]. Free radicals, nitric oxide (NO), and superoxide anions can be produced in macrophages to kill microbes. However, an excessive generation of the free radicals under pathological conditions is associated with a wide range of illnesses. Plants are known to be rich in antioxidant phytochemicals. Chiang and colleagues evaluated the free radical scavenging activity of crude extract, fractions, and compounds of *B. pilosa* using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hypoxanthine/xanthine oxidase assays [47]. Using DPPH and hypoxanthine/xanthine oxidase assays, they found that the *B. pilosa* crude extract and the ethyl

acetate, butanol, and water fractions had free radical scavenging activity. Nine compounds, Heptyl-2-O- β -xylofuranosyl-(1 \rightarrow 6)- β -glucopyranoside (199), 3-O-Rabinobioside (124), Quercetin 3-O-rutinoside (130), Chlorogenic acid (167), 3,4-Di-O-caffeoquinic acid (169), 3,5-Di-O-caffeoquinic acid (170), 4,5-Di-O-caffeoquinic acid (171), Jacein (119), and Centaurein (110) had DPPH radical scavenging activity [47]. The IC₅₀ values of the *B. pilosa* crude extract/fractions and compounds are summarized in Tables 14 and 15, respectively.

Measurement of free radical scavenging activities is one way of assessing the antioxidant activities of *B. pilosa* and its fractions and compounds. It is interesting that the ethyl acetate and butanol fractions are more active than the water fraction and *B. pilosa* crude extract [47]. Of the secondary metabolites, only phenolic compounds 124, 130, 167, 169, and 171 showed significant DPPH-radical scavenging activities. Further analysis of the structure-activity relationship of the compounds suggested that substitution of the C3 hydroxyl group with glycosides increased the activity approximately 2-fold (for example, in compounds 124 and 130) relative to quercetin (-OH in its C3) [47]. Further modification of the structures of the active compounds needs to be performed to test the effects of various substituents on activity. The reason why most of the antioxidant compounds contain phenol moieties in their structure could be that the reduction-oxidation (redox) properties of phenols allow them to act as reducing agents, singlet oxygen quenchers, and hydrogen donors.

A complementary study by Muchuweti and colleagues determined phenolic content, antioxidant activity, and the phenolic profile of *B. pilosa* methanol extract [48]. They estimated that the phenolic content of the methanol extract of *B. pilosa* was 1102.8 ± 2.2 mg/g [48]. Vanillin, hydroxybenzaldehyde, caffeic acid, coumaric acid, and ferulic acid were found in this extract. The *B. pilosa* extract also showed DPPH radical scavenging activity. Furthermore, the antioxidant activity of the flavonoids found in *B. pilosa* was correlated with its hepatoprotective effects through their inhibition of NF- κ B activation which may lessen the oxidative stress caused by the production of free radicals during liver injury [81]. This activity might also be due to the anti-inflammatory effects of the aqueous extracts of *B. pilosa* aerial parts on the inhibition of COX-2 and PGE₂ production [42].

Essential oils from *B. pilosa* flowers and leaves are also reported to possess antioxidant activity. With the aim of replacing chemically synthesized additives, Deba and colleagues [53] worked on the antioxidant, antibacterial, and antifungal activities of essential oils and water extracts of *B. pilosa*'s leaves and flowers. Table 16 summarizes the results obtained from DPPH free radical scavenging assay.

It can be inferred from Table 16 that essential oils from the leaves possessed the highest activity. It is reported elsewhere that monoterpenes present in essential oils such that of *B. pilosa* have protective effects and antioxidant properties [53]. Beta-carotene bleaching method was also performed. Leaves essential oils and aqueous extracts of the leaves and flowers showed higher activity than the flower essential oils. This is due to the volatility of the flower essential oils. The activity exhibited by the aqueous extracts was accounted to the

TABLE 14: Radical scavenging activities of *B. pilosa* extracts [47].

Extracts/control	DPPH assay, IC ₅₀ (μ g/mL)	NBT/hypoxanthine superoxide assay, IC ₅₀ (μ g/mL)
Quercetin	1.98	1.5
Ascorbic acid	6.34	Not determined
α -tocopherol	8.97	Not determined
Ethyl acetate extract	13.83	59.7
Butanol extract	16.69	11.4
Water extract	>100	>100

TABLE 15: Radical scavenging activity of secondary metabolites from *B. pilosa* [47].

Metabolite (Table II)/Control	DPPH assay, IC ₅₀ (μ g/mL)
199	Not determined
124	5.3
130	6.8
167	10.5
169	3.3
171	3.8
119	Not determined
110	Not determined
Quercetin	2.56
Caffeic acid	8.90

TABLE 16: Antioxidant activity of the essential oils and water extracts from *B. pilosa* [53].

Extract	IC ₅₀ (μ g/mL)
Leaf essential oils	47
Flower essential oils	50
Leaf extract	61
Flower extract	172

presence of phenolic compounds that are reported to donate a hydrogen atom to free radicals such that the propagation of the chain reaction during lipid oxidation is terminated [53]. Overall, essential oils and phenolics present in *B. pilosa* can be thought of as major antioxidant compounds.

3.5. Immunomodulatory Activity. *B. pilosa* is thought to be an immunomodulatory plant and is reported to be effective in the treatment of immune disorders such as allergy [37], arthritis [37], and T1D [46, 50, 73].

As pointed out in the discussion of its anti-diabetic activities (Section 3.3), a combination of phytochemicals method and T-cell activation assays was used to study immunomodulatory properties of *B. pilosa*. IFN- γ is a key cytokine released by T and NK cells that mediates immune cells and sustains immunity against pathogens. Defects in IFN- γ expression, regulation, and activation result in vulnerability to diseases caused by bacteria and viruses [29]. An elegant study, performed by Chang and colleagues using IFN- γ promoter-driven luciferase reporter construct in Jurkat T

cells, showed that hot water crude extracts of *B. pilosa* increased IFN- γ promoter activity two-fold [29]. Out of the subfractions of this extract, the butanol fraction, but not the water or ethyl acetate, fractions, increased IFN- γ promoter activity six-fold. Centaurein (110) and centaureidin (109) were identified from the butanol fraction and were stated to cause a four-fold increase in IFN- γ promoter activity with EC₅₀ values of 75 μ g/mL and 0.9 μ g/mL, respectively. The mechanism of action of centaurein was determined using transcription factors such as AP-1, NFAT, and NF κ B which are reported to bind to IFN- γ promoter and regulate IFN- γ transcription. Unlike with the activity of the positive control, PHA, centaurein caused a four-fold increase in NFAT, a 3-fold increase in NF κ B and had little, if any, effect on AP-1 enhancer activities (23-fold, three-fold, and ten-fold increases, respectively, were seen with PHA) [29]. The authors concluded that centaurein modulates IFN- γ expression by the NFAT and NF κ B pathways. The article only determined the mechanism of action of centaurein. Its aglycone centaureidin may act through the same mechanism though this conclusion needs to be further verified.

B. pilosa extract and its compounds are reported to inhibit differentiation of naïve CD4 $^{+}$ helper T (Th0) cells into Th1 cells [44]. Using the Th cell differentiation assay as a screening platform, 3 polyynes, 2- β -D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (53), 3- β -D-glucopyranosyl-1-hydroxy-6(E)-tetradecene-8,10,12-triyne (69), and 2- β -D-glucopyranosyloxy-1-hydroxy-5(E)-tridecene-7,9,11-triyne (49) were discovered from *B. pilosa* [44, 46]. The data shows that cytopiloyne and other two polyynes suppressed the differentiation of type 1 helper T (Th1) cells and production of Th1 cytokines and promoted that of type 2 helper T (Th2) cells and production of Th2 cytokines, thus explaining the immunomodulatory and anti-inflammatory effects of *B. pilosa* and its polyynes.

Chang and colleagues were the first to report the effect of the butanol extract of *B. pilosa* on the autoimmune diabetes and airway inflammation in mice [73]. Imbalance in the levels of Th1 and Th2 and of various cytokines leads to autoimmune diseases. T1D and other autoimmune diseases (rheumatoid arthritis, Crohn's disease, among others) are exacerbated by an increase Th1 levels (specifically, CD4 $^{+}$ Th1 cells) while Th2 cells antagonize this effect [56]. Moreover, Th2 cells mediate asthma in ovalbumin-induced hypersensitivity in BALB/c mice [73]. In their work [73], Chang and colleagues showed that 10 mg/kg butanol extracts with 1.5% (w/w) compound 69 and 1.1% (w/w) compound 49 (Table II) ameliorated the development of Th1-mediated diabetes in NOD mice through inhibition of β cell death and leukocyte infiltration. At the same dosage, the butanol extracts also exacerbated ovalbumin-induced pulmonary inflammation in BALB/c mice with an increase in the infiltration of eosinophils and mast cells into the airway of the mice [73]. Despite the different outcomes, both mouse models proved the concept that control over the Th1/Th2 shift is associated with autoimmune diseases and that the *B. pilosa* butanol extract can shift the differentiation of Th0 cells to Th2 cells [44, 73].

An extended study presented by Chiang and colleagues showed that compound **53** modulates T-cell functions [50]. Using CD4⁺ T cells from BALB/c mice, they demonstrated that cytopiloyne decreased levels of IFN- γ producing cells (Th1) by 12.2% (from 72% to 59.8%). Since Th1 and Th2 cell differentiation is antagonistic, it was expected that compound **53** increased the percentage of mouse IL-4 producing cells (Th2) by 7.2% (from 23.7% to 30.9%). Subsequent assessment of effect of compound **53** on the modulation of the transcription of IL-4 and IFN- γ showed that cytopiloyne, as expected, decreased the splenocyte levels of IFN- γ mRNA and increasing that of IL-4 in a dose-dependent manner. In the range of 0.1 to 3 μ g/mL of compound **53**, the effects were not attributed to its cytotoxicity. Consequently, using 3 μ g/mL cytopiloyne for 72 and 96 hours, the protein concentration of IFN- γ decreased to 18.6% and 44.4%, respectively. Under the same conditions, cytopiloyne increased IL-4 concentrations to 198.5% and 247.0% (for 72 and 96 hours, resp.). This modulation of T-cell differentiation exhibited by compound **53** was used to explain its anti-diabetic activity [50].

The anti-diabetic role of cytopiloyne was extensively discussed above (Section 3.3, anti-diabetic activity). The molecular basis of the regulation of cytokine expression by cytopiloyne has been described. Cytopiloyne directly elevated the expression level of IL-4 via GATA-3 upregulation in T cells [46]. However, reduction of IFN- γ expression in T cells seemed to come from the indirect opposing effect IL-4 cytokine because the expression level of T-bet was unaltered [46]. In this way, cytopiloyne skewed Th1 polarization into the Th2 state, conferring protection against T1D in NOD mice. Aside from polarization of Th cell differentiation, cytopiloyne also activated the expression of Fas ligand in pancreatic b cells, this increase leading to the partial depletion of T cells and reduction of immune response in local areas such as the pancreas. Of note, cytopiloyne also inhibited T-cell proliferation and activation. By targeting T cells from three immunomodulatory actions, cytopiloyne protects against T1D and probably other Th1-mediated autoimmune diseases [56].

The phytochemical constituents of *B. pilosa* exert their functions on different immune cells to modulate immune response. It is possible that some of the compounds may have agonistic or antagonistic effects on immune response. Immune function of *B. pilosa* may depend on its composition and amount of compounds, which could explain the apparently conflicting report of *B. pilosa* butanol extract aggravating allergy in mice [73] while cellulosine-treated extract ameliorated allergy [37, 73]. IFN- γ promoter reporter assays and T-cell differentiation assays were used to isolate 2 flavonoids [29] and 3 polyynes [44, 50] as immunomodulatory compounds from the butanol fraction of *B. pilosa*. Interestingly, the flavonoids promote IFN- γ expression in NK and T cells. In marked contrast, the polyynes promote IL-4 expression and indirectly inhibit IFN- γ expression in differentiating T cells. Sensitivity appears to be the key to identifying structure- and bioactivity-related phytochemicals from medicinal plants.

3.6. Antimalarial Activity. The use of chemical drugs against pathogens has resulted in drug-resistant mutants. Examples of drug resistance can be found in the species of the *Plasmodium* that cause malaria. It is important to search for new compounds to combat *Plasmodium* parasites [51]. A study of the anti-malarial activity of the leaf extracts of *B. pilosa* using a combination of phytochemistry and bioassays showed that compound **49** (Table 11) showed activity against a malaria parasite (*P. falciparum* NF54 strain) with an IC₅₀ value of 6.0 μ g/mL [30]. In addition, compound **49**, isolated from the aerial parts of *B. pilosa*, inhibited growth of the *P. falciparum* FCR-3 strain with an IC₅₀ value of 0.35 μ g/mL. This compound was tested for its *in vivo* effect in mice infected with *P. berghei* NK-65 strain. Results showed that compound **49** decreased the average parasitemia in the red blood cells by 20.7 (from 32.8% of that of the control to 12.1%) after an intravenous injection of 0.8 mg/kg BW/day for four days [51]. Further studies addressing the anti-malarial mechanism underlying both polyynes and clinical studies are needed.

3.7. Antibacterial Activity. Emergence of multiple antibiotic-resistant microbes is becoming a global threat to public health and a challenge to disease treatment. For instance, penicillin is commonly used to combat a food-borne intracellular bacterium *Listeria*; however, penicillin-resistant bacteria have been discovered recently [29]. Chang and coworkers isolated centaurein (**110**) and centaureidin (**109**) from *B. pilosa* extract [29, 47]. Centaurein enhances expression of IFN- γ , a key cytokine for macrophage activation and, consequently, enhances bactericidal activity in macrophages [29, 47]. In agreement with observed *in vitro* effects, centaurein was reported to prevent and treat *Listeria* infection in C57BL/6J mice [52]. Mechanistic studies confirmed that centaurein exerted antilisterial action via IFN- γ expression in wild-type mice but not IFN- γ knockout mice [52]. Further *in vitro* studies on centaurein showed that this compound increased IFN- γ expression by 13% (from 17% to 20%), 20% (from 21% to 41%), and 11% (from 6% to 17%) in CD4⁺ T cells, CD8⁺T cells, and NK cells, respectively. That is to say, there was an increase in IFN- γ producing immune cells. As expected, centaurein also enhanced the expression level of T-bet, a key nuclear factor for IFN- γ expression. Consistently, centaurein augmented the serum IFN- γ levels in C57BL/6J mice, and this augmentation peaked 24 hours after compound injection. The quantity of mouse serum IFN- γ was sufficient to activate macrophages *in vitro* and eradicated GFP-producing *Listeria* inside macrophages. However, the entry of *Listeria* into macrophages was not affected by centaurein-treated mouse sera. Centaurein treatment at 20 μ g per mouse rescued 30% of the mice infected with a lethal dose of *Listeria* (2×10^6 CFU). It is noteworthy that in the presence of ampicillin (5 μ g/mouse), centaurein (20 μ g/mouse) rescued 70% of the mice, suggesting an additive effect between ampicillin and centaurein [52]. Despite lower abundance, centaureidin was 30 times more active than centaurein in terms of IFN- γ production [52].

Aside from the indirect antibacterial action mentioned above, extract and/or compounds of *B. pilosa* also showed

TABLE 17: Antibacterial activity of essential oils and flower extracts from *B. pilosa* [53].

Strain	Leaf essential oil	Flower essential oil	Mean zone of inhibition (mm)	Leaf extract	Flower extract
<i>Micrococcus flavus</i>	12.7 ± 0.3	8.7 ± 0.3	10.2 ± 0.2	10.8 ± 0.3	
<i>Bacillus subtilis</i>	17.3 ± 1.9	11.7 ± 0.2	10.9 ± 0.2	10.3 ± 0.2	
<i>Bacillus cereus</i>	19.0 ± 1.4	11.2 ± 0.3	11.8 ± 0.4	18.5 ± 1.0	
<i>Bacillus pumilus</i>	12.3 ± 0.7	10.8 ± 0.2	10.5 ± 0.4	7.7 ± 0.2	
<i>Escherichia coli</i>	13.7 ± 0.4	20.3 ± 0.7	10.2 ± 1.1	14.0 ± 1.3	
<i>Pseudomonas ovalis</i>	12.5 ± 0.8	13.7 ± 1.5	10.2 ± 0.6	12.5 ± 0.6	

direct bacteriostatic and/or bactericidal action. One study reported that essential oils and leaf/flower extracts of *B. pilosa* could suppress the growth of gram positive and gram negative bacteria as evidenced by zone of inhibition assays. In this study, antibacterial activity of the essential oils from the leaves and flowers of *B. pilosa* was determined in an attempt to identify natural products as food preservatives for prevention of microbial multiplication and food oxidation. The essential oils and extracts of *B. pilosa* leaves and flowers showed moderate but different extents of antibacterial activity (Table 17). In general, essential oils had higher antibacterial activity than crude extracts. One explanation for this could be that monoterpenes in the essential oils destroy cellular integrity and, subsequently, inhibit the respiration and ion transport processes. The presence of antibacterial β-caryophyllene could be another explanation as reported elsewhere [53]. Another study reported that the methanol and acetone extract of *B. pilosa* roots displayed antibacterial activities against the bacteria listed in Table 18 [54] and methanol extracts from the roots seemed to be the most effective.

Another study indicated that the polyyne, (*R*)-1,2-dihydroxytrideca-3,5,7,9,11-pentayne (**49**), from this plant also suppressed bacterial growth as shown by the minimum inhibitory concentration required to inhibit 50% bacterial growth (MIC₅₀) in Table 19. This compound was highly effective against several Gram positive and Gram negative bacteria including the drug-resistant bacteria *Staphylococcus aureus* N315 (MRSA) and *Enterococcus faecalis* NCTC12201 (VRE) [51]. Strikingly, compound **49** had a similar MIC₅₀ value to antibiotics (ampicillin, tetracycline, norfloxacin, and amphotericin B) in most of the bacteria tested.

Antibacterial activity of *B. pilosa* extracts and components, expressed as MIC₅₀ and the mean zone of inhibition, is tabulated in Tables 17, 18, and 19. The zone of inhibition for Ampicilline (positive control) ranges from 15.3 ± 0.3 mm to 44.3 ± 0.2 mm [53].

3.8. Antifungal Activity. *B. pilosa* has traditionally been used to treat microbial infection. Recently, different parts of *B. pilosa* have been tested for antifungal activities. Deba and colleagues first evaluated the antifungal effect of the hot water extracts of the *B. pilosa* roots, stems, and leaves against *Corticium rolfsii*, *Fusarium solani*, and *Fusarium oxysporum*. They discovered that *C. rolfsii* was most suppressed by treatment with *B. pilosa* as its growth was reduced at almost

TABLE 18: Antibacterial activity of root extracts from *B. pilosa* [54].

Strain	MIC ₅₀ (mg/mL)	
	Methanol extract	Acetone extract
<i>Bacillus cereus</i>	10	—
<i>Escherichia coli</i>	5	5
<i>Klebsilla pneumonia</i>	5	10
<i>Micrococcus kristinae</i>	10	—
<i>Pseudomonas aeruginosa</i>	10	10
<i>Staphylococcus aureus</i>	5	10
<i>Sraphylococcus epidermidis</i>	5	5
<i>Serratia marcescens</i>	10	—
<i>Shigelea flexneri</i>	10	—
<i>Streptococcus faecalis</i>	10	—

TABLE 19: Antibacterial activity of *B. pilosa* of compound **29** [51].

Strain	MIC ₅₀ (μg/mL)
<i>Escherichia coli</i> NIHJ	1
<i>Escherichia coli</i> ATCC25922	1
<i>Klebsiella pneumoniae</i> ATCC700603	128
<i>Serratia marcescens</i> ATCC13880	16
<i>Pseudomonas aeruginosa</i> ATCC27853	8
<i>Staphylococcus aureus</i> FDA209P	0.5
<i>Staphylococcus aureus</i> ATCC29213	0.25
<i>Staphylococcus aureus</i> N315 (MRSA)	0.5
<i>Enterococcus faecalis</i> ATCC29212	2
<i>Enterococcus faecalis</i> NCTC12201 (VRE)	1
<i>Bacillus subtilis</i> ATCC6633	0.5
<i>Candida albicans</i> ATCC10231	0.25

all the tested doses, followed by *F. oxysporum* and *F. solani* [97]. However, the fungicidal activities of the stems, and roots were greater than the leaves [97]. Moreover, the same group assessed the antifungal activity of the essential oils and aqueous extracts from *B. pilosa* flowers and leaves [53]. They showed that the extracts and oils had antifungal activity against *C. rolfsii*, *F. solani*, and *F. oxysporum*. Essential oils appeared to have better fungicidal activity than water extracts as summarized in Table 20.

Another study by Ashafa and colleagues showed that acetone, methanol, and water extracts of the *B. pilosa* roots showed antifungal activities against *Aspergillus niger*, *A.*

TABLE 20: Antifungal activity of *B. pilosa* [53].

Part/extract	Concentration (ppm)	Strain, % Inhibition		
		<i>Corticium rolfsii</i>	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>
Leaves	Essential oils	100	85.7 ± 0.9	68.2 ± 0
		250	96.0 ± 0.8	77.9 ± 1.8
	Aqueous Extracts	100	44.6 ± 1.7	60.5 ± 2.1
		250	94.2 ± 0.3	68.9 ± 0.7
Flowers	Essential oils	100	60.4 ± 0.9	89.2 ± 0.4
		250	89.4 ± 1.2	98.0 ± 0.3
	Aqueous Extracts	100	33.1 ± 1.1	71.4 ± 0.7
		250	66.1 ± 1.4	91.2 ± 0

TABLE 21: Antifungal activity of *B. pilosa* root extracts [54].

Strain	LC ₅₀ (mg/mL)		
	Acetone extracts	Methanol extracts	Water extracts
<i>Aspergillus niger</i>	0.14	0.06	0.07
<i>Aspergillus flavus</i>	10.91	6.58	0
<i>Penicillium notatum</i>	0.05	0.05	0.05

flavus, and *Penicillium notatum* using the agar dilution method. The results are tabulated in Table 21 [54]. Negative controls showed 0% growth inhibition. The methanol extract of the *B. pilosa* roots at 10 mg/mL was also effective against *Candida albicans* [54]. Of note, *B. pilosa* obtained from Papua New Guinea had no activity against *A. niger* and *C. albicans* [125], but the South African (Eastern Cape) ecotype exhibited moderate activity against *C. albicans* [54]. This discrepancy may depend on extraction solvents, extraction procedure, assay techniques, different plant parts, and abundance of active compounds.

B. pilosa produces a variety of secondary metabolites such as flavonoids, phenylacetylenes, alkaloids, sterols, terpenoids, and tannins [53, 54]. However, none of them have been confirmed as active compounds against fungi. Further investigation of active compounds from *B. pilosa* is necessary to further understand the antifungal efficacy of this plant.

3.9. Hypotensive and Vasodilatory Activities. In early studies, Dimo and colleagues used three rat models, normotensive Wistar rats (NTR), salt-loading hypertensive rats (SLHR), and spontaneous hypertensive rats (SHR) to investigate the hypotensive effect of the methanol crude extract of *B. pilosa* leaves [19, 126]. The extract lowered systolic blood pressure in hypertensive rats (SLHR and SHR) to a greater degree than NTR [19, 126]. In addition, a decrease in urinary sodium ions and an increase in urinary potassium ions were observed after treatment with the methanol extract of *B. pilosa* leaves although neither differences were statistically significant [19, 126]. Taking the data together, the study proposed that *B. pilosa* leaf extract reduced blood pressure via vasodilation [19, 126]. The same group continued to test the antihypertensive effect of aqueous and methylene chloride extracts of *B. pilosa* leaves in a hypertensive rat model [19, 126]. To establish

a fructose-induced hypertension model, male Wistar rats were given 10% fructose solution to drink *ad libitum* for three weeks. In addition to free access to 10% fructose, the rats were treated with the aqueous (150 mg/kg) or methyl chloride (350 mg/kg) extracts of *B. pilosa* for additional three weeks [19, 126]. Both extracts of *B. pilosa* leaves had a hypotensive effect on rats. However, neither extracts reversed the elevation of serum insulin in fructose-fed rats. Therefore, *B. pilosa* lowered blood pressure irrespective of insulin [19, 126]. To better understand the hypotensive mechanism, the authors investigated the effect of a neutral extract of *B. pilosa* (NBP), a mixture of methanol and methylene chloride (1:1) extract after neutralization with NaOH and HCl, on the heart and the blood pressure of NTR and SHR [109]. This study showed that an intravenous injection of the NBP resulted in a biphasic reduction in systolic blood pressure. In addition, one intravenous dose of the extract at 10, 20, and 30 mg/kg BW decreased systolic blood pressure in normal rats by 18.3%, 42.5%, and 30%, respectively, and the same doses reduced the blood pressure in hypertensive rats by 25.8%, 38.9%, and 28.6%, respectively. Only the highest dose (30 mg/kg) affected the force of the contraction of the heart. Atropine and propranolol were used to interfere with the hypotensive action of the NBP. Atropine reduced the initial phase of the hypotensive response in NBP and completely abolished the second phase of hypotensive response in NBP. In contrast, propranolol increased the first hypotensive response but partially abolished the second hypotensive response provoked by the NBP [109]. This mechanistic study suggested that *B. pilosa* invokes the biphasic hypotensive responses via targeting cardiac pump efficiency during the first phase and vasodilation at the second phase [109].

A further study was performed to investigate the relaxing effect of a neutral extract of *B. pilosa* (NBP) on rat aorta contracted with KCl (60 mM) and norepinephrine (0.1 mM) [55]. Cumulative addition of NBP relaxed the rat aorta previously contracted by KCl in a dose-dependent manner. The EC₅₀ value of the NBP for vasorelaxation was 0.32 mg/mL. The data also showed that the NBP reduced the contraction of aorta previously contracted by KCl irrespective of the presence of aortic endothelium [55].

Pretreatment with glibenclamide, an ATP-dependent K⁺ channel blocker, did not considerably affect the relaxant effect of the NBP on KCl-induced contraction, suggesting that

the vasodilatory effect of *B. pilosa* was not related to the opening of this ATP-dependent K⁺ channel [55]. On the other hand, in the presence of indomethacin or pyrilamine maleate, the relaxant response induced by the plant extract was significantly inhibited at the lower concentrations. The plant extract was able to reduce the aorta resting tone, inhibit the KCl-induced contractions by 90% at 1.5 mg/mL and the CaCl₂-induced contractions by 95% at 0.75 mg/mL. These results demonstrate that *B. pilosa* can act as a vasodilator probably via acting as a calcium antagonist [55].

However, no specific compound for the above activity has been identified from *B. pilosa* to date. A bioactivity-guided identification approach may be adopted to identify the active compounds in *B. pilosa* that possess hypotensive and vasodilatory effects and understand their mechanism of action.

3.10. Wound Healing Activity. *B. pilosa* has been traditionally used to treat tissue injury in Cameroon, Brazil, and Venezuela [17]. Hassan and colleagues investigated the wound healing potential of *B. pilosa* in Wistar rats [127]. Mirroring the positive control neomycin sulfate, the ethanol extract of *B. pilosa* had faster wound closure than control rats 3, 6, and 9 days after topical application. Histological examination also revealed better collagenation, angiogenesis, and organization of wound tissue seven days after application. Epithelialization and total healing time in *B. pilosa*-treated rats were comparable to those of neomycin sulfate. Together, these data suggest that *B. pilosa* may be a viable alternative to neomycin lotion for the treatment of wounds.

In addition to studying the wound healing effect of *B. pilosa* on external ulcers, Tan and colleagues also examined the effect of methanol, cyclohexane, and methyl chloride extracts of *B. pilosa* on gastric ulcers in Wistar rats fed with 1 mL HCl/ethanol gastric necrotizing solution (150 mM HCl in 60% ethanol), and macroscopically visible lesions were scored [17]. Among the three extracts, methylene chloride extracts exhibited the highest activity showing 46.4% inhibition of lesion formation at a dose of 500 mg/kg BW and complete inhibition at 750 mg/kg [17]. The efficacy of the ethylene chloride extract was followed by that of the methanol extracts which had inhibition ranging from 30.4% to 82.2% at concentrations of 500 mg/kg and 1000 mg/kg BW, respectively [17]. The cyclohexane extracts showed the lowest activity against gastric ulcers in rats with 13.3%, 40%, and 79.7% inhibition at 500, 750, and 1000 mg/kg BW, respectively [17]. To better understand the mode of action of the methylene chloride extract of *B. pilosa*, rats were pretreated with indomethacin, a COX-2 inhibitor involved in prostaglandin synthesis. Pretreatment significantly reduced the protection against HCl/ethanol-induced ulcers to 31.3% inhibition at 750 mg/kg BW, suggesting a link between the antiulcerative activity of *B. pilosa* and prostaglandin synthesis. Unexpectedly, the methylene chloride extract of *B. pilosa* showed little gastric mucosal protection against gastric lesions induced by 95% ethanol (1 mL) [17]. Absolute alcohol is known to cause mucosal/submucosal tissue destruction via cellular necrosis and the release of tissue-derived mediators

(histamine and leukotriene C4). Thus, these data imply that *B. pilosa* did not prevent the generation or the necrotic action of these mediators on the gastric microvasculature. In addition, pylorus ligation can increase gastric acid secretion without an alteration of mucosal histamine content. The methylene chloride extract of *B. pilosa* did not possess antisecretory activity. On the contrary, it was observed that increases in the dose of the extract led to elevated gastric juice acidity [17]. Results with both absolute ethanol and pylorus ligation rat models suggested the possibility that the ineffectiveness of *B. pilosa* against gastric ulcers was due to lack of antihistaminic activity in the plant. In summary, overall the data suggest that *B. pilosa* protects against HCl/ethanol-mediated ulcers via inhibition of prostaglandin biosynthesis.

Previous phytochemical studies showed that a group of flavonoids, acyclicchalcones, are present in *B. pilosa* [128, 129] and the chalcones were proposed to have anti-ulcerative activity [130]. Moreover, nine hydroxychalcones were reported to possess gastric cytoprotective effects with 2,4-dihydroxychalcone being the most active [131]. Since methylene chloride extracts appear to be the most active *B. pilosa* extracts, next, the specific anti-ulcerative phytochemicals in the methylene chloride extracts of *B. pilosa* and their modes of action needs to be probed.

Despite the claims listed in Table 3, relatively few scientific studies have been conducted *in vitro* and *in vivo* to address the traditional ethnomedical uses of *B. pilosa*. Information about the use of *B. pilosa* as a botanical therapy recorded so far is far from complete. Studies conducted thus far only serve as a starting point for further investigation of *B. pilosa*, and the ultimate efficacious use of the herb in clinical applications.

4. Toxicology

Despite its use as an ingredient in food for human consumption, studies on systemic toxicity (e.g., acute, subacute, chronic and subchronic toxicities) of *B. pilosa* in humans and animals are still inadequate and insufficient. So far, acute, and/or subchronic toxicities have been evaluated in rats and mice. Oral acute and 28-day toxicities of water extract of *B. pilosa* leaves were evaluated in Wistar rats [132]. An oral dose of water extract of *B. pilosa* leaves at 10 g/kg BW showed no obvious mortality or changes in the appearance in rats [133]. The same extract at 0.8 g/kg BW/day, once a day, showed no obvious sub-chronic toxicity in rats over 28 days, as measured by survival rate, body weight, and gross examination of organs [133]. These data are consistent with our data indicating that oral delivery of the water extract of the *B. pilosa* whole plant at 1 g/kg BW/day, once a day, is safe in rats over 28 days (unpublished data). Taken together, these studies suggest that ingestion of *B. pilosa* aqueous extract at up to 1 g/kg BW/day, once a day, is highly safe in rats. In addition, the acute toxicity of aqueous and ethanol extracts of *B. pilosa* in mice have been reported [133]. Five- to six-week-old mice with weights between 28 and 35 g received a peritoneal injection of both extracts at the different doses. The LD₅₀, the dose that causes 50% lethality, of the aqueous and ethanol extracts in mice

was 12.30 g/kg BW and 6.15 g/kg BW, respectively [133]. A complete toxicological study has not been completed for humans. Furthermore, the drug interactions of *B. pilosa* with other drugs are unknown. Further safety verification and clinical trials should be performed before *B. pilosa* can be considered for medicinal use.

5. Conclusions

B. pilosa is an erect, perennial plant with green leaves, white or yellow flowers and tiny black seeds. As it is distributed worldwide and is widely used as a folk remedy, *B. pilosa* can be thought of as an extraordinary source of food and medicine. However, a comprehensive up-to-date review of research on *B. pilosa* has hitherto been unavailable. In this article, scientific studies on *B. pilosa* have been summarized and critically discussed from the perspectives of botany, ethnomedicine, phytochemistry, pharmacology, and toxicology. *B. pilosa* is claimed to treat more than 40 disorders, and 201 compounds have been identified from this plant. The medicinal utility of *B. pilosa* and its modes of action in relation to its known phytochemicals were discussed herein. Polyyne, flavonoids, phenylpropanoids, fatty acids, and phenolics are the primary bioactive compounds of *B. pilosa*, and they have been reported to be effective in the treatment of tumors, inflammation/immune modulation, diabetes, viruses, microbes, protozoans, gastrointestinal diseases, hypertension, and cardiovascular diseases. Caution should be exercised in the therapeutic use of *B. pilosa* for hypoglycemia, hypotension, bleeding, and allergy.

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Review Article

Indian Traditional Ayurvedic System of Medicine and Nutritional Supplementation

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Food is the major source for serving the nutritional needs, but with growing modernization some traditional ways are being given up. Affluence of working population with changing lifestyles and reducing affordability of sick care, in terms of time and money involved, are some of the forces that are presently driving people towards thinking about their wellness. There has been increased global interest in traditional medicine. Efforts to monitor and regulate traditional herbal medicine are underway. Ayurveda, the traditional Indian medicine, remains the most ancient yet living traditions. Although India has been successful in promoting its therapies with more research and science-based approach, it still needs more extensive research and evidence base. Increased side effects, lack of curative treatment for several chronic diseases, high cost of new drugs, microbial resistance and emerging, diseases are some reasons for renewed public interest in complementary and alternative medicines. Numerous nutraceutical combinations have entered the international market through exploration of ethnopharmacological claims made by different traditional practices. This review gives an overview of the Ayurvedic system of medicine and its role in translational medicine in order to overcome malnutrition and related disorders.

1. Introduction

India is known for its traditional medicinal systems—Ayurveda, Siddha, and Unani. Medical systems are found mentioned even in the ancient Vedas and other scriptures. The Ayurvedic concept appeared and developed between 2500 and 500 BC in India [1]. The literal meaning of Ayurveda is “science of life,” because ancient Indian system of health care focused on views of man and his illness. It has been pointed out that the positive health means metabolically well-balanced human beings. Ayurveda is also called the “science of longevity” because it offers a complete system to live a long healthy life. It offers programs to rejuvenate the body through diet and nutrition. It offers treatment methods to cure many common diseases such as food allergies, which have few modern treatments. However, one should be aware that Ayurvedic nutrition is not a “magic bullet” system but requires the full participation of the patient to succeed. It is an interactive system that is user-friendly and educational. It teaches the patient to become responsible and self-empowered. Ayurveda is not a nutritional system for those seeking an escape or

excuse to further abuse their body or mind. It is a system for empowerment, a system of freedom, and long life.

Food is the major source for serving the nutritional needs, but with growing modernization some traditional methods are being given up (Table 1). Hence, the modern food habits are affecting the balanced nutrition [2]. There is an ever widening gap in nutrient intake due to which normal life is no longer normal. However, affluence of working population with changing lifestyles and reducing affordability of sick care, in terms of time and money involved, are some of the forces that are presently driving people towards thinking about their wellness.

2. Medicinal Plants Used in Alternative/Traditional Medicines

Alternative medicines are being used by about 60 percent of the world’s population. These medicines are not only used by the rural masses for their primary health care in developing countries but are also used in developed countries where

TABLE 1: Impact of modern food concept in required nutrition.

Nutrients	Intake by traditional ways	Intake by modern ways	Effect on nutrient intake
Water soluble vitamins (vitamins B and C) and minerals	Vegetables used for cooking were/are fresh	Freezing and packaging of the cut vegetables	Loss of ascorbic acid, water soluble vitamins, and minerals
Proteins, minerals, and vitamin B complex	Manual processing of cereals, without polishing	Milling and polishing of cereals	Reduces protein, minerals, and vitamin B complex
Calcium, iron, thiamine, and niacin	Fresh grinding at home	Heavy milling and poor storage conditions	Loss of calcium, iron, thiamin, and niacin
Iron	Cooking in iron pot	Food generally cooked in cookware like nonstick and Teflon-coated utensils	The benefit of organic iron from the conventional iron pot is not obtained by using modern cookware
Copper	Storing of water and cooking use of copper vessels	Stainless steel utensils and plastic wares	Copper required in minor amount which is not gained from modern utensils used today. Deficiency is known to cause chronic diarrhea, malabsorption problems, and reduce immunity. Use of plastic containers is also harmful

modern medicines dominate [3]. The Indian subcontinent is a vast repository of medicinal plants that are used in traditional medical treatments. The alternative medicines in the traditional systems are derived from herbs, minerals, and organic matter, while for the preparation of herbal drugs only medicinal plants are used. Use of plants as a source of medicine has been an ancient practice and is an important component of the health care system in India. In India, about 70 percent of rural population depends on the traditional Ayurvedic system of medicine. Most healers/practitioners of the traditional systems of medicine prepare formulations by their own recipes and dispense to the patients. In the Western countries, approximately 40 per cent of people are using the herbal medicine for the treatment of various diseases. This interest in traditional medicines is growing rapidly due to the attention being given to it by the governmental agencies and different NGO's comprising of general public and researchers as well as the increased side effects, adverse drug reactions, and cost factor of the modern medicines.

India is the largest producer of medicinal plants. There are currently about 250,000 registered medical practitioners of the Ayurvedic system, as compared to about 700,000 of the modern medicine. In India, around 20,000 medicinal plants have been recorded; however, traditional practitioners use only 7,000–7,500 plants for curing different diseases. The proportion of use of plants in the different Indian systems of medicine is Ayurveda 2000, Siddha 1300, Unani 1000, Homeopathy 800, Tibetan 500, Modern 200, and folk 4500. In India, around 25,000 effective plant-based formulations are used in traditional and folk medicine. More than 1.5 million practitioners are using the traditional medicinal system for health care in India. It is estimated that more than 7800 manufacturing units are involved in the production of natural health products and traditional plant-based formulations in India, which requires more than 2000 tons of medicinal plant

raw material annually [4]. More than 1500 herbals are sold as dietary supplements or ethnic traditional medicines [5].

Alternative medicines are being used by those people who do not use or cannot be helped by conventional medicinal system. Some common medicinal plants having nutraceutical potential and their primary use in traditional medicine [6–26] are being given in Table 2.

3. Expanding Complementary and Alternative (CAM) Approaches

More than 80 percent of people in developing countries cannot afford the most basic medical procedures, drugs, and vaccines. Among wealthier populations in both developed and developing countries, complementary and alternative practices are popular although proof of their safety and effectiveness is modest. Evidence-based research in Ayurveda is receiving larger acceptance in India and abroad [27–30]. The National Center for Complementary and Alternative Medicine has been inaugurated as the United States Federal Government's lead agency for scientific research in this arena of medicine. Its mission is to explore complementary and alternative healing practices in the context of rigorous science, support sophisticated research, train researchers, disseminate information to the public on the modalities that work, and explain the scientific rationale underlying discoveries. The center is committed to explore and fund all such therapies for which there is sufficient preliminary data, compelling public health need and ethical justifications [31, 32].

Complementary and alternative practices are adjuncts or alternatives to Western medical approaches. Economic factors influence user behavior. Although social, cultural, and medical reasons account for most of the appeal of traditional

approaches, economic factors also play a role. It is assumed that users of these approaches choose them because they are cheaper than conventional therapies or systems. However, several studies have found that CAM approaches cost the same or more than conventional treatments for the same conditions; thus, people seek them out for reasons other than cost. At least one study showed that financial factors ranked behind such reasons as confidence in the treatment, ease of access, and convenience, in the choice of a traditional healer. Another common misconception is that the poor are more likely to use traditional medicine, but this is not always true. Nowadays people seek CAM techniques because they believe the side effects will be lower. In both developed and developing countries, users of complementary methods also commonly seek conventional care [33]. Table 3 enlists some important Ayurvedic herbal formulations [34].

4. Nutraceuticals an Evolving Alternative Approach

Nutrition is a fundamental need. Various risk factors related to health result from an imbalance in nutrition. These imbalances in India are widely prevalent leading to adverse outcomes. A certain section of the population consumes diet which does not provide sufficient calories, let alone sufficient nutrients. In India, nearly 20% of the total population and 44% of young children (below 5 years of age) are undernourished and underweight. On the other hand, there is a huge population that is nourished in calorie intake but not in terms of nutrient intake. This segment would typically include lower middle to upper class population with sufficient purchasing capacity but probably less awareness about their nutrient requirements, leading to imbalanced nutritional uptake. In fact, in our population about 30% in urban and 34% in rural areas consume more than the recommended number of calories with higher than recommended levels of dietary fats and could be the largest contributor in making India the future cardiovascular and diabetes capital of the world. The third population segment, which is about 80 million, consumes nutrients and calories more than those recommended for the lifestyle they have opted for. The main risk factors in developing countries like India are related to nutrition and contribute to nearly 40% of total death and 39% of total disease burden. The main leading risk factors in developing countries [2] are shown in Figure 1.

According to WHO report, India has the largest burden of cardiovascular diseases and largest number of diabetes patients in the world. The number of cardiovascular diseases patients in Brazil, Russia, China, and India are 4.1, 11.8, 24.5, and 28.9 million, respectively. Likewise the numbers of diabetes patients in same countries are 4.6, 4.6, 20.8, and 31.7 million, respectively. An estimate of the cost of productivity lost on account of mortality due to nutrition-related disorders was estimated to be 0.85% of the GDP in 2004 and is expected to increase up to 1.2% for India's GDP by 2015. Nearly 340 million people, 30% of the population in urban areas and 34% of the population in rural areas, consume calories more than the norms. Even in the population that shows

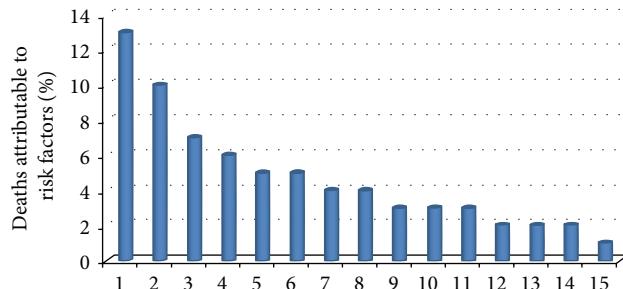


FIGURE 1: Risk factors related to nutrition—1: underweight, 2: unsafe sex, 3: blood pressure, 4: unsafe water, sanitation and hygiene, 5: cholesterol, 6: tobacco, 7: indoor smoke from solid fuels, 8: low fruit and vegetable intake, 9: zinc deficiency, 10: iron deficiency, 11: vitamin A deficiency, 12: physical inactivity, 13: alcohol, 14: overweight, and 15: unsafe healthcare injections.

sufficient calorie intake, the micronutrient consumption is not at desired levels. While the intake of calorie-rich foods may be high, micronutrient-rich foods are being consumed in low proportions. As a result, significant micronutrient deficiencies exist in urban as well as rural areas [35].

Hence, the requirement of external intervention, that can supplement diet to help prevent nutrition-related disorders and promote wellness over treatment of various diseases, has become a necessity, and such products are known as nutraceuticals. A nutraceutical is a food or food component that claims to have health benefits, including treatment and prevention of disease. Nutraceuticals, an emerging concept, can be broadly categorized as products which are extracted from natural sources (nature-like) or manufactured synthetically (man-made), which supplement the diet to provide nutrition over and above regular food and help prevent nutrition-related disorders. Nutraceuticals, foods or food components that help in prevention or treatment of disease, are made from herbal/botanical raw materials. They do more than just supplement the diet. They, as was pointed out, help with disease prevention and treatment. Theoretically, the appeal of nutraceuticals is to accomplish treatment goals without side effects.

The nutraceutical industry is rapidly growing (7%–12% per year). With extensive anecdotal data on exciting health results, nutraceuticals promise significant contributions to disease prevention. The global nutraceuticals market is estimated at 117 billion US dollar of which India's share is a meager 0.9%. United States and Japan are key markets for nutraceutical consumption. Indian nutraceuticals market is about 1 billion USD which is increasing day by day. Globally, this market is expected to reach 177 billion USD in 2013. The dietary supplements category is expected to be the fastest growing product category globally [2].

5. Herbal Medicines in Dietary Supplements

Dietary supplements and herbal remedies are popular complementary or alternative products for people. These are the supplements that are intended to supplement the diet and

TABLE 2: Some common medicinal plants having nutraceutical potential and their primary use in traditional medicine.

Plant name	Common name	Uses
<i>Asparagus racemosus</i> Willd	Shatavari	A potent Ayurvedic rejuvenative. It supplies many female hormones and mostly recommended for those women who have hysterectomies. It also helps to maintain urinary tract and strengthens the immune system and also purifies the blood.
<i>Commiphora mukul</i> Engl.	Guggul	A major ingredient in joint and immunocare and regarded as a remedy in Ayurvedic medicine; it increase white blood cell count to possess strong immuno-modulating properties. It also protects against the common cold as well as used in various other conditions like lower cholesterol and triglycerides, while maintaining the HDL to LDL ratio.
<i>Cyperus scariosus</i> Br.	Nagarmusta	Useful in supporting healthy genitourinary system and have hepatoprotective properties.
<i>Garcinia cambogia</i> Dr	Garcinia	Fruits contain biologically active compounds (-) hydroxycitric acid, which is known to inhibit the synthesis of lipids and fatty acids. HCA inhibits the enzyme ATP-citrate lyase that leads to reduce production of acetyl CoA, which is a key substance in fat and carbohydrate metabolism. Therefore, formation of LDL and triglycerides is very low. It also suppresses appetite by promoting synthesis of glycogen. That way the brain gets signals of fullness and satisfaction sooner. Garcinia contains significant amounts of vitamin C and used as a heart tonic.
<i>Glycyrrhiza glabra</i> L.	Yashtimadhu, Licorice	It is a versatile medicine in India and China, for gastrointestinal health. It is a mild laxative, soothes and tones the mucous membranes, and relieves muscle spasms. It is an antioxidant, cancer protecting, botanical boosting, and certain immune functions such as interferon production. Its mode of action is as an antimutagen, preventing damage to genetic material that can eventually result in cancer.
<i>Gymnema sylvestre</i> R. Br.	Gurmarar	Its Sanskrit name means literally “sugar destroyer,” has a glycolytic action, and reduces the strength of a glucose solution. It has been used in Ayurveda to regulate sugar metabolism for several centuries. It increases insulin production, regeneration of pancreas cells, and the site of insulin production. Another property is abolishing the taste of sugar, so that Gurmarar has been effective to suppress and neutralize the craving for sweets.
<i>Melia azadirachta</i> L.	Nimba, Neem	It has strong health alleviating activity, used as a tonic and astringent that promotes healing. The extract has antispasmodic action. Its usage in Ayurvedic medicine for thousands of years has proved its detoxifying properties. It has shown most beneficial effects for the circulatory, digestive, respiratory, and urinary systems.
<i>Momordica charantia</i> L.	Karela, Bitter melon	Karela has been widely used in Ayurvedic medicine. It contains Gurmarin, a polypeptide considered to be similar to bovine insulin, and has a strong sugar regulating effect by suppressing the neural responses to sweet taste stimuli.
<i>Moringa pterygosperma</i> Gaertn	Shigru, Horseradish tree	Shigru contains physiologically active principles that is effective in a broad range of health needs. It contains “Pterygospermin,” an antibiotic-like substance.
<i>Mucuna pruriens</i> Baker	Kiwanch, Kapikachchhu, Cow-itch plant	It is a good natural source of <i>L. dopa</i> . In the Ayurvedic system it is reported as an effective tonic for nervous system. Studies have demonstrated its usefulness maintaining optimum performance of the nervous system.
<i>Nardostachys jatamansi</i> DC.	Jatamansi, Musk root	Jatamansi is a relaxing plant, effectiveness for mental health. It is used in various Ayurvedic formulations as a potent ingredient. It has been shown effective in maintaining a restful sleep and with many menopausal symptoms.

TABLE 2: Continued.

Plant name	Common name	Uses
<i>Piper longum</i> L.	Pippali, Indian Long Pepper	Pippali is a powerful stimulant for both the digestive and the respiratory systems and has a rejuvenating effect on lungs. It plays an important role in release of metabolic heat energy. This effect is the result of increased thyroid hormone level in the body. Pippali a typical Ayurvedic complementary component whose benefit is to increase the bioavailability and enhance absorption of the other active ingredients.
<i>Piper nigrum</i> L.	Maricha, Black pepper	The black pepper is one of the most important spices which is widely used to amplify the body's ability to absorb nutrients contained in the food and aid the digestive process.
<i>Bergenia ligulata</i> Wall	Pasanavheda	It has the unique property like diuretic action with optimum urinary tract health. This important drug supports bladder by acting on the crystalloid-colloid balance and keeping calcium salts in solution.
<i>Terminalia chebula</i> Retz.	Haritaki	Haritaki is a safe and effective purgative, expectorant, and tonic. It is an important ingredient of the classical Ayurvedic formulation "Triphala" which has a combination of three fruits. Triphala is an important Ayurvedic medicine, which promotes health through successive steps of purification and detoxification. It is known to have strong antimutagenic activity, because of its very rich content vitamin C.
<i>Tinospora cordifolia</i> Miers	Guduchi	Guduchi is a rich source of natural vitamin C and effective in inhibiting the growth of bacteria and in building up the immune resistance and has immune-boosting ability. Use of this plant increases white blood cells the killing ability of macrophages, the immune cells responsible for fighting invaders.
<i>Withania somnifera</i> (L.) Dunal	Ashwagandha	In Ayurvedic medicines Ashwagandha holds a place similar to Ginseng in traditional Chinese medicinal therapies. It is also called the "Indian Ginseng." It has been used for thousands of years as a popular remedy in Ayurvedic systems for many conditions. It is one of the best health tonics and restorative agents that have been used to treat general debility.
<i>Zingiber officinale</i> Rosc	Sunthi, Ginger	Ginger is considered an adjuvant in many Ayurvedic formulas in which it enhances absorption and prevents gastrointestinal side effects. It is a very common spice which is used in Ayurvedic medicine to improve digestion and to prevent nausea. These properties help bowel movements and relax the muscles which control the digestive system.

contain one or more dietary ingredients (including vitamins, minerals, herbs or other botanicals, amino acids, and other substances) or their constituents. These are intended to be taken by mouth as a pill, capsule, tablet, or liquid and are labeled on the front panel as being a dietary supplement. Such products may range from isolated nutrients, dietary supplements, and diets to genetically engineered "designer" foods, herbal products, and processed foods such as cereals, soups, and beverages. These botanicals are sold in many forms as fresh or dried products, liquid or solid extracts, tablets, capsules, powders, tea bags, and so forth. For example, fresh ginger root is often used in various food stores; dried ginger root is sold packaged in tea bags, capsules, or tablets, and liquid preparations made from ginger root are also sold in the market. A particular group of chemicals or a single chemical may be isolated from a botanical and sold as a dietary supplement, usually in tablet or capsule form. An example is phytoestrogens from soy products [36].

6. Nutraceutical Concept with Varying Definition

The nomenclature for nutraceuticals is based on the segments it constitutes. In Canada, this term is natural health products; in USA, it is called dietary supplements, and in Japan it is called foods for special health use. There are distinct definitions and regulations for dietary supplements and functional foods in USA, Canada, and Europe. In Japan, dietary supplements and functional foods are governed under the same set of regulations. USA and Canada actually list the constituents that a product must have to be called a nutraceutical, whereas Europe and Japan just provide general guidelines on the properties that a product should have to be called a nutraceutical. Traditional and herbal medicines are included in the definition of dietary or nutritional supplements in Canada. Japan does not mention traditional herbal medicines under functional foods for special health use. USA includes

TABLE 3: Some important herbal formulations frequently used in traditional Ayurvedic system in India.

Disease	Formulation's ingredients/ratio	Dose/method of use
Anemia	<i>Asparagus racemosus</i> (roots) 20%	
	<i>Withania somnifera</i> (roots) 20%	
	<i>Phyllanthus emblica</i> (fruits) 15%	
	<i>P. amarus</i> (leaves) 10%	4 gm of powder is given to the patient, twice daily with water
	<i>Tephrosia purpurea</i> (leaves) 10%	
Asthma/bronchitis	<i>Plumbago zeylanica</i> (roots) 5%	
	<i>Glycyrrhiza glabra</i> (roots) 15%	
	<i>Piper longum</i> (fruits) 5%	
	<i>Solanum xanthocarpum</i> (whole plant) 25%	
	<i>Piper longum</i> (fruits) 10%	
Arthritis	<i>Adhatoda vasica</i> (leaves) 25%	
	<i>Zingiber officinale</i> (roots) 10%	4 gm (one teaspoonful) of mixed powder given to the patient, twice a day (morning and at bedtime) with water
	<i>Curcuma zedoaria</i> (roots) 10%	
	<i>Ocimum sanctum</i> (leaves) 10%	
	<i>Phyllanthus emblica</i> (fruits) 10%	
Blood circulation	<i>Piper longum</i> (fruits) 10%	
	<i>S. xanthocarpum</i> (whole plant) 15%	
	<i>Withania somnifera</i> (roots) 10%	
	<i>Terminalia chebula</i> (fruits) 10%	4 gm of mixed powder should be given to the patient, twice daily (morning and evening, one hour before meals) with ginger juice for rheumatic problems
	<i>T. bellerica</i> (fruits) 10%	
Cancer	<i>Curcuma zedoaria</i> (roots) 15%	
	<i>Phyllanthus emblica</i> (fruits) 15%	
	<i>Ricinus communis</i> (roots) 15%	
	<i>Zingiber officinale</i> (roots) 20%	
	<i>Piper longum</i> (roots) 10%	
Chronic constipation	<i>Withania somnifera</i> (roots) 10%	
	<i>Phyllanthus emblica</i> (fruits) 10%	
	<i>Curcuma longa</i> (roots) 10%	4 gm of mixed powder is given to the patient, twice daily with water
	<i>Terminalia bellerica</i> (fruits) 10%	
	<i>T. chebula</i> (fruits) 10%	
Chronic fever	<i>Ocimum sanctum</i> (leaves) 10%	
	<i>Tephrosia purpurea</i> (leaves) 10%	
	<i>Azadirachta indica</i> (bark) 20%	
	<i>Bauhinia variegata</i> (bark) 15%	
	<i>Crataeva nurvala</i> (bark) 15%	4 gm of mixed powder should be given to the patient, twice a day (morning and night) with lukewarm honey for cancer cure
Chronic fever	<i>Terminalia chebula</i> (fruits) 15%	
	<i>T. bellerica</i> (fruits) 10%	
	<i>Holarrhena antidyserterica</i> (bark) 10%	
	<i>Tinospora cordifolia</i> (stems) 15%	
	<i>Holarrhena antidyserterica</i> (bark) 10%	
Chronic fever	<i>Plumbago ovata</i> (husk) 20%	
	<i>Terminalia bellerica</i> (fruits) 10%	
	<i>T. chebula</i> (fruits) 15%	4 gm of mixed powder is given to the patient, at night before going to bed, with water
	<i>Phyllanthus emblica</i> (fruits) 15%	
	<i>Cassia angustifolia</i> (leaves) 20%	
Chronic fever	<i>Glycyrrhiza glabra</i> (roots) 10%	
	<i>Tinospora cordifolia</i> (stems) 15%	
	<i>Ocimum sanctum</i> (leaves) 15%	
	<i>Adhatoda vasica</i> (leaves) 15%	
	<i>Azadirachta indica</i> (leaves) 15%	4 gm of mixed powder is given to the patient, twice daily before meals with water
Chronic fever	<i>Holarrhena antidyserterica</i> (bark) 10%	
	<i>Piper longum</i> (fruits) 10%	
	<i>Zingiber officinale</i> (roots) 10%	
	<i>Terminalia bellerica</i> (fruits) 10%	

TABLE 3: Continued.

Disease	Formulation's ingredients/ratio	Dose/method of use
Cough	<i>Phyllanthus emblica</i> (fruits) 25% <i>Ahatoda vasica</i> (leaves) 20% <i>Ocimum sanctum</i> (leaves) 10% <i>Piper longum</i> (fruits) 10% <i>Zingiber officinale</i> (roots) 10% <i>Glycyrrhiza glabra</i> (roots) 15% <i>Solanum xanthocarpum</i> (whole plant) 10%	3 gm of mixed powder should be given to the patient twice daily (morning and at night before going to bed) with lukewarm mixed with honey to cure cold
Cysts	<i>Terminalia chebula</i> (fruits) 20% <i>Azadirachta indica</i> (bark) 20% <i>Holarrhena antidysenterica</i> (bark) 10% <i>Terminalia bellerica</i> (fruits) 10% <i>Withania somnifera</i> (roots) 20% <i>Tinospora cordifolia</i> (stems) 20%	4 gm of mixed (one teaspoonful) powder is given to the patient, twice a day (morning and evening) with water
Dental diseases	<i>Azadirachta indica</i> (leaves) 15% <i>A. arabia</i> (bark) 15% <i>Areca catechu</i> (bark) 15% <i>Achyranthes aspera</i> (leaves) 10% <i>Ficus benghalensis</i> (bark) 15% <i>Quercus infectoria</i> (fruits) 15% <i>Symplocos racemosa</i> (bark) 15%	The powder is applied to the gums and teeth, two times a day. Additionally a gargle of the decoction (3 gm of powder mixed in 150 mL of water)
Diarrhoea	<i>Holarrhena antidysenterica</i> (bark) 25% <i>Aegle marmelos</i> (fruits) 25% <i>Zingiber officinale</i> (roots) 10% <i>Terminalia chebula</i> (fruits) 10% <i>Cyperus rotundus</i> (roots) 10% <i>Syzygium cumini</i> (seeds) 10% <i>Phyllanthus emblica</i> (fruits) 10%	3 gm of mixed powder is given to the patient, three times a day, with curd for dysentery and diarrhoea
Dislocation of bones	<i>Asparagus racemosus</i> (roots) 15% <i>Withania somnifera</i> (roots) 15% <i>Azadirachta arabica</i> (bark) 20% <i>Terminalia arjuna</i> (bark) 20% <i>T. chebula</i> (fruits) 10% <i>T. bellerica</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 10%	3 gm of mixed powder is given to the patient, twice a day with water for dislocation of bones and fractures
Diabetes	<i>Gymnema sylvestre</i> (leaves) 30% <i>Tinospora cordifolia</i> (stems) 15% <i>Azadirachta indica</i> (leaves) 10% <i>Phyllanthus emblica</i> (fruits) 20% <i>Curcuma longa</i> (roots) 10% <i>Aegle marmelos</i> (leaves) 15%	4 gm of mixed powder should be given to the patient, twice a day with water
Fistula	<i>Glycyrrhiza glabra</i> (roots) 20% <i>Terminalia chebula</i> (fruits) 20% <i>T. bellerica</i> (fruits) 15% <i>Tinospora cordifolia</i> (stems) 15% <i>Azadirachta indica</i> (leaves) 15% <i>Withania somnifera</i> (roots) 15%	3 gm of mixed powder should be given to the patient, twice daily with water to treat fistula
Female sterility	<i>Asparagus racemosus</i> (roots) 20% <i>Withania somnifera</i> (roots) 20% <i>Glycyrrhiza glabra</i> (roots) 20% <i>Phyllanthus emblica</i> (fruits) 10% <i>Ficus glomerata</i> (bark) 10% <i>F. religiosa</i> (bark) 10%	3 gm of mixed powder is given to the patient twice daily, half an hour before meals with milk
General health tonic	<i>Withania somnifera</i> (roots) 20% <i>Asparagus racemosus</i> (roots) 10% <i>Glycyrrhiza glabra</i> (roots) 10% <i>Tribulus terrestris</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 15% <i>Terminalia arjuna</i> (bark) 15% <i>Centella asiatica</i> (leaves) 10%	4 gm of powder is given to the patient, twice daily (morning and evening) with milk

TABLE 3: Continued.

Disease	Formulation's ingredients/ratio	Dose/method of use
Gastritis	<i>Zingiber officinale</i> (roots) 10% <i>Piper longum</i> (fruits) 10% <i>Mentha piperita</i> (leaves) 10% <i>Terminalia chebula</i> (fruits) 15% <i>T. bellerica</i> (fruits) 15% <i>Phyllanthus emblica</i> (fruits) 15% <i>Plumbago zeylanica</i> (roots) 10% <i>Tinospora cordifolia</i> (stems) 15%	4 gm of (one teaspoonful) mixed powder is given to the patient twice daily, half an hour before meals with water
Hair problems	<i>Eclipta alba</i> (leaves) 15% <i>Centella asiatica</i> (leaves) 15% <i>Terminalia chebula</i> (fruits) 10% <i>T. bellerica</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 15% <i>Glycyrrhiza glabra</i> (roots) 15% <i>Tinospora cordifolia</i> (stems) 10% <i>Tribulus terrestris</i> (fruits) 10%	4 gm of mixed powder is given to the patient, twice a daily with honey
High blood pressure	<i>Terminalia arjuna</i> (bark) 35% <i>T. chebula</i> (fruits) 15% <i>Asparagus racemosus</i> (roots) 15% <i>Zingiber officinale</i> (roots) 10% <i>Withania somnifera</i> (roots) 25%	4 gm of powder is given to the patient, twice a day (morning and night) with honey
Heart tonic	<i>Withania somnifera</i> (roots) 10% <i>Terminalia arjuna</i> (bark) 30% <i>T. bellerica</i> (fruits) 10% <i>T. chebula</i> (fruits) 10% <i>Cyperus rotundus</i> (roots) 10% <i>Phyllanthus emblica</i> (fruits) 10% <i>Ocimum sanctum</i> (leaves) 10%	3 gm of mixed powder is given to the patient, twice a day with water
Intestinal worms	<i>Holarrhena antidysenterica</i> (bark) 10% <i>Mentha piperita</i> (leaves) 10% <i>Tinospora cordifolia</i> (stems) 20% <i>Butea monosperma</i> (seeds) 20% <i>Azadirachta indica</i> (leaves) 10% <i>Phyllanthus emblica</i> (fruits) 20% <i>Tribulus terrestris</i> (fruits) 10%	3 gm of mixed powder is given to the patient, twice daily (morning and night) with water
Epilepsy	<i>Centella asiatica</i> (leaves) 30% <i>Withania somnifera</i> (roots) 20% <i>Tribulus terrestris</i> (fruits) 15% <i>Piper longum</i> (roots) 10% <i>Achyranthes aspera</i> (leaves) 15% <i>Plumbago zeylanica</i> (roots) 10%	3 gm mixed powder is given to the patient, twice daily (morning and evening) with fruit juice to treat Hysteria
Leucorrhoea	<i>Symplocos racemosa</i> (bark) 35% <i>Asparagus racemosus</i> (roots) 15% <i>Adhatoda vasica</i> (leaves) 10% <i>Aegle marmelos</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 10% <i>Azadirachta indica</i> (bark) 10%	3 gm of mixed powder is given to the patient, twice daily with water
Leucoderma	<i>Psoralea corylifolia</i> (seeds) 20% <i>Terminalia chebula</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 20% <i>Azadirachta indica</i> (bark) 20% <i>Areca catechu</i> (bark) 10% <i>Tinospora cordifolia</i> (stems) 10% <i>Eclipta alba</i> (leaves) 10%	3 gm of mixed powder should be given to the patient, twice a day before meals with water

TABLE 3: Continued.

Disease	Formulation's ingredients/ratio	Dose/method of use
Liver tonic	<i>Holarrhena antidysenterica</i> (bark) 10% <i>Eclipta alba</i> (leaves) 20% <i>Tephrosia purpurea</i> (leaves) 20% <i>Tinospora cordifolia</i> (stems) 10% <i>Azadirachta indica</i> (bark) 10% <i>Phyllanthus amarus</i> (whole plant) 20% <i>Plumbago zeylanica</i> (roots) 10%	4 gm of mixed powder is given to the patient twice daily, half an hour before meals with water
Lack of appetite	<i>Zingiber officinale</i> (roots) 10% <i>Piper longum</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 30% <i>Terminalia chebula</i> (fruits) 15% <i>Tinospora cordifolia</i> (stems) 15% <i>Cassia angustifolia</i> (leaves) 10% <i>Mentha piperita</i> (leaves) 10%	4 gm of mixed powder is given to the patient, two times a day after meals with water for indigestion
Male sterility	<i>Withania somnifera</i> (roots) 15% <i>Mucuna pruriens</i> (seeds) 25% <i>Tribulus terrestris</i> (fruits) 20% <i>Glycyrrhiza glabra</i> (roots) 10% <i>Terminalia arjuna</i> (bark) 10% <i>Phyllanthus emblica</i> (fruits) 10% <i>Zingiber officinale</i> (roots) 5% <i>Piper longum</i> (fruits) 5%	4 gm of mixed powder is given to the patient, twice a day with honey
Migraine	<i>Curcuma longa</i> (roots) 15% <i>Glycyrrhiza glabra</i> (roots) 15% <i>Azadirachta indica</i> (bark) 15% <i>Tinospora cordifolia</i> (stems) 15% <i>Terminalia chebula</i> (fruits) 10% <i>Ocimum sanctum</i> (leaves) 15% <i>Eclipta alba</i> (leaves) 15%	4 gm of mixed powder is given to the patient, twice a day with honey
Obesity	<i>Terminalia chebula</i> (fruits) 15% <i>Terminalia bellerica</i> (fruits) 15% <i>Phyllanthus emblica</i> (fruits) 10% <i>Crataeva nurvala</i> (bark) 25% <i>Tribulus terrestris</i> (fruits) 25% <i>Zingiber officinale</i> (roots) 10%	4 gm of powder is given to the patient, twice a day with warm water
Paralysis	<i>Curcuma zedoaria</i> (roots) 20% <i>Withania somnifera</i> (roots) 20% <i>Tribulus terrestris</i> (fruits) 20% <i>Zingiber officinale</i> (roots) 20% <i>Piper longum</i> (fruits) 5% <i>Crataeva nurvala</i> (leaves) 10% <i>Plumbago zeylanica</i> (roots) 5%	3 gm of mixed powder is given to the patient, three times a day with honey
Prostate enlargement	<i>Tinospora cordifolia</i> (stems) 15% <i>Tribulus terrestris</i> (fruits) 15% <i>Phyllanthus emblica</i> (fruits) 15% <i>Zingiber officinale</i> (roots) 10% <i>Butea monosperma</i> (seeds) 10% <i>Adhatoda vasica</i> (leaves) 5% <i>Terminalia chebula</i> (fruits) 10% <i>T. bellerica</i> (fruits) 10% <i>Glycyrrhiza glabra</i> (roots) 10%	4 gm of mixed powder is given to the patient twice a day, morning and evening before meals with water
Piles	<i>Eclipta alba</i> (leaves) 35% <i>Terminalia chebula</i> (fruits) 15% <i>Terminalia bellerica</i> (fruits) 10% <i>Phyllanthus emblica</i> (fruits) 10% <i>Adhatoda vasica</i> (leaves) 10% <i>Plumbago zeylanica</i> (roots) 5% <i>Piper longum</i> (fruits) 5% <i>Aegle marmelos</i> (fruits) 10%	4 gm of mixed powder is given to the patient, twice daily (morning and at bedtime) with water

TABLE 3: Continued.

Disease	Formulation's ingredients/ratio	Dose/method of use
Sleeplessness	<i>Withania somnifera</i> (roots) 20% <i>Centella asiatica</i> (leaves) 30% <i>Piper longum</i> (roots) 20% <i>Glycyrrhiza glabra</i> (roots) 10% <i>Terminalia bellerica</i> (fruits) 10%	3 gm mixed powder is given to the patient, at night before going to bed, with milk
Skin diseases	<i>Cyperus rotundus</i> (roots) 10% <i>Tinospora cordifolia</i> (stems) 20% <i>Azadirachta indica</i> (bark) 20% <i>Terminalia chebula</i> (fruits) 10% <i>T. bellerica</i> (fruits) 10% <i>Curcuma longa</i> (roots) 10% <i>Phyllanthus emblica</i> (fruits) 10% <i>Centella asiatica</i> (leaves) 10%	3 gm of powder is given to the patient, twice a day before meals with water to cure allergy problems
Sexual debility	<i>Withania somnifera</i> (roots) 10% <i>Mucuna pruriens</i> (seeds) 20% <i>Asparagus racemosus</i> (roots) 10% <i>Sida cordifolia</i> (seeds) 10% <i>Tribulus terrestris</i> (fruits) 20% <i>Glycyrrhiza glabra</i> (roots) 10%	About 4 gm of mixed powder should be given to the patient, twice daily (morning and at night before going to bed) with milk
Throat diseases	<i>Glycyrrhiza glabra</i> (roots) 30% <i>Terminalia chebula</i> (fruits) 10% <i>T. bellerica</i> (fruits) 10% <i>Solanum xanthocarpum</i> (whole plant) 20% <i>Piper longum</i> (fruits) 10% <i>Sida cordifolia</i> (roots) 10% <i>Phyllanthus emblica</i> (fruits) 10%	4 gm of mixed powder is given to the patient twice daily, morning and at bedtime with honey
Thyroid problems	<i>Crataeva nurvala</i> (bark) 20% <i>Bauhinia variegata</i> (bark) 20% <i>Sida cordifolia</i> (leaves) 15% <i>Terminalia chebula</i> (fruits) 10% <i>T. bellerica</i> (fruits) 10% <i>Glycyrrhiza glabra</i> (roots) 15% <i>Zingiber officinale</i> (roots) 10%	3 gm of mixed powder is given to the patient, twice daily with lukewarm water
Urinary tract	<i>Tribulus terrestris</i> (fruits) 25% <i>Zingiber officinale</i> (roots) 10% <i>Solanum xanthocarpum</i> (whole plant) 10% <i>Crataeva nurvala</i> (bark) 25% <i>Tinospora cordifolia</i> (stems) 10% <i>Asparagus racemosus</i> (roots) 10% <i>Tephrosia purpurea</i> (leaves) 10%	4 gm of mixed powder is given to the patient, twice a day with water

herbal and botanical in its definition. The Indian definition lists down the ingredients that a product should have, and it also specifies general properties of nutraceutical. Traditional medicines though have been excluded from the definition. There are three categories which have been considered under the nutraceuticals [2].

Functional Foods. Foods that have specific physiological benefits and/or reduce the risk of chronic disease, that is, nutrition fortified foods like fortified flour, fortified oil, fortified malt-based powder and probiotic foods like yogurt.

Dietary Supplements. Supplements provide nutrients that are missing or are not consumed in sufficient quantity

in a person's diet, that is, vitamin supplements, mineral supplements, macronutrients, antioxidants, tonics, herbal formulations like Chyawanprash, Musli pak, Ashwagandha leh, and nonherbal products like cod liver oil.

Functional Beverages. Liquids that quench thirst along with replenishing minerals provide energy, prevent ailments, and promote healthy life style, that is, sports and energy drinks, fortified juices, and glucose drinks and powder.

A product category can be classified into a specific need-segment based on its predominant use. The product segments catering to foundation and condition specific need are the largest and growing the fastest. Nutraceutical products aim

to fulfill specific needs of the persons based on which they may be classified as follows.

enhancement segments: high protein supplements, energy drinks, sports drinks, glucose drinks, and so forth.

specific condition segments: antioxidants, vitamin supplements, and mineral supplements.

foundation segments: macronutrient supplements, nutrition fortified foods (fortified flour, soups, biscuits, etc.), probiotic foods (yogurt), and herbal formulations (chyawanprash, Ashwagandhadi leh).

7. Conclusion

Although some uncertainty exists about the safety, effectiveness, and cost-effectiveness of CAM methods, expanding their use, where reasonable evidence of their effectiveness and good evidence of their safety exists, might yield health, social, and economic benefits [35]. For example, improving the information and services provided in local pharmacies, that are the primary source of treatment for many ailments in rural areas, might serve as an effective substitute for allowing unregulated use of conventional medical treatment. Thus, expanding CAM would require significant investment of time and resources if it is to be done appropriately and have an impact on population health. An important role exists for CAM. However, more evidence is needed before CAM approaches can be broadly integrated into national health systems for diseases for which they have promise.

Also, numerous nutraceutical combinations have entered the international market through exploration of ethnopharmacological claims made by different traditional practices. To truly consume a healthy diet, the vast majority of the diet must be composed of health-promoting foods and nutraceuticals but disease-promoting foods or junk food must be avoided. Ninety percent of the daily diet should be made up of nutrient rich plant foods, whose calories are accompanied by health-promoting phytochemicals, vegetables, fresh fruits, beans and legumes, raw nuts, seeds, and avocados, starchy vegetables, and whole grains. These foods or nutraceuticals construct a health-promoting, disease-preventing diet with protective substances. The rich nutrient food intake will provide maximum protection against not only infections, asthma, and allergies but also against heart disease and cancer in adulthood.

Conflict of Interests

The authors do not have any conflict of interests.

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Review Article

Botanical Compounds: Effects on Major Eye Diseases

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Botanical compounds have been widely used throughout history as cures for various diseases and ailments. Many of these compounds exhibit strong antioxidative, anti-inflammatory, and antiapoptotic properties. These are also common damaging mechanisms apparent in several ocular diseases, including age-related macular degeneration (AMD), glaucoma, diabetic retinopathy, cataract, and retinitis pigmentosa. In recent years, there have been many epidemiological and clinical studies that have demonstrated the beneficial effects of plant-derived compounds, such as curcumin, lutein and zeaxanthin, danshen, ginseng, and many more, on these ocular pathologies. Studies in cell cultures and animal models showed promising results for their uses in eye diseases. While there are many apparent significant correlations, further investigation is needed to uncover the mechanistic pathways of these botanical compounds in order to reach widespread pharmaceutical use and provide noninvasive alternatives for prevention and treatments of the major eye diseases.

1. Introduction

Botanical compounds have a long history of medicinal use. The earliest record of plants in medicine was found on clay tablets from Mesopotamia around 2600 B.C. This depiction showed the use of oils from *Cupressus sempervirens*, also known as cypress, in the treatment of coughing, colds, and inflammation [1]. Today, many natural compounds from plants are active ingredients in the fabrication of modern drugs. For example, the active ingredient of aspirin is acetyl-salicylic acid, which is derived from a compound called salicin that is isolated from the bark of *Salix alba L*, a species of willow tree. Similarly, analyses of *Papaver somniferum L.*, commonly called opium poppy, lead to the discovery of numerous alkaloids, including morphine [2]. Currently, 74% of modern drugs directly used in traditional medicine have their origins in natural compounds. Numerous botanical compounds contain active ingredients or produce secondary metabolites that have beneficial properties, including anti-inflammation, antioxidation, protection against apoptosis,

and restoration of the body's homeostasis. As the pathologic mechanisms of major blinding diseases, such as age-related macular degeneration (AMD), diabetic retinopathy (DR), cataracts, and glaucoma, often involve inflammation- and oxidative stress-mediated cell death, evidences are accumulating on the potential benefits of botanical compounds in diets to improve or prevent these vision threatening eye diseases [3]. It is estimated that 35 million Americans combined suffer from glaucoma, cataracts, and AMD, the leading causes of blindness in the country [4, 5]. Despite this high prevalence, there are few or no treatments currently available for diseases such as AMD and glaucoma. Preventive intervention may, therefore, be the most effective course of action against these age-related ocular diseases.

According to the World Health Organization, traditional medicines remain the principal source of health care for 80% of the world's population [6], and there is an increased interest in the Western world for functional foods, or "nutraceuticals." Nutraceuticals are not defined as food but rather as derived products from botanicals and other natural products in

the forms of pills, powders, and other types of dietary supplements, which contain active ingredients that have shown potential benefits for human health. The ingredients of botanical functional food and nutraceuticals are classified upon their biochemical properties and benefits, such as antioxidants, anticarcinogens, inflammation-inhibitors, blood pressure reducing agents, or antidiabetics [3]. Recent research studies have identified molecular mechanism of action of many of the botanical compounds, which are in use as nutraceuticals or supplements as summarized in Figures 1 and 2. However, the detail mechanisms of most plant-derived active ingredients are still under investigation, and few research studies have been performed on the efficacy of botanical compounds' effects on human diseases, especially for eye diseases. In this paper, we reviewed the evidence that certain botanical ingredients, including curcumin, lutein, zeaxanthin, saffron, catechin, *Ginkgo biloba* extract, ginseng, resveratrol, danshen, and quercetin, may be used as dietary supplements to have therapeutic benefits for many common ocular diseases.

2. Major Eye Diseases Share Common Mechanistic Pathways

AMD, glaucoma, cataract, and other retinal diseases, including diabetic retinopathy (DR) and retinitis pigmentosa (RP), are the major causes of blindness around the world [4, 5, 7]. An epidemiologic survey conducted by the Eye Diseases Prevalence Research Group indicated that by 2020 an estimated 30.1 million Americans will suffer from cataract [7]. Moreover, it was predicted that during that same year, 2.95 million and 2.2 million Americans will be diagnosed with AMD and glaucoma, respectively, and an estimated 4.1 million Americans aged 40 years and older will be suffering from diabetic retinopathy [4, 5, 8]. Interestingly, all these diseases are associated with aging, and their etiology or pathophysiologies share some common mechanistic pathways. These pathways include oxidative stress, inflammation, and apoptotic factors, which provide insight for potentially targetable areas. Indeed, in many cases of eye diseases, oxidative stress due to reactive oxygen or nitrogen species and lipid peroxidation lead to ocular cell death. In addition, many pathogenic pathways include inflammatory factors such as the tumor necrosis α (TNF- α) and nuclear factor-kappa B (NF- κ B). Interestingly, these pathways often intersect with the mechanism of action of many botanical compounds (Figures 1 and 2). Oxidative stress induces the formations of reactive oxygen species, which interact with the mitochondria and activates the JNK pathway leading to apoptosis. Since AMD, DR, RP, and glaucoma all have a significant impact on populations worldwide, this review will focus on these pathologies and the potential benefits of botanical compounds in their prevention and treatment.

2.1. Age-Related Macular Degeneration (AMD). AMD is a chronic retinal disease, commonly present among populations of age 50 or older, resulting in loss of central vision due to degeneration of photoreceptor and RPE cells in the macula,

which are essential in providing sharp and clear vision. Many epidemiologic surveys describe AMD as the major cause of blindness in elderly populations worldwide; risk factors include smoking, race, and family history. The major mechanistic pathways of the pathology include oxidative stress and inflammation [9–11]. The results from the age-related eye disease study (AREDS) of the National Eye Institute suggested that dietary supplements with antioxidants and zinc may decrease the risk of developing advanced AMD and could significantly prevent vision loss [12, 13]. This study opens the horizon for promising natural compounds in the prevention of AMD. Many plants' active ingredients have already been investigated for their antioxidant and anti-inflammatory properties, which may be critical in the treatment and prevention of AMD and other ocular diseases.

2.2. Glaucoma. Glaucoma is described as a group of eye conditions leading to the interruption of visual information from the eye to the brain [14]. In most cases of glaucoma, an increased pressure in the eye, commonly known as intraocular pressure (IOP), causes damage to the optic nerve via retinal ganglion cell (RGC) apoptosis [15]. The major treatment procedure for glaucoma consists of lowering the IOP through eye drops, oral drugs, and even sometimes surgery [16]. Although IOP is one of the main factors in glaucoma, many cases progress despite the lowering of eye pressure to standard levels. In those cases, it is necessary to find new and innovative ways to prevent or limit the damages other than lowering the IOP. Since apoptosis plays a significant role in glaucoma, investigations of compounds described as neuroprotectants may lead to promising results. Numerous botanical compounds possess such neuroprotective properties, which may be effective in the prevention and treatment of glaucoma.

2.3. Diabetic Retinopathy (DR) and Retinitis Pigmentosa (RP). Diabetic Retinopathy occurs in individuals suffering from both type 1 and type 2 diabetes. Along with AMD and glaucoma, DR is a leading cause of blindness worldwide, and it is estimated that 140 million individuals are affected with DR [17]. The pathology is triggered by changes in the retinal blood vessels. Blood vessels may swell or leak and growth of new abnormal vessels may be detected on the retinal surface. There are four stages in the DR pathology: mild nonproliferative retinopathy, moderate nonproliferative retinopathy, severe nonproliferative retinopathy, and proliferative retinopathy. There are no treatments required for the first three stages; however, in order to prevent progression of the disease in these stages, patients are required to control their blood sugar, blood pressure, and blood cholesterol [18]. In cases of proliferative retinopathy, laser surgery is needed to control leaking fluids. The laser treatment helps shrink the abnormal blood vessels, but in cases of severe bleeding a vitrectomy is required to remove the blood from the center of the eye [17, 18].

Retinitis pigmentosa (RP) is another retinal disease in which retinal rod and cone cells are affected, leading to decreased vision and, in severe cases, blindness. RP's main

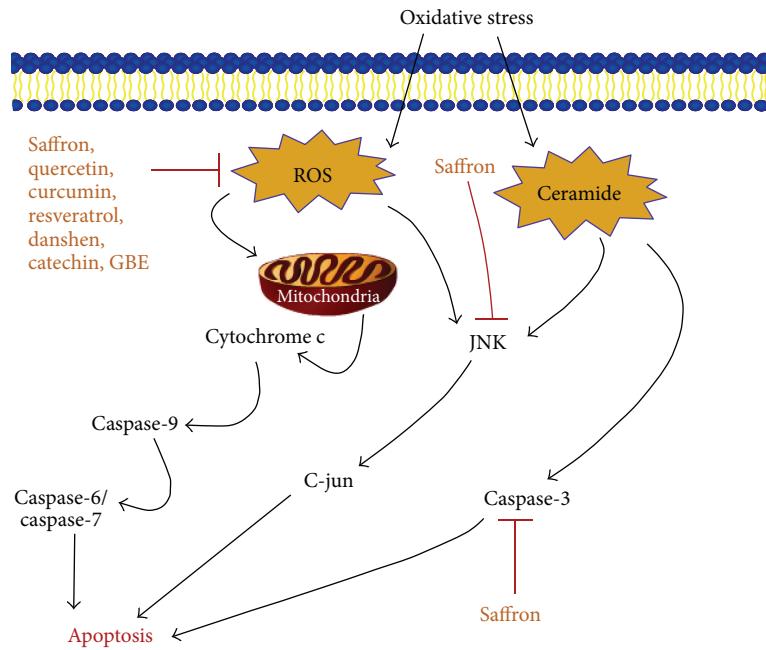


FIGURE 1: Oxidative stress pathway and botanicals. A schematic representation of cellular oxidative stress pathway and the effects of the botanical compounds discussed in this review that prevent the formation of reactive oxygen species (ROS) and protect the cell from apoptosis. Saffron particularly affects the JNK pathway and the production of caspase-3 from ceramide, which also lead to apoptosis.

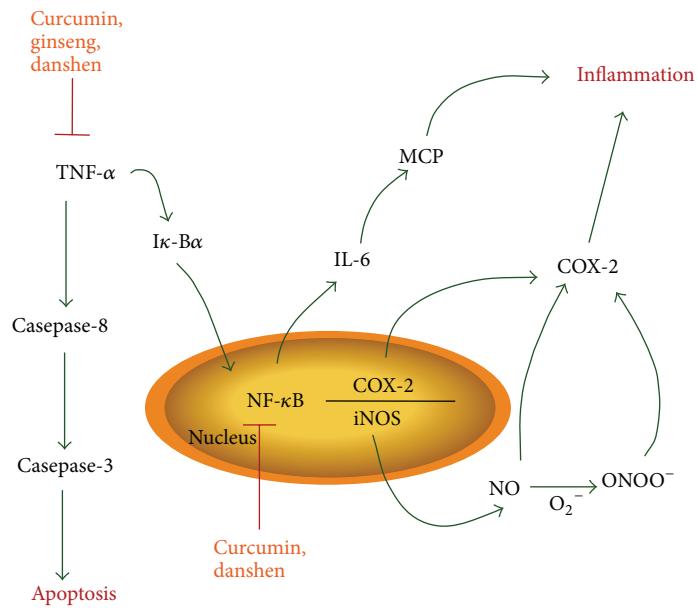


FIGURE 2: Effects of selected botanical compounds on TNF- α and NF- κ B pathways. TNF- α and NF- κ B pathways are the major pathways of cellular stress and inflammation. Botanical compound curcumin and danshen inhibit the activation of TNF- α and NF- κ B. Ginseng also interferes with the activation TNF- α and protects the cell from apoptosis.

risk factor is genetic predisposition, and it is a less common disease, affecting only 1 in 4000 people in the United States [19, 20]. Although it is not as prevalent as other ocular diseases, RP is a significant problem because there are no current treatments. However, previous studies in mice suggested that

high doses of antioxidants, such as vitamin A palmitate, may slow the disease [20]. For both RP and DR, many plant extracts may have significant effects on treatment and prevention. However, the lack of clinical trials leaves many uncertainties on the possible benefits of such supplements.

Current studies suggest interesting links between dietary supplements and positive effects on subjects with RP and similar retinal diseases.

2.4. Cataract. Cataract is an age-related eye disease that usually occurs starting at age 60. This pathology affects the lens due to the breakdown of proteins, leading to the clouding of the lens. Because the lens is necessary to focus on close or far away objects, damage to it leads to blurry vision with decreased color and shape sensitivity. Several factors, such as smoking, diabetes, eye injury, exposure to ultraviolet (UV) light, and family history, are known to favor cataract formation [21]. Patients with cataract can easily be treated by having surgery, which will remove the opaque lens and replace it with an artificial intraocular lens. While the surgery successfully restores sight in most patients, many countries do not have access to such eye care, and it is estimated that 51% of world blindness is caused by cataract [22]. Fortunately, recent studies have shown that consumption of botanical compounds containing strong antioxidants may prevent the degradation of proteins in the eye and minimize the effects of cataract.

3. Botanical Compounds and Their Effects on Eye Diseases

3.1. Curcumin. Curcumin, also known as *Curcuma longa*, is a spice widely prevalent in the South Asian diet. It is extracted from the turmeric plant and has a long history of use against inflammatory diseases. [23]. Curcumin is a lipophilic polyphenol and is insoluble in water, but it can remain stable in acidic pH environments like the human stomach [24, 25]. The mechanisms of curcumin involve interaction with many molecular targets for inflammation. For example, it regulates inflammatory processes by controlling the activity of cyclooxygenase-2 (COX-2), lipoxygenase, and nitric oxide synthase (iNOS) enzymes (Figure 2). Curcumin also inhibits the production of the inflammatory cytokine TNF- α as well as interleukins, monocyte chemoattractant protein (MCP), and migration inhibitory protein (Figure 2) [26, 27]. Since curcumin is an anti-inflammatory and antioxidative agent, we analyzed its effect on light-induced retinal degeneration (LIRD) in rat models and on retina-derived cell lines. We observed retinal neuroprotection in rats supplemented with 0.2% curcumin in their diet for two weeks [28]. Curcumin protected the retina from LIRD through inhibition of NF- κ B activation and downregulation of inflammatory genes. Experiments on pretreatment of retina-derived cell lines, 661W and ARPE-19, with curcumin lead to protection of the cells from hydrogen peroxide (H_2O_2) induced cell death by activating cellular protective enzymes, such as HO-1 and thioredoxin [28]. Further studies in human retinal cells showed that incubation with 15 μ M curcumin increased the cytoprotective effects against H_2O_2 oxidative stress through reduction of reactive oxygen species (ROS) levels mediated by an increase in HO-1 expression (Figure 1) [29]. In addition, curcumin has the ability to modulate the expression of cellular regulatory proteins, including NF- κ B, AKT, NRF2, and

growth factors, leading to an inhibition of inflammation and protection of the cells. Several studies of *in vivo* rat models have shown occurrence of direct benefits on ocular diseases from curcumin administration. Recent DR studies on Wistar albino rats with streptozotocin-induced diabetic retinopathy showed evidence of protective effects through oral administration of curcumin [30]. Similarly, other studies in rat models suggest that curcumin is effective against the development of galactose-induced cataract, naphthalene-induced cataract, selenite-induced cataract, and diabetic cataract [31–34]. In addition to this, dietary supplementation of curcumin prevented the loss of chaperone-like activity of eye lens α -crystallin concerning cataractogenesis caused from diabetes, thus preventing the formation of cataract in the rat lens [35]. While curcumin shows promise as a potential natural treatment, negative side effects have continuously been shown as early as 1976 [36]. These primarily include chromosomal and DNA alterations at higher doses of curcumin [36–46]. Although it has the potential for side effects at high doses, the anti-oxidative and anti-inflammatory properties of curcumin still make it a compound of choice in the treatment and prevention of AMD, DR, and cataract.

3.2. Lutein and Zeaxanthin. Lutein and zeaxanthin are carotenoids that are referred to as macular pigments due to their increased presence in the human macula and retina. They are commonly found in many fruits and vegetables, such as kale, spinach, corn, kiwi, or red grapes [47]. Many epidemiologic studies on AMD, including AREDS and the Case Control Study Group for Eye Diseases of the United States, evaluated the correlation between increased blood levels of lutein and zeaxanthin and decreased risks of developing AMD [48]. While the mechanisms of action of lutein and zeaxanthin are still under investigation, it is suggested that these carotenoids may protect the macula and photoreceptor outer retinal segments from oxidative stress by triggering the antioxidant cascade that disables reactive oxygen species (Figure 1) [49]. In addition, lutein and zeaxanthin act as light filters in the eye and absorb blue-light entering the retina, hence effectively protecting the retina during acute light exposure and high light levels from LIRD [50]. Studies on cultured ARPE-19 cells showed evidence that supplementation of lutein and zeaxanthin reduced photo-oxidative damages and inhibited the expression of inflammation-related genes in RPE cells [51, 52]. In addition, results from various epidemiological studies have shown inverse associations between the amount of macular pigment and the incidence of AMD. A recent clinical study showed that supplementation with lutein and zeaxanthin improved visual function and prevented progression of the pathology in patients with early AMD [53, 54]. Similarly, clinical studies showed that zeaxanthin improves visual function in older male patients with AMD [55]. Therefore, increase of macular pigments through dietary supplements of lutein and zeaxanthin may provide a valuable option in the prevention of macular degeneration [56].

3.3. Saffron. Saffron is a spice frequently used in traditional medicine for its antitoxic properties. Its active ingredients,

crocin and crocetin, are known antioxidant carotenoids and have antiapoptotic properties through protection of cells against reactive oxygen species [82, 83]. Crocin suppresses apoptosis, membrane lipid peroxidation, and caspase-3 activation in serum-deprived PC12 cells in hypoxic conditions. It also increases glutathione (GSH) levels and averts the activation of the JNK pathway, which contributes to the downstream signaling cascade of ceramide (Figure 1) [84]. Decreased levels of GSH lead to a higher sensitivity of the cell to apoptosis-inducing agents; thus, maintenance of the GSH levels through dietary supplementation of saffron may protect the cells from damages and death [85]. Studies on the retinal ganglion cell line RGC-5 showed that supplementation with crocin inhibited oxidative stress by decreased production of caspase-3 and -9, therefore preventing RGC-5 cell death [86]. Several studies analyzed the effects of saffron as a dietary complement in rats and in human clinical trials. In rats fed with saffron supplements, the effects of continuous bright light exposure were significantly diminished [84]. In human clinical trials of patients with early AMD, 20 mg per day saffron supplementation for 90 days showed a significant improvement of macular photopic flash electroretinogram (fERG) parameters, such as amplitude and modulation threshold [58]. While the mechanisms of the beneficial effects of saffron on the photoreceptors and bipolar cells are not yet elucidated, oral supplementation of saffron displayed a significant improvement in macular function. Preclinical studies have shown that saffron exhibits neuroprotective properties, and previous studies on rats provide evidences of cell death inhibition when exposed to intense light [84]. The results of the clinical trials indicated that dietary supplementation of saffron may induce a short-term improvement in retinal function in early AMD. In addition, the antiapoptotic and antioxidative properties of saffron have been shown to prevent formation of selenite-induced cataract in Wistar rats through inhibition of proteolysis of the lens's water-soluble protein fractions [57]. Although the studies do not conclusively prove saffron's neuroprotection in AMD and prevention of cataracts, this data seems promising in the developing of preventive and therapeutic uses of dietary supplements against the diseases.

3.4. Catechin. Catechin is a polyphenolic antioxidant commonly found in green tea [87, 88]. The most abundant catechin in green tea is epigallocatechin gallate (EGCG), which has extremely strong antioxidative properties [87, 88]. Previous studies involving intraocular injection of EGCG with sodium nitroprusside showed a protective effect on the retinal photoreceptors, indicating that EGCG may benefit patients suffering from ocular diseases in which oxidative stress is involved [89]. Mechanisms of catechin's action include the destruction of oxygen free radicals, oxidative alterations of LDL, and reduction of glutamate toxicity through LPO and protein modification [90]. Studies performed on rat models involving oral administration of EGCG reduced light-induced retinal neuronal death, suggesting that EGCG may be used in preventing photoreceptor cell death [67, 91, 92]. Similarly, supplementation of catechin on N-methyl-N-nitrosourea-induced cataracts in Sprague-Dawley

rats displayed inhibition of cataract-induced apoptosis in the lens epithelium, which may prove beneficial in the treatment or prevention of cataract in human patients [61]. In addition, EGCG has the ability to inhibit RPE cell migration and adhesion, thereby providing potential preventive actions against AMD [59, 60]. Therefore, catechin would be a compound of choice for prevention and treatment of diseases such as AMD.

3.5. *Ginkgo Biloba Extract.* *Ginkgo biloba* is one of the oldest living tree species, and its leaves have been extensively studied for their potential therapeutic properties. Ginkgo leaves contain two main active ingredients, flavonoids and terpenoids. *Ginkgo biloba* extract (GBE) is the most commonly used natural supplement in Europe and the United States, and its main properties are protection against free radical damage and lipid peroxidation. Studies suggest that GBE conserves mitochondrial metabolism and ATP production in tissues, thus partially inhibiting morphologic distortion and signs of oxidative damages due to mitochondrial aging [93–95]. Studies on mammalian cells indicate that GBE has the ability to scavenge nitric oxide and may prevent its production, consequently protecting mammalian cells against nitric-oxide reactivity [96]. Through preventing the loss of retinal ganglion cells and atrophy of the optic nerve, these properties of GBE may protect the optic nerve from degeneration, thus preventing blindness in patients suffering from glaucoma, DR, and RP [62]. Ma et al. performed studies on Sprague-Dawley rats by injecting GBE, followed by crushing the optic nerve. Animals that received the GBE extract via intraperitoneal injection prior to the optic nerve crush displayed a significantly higher survival rates of retinal ganglion cells than the controls [97]. Recent studies on Kunming mice showed that EGB761, the most widely studied GBE in clinical research [58], inhibited apoptosis of photoreceptor cells and increased cell survival after damaging or intense light exposure [98]. In addition, EGB761 was found to prevent inflammation associated with retinal detachment following the induction of vitreoretinopathy, therefore decreasing the occurrence of retinal detachment [62]. *Ginkgo biloba* is also believed to have good therapeutic potential in cases of normal tension glaucoma, where the disease continues to progress despite normalized IOP via surgery [99]. Thus, GBE could have a significant impact both for patients with glaucoma and with normal IOP. While GBE does not seem to have apparent negative side effects when used independently, evidence suggests that it can result in negative interactions when combined with some modern drugs; however, this is still under investigation [100]. Since GBE may act as a neuroprotectant and prevent damage to retinal ganglion cells, this plant extract would be an interesting component for prevention and treatment of ocular diseases such as glaucoma and other major neurodegenerative retinal pathologies.

3.6. *Ginseng.* Ginseng is the root of *Panax ginseng* and was widely used in traditional Chinese medicine. The main active ingredients of ginseng are ginsenosides, which is a group of steroid saponins that have the ability to target many tissues and lead to a high variety of pharmacological

responses [101]. Ginsenoside saponins Rb1 and Rg3 displayed inhibition of activities that lead to the apoptotic cascades, such as glutamate-induced neurotoxicity, lipid peroxidation, and calcium influx into cells when excess glutamate is present [15]. Rb1 and Rg3 have been known to suppress TNF- α and provide neuroprotective effects to cultured cortical cells by inhibition of the NMDA glutamate-receptor activity (Figure 2) [102, 103]. In clinical trials on patients with glaucoma, oral administration of Korean Red Ginseng (KRG) showed considerable increases in retinal blood flow in the temporal peripapillary areas. Since swelling of blood vessels and reduction of blood flow are the important risk factors for the optic nerve damage in glaucoma; increasing the retinal blood flow may be helpful in its prevention [63]. Since ginsenosides are known to inhibit TNF- α , ginseng may also be important in the prevention of AMD, since inflammation is one of the major risk factors for the disease [64]. Thus, ginseng's antiapoptotic and antioxidative properties show promising benefits for patients with diseases such as AMD, glaucoma, or cataract. Significant research has been done on the beneficial effects of ginseng in diabetes as well, including blood glucose reduction, weight gain control, and increased insulin production [104–106]. Very recent studies showed that, through its anti-oxidative properties, ginseng treatment significantly reduced retinal oxidative stress in diabetic mouse models [65]. While not as extensively researched as its effects on AMD and diabetes, ginseng has also demonstrated reduction of selenite-induced cataracts in rat models. Korean researchers, Lee et al., 2010, were even able to isolate the nonsaponin component of ginseng as the particular cataract-reducing agent [66]. Due to its strong anti-oxidative properties, ginseng is a highly promising compound for further research in the treatment of AMD, DR, and even cataracts.

3.7. Resveratrol. After an observation of low mortality due to lack of cardiovascular pathologies in France, as compared to other countries, researchers suggested that consumption of red wine might account for protective effects for human health. Red wines contain large amounts of polyphenols, a class of compounds exhibiting various properties such as inhibition of platelet aggregation, synthesis of proinflammatory and procoagulant eicosanoids, and inhibition of endothelin synthesis, which activates vasoconstriction [107–110]. In recent studies, resveratrol has significantly extended the health and survival of mice on a high calorie diet through increased sensitivity to insulin, reduced insulin-like growth factor-1 (IGF-I) levels, increased AMP-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PCG-1alpha) activity [111]. In addition, many studies suggested that supplementation of resveratrol reduced diabetes-induced early vascular lesion, vascular endothelial growth factor, and oxidative stress in rat and mice models [71–73]. Thus, this property may be beneficial for patients with diabetic retinopathy through prevention of cell death. Resveratrol also showed protection against injury-induced capillary degeneration and against endoplasmic reticulum stress through the inhibition of CHOP and IRE1 α expression [68]. Since retinal ischemia is a major factor for close-angle glaucoma and diabetic

retinopathy, resveratrol could be a potential novel drug for vascular dysfunction in the retina [68, 74]. There are significant evidences that resveratrol is an effective antioxidant and has the ability to inhibit lipid peroxidation of low-density lipoproteins (LDLs), prevent the cytotoxicity of oxidized LDL, and ultimately protect cells against lipid peroxidation [112–114]. In addition, resveratrol showed evidence of induction of blood flow elevation, which could prevent damages to vessels and apoptosis of optic nerve cells in patients suffering from glaucoma [69, 70]. Resveratrol may provide neuroprotection by inducing heme-oxygenase-1 and inhibits the effects of the pro-oxidant intracellular heme present in neuronal cell cultures after strokes, thus demonstrating an innovative pathway for cellular neuroprotection [115]. Studies on rats with supplementation of resveratrol demonstrated suppression of both selenite-induced oxidative stress and cataract formation through increased glutathione and decreased malonyl dialdehyde levels in the lens [116]. These properties of resveratrol could be essential in the establishment of innovative treatments and preventive interventions in major ocular diseases such as AMD, glaucoma, cataract, and diabetic retinopathy since oxidative stress is an integral part of the pathophysiology of those diseases.

3.8. Danshen (*Salvia miltiorrhiza*). *Salvia miltiorrhiza* (SM), commonly known as Asian red sage or danshen, is composed of salvianic acid B, which is a strong, water-soluble, polyphenolic antioxidant with anti-inflammatory properties [117, 118]. A recent study investigated the effect of injected danshen on diabetic retinopathy mice. A major injury in DR is blood capillary ischemia, characterized by a change of structure, due to the thickening of the capillary basement membrane. In such cases, oxygen radicals could not be eliminated soon enough after ischemia, leading to a destruction of the permeability membrane and edema formation due to lipid peroxidation of nerve cells [77]. Injection of danshen into the retinal hypoxia-ischemia tissues may improve the recovery of blood-oxygen transport, promote the absorption of retinal hemangioma, and therefore prevent loss of vision. In addition, danshen has the ability to scavenge free radicals and may help regulate blood sugar levels in DR patients [77]. It has also shown benefits in glaucoma, reducing the damage to retinal ganglion cells after intravenous treatment with danshen [75]. Previous clinical trials suggested that danshen may stabilize the visual field in middle to late stages of glaucoma [76]. Other studies demonstrated that danshen has the ability to inhibit TNF- α -induced activation of NF- κ B and protect against the loss of retinal ganglion cells in rabbits (Figure 2) [75]. Similar to ginseng, danshen's mechanism of neuroprotection may involve inhibition of the NMDA receptor antagonist activity [119]. Preclinical studies on danshen showed evidence of promising results for patients suffering from ocular diseases involving oxidative stress such as diabetic retinopathy, AMD, and cataract.

3.9. Quercetin. Quercetin is one of the most widely studied flavonoids and is found in a variety of plant foods, including black and green teas, *Brassica* vegetables, and

TABLE 1: Clinical and preclinical trials of major botanical compounds for ocular diseases. Most of the botanical compounds investigated for eye diseases are still on the pre-clinical stage with studies focusing on cells or animal models. However, several compounds such as lutein, zeaxanthin, saffron, *Ginkgo biloba* extract, and danshen have been tested in clinical trials.

	Preclinical	Clinical
Curcumin	Diabetic retinopathy: Gupta et al. [30] Cataract: Suryanarayana et al. [31], Raju et al. [32], Pandya et al. [33], Manikandan et al. [34], Kumar et al. [35]	
Lutein and zeaxanthin		AMD: Ma et al. [53, 54], Richer et al. [55], Bone et al. [56]
Saffron	Cataract: Makri et al. [57]	AMD: Falsini et al. [58]
Catechin	AMD: Alex et al. [59], Chan et al. [60] Cataract: Lee et al. [61]	
<i>Ginkgo biloba</i> Extract	Diabetic retinopathy: MacLennan et al. [62] Retinitis pigmentosa: MacLennan et al. [62]	Glaucoma: Kim et al. [63]
Ginseng	AMD: Cho et al. [64] DR: Sen et al. [65] Cataract: Lee et al. [66]	
Resveratrol	Glaucoma: Osborne [67], Li et al. [68], Kwok et al. [69], Losa [70] DR: Yar et al. [71], Kim et al. [72], Hua et al. [73], Li et al. [68], Osborne et al. [74]	
Danshen	Glaucoma: Zhu and Cai [75] DR: Zhang et al. [77]	Glaucoma: Wu et al. [76]
Quercetin	AMD: Chen et al. [78] Cataract: Stefek and Karasu [79], Shetty et al. [80], Gacche and Dhole [81]	

AMD: age-related macular degeneration; DR: diabetic retinopathy.

many types of berries [120]. Interest in flavonoids arose from the decreased incidence of cardiovascular disease and increased longevity found in populations with flavonoid-rich diets, such as the Mediterranean [121]. While there are no current FDA approved quercetin-based medications, its anti-inflammatory and anti-oxidative properties have been widely investigated [122]. Recent studies on the retinal cell line ARPE-19 demonstrated the protective effects of quercetin through inhibition of proinflammatory molecules as well as direct inhibition of the intrinsic apoptosis pathway [123]. *In vitro* studies using RF/6A rhesus choroids-retinal endothelial cells showed dose-dependent inhibition of cellular migration and tube formation, important steps of retinal angiogenesis, which is a characteristic of AMD, by treatment with quercetin [78]. Further studies on human cultured RPE cells showed similar results; quercetin treatment followed by oxidative damage dose-dependently reduced cellular damage and senescence [124]. Similarly, quercetin (and other natural flavonoids) significantly reduced reactive oxygen species' (ROS) production by ascorbate/Fe²⁺-induced oxidative stress in retinal cell cultures [125]. However, a study by Zhuang et al. (2011) using human umbilical vein endothelial cells (HUVECs) with oxidants found a decrease in HUVECs viability when followed by treatment with quercetin. The same

study also investigated quercetin treatment effects on laser-induced choroidal neovascularization *in vivo*; contrary to the untreated controls, choroidal neovascularization size was significantly diminished by administration of quercetin [126]. However, in glaucoma rat models, quercetin was found to inhibit the expression and thus blocked the neuroprotective effects of heat shock proteins (more specifically, HSP72) [127]. Quercetin is also known to have strong anticataract properties [79] through multiple pathways; however, there is no current comprehensive knowledge of all the exact mechanisms [80]. In addition to its already mentioned anti-oxidative role, it also affects sorbitol-aldoze reductase, calpain protease, glycation, and epithelial cellular signaling [80]. In fact, in 2011 Gacche and Dhole used quercetin as a standard for cataract inhibition, due to its aldose reductase inhibition, when testing for similar abilities among other flavonoids [80, 81]. While quercetin is believed to be the most abundant flavonoid in the human diet [128, 129], and although there are several potential pathways, the exact mechanism by which quercetin is processed and metabolized to affect the lens and cataract is uncertain [79]. Even though there is significant evidence linking quercetin and other flavonoids to ocular and other medical benefits, further investigation is needed to determine if this promising compound is suitable as

a treatment for ocular inflammatory diseases and cataract treatment.

4. Conclusion

Botanical compounds have been used throughout history for the prevention and treatment of various diseases. Previously, botanical supplements had not been awarded much scientific consideration; however, in the recent years, researchers and pharmaceutical companies have raised increasing interest for active ingredients from plants and nutraceuticals. Several major eye diseases in particular, AMD, glaucoma, cataract, and other retinal pathologies, are under investigation for potential beneficial effects of botanicals. These diseases can lead to ocular damage and visual problems primarily through oxidative stress, inflammation, and ocular pressure. Similarly, the active chemical ingredients in many botanicals contain strong antioxidative, anti-inflammatory, and anti-apoptotic properties. Although this review only addresses some of the well-studied and common botanical compounds for treatment of ocular diseases, there are numerous other compounds that may help with treatment of these diseases as well. While some botanical compounds, such as curcumin or quercetin, have been the subject of several studies and clinical trials, the benefits of many compounds have not been examined as extensively. In Table 1, we have summarized the compounds that have been used for either pre-clinical or clinical trials. There are several studies that indicated the mechanistic pathways of these compounds as being effective in cellular stress shown in Figures 1 and 2; however, extensive characterization is still required to bring these compounds for therapeutic research and human clinical trials. Thus, further investigation of natural plant-derived compounds, and especially their mechanisms of action, is necessary to harness the full potential of natural compounds to be used as a non-invasive and preventative complementary and/or alternative for major eye diseases.

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Research Article

Neurite Outgrowth and Neuroprotective Effects of Quercetin from *Caesalpinia mimosoides* Lamk. on Cultured P19-Derived Neurons

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Quercetin has been isolated for the first time from ethyl acetate extract of *Caesalpinia mimosoides* Lamk. *C. mimosoides* Lamk. (Fabaceae) or Cha rueat (Thai name) is an indigenous plant found in mixed deciduous forest in northern and north-eastern parts of Thailand. Thai rural people consume its young shoots and leaves as a fresh vegetable, as well as it is used for medicinal purposes. The antioxidant capacity in terms of radical scavenging activity of quercetin was determined as IC₅₀ of 3.18 ± 0.07 μg/mL, which was higher than that of Trolox and ascorbic acid (12.54 ± 0.89 and 10.52 ± 0.48 μg/mL, resp.). The suppressive effect of quercetin on both purified and cellular acetylcholinesterase (AChE) enzymes was investigated as IC₅₀ 56.84 ± 2.64 and 36.60 ± 2.78 μg/mL, respectively. In order to further investigate the protective ability of quercetin on neuronal cells, P19-derived neurons were used as a neuronal model in this study. As a result, quercetin at a very low dose of 1 nM enhanced survival and induced neurite outgrowth of P19-derived neurons. Furthermore, this flavonoid also possessed significant protection against oxidative stress induced by serum deprivation. Altogether, these findings suggest that quercetin is a multifunctional compound and promising valuable drugs candidate for the treatment of neurodegenerative disease.

1. Introduction

Caesalpinia mimosoides Lamk., a small spiny tropical trees or climbing shrubs belonging to family Fabaceae (subfamily: Caesalpinoideae), is mainly distributed in the south of China and grows in countries like India, Myanmar, as well as in northern and north-eastern parts of Thailand [1, 2]. Young sprouts and leaves are edible and sour and are traditionally used as a carminative and a remedy for dizziness [1]. In addition, the folk practitioners of Udupi district of India used the roots for ulcer and wound management, as well as for the treatment of arthritis [2]. Furthermore, this plant showed *in vivo* antiarthritic and analgesic activities [2]. The methanolic extract of *C. mimosoides* shoot tips was reported to exhibit antioxidant activity [3]. Moreover, the aqueous and

the ethanol extracts contained gallic acid, the antioxidative compound [1]. Based on these data, we postulated that this plant may compose of constituents that could exert some neuroprotective effects analogous to that of antioxidants.

Alzheimer's diseases (AD), a neurodegenerative disease, is the most common cause of senile dementia adversely affecting a significant proportion of the world's population. AD is characterized clinically by the progressive and irreversible loss of neurons in the brain [4–8]. On the other hand, brain amyloid-β plaques and neurofibrillary tangles, in addition to loss of neurons and their synaptic projections, are the main pathological features of AD [4–8]. Though the onset of this disease is still unclear, many studies have provided evidence for the deleterious consequences of oxidative stress (OS), which plays a significant causative role in the disease

process [5–9]. So far, one of the most promising approaches for treating this disease is to enhance the acetylcholine (ACh) level in the brain using AChE inhibitors which merely provide short-lived symptomatic relief. More recently, therapeutic approach, in which drug candidates are designed to possess diverse pharmacological properties and act on multiple targets, has stimulated the development of the multifunctional drugs [7–11].

On the other hand, quercetin (3,3',4',5,7-pentahydroxyflavone) is the major representative flavonol-type flavonoid found in various plants including fruits, vegetables, tea, wine, and honey [12, 13]. Recently, quercetin has been marketed in the United States primarily as a dietary supplement [14]. Quercetin is a well-known potent antioxidant, which could be a result of scavenging of radicals, metal chelation, enzyme inhibition, and/or induction of the expression of protective enzymes [12, 13]. Accordingly, OS and production of free radicals tend to increase with age, whereas the body's natural antioxidant defenses decline. Cell damage caused by OS is thought to contribute to the development of certain disorders such as cancer and neurodegenerative disorders, including AD [6–9]. Particularly, oxidative damage is the most marked in the brain due to its high oxygen consumption, high fatty acids levels, and low antioxidant enzyme levels. Moreover, neurons are largely postmitotic, so they cannot be replaced readily via mitosis when damaged [15, 16]. Thus, the antioxidant properties certainly contribute to their neuroprotective effects. To combat with such complex disease like AD, the search for multifunctional agents that simultaneously possess anticholinesterase, antioxidant, and neuroprotection activities emerges as a new strategy for the development of new drugs. In this study, quercetin, an active compound from *C. mimosoides*, was investigated for antioxidant, anti-AChE, and neuroprotective effects. In order to further investigate the benefit of quercetin on neuronal cells, P19-derived neurons were used as a neuronal model in this study.

2. Materials and Methods

2.1. Plant Material. The young sprouts and leaves of *C. mimosoides* Lamk. were collected from Maha Sarakham Province, then authenticated by Professor Dr. Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. A voucher specimen (BKF number 173175) was deposited in the herbarium, Royal Forest Department, Bangkok, Thailand. The plant material was washed thoroughly and dried in a hot air oven at 40°C before ground to a fine powder.

2.2. Chemicals and Enzymes. Acetylthiocholine iodide (ATCI), lyophilized powder of AChE (a purified enzyme from eel (*Electrophorus electricus*) type VI-s, 425.94 units/mg, 687 U/mg protein), 5,5-dithiobis [2-nitrobenzoic acid] (DTNB), galanthamine, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Alpha minimal essential medium (α -MEM), newborn calf serum (NCS), fetal bovine serum (FBS), and antimycotic solution were purchased from Gibco, USA. All transretinoic acids (RAs), cytosine-1- β -D-arabinoside (Ara-C), 1:250 porcine trypsin,

poly-L-lysine (MW > 300,000), dimethyl sulfoxide (DMSO), XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt), phenazine methosulphate (PMS), and phosphate buffer saline (PBS) were obtained from Sigma, USA. Analytical grade methanol (MeOH) was purchased from Merck, Germany. T-25 flask, 6-well, and 96-well plates were purchased from Corning, USA. Bacteriological grade culture dishes were obtained from Hycon Plastic Inc., USA.

2.3. Extraction and Isolation. Powdered plant material was macerated with hexane, ethyl acetate (EtOAc), and MeOH, respectively. The extracts were filtered and then evaporated to dryness under reduced pressure at 40°C and screened for antioxidant activity. The active EtOAc extract (45 g) was chromatographed on flash column packed with silica gel (grade 7734, 70–230 mesh) and eluted with hexane and increasing polarities with EtOAc and MeOH, respectively, to yield 8 fractions. Fraction F7 (1.20 g), the active fraction eluted with 50–80% EtOAc hexane, was recrystallized and further purified on a Sephadex LH-20 column using 100% MeOH as eluent to give compound **1** (518 mg) and compound **2** (24 mg).

2.4. Determination of Antioxidant Activity: DPPH Microplate Assay. The antioxidant capacity was estimated in terms of radical scavenging activity according to a modified version of Brand-Williams method [17]. Briefly, 100 μ L of tested compounds at least 5 concentrations (dissolved in MeOH) was thoroughly mixed with 100 μ L of freshly prepared DPPH solution (3×10^{-5} M dissolved in MeOH). The reaction mixture was incubated for 30 min, then, the absorbance was read at 517 nm. Each assay was done in triplicate. IC₅₀ value was determined by a linear regression analysis between the inhibition percentages against the concentration of tested compounds by using the Excel program.

2.5. Determination of Anticholinesterase Activity: Microplate Assay. Ellman's colorimetric method [18, 19] and modified method using 96-well microplates [20] were used. Briefly, acetylthiol, the product of hydrolysis reaction of ACh by cholinesterase, will react with DTNB to give 2-nitro-5-thiobenzoate (NTB[−]), which ionizes to the NTB^{2−} dianion (in water at pH 8). This NTB^{2−} ion appears as yellow, which is quantified by measuring the absorbance of visible light at 405 nm. Briefly, tested compound was prepared in buffer a containing 50% MeOH. In a 96-well microtiter plate, 25 μ L of tested sample was added to 200 μ L reaction medium that consisted of 50 μ L of buffer (50 mM TrisHCl pH 8.0) containing 0.1% BSA, 125 μ L of 3 mM DTNB in buffer containing 0.1 M NaCl, and 0.02 M MgCl₂·2H₂O and 25 μ L of 15 mM ATCI in deionized water. These contents were mixed and preincubated for 5 min at 37°C. The plate was pre-read at 405 nm using a microplate reader (TECAN M200, Switzerland). Thereafter, the reaction was initiated by the addition of 25 μ L of AChE (0.22 U/mL). After 20 min incubation at 37°C, absorbance was measured again within 4–7 min. The reaction control consisted of reaction medium

(200 μ L), 25 μ L of enzyme, and 25 μ L of 50% MeOH in buffer. Two blanks were also carried out (with and without sample), and MeOH was used as the solvent control. MeOH was controlled at 5% of the final volume, since it had been found that in this proportion it does not affect the AChE activity as well as thiols determination and the degree of AChE inhibition [21]. Galanthamine (50% MeOH) served as the positive control. Each assay was done in triplicate.

2.6. Determination of Anticholinesterase Activity in Neuroblastoma Cells

2.6.1. Cell Culture. The human neuroblastoma cell line SK-N-SH was purchased from the American Type Culture Collection (Manassas, VA, USA). The SK-N-SH cells were cultured in minimum essential medium from Gibco (California, USA), supplemented with 10% heat inactivated FBS, 2% supplementary amino acid solution, and 1% Glutamax (Gibco, California, USA), 1% penicillin/streptomycin. Incubation was carried out at 37°C in a humidified atmosphere of 5% CO₂-95% O₂ atmosphere. Cells were seeded into 96-well microplates (Nunc, Roskilde, Denmark) at a density of 1 × 105 cells/mL (100 μ L in each well). Experiments were carried out after 24 h of seeding.

2.7. Measurement of Anticholinesterase Activity on Cellular AChE. One of the important neuronal properties of SK-N-SH cells is the synthesis of neurotransmitter enzymes [22, 23]. The inhibition activity of samples on this cell line was investigated by the modified Ellman's colorimetric method [17, 18]. The condition was slightly modified to enable the cellular enzyme to work properly [19]. Briefly, 25 μ L of sample dissolved in buffer containing 50% methanol, 100 μ L of 3 mM DTNB in buffer containing 0.1 M NaCl and 0.02 M MgCl₂·2H₂O, and 25 μ L of 15 mM ATCI in deionized water were added to the well containing 100 μ L of the cells. The absorbance at 405 nm was measured by a microplate reader (TECAN M200, Switzerland). All assays were done in triplicate.

2.8. Neuroprotective Activity on P19 Embryonic Carcinoma Cells

2.8.1. Cell Culture. Murine P19 embryonic carcinoma cells were purchased from American Type Culture Collection (ATCC), USA. P19 cells were cultured as described by Mcburney [24, 25] in a slightly modification [26, 27]. In brief, the undifferentiated P19 cells were cultured as monolayers in α -MEM with 7.5% NCS, 2.5% FBS, and 1% antibiotics-antimycotic solution in a 25-cm² tissue culture flask, incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells in monolayer cultures were maintained in exponential growth phase by subculturing every 2 days until use.

2.9. Neuronal Differentiation of P19 Cells. The exponentially grown cultures indicated the cells density by trypan blue exclusion assay. Then, neuronal differentiation was induced by seeding 2 × 10⁶ cells/mL cell in a 100 mm bacteriological

grade culture dish containing 10 mL α -MEM supplemented with 5% FBS, 1% antibiotics-antimycotic solution, and 0.5 μ M RA. Under these conditions, cells did not adhere to dishes but instead formed large aggregates in suspension (neurospheres). After 4 days of RA treatment, aggregates were dissociated with trypsin, washed, resuspended on poly-L-lysine-precoated 96-well plates (plates were previously coated with 50 μ g/mL poly-L-lysine dissolved in PBS for overnight and sterilized under UV light for 30 min) at a cell density of 7 × 10⁴ cells/mL (150 μ L/well) in α -MEM supplemented with 10% FBS and 1% antibiotics-antimycotic solution, and incubated for 24 h. Ara-C (10 μ M) was added at day one after plating to inhibit the proliferation of nonneuronal cells. The medium was changed every 2-3 days. The differentiated P19-derived neurons were used after day 14 of the differentiation process.

2.10. Evaluation of Cell Viability (Measurement of Cell Density). The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Because viable P19 cells maintained membrane integrity, the cells did not allow trypan blue dye to pass through the cell membrane. Only cells with damaged membrane appeared blue because their accumulations of dye were counted as dead [28].

The embryo body (aggregated neuron cells), which is treated with RA in Petri dishes, was collected into a centrifuge tube. Samples were centrifuged at 1500 rpm for 20 min and the temperature was controlled at 25°C; the supernatant was gently removed and the cell pellet was then resuspended. The aggregated cells were rewash again two times, the medium was changed and centrifuged at 1500 rpm for 5 min, and then the precipitate was resuspended in corresponding medium. The cell suspension was stained with equal volume of 0.2% trypan blue in PBS, incubated at room temperature for 3 min, and loaded into a hemocytometer. The viable cells were counted under an inverted microscope, and the cell density was calculated.

2.11. XTT Reduction Assay for Neuronal Cells Viability. The procedure was carried out on P19-derived neurons cultured in a 96-well plate [29]. After 14 days of differentiation process, the α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution was removed and replaced with DMSO solutions of samples, diluted with the α -MEM supplemented with 10% FBS, and 1% antibiotics-antimycotic solution in the presence of 10 μ M Ara-C was added to give the concentrations of 0.0001, 0.001, 0.01, 0.1, 1, and 10 μ M. The final DMSO concentration on the assay was kept at 0.5% which had no effect on cell viability. The blank control wells contained the corresponding medium (α -MEM supplemented with 10% FBS, 10 μ M Ara-C, and 1% antibiotics-antimycotic solution). The cells were incubated for 18 h at 37°C. Then 150 μ L of the medium was removed, and after that 50 μ L of XTT reaction solution (12 mL of XTT 1 mg/mL in α -MEM, 30 μ L of PMS (an intermediate electron acceptor), and 10 mM in PBS were added. Blank control was performed by adding XTT reaction solution without cells. Shake the plate gently to evenly

distribute the dye in the wells. After incubated at 37°C for 4 hours, 150 µL of PBS was added to each well. The optical density (OD) value was determined on a microplate reader at wavelength of 450 nm. Absorbance values that were higher than control conditions indicate an increase in cell viability. The data were expressed as the mean ± SEM ($n = 3$), with the medium as a control representing 100% cell viability. The concentration that enhanced survival of cultured neurons more than control will be further investigated for neuritogenic and neuroprotective activity against OS induced by serum deprivation.

2.12. Neuritogenic Assay. The assay was carried out with P19-derived neurons cultured in a poly-L-lysine-precoated 6-well plate [26]. After 14 days of differentiation process, the α -MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution was removed, and DMSO solution of quercetin, diluted with the α -MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution, was added. The concentration of DMSO was added to the cultures at 0.5%. The α -MEM supplemented with 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution was added into control wells. The cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. Geldanamycin 1nM was used as positive control. The morphology under a phase-contrast microscope was observed. The appearance of P19-derived neurons was compared to the control (vehicle without quercetin) and measured for the length and number of neurites. Average length and number of neurites of 30 neurons from the assay were measured. The assay was performed in a replicate. The data were expressed as the mean ± SEM from three independent experiments.

2.13. Neuroprotective Activity against OS Induced by Serum Deprivation. For serum withdrawal-induced oxidative stress [27], cells were seeded and cultured in the α -MEM supplemented with 10% fetal bovine serum for 24 h, washed with α -MEM three times, and cultured in serum-free α -MEM (α -MEM plus 10 µM Ara-C) in the absence or the presence of quercetin for 18 h. The blank control was cultured in α -MEM supplemented with 10% FBS and 10 µM Ara-C. The cell survival ability was measured using the XTT method.

3. Results

3.1. Extraction and Isolation. The yields of hexane, ethyl acetate, and methanol extracts were 1.35, 4.72, and 17.50% w/w, respectively. Bioactivity-guided isolation of the active compounds from the EtOAc extract which exhibited potent DPPH radical scavenging activity led to the isolation of two known compounds. Through the comparison of the physical property and spectroscopic data comparing with the literature values [30–32], the isolated compounds were identified as gallic acid (1) and quercetin (2, Figure 1). This is the first report on the isolation of quercetin from this plant.

Gallic acid (1). white crystal, mp 253–256°C; IR (KBr): 3492, 3368, 3288, 1703, 1619, 1541, 1450, 1247, 1027 cm⁻¹; ¹H

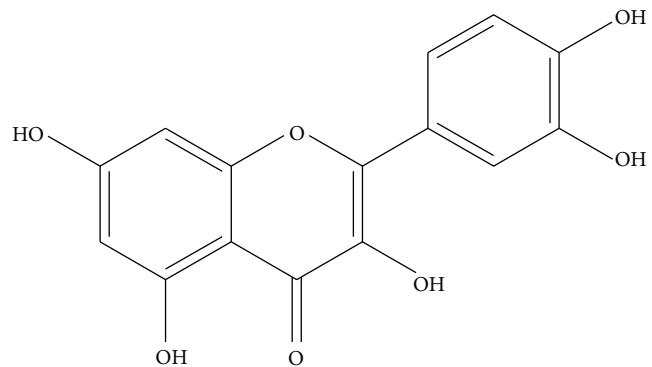


FIGURE 1: Chemical structure of quercetin.

NMR (300 MHz, DMSO-*d*₆) δ_H 6.90 (2H, s, H-2 and H-7); ¹³C NMR (75 MHz, DMSO-*d*₆) δ_C 108.9 (C-3 and C-7), 120.7 (C-2), 138.3 (C-5), 145.6 (C-4 and C-6), 167.7 (C-1); ESI-MS *m/z* 169.20 [M-H]⁻.

Quercetin (2). yellow crystal, mp 318–320°C; IR (KBr): 3282, 1743, 1666, 1610, 1517, 1430, 1211, 1094 cm⁻¹; ¹H NMR (300 MHz, DMSO-*d*₆) δ_H 6.14 (1H, d, *J* = 2.0, H-6), 6.36 (1H, d, *J* = 2.0, H-8), 7.65 (1H, d, *J* = 2.0 H-2'), 6.87 (1H, *J* = 9.0 Hz, H-5'), 7.53 (1H, dd, *J* = 2.0, 9.0, Hz, H-6'); ¹³C NMR (75 MHz, DMSO-*d*₆) δ_C 148.6 (C-2), 137.2 (C-3), 176.6 (C-4), 161.5 (C-5), 99.2 (C-6), 165.5 (C-7), 94.3 (C-8), 157.0 (C-9), 103.6 (C-10), 122.8 (C-1'), 116.4 (C-2'), 146.0 (C-3'), 153.4 (C-4'), 115.8 (C-5'), 120.8 (C-6'); ESI-MS *m/z* 301.39 [M-H]⁻.

3.2. Free Radical Scavenging Property. The antioxidant activity was evaluated in terms of radical scavenging property. Quercetin and gallic acid strongly possessed antioxidant activity with IC₅₀ of 3.18 ± 0.07 and 4.83 ± 0.03 µg/mL, respectively, which were stronger than that of Trolox and ascorbic acid (12.54 ± 0.89 and 10.52 ± 0.48 µg/mL, resp.).

3.3. Anticholinesterase Activity. Quercetin and gallic acid possessed anticholinesterase activity both upon purified and cellular AChE with respective IC₅₀ values of 56.84 ± 2.64 and 36.60 ± 2.78 µg/mL for quercetin, and 12.73 ± 0.56 and 2.97 ± 0.17 µg/mL for gallic acid, whereas IC₅₀ of galanthamine (positive control) was 1.73 ± 0.12 µg/mL and 0.23 ± 0.02 µg/mL, respectively, (Table 1).

3.4. Effects on P19-Derived Neuron Viability. Two isolated compounds, gallic acid and quercetin, which possessed potent antioxidant and anticholinesterase activity, were selected to determine their neuroprotective capacity. The P19 cells, isolated from an experimental embryo-derived teratocarcinoma in mice, are widely used as *in vitro* model because of their specific characteristics. Exposing aggregated P19 cells to retinoic acid (RA) effectively induces the development of neurons, astroglia, and microglia cell types [24–27]. Unlike P12 cell line, P19-derived neurons are irreversibly postmitotic; moreover, these neurons exhibit many characteristics of

TABLE 1: Antioxidant and anticholinesterase activities of isolated compounds.

	Antioxidant		Anticholinesterase (using purified enzyme from <i>E. electricus</i>)		Anticholinesterase (using neuroblastoma cell)	
	% inhibition (at 100 μ g/mL)	IC ₅₀ (μ g/mL)	% inhibition (at 100 μ g/mL)	IC ₅₀ (μ g/mL)	% inhibition (at 100 μ g/mL)	IC ₅₀ (μ g/mL)
Gallic acid	94.76 \pm 0.51	4.83 \pm 0.02	73.64 \pm 0.06	12.73 \pm 0.56	90.67 \pm 2.66	2.79 \pm 0.17
Quercetin	92.61 \pm 0.99	3.18 \pm 0.07	80.94 \pm 2.52	56.84 \pm 2.64	82.0 \pm 3.34	36.60 \pm 2.78
Positive control	Trolox: IC ₅₀ 12.54 \pm 0.89 Ascorbic acid: IC ₅₀ 10.52 \pm 0.48		Galanthamine IC ₅₀ 0.81 \pm 0.04		Galanthamine IC ₅₀ 0.23 \pm 0.02	

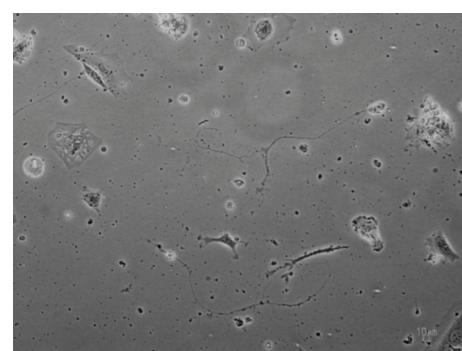
Values are mean \pm SEM ($n = 3$).

mature CNS neurons containing particular neurotransmitters such as γ -aminobutyric acid (GABA) and acetylcholine [25–27].

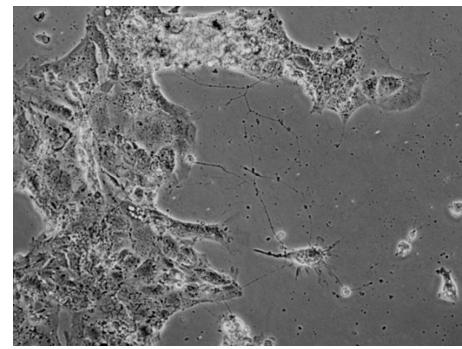
The biological effects on P19-derived neurons of two compounds at a serial of dilutions in a microplate were identified and quantified; the survival of cells was determined by using a XTT reduction assay. Viable cells with active mitochondrial dehydrogenase caused cleavage of the tetrazolium ring into a visible red-orange product through a formazan reaction, while dead cells remained a light orange color of which the OD was measured in a microplate reader at 450 nm. After 24 h of quercetin treatment, the result indicated that a very low dose of 1 nM quercetin promoted high cell viability of cultured neurons (% cell viability $>$ 100%) more than control without any cytotoxicity (IC₅₀ $>$ 10 μ M), while gallic acid was found to be cytotoxic to the cell. Therefore, only quercetin was chosen for further evaluation of neuritogenic and neuroprotective activity.

3.5. Neuritogenic Activity. The characterization of neurite formation, maturation, and collapse/resorption is an area of intense interest; particularly, it is a readjustment in the normal neuronal functions and local circuits in the damaged CNS. Measurement of the length of outgrowth per cell is the most commonly used to assess the ability of a compound that affects the growth of neurite [33, 34]. P19-derived neurons grown in a poly-L-lysine-precoated 6-well plate were treated with or without 1 nM quercetin for 24 h. The morphology of 30 neuronal cells was examined using phase-contrast micrographs. The result showed that neurite outgrowth was induced in P19-derived neurons by quercetin compared with an active control, geldanamycin. Quercetin not only significantly increased the amount of neurites (3.23 \pm 1.55), but also the neurite length (139.00 \pm 108.80 μ m) (Figure 2), whereas geldanamycin exhibited the amount and length of neurite at 2.03 \pm 1.30 and 3.23 \pm 1.55 μ m, respectively.

3.6. Neuroprotective Activity against OS Induced by Serum Deprivation. To determine whether quercetin protects neurons from oxidative stress and induces cell death triggered by serum deprivation, six experiments were performed, cultured in serum-free or complete medium, alone or with 1 nM quercetin. After 24 h, the amount of survival cells in each experiment was measured by XTT assay and typical histograms were shown in Figure 3. A dramatic decrease



(a)



(b)

FIGURE 2: Effect of quercetin on neurite outgrowth from P19-derived neurons. Cells were treated without or with quercetin 1 nM for 24 h. Phase-contrast micrographs of control (a) and treatment with quercetin (b); scale bar, 10 μ m.

in cell viability was observed, in which P19 neuronal cells were cultured in α -MEM. Serum deprivation reduced the cell survival to 31.16% as compared to the untreated control (P19SM). However, pretreatment of P19 neuronal cells with 1 nM quercetin was effective in increasing the survival in both serum-supplemented and serum-deprived cultures up to 128.01 and 88.29% as compared to the control and α -MEM-treated cell, respectively.

4. Discussion

From the present study, quercetin was isolated for the first time from the EtOAc extract of *C. mimosoides*. The strong antioxidant activity of quercetin was evaluated in

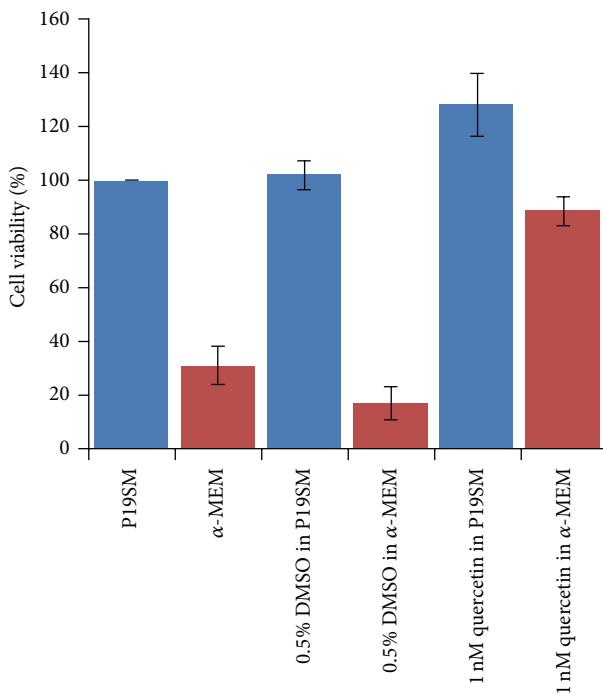


FIGURE 3: The effect of quercetin in neuronally differentiated P19 cells against oxidative stress induced by serum deprivation. The histogram shows the percentage of cell viability relative to vehicle-treated control cultures. Each bar represents mean \pm SEM from three individual measurements.

terms of free radical scavenging capacity which was more potent than Trolox and ascorbic acid. Quercetin also exerted cholinesterase inhibitory activity on both purified and cellular AChE enzymes. Furthermore, quercetin at a very low dose of 1 nM enhanced the survival of P19-derived neurons, significantly increased neurite outgrowth, as well as showed powerful neuroprotective action against OS induced by serum deprivation. Since the damaged brain lacks the reconstructive capacity, the use of compounds that are capable of enhancing the action of neurotrophic factors to stimulate neurite outgrowth seems to be an important step in the process of neuronal regeneration [34]. To assess neuritogenicity of quercetin, we quantified the neurite outgrowth from P19-derived neurons which is dependent on microtubule formation and a key to restoring proper function. Our result showed that quercetin produced a significant neurite outgrowth from P19-derived neurons.

Considerately, serum is a mixture that consists of hundreds of proteins and some vital growth factors needed for proliferation of cells in culture which functions as hormonal factors that stimulate cell growth transport proteins that carry hormones, lipids, minerals, and trace elements, and stabilizing and detoxifying factors [35]. Mounting evidence suggests that serum deprivation induced oxidative stress due to a lack of necessary nutrients and trophic factors, triggered mitochondrial ROS generation, with activation of the intrinsic (caspase 9-dependent) apoptotic pathway and release of cytochrome *c*, resulting in cell apoptosis [36, 37]. An increasing number of studies have also revealed that

drugs or other therapeutics can prevent serum deprivation-trigger cell death by scavenging the intracellular ROS or implicating it in apoptosis signaling under those conditions [36]. Our examination found that quercetin at a very low dose of 1 nM effectively reduced neuronal cell death caused by serum deprivation-triggered OS, comparison to its antioxidant activity that exhibiting IC₅₀ in micromolar range (9.4 μ M). These observations imply that the neuroprotective effect of quercetin may act as a modulator of cell signaling, not an antioxidant. However, further studies are also required to understand the mechanism of its neuroprotective action.

5. Conclusion

The present study described the isolation of quercetin from *C. mimosoides* Lamk. The results indicated that quercetin, formerly thought to be a radical scavenger, is now considered as an anticholinesterase as well as a significant neuroprotective agent. In view of its multiple biological activities, quercetin holds a great promise as a potential therapeutic agent for Alzheimer's diseases and other neurodegenerative diseases.

Conflicts of Interests

The authors declare that they do not have any conflict of interests.

Acknowledgments

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Research Article

Inhibitory Action of Quercetin on Eosinophil Activation *In Vitro*

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The influence of quercetin on eosinophil functions was examined *in vitro* and *in vivo*. The first set of experiments was undertaken to examine whether quercetin could suppress eosinophilia and IgE hyperproduction induced by *Mesocestoides corti* infection in BALB/c mice. The number of peripheral blood eosinophils and IgE levels were examined 21 days after infection. Oral administration of quercetin for 21 days could not suppress both peripheral blood eosinophilia and IgE hyperproduction, even when 20.0 mg/kg quercetin was used for treatment. The second part of the experiment was designed to examine the influence of quercetin on eosinophil activation induced by SCF stimulation *in vitro*. Eosinophils were obtained from *M. corti*-infected mice and stimulated with SCF in the presence of various concentrations of quercetin for 24 h. The addition of quercetin into cell cultures could suppress eosinophil activation induced by SCF stimulation as assessed by measuring the contents of RANTES, MIP-1 β , ECP, and MBP in culture supernatants. The minimum concentration of quercetin which caused significant suppression of factor secretion was 5.0 μ M. These results may suggest that quercetin will be a good candidate for the supplement on the management of eosinophil-mediated diseases, such as allergic rhinitis and asthma.

1. Introduction

The inflammatory responses in airway diseases such as allergic rhinitis and asthma involve a complex network of several inflammatory cells and mediators. These include antigen-presenting cells, Th2 type helper T cells, mast cells, eosinophils, and fibroblasts [1, 2]. Although mast cells and Th2 type helper T cells largely contribute to the induction and the early phase of response of the allergic reaction, eosinophils are believed to be the main type of cells recruited during the late phase and play an essential role as final effector cells in the development of persistent inflammation and tissue damages through the secretion of cationic proteins, lipid mediators, cytokines, and chemokines [2, 3]. This was confirmed by histological examination, which showed extensive degranulation of eosinophils in airway tissues during active diseases [4]. The presence of much higher levels of granule proteins in the damaged tissues is also observed [5]. These

reports may suggest that the manipulation of eosinophil functions, such as activation and degranulation, will be a good therapeutic target in the treatment and prevention of allergic diseases.

Quercetin belongs to a group of plant compounds called flavonoids, which give many fruits, flowers, and vegetables their color [6]. For many years, quercetin has been studied for the possible health benefits, and it has been revealed that quercetin plays the role of scavenger for free radicals, which damage cell membrane, tamper with DNA, and even cause cell death [7]. It is also reported that quercetin can inhibit the release of histamine and other mediators responsible for the development of allergic reaction from mast cells [8, 9]. Furthermore, oral administration of quercetin is reported to attenuate the clinical symptoms (e.g., bronchial hyperreactivity among others) observed in a murine [10] and guinea pig asthma model [11]. However, the influence of quercetin on eosinophil functions is poorly understood.

Infection with parasites, except protozoa, is well known to induce IgE hyperproduction and peripheral blood eosinophilia in mammalian hosts [12, 13]. Because these immune responses are quite similar to those in allergic diseases [12, 13], the parasite/host system may be considered to provide a suitable model to examine the therapeutic mechanisms of antiallergic agents. *Mesocestoides corti* is a common parasite in dogs and humans in North and Central America. The larval worm of this parasite is called tetrathyridium larva and has been observed in the peritoneal cavity of wild rodents. Tetrathyridium larva infection in rodents is accepted to produce peripheral blood eosinophilia and IgE hyperproduction [12].

In the present study, therefore, we have used the *M. corti*/mouse system and examined the influence of quercetin on both peripheral blood eosinophilia and IgE hyperproduction *in vivo*. We also examined the influence of quercetin on the activation of eosinophils obtained from mice infected with *M. corti* by using an *in vitro* cell culture technique.

2. Materials and Methods

2.1. Mice. Specific pathogen-free male BALB/c mice, 5 weeks of age, were purchased from Charles River Japan Inc. (Atsugi, Japan). They were maintained in our animal facilities under a controlled environment ($25 \pm 3^\circ\text{C}$, $55 \pm 5\%$ humidity, and a 12 h light/dark cycle). All animal experimental procedures were approved by the Animal Care and Use Committee of Showa University and were carried out in accordance with the guidelines of the Physiological Society of Japan [12, 14].

2.2. Agent and Treatment. Quercetin was purchased from Sigma Chemicals Co. Ltd. (St Louise, MO, USA). For *in vivo* use, quercetin was well mixed with 5% gum tragacanth solution. The mice (5 mice/group) were given various doses (5.0, 7.5, 10.0, 15.0, 17.0, and 20.0 mg/kg) of quercetin once a day for 3 weeks via a stomach tube in a volume not exceeding 0.25 mL, starting on the day of infection. The control mice were administered orally with 5% of gum tragacanth solution alone. For *in vitro* used, quercetin was dissolved in dimethyl sulfoxide at a concentration of 1.0 M, then diluted at RPMI-1640 medium (sigma chemicals co. ltd.), and supplemented with 10% fetal calf serum (Nihon Bio-Supply Center, Tokyo, Japan; RPMI-FCS) at appropriate concentrations for experiments.

2.3. Parasitological Technique. *M. corti* kindly donated by Dr. A. Niwa (School of Medicine, Kinki University, Osaka, Japan) was maintained in mice by intraperitoneal injection of 500 tetrathyridia according to the method described previously [12].

2.4. Assay for IgE. Blood was obtained from retro-orbital plexus in a volume of 100 μL . After clotting, the serum was obtained, and the total IgE levels were assayed by mouse IgE ELISA test kits (Yamasa Co. Ltd., Chiba, Japan). The ELISA was done in duplicate according to the manufacturer's recommended instructions. The minimum detectable level of these kits is 10.0 ng/mL.

2.5. Counting for Peripheral Blood Eosinophils. The number of eosinophils in peripheral blood was examined according to the method described previously [14]. Briefly, 5 μL of the blood taken from retro-orbital plexus was mixed with 20 μL of Hinkelmann's solution (muto pure chemicals co. ltd., Tokyo, Japan). Eosinophils were counted using haemocytometers in triplicate.

2.6. Culture of Eosinophils. Mice were killed by ether anesthesia 21 days after intraperitoneal injection with 500 tetrathyridia. Peritoneal cells were obtained by washing the mouse peritoneal cavity with 10 mL of sterile phosphate buffered saline. The cells were washed 3 times with RPMI-FCS and incubated in plastic tissue culture plates to remove plastic adherent cells in a humidified atmosphere with 5% CO_2 at 37°C . After two hours, nonadherent cells (eosinophils) were collected and suspended in RPMI-FCS at a concentration of 5×10^5 cells/mL. Eosinophils (1.0 mL) were then treated with various doses of quercetin for one hour and stimulated with 200.0 ng/mL stem cell factor (R & D Corp., Minneapolis, MN, USA) for 24 h in a final volume of 2.0 mL [14]. The culture supernatants were collected after pelleting cells by centrifugation at 3000 rpm for 15 min at 25°C and stored at -40°C until used. The purity of eosinophils was $>95\%$ as judged by Giemsa stain.

2.7. Assay for Eosinophil-Derived Chemokines. Levels of eosinophil-derived chemokines, regulated on activation, normal T-cell expressed and secreted (RANTES) and macrophage inflammatory protein-1 β (MIP-1 β), in culture supernatants were examined by commercially available ELISA test kits (R & D Corp.). The ELISA was done in duplicate according to the manufacturer's recommendations. The minimum detectable levels of these ELISA kits were 2.0 pg/mL and 1.5 pg/mL, respectively.

2.8. Assay Degranulation. Levels of eosinophil granule proteins, eosinophil cationic protein (ECP), and major basic protein (MBP) in culture supernatants were examined by commercially available ELISA test kits, which were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China) and Uscn Life Science Inc. (Wuhan, China), respectively. The minimum detectable levels were 0.156 ng/mL for ECP and 0.225 ng/mL for MBP. The ELISA was done in duplicate according to the manufacturer's recommendations.

2.9. Statistical Analysis. Data were analyzed with analysis of variance (ANOVA) followed by Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Quercetin on Eosinophilia and IgE Hyperproduction Induced by *M. corti* Infection. The first set of experiments was carried out to examine the influence of quercetin on eosinophilia and IgE hyperproduction. BALB/c mice were infected intraperitoneally with 500 *M. corti* larvae on day 0. These mice were then treated with various doses of quercetin once a day for 21 days, starting on the day of infection. The

number of peripheral blood eosinophils and IgE levels were examined 21 days after infection. As shown in Figure 1(a), quercetin could not suppress the increase in the number of eosinophils induced by *M. corti* infection, even when the mice were treated with quercetin at more than 15 mg/kg/day. We then examined the influence of quercetin on IgE production caused by *M. corti* infection. The data in Figure 1(b) clearly showed the negative suppressive effect of quercetin on IgE production: the IgE levels in serum from mice treated with quercetin at 20 mg/kg were nearly identical (not significant) to that from nontreated, *M. corti*-infected mice.

3.2. Influence of Quercetin on Eosinophil Activation In Vitro. The second set of experiments was designed to examine whether quercetin could suppress eosinophil activation in response to immunological stimuli. To do this, eosinophils were pretreated with various doses of quercetin for one hour and then stimulated with 200.0 ng/mL SCF. After 24 h, culture supernatants were collected, and RANTES and MIP-1 β levels were examined by ELISA. Quercetin could inhibit the ability of eosinophils to produce RANTES (Figure 2(a)) and MIP-1 β (Figure 2(b)), which was enhanced by SCF stimulation. The minimum concentration of quercetin, which causes significant suppression of factor productions, was 5.0 μ M (Figures 2(a) and 2(b)).

3.3. Influence of Quercetin on Eosinophil Degranulation In Vitro. The third set of experiments was undertaken to examine whether quercetin could inhibit eosinophil degranulation induced by immunological stimuli. Eosinophils were pretreated with various doses of quercetin for one hour and then stimulated with 200.0 ng/mL SCF for 24 hours. ECP and MBP contents in culture supernatants were examined by ELISA. Quercetin lower than 2.5 μ M scarcely affected eosinophil degranulation: the levels of both ECP (Figure 3(a)) and MBP (Figure 3(b)) in experimental culture supernatants were nearly identical ($P > 0.05$) to the control cultures. On the other hand, higher concentrations of quercetin (5.0 μ M and 10.0 μ M) significantly inhibit the degranulation induced by SCF stimulation (Figures 3(a) and 3(b)).

4. Discussion

Quercetin is reported to exhibit a wide variety of biological activities such as anticancer and antihypertensive effects [6]. It is also reported that quercetin inhibits mast cell activation, including inflammatory cytokine production and histamine release after immunological stimulation [8, 9, 11]. Furthermore, treatment of experimental animal models of asthma with quercetin caused not only a decrease in interleukin (IL)-5 levels and eosinophil counts in both bronchoalveolar lavage fluid and lung tissues but also bronchial hypersensitivity, which are induced by specific allergen challenge [11]. Although these reports strongly suggest that quercetin is an effective allergic inflammation suppressor, the mode of action of quercetin on allergic immune responses is not well understood.

Immunocytochemical studies of allergic diseases have shown the presence of numerous numbers of activated

inflammatory cells, such as Th2 type helper T cells, macrophages, and eosinophils [15]. Of these, eosinophils are believed to be a key cell and play an essential role in the development and maintenance of allergic diseases through the secretion of lipid mediators and proteins [2, 3, 5], suggesting that eosinophils will be an important target for the treatment and the management of allergic diseases. The experiments presented here characterized firstly the effects of quercetin on allergic immune responses, especially eosinophilia and IgE hyperproduction, during normal *in vivo* immune responses. The model used is that of eosinophilia and IgE hyperproduction during *M. corti* infection. The data obtained clearly show that quercetin cannot suppress eosinophilia and IgE hyperproduction induced by *M. corti* infection, even when 20.0 mg/kg of quercetin was used for treatment. There is much evidence that eosinophilia and IgE hyperproduction caused by infection with tissue-invasive helminth species are controlled by several types of cytokines, especially IL-4 and IL-5, which are produced by Th2 type helper T cells in response to the stimulation with larval excretory/secretory antigen(s) [16–18]. The production of both IL-4 and IL-5 from Th2 type helper T cells after stimulation is also accepted to require the activation of transcription factor, such as nuclear factor-kappa B (NF- κ B), which controls the expression of genes encoding inflammatory cytokines [19, 20]. Although the treatment of cells with quercetin has been reported to be able to inhibit NF- κ B activation after inflammatory stimulation *in vitro* [21, 22], oral administration of quercetin cannot downregulate NF- κ B activation *in vivo* [21, 22]. Taken together, it is reasonable to speculate that the present results strongly suggest that treatment of mice with quercetin cannot suppress NF- κ B activation in Th2 type helper T cells induced by *M. corti* infection and that it causes the production of both IL-4 and IL-5 *in vivo*, resulting in eosinophilia and IgE hyperproduction in *M. corti*-infected, quercetin-treated mice. On the other hand, in experimental murine allergic asthma, oral administration of quercetin at a single dose of 10 mg/kg once a day for 5 days is reported to be able to suppress the increase in eosinophil numbers in both bronchoalveolar lavage fluids and peripheral blood, which are induced by relevant antigenic challenge [10]. This discrepancy may arise from the difference in models used for the experiments. Further experiments are required to clarify this point.

Eosinophils secrete a number of harmful mediators, including ECP and MBP, which have been implicated in airway reactivity, vascular leak syndromes, destruction of epithelium, and other inflammatory changes that underlie allergic diseases [3, 5]. Eosinophils are also able to produce certain cytokines and chemokines such as IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF), and RANTES which exert an autocrine effect on eosinophil survival, differentiation, and accumulation [3, 5]. SCF is a primary cytokine involved in hematopoiesis, mast cell differentiation, and mast cell activation [23]. SCF has also been shown to play a significant role in development of eosinophil-associated inflammatory responses [14, 24]. Therefore, the second part of the experiment was carried out to examine the influence of quercetin on eosinophil activation by using SCF

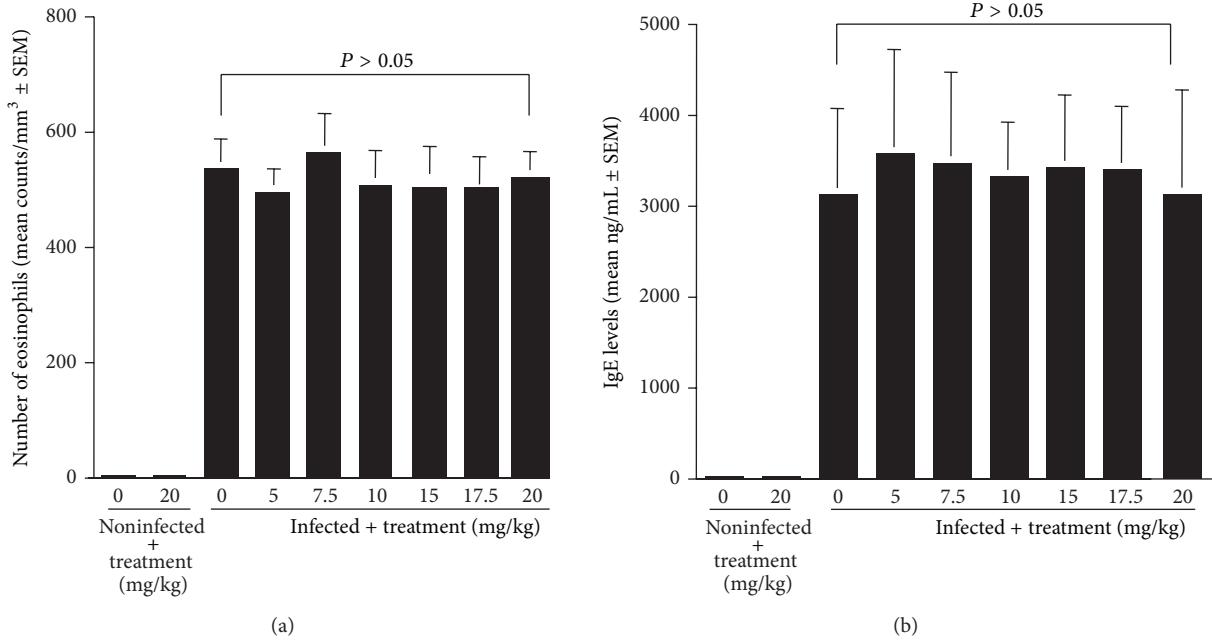


FIGURE 1: Influence of quercetin on peripheral blood eosinophilia (a) and IgE hyper-production (b) induced by *Mesocestoides corti* infection in mice. BALB/c mice were injected intraperitoneally with 500 *M. corti* larvae. These mice were then treated with various doses of quercetin once a day for 21 days, starting on the day of infection. After 21 days, the number of peripheral blood eosinophils and IgE levels were examined. Values are means \pm standard errors of the mean (SEM) for five mice/group.

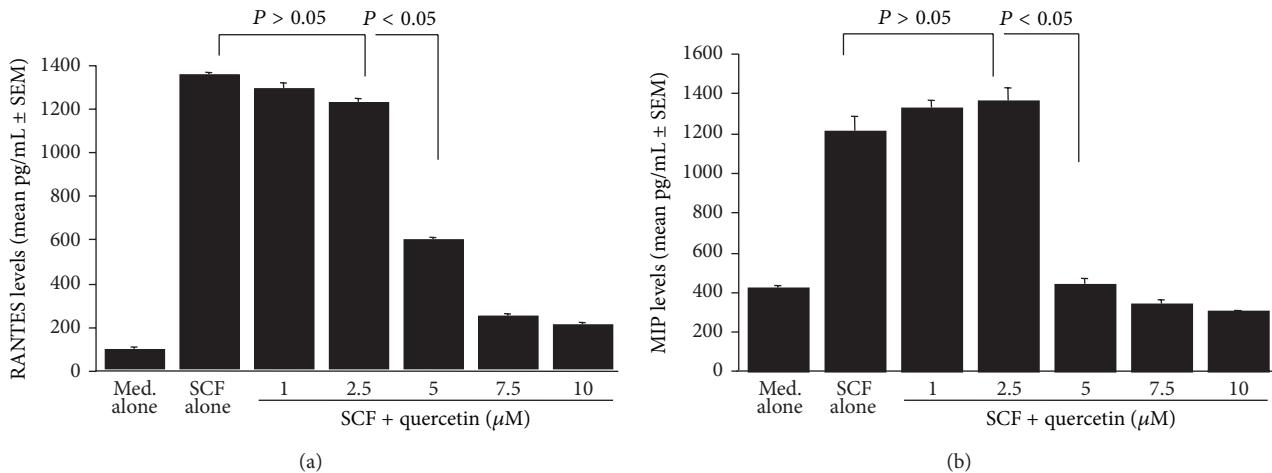


FIGURE 2: Influence of quercetin on eosinophil activation induced by stem cell factor (SCF) stimulation *in vitro*. Eosinophils (5×10^5 cells/mL) obtained from mice infected with *Mesocestoides corti* (500 larvae/mouse) were stimulated in triplicate with 200 ng/mL SCF in the presence of various concentrations of quercetin for 24 h. Factor levels in culture supernatants were examined by ELISA. The data are expressed as the mean \pm standard errors of the means (SEM). Med. alone: medium alone; (a) RANTES; (b) MIP-1 β (MIP).

and a cell culture technique *in vitro*. As assessed by examining the levels of RANTES and MIP-1 β , quercetin could suppress these factor productions from eosinophils after SCF stimulation. A significant suppression of the production was firstly observed at 5.0 μ M. Airway mucosal eosinophilia is a prominent feature of allergic airway diseases such as asthma and rhinitis [5, 25]. Although IL-5 is accepted to be a central factor mediating eosinophil recruitment in response to allergic stimuli, chemokines, eotaxin, and RANTES are

powerful chemotactic factors for eosinophils [5, 25]. MIP-1 β is a member of the CC subfamily of chemokines, which induce the migration and recruitment of monocytes and T cells to the sites of inflammation [26]. MIP-1 β has also been reported to enhance eosinophil effector functions by inducing the production of superoxide, which is the most important final effector molecule in inflammatory diseases [27]. In addition to the ability of eosinophils to produce these chemotactic factors, eosinophils undergo degranulation and

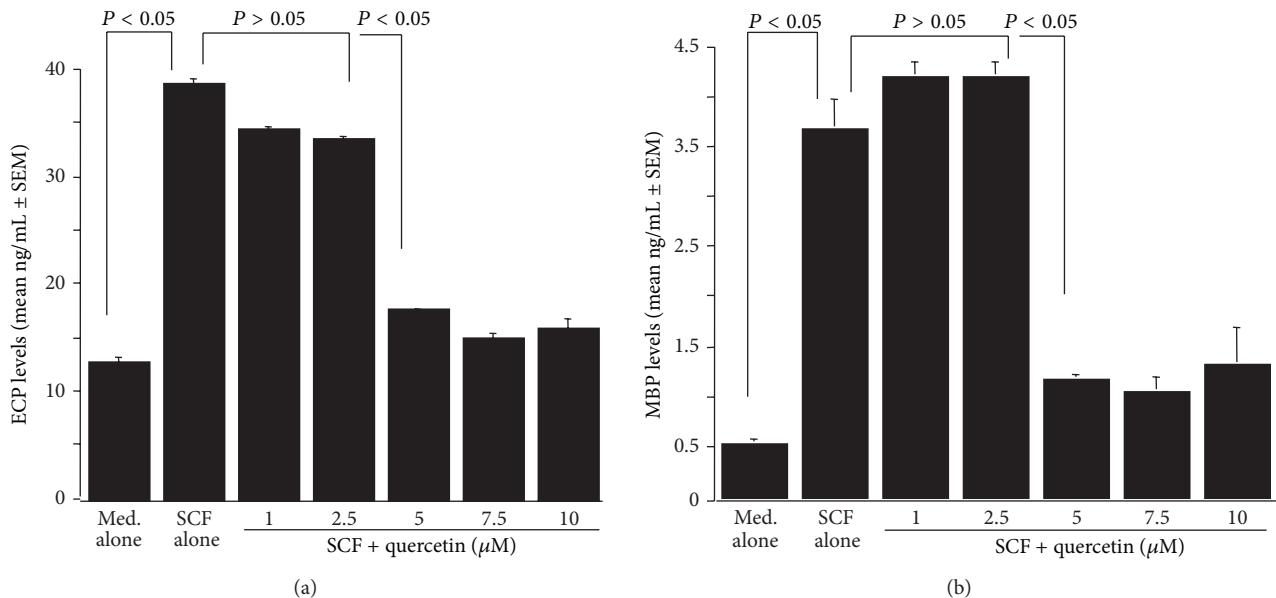


FIGURE 3: Influence of quercetin on eosinophil degranulation induced by stem cell factor (SCF) stimulation *in vitro*. Eosinophils (5×10^5 cells/mL) obtained from mice infected with *Mesocestoides corti* (500 larvae/mouse) were stimulated in triplicate with 200 ng/mL SCF in the presence of various concentrations of quercetin for 24 h. Factor levels in culture supernatants were examined by ELISA. The data are expressed as the mean \pm standard errors of the means (SEM). Med. alone: medium alone; (a) ECP; (b) MBP.

release cytotoxic granule contents, such as ECP and MBP, which are responsible for tissue remodeling and symptoms, in response to allergic stimuli [5, 9]. Taken together, the present results showing the suppressive activity of quercetin at more than 5.0 μ M to produce chemokines and degranulation of eosinophils provide possible mechanisms that could explain the favorable effects of quercetin on allergic diseases.

Although the present results clearly indicate the suppressive mode of action of quercetin on eosinophil-mediated allergic immune responses, the precise mechanisms by which quercetin could suppress eosinophil activation by SCF stimulation are not fully understood. SCF exerts its biological effect through a specific interaction with the cell surface receptor c-kit, which is a member of the receptor tyrosine kinase family [28]. SCF binding to c-kit causes the receptor to homodimerize and autophosphorylate at tyrosine residues [28]. The stimulation of c-kit leads to the activation of multiple signaling cascade (e.g., extracellular signal-regulated kinase and c-Jun N-terminal kinase, among others), which is responsible for the activation of inflammatory cells such as mast cells and eosinophils, through Ca^{2+} -dependent mechanisms [28]. It is reported that quercetin could inhibit the increase in intracellular Ca^{2+} levels induced by compound 48/80 in human mast cell line *in vitro* [29], suggesting that quercetin inhibits changes in Ca^{2+} concentration in cytosol induced by SCF stimulation and results in the inhibition of release of inflammatory mediators examined. Quercetin is reported to inhibit the activation of tyrosine kinases [30–32], which are responsible for the production of several types of cytokines, including IL-4 and IL-5 [19, 20]. From these reports, there is another possibility that quercetin inhibits

the activation of tyrosine kinases in eosinophils after SCF stimulation and results in inhibition of mediator release from eosinophils *in vitro*.

Dietary flavonoid glycosides are hydrolyzed in the intestine, absorbed as aglycones, and metabolized to methylated, glucurono-sulfated derivatives [33]. It is also observed that, after oral administration of 64 mg quercetin into human, hydrolyzed plasma levels of quercetin gradually increased and peaked at 650 nM and that the elimination half-life of quercetin was 17 to 24 h [34]. Commercially available preparations of quercetin recommend dosages of 400–1200 mg daily as a dietary supplement [35]. Assuming first order kinetics, a 1200 mg dose of quercetin could lead to plasma concentration up to 12 μ M [34], which is a much higher level of quercetin that showed suppressive effects of eosinophil activation *in vitro*. From these reports, it is strongly suggested that the findings of the present *in vitro* study may reflect the biological function of quercetin *in vivo*.

5. Conclusion

The results from the present study clearly demonstrate that quercetin exerts suppressive effects on eosinophil activation, but not eosinophil growth and IgE hyperproduction. These results indicate that quercetin will be a useful supplement for the management of eosinophil-mediated diseases, such as allergic rhinitis and asthma.

Conflict of Interests

The authors report no conflict of interests in this work.

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Research Article

Total Phenolics and Total Flavonoids Contents and Hypnotic Effect in Mice of *Ziziphus mauritiana* Lam. Seed Extract

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The seeds of *Ziziphus mauritiana* Lam. have been traditionally used for treatment of various complications including insomnia and anxiety. They are popularly used as sedative and hypnotic drugs in China, Korea, Myanmar, Vietnam, and other Asian countries. However, no scientific proof on hypnotic activity of *Z. mauritiana* seeds (ZMS) was reported. In this study, the hypnotic activity of 50% ethanolic extract from ZMS was observed on the loss of righting reflex in mice using pentobarbital-induced sleep mice method. The contents of total phenolics and total flavonoids in the extract were also determined. The results showed that the 50% ethanolic extract from ZMS contained total phenolics 27.62 ± 1.43 mg gallic acid equivalent (GAE)/g extract and total flavonoids 0.74 ± 0.03 mg quercetin equivalent (QE)/g extract. Oral administration of the extract at the dose of 200 mg/kg significantly increased the sleeping time in mice intraperitoneally administered with sodium pentobarbital (50 mg/kg body weight). These results supported the traditional use of ZMS for the treatment of insomnia. The seeds of *Z. mauritiana* should be further developed as an alternative sedative and/or hypnotic product.

1. Introduction

Ziziphus is a genus in the family Rhamnaceae which contains about 40 species. Fruits and seeds of *Ziziphus* have been widely used as traditional medicines since ancient time. *Z. mauritiana* Lam. is a tropical or subtropical fruit tree widely distributed in many Asian countries such as Afghanistan, Bhutan, India, Indonesia, Malaysia, Myanmar, Nepal, Sri Lanka, Vietnam, Africa, Australia, and Thailand [1]. The seeds of *Z. mauritiana*, a species close to *Z. jujuba* Mill., have been reported as anticancer, antidiabetic, and hypoglycemic agents [2, 3] while the seeds of *Z. jujuba*, which are known as jujube or Chinese date, are popularly used to treat insomnia and reduce the body temperature and sweat [4, 5]. *Z. mauritiana* seeds have been also used as sedative and hypnotic drugs in many Asian countries. *Ziziphus* seeds contain large amounts of fatty oil and proteins, sterols, and triterpenoid compounds

(betulin and betulinic acid) and also contain a large amount of vitamin C [6].

Sleep is one of the most deeply healing and revitalizing known experiences. Insomnia, a common problem, is a lack of healthful and restful sleep. Thirty to fifty percent of the populations are reported to be affected by insomnia while 10% of them have chronic insomnia. Fewer than 15% of patients with insomnia receive treatment [7]. Most patients are engaged in long-term use of benzodiazepines (BZDs) analogs to treat insomnia. There are some deficiencies on impaired cognitive function, memory, and general daytime performance in patients treated with these drugs. Moreover, tolerance and dependence are the obvious side effects of the drug's long-term administration [8]. Natural sleep aids, which contain specific constituents of foods and herbal plants, have recently become popular as alternatives to prescription sedative-hypnotics to improve sleep quality and

avoid side effects [9]. Therefore, there has been a growing demand for a new class of food constituents and natural products with hypnotic effects. GABAergic neurotransmission plays a key role in sleeping regulation and the BZD-binding site on the GABA-A receptor which is a target for the most sedative-hypnotics [10]. It has been widely reported that polyphenols and flavonoids have sedative-hypnotic effects based on positive allosteric modulation of GABA-A receptors [11]. ZMS have been popularly used as sedative and hypnotic drugs in many Asian countries without any scientific report on hypnotic activity. Our study was aimed to explore the hypnotic activity of 50% ethanolic extract from ZMS, which was previously found to promote high radical scavenging activity [12] through the loss of righting reflex on pentobarbital-induced sleep mice. The contents of total phenolics and total flavonoids in the extract were also determined.

2. Materials and Methods

2.1. Plant Material. Dried ZMS was purchased from Kaung Su Aung Co., Ltd. Herbs Plantation in Myanmar in June, 2010. The sample was identified by comparison with the voucher specimens at the Bangkok Herbarium, Botanical Section, Botany and Weed Science Division, Department of Agriculture, Bangkok. The voucher specimen (ZM0610) was deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The seeds were dried at 55°C for 6 hours and ground with an electronic mill to give moderate powder. The powdered sample was kept in an air tight container protected from light in a cool place until extracted.

2.2. Preparation of 50% Ethanolic Extract from ZMS. According to our former report the soxhlet extraction with 50% ethanol was the appropriate extraction method and solvent to promote the ZMS extract with the strongest antioxidant activity [12]. Therefore, the 50% ethanolic extract was prepared from ZMS by this procedure. The powdered sample was placed into a thimble in a soxhlet apparatus and was extracted with 50% ethanol (1 : 35, w : v) at 55°C until exhaust (15 hours). The extract was filtered through a Whatman No. 1 filter paper and the filtrate was concentrated under reduced pressure at 50°C using a rotary vacuum evaporator. The concentrated extract was then evaporated on a boiling water bath until a constant weight was obtained. The dried extract was weighed and the yield was calculated. The extract was kept in air tight container protected from light until used.

2.3. Determination of Total Flavonoids Content. Total flavonoids were analyzed using aluminum chloride colorimetric method. Sample (500 µg/mL) of 500 µL was mixed with 500 µL of 2% aluminum chloride solution. The mixture was allowed to stand at room temperature (28 ± 2°C) for 10 min with intermittent shaking. The absorbance of the mixture was measured at 415 nm against a blank sample (methanol) without aluminum chloride using a UV-vis spectrophotometer (PerkinElmer, USA). The total flavonoids content

was determined using a standard curve of quercetin (0.5–12.5 µg/mL). The content was calculated as mean ± SD ($n = 3$) and expressed as milligrams of quercetin equivalents (QE) in 1 g of the extract and dried powder.

2.4. Determination of Total Phenolic Compounds Content [13]. The content of total phenolic compounds was determined using Folin-Ciocalteu procedure. The sample (250 µg/mL) or standard gallic acid solution (10–100 µg/mL), 0.2 mL was mixed with 0.5 mL of the Folin-Ciocalteu reagent (diluted 1 : 10 with deionized water) and 0.8 mL of sodium bicarbonate solution (7.5% w/v). The mixture was allowed to stand at room temperature (28 ± 2°C) for 30 min with intermittent shaking. The absorbance of the mixture was measured at 765 nm using a UV-vis spectrophotometer (PerkinElmer, USA). The content of total phenolic compounds was calculated as mean ± SD ($n = 3$) and expressed as milligrams of gallic acid equivalent (GAE) in 1 g of the extract and dried powder.

2.5. Animals. Twenty male (*Mus musculus*) ICR mice (25–35 g), 5 weeks of age, were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand. The animals were habituated to the laboratory animal room for at least 1 week before the experiment. They were housed in groups of four or five animals in standard cages containing a supply of pellet diet and ad libitum water. The animal room was maintained at 25 ± 2°C with constant humidity (65%) and a 12 h of dark-light cycle. All studies were conducted between 9 a.m. and 1 p.m. The experiment was approved by the Institutional Animal Care and Use Committee, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand (Proof no. PYR002/2554).

2.5.1. Drugs. Diazepam (Roche, Italy) was obtained from Yangon General Hospital in a tablet form. It was dissolved in distilled water to the concentration of 0.2 mg/10 mL. Sodium pentobarbital was purchased from Sigma-Aldrich (USA), dissolved in 0.9% normal saline, and adjusted to the concentration of 50 mg/10 mL.

2.5.2. Pentobarbital-Induced Sleeping in Mice. Mice were randomly divided into 4 groups of 5 mice each and treated as follows: group 1 was orally administered with a single dose of distilled water (10 mL/kg body wt.) as the normal control group; groups 2 and 3 were orally administered with the single dose of 50% ethanolic extract from ZMS at 100 and 200 mg/kg, respectively. Group 4 was the positive control group, which was orally treated with diazepam (0.2 mg/kg). Pentobarbital-induced sleeping effect was performed as previously reported [14]. Briefly, the animals were orally administered with distilled water, the ZMS extracts, or diazepam. Thirty minutes later, sodium pentobarbital at the doses 50 mg/kg was intraperitoneally injected into each mouse to induce sleeping effect. Mice that remained immobile for more than 30 min were judged to be asleep. The interval between loss and recovery of righting reflex was used as index of hypnotic effect. The animals were observed

TABLE 1: Yield, total flavonoids, and total phenolics contents of 50% ethanolic extract from *Z. mauritiana* seeds.

Yield of crude extract (% dry weight)	Total flavonoids content (mg QE/g)		Total phenolics content (mg GAE/g)	
	In extract	In dried seeds	In extract	In dried seeds
30.20 ± 0.02	0.74 ± 0.03	0.22 ± 0.01	27.62 ± 1.43	8.34 ± 0.43

constantly and the time of awakening as characterized by righting of animals was noted.

2.5.3. Statistical Analysis. The results are expressed as mean ± standard error of the mean (SEM). The values were compared using the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. *P* values less than 0.05 were considered to be statistically significant.

3. Results and Discussion

Yield, total flavonoids, and total phenolics contents in the 50% ethanolic extract of ZMS are shown in Table 1. Fifty percent ethanol extract, which was previously reported to be the appropriate extract providing high *in vitro* antioxidant activity, was used for testing the hypnotic effect in this experiment. As shown in Figure 1, the sleep duration induced by 50 mg/kg sodium pentobarbital in the control group was 56.20 ± 3.35 min. The ZMS extract potentiated pentobarbital-induced sleeping behaviors in mice in a dose dependent manner. ZMS extract at doses of 100 and 200 mg/kg significantly prolonged the sleeping time (60.25 ± 1.44 and 71.60 ± 2.72 min, $P < 0.05$ versus control group). The hypnotic effect of ZMS extract (200 mg/kg) was comparable to those of diazepam (0.2 mg/kg). Sodium pentobarbital is a drug in barbiturate group that can induce the sleep in both rodents and humans [15]. It is well known that many drugs such as benzodiazepines and sodium pentobarbital possess anxiolytic and sedative effects [16]. The classic method of pentobarbital-induced sleeping in mice is often used to screen sedative-hypnotic drugs. Previous pharmacological studies reported that ZMS reduced locomotor activities in animals [17]. The present study showed that the 50% ethanolic extract of ZMS (200 mg/kg) as well as diazepam (0.2 mg/kg) significantly increased sleeping times in mice from 56.20 ± 3.35 min in control group to 71.60 ± 2.72 and 67.60 ± 2.40 min, respectively. Our results demonstrated for the first time that ZMS extract potentiated pentobarbital-induced sleeping behavior in mice. This supports the traditional use of ZMS for insomnia and anxiety treatments [1]. The mechanism of hypnotic effect of ZMS extract may be similar to diazepam, so the further study should be investigated by using benzodiazepine antagonist drug to see a reversal sleeping time.

The hypnotic activity of herbal medicines has been attributed to different phytochemical compounds such as flavonoids, terpenes, and saponins. The active sedative components in *Z. jujuba* seeds were reported to be flavonoids and saponins [18]. Therefore, the active sedative components in

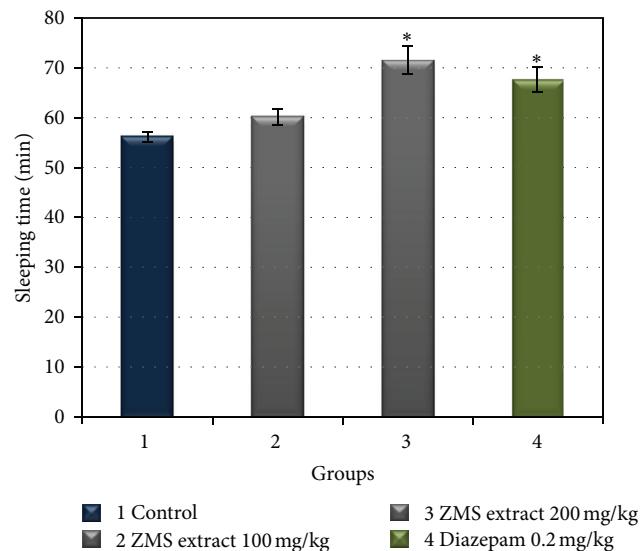


FIGURE 1: Effects of *Z. mauritiana* seed extracts (ZMS, 100 and 200 mg/kg) or diazepam (0.2 mg/kg) on sleeping time in mice induced by sodium pentobarbital ($n = 5$). All values represent mean ± SEM, * $P < 0.05$ compared with distilled water treated control group.

the seeds of *Z. mauritiana* should be further identified and quantitatively analysed.

4. Conclusion

This study demonstrated the hypnotic effect of the ethanolic extract from *Z. mauritiana* seeds. The standardized extract might be developed as an alternative sedative/hypnotic product. The major active components for hypnotic effect in ZMS should be separated and identified.

Conflict of Interests

The authors have no conflict of interests with the following trademarks: "Kaung Su Aung Co," PerkinElmer," "Sigma-Aldrich," and "ANOVA."

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Research Article

Two New 3,4;9,10-seco-Cycloartane Type Triterpenoids from *Illicium difengpi* and Their Anti-Inflammatory Activities

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A pair of new 3,4;9,10-seco-cycloartane type triterpenoid stereoisomerides: 24R,25-dihydroxy-3,4;9,10-seco-4(28)-cycloarten-10,3-olide (**1**) named Illiciumolide A and 24S,25-dihydroxy-3,4;9,10-seco-4(28)-cycloarten-10,3-olide (**2**) named Illiciumolide B were isolated from the stem bark of *Illicium difengpi*, as well as five known biogenetically related triterpenoids, including sootepin E (**3**), betulinic acid (**4**), lupeol (**5**), (all-Z)-1,5,9,13,17,21-hexamethyl-1,5,9,13,17,21-cyclotetracosahexaene (**6**), and (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene (**7**). The structures of two new compounds were determined on the basis of spectroscopic analysis including 1D-, 2D-NMR, and MS techniques. Two assays were conducted: inhibition of tumor necrosis factor-alpha (TNF- α) and inhibition of nuclear factor kappa B (NF- κ B) in RAW264.7 cells induced by lipopolysaccharide (LPS). It was observed that compounds **1**, **2** and **7** showed significant inhibition of TNF- α production and NF- κ B release. The molecule docking results showed that compounds **1** and **2** got high fitness scores with dual specificity mitogen-activated protein kinase kinase 1 (MPKK1), whose activation plays a pivotal role between TNF- α and activation of NF- κ B. The anti-HIV-1 potency of compounds **1–5** was also discussed, in addition to the results of computer-aided screening for targets.

1. Introduction

Natural products have been, and continue to be, a major source of pharmacologically active substances from which drugs can be developed [1]. Medicinal plants that can relieve rheumatism, chills, and pains according to traditional Chinese medicine theory are fit for use by rheumatism sufferers. From these plants, many constituents with proved anti-inflammatory activity have been isolated and their structures were determined by spectroscopic methods.

I. difengpi (Illiciaceae), the stem bark of which has been applied for treatment of rheumatoid arthritis as a traditional Chinese medicine, is a small shrub growing in mountain areas of Guangxi province in China. *I. difengpi* is listed in Chinese Pharmacopoeia. In previous phytochemical investigation of barks of *I. difengpi* thirty compounds were isolated including dominant phenylpropanoids and neolignans [2–4] together with four sesquiterpene lactones [5] and three triterpenoids [6]. The anti-inflammatory activities of several

neolignans were assayed by measuring the inhibitory ratio of β -glucuronidase release in rat PMNs induced by PAF *in vitro* [2, 5]. The sesquiterpene lactones were predicted to exhibit neurotrophic activity [7–9]. However, there have been limited studies that focus on the triterpenoids from *I. difengpi* and of the family Illiciaceae. Until now, only six cycloartanes [6, 10, 11] were reported to be isolated from family Illiciaceae, while from family Schisandraceae (*Schisandra* and *Kadsura*, order Illiciales) more than 150 triterpenoids [12] have been isolated. In particular, they both contain *seco*-cycloartane triterpenoids. These characteristic chemical structures from the Schisandraceae and their activities were widely studied. Most of the *seco*-cycloartanes from the family Schisandraceae were demonstrated to possess anti-HIV-1 activity.

As a part of our study to find the active constituents, an investigation of *I. difengpi* was undertaken, leading to isolation and structural elucidation of two new 3,4;9,10-seco-cycloartane triterpenoids and three known triterpenoids together with two squalenes. In order to shed some light on

biological activities, the anti-inflammatory and anti-HIV-1 properties of the isolates were evaluated and discussed.

2. Materials and Methods

2.1. General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 341 polarimeter. IR spectra were recorded on a NEXUS 470 FT-IR spectrometer (Thermo Nicolet, USA). 1D (^1H , ^{13}C , DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra were acquired on a Bruker Avance 600 NMR spectrometer operating at 600 (^1H) or 150 (^{13}C) MHz using the residual solvent signals as an internal reference (CDCl_3 , δ_{H} 7.26 ppm, δ_{C} 77.0 ppm). NMR samples were in 3 mm Shigemi tubes during NMR analyses. High-resolution mass spectrometric data were obtained on an Agilent 6220 TOF LC/MS instrument (Agilent Technologies, MA, USA) with ESI ionization in the positive mode. Column chromatography (CC) was performed on Sephadex LH-20 gel (40–70 μm , Amersham Pharmacia Biotech AB, Uppsala, Sweden), YMC-GRL ODS-A (50 μm ; YMC, MA, USA), and silica gel H (100–200 and 200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, China). TLC analyses were performed on $\text{Si}_{60}\text{F}_{254}$ plates and visualized under UV light or by heating after spraying with 10% H_2SO_4 /EtOH solution. Semipreparative RP-HPLC isolation was achieved with an Agilent 1200 instrument using a YMC 5 μm C18 column (250 mm \times 10 nm) eluted with 80% MeCN/ H_2O at 1–2 mL/min. Peak detection was made with a refractive index detector (RID). The positive controls in the anti-inflammatory assay were tripterygium tablets (TRT) and total glucosides of paeony (TGP). Purity was assessed by HPLC and determined to be 95% or greater for compounds 1–7 at the time of testing.

2.2. Plant Material. The stem barks of *Illicium difengpi* were purchased from Caitongde Pharmacy, Shanghai, China, in January 2010. Plant material was authenticated by Professor Lianna Sun (Department of Pharmacognosy, School of Pharmacy, Second Military Medical University) based on morphological characters. Voucher specimen (No. 20100110) has been deposited at the Herbarium of Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai, China.

2.3. Extraction and Isolation. The air-dried stem bark of *I. difengpi* (40 kg) was powdered and extracted three times with 80% ethanol under reflux. The solvent was concentrated to obtain a crude extract (1200 g) which was suspended in water (10 L) and extracted with petroleum ether (10 L \times 3), EtOAc (10 L \times 3), and BuOH (10 L \times 3), affording 40, 560, and 300 g of each dried fraction, respectively.

The dried petroleum ether fraction (Fr.₁) (40 g) was chromatographed on silica gel column (CC) (80 \times 5 cm, gradient with petroleum ether: EtOAc = 100 : 0 \rightarrow 0 : 100) to give eight main fractions (Fr.₁₋₁–Fr.₁₋₈), among which Fr.₁₋₂ gave compound 7 (27 mg), Fr.₁₋₇ provided compound 6 (85 mg), and Fr.₁₋₈ afforded compound 3 (34 mg) through Sephadex LH-20 CC (150 \times 2 cm, CH_2Cl_2 : MeOH = 1 : 1). The EtOAc extract

(Fr.₂) (560 g) was chromatographed on silica gel CC (150 \times 10 cm, gradient with CH_2Cl_2 : MeOH = 300 : 1 \rightarrow 0 : 100) to give four main fractions (Fr.₂₋₁–Fr.₂₋₄). Fr.₂₋₂ was subjected to silica gel CC (20 \times 2 cm, gradient with petroleum ether: EtOAc = 100 : 1 \rightarrow 1 : 1) affording a mixture of compounds 4 and 5. The mixture was rechromatographed using silica ODS-A gel CC (20 \times 2 cm, gradient with MeOH: H_2O = 1 : 1 \rightarrow 1 : 0) to give pure compounds 4 (45 mg) and 5 (21 mg). Fr.₂₋₃ was fractionated by silica gel CC (35 \times 3 cm, gradient with petroleum ether: EtOAc = 100 : 1 \rightarrow 5 : 1) to yield three subfractions (Fr.₂₋₃₋₁–Fr.₂₋₃₋₃). Fr.₂₋₃₋₃ was rechromatographed using semipreparative HPLC system (Agilent 1200 series; YMC HPLC C18 column-5 μm , 250 \times 10 mm, refractive index detector; flow 2 mL/min; mobile phase MeCN: H_2O = 80 : 20) to give pure compounds 1 (8 mg) and 2 (2.5 mg).

2.4. Characterization of Compounds. Compound 1: yellowish solid; $[\alpha]_D^{25} + 41.2$ (c 0.5, MeOH); IR (KBr) ν_{max} 3402, 2929, 2872, 1763, 1458, 1377, 1273, 1238, 1194, 1172, 1074, 1041, 1007, 899 cm^{-1} ; ^1H NMR and ^{13}C NMR data see Table 1; HRESIMS m/z 475.3774 [M+H]⁺ (calcd. for $\text{C}_{30}\text{H}_{51}\text{O}_4$, 475.3784).

Compound 2: yellowish solid; $[\alpha]_D^{25} + 94.0$ (c 0.5, MeOH); IR (KBr) ν_{max} 3454, 2931, 2873, 1764, 1461, 1452, 1379, 1259, 1195, 1171, 1080, 1039, 1009, 920, 870 cm^{-1} ; ^1H NMR and ^{13}C NMR data see Table 1; HRESIMS m/z 475.3765 [M+H]⁺ (calcd. for $\text{C}_{30}\text{H}_{51}\text{O}_4$, 475.3784).

2.5. Inhibition of TNF- α Release Assay. Isolated compounds were tested for their ability to inhibit TNF- α release from LPS-stimulated RAW 264.7 macrophages using enzyme-linked immunosorbent assay (ELISA) as a quantitative assay. Tripterygium tablets (TRT) and total glucosides of paenia (TGP) were used as positive controls. The ELISA Max set standard (BioLegend, San Diego, CA, USA) was performed according to the manufacturer's instruction strictly. The inhibition ratio (IR) was calculated as IR (%) = $(A_{\text{LPS}} - A_{\text{S}})/A_{\text{LPS}} \times 100\%$, where A_{LPS} and A_{S} refer to the amount of TNF- α in cells pretreated with LPS and samples, respectively.

2.6. Inhibition of NF- κ B Release Assay. The effect of compounds 1–7 on NF- κ B production from LPS-treated RAW264.7 cells was monitored. Tripterygium tablets (TRT) and total glucosides of paeony (TGP) were used as positive controls. Cells were placed on 96-well plates (Costar) at a density of 1.0×10^5 cells mL^{-1} , maintained in DMEM containing 10% FBS for 24 h. After changing the medium, the samples were incubated at 37°C with 5% CO₂ for 4 h, and 1 $\mu\text{g}/\text{mL}$ LPS was then added and incubated for another 48 h. After 48 h, cells were collected and completely cracked using 1× lysis buffer. After centrifugation at 1500 rpm for 5 min, the supernatant was obtained. 100 μL luciferase assay reagent and 20 μL cell lysate were added to it in order rapidly. The detection results were read in 10 s. The IR was calculated in a similar way to the above formula, IR (%) = $(I_{\text{LPS}} - I_{\text{S}})/I_{\text{LPS}} \times 100\%$, where I_{LPS} and I_{S} refer to the fluorescence intensity of cells pretreated with LPS and samples, respectively.

TABLE 1: ^1H -NMR (600 MHz) and ^{13}C -NMR (150 MHz) data for compounds **1** and **2** (CDCl_3 , δ_{H} in ppm, J in Hz).

Position	1		2	
	δ_{C} , mult.	δ_{H}	δ_{C} , mult.	δ_{H}
1	31.5, CH ₂	2.33 (m, H _b -1) 1.77–1.84 (m, H _a -1)	31.5, CH ₂	2.30–2.36 (m, H _b -1) 1.70–1.76 (m, H _a -1)
2	29.6, CH ₂	2.41–2.48 (m, H _a -2) 2.41–2.48 (m, H _b -2)	29.6, CH ₂	2.42–2.48 (m, H _a -2) 2.42–2.48 (m, H _b -2)
3	177.3, qC	—	177.3, qC	—
4	146.6, qC	—	146.6, qC	—
5	54.8, CH	2.54 (d, 9.6, H-5)	54.8, CH	2.56 (d, 9.8, H-5)
6	31.4, CH ₂	1.65–1.70 (m, H _a -6) 1.82–1.89 (m, H _b -6)	31.4, CH ₂	1.61–1.67 (m, H _a -6) 1.82–1.89 (m, H _b -6)
7	30.5, CH ₂	1.46–1.51 (m, H _a -7) 1.79–1.83 (m, H _b -7)	30.5, CH ₂	1.41–1.46 (m, H _a -7) 1.72–1.77 (m, H _b -7)
8	48.4, CH	1.43 (dd, 3.2, 7.0, H-8)	48.4, CH	1.42–1.47 (m, H-8)
9	31.7, CH	1.90 (td, 7.4, 14.0, H-9)	31.7, CH	1.85–1.92 (m, H-9)
10	91.7, qC	—	91.7, qC	—
11	29.6, CH ₂	1.23 (m, H _a -11) 1.73 (m, H _b -11)	29.6, CH ₂	1.46 (m, H _a -11) 1.73 (m, H _b -11)
12	32.7, CH ₂	1.61–1.64 (m, H _a -12) 1.75–1.80 (m, H _b -12)	32.7, CH ₂	1.61–1.64 (m, H _a -12) 1.75–1.80 (m, H _b -12)
13	45.5, qC	—	45.5, qC	—
14	49.2, qC	—	49.2, qC	—
15	33.3, CH ₂	1.06–1.14 (m, H _a -15) 1.23–1.28 (m, H _b -15)	34.0, CH ₂	0.97–1.02 (m, H _a -15) 1.23–1.25 (m, H _b -15)
16	27.9, CH ₂	1.37–1.42 (m, H _a -16) 1.92–1.98 (m, H _b -16)	27.8, CH ₂	1.47 (m, H _a -16) 1.92–1.98 (m, H _b -16)
17	51.0, CH	1.49–1.53 (m, H-17)	50.9, CH	1.49–1.55 (m, H-17)
18	14.7, CH ₃	0.80 (s, H ₃ -18)	14.7, CH ₃	0.80 (s, H ₃ -18)
19	49.2, CH ₂	1.64–1.70 (m, H _a -19) 1.77–1.83 (m, H _b -19)	49.2, CH ₂	1.64–1.70 (m, H _a -19) 1.78–1.83 (m, H _b -19)
20	35.9, CH	1.37–1.47 (m, H-20)	36.4, CH	1.51–1.55 (m, H-20)
21	18.3, CH ₃	0.86 (d, 7.22, H ₃ -21)	18.6, CH ₃	0.88 (d, 6.5, H ₃ -21)
22	33.1, CH ₂	1.13–1.20 (m, H _a -22) 1.35–1.40 (m, H _b -22)	33.3, CH ₂	1.13–1.20 (m, H _a -22) 1.35–1.40 (m, H _b -22)
23	28.3, CH ₂	1.36 (m, H _a -23) 1.14–1.22 (m, H _b -23)	28.6, CH ₂	1.36 (m, H _a -23) 1.09–1.14 (m, H _b -23)
24	78.7, CH	3.31 (t, 6.2, H-24)	79.6, CH	3.27 (dd, 9.5, 1.6, H-24)
25	73.2, qC	—	73.2, qC	—
26	23.2, CH ₃	1.14 (s, H ₃ -26)	23.2, CH ₃	1.15 (s, H ₃ -26)
27	26.6, CH ₃	1.19 (s, H ₃ -27)	26.5, CH ₃	1.21 (s, H ₃ -27)
28	16.6, CH ₃	0.83 (s, H ₃ -28)	16.7, CH ₃	0.84 (s, H ₃ -28)
29	22.6, CH ₃	1.76 (s, H ₃ -29)	22.6, CH ₃	1.77 (s, H ₃ -29)
30	115.0, CH ₂	4.80 (s, H _a -30) 4.91 (s, H _b -30)	115.0, CH ₂	4.81 (s, H _a -30) 4.92 (s, H _b -30)

2.7. Statistical Analysis. The statistical significance of differences was determined by two-tailed Student's *t*-test for unpaired data.

3. Results and Discussion

3.1. Structure Analysis. Illiciumolide A (**1**) was isolated as a yellowish solid. The positive HRESI-MS analysis showed

a pseudo-molecular ion at *m/z* 475.3774 [M+H]⁺, consistent with the formula $\text{C}_{30}\text{H}_{50}\text{O}_4$ (calcd. 474.3709), which accounted for six degrees of unsaturation. IR (KBr) absorption bands were observed as 3402 cm⁻¹ (OH), 2929 cm⁻¹ (CH₃), 1763 cm⁻¹ (C=O), 1377 cm⁻¹ (CH₂), and 899 cm⁻¹ (C=CH₂). The ¹³C NMR and DEPT spectra exhibited thirty resonances, including six quaternary carbons, six methines, twelve methylenes, and six methyls. Among these, five

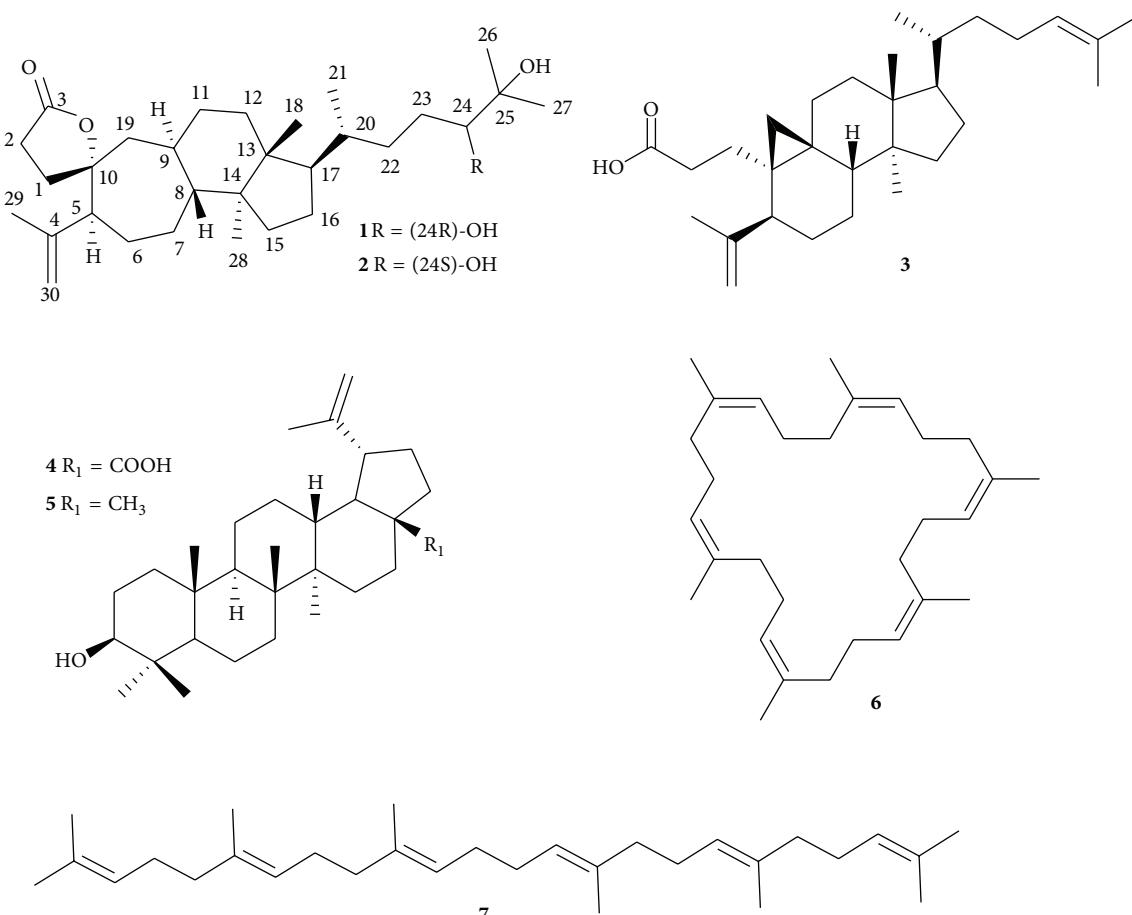


FIGURE 1: Structures of compounds 1–7.

primary methyls (δ_{C} 14.7, H₃-18; δ_{C} 16.6, H₃-28; δ_{C} 22.6, H₃-29; δ_{C} 23.2, H₃-26; δ_{C} 26.5, H₃-27) and a secondary methyl (δ_{C} 18.3, H₃-21) were determined as evident from their multiplicities in the ¹H NMR spectrum (Table 1). The ¹³C NMR spectrum of **1** revealed the presence of one lactone carbonyl carbon (δ_{C} 177.3) and a pair of olefinic carbons (δ_{C} 146.6, δ_{C} 115.0). Taking into consideration the presence of a pair of methylene protons (δ_{H} 4.80 and 4.91) in the ¹H NMR spectrum of **1** (Table 1), these features indicated the existence of a terminal methylene group in accordance with the IR spectrum absorption. The oxygenated quaternary carbon appeared at the low field δ_{C} 91.7, together with the lactone carbonyl carbon (δ_{C} 177.3), suggesting that the C-3 (δ_{C} 177.3) may lactonize to C-10 (δ_{C} 91.7) forming a five-membered lactone ring, which required future analysis of HMBC and ¹H-¹H COSY. Apart from two degrees of unsaturation occupied by one double bond and one carbonyl, the remaining four degrees of unsaturation indicated that **1** should possess a tetracyclic system. Detailed comparison of the ¹H and ¹³C NMR spectra of **1** with those of schisanterpene A [13] suggested a similar structure for rings A–D in both compounds and might be derived from cycloartane type triterpenoids, but a pair of double bonds between C-24 and C-25 and the carboxyl group at C-26 in schisanterpene A

was absent in **1**, which instead exhibited vicinal diol (C-24, δ_{C} 78.7; C-25, δ_{C} 73.2) and two methyls (C-26, δ_{C} 23.2; C-27, δ_{C} 26.5). As a result, **1** was tetracyclic and belonged to 3,4,9,10-seco-type triterpenoids [13, 14]. Subsequently, the structure was fully elucidated by 2D NMR spectroscopy. The H₂-1, H₂-2, and H-5 showed distant correlations with C-3, C-10, and C-19, coupled with ¹H-¹H COSY correlation of H-1/H-2 (Figure 2), consistent with the lactone ring (A) substructure. A detailed analysis of its HSQC, HMBC and ¹H-¹H COSY spectra confirmed that **1** contained a seven-membered ring (B) evident as the HMBC correlations from H-5 to C-10, from H-6 to C-8, from H-7 to C-5, and C-9 and from H-19 to C-9 and C-10, as well as ¹H-¹H COSY correlations of H-5/H-6/H-7 and H8/H-9 (Figure 2). The structure of rings C and D was deduced from the ¹H-¹H COSY correlations of H-9/H-11/H-12 and H-15/H-16/H-17 and HMBC correlations from H₃-13 to C-8 and C-15, H₃-14 to C-12 and C-17, and H-11 to C-19 and C-14. From the above deduction, compound **1** and schisanterpene A were confirmed to have the similar structure in rings A–D, while the chain from C-17 was verifiably different. Further evidence supporting chain structure was provided by the presence of the correlations of H-15/H-16, H-21/H-20, H-22/H-23, and H-23/H-24 as deduced from the COSY spectrum and HMBC

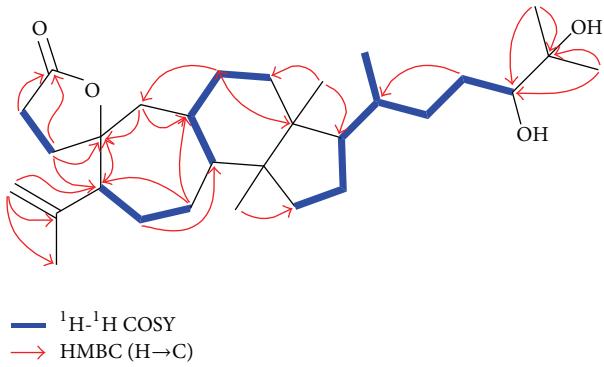


FIGURE 2: Key ^1H - ^1H COSY (bold lines) and HMBC ($\text{H} \rightarrow \text{C}$) correlations of compound **1**.

correlation from H-23 to C-20. Furthermore, the vicinal diol with two methyls termination was proved from the HMBC evident correlations from H₃-26 (H₃-27) to C-24 and C-25 (Figure 2). The relative stereochemistry of compound **1** was deduced from NOESY correlations (Figure 3). The oxygen atom of the spiroring on C-10 was in the α -orientation, as the NOESY correlations between H-2 β and H₂-19 and H-1 β and H₃-29 are the same as those of schinalactone B. Correlations between H-5 α with H-7 α and H-9 clearly showed that these protons were on the same face. Other important NOESY correlations were observed between H-9 and H-11 α and H₃-28, and H-7 α /H-16 α , indicating that H₃-28 was α -orientation while H₃-18 was on the other side. Finally about the C-17 side chain, the intense cross-peaks between H-16 α , H-17, and H₃-21 suggested that both H-17 and C-21 have α orientation in **1**. Except for 24-OH, the relative configurations of **1** were the same as schinalactone B. The 24-OH configuration was deduced by the resonances of protons and carbons at C-23, C-24, and C-25 and by the *J* values between H-23 and H-24 (*J* = 6.2 Hz). In the previous literature, *J* value between H-23 and H-24 in 24R-configuration was reported to be around 6.5 and 1.0 Hz, while that in 24S-configuration was around 10.5 and 1.9 Hz [15, 16]. Comparison with the literature data and significant NOE correlations between H-24 and H-23 α , alone with modeling in Chem3D 11.0 (Cambridge Soft, Inc.) as shown in Figure 3, both suggested 24-OH was R-configuration. Based on these evidences, the structure of **1** was determined to be 24R,25-dihydroxy-3,4;9,10-seco-4(28)-cycloarten-10,3-olide (Figure 1).

Illiciumolide B (**2**) was obtained as a yellowish solid with molecular formula C₃₀H₅₀O₄ established by positive HRESI-MS (*m/z* 475.3765 [M+H]⁺, calcd. for 475.3784). Both compounds **1** and **2** have the same molecular formula, suggesting that they are isomer. The ^1H and ^{13}C NMR spectra of **1** were quite similar to those of **2**. In comparison with their ^1H and ^{13}C NMR data (Table 1), it was found that the chemical shifts of C-20, C-21, C-22, C-23, and C-24 were slightly different, while the other chemical shifts remained unchanged. These suggested that compound **2** was a stereoisomer of **1**, which was confirmed by the NOE spectrum. In the NOE experiments, correlation signal from

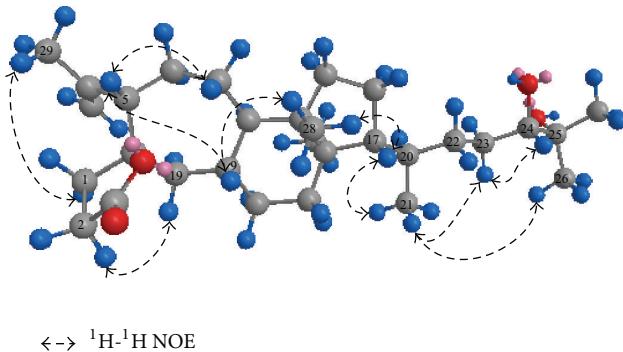


FIGURE 3: Key NOE correlations of compound **1**.

H-24 to H-23 β (δ_{H} 1.09–1.14) in **2** was observed, while H-24 to H-23 α (δ_{H} 1.36) in compound **1** was observed. Besides, the *J* value between H-23 and H-24 (*J* = 9.5, 1.6 Hz) was different with that (*J* = 6.2 Hz) of compound **1**. By comparison with the literature [15, 16], compound **2** was deduced to be 24S-configuration. Therefore, compound **2** was elucidated as 24S,25-dihydroxy-3,4;9,10-seco-4(28)-cycloarten-10,3-olide (Figure 1). The spectra of Illiciumolide A and B are presented in Supplementary Materials available online at <http://dx.doi.org/10.1155/2013/942541>, including MS, IR, and NMR spectra.

In addition to the two new compounds, the known triterpenoids **3** [15, 17, 18], **4** [19, 20], and **5** [21, 22], and squalenes **6** [23] and **7** [24] were also isolated from the *I. difengpi*. This is the first report on isolation of compounds **3**, **6**, and **7** and 3,4-seco- and 3,4;9,10-seco-type triterpenoids from *I. difengpi*.

3.2. Bioactivities Analysis

3.2.1. Inhibition of TNF- α Release Assay. The antiinflammatory activities of compounds **1**–**7** at 25 $\mu\text{g}/\text{mL}$ were assessed by determining the inhibitory ratio of TNF- α release in LPS-stimulated RAW 264.7 macrophages *in vitro*. Tripterygium tablets (TRT) and total glucosides of paenia (TGP) were used as positive controls. As shown in Figure 4, the concentrations of TNF- α in the RAW 264.7 cells pretreated with compounds **1**, **2**, and **7** were reduced by 90%, 85%, and 91%, respectively, compared to LPS-stimulated RAW 264.7 cells, while the inhibitory rates of two positive controls TRT and TGP were 59% and 49%, respectively. These results demonstrated that compounds **1**, **2**, and **7** had a significant inhibitory effect on TNF- α release from macrophages.

3.2.2. Inhibition of NF- κ B Release Assay. Based on the results obtained from TNF- α release experiments, compounds **1**–**7** were further assessed for their possible effect on NF- κ B production from RAW 264.7 cells stimulated with LPS. The cytotoxic effects of tested compounds on LPS-stimulated RAW 264.7 cells were determined initially. The results showed that compounds **1**–**5** did not affect cell viability at concentrations up to 25 $\mu\text{g}/\text{mL}$ and **6** and **7** did up to 100 $\mu\text{g}/\text{mL}$. Compound **1** at concentration 10 $\mu\text{g}/\text{mL}$ and

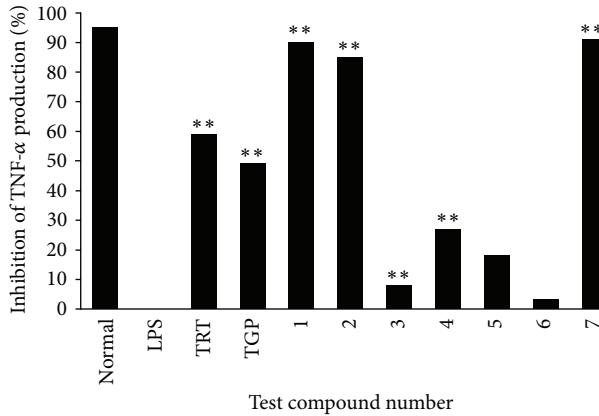


FIGURE 4: Inhibitory rate of TNF- α production from LPS-stimulated RAW 264.7 cells by compounds 1–7 at a concentration of 25 mg/mL. ** $P < 0.01$ for TNF- α levels in RAW 264.7 cells treated with LPS in the presence of the test compounds versus that in the absence of the test compounds.

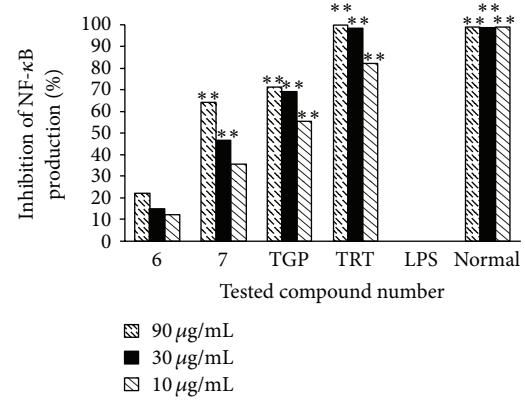
20 μ g/mL, 2 at 20 μ g/mL, and 7 at 90 μ g/mL greatly reduced the NF- κ B production stimulated by LPS ($P < 0.01$). It was observed that these compounds showed a dose-dependent inhibition of NF- κ B release in LPS-stimulated RAW264.7 cells. The remaining compounds showed slight activities against NF- κ B release (Figure 5).

3.2.3. Molecule Docking Screening for Targets. Binding properties for compounds 1, 3, 4, and 5 on various inflammation related ligands were estimated by computer-aided molecular docking. The results showed that all these compounds had good binding with dual specificity mitogen-activated protein kinase kinase 1 (MPKK1), whose activation is involved in the upstream of NF- κ B signal pathway [1], followed by production of many proinflammatory cytokines as well as other important inflammation-released proteins (see Table 2).

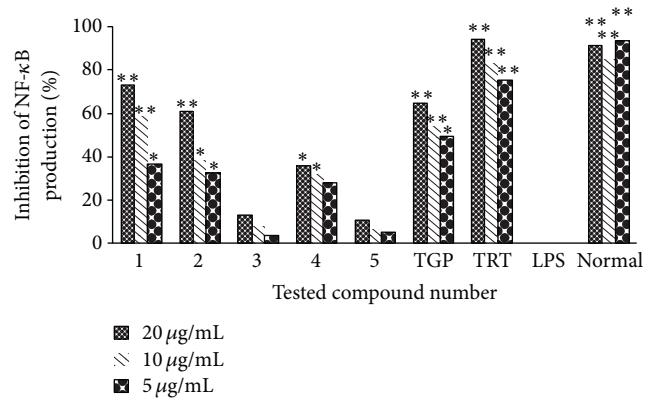
Similar computer-aided molecule docking to screen targets had been calculated and several ligands, including gag-pol polyprotein, protease, androgen receptor, and renin, exhibited high fit score and norm fit score (Table 3).

3.3. Discussion. This is the first time that 3,4;9,10-*seco*-type cycloartane triterpenoids were isolated from *I. difengpi* and from genus *Illicium*. There has been only five cycloartane triterpenoids [10], two of which were 3,4-*seco*-cycloartane type triterpenoids [11], isolated from family Illiciaceae. These *seco*-cycloartane types of triterpenoids have been extensively isolated from family Schisandraceae, especially 46 compounds of 3,4-*seco*-cycloartane type out of 166 triterpenoids totally [22]. It was widely accepted that family Illiciaceae has close relationship with the Schisandraceae (*Schisandra* and *Kadsura*) and both of them were under the Illiciales. The common possession of the *seco*-cycloartanes in both *I. difengpi* and the Schisandraceae is of great significance.

I. difengpi is included in Chinese Pharmacopoeia for its traditional treatment of rheumatoid arthritis (RA). In RA patients, TNF- α levels are elevated in RA synovial



(a)



(b)

FIGURE 5: Inhibitory rate of NF- κ B production from LPS-stimulated RAW 264.7 cells by (a) Compounds 6–7 at three concentrations 90 μ g/mL, 30 μ g/mL, and 10 μ g/mL. (b) Compounds 1–5 at three concentrations 20 μ g/mL, 10 μ g/mL, and 5 μ g/mL. * $P < 0.05$ and ** $P < 0.01$ for TNF- α levels in RAW 264.7 cells treated with LPS in the presence of the test compounds versus that in the absence of the test compounds.

fluid, serum, and synovial fibroblasts [25]. Macrophages are important cells implicated in the initiation of inflammatory responses, so the agents that inhibit TNF- α production have been studied almost exclusively in these cells. The objective of this present study was to investigate the potential activities against RA of triterpenoids isolated from the *I. difengpi* against RA. The main focus was to explore the attenuation of LPS-induced acute inflammatory response under *in vitro* conditions. Our research results suggested that triterpenoids 1, 2, and 7 may modulate macrophages responsiveness to LPS. These three compounds reduced the production of TNF- α stimulated with LPS. However, the molecular mechanisms of the induction of TNF- α production in RAW264.7 cells in response to LPS remain incompletely understood. Another important anti-inflammatory activity assay results showed that compounds 1, 2, and 7 have the potential effect on suppression of NF- κ B in a concentration-dependent manner in LPS-stimulated RAW 264.7 cells. These similar inhibitions on TNF- α and NF- κ B suggested some close relationship through

TABLE 2: Screening anti-inflammation targets over molecule docking of compounds 1–5.

Compound number	Target name	Molecules docking results	Fit score	Norm fit score
1/2	Dual specificity mitogen-activated protein kinase kinase 1 (MPKK1)		4.111	0.4567
	Glucocorticoid receptor (GR)		4.010	0.5012
	Prothrombin		3.843	0.4271
	Alpha-1-antitrypsin		3.834	0.6390
	Glycogen synthase kinase-3 beta		3.786	0.5409
	ADAM 17		3.726	0.9315
	Tyrosine-protein kinase SYK		3.712	0.6187
3	Protein kinase C theta type		3.701	0.5287
	Dual specificity mitogen-activated protein kinase kinase 1 (MPKK1)		5.171	0.5745
	Proto-oncogene tyrosine-protein kinase LCK		4.370	0.4370
	Glucocorticoid receptor		4.332	0.5415
	Glycogen synthase kinase-3 beta		4.298	0.6140
	Cathepsin B		4.110	0.4566
	Peroxisome proliferator-activated receptor alpha		4.042	0.5053
4	Prothrombin		3.988	0.6646
	Dual specificity mitogen-activated protein kinase kinase 1 (MPKK1)		3.660	0.4067
	Proto-oncogene tyrosine-protein kinase LCK		3.596	0.5137
	Prothrombin		3.496	0.3885
	Leukotriene A-4 hydrolase		3.354	0.3727
	Dual specificity mitogen-activated protein kinase kinase 1 (MPKK1)		4.246	0.4718
	Glucocorticoid receptor		3.931	0.4913
5	Estrogen receptor		3.728	0.5325
	Leukocyte elastase		3.709	0.4121
	Protein kinase C theta type		3.689	0.5270
	Glycogen synthase kinase-3 beta		3.642	0.5203
	Proto-oncogene tyrosine-protein kinase LCK		3.626	0.6044
	Mitogen-activated protein kinase 10		3.596	0.5137
	Peroxisome proliferator-activated receptor alpha		3.576	0.4470

some signal pathway. Previous studies have elucidated some signal pathways leading to TNF- α in response to LPS. In particular, the activation of NF- κ B may play a significant role in LPS-induced expression of TNF- α [26, 27]. We deduced that compounds 1, 2, and 7 may inhibit LPS-induced TNF- α production through inhibition of NF- κ B signal pathway; similar conclusion has been reported in triterpenoids anti-inflammatory activity studies [28]. It was reported that the activation of MPKK1 is in the upstream of NF- κ B signal pathway [1]. The computer-aided molecule modeling results also showed compound 1 binding well with MPKK1. From this molecule docking, we further deduced that compounds 1 and 2 may interact with MPKK1 and consequently suppress the NF- κ B.

Some of the *seco*-cycloartanes have reported to have anti-HIV-1 activity [29] and anti-HBV (hepatitis B virus) activity

[30]. The earliest example was nigranoic acid from stems of *Schisandra sphaerandra* that has been demonstrated to be capable of inhibiting HIV viral reverse transcriptase with IC₅₀ = 74.1 μ g/mL [31]. Further structure-activation relation experiment were reported quite recently that cycloartane triterpenoids with *seco* structure in ring A shown to inhibit HIV integrase (IN), while cycloartane triterpenoids without *seco*-structure in ring A showed weak or no inhibition at all [32]. The *seco*-structure seems to be the crucial anti-HIV-1 activation related structural feature. Besides, betulinic acid and its derivatives were also extensively reported to have a potent inhibitory activity against human immunodeficiency virus type 1 (HIV-1) [33–36]. These reports suggest that compounds 1–5 may also possess certain antiviral activity. Molecule docking provided some well-binding targets, including gag-pol polyprotein, protease, androgen receptor

TABLE 3: Screening anti-HIV targets over molecule docking of compounds 1–5.

Compound number	Target name	Molecules docking results	
		Fit score	Norm fit score
1/2	Androgen receptor	4.294	0.7157
	Gag-pol polyprotein	4.281	0.3058
	Glucocorticoid receptor	4.010	0.5012
	Renin	3.781	0.5401
3	Androgen receptor	4.768	0.7947
	Glucocorticoid receptor	4.332	0.5415
	Renin	4.121	0.5151
4	Gag-pol polyprotein	3.549	0.2218
	Androgen receptor	3.359	0.4199
	Renin	3.347	0.4183
	Thymidine kinase	3.337	0.4171
5	Androgen receptor	4.022	0.5028
	Glucocorticoid receptor	3.931	0.4913
	Gag-pol polyprotein	3.674	0.2625
	Protease	3.556	0.4446

and renin. Among these, gag-pol polyprotein and protease have close relation with HIV infection. These results may offer more clues for further experiments for anti-HIV-1 potential and their mechanism.

4. Conclusions

We reported the first *secō*-triterpenoids isolated from the stem barks of *I. difengpi*. Two new 3,4;9,10-*secō*-cycloartane triterpenoids, as well as three known triterpenoids and two biosynthetic related squalenes were structurally elucidated through spectral methods, together with comparison with literature. Our study has demonstrated the anti-inflammatory activity of these compounds. They showed remarkable anti-inflammatory activity, especially compounds 1, 2, and 7 and deserve further considerations towards developing as an effective anti-inflammatory drug. As reported in the previous literatures, some *secō*-cycloartane triterpenoids are capable of inhibiting HIV-1. The computer-aided molecule docking provided clues for targets screening and further mechanism research. Our study suggested that the triterpenoids from *I. difengpi* are of great interest as potential leads for natural product-based candidates for further studies.

Conflict of Interests

They have not any possible conflict of interests with the trademarks included in the paper.

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Research Article

Antiproliferative Effect and the Isolated Compounds of *Pouzolzia indica*

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Previous report showed the high potent antiproliferative effect of the methanolic part extracted from the aerial parts of *Pouzolzia indica* on NB4 and HT93A acute leukemic cell lines with the IC₅₀ values of 28.5 and 49.8 μg/mL, respectively. The bioassay-guided fractionation of the methanolic part gave 5 fractions, that is, FFII–FFV. FFII, FFIII, and FFIV inhibited the above leukemic cell lines with the IC₅₀ values of 15.1 (FFII), 14.4 (FFIII), 32.1 (FFIV), and 31.0 (FFII), 9.7 (FFIII), 10.5 (FFIV) μg/mL, respectively. The compounds in these fractions were isolated using chromatographic technique. FFII contained friedelin 1, 28-hydroxy-3-friedelanone 2, and 7-methoxy-coumarin 3. FFIII contained 6,7-dimethoxy-coumarin 4, scopoletin 5, methyl caffeoate 6. FFIV contained sitosteryl glucoside 7 and a supposed glycosphingolipid 8. The chemical structures were elucidated by spectroscopic methods.

1. Introduction

Pouzolzia indica Gaudich.var. *angustifolia* Wedd. (local name “Non tai baihong”) is a Thai medicinal plant in the family Urticaceae [1, 2]. It was used as remedy for the ailments in female infertility, cancer, and inflammation and as emmenagogue and insecticide [3]. The chemical constituents in *P. indica* were scarcely reported. Only lanceolone, an isoflavone compounds, was isolated [4]. Previously, the antiproliferative effect of the methanolic part of this plant was reported [5]. It could inhibit the growth of NB4 and HT93A cells with the IC₅₀ values of 28.5 ± 0.1 and 49.8 ± 0.7 μg/mL [5], respectively. The apoptosis of NB4 cells treated with 75 μg/mL of this fraction for 24 hours increased from 3.2% to 22.2%, whereas HT93A cells underwent apoptosis from 3.0% to 51.3% when treated with the methanolic part at 150 μg/mL [5]. The previous results, therefore, showed high potent antiproliferative effect of this methanolic part on these acute leukemic cells. From this study, the active extract was further fractionated using column chromatography. The active fractions were

determined by bioassay. The compounds in each fraction were isolated and structurally identified.

2. Materials and Methods

2.1. Cytotoxicity Test

2.1.1. Cell Lines and Culture. NB4 promyelocytic cell line was kindly provided by Ms. Setsuko Miyanishi, Tenri Institute of Medical Research, Japan, and HT93A promyelocytic cell line was kindly provided by Dr. Kenji Kishi, Tokai University, Japan. Long and short types of PML-RAR α have been detected in NB4 and HT93A cells, respectively [6–8]. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (Stem cell Technology, Vancouver, BC, Canada) with 1% penicillin and streptomycin (Gibco, Life Technologies, Breda, the Netherlands). Cells were incubated at 37°C with 95% humidified atmosphere containing 5% CO₂ [9].

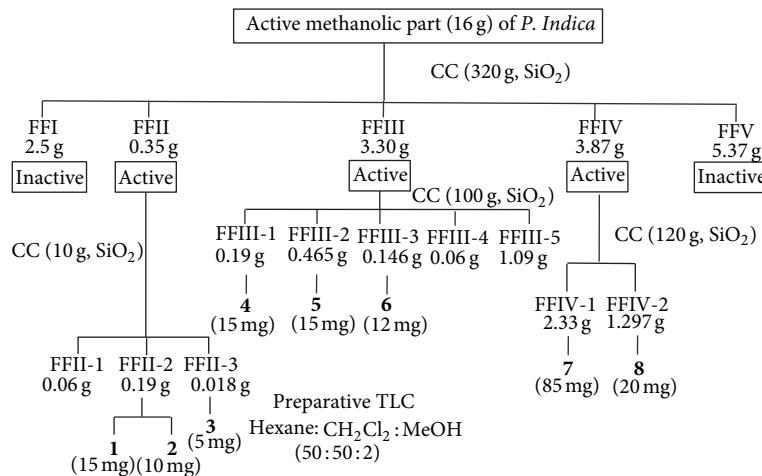


FIGURE 1: Isolation of compounds **1–8** from active methanolic part of *P. indica*.

2.1.2. Cell Viability Assay. Cell viability was assessed using MTT [(3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] assay [10]. In brief, 1×10^4 cells of NB4 and 1×10^5 cells of HT93A were seeded in each well in 96-well plate flat bottom. Cells were treated with each fraction isolated from this medicinal plant for 48 h at the concentrations varying from 0 to $50 \mu\text{g}/\text{mL}$. After 48 h incubation, $50 \mu\text{L}$ of 1 mg/mL of MTT in PBS (Sigma, Thailand) was added to each well, and cells were incubated for 4 hr. $100 \mu\text{L}$ of 10% SDS in 0.01 M HCl was added to stop the reaction and was incubated overnight. The absorbance at 590 nm was measured and read using the ELISA reader (Biorad, USA). Cell viability was calculated using the following formula [11]:

$$\text{Cell viability (\%)} = \left(\frac{\text{sample OD}}{\text{control OD}} \right) \times 100. \quad (1)$$

2.1.3. Statistical Analysis. The experiments were performed in triplicate with three independent experiments. Data were expressed as the mean \pm standard deviation. The *R*-square equation was used to calculate the IC_{50} value. A *P* value less than 0.05 was considered statistically significance [9].

2.2. Phytochemistry

2.2.1. General. ^1H and ^{13}C NMR spectra recorded on Bruker DPX-300, Switzerland, with deuterated solvents and TMS as a reference. APCI-MS atmospheric pressure chemical ionization mass spectra were measured on Perkin Elmer mass spectrometer. IR spectra were recorded on FT-IR spectrometer; Perkin-Elmer UV spectra were recorded on Hitachi, U 320 spectrophotometer. Melting points were measured on Digital Electromol 9100. Separation and isolation were performed by column chromatography (CC) using following adsorbents: Diaion HP20, size 250–850 μm , Mitsubishi Chemical Industry, silica gel for CC (63–200 μm , Merck 7734), and low pressure column chromatography (LiChroprep, Merck). TLC: precoated Kieselgel 60 F₂₅₄ (Merck). NP/PEG or NEU spray reagent was used to detect flavonoids and coumarins, 10%

H_2SO_4 in ethanol was used as universal spray reagent, and 10% FeCl_3 in ethanol was used to detect phenolic compounds.

2.2.2. Plant Material. The aerial parts of *P. indica* were collected from Ratchaburi Province in the central part of Thailand. The plant was identified by the expert from Forest Herbarium-BKF, Bangkok, Thailand. A plant specimen was deposited with the voucher number of BKF no. 106441 and SN 096588.

2.2.3. Extraction and Isolation. *P. indica* was extracted with ethanol and fractionated by Diaion HP20 column, eluted with water, water : methanol (1:1), methanol, and ethyl acetate. The methanol fraction showed significant antiproliferative effect [5]. This active methanolic part (16 g) was applied on a silica gel column, eluted with gradient solvent systems (ss) of hexane-ethyl acetate and ethyl acetate-methanol, to obtain 5 fractions (FFI–FFV). FFI (2.5 g, ss : hexane-EtOAc, 9:1) appeared as oily liquid. FFII (0.35 g, ss : hexane-EtOAc, 1:1), FFIII (3.30 g, ss : hexane-EtOAc, 1:9), FFIV (3.87 g, ss : EtOAc-MeOH, 8:2), and FFV (5.37 g, ss : EtOAc-MeOH, 7:3). The bioassay-guided fractionation showed the active fractions, that is, FFII–FFIV. FFII–FFIV were further separated by chromatographic column (Figure 1). FFII was chromatographed on silica gel column, eluted with gradient ss of hexane-acetone and acetone-methanol, to obtain 3 subfractions FFII-1–3. FFII-1 (0.06 g, ss : hexane-Me₂O, 95:5) appeared as oily liquid. FFII-2 (0.19 g, ss : hexane-Me₂O, 8:2) was recrystallized with Et₂O, and the white needles **1** (15 mg) was obtained. The filtrate was evaporated to dryness and recrystallized with methanol, and **2** (10 mg) was obtained. FFII-3 (0.018 g) was separated on the preparative TLC using solvent system of hexane : CH_2Cl_2 : methanol (50 : 50 : 2) giving four separated bands. The band with R_f value of 0.75 was isolated, recrystallized in ethyl acetate resulting **3** (5 mg).

FFIII was chromatographed on silica gel column, eluted with gradient ss of hexane- CH_2Cl_2 and CH_2Cl_2 -methanol, to obtain 5 subfractions FFIII-1–5. FFIII-1 (0.19 g, ss : hexane- CH_2Cl_2 , 7:3) was recrystallized in methanol giving **4** (15 mg).

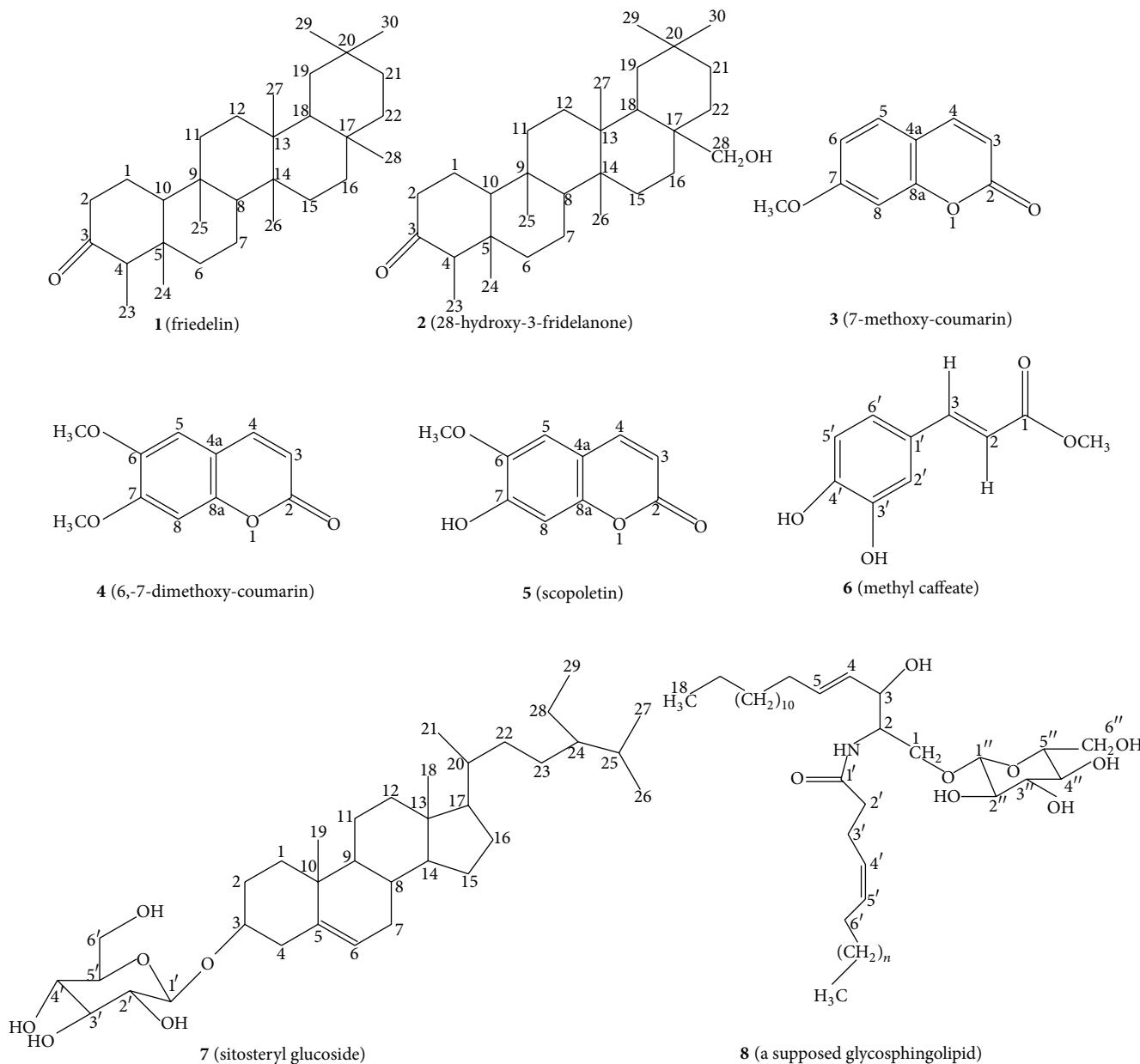


FIGURE 2: Structures of 1–8.

FFIII-2 (0.465 g, ss : hexane- CH_2Cl_2 , 1:1) was recrystallized with methanol giving **5** (15 mg). FFIII-3 (0.146 g, ss : CH_2Cl_2 -MeOH, 9.5 : 0.5) was recrystallized in methanol giving **6** (12 mg). FFIII-4 (0.06 g, ss : CH_2Cl_2 -MeOH, 9:1) was brown gum. FFIII-5 (1.09 g, ss : CH_2Cl_2 -MeOH, 8:2) appeared as yellowish gum.

FFIV was chromatographed on silica gel column, eluted with gradient ss of hexane-acetone and acetone-methanol. It produced 2 subfractions, that is, FFIV-1-2. FFIV-1 (2.33 g, ss : hexane : acetone, 7:3) was added with methanol, **7** (85 mg) was precipitated as white powder. FFIV-2 (1.297 g, ss : acetone : methanol, 9:1) was added with chloroform : methanol (95:5); **8** (20 mg) was obtained as white powder. Each of the purified compounds **1–8**

described above possessed the following physicochemical properties and the chemical structures were identified using spectroscopic methods (^1H NMR, ^{13}C NMR, Mass spectra, IR spectra, and UV-spectra). The structure of compounds **1–8** (Figure 2) were elucidated as the followings:

- (**1**) (friedelin): white needles (Me_2O). mp 258–260°C. UV (EtOH) λ_{\max} 220 nm. IR (CHCl_3) ν_{\max} 2980, 2927, 2870 (CH), 1706 (C=O), 1462, 1389 (CH) cm^{-1} . APCI-MS 427.4156 [$\text{M}+\text{H}]^+$ (calc. $\text{C}_{30}\text{H}_{50}\text{O}$ 426.7244). ^1H NMR (CDCl_3 , 300 MHz) δ 0.75 (3H, s, H-24), 0.90 (3H, s, H-23), 0.98 (3H, s, H-25), 1.04 (3H, s, H-29), 1.04 (3H, s, H-30), 1.04 (3H, s, H-26), 1.07 (3H, s, H-27), 1.20 (3H, s, H-28), 1.3–1.4 (18H, complex m, H-6,

- 7, 11, 12, 15, 16, 19, 21, 22), 1.4–1.6 (3H, complex m, H-8, 10, 18), 1.74 (2H, dd, *J* 5.5, 3.0 Hz, H-1a, H-1b), 2.2 (1H, m, H-4), 2.34 (1H, m, H-2b), 2.40 (1H, m, H-2a). ^{13}C NMR (CDCl_3 , 75 MHz) δ 213.1 (C3), 59.4 (C10), 58.2 (C4), 53.1 (C8), 42.8 (C18), 42.0 (C5), 41.5 (C6), 41.5 (C2), 39.7 (C13), 39.2 (C22), 38.3 (C14), 37.4 (C9), 36.0 (C16), 35.6 (C11), 35.3 (C19), 35.0 (C29), 32.8 (C21), 32.4 (C15), 32.1 (C28), 31.8 (C30), 30.5 (C12), 30.0 (C17), 28.1 (C20), 22.2 (C1), 20.2 (C26), 18.6 (C27), 18.2 (C7), 17.9 (C25), 14.6 (C24), 6.8 (C23) [12–14].
- (2) (28-hydroxy-3-friedelanone or canophyllool): white prisms (MeOH). mp 363–364°C. UV (EtOH) λ_{\max} 220 nm. IR (CHCl_3) ν_{\max} 3200–3513 (OH), 1709 (C=O), 2990, 2930, 2855 (C–H), 1466, 1378 (C–H) and 1039, 1116 (C–O). APCI-MS 443.4015 [$M+\text{H}]^+$ (calc. $\text{C}_{30}\text{H}_{50}\text{O}_2$ 442.5038). ^1H NMR (CDCl_3 , 300 MHz) δ 0.88 (3H, s, H-24), 0.91 (3H, d, *J* 7.8 Hz, H-23), 0.99 (3H, s, H-25), 1.02 (3H, s, H-26) 1.01 (3H, s, H-27), 1.19 (3H, s, H-29), 1.23 (3H, s, H-30), 1.3–1.4 (18H, complex m, H-6, 7, 11, 12, 15, 16, 19, 21, 22), 1.4–1.6 (3H, complex m, H-8, 10, 18), 1.96 (2H, dd, *J* 7.6, 3.7 Hz, H-1a, H-1b), 2.32 (1H, m, H-2b), 2.30 (1H, m, H-4), 2.40 (1H, m, H-2a) and 2.5 (2H, m, H-28), 3.71 (1H, 28-OH). ^{13}C -NMR (CDCl_3 , 75 MHz) δ 213.1 (C3), 68.3 (C28), 59.4 (C10), 58.2 (C4), 53.1 (C8), 42.0 (C5), 41.7 (C22), 41.5 (C2, C6), 39.7 (C13), 39.5 (C18), 38.3 (C14), 37.4 (C9), 35.6 (C11), 35.2 (C17), 34.4 (C29), 33.4 (C19), 32.9 (C30), 31.6 (C21), 31.3 (C16), 30.5 (C12), 30.3 (C20), 29.2 (C15), 23.4 (C1), 19.4 (C25), 19.1 (C26), 18.2 (C7), 18.1 (C27), 14.8 (C24), 6.8 (C23) [13, 14].
- (3) (7-methoxy-coumarin or herniarin): white needles (MeOH). mp 117–118°C. UV (MeOH) λ_{\max} 254, 366 nm. IR (CHCl_3) ν_{\max} 2927, 2870 (CH), 1682 (C=O), 1533 (C=C) cm $^{-1}$. APCI-MS 176.2015 [$M]^+$ (calc $\text{C}_{10}\text{H}_8\text{O}_3$ 176.1714). ^1H NMR (CDCl_3 , 300 MHz) δ 7.63 (1H, d, *J* 9.6 Hz, H-4), 6.31 (1H, d, *J* 9.6 Hz, H-3), 7.09 (1H, d, *J* 8 Hz, H-5), 6.95 (1H, s, H-8), 6.90 (1H, d, *J* 8 Hz, H-6) and 3.99 (3H, s, 7-OCH $_3$). ^{13}C NMR (CDCl_3 , 75 MHz) δ 162.02 (C2), 156.3 (C7), 150.0 (C8a), 142.7 (C4), 121.1 (C3), 115.2 (C4a), 113.4 (C6), 111.4 (C8), 109.1 (C5), 56.3 (OCH $_3$) [15, 16].
- (4) (6, 7-dimethoxy-coumarin or scoparone): pale yellow needles (MeOH). mp 146–147°C. UV (MeOH) λ_{\max} 345 nm. IR (CHCl_3) ν_{\max} 2985, 2925, 2855 (CH), 1684 (C=O), 1535 (C=C), 1462, 1389, 1363 and 1311 (CH). APCI-MS 206.1245 [$M]^+$ (calc. $\text{C}_{11}\text{H}_{10}\text{O}_4$ 206.1576). ^1H NMR (CDCl_3 , 300 MHz) δ , 7.63 (1H, d, *J* 9.5 Hz, H-4), 6.9 (1H, s, H-5), 6.85 (1H, s, H-8), 6.25 (1H, d, *J* 9.5 Hz, H-3), 3.90 (3H, s, 7-OCH $_3$), 3.85 (3H, s, 6-OCH $_3$). ^{13}C -NMR (CDCl_3 , 75 MHz) δ 161.3 (C2), 152.9 (C7), 150.1 (C6), 146.4 (8a), 143.2 (C4), 113.6 (C3), 111.5 (C4a), 108.20 (C5), 100.1(C8), 56.40 (2xOCH $_3$) [15, 17].
- (5) (6-methoxy-7-hydroxy-coumarin or scopoletin): pale yellow needles (CH_2Cl_2 : MeOH: 9.5 : 0.5). mp 203–204°C. UV (MeOH) λ_{\max} 254, 366 nm. IR (CHCl_3) ν_{\max} 3400–3550 (OH), 1685 (C=O), 2998, 2938, 2856 (CH), 1589, 1511 (C=C) cm $^{-1}$. APCI-MS 192.2008 [$M]^+$ (calc. $\text{C}_{10}\text{H}_8\text{O}_4$ 192.1708). ^1H NMR (CDCl_3 , 300 MHz) δ 7.61 (1H, d, *J* 9.5 Hz, H-4), 6.90 (1H, s, H-5), 6.85 (1H, s, H-8), 6.28 (1H, d, *J* 9.5 Hz, H-3) and 3.85 (3H, s, 6-OCH $_3$). ^{13}C NMR (CDCl_3 , 75 MHz) δ 161.5 (C2), 150.2 (C6), 149.7 (C7), 144.0 (C8a), 143.4 (C4), 113.4 (C3), 111.5 (C4a), 107.5 (C5), 103.2 (C8), 56.4 (OCH $_3$) [16, 17].
- (6) ((E)-methyl-3 (3',4'-dihydroxy-phenyl) acrylate or methyl caffeate): pale yellow crystalline powder (MeOH). mp 157–159°C. UV (MeOH) λ_{\max} 354 nm. IR (CHCl_3) ν_{\max} 3530 (OH), 2995, 2927, 2870 (CH), 1516, 1643 (C=C) and 1690 (C=O). APCI-MS 194.0623 [$M]^+$ (calc. $\text{C}_{10}\text{H}_{10}\text{O}_4$ 194.1866). ^1H NMR (CDCl_3 , 300 MHz) δ 7.50 (1H, d, *J* 16.1 Hz, H-3), 7.17 (1H, d, *J* 2 Hz, H-2'), 6.93 (1H, d, *J* 7 Hz, H-5'), 6.88 (1H, dd, *J* 7, 2 Hz, H-6'), 6.15 (1H, d, *J* 16.1 Hz, H-2), 3.85 (3H, s, OCH $_3$). ^{13}C NMR (CDCl_3 - CD_3OD , 75 MHz) δ 168.3 (C1), 147.4 (C4'), 145.5 (C3), 144.8 (C3'), 126.6 (C1'), 121.8 (C2), 115.1 (C2'), 114.1 (C5'), 113.9 (C6'), 51.4 (OCH $_3$) [18, 19].
- (7) (sitosteryl glucoside): white needles (MeOH). mp 258–260°C. UV (EtOH) λ_{\max} 220 nm. IR (KBr) ν_{\max} 3200–3450 (OH), 2980, 2927, 2870 (CH), 1560 (C=C). APCI-MS 412.4002 [$M - 180]^+$ (calc. $\text{C}_{35}\text{H}_{60}\text{O}_6$ 576.8854). ^1H NMR (CDCl_3 + CD_3OD , 300 MHz) δ 0.59 (3H, s, H-18), 0.85 (3H, d, *J* 7.3 Hz, H-27), 0.90 (3H, t, *J* 6.5, 6.5 Hz, H-29), 0.91 (3H, s, H-19), 0.92 (3H, d, *J* 7.3 Hz, H-26), 1.12 (3H, d, *J* 7.0 Hz, H-21), 1.30 (2H, m, H-28), 1.74 (1H, m, H-2a), 1.94 (2H, m, H-1), 2.18 (1H, m, H-2b), 2.21 (1H, m, H-4), 3.28–3.5 (5H, m, H-1', H-2', H-3', H-4', H-5'), 3.50–3.70 (2H, m, H-6'). 3.74 (1H, m, H-3), 5.31 (1H, m, H-6), ^{13}C NMR (CDCl_3 + CD_3OD , 75 MHz) δ 140.1 (C5), 121.9 (C6), 100.9 (C1'), 79.0 (C3, C3'), 76.2 (C5'), 75.7 (C2'), 73.4 (C4'), 61.8 (C6'), 56.6 (C20), 55.9 (C17, C24), 50.0 (C9), 45.6 (C8), 42.1 (C13), 40.0 (C12), 39.9 (C4), 37.5 (C1), 36.5 (C10), 33.8 (C7, C22), 31.7 (C14), 29.8 (C2), 29.4 (C16), 29.0 (C25), 28.0 (C23), 24.1 (C15), 23.0 (C28), 22.9 (C11, C21), 20.9 (C26), 19.5 (C27), 19.0 (C19), 11.7 (C29), 11.6 (C18) [16].
- (8) (a supposed glycosphingolipid): white amorphous powder (CHCl_3 : MeOH, 3:97). mp 252–254°C (MeOH). UV (EtOH) λ_{\max} 236 nm, IR (KBr) ν_{\max} 3230–3450 (OH), 3384 (NH), 2998, 2935, 2851(CH), 1642 (C=O). APCI-MS 751.2596 [$M]^+$ (calc. $\text{C}_{44}\text{H}_{81}\text{NO}_8$ 751.2539). ^1H NMR (pyr-d $_5$, 300 MHz) δ 0.90 (2 \times 3H, t, J6, 6 Hz, acyl-CH $_3$), 1.20 (2H, m, H-17), 1.30 (complex m, H7-16, (CH $_2$) $_n$), 1.8 (6H, m, H-6, H-3', H-6'), 2.4 (2H, m, H-2'), 2.6–2.8 (1H, m, OH), 3.90–4.50 (7H, m, H-1'', H-2'', H-3'', H-4'', H-5'', H-6''). 4.24 (1H, m, H-1a), 4.66 (1H, m, H-1b), 4.70 (1H, m, H-5'), 4.75 (2H, m, H-2, H-3), 5.30 (1H, m, H-5, H-4'), 5.50 (1H, dd, J6.6, 1.0 Hz, H-4), 6–8 (4H, br, s, 4xOH), 8.59 (1H, d, J9.2 Hz, NH). ^{13}C NMR (pyr-d $_5$, 75 MHz) δ 175.7 (C1'), 130.9 (C4), 130.7 (C4'), 123.8 (C5), 122.0 (C5'), 105.6 (C1''), 78.6 (C3''), 78.4 (C5''), 75.4 (C2''), 72.5 (C3), 71.8 (C4''),

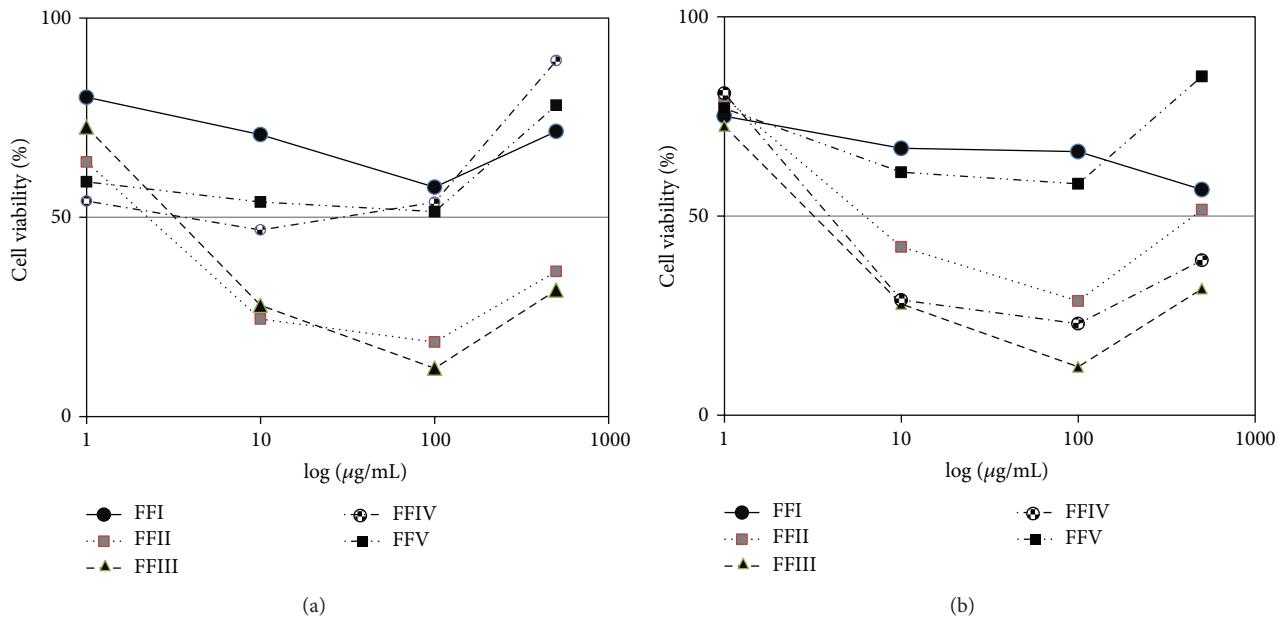


FIGURE 3: Percentage viable cell of FFI–FFV on leukemic cell lines (a) NB4 and (b) HT93A.

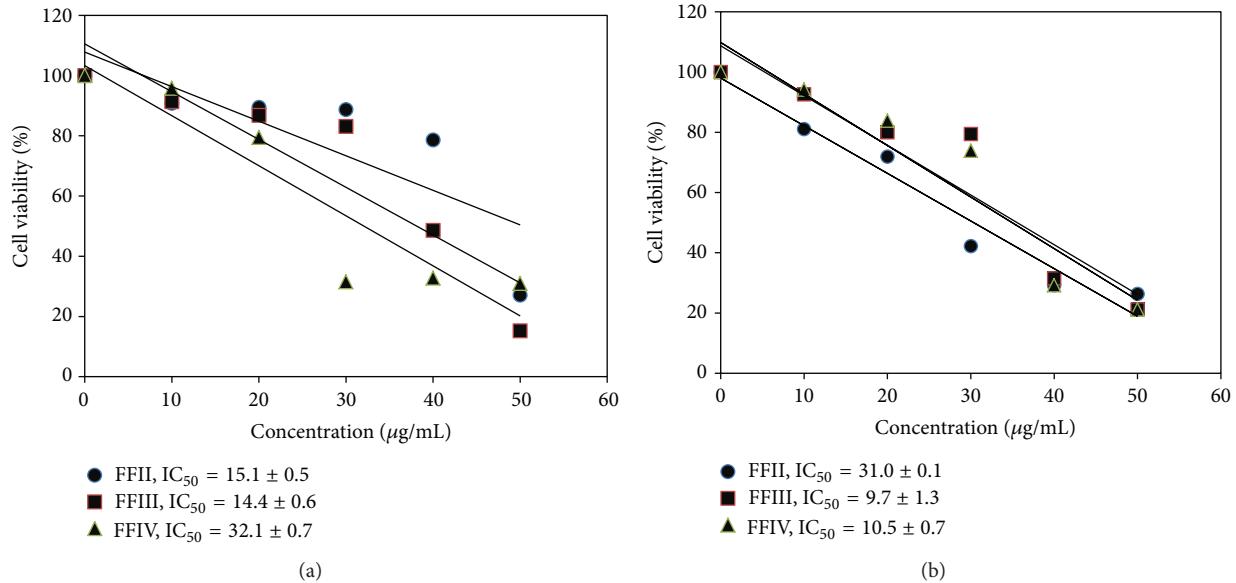


FIGURE 4: Antiproliferative effect of FFII–FFIV on leukemic cell lines (a) NB4 and (b) HT93A.

70.5 (C1), 63.0 (C6''), 57.7 (C2), 34.3 (C2'), 33.3 (C3'), 32.2 (C6, C6'), 29.6 (C7-C16, C7'-(CH₂)_n), 22.9 (C17), 14.3 (acyl CH₃), 14.1 (acyl CH₃) [20-23].

3. Results

The antiproliferative effect of FFI–FFV on human leukemic cell lines was investigated as shown in Figure 3. It was found that FFII, FFIII, and FFIV could inhibit growth of NB4 and HT93A (Figure 4). Therefore, FFII, FFIII, and FFIV were continued to evaluate the IC₅₀ values on these cell lines at varying concentrations ranging from 0 to 50 $\mu\text{g/mL}$.

The results showed that FFII, FFIII, and FFIV had the IC₅₀ values on NB4 cell line at $15.1 \pm 0.5 \mu\text{g/mL}$, 14.4 ± 0.6 , and $32.1 \pm 0.7 \mu\text{g/mL}$, respectively, whereas the IC₅₀ values of the HT93A cell line were 31.0 ± 0.1 , 9.7 ± 1.3 , and $10.5 \pm 0.7 \mu\text{g/mL}$, respectively, as shown in Figure 4. Additionally, FFIII inhibited growth strongly on both NB4 and HT93A cell lines, while FFII inhibits growth strongly on NB4 more than HT93A. FFIV showed strong growth inhibition on HT93A more than NB4.

The active fractions FFII, FFIII, and FFIV were further chromatographed on the silica gel columns repeatedly, and the isolated compounds were identified using spectroscopic

methods. FFII was composed of friedelin **1** and 28-hydroxy-3-friedelanone **2** and 7-methoxy-coumarin or herniarin **3**. FFIII was composed of 6,7-dimethoxy-coumarin or scopoletin **5**, and methyl caffeate **6**. FFIV was composed of sitosteryl glucoside **7** and a supposed glycosphingolipid **8**. Sitosteryl glucoside **7** (85 mg) was isolated which was the highest yield as shown in Figure 1.

4. Discussion

P. indica, which has been long used in Thai traditional medicine for treating various diseases including malignancies, was investigated in this study. Based on our previous report [5], the methanolic part of this plant showed high potent antiproliferative effect on NB4 and HT93A acute promyelocytic cell lines. Here, in this study, we demonstrated that eight compounds were isolated from this active methanolic part. It was chromatographed on CC repeatedly as shown in Figure 1. The bioassay determined the active fractions; they were FFII, FFIII, and FFIV. We found that FFII could inhibit growth on NB4 stronger than HT93A, while FFIII showed growth inhibition on both NB4 and HT93A. Interestingly, FFIV exhibited dominantly growth inhibition on HT93A. The differences in the antiproliferative effects of these fractions might arise from the different active compounds themselves and the interactions with the oncogenes in these acute promyelocytic cell lines, that is, the long and short types of PML-RAR α in NB4 and HT93A, respectively.

The antiproliferative effect of FFII might be caused by the presence of 2 triterpenes, that is, friedelin **1**, and 28-hydroxy-3-friedelanone **2**, and one coumarin **3**, namely, 7-methoxy-coumarin. Previously, the cytotoxicity of 28-hydroxy-3-friedelanone against A549-human lung cancer cell line, LLC-mouse Lewis lung carcinoma, HL60-human promyelocytic cell line, and MCF7-human breast cancer cell line were demonstrated. Hence, some triterpenes could strongly induce apoptosis by attending the mitochondrial membrane potential and regulating the expression of Bcl-2 different compasses [14, 20]. The IC₅₀ ($\mu\text{g}/\text{mL}$) of 7-methoxy-coumarin **3** on HL60 and K562 human chronic leukemia cells was also demonstrated with the values of 28.9 and 19.3 $\mu\text{g}/\text{mL}$, respectively [21, 22].

For FFIII, the cytotoxic activity of this fraction might result from coumarins (**4**, and **5**, namely, 6,7-dimethoxy-coumarin, and scopoletin, resp.) including methyl caffeate **6**. The previous reports demonstrated that coumarins could inhibit several human cancer cell lines such as QU-DB large cell lung cancer, and human leukemia HL60 cells [22, 23]. The mechanism of action of coumarins was exerted from the inhibition of tubulin polymerization and the induction of cell cycle arrest at G2/M phase [23]. The involvement of cell cycle inhibition might be due to the inhibition of the release of cyclin D1, an essential enzyme in cell cycle progression [24]. Interestingly, high concentration of scopoletin can have antiproliferative effect on lymphoma cell line by inducing apoptosis [25]. In addition, methyl caffeate can inhibit growth of human cervical adenocarcinoma cell line (HeLa) [26]. Notably, methyl caffeate, which contains 2 hydroxyl groups

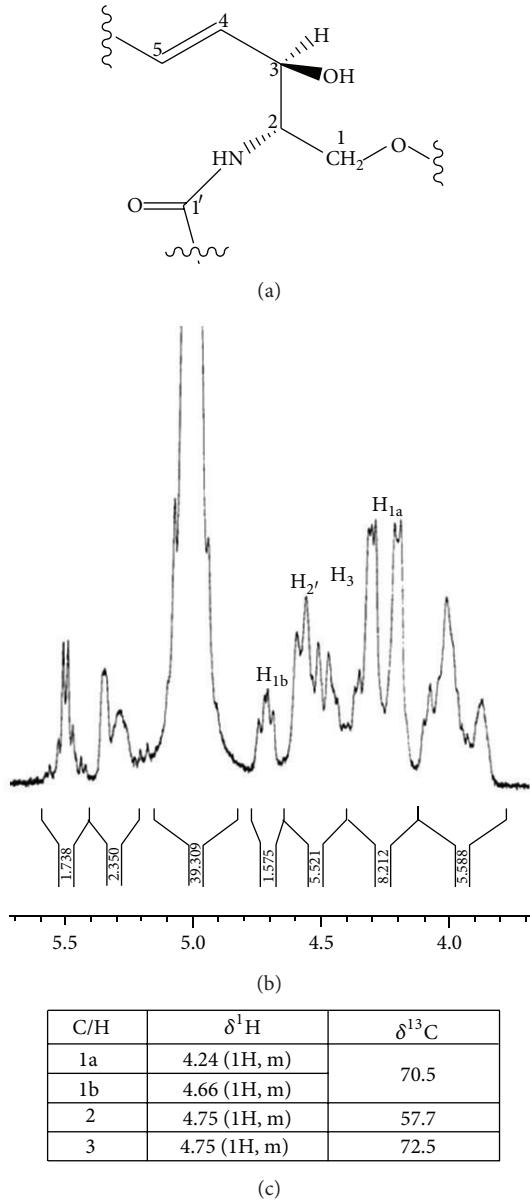


FIGURE 5: Amino alcohol part of glycosphingolipid (a) chemical structure (b) ^1H NMR spectrum, and (c) ^1H and ^{13}C assignments.

on aromatic ring, can induce cytotoxic activity via the strong antioxidant activity from these hydroxyl groups [27].

FFIV inhibited HT93A stronger than NB4 cells. It contained sitosteryl glucoside **7** and a supposed glycosphingolipid **8**. The partial structures of **8** included two acyl chains, one of which was palmitic acid, β -D-glucose, and an amino alcohol. ^1H -NMR spectrum of **8** showed that the typical resonances of amino alcohol part of glycosphingolipid were H-1a at δ 4.24 (1H, m), H-1b 4.66 (1H, m), and H-2 and H-3 4.75 (2H, m) [3, 28]. One acyl chain was biosynthetically originated from palmitoyl-CoA which was shown by the long chain methylene protons of **8** appearing as multiplets at δ 1.1–1.3 [29]. The presence of sugar protons as complex multiplets at δ 3.90–4.50 ppm (7H, m, from H-1"

to H-6'') was substantiated by carbon signals at δ 105.6 (C1''), 75.4 (C2''), 78.6 (C3''), 71.8 (C4''), 78.4 (C5''), and 63.0 (C6''). The structure of **8** was thus supposed to be a glycosphingolipid. The sitosteryl glucoside **7** was previously reported to have the antiproliferative effect on human colon cancer cell by inducing the apoptotic pathway [27]. The glycosphingolipid **8**, which contains sphingosine, can induce apoptosis involving with the ceramide and sphingosine-1-phosphate-mediated pathway [30, 31]. The result from our study pointed out that coumarins were promising anticancer agent [32]. The extract fraction containing mainly coumarins like FFIII could be developed as a drug material for anticancer phytopharmaceutical.

5. Conclusion

The methanolic part of *P. indica* extract inhibited the acute promyelocytic leukemia cell lines, NB4, and HT93A. The bioassay-guided fractionation of the active part got three different active fractions. They were FFII, FFIII, and FFIV. The FFII showed strong growth inhibition on NB4, whereas the FFIII exhibited strong growth inhibition on both NB4 and HT93A. The FFIV demonstrated strong growth inhibition on HT93A. The active compounds isolated from the FFII contained mainly triterpenoids (friedelin **1** and 28-hydroxy-3-friedelanone **2**) and some coumarins (7-methoxy-coumarin **3**). The FFIII contained mainly phenolic compounds (scoparone **4**, scopoletin **5**, and methyl caffeoate **6**), and the FFIV contained mainly glycosides (sitosteryl glucoside **7** and glycosphingolipid **8**). *P. indica* was the first report about antiproliferative effect on human leukemic cell lines, and the structures of compounds **1–8** were elucidated. The further investigation including drug development will be studied on these fractions especially the FFIII which demonstrated the best antiproliferative effect on both human leukemic cell lines (NB4 and HT93A).

Conflict of Interests

The authors declare that they do not have conflict of interests.

Acknowledgments

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Research Article

Inclusion Complex of Zerumbone with Hydroxypropyl- β -Cyclodextrin Induces Apoptosis in Liver Hepatocellular HepG2 Cells via Caspase 8/BID Cleavage Switch and Modulating Bcl2/Bax Ratio

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Zerumbone (ZER) isolated from *Zingiber zerumbet* was previously encapsulated with hydroxypropyl- β -cyclodextrin (HP β CD) to enhance ZER's solubility in water, thus making it highly tolerable in the human body. The anticancer effects of this new ZER-HP β CD inclusion complex via apoptosis cell death were assessed in this study for the first time in liver hepatocellular cells, HepG2. Apoptosis was ascertained by morphological study, nuclear stain, and sub-G1 cell population accumulation with G2/M arrest. Further investigations showed the release of cytochrome c and loss of mitochondrial membrane potential, proving mitochondrial dysfunction upon the ZER-HP β CD treatment as well as modulating proapoptotic and anti-apoptotic Bcl-2 family members. A significant increase in caspase 3/7, caspase 9, and caspase 8 was detected with the depletion of BID cleaved by caspase 8. Collectively, these results prove that a highly soluble inclusion complex of ZER-HP β CD could be a promising anticancer agent for the treatment of hepatocellular carcinoma in humans.

1. Introduction

The use of plants as the preferential treatment for cancer has been known for centuries. To date, about 3000 plant species have been identified as possessing anticancer properties. This piece of information is later utilized and investigated by

scientists in search for better compounds to act as anticancer agent [1]. One of the most important benefits of using these plants for health purposes is the availability of anticancer agents useful for treatments [2]. This is proven by the fact that more than 60 types of clinical chemotherapy drugs used as anticancer agents are plant derived. Amongst this, a number

of new, promising anticancer agents in clinical drug development based on selective activity against cancer-related molecular targets include flavopiridol and combretastatin-A4-phosphate, while other anticancer agents which failed in earlier clinical studies have stimulated renewed interest [1].

Zingiber zerumbet is a member of the family Zingiberaceae. Other plants that belong to this family include *Zingiber cassumunar* and *Zingiber officinale* [3]. The plant is also known as lempoyang in Malaysia, Ghatian and Yaiimu in India, and Jangli adha in Bangladesh [4–6]. It can be found in the moist forest, beach thickets and mangrove margin. *Z. Zerumbet* extracts have been used traditionally for treating fever, headaches, asthma, indigestion, diarrhea, severe sprains, inflammation, constipation, and toothache, while the Malays use the rhizomes extract to cure edema and worm infestation in children [7, 8]. It was found that the oils obtained from the rhizomes were rich in zerumbone (37%), α -humulene (14.4%), and camphene (13.8%) [9].

Zerumbone (ZER) is a crystalline monocyclic sesquiterpene isolated from the rhizomes of *Z. zerumbet*. This bioactive component has a unique structure consisting of a cross-conjugated ketone in an 11-membered ring responsible for its entire biological activities (Figure 1). ZER was previously reported to produce cytotoxicity through apoptosis in various cancers including skin tumor, colon and lung carcinogenesis, hepatocarcinogenesis, leukemic cancer, and cervical intraepithelial neoplasia [10–14].

Hydroxypropyl- β -cyclodextrin (HP β CD) is a cyclodextrin derivative that is used widely in drug encapsulation due to its inclusion ability as well as its high water solubility (Figure 2). HP β CD is well tolerated by the human body both by intravenous and oral administrations. Previously, we investigated the inclusion complex between ZER and HP β CD and its physical characterizations. The study provided evidence that ZER penetrates completely into the cavity of HP β CD, allowing the solubility of ZER to be enhanced with >30 fold after complexation. The study further showed that HP β CD is a suitable encapsular capable of forming a thermodynamically stable complex with ZER for the intended delivery of ZER as an anticancer [15]. Hence, this study further warrants the importance of ZER-encapsulated HP β CD as a promising biopharmaceutical drug, which encourages the current study to further investigate the antiproliferative effects of the new ZER-HP β CD inclusion complex against liver hepatocellular cells, HepG2, as a potential anticancer. This is the first documented report of ZER-HP β CD inclusion complex as an anticancer in HepG2 cells, together with its preliminary investigations of probable molecular mechanism in apoptosis induction.

2. Materials and Methods

2.1. Cell Lines and Reagents. HepG2, HeLa, MCF-7, MDA-MB-231, CEMss and WRL-68 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI1640 (PAA, Germany) media supplemented with 10% fetal bovine serum (FBS) (PAA, Germany) and 1% 100 IU penicillin and 100 μ g/mL streptomycin (Sigma, USA).

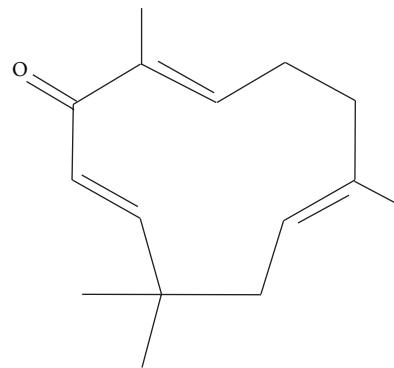


FIGURE 1: Molecular structure of Zerumbone (2,6,10-humulatrien-1-one).

Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂.

2.2. Inclusion Complex of ZER with HP β CD. The inclusion complex of ZER with HP β CD was obtained from the UPM-MAKNA Cancer Research Laboratory, University Putra Malaysia. Briefly, the thermodynamic parameters (ΔH , ΔS , ΔG) for the formation of the complex were obtained from the van't Hoff equation. ZER complex with HP β CD was characterized by differential scanning calorimetry (DSC), X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FT-IR), Transmission Emission Microscopy (TEM), and molecular modelling using PM6. Calculations show that ZER penetrates completely into the cavity of HP β CD [15].

2.3. Cytotoxicity Assay. The cytotoxicity profiles of the ZER-HP β CD inclusion complex were assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay as previously described by Mosmann (1983) with slight modifications [16]. Briefly, cells were seeded in 96-well microplates at a density of 2×10^5 cells mL⁻¹. After a 24-hour incubation, the cells were treated at various concentrations of ZER-HP β CD inclusion complex (1.563, 3.125, 6.25, 12.5, 25, and 50 μ g mL⁻¹) for 72 h. After a 68-hour incubation, 20 μ L of MTT solution (5 mg mL⁻¹) (Amresco, USA) was added into each well and the plate was then incubated for 4 h. Subsequently, the media was removed and the formed formazan crystals were dissolved with 100 μ L of DMSO (Sigma, USA). The absorbance was measured at wavelength of 595 nm using a microtiter plate reader (Tecan Sunrise basic, Austria). The percentage of cellular viability was calculated with appropriate controls taken into account. The concentration which inhibits 50% of cellular growth (IC₅₀ value) was determined. Three independent experiments performed in triplicates were used for the calculations and statistical analysis.

2.4. Morphological Evaluation by Phase Contrast Inverted Microscopy. Treatment was carried out in 25 mL culture flask. HepG2 cells were plated at concentration of 2×10^5 cells mL⁻¹

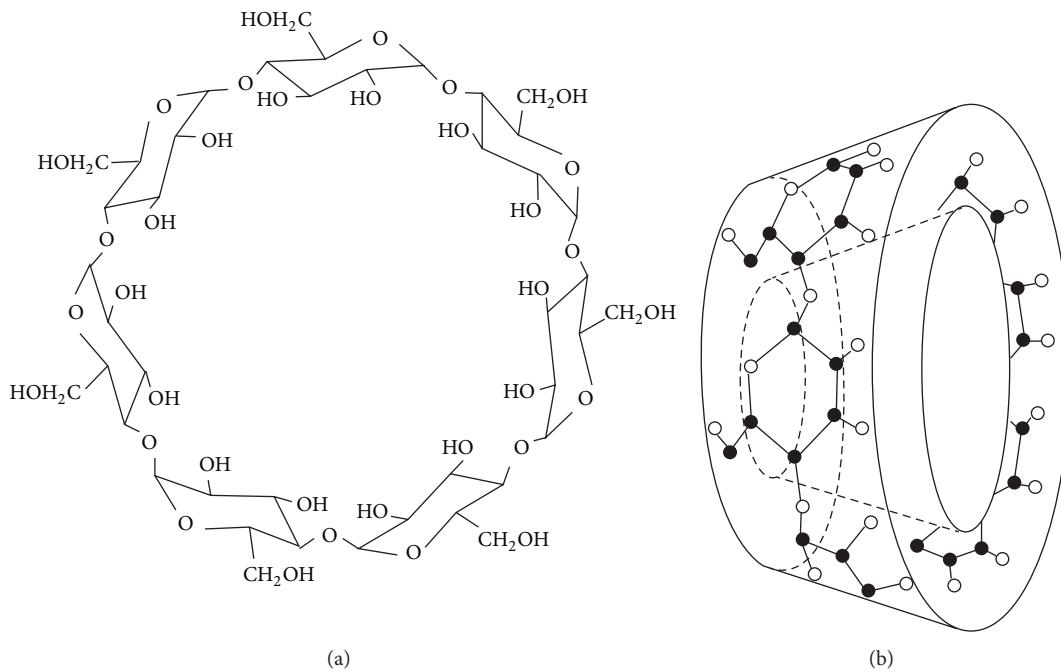


FIGURE 2: Structure (a) and torus-like shape (b) of β -CD molecule.

and treated with the ZER-HP β CD inclusion complex at 11.43 μ g/mL concentration for 24, 48, and 72 h. Morphological appearances of treated cells were compared concurrently with control untreated cells by observing under phase contrast inverted microscopy. Morphological changes that included appearances such as rounding up of cells, plasma membrane blebbing, and cell detachment were observed in treated HepG2 cells.

2.5. Propidium Iodide (PI) and Acridine Orange (AO) Double Staining Assay. The AO/PI viability assay is a rapid, highly linear, and functionally correlated assay that has advantages to conventional viability measurement [17]. This analysis examines whether apoptosis may be implicated in mediating cell death in HepG2 cells treated by the ZER-HP β CD inclusion complex. After treatment of HepG2 cells with inclusion complex (at 11.43 μ g/mL concentration for 24, 48, and 72 h), the cells were harvested and washed with cold PBS twice. The cells were later added with 10 μ L of fluorescent dyes, (AO/PI) containing acridine orange (AO, 10 μ g/mL), and propidium iodide (PI, 10 μ g/mL) at equal volumes of each. Freshly stained cell were observed under confocal microscope (LSM 5 Pascal Zeiss, Germany) within 30 minutes before the fluorescence colour starts to fade.

2.6. Assessing Apoptosis Using Annexin V-FITC Assay. Annexin V-FITC Assay was done using the AbD Serotec Annexin V-FITC assay kit (ANNEX100F, USA) in which staining of Annexin V-FITC and propidium iodide was done towards the cells specifically. Fluorochrome FITC-labelled Annexin V is a sensitive protein probe which possesses high

affinity towards phosphatidylserine (PS). Briefly, the assay was done according to the manufacturer's instruction. The binding buffer was diluted in 1 : 4 ratios (50 mL binding buffer + 150 mL distilled water). HepG2 cells, at concentration of 2×10^5 cells mL^{-1} , were seeded into 25 mL culture flask and treated with the ZER-HP β CD inclusion complex at 11.43 μ g/mL concentration. After 6, 12, and 24 h of incubation, the cells were harvested and washed with cold PBS twice. Cells were resuspended in prediluted binding buffer and 5 μ L Annexin V-FITC was added to 195 μ L of the cell suspension, mixed well, and incubated for 10 minutes in the dark at room temperature. The cells were then washed with 190 μ L of prediluted binding buffer, followed by the addition of 10 μ L of the PI solution, and analysed immediately with flowcytometer (BD FACS Canto II, USA).

2.7. Cell Cycle Arrest Analysis. HepG2 cells at concentration of 2×10^5 cells mL^{-1} was seeded into 25 mL culture flask and treated with the ZER-HP β CD inclusion complex at 11.43 μ g/mL concentration for 24, 48, and 72 h. Untreated cells serve as negative control. The cells were washed with PBS twice to remove any remaining media. To restore cell integrity, fixation of cell population for flowcytometry analysis was performed. Cell pellets were fixed with 90% cold ethanol by mixing 700 μ L of 90% cold ethanol and kept for overnight observation at -20°C. The cells were washed using 2 mL PBS twice. Cell pellet was resuspended with 600 μ L of PBS + 10 mg/mL RNase + 1 mg/mL propidium iodide (PI). PI has the ability to bind to RNA, and hence, RNase enzyme was added in order to allow PI to bind directly to DNA. The cells were then incubated between 30 min to 1 h at 37°C. Finally,

the cell cycle kinetics was examined using flowcytometer (BD FACS Canto II, USA). The fluorescence intensity of sub-G₁ cell fraction represents apoptotic cell population.

2.8. High Content Screening Assay. This study was conducted using Cellomics Multiparameter Cytotoxicity 3 Kit as described previously [18–20]. Briefly, cells were treated with ZER-HPβCD inclusion complex at 11.43 μg mL⁻¹ concentration. Untreated cells serve as negative control, whilst paclitaxel (3.68 μg/mL) was used as the positive control. The 96-well microplate was then incubated for 24, 48, and 72 h. In this assay, four important parameters related to apoptosis characteristics were observed simultaneously, which included nuclear morphology changes; changes in cell permeability, mitochondrial membrane potential changes, and cytochrome c release. Plates were analyzed using the ArrayScan HCS system (Cellomics, USA).

2.9. Human Apoptosis Proteome Profiler Array. Detection of the relative levels of apoptosis-related markers was done using Human Apoptosis Antibody Array Kit (RayBio, USA), according to the manufacturer's instruction. Briefly, cells were treated with ZER-HPβCD inclusion complex at 11.43 μg/mL concentration. Untreated cells serve as negative control. The human apoptosis array was incubated overnight with 300 μg proteins from each sample. Quantification of the apoptosis array was done using the BiOpectrum AC ChemiHR40 (UVP, Upland, CA, USA) via scanning the membrane, and analysis was done using image analysis software according to the manufacturer's instruction.

2.10. Caspase Bioluminescent Assay. Caspase 3/7, caspase 8, and caspase 9 activities were measured in HepG2 cells using a Caspase-Glo assay kit (Promega, USA). HepG2 cells were plated at concentration of 2×10^5 cells mL⁻¹ in a white walled 96-well plate and treated with ZER-HPβCD inclusion complex at 11.43 μg/mL concentration for 24, 48, and 72 h. Untreated cells serve as negative control. The Caspase-Glo Reagent was mixed well and allowed to equilibrate to room temperature before starting the assay. The 96-well plate containing cells was removed from the incubator and allowed to equilibrate to room temperature as well. Then, 100 μL of Caspase-Glo Reagent was added to each well of the 96-well plate containing 100 μL of blank (vehicle only), negative control cells, or treated cells in culture medium. The contents of wells were gently mixed by using a plate shaker at 300–500 rpm for 30 seconds and incubated at room temperature between 30 min to 3 h. The luminescence of each sample was measured in a luminescence microplate reader (Infinite M200 PRO Tecan, Austria). Concisely, the proluminescent substrate containing the DEVD, LETD, and LEHD (sequence is in a single-letter amino acid code) is cleaved by caspase 3/7, caspase 8, and caspase 9, respectively. After the caspase cleavage, a substrate for luciferase (aminoluciferin) is released which eventually results in the luciferase reaction and the production of luminescent signal analysed in the luminescence microplate reader.

2.11. Western Blot Analysis. HepG2 cells were treated with ZER-HPβCD inclusion complex for 3, 6, 12, and 24 h. Untreated cells serve as negative control. The total proteins of cells were extracted using cell lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM PMSF), and 40 μg of the protein extract was separated by 10% SDS PAGE then transferred into a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) using semidry transfer unit (Hoefer TE 70X, USA) blocked with 5% nonfat milk in TBS-Tween buffer (0.12 M tris-base, 1.5 M NaCl, 0.1% Tween20) for 1 hour at room temperature. PVDF membrane was then incubated with the appropriate primary antibody overnight at 4°C, then it was incubated with horseradish peroxidase conjugated secondary antibody for 30 minutes at room temperature. The bound secondary antibody was detected with peroxidase-conjugated anti-rabbit antibody (1:10000) or anti-mouse antibody (1:10000) followed by detection using colorimetric method. The following primary antibodies β-actin (1:10000), Bcl-2 (1:1000), Bax (1:1000), and Hsp-70 (1:1000) used in this study were purchased from Santa Cruz Biotechnology, Inc., California, USA.

2.12. Statistical Analysis. Statistical analysis of all experimental data was performed using Student's *t*-test where *P* < 0.05 was considered statistically significant where results were presented as mean ± SD for at least three analyses for each sample.

3. Results

3.1. Antiproliferative Activity. A few types of cell viability were determined by conducting the MTT assay. The IC₅₀ values of the ZER-HPβCD inclusion complex against five tested cancer cell lines (HepG2, MCF-7, MDA-MB-231, CEMss, and HeLa) including one normal hepatic cell line (WRL-68) are shown in Table 1. The treated HepG2 cells showed a decrement of metabolic activity with an IC₅₀ value of 11.43 ± 0.31 μg mL⁻¹. The IC₅₀ value obtained for pure ZER alone against HepG2 cells was 15.54 ± 0.15 μg mL⁻¹, and it shows a similar cytotoxicity effect as exhibited by the ZER-HPβCD inclusion complex. Paclitaxel, a commercial anticancer drug, was evaluated as positive control to demonstrate concurrent cytotoxicity with an IC₅₀ of 3.68 ± 0.22 μg mL⁻¹. According to the American National Cancer Institute, a bioactive compound with an IC₅₀ value of ≤ 30 μg/mL has the potential to be an anticancer agent.

3.2. ZER-HPβCD Inclusion Complex Showing Morphological Changes on HepG2 Cells That Associate with Apoptosis. Treatment of ZER-HPβCD inclusion complex (11.43 μg/mL) on HepG2 cells showed a cell degenerative in a time-dependent manner while untreated HepG2 control cells were viable and showed normal morphology under normal inverted microscopy (Figure 3). To confirm the apoptosis mechanism of cell death, we examined the nuclear morphological changes using AO/PI double staining. HepG2 cells treated with ZER-HPβCD inclusion complex showed cell blebbing and nuclear

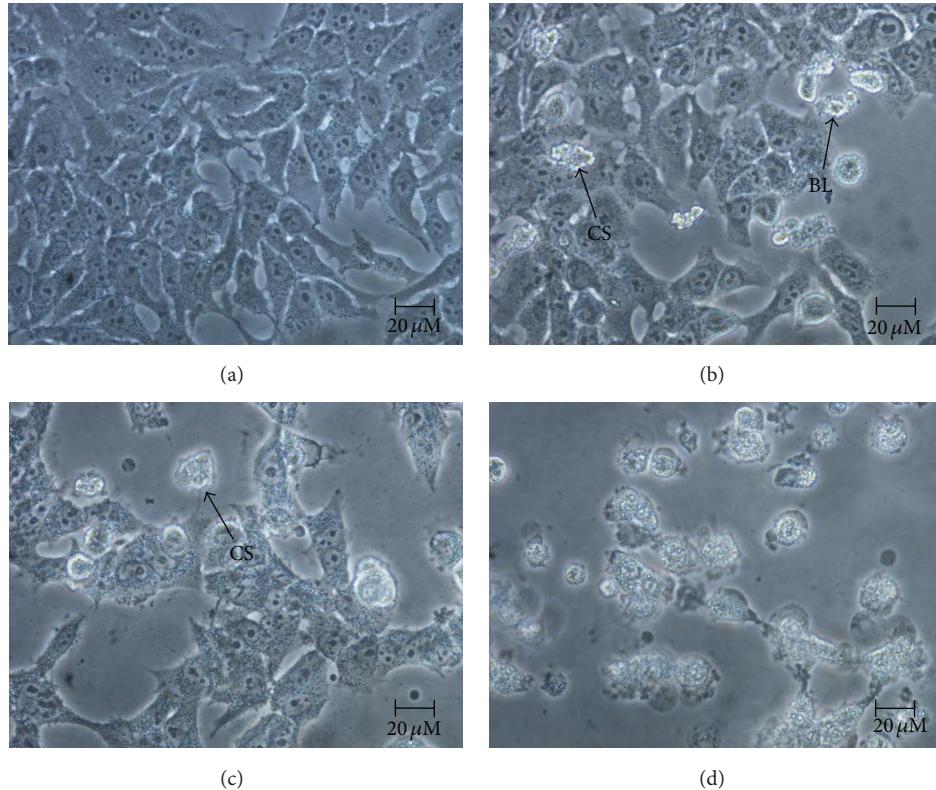


FIGURE 3: Normal phase contrast inverted micrographs of HepG2 cells treated with $11.43 \mu\text{g}/\text{mL}$ ZER- $\text{HP}\beta\text{CD}$ inclusion complex. (400x magnification) (a) Control untreated cells; (b) 24 h treatment (most of the cells having normal morphology with few cells showing membrane blebbing); (c) 48 h treatment (detachment of cells, prominent growth inhibition and membrane blebbing showing apoptogenic morphology); (d) 72 h treatment (most of the cells were detached with obvious cell shrinkage). CS: cell shrinkage; BL: cell membrane blebbing.

chromatin condensation of moderate apoptosis after a 24-hour treatment, with subsequent increase at 48 h of treatment followed by the presence of reddish-orange colour after 72 h of treatment due to AO binding towards denatured DNA, thus confirming late stage apoptosis (Figure 4).

3.3. ZER- $\text{HP}\beta\text{CD}$ Inclusion Complex Triggers Early Apoptosis Cell Death in HepG2 Cell Line. Early apoptotic population is indicated by positive Annexin V and negative PI whilst late apoptotic population is indicated by both positive Annexin V and PI. The result obtained is summarized in Table 2. After 6 h of HepG2 cells treatment with ZER- $\text{HP}\beta\text{CD}$ inclusion complex ($11.43 \mu\text{g}/\text{mL}^{-1}$), early apoptotic population significantly increased to 9.0%. After 12 h of treatment, early apoptotic population was significantly higher than untreated HepG2 control cells at 8.80% with a decrement of viable cells at 88.80%. After 24 h of treatment, decrement of viable cells continued with 79.30%, and both early and late apoptotic cell populations rose significantly at 15.20% and 5.50%, respectively ($P < 0.05$). These results prove that the treatment of ZER- $\text{HP}\beta\text{CD}$ inclusion complex on HepG2 cells induced apoptosis, with possible translocation of phosphatidylserine from cytoplasm to the transmembrane of the HepG2 cells.

TABLE 1: Cytotoxicity of ZER- $\text{HP}\beta\text{CD}$ inclusion complex on different cancer and normal cells *in vitro*.

Cell line	Origin of cells	$\text{IC}_{50} (\mu\text{g}/\text{mL})$
HepG2	Liver hepatocellular cells	11.43 ± 0.31
MCF-7	Oestrogen receptor-positive breast adenocarcinoma cells	15.32 ± 0.61
MDA-MB-231	Oestrogen receptor-negative breast adenocarcinoma cells	17.20 ± 0.17
CEMss	T-acute lymphoblastic leukemia	9.13 ± 0.38
HeLa	Cervical cancer cells	14.47 ± 0.26
WRL-68	Normal hepatic cell line	>30

IC_{50} values were obtained from MTT assay. Data are reported as means \pm SD for measurements in triplicate.

3.4. ZER- $\text{HP}\beta\text{CD}$ Inclusion Complex Induces Apoptosis with G2/M Phase Cell Cycle Arrest. HepG2 cells treated with ZER- $\text{HP}\beta\text{CD}$ inclusion complex ($11.43 \mu\text{g}/\text{mL}$) revealed a significant time-dependent increase ($P < 0.05$) in hypodiploid sub-G0/G1 DNA fraction, which corresponds to the presence of apoptotic cells (Figure 6). Sub-G0/G1 fraction in untreated control cells was 0.20%, and this value subsequently increased to 24.20% after 72 h of treatment with ZER- $\text{HP}\beta\text{CD}$ inclusion

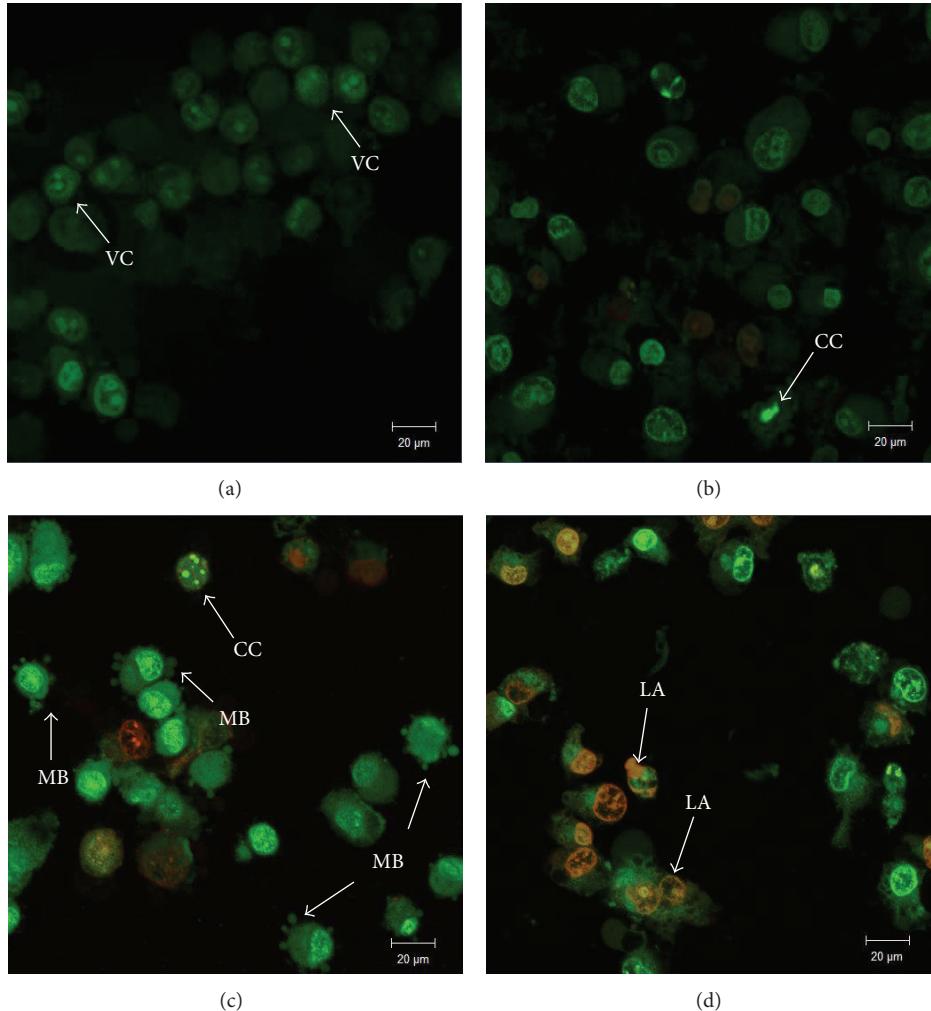


FIGURE 4: Confocal micrographs of acridine orange and propidium iodide double-stained HepG2 cells treated with $11.43 \mu\text{g}/\text{mL}$ ZER- $\text{HP}\beta\text{CD}$ inclusion complex. (400x magnification) (a) Untreated cells showed normal structure without prominent apoptosis; (b) early apoptosis features were seen after 24 h representing intercalated acridine orange (bright green) amongst the fragmented DNA; (c) more cells showing membrane blebbing after 48 h; (d) presence of reddish-orange colour at 72 h. VC: viable cells; MB: cell membrane blebbing; CC: chromatin condensation; LA: late apoptosis.

TABLE 2: Flow cytometric analysis of Annexin V in HepG2 cells which were treated with $11.43 \mu\text{g}/\text{mL}$ ZER- $\text{HP}\beta\text{CD}$ inclusion complex for 6, 12, and 24 hours.

Cell condition	Percentage of cells (%)			
	Control	6 h	12 h	24 h
Viable	93.90 ± 1.20	89.70 ± 1.50	88.80 ± 3.20	79.30 ± 5.80
Early apoptosis	4.20 ± 0.27	$9.00 \pm 0.31^*$	$8.80 \pm 0.44^*$	$15.20 \pm 0.40^*$
Late apoptosis	1.90 ± 0.12	1.30 ± 0.53	2.30 ± 0.35	$5.50 \pm 0.27^*$

Data are shown as mean \pm SD ($n = 3$); *Indicates a significant difference compared with control ($P < 0.05$).

complex. The results further indicated that ZER- $\text{HP}\beta\text{CD}$ inclusion complex treatment caused mitotic block and cell cycle delay in G2/M phase (Figure 5). The proportion of accumulated cells blocked at G2/M phase significantly ($P < 0.05$) increased to 59.84% as opposed to 16.44% for the untreated HepG2 control cells in G2/M phase after a 72-hour treatment (Figure 6).

3.5. ZER- $\text{HP}\beta\text{CD}$ Inclusion Complex Reduces Mitochondrial Membrane Potential and Translocates Cytochrome c in HepG2 Cells. The nuclear intensity which corresponds to apoptotic chromatin changes (Figures 7(a) and 8(a)) and cell permeability (Figures 7(a) and 8(a)) were both found to be increased significantly ($P < 0.05$) compared to the untreated control cells. Fluorescence intensity in the membrane potential area

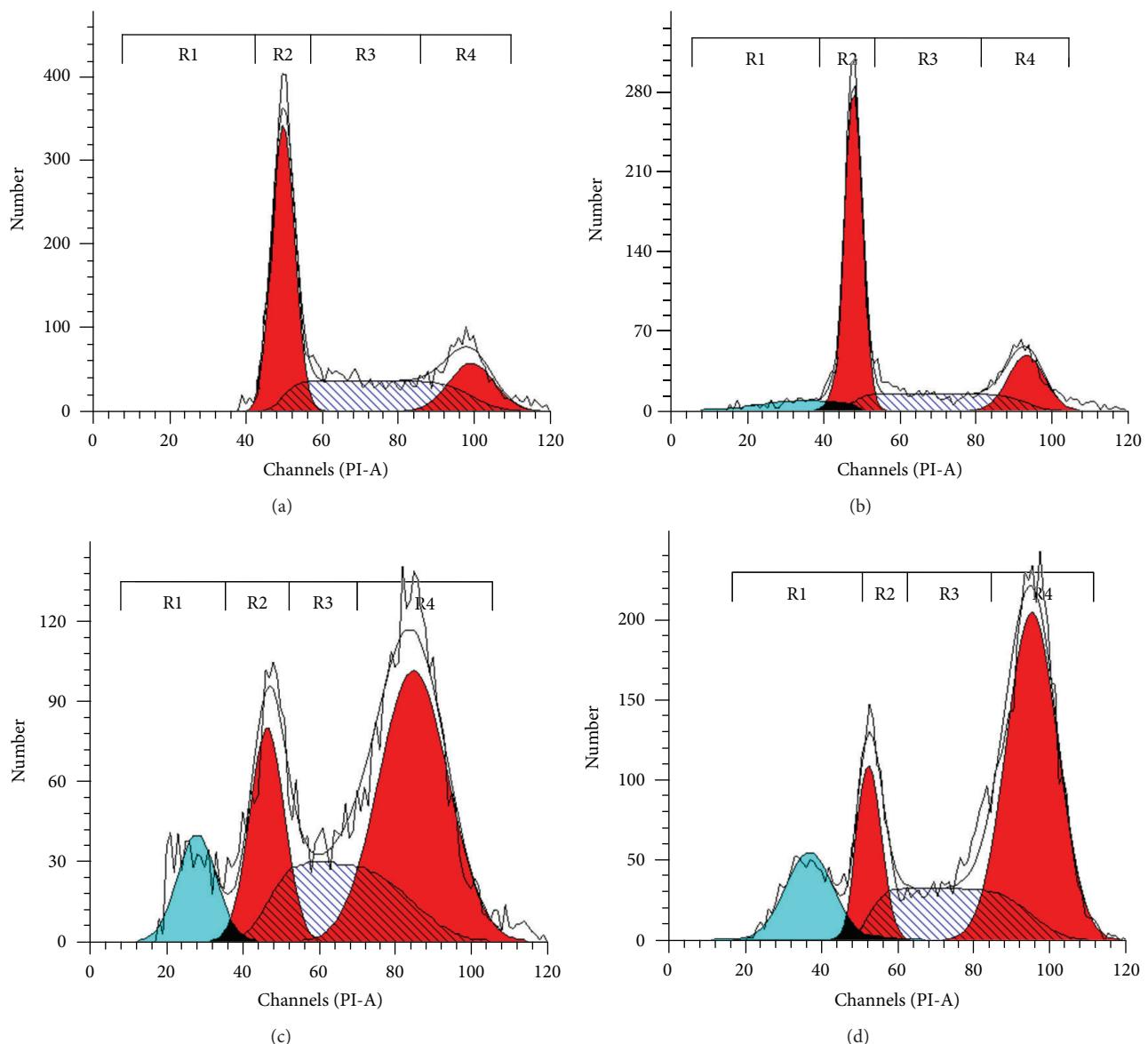


FIGURE 5: DNA analysis of ZER-HP β CD inclusion-complex-treated HepG2. Rapidly proliferating HepG2 cells exposed to 11.43 μ g/mL at (b) 24 h, (c) 48 h, (d) 72 h and (a) control were tested for DNA content. Panels are representative of DNA histograms obtained and subsequently analyzed for DNA content; whereby R1, R2, R3, and R5 indicate sub-G0/G1 (apoptosis cells), G0/G1, S, and G2/M phase, respectively.

of HepG2 cells treated with ZER-HP β CD inclusion complex (11.43 μ g/mL) was shown to be reduced compared to the untreated control cells (Figure 7(c)), reflecting the collapsing of mitochondrial membrane potential with a significant decrease ($P < 0.05$) and a drastic drop after 72 h of treatment (Figure 8(c)). Cytochrome c was released into the cytosol upon treatment, demonstrating possible involvement of the mitochondrial pathway with a significant increase of cytosolic cytochrome c ($P < 0.05$) in a time-independent manner (Figures 7(d) and 8(d)).

3.6. ZER-HP β CD Inclusion Complex Involves Extrinsic and Intrinsic Apoptosis Pathways.

The result obtained from our Human Apoptosis Antibody Array analysis showed an upregulation of the proapoptotic Bax protein, which correlated to the downregulation of the antiapoptotic Bcl-2 protein (Figure 9). Caspase 8, caspase 3, and cytochrome c showed significant increase while BID was seen to be depleted significantly after the treatment of HepG2 with ZER-HP β CD inclusion complex compared to untreated control cells ($P < 0.05$). The upregulation of caspases 8 and 3 supports the possibility that the ZER-HP β CD inclusion complex induces cell death in HepG2 cells via apoptosis with the involvement of both intrinsic and extrinsic pathways. In addition, p53 protein showed no significant change, proving that the apoptosis mechanism was p53 independent ($P > 0.05$). Hsp-70, also an antiapoptotic protein, was found to be significantly

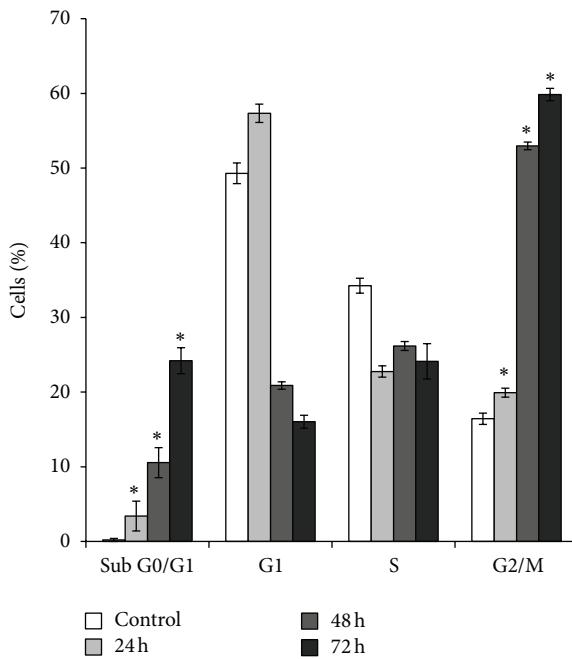


FIGURE 6: Percentages of cell cycle distribution in sub-G0/G1 (apoptosis cells), G0/G1, S, and G2/M phase of HepG2 cells treated with 11.43 $\mu\text{g}/\text{mL}$ of ZER-HP β CD inclusion complex for 24, 48, and 72 h. Induction of G2/M arrest in cell cycle progression of HepG2 cells by ZER-HP β CD inclusion complex. *Indicates a significant difference ($P < 0.05$) compared with control.

decreased ($P < 0.05$). XIAP, an inhibitor of apoptosis protein (IAP) was found to be down-regulated while SMAC that works as the inhibitor to IAP was shown to be up-regulated, providing evidence that both XIAP and SMAC correspond to each other to promote cell death in HepG2 cells.

The luminescence assay was further used to confirm the involvement of caspases proteins in apoptosis induction. The effector caspase, caspase 3/7, was found to be increased as well as the initiator caspases, caspase 9 and caspase 8 (Figures 10(a), 10(b), and 10(c)). As shown in our results, ZER-HP β CD inclusion complex significantly stimulated all three caspases ($P < 0.05$) compared to untreated control cells, further suggesting that both intrinsic (mitochondrial pathway) and extrinsic pathways were involved. These results correspond well with our previous results using the Human Apoptosis Antibody Array analysis.

The involvement of Hsp-70, Bcl-2, and Bax apoptosis proteins was confirmed using Western blot analysis (Figure 11). Protein Hsp-70 could be seen to decrease gradually after 12 h of treatment without significant changes at 3 h and 6 h of treatment with ZER-HP β CD inclusion complex. Similar to Hsp-70, Bcl-2 protein exhibited decreased band intensity in a time-dependent manner, showing obvious fading of the colour after 6 h of HepG2 cells treatment with ZER-HP β CD inclusion complex. On the other hand, Bax that acted as proapoptotic protein increased with the increment of treatment time with the highest band intensity after 24 h, providing evidence that treated HepG2 cells initiated the

induction of apoptosis cascade mechanism, which finally resulted in cell death. β -Actin, used as the loading control, showed equal intensity to all bands, confirming equal protein concentration in all loaded samples.

4. Discussion

Due to the growing use of natural-derived substances all around the world, a detailed evaluation of their pharmacological qualities and safety issues is critically needed, since traditional beliefs and remedies cannot stand alone without undergoing detailed scientific studies [21]. Consuming such herbal medicines without proper scientific approval may invite other conflicts which are not favourable to consumers. *Z. zerumbet* is one of the most well-known compounds for its role in traditional medicines as well as in pharmacological activities. ZER is one of the plant bioactive compounds, which can be found mostly in the rhizome of *Z. Zerumbet*, that was previously reported to produce cytotoxicity through apoptosis induction in various cancers including skin tumor, colon, and lung carcinogenesis, hepatocarcinogenesis, leukemic cancer, and cervical intraepithelial neoplasia [10–14].

Despite the apoptotic effects that ZER demonstrates, ZER was reported to be poorly soluble in water and requires organic solvent to solubilize it. Hence, in our previous research, we prepared an inclusion complex of ZER with HP β CD and characterized its physicochemical properties. The results provided evidence that ZER fits well inside the nanocavity of HP β CD. The study further concluded that the complexation of ZER with HP β CD leads to crucial modifications pertaining to the physicochemical properties of ZER which includes its solubility, stability, and bioavailability in blood [15]. Thus, taking into consideration the importance of ZER-HP β CD new findings as a potential biopharmaceutical drug, the antiproliferative effects of the inclusion complex was investigated to determine its bioactivity in HepG2 cells. This current study is an initiative to investigate the anticancer activity of the inclusion complex that would eventually lead to preclinical studies of ZER-HP β CD inclusion complex as a potential anticancer for future treatment of hepatocarcinoma in humans.

The ZER-HP β CD inclusion complex was screened against five different human cancer cell lines: HepG2, MCF-7, HeLa, MDA-MB-231, and CEMss. The results obtained showed that ZER-HP β CD inclusion complex exhibits cytotoxic activity on all cancer cell lines screened, with IC₅₀ values <30 $\mu\text{g}/\text{mL}$. Pure ZER alone were screened and its activity in HepG2 cells were compared concurrently to that of ZER-HP β CD inclusion complex. The determined IC₅₀ values confirmed the previous study of pure ZER alone against cell lines (MCF7, HeLa, MDA-MB-231, and CEMss), where it was found to be nearly similar to the IC₅₀ values obtained for the ZER-HP β CD inclusion complex [15]. These results are consistent with our current study, proving that the cytotoxic activities of ZER-HP β CD inclusion complex in these cancer cells may possibly resemble that of pure ZER alone. Interestingly, the ZER-HP β CD inclusion complex gave an IC₅₀ value of more than 30 $\mu\text{g}/\text{mL}$ in the normal hepatic cell line (WRL-68), showing that the cytotoxicity

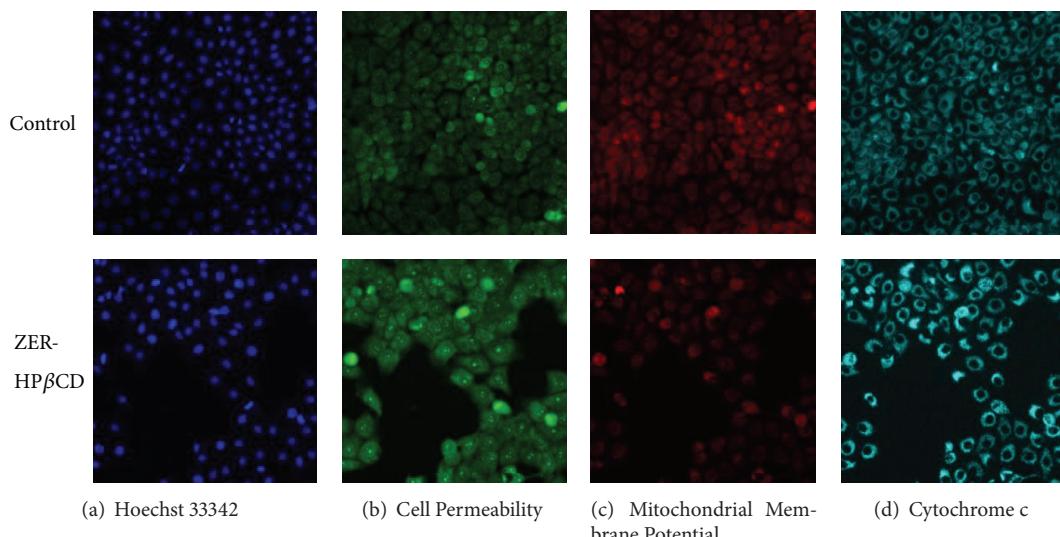


FIGURE 7: Fluorescent images of HepG2 cells treated with medium alone and $11.43 \mu\text{g}/\text{mL}$ of ZER-HP β CD inclusion complex, stained with (a) Hoechst for nuclear, (b) cell permeability dye, (c) mitochondrial membrane potential dye, and (d) cytochrome c. The images from each row were obtained from the same field of the same treatment sample. ZER-HP β CD inclusion complex showed marked increases in permeability dye, marked reduction in mitochondrial membrane potential, and marked increases in cytochrome c.

produced by this inclusion complex is selective in cancer cells only.

The mode of HepG2 cell death was determined based on the characteristics of the cells exerted after treatment. Microscopy analysis has been used as the gold standard for precise detection of cell death, particularly apoptosis, according to the morphological criteria stated by Wyllie et al. (1980) [22] and Yasuhara et al. (2003) [23]. Indications of apoptosis in HepG2 cells treated with ZER-HP β CD inclusion complex such as a reduction in the number of cells, detachment of cells, cytoplasmic shrinkage, and membrane blebbing were observed using phase contrast inverted microscopy and confocal microscopy (with AO/PI double staining). The significant increment of apoptotic scores of HepG2 cells treated with ZER-HP β CD inclusion complex correlates with a previous study, which reported that HepG2 cells treated with pure ZER lead to a large increase of apoptotic scores at approximately 80% by 48 h and 90% after 72 h [24].

However, morphological studies alone is not sufficient to validate the early and late apoptosis; thus, the finding was further evaluated using the Annexin V assay, which demonstrated that early apoptosis in HepG2 cells was significantly increased in a time-dependent manner after treatment with ZER-HP β CD inclusion complex. The critical event during apoptosis is when the changing plasma membrane signals phagocytes to allow them to engulf cells undergoing apoptosis before rupturing [25]. Modification of the apoptotic cell surface included the exposure of phosphatidylserine (PS), which normally dominates surface membrane facing the cytosol [26]. PS externalization occurs in early apoptosis event before the cell undergoes nuclear changes, regardless of the initiator apoptosis catalyst [27]. Hence, early phases of apoptosis (prior to the loss of cell membrane integrity) were detected using the fluorescein isothiocyanate (FITC)

conjugated Annexin V (protein with high affinity for PS) binding assay of PS, allowing measurement and therefore scoring of individual apoptotic cells [25]. Our results in this current study are parallel with the significant increase of apoptosis in HepG2 cells at 6, 12, and 24 h treatments, which proved that the ZER-HP β CD inclusion complex is able to trigger early apoptosis with the exposure of PS at the external surface of treated HepG2 cells.

To further elucidate the probable mechanisms of apoptosis induced by ZER-HP β CD inclusion complex in HepG2 cells, cell cycle analysis was performed and evaluated. The observed results showed a hypodiploid sub-G0/G1 DNA accumulation, which concluded that the ZER-HP β CD inclusion complex is able to induce apoptosis in HepG2 cells in a time-dependent manner and simultaneously induce cell cycle arrest at G2/M. This mitotic blockage has been observed as a result of treating HepG2 cells with the accumulation of ZER-HP β CD inclusion complex at G2/M. It has been reported that the G2/M arrest triggers possible phosphorylation of apoptosis-associated proteins in the mitotic phase of cell cycle, which further explains the involvement of G2/M arrest to be associated with apoptosis [28]. This result was confirmed by previous studies done on pure ZER alone, which was found to inhibit interleukin-6 and induces apoptosis and cell cycle arrest at G2/M phase in ovarian (Caov-3) and cervical (HeLa) cancer cells [29]. It was also previously reported elsewhere that ZER concurrently showed apoptogenic effects to induce G2/M cell cycle arrest in promyelocytic leukemia NB4 cells and colon adenocarcinoma HT-29 cell line [30, 31].

A molecular study was conducted to clarify probable mechanisms of apoptosis induced by ZER-HP β CD inclusion complex using high-content screening assay, proteome profiler array, caspase luminescence assay, and Western blot analysis. The study confirmed that ZER-HP β CD inclusion

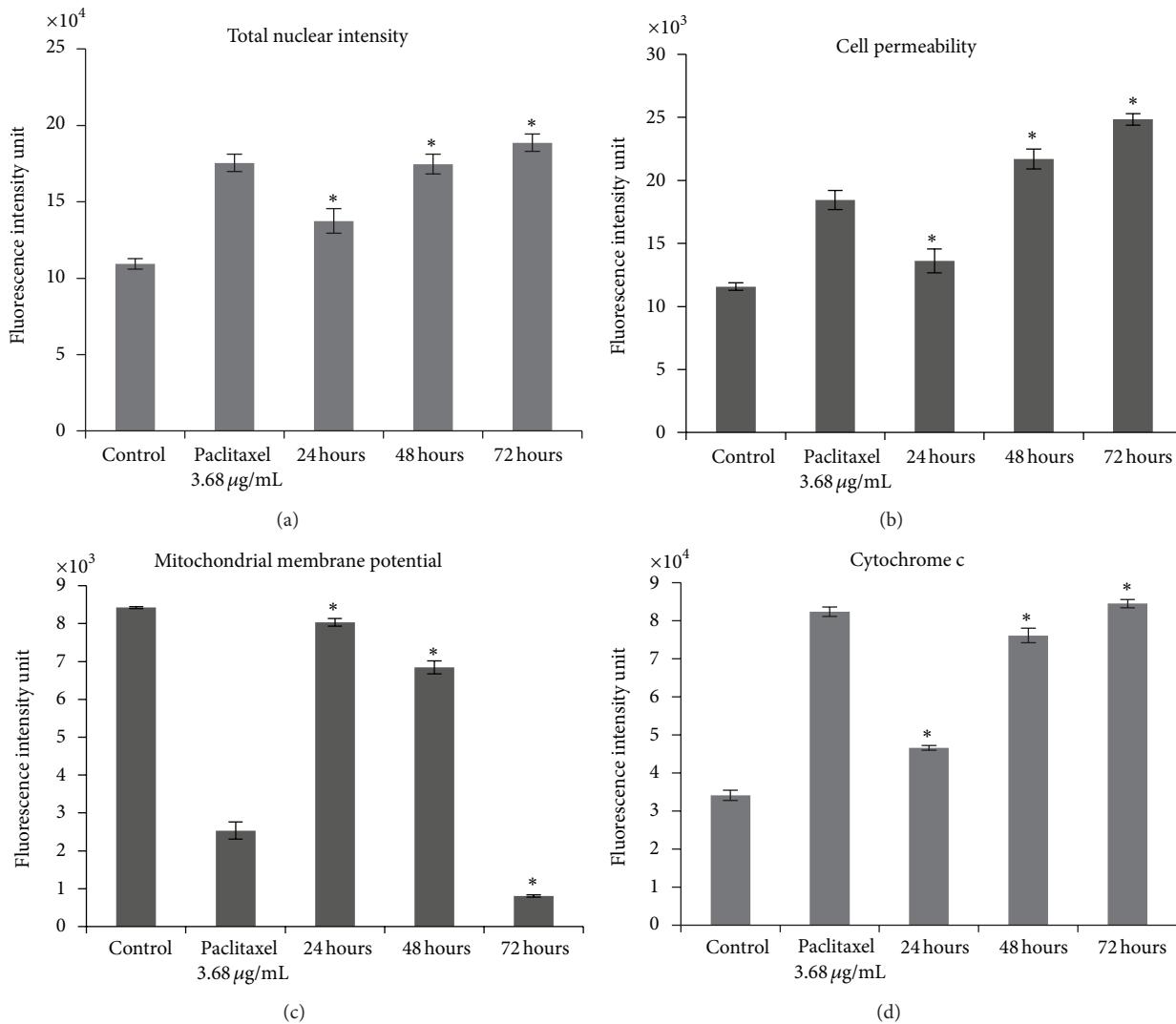


FIGURE 8: Quantitative analysis of ZER-HP β CD inclusion complex mediated apoptosis parameter. Changes in (a) total nuclear intensity, (b) cell permeability, (c) mitochondrial membrane potential, and (d) cytochrome c localization were all measured simultaneously in HepG2 treated cells. Treatment with ZER-HP β CD inclusion complex showing statistically significant cell loss (data not shown), increased total nuclear intensity, increased cell permeability, loss of mitochondrial membrane potential, and cytochrome c release from the mitochondria with good P values. Each experiment was performed at least two times. *Indicates a significant difference ($P < 0.05$) compared with control.

complex stimulates apoptosis signal, causing a decrement of membrane permeability in HepG2 cells followed by mitochondrial transmembrane potential ($\Delta\Psi_m$) changes. The structure and dynamics of the altered cell membrane in HepG2 cells are due to this increment of susceptibility to hydrolysis; thus, increasing cell permeability and finally events leading to apoptosis [32]. Any stimulus causing apoptosis affects HepG2 cells in such a way that will cause permeability transition pores to open to the inner mitochondrial membrane, subsequently causing increased permeability of the inner mitochondrial membrane capable of soluting molecular mass of less than about 1500 Da, thus resulting in the depletion of $\Delta\Psi_m$, and in turn, the release of proapoptotic proteins and the arrest of the bioenergetic function of the organelle [33–35].

The present study further demonstrated that the exposure of HepG2 cells to ZER-HP β CD inclusion complex showed an upregulation of proapoptotic protein, Bax, and a downregulation of antiapoptotic, Bcl-2 protein. Bax and other related proapoptotic proteins activities were affected by the formation of Bcl-2 antiapoptotic protein. Bax will be translocated to the mitochondria and other membrane sites, which activates the transformation of mitochondrial function. This mitochondrial transformation will cause loss of transmembrane potential and release of cytochrome c to the cytosol, resulting in apoptotic cell death [36]. Overexpression of Bcl-2 will prevent the release of cytochrome c, causing HepG2 cells to be resistant to apoptotic induction [37]. Contrary to this, ZER-HP β CD inclusion complex was able to suppress Bcl-2 expression, thus allowing Bax to form a homodimer

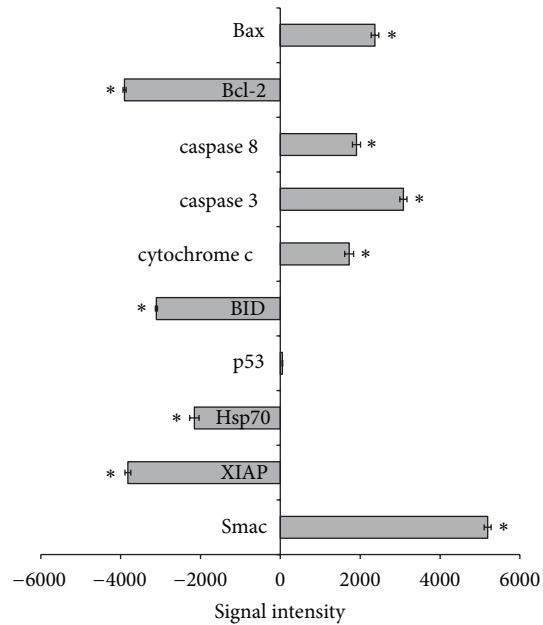


FIGURE 9: Human apoptosis proteome profiler array analysis in HepG2 cells treated with $11.43 \mu\text{g/mL}$ of ZER- $\text{HP}\beta\text{CD}$ inclusion complex for 48 h. Graph shows the difference between treated and control untreated cells. *Indicates a significant difference ($P < 0.05$) compared with control.

with another Bax instead of forming a heterodimer with Bcl-2. These upregulation and downregulation of Bax and Bcl-2 ratio reaffirm a previous study which reported that pure ZER alone induced apoptosis in HepG2 cells via modulation of Bax/Bcl-2 ratio [36].

Results of the caspase luminescence assay and apoptosis proteome profiler array further demonstrated that ZER- $\text{HP}\beta\text{CD}$ inclusion complex treatment induces the upregulation of caspase 8, caspase 9, caspase 3, and cytochrome c, and degradation of BID in treated HepG2 cells. The upregulation of caspase 8 was due to the proteolysis of procaspase 8, forming activated caspase 8 and therefore allowing the amplification of caspase signal for apoptosis [38]. In a previous study elsewhere, commercial drugs such as daunorubicin, doxorubicin, etoposide, and mitomycin C induced apoptosis in Jurkat leukemic T cells involving the activation of caspase 8 [39]. Paclitaxel was also reported to induce apoptosis involving caspase 8 activation in colon cancer cell (HT-29-D4) [40]. Activated caspase 8 will cleave BID into a proapoptotic fragment called truncated BID (tBID), which will later translocate to the mitochondrial membrane with a subsequent release of cytochrome c into the cytosol with the help of Bax [41]. Cleavage of BID is mediated by caspase 8, which is known to connect the extrinsic and mitochondrial pathways in apoptosis cell death [42]. Cytochrome c release will induce the oligomerization of cytochrome c/Apaf-1/caspase 9 complex that activates caspase 9 and finally leads to the cleavage of downstream effector caspase 3 and 7 [43].

The protein expression of p53 did not exhibit any significant changes in HepG2 cells treated with ZER- $\text{HP}\beta\text{CD}$ inclusion complex, providing evidence for the first time that

induced apoptosis upon treatment of HepG2 cells with ZER- $\text{HP}\beta\text{CD}$ inclusion complex is p53 independent. Although p53 is known as the “guardian of the genome” since it prevents proliferation of damage cells, several chemotherapy drugs such as paclitaxel, tamoxifen, and vinkristin were identified as able to induce apoptosis without the involvement of p53 [44–46]. This is probably due to the expression of Bax protein, which after achieving certain level is able to induce apoptosis without the presence of p53 [44]. Our current finding is crucial as most cancer tumours including those of the breasts, lung, colon, bladder, brain, bone, hematopoietic, and muscle tissues are initiated due to the abnormalities of or mutated p53 gene [47]. This would allow the ZER- $\text{HP}\beta\text{CD}$, a significant anticancer, complex to be able to trigger the induction of apoptosis in most human cancers that are p53 dependent.

Heat shock protein (Hsp) 70 will interfere with the apoptotic process of cell death as its role is to mediate cellular protection by preventing cytochrome c/dATP-mediated caspase activation but allowing the formation of Apaf-1 oligomers to be accessible. Hsp-70 binds to Apaf-1 but not to procaspase 9, therefore preventing the recruitment of caspases to the apoptosome complex. This directly blocks the assembly of functional apoptosome and prevents the cell from undergoing apoptosis [48]. In this current study, Hsp-70 was found to be suppressed in HepG2 treated with ZER- $\text{HP}\beta\text{CD}$ inclusion complex, resulting in the Apaf-1 functional assembly blockage to be prevented. Protein Hsp-70 was also found to be decreased gradually only after 12 h of treatment without any significant changes at 3 h and 6 h of treatment. In accordance with this, it may be suggested that the host stress response of HepG2 cells may be activated at 3 and 6 h of treatment but with Hsp-70 exhibiting no significant

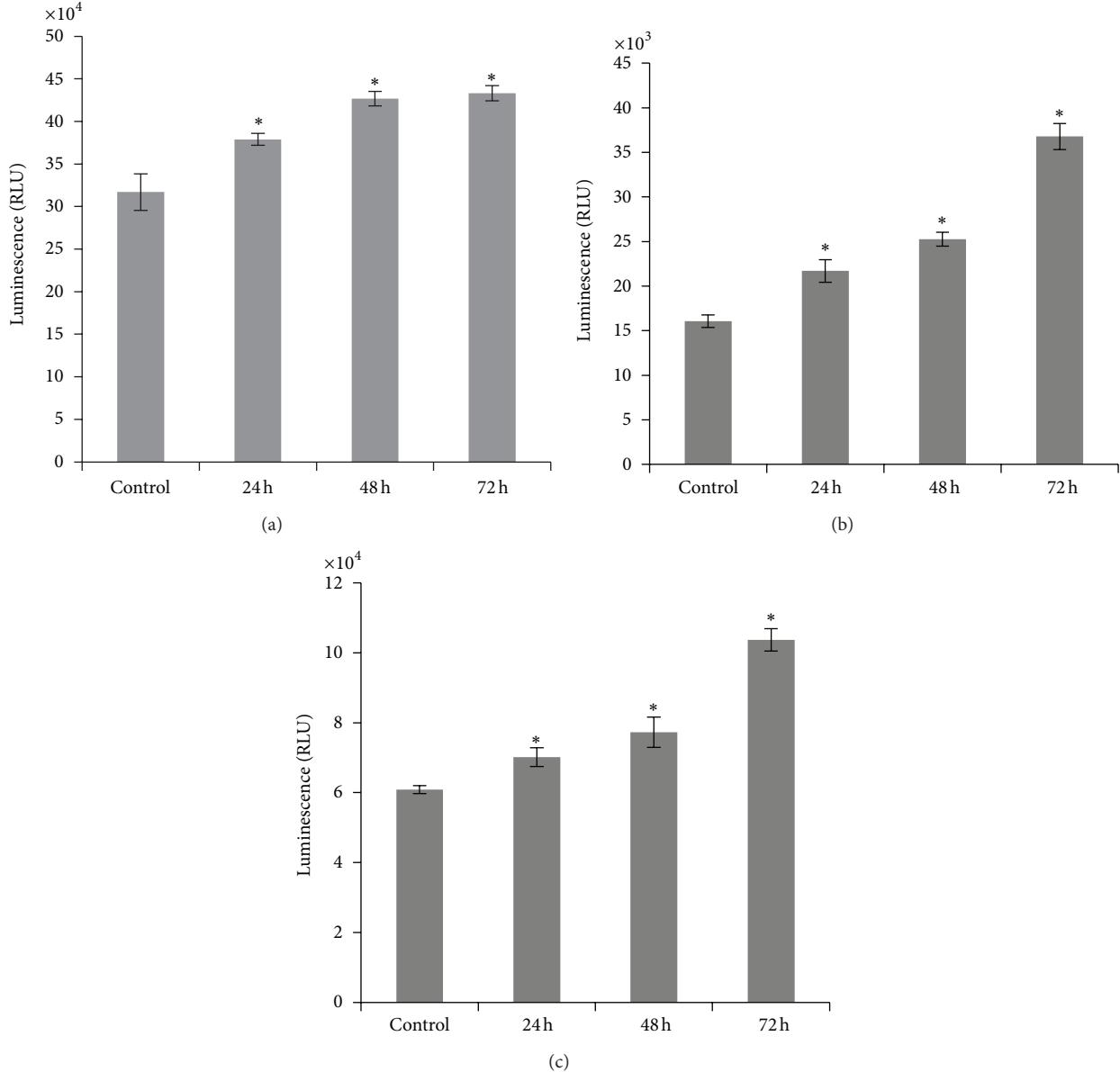


FIGURE 10: Relative luminescence expression of (a) caspase 3/7, (b) caspase 8, and (c) caspase 9 in the HepG2 cells treated with 11.43 μ g/mL of ZER-HP β CD inclusion complex for 24, 48, and 72 h. Caspases 3/7, 8, and 9 were significantly increased compared to the control untreated cells. *Indicates a significant difference ($P < 0.05$) compared with control.

increase during this period due to the treatment of ZER-HP β CD inclusion complex. It was reported previously that, in normal unstressed cells, Hsp-70 is expressed only at a very low level and nearly undetectable while in most human tumors, it is expressed at a very high level while adjusting to the unfavourable environmental conditions [49–51]. With the ability to suppress Hsp-70 in HepG2 cells, ZER-HP β CD inclusion complex will be able to stop the growth of HepG2 cells which finally leads to apoptosis of the cells.

Procaspsase activation and caspase activity need to be controlled in order to prevent overexpression of such proteins. In addition, the inhibition of apoptosis protein (IAPs), which acts as the modulator to directly control caspase activation,

inhibits caspase 3, caspase 7, and caspase 9 activities. Among all IAPs identified, X-linked inhibitor of apoptosis protein (XIAP) is the most crucial in suppressing procaspsase activation [52]. Hence, in order to induce cell death via apoptosis, protein suppression by XIAP must be crucially blocked. In this regard, the second mitochondria-derived activator of caspases (Smac)/DIABLO resumes the responsibility to suppress XIAP activity. As proapoptotic of Bcl-2 family induces the mitochondrial transmembrane potential ($\Delta\Psi_m$) changes, Smac/DIABLO protein is released from the mitochondria [53]. ZER-HP β CD inclusion complex has shown the ability to induce XIAP inhibition through Smac protein activation in HepG2 cells and therefore allowing the cascade caspases

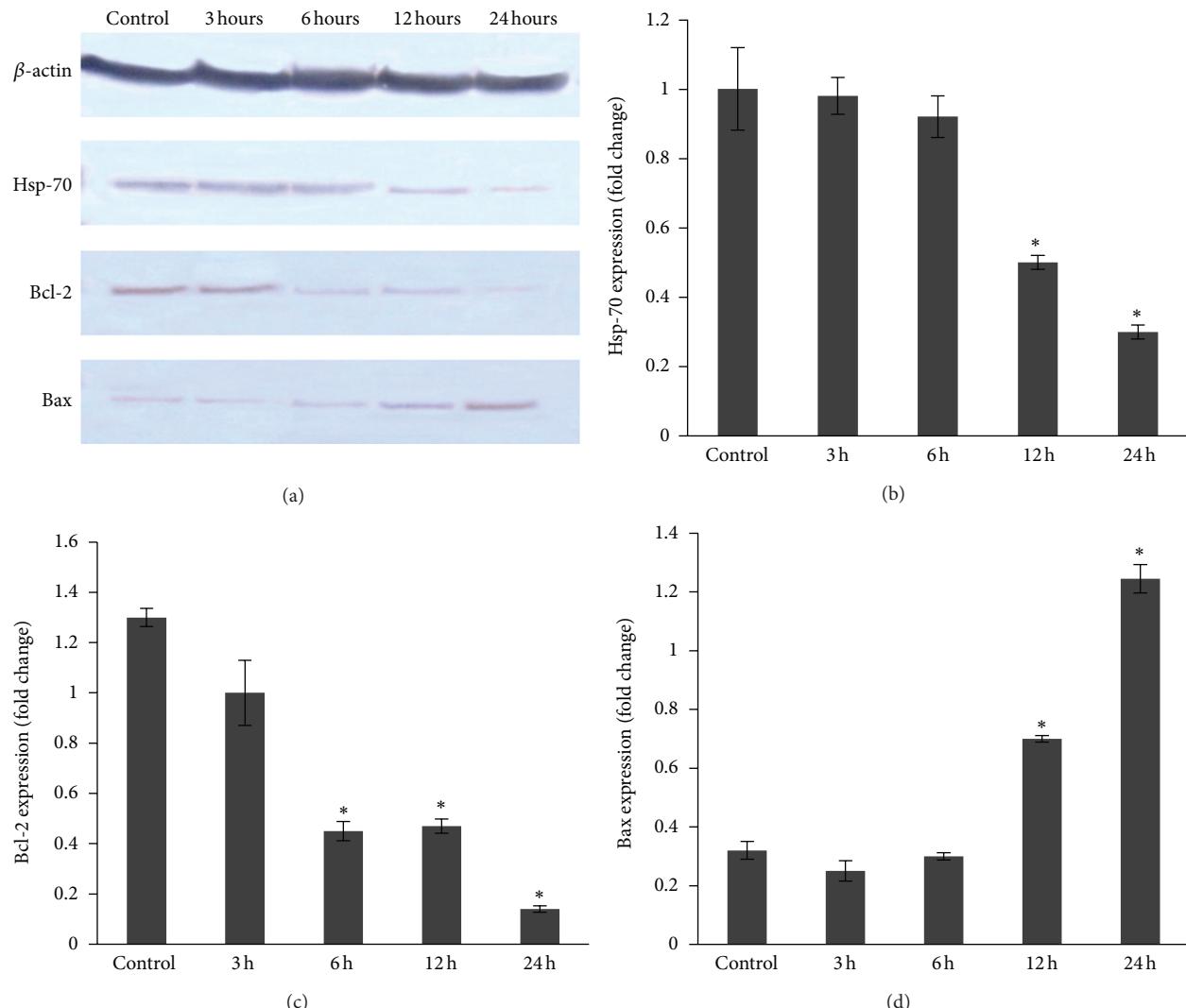


FIGURE 11: Western blot analysis on effect of ZER-HP β CD inclusion complex on the levels of apoptosis regulatory proteins in HepG2 cells. Bax was upregulated while Bcl-2 in contrast downregulated in a time-dependent manner. Hsp-70 was also found to be depleted as time increases. The blot densities are expressed as folds of control. Detection of proteins was done by specific antibodies with β -actin as a loading control. Data are mean \pm SD ($n = 3$). * $P < 0.05$ versus control.

to be activated downstream which in turn triggered apoptosis induction.

5. Conclusion

Collectively, our current findings showed that the highly soluble ZER-HP β CD inclusion complex possesses anticancer properties towards HepG2 cell line and the death receptor pathway may be involved in the induction of apoptosis. Our current finding can be extrapolated to postulate that caspase 8's activation is indirectly involved as an interconnection between the extrinsic and intrinsic pathways. This ZER-HP β CD inclusion complex not only demonstrated antiproliferative effects towards HepG2 cells, but its high solubility in water provided the advantage to pursue the complex as a therapeutic drug candidate in humans.

Future *in vivo* studies are recommended prior to the use of the ZER-HP β CD inclusion complex as an anticancer on hepatocarcinoma patients, and detailed toxicity studies of this complex are vital before pursuing human clinical trials.

Abbreviations

AO:	Acridine orange
Apaf-1:	Apoptotic protease-activating factor-1
Bax:	Bcl-2 associated X protein
Bcl-2:	B cell lymphoma 2
BID:	BH3 interacting domain death agonist
DMSO:	Dimethyl sulfoxide
FITC:	Fluorescein isothiocyanate
HP β CD:	Hydroxypropyl- β -cyclodextrin
Hsp-70:	Heat shock protein 70
IC ₅₀ :	Half maximal inhibitory concentration

- MMP: Mitochondrial membrane potential
 MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 PI: Propidium iodide
 PS: Phosphatidylserine
 SMAC: Second mitochondria-derived activator of caspases
 XIAP: X-linked inhibitor of apoptosis protein
 ZER: Zerumbone
 ΔVm : Mitochondrial transmembrane potential.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Treating Type 2 Diabetes Mellitus with Traditional Chinese and Indian Medicinal Herbs

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Type II diabetes mellitus (T2DM) is a fast-growing epidemic affecting people globally. Furthermore, multiple complications and comorbidities are associated with T2DM. Lifestyle modifications along with pharmacotherapy and patient education are the mainstay of therapy for patients afflicted with T2DM. Western medications are frequently associated with severe adverse drug reactions and high costs of treatment. Herbal medications have long been used in the treatment and prevention of T2DM in both traditional Chinese medicine (TCM) and traditional Indian medicine (TIM). This review examines *in vivo*, *in vitro*, and clinical evidence supporting the use of various herbs used in TCM and TIM. The problems, challenges, and opportunities for the incorporation of herbal frequently used in TCM and TIM into Western therapy are presented and discussed.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic illness due to endocrine dysfunction. Uncontrolled, diabetes is associated with various acute and chronic comorbidities. T2DM is a rapidly growing health concern in both developed and developing nations. T2DM accounts for over 90% of cases globally [1, 2]. According to the World Health Organization (WHO), in 2011, approximately 364 million people globally suffer from diabetes (DM), with projections that DM-related deaths will double from 2005 to 2030 [3]. In 2004, 3.4 million people died directly from the consequences of high blood glucose. The prevalence of DM worldwide was calculated as 2.8% in 2000. This is expected to increase to 4.4% by 2030 [4]. The growing concern is the epidemic growth in obesity and increase in the elderly population, which will continue to increase the prevalence of DM. Another study, using data from 91 countries, estimates that the prevalence can be as high as 7.7% (439 million adults) by 2030 [2]. Other estimates include a 70% increase in DM in developing countries and 20% increase in developed nations.

In the United States, T2DM is quickly becoming an epidemic. The Center for Disease Control (CDC) estimates that in the United States alone, 25.8 million Americans, or 8.3% of the population, suffer from DM, with 7 millions currently undiagnosed [5]. DM is higher, 26.9%, in the elderly (65 years or older). But it is also rapidly becoming a disease observed in younger patients with almost 2 millions over the age of 20 being newly diagnosed with DM in 2010. More alarmingly, 35% of adults over the age of 20 and 50% of elderly had prediabetes. This equates to 79 million people in the US. DM is the primary cause of renal failure, non-traumatic lower-limb amputations, and newly diagnosed retinopathy. DM is the 7th leading cause of mortality of Americans.

1.1. Current Pharmacological Agents in the Treatment of T2DM. T2DM is a chronic disease that affects millions of people globally and is associated with multiple comorbidities and complications. DM education, prevention, and care are complex and should be designed to be patient specific. Physicians, nurses (and nurse practitioners), pharmacists,

and dieticians are often recruited as a balanced health-care team in managing a patient's diabetes. The American Diabetes Association (ADA) promotes diabetes self-management education, a process in which the patient is equipped with the knowledge and skills to provide self-care, manage crisis (severe hyperglycemia and hypoglycemia), and make lifestyle changes [6, 7].

Primary non-pharmacological interventions include appropriate diet and exercise. Diet should be balanced and aimed to reduce weight. At least thirty minutes of moderate to intense exercise can improve T2DM and weight management. Intense lifestyle modifications (LSMs) are the mainstay of all treatment modalities and should be encouraged in both populations who are at risk for developing diabetes and patients who are suffering from diabetes. For patients requiring pharmacological interventions of T2DM, metformin, a biguanide, is first-line treatment for most patients who are unable to achieve their glycemic goals with LSM. Used for years, metformin increases glucose uptake by the skeletal muscles [8], inhibits hepatic gluconeogenesis [9], and increases insulin sensitivity [10] (summarized in Table 1). Not only is metformin the first-line recommendation for the treatment of T2DM, but there is also evidence of metformin being a useful agent in preventing T2DM in high-risk populations [11].

Sulfonylureas are a commonly used second-line class of antidiabetic drugs which increases insulin secretion by binding to K_{ATP} (potassium) channels of the β -islet cells in the pancreas [12]. Second-generation sulfonylureas are largely used due to their potency, fewer drug interactions, and less severe adverse reactions [6]. Insulin, which has long been considered last-line therapy in the treatment of T2DM and is the primary treatment of Type 1 Diabetes Mellitus (T1DM, insulin-dependent DM), is now a viable addition to metformin as a second-line agent in lieu of sulfonylureas [6, 7]. Insulin is effective in reducing blood glucose and HbA_{1C}. Insulin regimens are patient-specific and can involve various combinations.

Other less validated classes of medications that can be added to metformin include the thiazolidinediones (TZDs) and GLP-1 agonists (glucagon-like peptide-1). TZDs (pioglitazone, rosiglitazone) act by modulating peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear receptor involved in the regulation of glucose and lipid metabolism. Activation of PPAR γ leads to increased insulin sensitivity primarily in adipose tissue but has also shown to have an effect on skeletal muscle and liver [13]. In the United States, TZDs carry a black box warning of increased cardiovascular events due to a trial that demonstrated an increased risk of myocardial events with rosiglitazone [14]. GLP-agonists (exenatide, liraglutide) are peptides derived from naturally occurring incretin hormones produced in the small intestines after meals [15]. It binds to GLP-1 receptors in the pancreas to stimulate insulin secretion and suppress glucagon secretion. The meglitinides class (repaglinide, nateglinide) has a similar mechanism to sulfonylureas [16] but binds to a different site from sulfonylureas on the K_{ATP} channels of the β -islet cells in the pancreas, also stimulating insulin release. There is a reduced risk of hypoglycemia with the meglitinides. α -Glucosidase inhibitors (acarbose, miglitol)

work primarily in the gut by inhibiting α -glucosidase enzymes on the intestinal brush border. α -Glucosidase is a key enzyme for breaking down carbohydrates such as starch, dextrin, and disaccharides for absorption [17]. α -Glucosidase inhibitors may also stimulate GLP-1 secretion. Dipeptidyl peptidase-4 (DPP-4) is a protease enzyme responsible for the inactivation of hormones GLP-1 and GIP (gastric inhibitory peptide) [18]. Inhibition of DPP-4 by inhibitors (sitagliptin, saxagliptin, linagliptin) increases endogenous levels of GLP-1. Endogenous amylin and its analogues (pramlintide) bind to amylin receptors in the brain [19]. Endogenous amylin is secreted along with insulin from pancreatic β -islet cells. Pramlintide delays gastric emptying, reducing postprandial glucose levels [20].

1.2. Pharmacological Prevention of T2DM. The prevention of T2DM in patients primarily focuses on education, diet, and exercise. While the use of pharmacological approaches for prevention is not routinely practiced, the ADA recommends that health-care practitioners consider the use of metformin in patients who are at high risk for developing diabetes. In the Diabetes Prevention Program (DPP) trial, metformin 850 mg twice daily was given to female patients considered at risk for developing DM [11]. One group was administered metformin, another group underwent intensive LSM, the last group was given placebo medication. After a four-year study, the incidence of DM was decreased by 58% ($P < 0.001$) with the LSM group and 31% ($P < 0.001$) with the metformin-treated population when compared to placebo. As such, metformin is the only current medication that has been advocated to be used in the prevention of diabetes in high-risk populations such as those with a history of gestational diabetes, morbidly obese, and those with progressive hyperglycemia [6, 21].

The Troglitazone in Prevention of Diabetes (TRIPOD) study demonstrates preservation of pancreatic β -islet cell function [22]. TZD (troglitazone) was administered in high-risk Hispanic women as identified with the development of gestational diabetes within the previous four years. In women receiving 400 mg troglitazone for 30 months, the cumulative incidence of diabetes was reduced significantly in treated women (5.4%) compared to placebo (12.1%; $P < 0.01$). Troglitazone was discontinued in the USA in 1998 due to potential liver damage associated with the drug.

Over 1300 patients with impaired glucose tolerance in a multi-center study were selected for the STOP-NIDDM trial and given either acarbose three times daily or placebo [23]. After treatment for an average of 3.3 years, 17% of the patients in the acarbose-treated group developed diabetes compared to 26% in the placebo group ($P = 0.001$).

Native Asian Indians with impaired glucose tolerance (IGT) enrolled in the Indian Diabetes Prevention Programme (IDPP-1) study received placebo, LSM, metformin, or LSM plus metformin [24]. Patients were followed for three years, and the cumulative 3-year incidences of diabetes were 39.3% with LSM (relative risk reduction [RRR] = 28.5%, $P = 0.018$), 40.5% with metformin (RRR = 26.5%, $P = 0.029$), and 39.5% with LSM plus metformin (RRR = 28.2%, $P = 0.22$). Results demonstrated that LSM or metformin alone can significantly

TABLE I: Comparison of various mechanisms of action of Western medications with TCM and TIM herbs. Numbers in parenthesis corresponds with mechanisms of action depicted in Figure 1.

	Inhibition of carbohydrate absorption (1)	Increased peripheral glucose uptake (2)	Activation of PPAR (3)	Increased insulin receptor expression (4)	Increased insulin receptor sensitivity (4)	Decreased peroxidation or apoptosis of β -cells (5)	Stimulation of insulin secretion (6)	Decreased gluconeogenesis/glycogenolysis (7)	Suppression of glucagon (8)	Delayed gastric emptying
Western medications										
Biguanides	*			*			*			
Sulfonylureas				*			*			
Thiazolidinediones		*								
GLP-agonists			*				*			
Meglitinides				*			*			
α -glucosidase inhibitors	*									
DPP-4 inhibitors			*				*			
Amylin							*			
Herbs										
<i>G. sylvestre</i>				*						
<i>M. charantia</i>	*			*						
<i>F. Mori</i>		*		*			*			
<i>T. foenum-graecum</i>			*				*			
<i>Ridix Rehmanniae</i>				*			*			
<i>S. tetrandra</i>					*					
<i>Rhizoma coptidis</i>	*				*					
<i>Radix astragali</i>			*		*					
<i>E. japonica</i>				*						
<i>G. biloba</i>	*				*					
<i>Radix ginseng</i>				*			*			
<i>Fructus schisandrae</i>					*		*			
<i>P. lobata</i>	*					*				
<i>C. officinalis</i>	*					*				
<i>B. racemosa</i>	*					*				
<i>S. cuminii</i>	*					*				
<i>T. cordifolia</i>	*					*				
<i>O. basilicum</i>	*					*				
<i>B. aristata</i>						*				

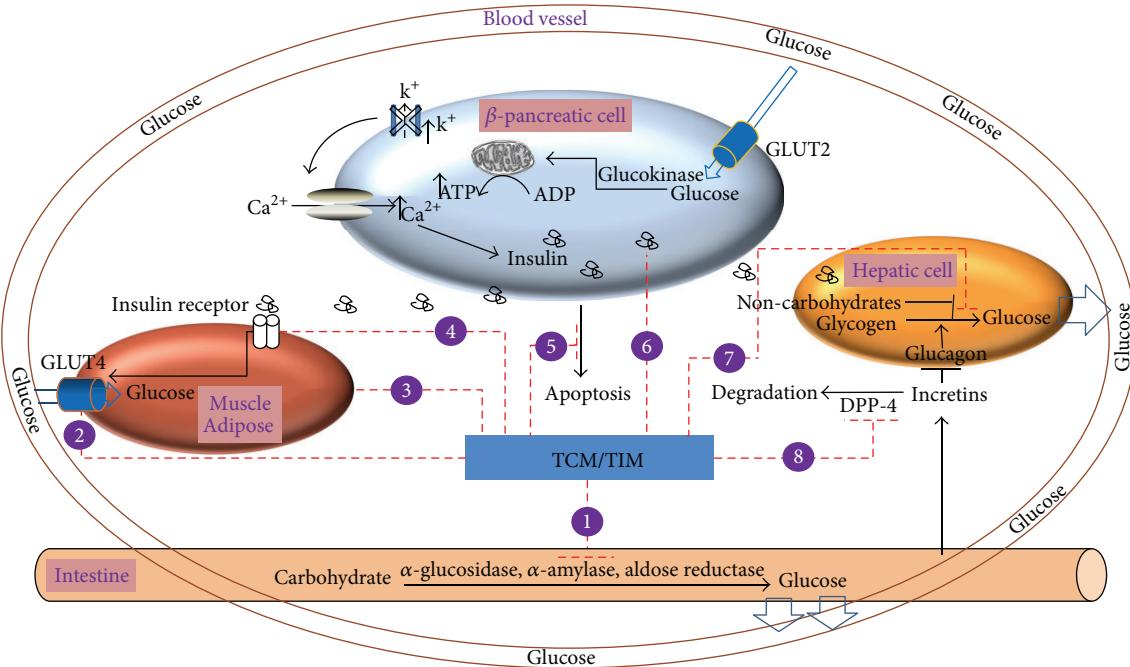


FIGURE 1: Mechanisms of antidiabetic effect of TCM and TIM herbs. (1) Reduced carbohydrate absorption, such as inhibition of α -glucosidase, α -amylase, and aldose reductase, (2) increased glucose uptake in muscle and adipose tissues, (3) activation of PPAR, (4) increased insulin sensitivity/upregulation of receptor expression, (5) exertion of antioxidant effects and decreasing β -cell apoptosis, (6) stimulation of β -cell insulin secretion, (7) inhibition of hepatic gluconeogenesis/glycogenolysis, and (8) prevention of endogenous incretins from degradation/suppression of glucagon. The numbered mechanisms of actions correspond to Table 1.

lower the incidence of diabetes, but the combination of LSM and metformin did not display any added benefit.

The IDPP-2 study recruited native Asian Indians with IGT and received LSM plus placebo or LSM plus pioglitazone. Followup three years later did not show improvements or reduction in the development of T2DM [25]. The cumulative risk was 29.8% in the pioglitazone group and 31.6% in the placebo group.

In the DREAM trial (Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication), rosiglitazone was administered in hopes of preventing T2DM [26] in patients with IGT or impaired fasting glucose (IFG). Patients were followed for a median of 3 years. The incidence of DM in the rosiglitazone treatment group was 10.6% and 25% in the placebo group ($P < 0.0001$). The risk of T2DM or death was reduced by 60% in patients who have a high risk of developing T2DM. Heart failure, which is a concern of rosiglitazone, was 0.5% in the rosiglitazone arm compared to 0.1% ($P = 0.01$) in the placebo arm.

The NAVIGATOR (Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research) study group randomized patients with IGT to receive nateglinide or placebo with a median followup of 5 years [27]. The cumulative incidence of diabetes was nonsignificant in the nateglinide group (36%) compared to the placebo group (34%; $P = 0.05$).

The effects of low-dose combination of metformin and rosiglitazone were examined in patients with IGT in the CANOE (Canadian Normoglycemia Outcomes Evaluation)

trial [28]. The median followup was 3.9 years and demonstrated that this combination was effective in reducing the incidence of developing DM in the treatment group (14%) compared to the placebo group (39%; $P < 0.0001$), with a relative risk reduction of 66%. A significant reduction in insulin sensitivity in the placebo group (-1.24) compared to the treatment group (-0.39 ; $P = 0.0006$) was also observed.

Orlistat, a gastrointestinal lipase inhibitor used in the treatment of obesity, was used in the XENDOS (Xenical in the Prevention of Diabetes in Obese Subjects) trial [29]. Patients were recruited on the basis of BMI (body mass index) $>30 \text{ kg/m}^2$, which is classified as obese. Approximately 21% of the patients exhibited IGT in both the orlistat treatment group and the placebo group. The results of the four-year study showed the cumulative incidence of diabetes to be 6.2% in the orlistat-treatment group and 9.0% in the placebo group (37.3% risk reduction; $P = 0.0032$).

1.3. Traditional Chinese Medicine (TCM) and Traditional Indian Medicine (TIM) for Treatment and Prevention of DM. Although there are currently a number of effective Western T2DM medications available for treatment, management of T2DM using medications with fewer side effects at lower costs is still a big challenge. These medications frequently have side effects, such as weight gain, bone loss, and increased risk of cardiovascular events [30]. These side effects could become more prevalent due to continuous use. Furthermore, treatment is very costly as well, since T2DM is a chronic disease and long-term medications are necessary. Herbal

medications can be a good alternative to replace or at least supplement to Western medications [31–34]. The Indian and Chinese cultures have had several thousand years of history and experience in the prevention and treatment of T2DM with herbal medicine. As later discussed, several herbal medications have been proven to be clinically effective. Because herbal medicines are usually derived from natural plants, they are considered to be relatively safe and have fewer side effects compared to the conventional drugs.

Herbal medications treating T2DM can target multiple mechanisms including enhancement of insulin sensitivity, stimulation of insulin secretion, or reduction of carbohydrate absorption [31]. Unlike Western medicine which usually contains a single active ingredient aiming for a specific mechanism, herbal concoctions may contain various active ingredients targeting multiple mechanisms. Herbal medicine is based on the holistic theory, which puts an emphasis on the integrated body. Western drugs are typically more potent than herbal medicine in lowering blood glucose levels. However, herbal supplements have shown to be able to treat diabetic complications [35]. Thus herbal medicine can also be used as supplementation or in combination with the Western medicine to improve better therapeutic outcomes.

In the Chinese and Indian cultures, traditional medicine has long been the foundation in the treatment and prevention of many diseases. Approximately 800 plants have been identified in the treatment or prevention of T2DM. Many formulations are present as a single herbal extract or in a complex formula. Over 400 extracts have shown to be effective *in vitro* or *in vivo* [32]. The pharmacological mechanisms of the herbs can be classified as (1) decreasing carbohydrate absorption, (2) improving insulin sensitivity, (3) increasing peripheral glucose uptake, (4) stimulating insulin secretion, (5) potentiating endogenous incretins, (6) exerting antioxidant effects and decreasing cell apoptosis, and (7) increasing the glycosogenesis or inhibiting hepatic glycogenolysis (Figure 1) [31, 32, 36]. Since many formulations contain multiple extracts and compounds, each herbal preparation may contain multiple mechanisms. In the following sections, we summarize the Chinese and English literature and list the most effective TCM and TIM herbal preparations under these identifiable mechanisms. Most of the studies have been conducted using *in vitro* systems and diabetic animals. However, there has been an increase in randomized placebo-controlled clinical trials testing the effectiveness of various TCM and TIM in both healthy and T2DM patients (Table 2).

2. Commonly Used TCM/TIM for T2DM

2.1. Herbs in Both TCM and TIM

2.1.1. *Gymnema sylvestre* Schult (syn. *Periploca sylvestris* Retz). *Gymnema sylvestre* Schult, belonging to genus *Gymnema* and family of Apocynaceae, grows in the tropical forests of southern and central India, southern China, Vietnam, Australia, and African countries. The leaves of *G. sylvestre* have been used for treatment of diabetes, hypercholesterolemia, joint pain, and snake bites in India and China [37, 38]. The leaf

extract of the *G. sylvestre* has also been marketed as herbal supplements for diabetic patients [39]. The major chemical components are gymnemic acids I–VII, triterpenoid saponins (gymnemosides A–F and gymnoside W1–2), conduritol A, and dihydroxy gymnemic triacetate.

The major bioactive constituents are gymnemic acids, a group of oleanane-type triterpenoid saponins including gymnemic acids I–VII, gymnema saponins and their derivatives such as deacylgymnemic acid (DAGA) which is the 3-O-glucuronide of gymnemagenin (3,16,21,22,23,28-hexahydroxy-olean-12-ene) [38].

In alloxan-induced diabetic mice, body weight as well as pancreas and liver weight were increased by oral administration of the leaf or callus extract of *G. sylvestre* at a dose of 200 mg/kg [39]. The effect of the extracts was similar to 4 unit/kg of insulin. Hepatic glycogen levels were also increased (2.15 to 2.47 mg/g versus 1.35 mg/g for the extracts and control, respectively), which in turn could stimulate the secretion of insulin. In streptozotocin (STZ)-induced DM rats, the hexane, acetone, and methanol extracts decreased plasma glucose levels. The acetone extract was found to be most potent. Oral administration of 600 mg/kg of the acetone extract for 45 days decreased the glucose level from 443 to 114 mg/L. Dihydroxy gymnemic triacetate was identified to be the major active component; at 5–20 mg/kg, it showed significant effects on lowering blood glucose level by increasing plasma insulin levels. *G. sylvestre* extracts were shown to be able to regenerate pancreatic β cells and increase circulating insulin level by stimulating its secretion [40].

In one small clinical study, the fasting blood glucose (FBG) and HbA_{1C} levels were improved in T2DM patients after receiving 200 mg of ethanolic extract of *G. sylvestre* either daily or their usual treatment for 18 to 20 months [41]. In a second clinical trial, the subjects showed reduced polyphagia, fatigue, blood glucose (fasting and postprandial), and HbA_{1C} in comparison to the control group following an oral dose of 500 mg of herbal extract for a period of 3 months [42]. In an uncontrolled trial involving 65 patients with T1DM and T2DM, the FBG and HbA_{1C} levels were decreased 11% and 0.6%, respectively, after oral dose of 800 mg daily of *G. sylvestre* extract [41].

2.1.2. *Momordica charantia*. *Momordica charantia*, a tendril-bearing vine belonging to the Cucurbitaceae family (also known as bitter melon/gourd, karela, or balsam pear), is a popular plant used for the treatment of diabetes in China, South America, India, the Caribbean, and East Africa [32, 43].

In *M. charantia* seeds, the major components have been identified to be eleostearic acid and stearic acid, which account for approximately 45% of total weight. Several glycosides, such as charantin and vicine, were isolated from the *M. charantia* stem and fruit. Other components include polypeptide-p, lipids, triterpenoids, and alkaloids [43].

The methanol extract of *M. charantia* exhibited hypoglycemic effects in diabetic male ddY mice at a dose of 400 mg/kg. *M. charantia* can also suppress glucose tolerance and postprandial hyperglycaemia in rats [43] by inhibiting

TABLE 2: Recently completed and current clinical trials of herbs.

Herb	Trial name	Status	Sponsors	Clinical trial no.
<i>Gymnema sylvestre</i> Schult.	Double Blind Randomized Trial to Compare Gurmar (<i>Gymnema sylvestre</i>) with Metformin in Type 2 Diabetes	Status currently unknown	(1) Postgraduate Institute of Medical Education and Research (2) Indian Council of Medical Research (3) International Clinical Epidemiology Network (INCLEN) TRUST	NCT00396851
<i>Momordica charantia</i>	The Effect of Metamin 3D on the Lipid and Glucose in Subjects with Metabolic Syndrome	Completed 2009	Taichung Veterans General Hospital, Taiwan	NCT01120873
<i>Folium mori</i> (1-deoxynojirimycin extract)	Effect of Mulberry Leaf Extract on Blood Glucose	Completed 2011	(1) Ewha Womans University (2) Bundang CHA Medical Center (3) Ministry of Knowledge Economy, Korea	NCT01385865
<i>Trigonella foenum-graecum</i> L.	Effect of Fenugreek on Blood Sugar and Insulin in Diabetic Humans	Completed 2008	(1) Pennington Biomedical Research Center (2) Louisiana State University Health Sciences Center in New Orleans	NCT00597350
<i>Rhizoma coptidis</i>	Trial of Different Dosages' Ge Gen Qin Lian Decoction in the Treatment of Type 2 Diabetes	Currently recruiting patients	Guang'anmen Hospital of China Academy of Chinese Medical Sciences	NCT01219803
<i>Rhizoma coptidis</i> (berberine extract)	Efficacy and Safety of Berberine in the Treatment of Diabetes with Dyslipidemia	Completed 2006	Shanghai Jiao Tong University School of Medicine, China	NCT00462046
	Therapeutic Effects of Berberine in Patients with Type 2 Diabetes	Completed 2004	(1) Shanghai Jiao Tong University School of Medicine (2) National Institutes of Health (NIH)	NCT00425009
<i>Ginkgo biloba</i>	Ginkgo Biloba Extract and the Insulin Resistance Syndrome	Completed 2005	National Center for Complementary and Alternative Medicine (NCCAM)	NCT00032474
<i>Radix ginseng</i> Mey (ginsenosides extract)	A Clinical Trial of Ginseng in Diabetes	Completed 2008	Washington University School of Medicine	NCT00781534

Data retrieved from the U.S. National Institutes of Health (<http://clinicaltrials.gov/>).

the absorption of carbohydrates from the gastrointestinal tract.

Leung et al. reviewed clinical trials examining the hypoglycemic effects with *M. charantia* in T2DM patients. However, contradictory clinical outcomes were observed among these trials, probably due to poor methodological design without baseline characterizations along with non-standardized extraction method [43]. Nevertheless, the *M. Charantia* juice from the fresh fruit showed glucose-lowering effects in T2DM patients [44, 45], but not the extract from the dried fruit [46].

The extract of *M. charantia* using ethyl acetate was able to activate peroxisome proliferator-activated receptors (PPAR α and γ) and upregulate the expression of the acyl CoA oxidase gene in H4IIEC3 hepatoma cells. The suppression of peroxidation and apoptosis resulted in improvements in β -cell function and enhanced insulin excretion. In adipocytes, the momordicosides from *M. charantia* stimulated glucose

transporter-4 (GLUT4) translocation to the cell membrane and increased the activity of adenosine monophosphate-activated protein kinase (AMPK), which could enhance glucose uptake from the blood. Animal studies showed that the extract could also enhance insulin sensitivity and lipolysis. In STZ rats, gluconeogenesis was inhibited by *M. charantia* via downregulation of hepatic glucose-6-phosphatase (G6P) and fructose-1,6-bisphosphatase activities [47].

2.1.3. *Morus alba* L. The mulberry tree (*Morus alba* L.) grows widely in Asian countries, and various constituents of its leaves, *Folium mori*, have been applied clinically in TCM [48] as hypoglycemic, hypotensive, and diuretic agents. *Folium mori* have been traditionally used to treat hyperglycemia. The main bioactive components are flavonoids, alkaloids (1-deoxynojirimycin), and polysaccharides [33].

In T2DM mice (high sucrose-fed KK-Ay mice), *Folium mori* extract reduced insulin resistance following 8-week

treatment. Both FBG levels and urinary glucose levels were significantly lowered in mice fed with a diet supplemented with *Folium mori* extract in a dose-dependent manner [49]. In Goto-Kakizaki rats, a spontaneous nonobese animal model for T2DM, the *Folium mori* extract demonstrated reduced postprandial blood glucose levels [50]. In human subjects, it showed that a food-grade mulberry powder enriched 1-deoxynojirimycin suppressed postprandial blood glucose surge [50, 51].

In vitro cell studies showed that in adipocytes, *Folium mori* extract increased glucose uptake and thus enhanced the translocation of GLUT-4 with concentrations ranging from 5 to 45 mcg/mL [52, 53]. In db/db mice, the extract ameliorated adipocytokines in white adipose tissue possibly due to the inhibition of oxidative stress [52]. One of the alkaloids, 1-deoxynorimycin, is also a potent inhibitor of α -glucosidase [54].

2.1.4. *Trigonella foenum-graecum* L. The fenugreek is an annual plant in the family Fabaceae. The fenugreek seed was a traditional remedy used by ancient Egyptians and spread to Asian countries such as China and India [55]. Fenugreek seeds are a rich source of the polysaccharide galactomannan and also contain saponins such as diosgenin, yamogenin, gitogenin, tigogenin, and neotigogens. Other active constituents include mucilage, volatile oils, and alkaloids [56, 57].

The hypoglycemic effects of *T. foenum-graecum* in rats were firstly reported in 1974 [58]. Soon afterwards, the amino acid 2S,3R,4S, 4-hydroxyisoleucine, purified from fenugreek seeds, showed insulinotropic effects which increased peripheral glucose uptake *in vitro* [59–61]. The activities of hepatic enzymes hexokinase, glucokinase, G6P, and fructose-1,6-bisphosphatase were reduced in DM rats [62, 63]. Plasma glucose levels decreased after receiving the *T. foenum-graecum* extract in both non-DM patients and DM patients [64, 65]. Insulin levels were significantly higher in the fenugreek treatment group in comparison to the placebo treatment [66]. A meta-analysis of *T. foenum-graecum* showed that the herb may reduce HbA_{1C} by 1.13% ($P = 0.03$) [67].

2.2. Other Herbs in TCM

2.2.1. *Radix rehmanniae*. *Radix rehmanniae* is the root of *Rehmannia glutinosa* Libosch, under the family of Scrophulariaceae or Gesneriaceae. It has been widely used for treatment of diseases relating to blood, immune, endocrine, nervous, and cardiovascular systems.

The bioactive components of *Radix rehmanniae* include catalpol, rehmannioside A, B, C, and D, phenethyl alcohol derivatives such as leucosceptoside A and purpureaside C, monocyclic sesquiterpenes as well as their glycosides [68].

Radix Rehmanniae showed hypoglycemic activity in normal and STZ-induced DM mice. In Chinese medicine, it is usually prepared in combination with other herbs such as *Radix ginseng*, *Radix scutellariae* [69], and *Radix astragali* [70]. These combinations stimulated insulin secretion and β -cell proliferation through insulin receptor substrate 2 induction. It also showed improvements in diabetic foot ulcer

healing in rats through the processes of tissue regeneration, angiogenesis, and inflammation control [70]. The postulated mechanisms of action are stimulation of insulin secretion, regulation of glucose metabolism in DM rats, and reduction of hepatic glycogen content of non-DM mice [31, 71].

2.2.2. *Stephania tetrandra* Moore. *Stephania tetrandra* Moore is an herbaceous perennial vine of the Menispermaceae family, which is a fundamental herb used in TCM for the reduction of swelling and also providing an analgesic effect. The root of *S. tetrandra* has demonstrated to have anti-inflammatory, anti-allergic and hypotensive effects in experimental animal studies [72].

The major components are alkaloids, including tetrandrine, fangchinoline, bisbenzylisoquinoline, protoberberine, morphinan, and phenanthrene [73]. At 0.3–3 mg/kg, fangchinoline significantly decreased blood glucose and increased blood insulin in STZ-mice by potentiating insulin release [31]. In another study, formononetin, one of the active components in *Radix astragali*, potentiated the effect of *S. tetrandra* on lowering the blood glucose level and increasing the blood insulin level, although no direct anti-hyperglycemic effect of formononetin was observed [72]. The postulated antidiabetic mechanism of *S. tetrandra* extract is the stimulation of insulin release in pancreatic β -cells [72, 74].

2.2.3. *Rhizoma coptidis*. *Rhizoma coptidis* is the rhizome of *Coptis chinensis* Franch that belongs to the Ranunculaceae family, recorded as *Coptidis Rhizoma* (CR) in the Chinese Pharmacopeia with the Chinese name of Huang Lian. It has been widely used to clear heat, dry dampness, and eliminate toxins from the body. It is also a commonly used herb in various formulas against intestinal infections, diarrhea, inflammation, hypertension, and hypoglycemia.

The most well-known components of *Rhizoma coptidis* are isoquinoline alkaloid and berberine [75] which has variety of biological activities such as tumor reduction, antimicrobial, anti-Alzheimer's disease, anti-hyperglycemic, anti-inflammatory, and anti-malarial [76]. The berberine compounds of *Rhizoma coptidis* have been studied for its anti-hyperglycemic effects. The other alkaloids include palmatine, jateorrhizine, epiberberine, and coptisine.

Both the extract and pure berberine significantly decreased blood glucose and serum cholesterol levels in high fat diet-fed mice at the dose of 200 mg/kg by gavage. In alloxan-induced diabetic mice, berberine showed an anti-hyperglycemic effect and also blunted blood glucose increase induced by intraperitoneal glucose or adrenaline administration in normal mice. The activity of berberine was similar to sulfonylureas or biguanides [31, 77].

The anti-hyperglycemic effects of berberine could be due to the improvement of insulin sensitivity by activating the AMPK pathway or inducing insulin receptor expression. Berberine could also improve fatty acid oxidation via activation of AMPK and acetyl-CoA carboxylase. Furthermore, six quaternary protoberberine-type alkaloids of berberine inhibited aldose reductase activity *in vitro* with an IC₅₀ less

than 200 μM [77, 78]. However, no evidence from *in vivo* studies is available to verify this mechanism.

2.2.4. *Radix astragali*. *Radix astragali*, Chinese name of Huang Qi, is the dried root of perennial herbs *Astragalus membranaceus* (Fisch.) Bunge and *Astragalus mongholicus* (Fabaceae) Bunge of the Leguminosae family and grows in northern China. The major active compounds in *Radix astragali* are isoflavones and isoflavanoids (formononetin, calycosin, and ononin), saponins (astragaloside IV, astragaloside II, astragaloside I and acetylastragaloside), and astragalus polysaccharides [79].

Radix astragali possesses a broad spectrum of effects such as immunostimulation, hepatoprotection, diuresis, analgesia, expectorant, and sedation. In traditional Chinese medicinal theory, the herb is capable of consolidating the exterior of the body and can alleviate heat in the muscles by ascending positive qi [70, 80].

After treating DM Sprague-Dawley rats with *Radix astragali* decoction (500 mg/kg IP daily) for two months, improvements in insulin sensitivity and attenuation of fatty liver development were observed. However, blood glucose levels, β -cell function, and glucose tolerance were not substantially improved. *Radix astragali* polysaccharides reduced hyperglycemia and led to indirect preservation of β -cell function and mass via immunomodulatory effects in T1DM mice. In addition, its polysaccharides restored glucose homeostasis in T2DM mice/rats by increasing insulin sensitization. Formononetin, calycosin and ononin might exert a synergistic hypoglycemic effect with fangchinoline in STZ-diabetic mice, most likely by increasing insulin release [70, 80].

2.2.5. *Eriobotrya japonica* Lindl. The loquat *Eriobotrya japonica* Lindl., a fruit tree in the family Rosaceae, is indigenous to central and south China. The dried leaves of *E. japonica*, also called *Folium eriobotryae*, have been used for treatment of chronic bronchitis, cough, and diabetes. The active compounds in *E. japonica* are identified to be triterpenes, sesquiterpenes, flavonoids, megastigmane glycosides and polyphenolic compounds including ursolic acid, oleanolic acid, cinchonain Ib, procyanidin B-2, chlorogenic acid, and epicatechin [81–83].

In an *in vitro* study using insulin receptor substrate-1 cells, the aqueous extract and the cinchonain Ib (one of the components in *E. japonica*) enhanced insulin secretion in a dose-dependent manner [84]. *In vivo* studies showed that the aqueous extract of *E. japonica* could transiently reduce blood glucose levels [84]. The 70% ethanol extract exerted a significant hypoglycemic effect on alloxan-diabetic mice following oral doses of 15, 30, and 60 g/kg (crude drug). The total sesquiterpenes were found to significantly lower blood glucose levels in both normal and alloxan-diabetic mice [85]. Shih et al. found that the extract, with major components of tormentic acid, maslinic acid, corosolic acid, oleanolic acid, and ursolic acid, could ameliorate high fat induced hyperglycemia, hyperleptinemia, hyperinsulinemia and hypertriglyceridemia [86]. Another study found that the co-fermentation of *Folium eriobotryae* and green tea leaf

reduced the blood glucose level by 23.8% within 30 min in maltose-loaded SD rats at a dose of 50 mg/kg, although this effect was not observed in the sucrose- and glucose-loaded rats [87].

2.2.6. *Ginkgo biloba*. The *Ginkgo biloba* tree, native to China, dates back to the prehistoric ages of 250–300 million years and is frequently called a “living fossil” [88]. Today, the biloba tree can live more than 1,000 years [89]. In the United States, *G. biloba* is one of the most frequently used over-the-counter (OTC) herbal supplements [90]. Extract from its leaves contains ginkgo flavonoid glycosides, terpene lactones, and ginkgolic acids. Many human clinical trials have examined possible ginkgo uses in cerebrovascular disease, tinnitus, sexual dysfunction, intermittent claudication, migraine prophylaxis, and alleviating symptoms of the common cold [15, 91–94]. However, the most common use of ginkgo is the prevention and treatment of Alzheimer’s disease and dementia [95–100].

The administration of the ginkgo extract, EGb 761, in rats with DM increased glucose uptake into hepatic and muscle tissues [101] and decreased atherogenesis, a common comorbidity of DM [102]. *In vitro* assays determined the possible anti-diabetic effect of ginkgo to be through the inhibition of α -glucosidase and amylase activities [103]. Clinical investigations of the anti-diabetic properties of ginkgo in humans have produced mixed results. Healthy human subjects showed no reduction in blood glucose levels with an accompanying significant increase in plasma insulin levels [104]. The randomized double-blinded clinical study involving non-DM, pre-T2DM, and T2DM patients showed that ginkgo did not increase insulin sensitivity nor reduced blood glucose levels [105, 106]. However, in T2DM patients, ingestion of *G. biloba* extract showed increased clearance of insulin, resulting in a reduction plasma insulin levels and elevated blood glucose. Gingko may improve endothelial function in T2DM patients with early stages of nephropathy, but without affecting blood glucose levels [107]. While popular for its many possible indications, gingko appears to have limited anti-diabetic properties to warrant its use in diabetes.

2.2.7. *Radix ginseng*. *Radix ginseng* is native in the northern hemisphere, most notably, in eastern Asia (northern China, Korea, and eastern Siberia) and northern America. Subsequently, ginseng is often named from its origin—Asian ginseng, American ginseng, Chinese ginseng, to name a few. More than 700 compounds have been identified in ginseng, with the most active components being identified as the ginsenosides (Rb1, Re, Rd), polysaccharides, peptides, and polyacetylenic alcohols [108]. The geographical origin of ginseng, in combination with the extraction and processing method, produces variable anti-diabetic results [32, 108, 109].

The anti-diabetic activity of *Ridix ginseng* has been explored in both animal and human studies. Hypoglycemic activity is greater in lipophilic extracts than aqueous extracts. In DM rats, Korean ginseng (0.1–1.0 g/mL) stimulated the release of insulin from isolated pancreatic islets. American

ginseng (100 mg/kg) produced lowered levels of serum glucose and HbA_{1C} in DM rats [110]. Vuksan and colleagues have conducted a number of human clinical trials demonstrating that ginseng reduced postprandial blood glucose, fasting blood glucose, and HbA_{1C} levels [111–115]. Similar results were presented at American Diabetes Association Annual Meeting in 2003 [116].

Pharmacologically, ginseng has antioxidant properties. It also reduces β -cell apoptosis by upregulating adipocytic PPAR- γ protein expression [117]. Ginseng impairs glucose absorption by decreasing glucosidase activity [118]. It may also increase insulin sensitivity in peripheral tissues [32]. One of the active components, ginsenoside Rb1, can enhance glucose transport by inducing the differentiation of adipocytes via upregulating the expression of PPAR- γ and C/EBP- α [119]. In addition, ginsenoside Rb1 can increase GLUT-4 activity leading to increased uptake of glucose from blood by adipocytes [120].

2.2.8. *Fructus schisandrae*. *Fructus schisandrae* (also known as “five-flavor berry” in China), the fruit of a deciduous woody vine native to forests of northern China, is traditionally used as a tonic or sedative agent. It has been used in TCM to astringe the lungs and nourish the kidneys. It was reported that *Fructus schisandrae* can enhance hepatic glycogen accumulation and decrease hepatic triglycerides. It has also been used in various TCM formulas, such as the modified Ok-Chun-San and modified Huang-Lian-Jie-Du-Tang, to treat diabetes [121]. The major chemical components include lignans such as schizandrins (schizandrin A) and gomisins (gomisin A, J, N, and angeloylgomisin H), and polysaccharides [72].

In vitro, gomisin J, gomisin N and schizandrin A increased basal glucose uptake in HepG2 cells [122]. Several other schizandrins were found to be able to prevent β -cell apoptosis and decrease insulin resistance [123]. In an *in vitro* study using 3T3-L1 adipocytes, several fractions of ethanol extract showed the stimulation effect of PPAR- γ . Among these fractions, FS-60, a subfraction from the 70% ethanol extract, was identified to be most potent with the major components of schizandrin A, gomisin A, and angeloylgomisin H. In an *in vivo* study using pancreatectomized DM rats, FS-60 lowered serum glucose levels during the OGTT similar to the level of the fasting stage. During hyperglycemic clamp, FS-60 increased the first phase insulin secretion in diabetic animals [121].

The major mechanisms are hypothesized to be the stimulation of insulin secretion and increased insulin sensitivity by ameliorating insulin resistance via increased PPAR- γ activity. *Fructus schisandrae* can also improve glucose homeostasis in DM mice by inhibiting aldose reductase [121].

2.2.9. *Pueraria lobata* (Gegen). Gegen is the dried root of *Pueraria lobata* (Willd.) Ohwi, a semiwoody, perennial and leguminous vine native to South east Asia, and also known as yegen, kudzu root, and kudzu vine root [124, 125]. For more than 2000 years, gegen has been used as an herbal medicine for the treatment of fever, acute dysentery,

diarrhea, DM, and cardiovascular diseases [126, 127]. Over seventy compounds have been identified in gegen, with isoflavonoids (puerarin) and triterpenoids being the major constituents.

In vitro studies showed that puerarin contained in *P. lobata* can enhance the glucose uptake in a dose-dependent manner performed in high glucose-treated preadipocytes [128]. Puerarin also promoted insulin-induced preadipocyte differentiation and upregulated mRNA expression of PPAR γ , which can regulate glucose homeostasis, adipocyte differentiation, and lipid metabolism [129]. In China, a clinical trial was conducted in DM patients using the Gegen Qin Lian decoction which showed a dose-dependent effect on reducing HbA_{1C} and FBG [130]. Possible mechanisms of action from *in vitro* and *in vivo* studies include α -glucosidase inhibition, increased expression and activity of PPAR- γ , upregulation of GLUT-4 mRNA, increased plasma endorphins, and preservation of pancreatic islets [131–133].

2.2.10. *Cornus officinalis* Sieb. et Zucc. *Cornus officinalis* Zucc., native to China, Japan, and Korea, is a common herbal medicine of the family of Cornaceae. *Fructus corni* is the dried ripe sarcocarp of *C. officinalis* Sieb. et Zucc. Cornaceae, which has been widely prescribed as a tonic agent in Chinese medicinal formula and possess activities of improving the function of the liver and kidney [134]. The major active components are iridoid glycosides, morroniside, loganin, mevaloside, loganic acid, ursolic acid and oleanolic acid, 5-hydroxymethyl-2-furfural, and 7-O-galloyl-D-sedoheptulose [135].

The ethanol extract of *Fructus corni* induced the expression of GLUT-4 by stimulating the proliferation of pancreatic islets, resulting in increased insulin secretion [136]. One of the active components, ursolic acid, was found to be an inhibitor of protein tyrosine phosphatase (PTP) 1B, which sensitizes the effects of insulin [137]. The *Fructus corni* extract decreased blood sugar in STZ mice and reduced renal oxidative stress and glycation products in STZ-induced diabetic rats. The underlying mechanisms include the inhibition of glucosidase, reduction of gene expression for hepatic gluconeogenesis, protection of β -cells against toxic challenges, and enhancement of insulin secretion.

2.3. Other Herbs in TIM

2.3.1. *Barringtonia racemosa*. *Barringtonia racemosa* is an evergreen mangrove tree that grows in Bangladesh, Sri Lanka, and the west coast of India, with the bark and leaves used for snake bites, rat poisoning, boils, and gastric ulcers. The extracts from different parts have various biological activities including anti-cancer, analgesic, anti-DM, anti-bacterial, and anti-fungal activities. Its seeds are aromatic and useful in colic and ophthalmic disorders [138].

Several diterpenoids and triterpenoids have been identified in *B. racemosa* extract and a pentacyclic triterpenoid, bartogenic acid, is the major active component [138, 139]. The hexane, ethanol and methanol extracts as well as

the pure compound of bartogenic acid inhibited intestinal α -glucosidase activity at concentrations ranging from 0.02–0.2 $\mu\text{g}/\text{mL}$ in an *in vitro* enzymatic study. In an *in vivo* rat study, the methanol extract was found to suppress the rise of blood glucose level after receiving maltose [140].

2.3.2. *Syzygium cumini* (L.) Skeels. *Syzygium cumini* (L.) Skeels, frequently referred to as Skeels, is a tropical tree native to India, China, and Indonesia. Skeels is also known as *Eugenia jambolana*, Jamun, Jambu, Black Plum, or Black Berry and has been frequently used to treat DM in India [140] and Brazil [141]. Studies using DM rats showed reductions in blood glucose, post prandial glucose, cholesterol, and free fatty acid [142, 143]. Pharmacologically, the extracts of *S. cumini* have shown α -glucosidase inhibitory activities [144, 145]. Hepatic enzymatic activities of glucokinase and phosphofructokinase, hepatic enzymes which play a role in glucose metabolism, were significantly reduced in DM animals [145, 146]. Adenosine deaminase activity was inhibited in Skeels-treated erythrocytes procured from both DM and non-DM patients. However, clinical trials have not produced favorable results. Two double-blind, randomized trials involving non-DM and DM patients did not support the use of Skeels in DM [147, 148]. In patients with DM consumed tea prepared from *S. cumini* leaves, FBG levels were not reduced significantly.

2.3.3. *Tinospora cordifolia*. *Tinospora cordifolia*, also called Guduchi of the Menispermaceae family, is a succulent climbing shrub, indigenous to the tropical areas of India, Myanmar, and Sri Lanka. The aqueous stem extract is used for curing gastrointestinal pain [149]. *T. cordifolia* also has anti-spasmodic, anti-pyretic, anti-allergic, anti-inflammatory, immunomodulatory, and anti-leprosy activities [150]. The bioactive ingredients are alkaloids (palmatine, jatrorrhizine and magnoflorine), diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds, and polysaccharides.

The anti-DM activity of *T. cordifolia* has been investigated in DM mice. The aqueous and alcoholic extract of the plant can improve glucose tolerance in DM rats. Grover and coworkers found that *T. cordifolia* ameliorated diabetic neuropathy at a dose of 400 mg/kg [151]. The 70% ethanol extract significantly decreased blood glucose levels and attenuated the rate of blood glucose elevation after 2 g/kg glucose loading following an oral dose of 100 or 200 mg/kg of *T. cordifolia* for 14 days [152]. The extract was also found to be able to prevent diabetic retinopathy in STZ diabetic rats at a dose of 250 mg/kg [153].

The anti-DM effect is related to the amelioration of oxidative stress by reducing the production of thiobarbituric acid-reactive substances. The extract can increase the expression of thioredoxin and glutaredoxin. *T. cordifolia* extract can also adjust alter carbohydrate metabolism and reduce gluconeogenesis via inhibiting G6P and fructose 1,6-diphosphatase [154]. Other possible mechanisms examined are the enhancement of the insulin release and inhibition of α -glucosidase [155].

2.3.4. *Ocimum basilicum*. *Ocimum basilicum*, with the common name of basil, or sweet basil, is a culinary herb of the family Lamiaceae (mints), which is sometimes known as Saint Joseph's Wort. Basil was originally from India and widely used in Southern Asian.

Basil is a potent anti-septic and preservative agent and also demonstrates slight sedative effects, regulation of digestion, and diuresis. Clinically, it has been used to treat headache, cough, upper respiratory tract infection, and kidney dysfunction. Laboratory studies have found that basil also has activities in lowering blood sugar, stimulating nervous system, and protection from radiation [156, 157].

The major components of basil consist of apigenin, linalool, and ursolic acid, which previously demonstrated anti-viral activity [158]. Basil improved lipid metabolism in hypercholesterolemic rats [159]. In an *in vitro* cell line study using human macrophages, the ethanol extract of basil reduced cholesterol synthesis [160]. The aqueous extract of *O. basilicum* can inhibit rat intestinal sucrase, maltase, and porcine pancreatic α -amylase activities which may have positive effect for treatment of DM [161]. In a clinical trial in DM patients in India, the basil leaf extract decreased the fasting blood glucose by 21.0 mg/dL, and postprandial blood glucose fell by 15.8 mg/dL. The results suggest that *O. basilicum* may be used as a dietary therapy in mild to moderate T2DM [162].

2.3.5. *Berberis aristata*. *Berberis aristata* (also known as Zarshik, Daruharidra) of the family Berberidaceae is an Ayurvedic herb which has been used since ancient times in South Asia as an herbal tonic agent to improve hepatic and cardiac functions [163, 164].

The main constituents of the root have been identified as berberine, berbamine and palmatine [165]. The extract of *B. aristata* (root) has a strong potential to regulate glucose homeostasis by decreasing gluconeogenesis and oxidative stress. In DM rats, the extract increased the glucokinase and G6P dehydrogenase activities but decreased G6P activity [165]. In patients with sub-optimal glycemic control, HbA_{1C}, basal insulin, insulin resistance, total and low-density lipoprotein cholesterol, and triglycerides were significantly reduced after 90-day treatment with combination of *B. aristata* extract and *Silybum marianum* extract [166].

3. Problems, Challenges, and Opportunities

There are two entirely different approaches in the future research on TCM/TIM. The “sharp shooter” approach is to select a particular plant with a specific activity or a biological target. By using bioactivity-guided isolation and structural elucidation, one can discover a new chemical entity for a specific disease target. Many Western drugs, such as chemotherapeutic agent paclitaxel, were discovered this way, while some others such as metformin were developed upon further structural modification. Obviously, this is a validated approach for drug discovery and development for the treatment of human diseases. On the other hand, instead of targeting a specific receptor or mechanism, one can select

proper combinations of herbs or ingredients, and optimize the outcome of treatment by different combinations and dose regimens. This approach is termed the “shotgun” approach. While it is not necessary to identify the exact active ingredient(s), it is still necessary to have good quality control of the preparations to ensure reproducibility. Certain chemical markers in the preparations can be selected as markers to standardize the raw materials and processing procedures. Once a reproducible preparation is obtained and its biological activity is established, it can be used to treat certain given disease in the general patient population. Because the extract contains multiple components which may interact with multiple disease targets, it might be advantageous to a single chemical entity, especially in the area of disease prevention.

However, pharmaceutical scientists are facing unique challenges in developing herbal products as anti-DM agents.

- (1) *Patentability.* Because herbal medicines derive from natural plants, their active components cannot be patented as novel materials. It is also difficult to patent their usage since much information is already in the public domain. But it is possible to patent a unique combination and/or the extraction process.
- (2) *Product standardization.* This should be achieved via proper control on raw material, extract process and final formulation. Without effective quality control, consistency of the herbal product may be compromised. Improved methods for quality control of herbal products, such as bioactivity-guided pharmacokinetic methods and genomic fingerprinting techniques are promising.
- (3) *Placebo-controlled, randomized clinical trials.* TCM/TIM physician's philosophy to individualize formula for different patients has significantly hindered the systematic scientific investigation according to Western medicine standards. In comparison to their Western counterparts, the anti-DM efficacies of TCM/TIM herbs have not been well studied using randomized, double-blinded clinical trials, although many animal studies have been carried out. However, the implementation of placebo-controlled, randomized clinical trials is a prerequisite for the evidence-based practice of using TCM/TIM preparations for DM prevention.
- (4) *Toxicity and herb-drug interaction.* TCM herbs are generally thought to be relatively safe and with milder side effects. However, their activities are usually not as potent as Western medications. Thus high doses (sometimes as high as 10 g per day) are usually required to achieve optimal therapeutic efficacy. In addition, the toxicity of herbal products cannot be ignored. Most common complaints of herbal supplements ingestion are gastrointestinal related, including stomach upset, diarrhea, constipation, nausea, and vomiting. In addition, more serious adverse effects may also occur. For example, ginseng abuse syndrome is a result of chronic ingestion of excessive amounts of ginseng. This is characterized by hypertension and CNS stimulation, insomnia, and nervousness. There

is also an increased awareness of herb-drug interaction in pharmacokinetics and pharmacodynamics. When used in combination with established anti-DM medications, herbal supplementation may predispose patients at an increased risk of hypoglycemia. For example, *Ginkgo biloba* extract interacts with selective serotonin reuptake inhibitors used in the treatment of depression, resulting in the serotonin syndrome, and with thiazide diuretics resulting in decreased efficacy.

It is encouraging to note that two drugs based on plant extract have been approved by the FDA for the treatment of human diseases. Veregen (Polyphenon E) Ointment is the first prescription botanical drug approved by FDA in 2006. It is an extract of green tea as a prescription drug for the topical (external) treatment of genital warts caused by the human papilloma virus (HPV). More recently, FDA's approval of crofelemer (Fulyzaq) signals the first time an orally administered botanical has received drug approval from the Administration. Crofelemer derived from the latex of the South American sangre de drago tree (dragon's blood, *Croton lechleri*) is the first drug to be approved in the United States to treat HIV-associated diarrhea. With experience gained through the developmental and regulatory processes comes high hope that many TCM/TIM-based anti-DM products will be available for the general population.

4. Conclusions

It is evident that many TCM/TIM herbs possess anti-DM activities by interacting with various proven drug targets where Western drugs interact. Because of their empirically known oral efficacy and safety profiles, nutritional supplement status, multiple components for multiple drug targets, low cost, and easy access, TCM/TIM herbs such as ginseng, mulberry, and *Radix coptidis* are excellent candidates for long-term use for the prevention and treatment of T2DM. During the development stage, product standardization, quality control and assurance, placebo-controlled and randomized clinical trials are essential components that need to be perfected in order to translate their potential into a reality that millions of people could benefit upon.

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Research Article

Phytochemicals and Antioxidant Capacity from *Nypa fruticans* Wurmb. Fruit

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Nypa fruticans Wurmb. is one of the important underutilized fruit of Malaysia, which lacks scientific attention. Total phenolics, flavonoid content, and antioxidant capacities from endosperm extracts of *Nypa fruticans* (unripe and ripe fruits) were evaluated. Endosperm extract of unripe fruits (EEU) exhibited the highest phenolics (135.6 ± 4.5 mg GAE/g), flavonoid content (68.6 ± 3.1 RE/g), and antioxidant capacity. Free radical scavenging capacity of EEU as assessed by 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals showed inhibitory activity of $78 \pm 1.2\%$ and $85 \pm 2.6\%$, respectively. Beta carotene bleaching coefficient of EEU was higher (2550 ± 123), when compared to endosperm extract of ripe fruits (1729 ± 172). Additionally, EEU exhibited high antioxidant capacity by phosphomolybdenum method and ferric reducing antioxidant power values. Eight phenolic compounds from *Nypa fruticans* endosperm extracts were identified and quantified by ultra-high-performance liquid chromatography. Chlorogenic acid, protocatechuic acid, and kaempferol were the major phenolic compounds. Thus this fruit could be used as a potential source of natural antioxidant.

1. Introduction

Antioxidants from plant sources have been an increasing concern to consumers since synthetic antioxidants such as butylated hydroxy toluene (BHT) have restricted usage in foods, due to carcinogenic activity [1]. Epidemiological studies have shown that frequent consumption of fruits and vegetables high in natural antioxidants can lower the incidence of certain types of cancer, cardiovascular diseases, and diabetes [2]. These beneficial effects are related to bioactive

compounds like phenolic acids, flavonoids, anthocyanins, and carotenoids possessing antioxidant activity [3, 4].

The years 2011–2020 are recognized as the “Decade on Biodiversity” by the United Nations to promote the importance and utilization of underutilized foods. Recently, research and development activities on antioxidants from underutilized fruits have become a great priority [5, 6]. They are notable by the fact that they are locally available but universally erratic, and much related information is also limited. Many of these fruits have a wide range of color



FIGURE 1: *Nypa fruticans* (a) and fruit bunch (b).

for skin and pulp with health-promoting benefits. However, many of these fruits are still not familiar due to lack of publicity and promotional campaign [7].

Nypa fruticans Wurmb. (NF) belongs to *Araceae* family and is considered as “underutilized” plant [8]. Other synonym of this plant includes *Cocos nypa* Lour, *Nypa fruticans* Thunb., and *Nypa palm*. It is a monoecious palm found growing in brackish water with upright stem (Figure 1(a)), trunkless with fruits found commonly emerging from the soil (Figure 1(b)). This palm is typically found in India, Malaysia, Indonesia, Philippines, and in some parts of Queensland, Australia [9]. Sap (obtained from the inflorescence stalk) is used as a drink by the indigenous peoples, while young fruits are eaten [10]. The sap is a good source of sugar and used for making sweets, vinegar, beverage, and alcohol production [8]. The fruit is also rich in carbohydrates, fibers, minerals, and vitamin A [11]. Traditionally, leaves, stem, and roots of NF are used to treat asthma, leprosy, tuberculosis, sore throat, liver disease, snake bite, as a pain reliever, and can also be used as sedative and carminative [12, 13]. Recently, stem and leaf methanol extracts of NF have been shown to have antidiabetic and analgesic effect [14]. Till date, no information on antioxidant activity and identification of phytochemicals from endosperm extract of ripe (EER) and unripe fruits (EEU) of NF has been documented. This work is essential, since it would determine the potency of the extract at different maturity levels. Hence, the objective of the present work is to evaluate the antioxidant capacity of EER and EEU of NF and also to identify its phenolic compounds.

2. Materials and Methods

2.1. Chemicals and Reagents. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine (TPTZ), aluminium chloride, tween-20, linoleic acid, chlorogenic acid, protocatechuic acid, kaempferol, rutin, hydroxybenzoic acid, beta carotene, quercetin, gallic acid, BHT, trichloroacetic acid, thiobarbituric acid, sodium azide, tris-HCl buffer, phosphate buffer, and Hepes were obtained from Sigma-Aldrich Co. (MO, USA). Folin-Ciocalteu reagent, trifluoroacetic acid (HPLC Grade), acetonitrile (HPLC Grade), and hydrogen peroxide were

TABLE 1: Physical parameters of *Nypa fruticans* fruit.

	Unripe	Ripe
Whole fruit bunch		
Weight (kg)	7.2 ± 0.19 ^b	16.1 ± 0.16 ^a
Length (cm)	25.5 ± 2.5 ^b	34.5 ± 2.7 ^a
Perimeter (cm)	81.2 ± 4.5 ^b	108.9 ± 6.3 ^a
Individual fruit		
Weight (g)	138.1 ± 5 ^b	159.6 ± 7.7 ^a
Length (cm)	11.1 ± 0.5 ^b	12.9 ± 0.7 ^a
Breadth (cm)	7.8 ± 0.5	8.1 ± 0.3
Endosperm		
Weight (g)	3.6 ± 1.5 ^b	19.6 ± 0.8 ^a
Length (cm)	3.0 ± 0.2 ^b	4.5 ± 0.5 ^a
Perimeter (cm)	5.8 ± 0.7 ^b	10.1 ± 0.5 ^a

For each treatment means in a row followed by different letters are significantly different at $P < 0.05$.

obtained from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade.

2.2. Plant Material. Whole bunch of unripe (Figure 2(a)) and ripe fruits (Figure 2(b)) of *Nypa fruticans* at three and six month’s maturity, respectively, were collected on the 6th of February 2011 from Kedah, Malaysia, with the assistance of Muda Agricultural Development Authority (MADA), Malaysia. Voucher specimens (NFUR622011 and NFR622011) are preserved at MADA office. The fruits were immediately transported to the laboratory of Universiti Putra Malaysia. Upon arrival, the fruits were washed under running tap water and air dried. The individual fruits (Figures 2(c) and 2(d)) were separated from the bunch and physical parameters of the fruits were measured (Table 1). Later, the fruits were manually separated to obtain the edible endosperm (Figures 2(e) and 2(f)). The endosperm (150 g) was kept in an oven maintained at 60°C for drying until constant weight was obtained. The dried portion was allowed to cool and be powdered and sieved (particle size of 20 mesh) to get uniform particle size. The dry powder was used for extraction.



FIGURE 2: *Nypa fruticans* unripe (a) and ripe (b) whole fruit bunch, unripe (c) and ripe (d) individual fruit, and unripe (e) and ripe (f) endosperm.

2.3. Extraction. Dry powder (10 g) from unripe and ripe endosperms of NF was extracted separately with 50% ethanol (100 mL) using an orbital shaker (Unimax 1010, Heidolph, Germany). The shaker was maintained at a speed of 400 rpm and the extraction was carried out at 30°C for 1 h duration. The extracts were then filtered using Whatman filter paper (no. 4), concentrated, freeze dried using bench top freeze dryer (Virtis, NY, USA), and stored at -20°C until further analysis.

2.4. Determination of Total Phenolics Content. Total phenolics content (TPC) of the extracts was determined according to the method developed by Singleton and Rossi [15]. In brief, 100 μ L-aliquot of the extract was added to 2 mL of 20 g/L

Na_2CO_3 solution. After 2 min, 100 μ L of 50% Folin-Ciocalteu reagent was added and the mixture was allowed to stand for 2 h at 25°C. The absorbance was measured at 750 nm using a spectrophotometer (UV 1601, Shimadzu Co., Ltd., Kyoto, Japan). The total phenolics content was determined using the standard gallic acid calibration curve and the results were expressed as milligram gallic acid equivalents per gram sample dry weight (mg GAE/g DW).

2.5. Determination of Total Flavonoid Content. Total flavonoid content (TFC) was measured using the aluminum chloride colorimetric assay described by Liu et al. [16]. An aliquot (0.1 mL) of the extract was mixed with 0.2 mL of 5% sodium nitrite. After 5 min, 0.2 mL of 10% aluminum chloride

and 2 mL of 1 M sodium hydroxide were added and mixed vigorously. Absorbance was measured at 510 nm against a blank. The total flavonoid content was determined using a standard curve of rutin and the results were expressed as milligram rutin equivalent per gram sample dry weight (mg RE/g DW).

2.6. Analyses of Antioxidant Activities

2.6.1. ABTS Radical Scavenging Activity. Radical scavenging activity of the samples against ABTS was carried out according to the method described by Re et al. [17]. ABTS radical cation was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate and allowed the mixture to stand in the dark at room temperature for 16 h. The ABTS solution was diluted to obtain an absorbance of 0.70 at 734 nm and equilibrated at 30°C. ABTS solution (1 mL) was mixed with the extracts (100 µL) at different concentrations (10, 50, 100, and 200 µg/mL) and the decrease in absorbance after 6 min of incubation was monitored using spectrophotometer. Control received only ABTS solution, while distilled water was used as blank. The inhibition of ABTS radicals by the test samples was calculated as scavenging activity (%) = (Control optical density (OD) – sample OD/control OD)) × 100.

2.6.2. DPPH Radical Scavenging Activity. DPPH radical scavenging activities were determined based on a method developed by Prasad et al. [6] with some minor changes. An aliquot of 0.1 mL of the extract at different concentrations (10, 50, 100, and 200 µg/mL) mixed with 1 mL of DPPH (200 mM, dissolved in methanol). The reaction mixture was vortexed and incubated at 37°C in dark light for 30 min. The changes in absorbance were measured at 517 nm using a spectrophotometer. The inhibition of DPPH radicals was calculated as scavenging activity (%) = (Control OD – sample OD/control OD) × 100. BHT was used for comparison.

2.6.3. Ferric Reducing Antioxidant Power (FRAP) Assay. Ferric reducing antioxidant power assay of the extracts was performed according to the method of Re et al. [17]. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6) : 10 mM TPTZ solution in 40 mM HCl : 20 mM ferric chloride solution, in proportion of 10 : 1 : 1 (v/v/v). An aliquot (50 µL) of appropriately diluted extract was mixed with 3 mL of freshly prepared FRAP reagent and mixed thoroughly. The reaction mixture was then incubated at 37°C for 30 min. Absorbance of the reaction mixture was read at 593 nm against a blank. The results were then calculated based on the calibration curve plotted using ferrous sulphate and expressed as mmol Fe²⁺/100 g dry weight.

2.6.4. Beta Carotene Bleaching Assay. Beta carotene bleaching assay was performed according to the method of Velioglu et al. [18] with slight modifications. One milliliter of β-carotene solution (2 mg/mL dissolved in chloroform) was added into brown color round-bottom flask containing 0.02 mL of linoleic acid and 0.2 mL of Tween 20. The chloroform in the mixture was evaporated under vacuum

and 100 mL of deionized water was added. The mixture was shaken vigorously to form an emulsion. The emulsion (1 mL) and 100 µL of the extract at different concentrations (10, 50, 100, and 200 µg/mL) were pipetted in different test tubes and incubated at 45°C for 2 h. Control received only the emulsion without any sample, while blank consists of emulsion without β-carotene and the extract. Absorbance of the solution was monitored at 470 nm. The rate of β-carotene bleaching was calculated as antioxidant activity coefficient (AAC) and calculated using the equation:

$$\text{AAC} = \left[A_s(120) - \frac{A_c(120)}{A_c(0)} - A_c(120) \right] \times 1000, \quad (1)$$

where $A_s(120)$ is absorbance of the sample at time 120 min, $A_c(120)$ is absorbance of control at 120 min, $A_c(0)$ is absorbance of control at 0 min, and $A_c(120)$ is absorbance of control at 120 min. The higher the AAC values, the higher the antioxidant activity.

2.6.5. Antioxidant Capacity by Phosphomolybdenum Method. Antioxidant capacity by phosphomolybdenum method was determined by the method of Prieto et al. [19]. An aliquot (0.1 mL) of the extracts at various concentrations (10, 50, 100, and 200 µg/mL) was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixture was covered and incubated at 95°C for 90 min. After the mixture was cooled, it was centrifuged and absorbance of the supernatant was measured at 695 nm using a spectrophotometer. The antioxidant capacity was expressed as the absorbance value. A higher absorbance value indicates higher antioxidant capacity.

2.7. Identification and Quantification of Phenolic Compounds Using Ultra-High-Performance Liquid Chromatography (UHPLC). Individual phenolic compounds in EEU and EER of NF were identified using validated UHPLC method described by Kong et al. [20] on an Agilent 1290 Infinity LC system (Agilent Technologies, Waldbronn, Germany). The system was equipped with binary pump, diode array detector, and an autosampler. A C-18 Zorbax Eclipse column (50 mm × 2.1 mm, I.D: 1.8 µm, Agilent, Darmstadt, Germany) was used for polyphenol separation and it was maintained at 25°C. Five microliter of the sample was injected into the system and the elution program was set as follows. Mobile phase A is comprised of 0.1% trifluoroacetic acid (TFA) while mobile phase B contained acetonitrile, and the flow rate was set at 0.6 mL/min. The following linear gradient elution was carried out for separation of polyphenols, 15% B for 6 min; 25% B for 3 min; 60% B for 3 min; 80% B for 0.6; 100% B for 0.8 min. The total runtime was 14 min. UV-Vis absorption spectra were monitored by diode array detector (DAD) at 280 nm. The identification of phenolic compounds was achieved by comparison with retention times and UV-Vis absorption spectra with standards available. The phenolic compounds were quantified on the bases of their peak areas and calibration curves of the corresponding standards and then expressed as microgram per gram dry weight (µg/g DW).

2.8. Statistical Analysis. Data were expressed as means \pm standard deviations (SD) of three determinations and analyzed by SPSS V.13 (SPSS Inc., Chicago, USA). One way analysis of variance (ANOVA) and Duncan's multiple-range test were used to determine the differences among the means. *P* values of <0.05 were considered to be significantly different.

3. Results and Discussion

The fruit of NF was selected for the present investigation, since it is one of the important underutilized plants of Malaysia. Although the sap is often used as beverage [8], the fruits are discarded. Many underutilized fruits in Malaysia had been documented previously to be rich in antioxidants with many health benefits [5–7]. Weight (159.6 ± 7.7 g) and length (12.9 ± 0.7 cm) of ripe fruits were significantly higher ($P < 0.05$) than unripe ones (Table 1). Unripe endosperm is soft, juicy, and pale white in color (Figure 2(e)), while the ripe endosperm is hard and milky white in color (Figure 2(f)). These data are important for the food processing industry.

3.1. Total Phenolics and Flavonoid Contents. Phenolics and flavonoids present in fruits and vegetables have received considerable attention due to their potential antioxidant activities [21]. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphomolybdic acids present in Folin-Ciocalteu (FC) reagent. However, it should be also noted that some chemical groups of proteins, organic acids, and sugars present in the extracts might also react with FC reagent and therefore interfere with the result [22]. EEU of NF had significantly ($P < 0.05$) higher total phenolics content (135.6 ± 4.5 mg GAE/g), compared to EER (8.8 ± 2.0 mg GAE/g). In addition, higher total flavonoid content ($P < 0.05$) was also noticed in EEU (68.6 ± 3.1 mg RE/g), compared to EER (3.6 ± 0.4 mg RE/g).

3.2. Antioxidant Capacity Assay. Different antioxidant compounds could react through different mechanisms, and hence a single method alone cannot fully evaluate the antioxidant capacity of foods [23]. For this reason, different antioxidant capacity tests with different approaches and mechanisms were carried out in the present study.

3.2.1. ABTS Assay. ABTS method is developed based on a decolorization technique. Once added, the antioxidant causes a reduction of ABTS, which could be measured at 734 nm. This assay is performed to measure the ability of antioxidants to inhibit radical cation induced by persulfate. Strong antioxidants have the ability to change the blue color of ABTS into light blue color. The change in absorbance is proportional to the antioxidant concentration [22]. Figure 3 illustrates the effect of the NF extracts against ABTS radicals. EEU exhibited significantly ($P < 0.05$) higher scavenging activity of $78 \pm 1.2\%$ at a concentration of $200 \mu\text{g/mL}$, higher than BHT standard ($75 \pm 2.6\%$). EER exhibited moderate activity of $55 \pm 4.3\%$. The IC_{50} (concentration of the extract/sample to scavenge 50% ABTS radicals) values were also obtained. The lower the IC_{50} value the higher the antioxidant activity. The IC_{50}

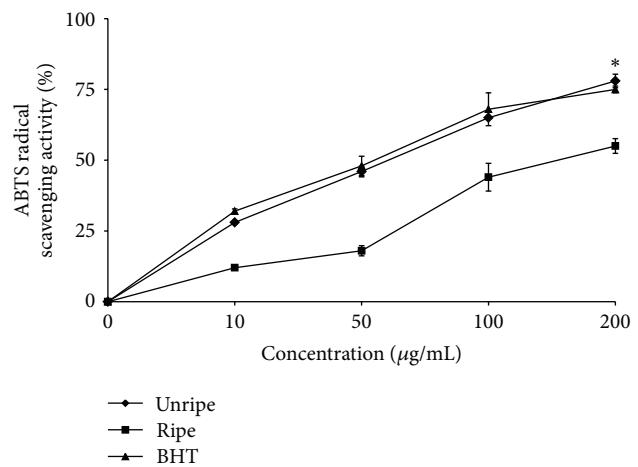


FIGURE 3: ABTS radical scavenging activity of unripe and ripe endosperm extracts of *Nypa fruticans*. *denotes significant differences at $P < 0.05$.

values of EEU ($52 \pm 2.7 \mu\text{g/mL}$) were parallel to BHT ($51 \pm 1.7 \mu\text{g/mL}$), but EER showed higher values ($187 \pm 4.7 \mu\text{g/mL}$). Our results are supported by similar findings by Gordon et al. [24], where unripe acacia fruits exhibited higher ABTS radical scavenging activity as compared to ripe fruits. Unripe cactus berry exhibited two times higher ABTS scavenging activity compared to ripe fruits [25]. The antioxidant activity of the extracts in the present investigation is probably due to the action of hydroxyl groups of phenolic compounds, which might act as hydrogen donors.

3.2.2. DPPH Radical Scavenging Activity. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity. In DPPH radical scavenging assay, antioxidants react with DPPH and convert it to yellow coloured α,α -diphenyl- β -picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the antioxidant [26]. DPPH radical scavenging activity of NF extracts and BHT increased as concentration increased (Figure 4). EEU exhibited the highest scavenging activity ($85 \pm 2.6\%$), significantly ($P < 0.05$) higher than EER ($32 \pm 2.8\%$) at a concentration of $200 \mu\text{g/mL}$, and was comparable to the scavenging activity of BHT ($88 \pm 1.2\%$). IC_{50} value of EEU ($36 \pm 1.2 \mu\text{g/mL}$) was more potent than EER ($312 \pm 4.8 \mu\text{g/mL}$). However, BHT showed the highest antioxidant activity with IC_{50} value of $12 \pm 0.7 \mu\text{g/mL}$. It has been found that phenolics, flavonoids, and tocopherols scavenge DPPH radicals by their hydrogen-donating ability [22]. The results obtained in this investigation also reveal that the sample extracts act as free radical scavengers, which might be attributed to their electron-donating ability.

3.2.3. Ferric Reducing Antioxidant Power (FRAP). Ferric reducing antioxidant power is widely used in evaluating antioxidant activity of plant polyphenols. Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox-linked colorimetric reaction [22]. This assay is

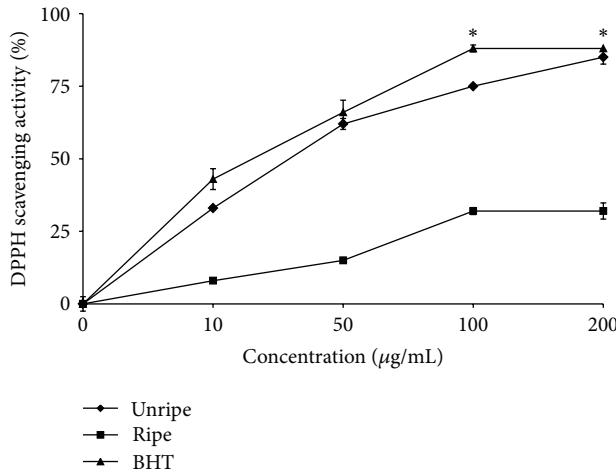


FIGURE 4: DPPH radical scavenging activity of unripe and ripe endosperm extracts of *Nypa fruticans*. * denotes significant differences at $P < 0.05$.

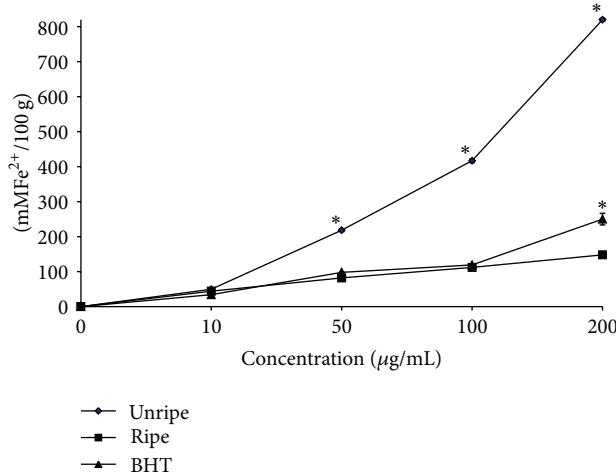


FIGURE 5: FRAP values of unripe and ripe endosperm extracts of *Nypa fruticans*. * denotes significant differences at $P < 0.05$.

relatively simple and easy to conduct. FRAP assay measures the reducing potential of antioxidant to react on ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex and produce blue color of ferrous form which can be detected at absorbance of 593 nm [15]. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction [27]. In the present study, EEU exhibited the highest reducing power followed by BHT and EER (Figure 5). At 200 $\mu\text{g}/\text{mL}$, the reducing power of EEU, BHT, and EER was 819 ± 4.3 , 250 ± 16 and 147 ± 0.7 mmol $\text{Fe}^{2+}/100 \text{ g}$ dry weight, respectively. The reducing power of fruit fractions is probably due to the action of hydroxyl group of the phenolic compounds which might act as electron donors.

3.2.4. Beta Carotene Bleaching Assay. Beta carotene bleaching method is widely used to measure antioxidant activity of plant

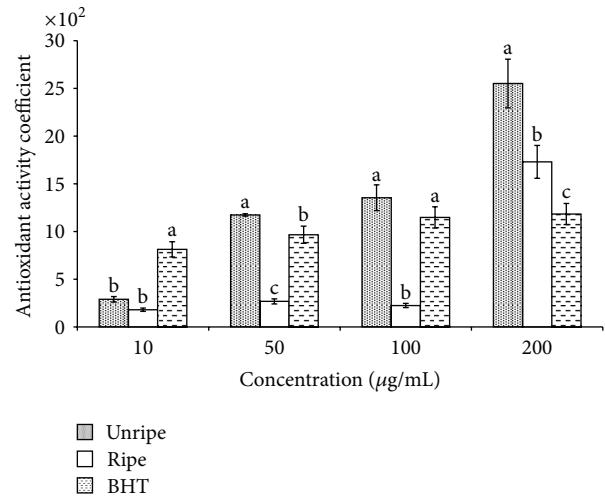


FIGURE 6: Antioxidant activity coefficient values determined by beta carotene bleaching method of unripe and ripe endosperm extracts of *Nypa fruticans*. For each treatment means in a row followed by different letters are significantly different at $P < 0.05$.

extracts. It is an *in vitro* assay that measures the inhibition of coupled autoxidation of linoleic acid and β -carotene. This method is based on lipid radicals as autoxidation products of linoleic acid which attack the double bonds of β -carotene, but in presence of antioxidants they can inhibit the oxidation and retain the yellowish-orange color of beta carotene and thus reduce their bleaching activity [28]. The bleaching activities of NF extracts were concentration dependent, and the activities increased as the concentration increased. EEU exhibited excellent antioxidant activity coefficient of 2550 ± 28 at a concentration of 200 $\mu\text{g}/\text{mL}$, significantly higher ($P < 0.05$) than EER (1729 ± 19) and BHT (1183 ± 34) (Figure 6). The antioxidant activity of the extracts in the present investigation is probably due to the transfer of hydrogen atom from phenolic compounds to the free radical, therefore inhibiting bleaching of beta carotene.

3.2.5. Antioxidant Capacity by Phosphomolybdenum Method. Antioxidant capacities of NF extracts were measured spectrophotometrically using phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm [19]. A high absorbance value of the sample indicates its strong antioxidant activity. Figure 7 shows the total antioxidant capacities of NF extracts and BHT. All the extracts showed a concentration-dependent activity. The total antioxidant activity of EEU at 200 $\mu\text{g}/\text{mL}$ was 0.9 (Figure 3), significantly higher ($P < 0.05$) than EEU (0.3) and BHT (0.7). However, the total antioxidant activity of BHT at all other concentrations tested was higher than other extracts. Previously, Jayaprakasha et al. [29] indicated that total antioxidant activity of citrus was due to the presence of bioactive compounds in the form of phenolics and flavonoids. Hence, probably in the present investigation, the antioxidant capacity might be attributed to the reducing activity of phenolic compounds.

TABLE 2: Phenolic compound composition of unripe and ripe endosperm extracts of *Nypa fruticans* ($\mu\text{g/g}$ DW of plant material).

Peak number	Compound	Retention time (min)	λ max (nm)	Unripe	Ripe
1	Gallic acid	2.1	272	0.47 ± 0.02	0.46 ± 0.01
2	Protocatechuic acid	2.9	294	$5.52 \pm 0.40^{\text{a}}$	$3.88 \pm 0.50^{\text{b}}$
3	Hydroxybenzoic acid	3.9	256	$1.10 \pm 0.07^{\text{a}}$	$0.87 \pm 0.06^{\text{b}}$
4	Chlorogenic acid	6.1	326	$14.5 \pm 1.30^{\text{a}}$	$9.7 \pm 1.10^{\text{b}}$
5	Rutin	9.5	354	$2.7 \pm 0.04^{\text{a}}$	$2.2 \pm 0.07^{\text{b}}$
6	Cinnamic acid	10.7	272	1.1 ± 0.01	0.9 ± 0.02
7	Quercetin	11.6	372	1.73 ± 0.02	1.74 ± 0.7
8	Kaempferol	11.9	364	3.2 ± 0.40	3.0 ± 0.10

For each treatment means in a row followed by different letters are significantly different at $P < 0.05$.

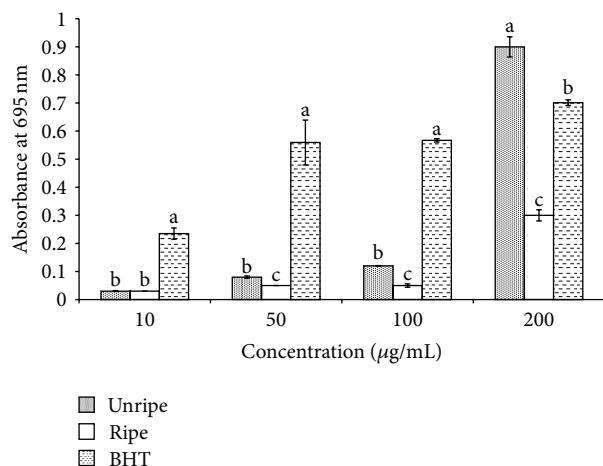


FIGURE 7: Antioxidant capacity of unripe and ripe endosperm extracts of *Nypa fruticans* determined by phosphomolybdenum method. For each treatment means in a row followed by different letters are significantly different at $P < 0.05$.

3.3. Identification and Quantification of Phenolic Compounds. HPLC chromatograms from EEU (Figure 8(a)) and EER (Figure 8(b)) of NF are provided. Eight phenolic compounds were identified where chlorogenic acid, protocatechuic acid, and kaempferol were the major compounds. Among all the phenolic compounds identified, chlorogenic acid was the highest in EEU ($14.5 \pm 1.30 \mu\text{g/g}$) and EER ($9.7 \pm 1.10 \mu\text{g/g}$), while the lowest was gallic acid (Table 2). Protocatechuic acid occupied the second major compound as noted in EEU ($5.5 \pm 0.40 \mu\text{g/g}$) and EER ($3.8 \pm 0.50 \mu\text{g/g}$), followed by kaempferol. In addition, hydroxy benzoic acid, rutin, cinnamic acid, and quercetin were detected in minor amount. Phenolic compounds (chlorogenic acid, protocatechuic acid, rutin, and hydroxybenzoic acid) of EEU were significantly higher ($P < 0.05$) than EER.

Our results are in good agreement with Monde et al. [30], where chlorogenic acid was reported as the major compound in oil palm fruits. In addition caffeic acid, rutin and quercetin were also detected. Da Silva Campelo Borges et al. [31] have identified gallic acid, hydroxy benzoic acid, ferulic acid, and quercetin from jussara palm plant. Gordon et al. [24] reported from acacia palm that protocatechuic acid, chlorogenic acid, hydroxy benzoic acid and gallic acid

decreased as fruit maturity increased. In support to current findings, Herrera-Hernández et al. [25] reported high gallic acid, ellagic acid, caffeic acid, and quercetin in unripe fruits, when compared to ripe fruits.

Antioxidant activity and phenolic content of EEU determined in the present study were higher compared to EER. This is in good agreement with other researchers, where they had reported parallel results [25, 32, 33]. Phenolic compounds are synthesized rapidly during the early stages of fruit maturity. Once the fruit matures, decrease in phenolic concentration is noticed due to the dilution caused by cell growth [34]. Reduction of primary metabolism in the ripe fruit due to lack of substrates necessary for the biosynthesis of phenolic compounds also results in decrease of phenolics compounds. In addition, polymerisation, oxidation, and conjugation of bound phenolics during maturation could also result in decrease of phenolic composition [32].

The total phenolics content of NF is higher when compared to acai palm (123.1 GAE/g) [24] and date palm (4.8 GAE/g) [35]. Statistical correlations between total phenolics content and antioxidant activities were also determined. Total phenolics content exhibited the highest associations with FRAP ($r = 0.9815$), total antioxidant capacity ($r = 0.9523$), and DPPH ($r = 0.8594$). For total flavonoid content, its correlation with FRAP and antioxidant capacity was low ($r = 0.249$ and 0.315 resp.), while negative correlation was observed for other antioxidant assays. Thus, the amount of phenolic compounds could be used as an important indicator of antioxidant capacity. This also clearly indicated that total phenolics in EEU and EER of NF are major contributors for antioxidant activities since they have a high correlation, while flavonoids are not the major contributors for antioxidant activities. In addition, antioxidant activity could also be contributed by synergistic action of other phytochemicals which are not determined in the current study such as carotenoids and polysaccharides. Previously, many authors have reported a positive correlation between total phenolics content and antioxidant activity [16, 34]. Liu et al. [16] reported negative correlation between antioxidant activity and total flavonoid content.

4. Conclusion

Unripe endosperm extract of *Nypa fruticans* showed high total phenolics, total flavonoid content, and antioxidant

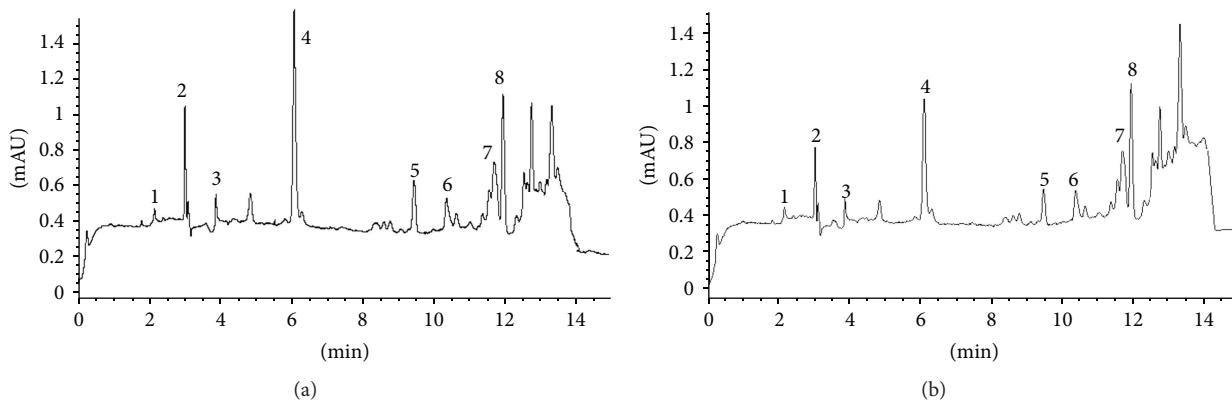


FIGURE 8: HPLC chromatogram of unripe (a) and ripe endosperm extract (b) of *Nypa fruticans*. The peak identifications are (1) gallic acid, (2) protocatechuic acid, (3) hydroxybenzoic acid, (4) chlorogenic acid, (5) rutin, (6) cinnamic acid, (7) quercetin, and (8) kaempferol.

capacities as compared to ripe endosperm extract. Chlorogenic acid, protocatechuic acid, and kaempferol were identified as major compounds in the extract. Thus, unripe endosperm extract of NF could be used as natural antioxidant. Further investigations on the evaluation of nutritional composition and other therapeutic uses of *Nypa fruticans* endosperm extracts are worth investigating.

Conflict of Interests

The authors have no conflict of interest to declare.

Acknowledgments

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Research Article

Total and Inorganic Arsenic Contents in Some Edible Zingiberaceous Rhizomes in Thailand

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The arsenic accumulation in rhizomes of Zingiberaceous plants was determined by atomic absorption spectrometry interfaced with hydride generation system (HG-AAS). The raw herbal materials, rhizomes, were collected from different regions of Thailand between December 2011 and January 2012. Six well-known Zingiberaceous plants, 16 samples from each and a total of 96 samples, were analyzed *Alpinia galanga* (Khaa), *Boesenbergia rotunda* (Kra-chaai), *Curcuma longa* (Khamin-chan), *Curcuma zedoaria* (Khamin-oi), *Zingiber cassumunar* (Plai) and *Zingiber officinale* (Ginger). Concentrations of total arsenic based on dry weight were 92.4 ± 9.2 , 103.5 ± 20.8 , 61.7 ± 12.5 , 89.8 ± 17.5 , 106.7 ± 19.5 and 69.3 ± 11.8 ng/g, respectively and inorganic arsenic were 48.8 ± 7.0 , 66.3 ± 12.7 , 25.5 ± 5.0 , 38.7 ± 4.7 , 71.2 ± 11.6 , and 38.5 ± 5.5 ng/g, respectively. Among these, Plai and Kra-chaai exhibited the highest levels of total arsenic and inorganic arsenic accumulation that remind consumers to be aware of excess consuming of these rhizomes. On the contrary, the lowest value found in Khamin-chan indicating natural dietary supplements and herbal medicines comprising Kamin-chan are safe from arsenic poison. All investigated amounts of total and inorganic arsenic were much lower than limits recommended by Thai Food and Drug Administration.

1. Introduction

Zingiberaceae, one of the largest families of the plant kingdom, is an important natural resource that provides many useful food products, spices, and traditional medicines to treat a variety of diseases [1, 2]. The consumption of herbal products for therapeutic purposes and to promote wellness is widely popular since people are greatly concerned about side effects of synthetic drugs [3]. Herbs are being increasingly used in the pharmaceutical industry as raw materials for the preparation of herbal medicines. The arsenic contamination of herbs may be due to environmental pollution [4]. Arsenic is widely distributed in the Earth's crust and present at an

average concentration of 2 mg/kg. Around one-third of atmospheric flux of arsenic is of natural origin [5]. Arsenic is one of the first chemicals designated as a group 1 carcinogen [6] and well known to be poisonous to organisms [7]. The inorganic arsenic species (As(III) and As(V)) are the most toxic forms of arsenic present in food [8]. Previous studies have indicated that ingested inorganic arsenic is strongly associated with a wide spectrum of adverse health outcomes, primary cancers, and other chronic diseases [9]. The primary route of arsenic exposure for the general population is via ingestion. The daily intake of total arsenic from food and beverages is generally in the range of 20–300 μ g/day [10]. Furthermore, Joint Food and Agriculture Organization/World Health Organization Expert

Committee on Food Additives note that benchmark dose lower confidence limit for a 0.5% (BMDL 0.5) of inorganic arsenic in human was $3.0 \mu\text{g}/\text{kg}$ body weight per day [11].

Root or rhizome is a part of the plant that has high opportunity to contaminate with arsenic. Current research demonstrated that plants absorbed heavy metals from soil [12, 13]. The contaminants and residues of toxic metal arsenic may cause harm to the consumers of herbal medicines. Plants that grow in an arsenic-affected area may have a high level of arsenic. The transfer of arsenic from soils to plants might be a key step in the route of arsenic entry into human body [14, 15]. Experimental data had shown that a variety of vegetable crops accumulate arsenic by root uptake from soil deposited on the leaves [16], and Gulz et al. [17] reported several edible plants grown in contaminated soils that accumulated high levels of arsenic. Arsenic species from soil can enter into edible tissues through absorption. The WHO [5] determined that arsenic concentration in plants grown in soils without arsenic-containing pesticides varied from 0.02 to 5 mg/kg (dry weight), and in the arsenic soil contamination indicated root can contain higher levels of arsenic than other parts of plant.

A previous study about rhizome of *Zingiber officinale* (Ginger) shows that arsenic level varied from not detected to $0.13 \mu\text{g}/\text{L}$ [18]. Other arsenic contamination reports of Karadas and Kara [19] show that arsenic levels in cumin and turmeric were 174 ± 14 and $39 \pm 5 \text{ ng/g}$, dry wt, respectively. Vegetable crops in the contaminated region were found high in arsenic level (dry wt) in *Arum* ranged from 74.3 to 89.2 mg/kg, in cabbage from 27.12 to 39.39 mg/kg and in pumpkin from 17.28 to 22.05 mg/kg [20]. Baroni et al. [21] analyzed sixty-four plant species, and the highest arsenic contents were found in roots of *Phragmites australis* (688 mg/kg). Roychowdhury et al. [22] investigated high arsenic levels in cumin and turmeric (47.86–209.75 and 297.33–280.9 ng/g, res.) Moreover, dry Ginger exhibited mean arsenic content of $77.9 \pm 8.7 \text{ ng/g}$ [14]. In general, the highest concentration of arsenic was found in plant roots, the intermediate level in vegetative tissues (leaves and stems), and the lowest level in reproductive tissue (fruits and seeds) [23, 24]. Plants absorb arsenic fairly easily, so that high-ranking concentration may be present. Among many of public researches, most studies had focused on foods and not much on the information that was available on plants especially in the part of rhizomes although it was a high opportunity for arsenic accumulation. For this reason, well-known rhizomes of Zingiberaceae family that are used as food, dietary supplements and alternative medicines in Thailand, that is, *A. galanga*, *B. rotunda*, *C. longa*, *C. zedoaria*, *Z. cassumunar*, and *Z. officinale*, were interesting to investigate their total and inorganic arsenic concentrations.

2. Materials and Methods

2.1. Chemicals. Standard reference material (SRM) 1568a (rice flour) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Nitric acid (HNO_3) and hydrochloric acid (HCl) were purchased from

Merck Chemicals (Darmstadt, Germany); dimethylarsinic acid (DMA), hydrazine sulfate, hydrobromic acid, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). All standard solutions, reagents, and samples were prepared using deionized water ($18 \text{ M}\Omega \text{ cm}$) throughout the study. To remove possible arsenic residue contamination, all glasswares were washed thoroughly with tap water, air-dried, soaked in 10% (v/v) HNO_3 for 20–24 h, and washed three times with deionized water.

2.2. Sample Collection. Six kinds of plants in the family Zingiberaceae of which rhizomes are widely used for consumption and medication purposes were selected. They were *Alpinia galanga* (L.) Willd. (Khaa), *Boesenbergia rotunda* (L.) Mansf. (Kra-chaai), *Curcuma longa* (L.) (Khamin-chan), *Curcuma zedoaria* (Berg.) Roscoe (Khamin-oi), *Zingiber cassumunar* Roxb. (Plai), and *Zingiber officinale* Roscoe (Ginger). Sixteen samples of each rhizome species were collected during December 2011 and January 2012 from eight provinces (Chiang Rai, Lampang, Khon Kaen, Ubon Ratchathani, Ratchaburi, Samut Prakan, Krabi, and Songkhla) in north, northeast, central and south Thailand. Totally, 96 samples were collected for investigation. These collections were grown in urban and agricultural fields. Growing period was around two years and harvesting time from December to January. The noticed appearances of mature rhizome were strong flavor and pungent odor. The samples were identified by Dr. W. Gritsanapan, and the voucher specimens (AGI11201-16, BR120101-16, CL111201-16, CZ111201-16, ZC120101-16, and ZO120101-16) were deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Thailand.

2.3. Sample Preparation. Rhizome samples were washed, cleaned, air-dried, and sliced. Freeze drying is used to dry sliced rhizomes to keep them in a stable condition. The dried samples were grinded into powder with a porcelain mortar and pestle and passed through a fine mesh sieve. Powder of lyophilized samples was kept in air tight containers at 4°C and protected from light until analysis. The moisture contents were calculated using weights of samples before and after lyophilization.

2.4. Determination of Total Arsenic. The lyophilized sample preparation for determination of total arsenic was performed by acid digestion procedure described by Muñoz et al. [25]. An accurate weight ($0.5 \pm 0.01 \text{ g}$) of each lyophilized sample was mixed with 1 mL of an ashing suspension (20% (w/v) $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and 2% (w/v) MgO in water) and 5 mL of 50% (v/v) HNO_3 . The mixture was evaporated on a hot plate to dryness and mineralized at 450°C in a furnace. The resulting white ash was dissolved in 5 mL of 6 N HCl and 5 mL of a freshly prepared reducing solution (5% (w/v) KI and 5% (w/v) ascorbic acid). The solution was left for 30 min, and then 10 mL of 50% (v/v) HCl was added to the solution. The solution was filtered through a Whatman no. 1 filter paper into a 25 mL volumetric flask and adjusted to volume with 50% (v/v) HCl. The resulting solution was used for

the determination of total arsenic. Duplicate analyses were performed for individual samples.

2.5. Determination of Inorganic Arsenic. Inorganic arsenic was determined by the nonchromatographic method described by Muñoz et al. [25]. An accurate weight (1.0 ± 0.01 g) of each lyophilized sample was placed in a 50 mL screw-capped centrifuge tube; 4.1 mL of water was added to the sample and mixed until completely moistened. In order to hydrolyze As(III) from the thiol groups of proteins, 18.4 mL of concentrated HCl was added to the moistened sample, shaken for 1 h, and left overnight (12 to 15 h). A reducing agent (1 mL of 1.5% (w/v) freshly prepared hydrazine sulfate and 2 mL of hydrobromic acid) was added to the sample tube and vortexed for 2 min. For extraction of inorganic arsenic, 10 mL of chloroform was added to the tube, vortexed for 3 min, and inverted for 1 min. To break the emulsion formed during the extraction, the tube was centrifuged at $1,000 \times g$ (measured in gravity \times force or g -force) for 10 min using an Eppendorf bench top centrifuge 5810 (Hamburg, Germany). The chloroform phase was aspirated into another centrifuge tube. The extraction process was repeated twice. To separate some residues from the extraction, the combined chloroform phase was filtered through a syringe filter with a 25 mm PTFE membrane, pore size $0.45 \mu\text{m}$ (Chrom Tech, Apple Valley, MN, USA), into another tube. Inorganic arsenic in the chloroform phase was back-extracted into an aqueous phase with 10 mL of 1N HCl and centrifuged at $1,000 \times g$ for 10 min. The aqueous phase was aspirated into a beaker. The chloroform phase was extracted one more time. The amount of inorganic arsenic in the combined acidic aqueous phase was quantified as described in Section 2.4, with the addition of 2.5 mL of the ashing suspension and 10 mL of 50% (v/v) HNO_3 . All samples were analyzed for inorganic arsenic in duplicate.

2.6. Instrumentation. A PerkinElmer (Waltham, MA, USA) AAnalyst 300 atomic absorption spectrometer (Norwalk, CT, USA) interfaced with an AS-90 autosampler and a FIAS-400 flow injection system was used to determine total and inorganic arsenic concentrations in the final solutions. The atomic absorption spectrophotometric conditions were as follows: wavelength, 193.7 nm; slit width, 0.70 nm; EDL current, 380 mA; loop sample, 0.5 mL. The hydride generation conditions were as follows: quartz cell, 16 cm path length, 90.7 cm i.d., electrothermal heating, cell temperature, 900°C , carrier gas (argon) flow rate, 50–100 mL/min, reducing agent (0.2% (w/v) NaBH_4 in 0.05% (w/v) sodium hydroxide solution) flow rate, 5–7 mL/min, and HCl flow rate, 9–11 mL/min [26].

2.7. Determination of Limit of Quantification. The Q2B analytical procedure described by the US FDA was used for determination of the limit of quantification (LOQ). Mixtures of lyophilized samples of all six species with equal weights were used for the determination of the limit of quantification (LOQ) of the method. For the determination of the LOQ for total arsenic, samples were fortified with a standard arsenic

mixture (As(III) : dimethylarsinic acid (DMA), 1:1 (w/w)) equivalent to total arsenic at concentrations of 250, 500, 1,000, and 2,500 ng/g; blank samples were not fortified with arsenic. All samples were analyzed in duplicates. A total of twelve regression lines (six regression lines each for intraday and interday analyses) were obtained by the least-square linear regression analyses of the residual peak heights of standard total arsenic versus fortified total arsenic concentrations. The residual peak heights were peak heights of total arsenic-fortified samples minus that of blank sample. The LOQ of the method was calculated using the equation $\text{LOQ} = 10\sigma/S$, where σ is the standard deviation of y -intercepts and S is the slope of linear regression analysis [27].

For the determination of the LOQ for inorganic arsenic, samples were fortified with an inorganic arsenic mixture (As(III) : As(V) 1:1 (w/w)) at concentrations of 50, 100, 500, and 1,000 ng/g; blank samples were not fortified with inorganic arsenic. Duplicate analyses were performed for individual samples. A total of twelve regression lines (six regression lines each for intraday and interday analyses) were obtained by the least-square linear regression analyses of the residual peak heights of standard inorganic arsenic versus fortified inorganic arsenic concentrations. The residual peak heights were the peak heights of fortified samples of inorganic arsenic minus the average peak height of blank sample. The LOQ of the method was calculated using the equation $\text{LOQ} = 10\sigma/S$, where σ is the standard deviation of y -intercepts and S is the slope of linear regression analysis [27].

2.8. Quality Assurance. The accuracy of determination of total arsenic was assessed by analyzing SRM 2568a (rice flour), because no commercial rice standard reference materials for inorganic arsenic are available. The amount of inorganic arsenic in SRM 1568a (rice flour) was determined and compared with the values previously reported.

2.9. Statistical Analysis. One-way analysis of variance and Tukey's test using the SPSS Statistics version 17.0 software were performed to determine differences in concentrations of total arsenic, inorganic arsenic (both on wet weight and dry weight basis), and percentages of inorganic arsenic with respect to total arsenic of six rhizome types. A significance level of $P < 0.05$ was accepted for all comparisons.

3. Results and Discussion

The calculation for LOQs was based on the standard deviation of y -intercepts of the linear regression analysis (σ) and the slope (S) by using the equation $\text{LOQ} = 10\sigma/S$ [27]. The LOQs for total and inorganic arsenic in rhizome samples were 19.7 and 15.7 ng/g, respectively. Concentrations of total arsenic and inorganic arsenic found in SRM 1568a (rice flour) were 283 ± 34 ng/g ($n = 10$, reference value of 290 ± 30 ng/g) and 102 ± 3.7 ng/g ($n = 10$), respectively [26]. The concentration of inorganic arsenic was in agreement with previous reports of 111 ± 6 ng/g [25] and 111 ± 3 ng/g [28], which was analyzed by the same method. The accuracy and precision for the determination of total and inorganic arsenic

TABLE 1: Accuracy and precision in the determination of total and inorganic arsenic in Zingiberaceae rhizomes.

Arsenic added (ng/g)	Intraday (n = 6)			Interday (n = 6)		
	Found (ng/g) mean \pm SD	% RSD ^a	Recovery (%)	Found (ng/g) mean \pm SD	% RSD ^a	Recovery (%)
Total						
250	244.1 \pm 8.5	3.5	97.6	237.4 \pm 6.7	2.8	94.9
500	473.1 \pm 13.5	2.9	94.6	477.8 \pm 21.2	4.4	95.6
1,000	944.7 \pm 32.6	3.5	94.5	972.2 \pm 48.7	5.0	97.2
2,500	2,366.3 \pm 41.5	1.8	94.7	2,382.0 \pm 40.6	1.7	95.3
Inorganic						
50	47.3 \pm 2.6	5.5	94.7	47.7 \pm 2.6	5.4	95.3
100	98.1 \pm 4.8	4.9	98.1	97.9 \pm 3.4	3.4	97.9
500	469.8 \pm 25.2	5.4	94.0	473.0 \pm 21.9	4.6	94.6
1,000	943.7 \pm 13.5	1.4	94.4	940.7 \pm 10.7	1.1	94.1

^a% RSD: percent relative standard deviation.

TABLE 2: Total arsenic, inorganic arsenic, and percentage of inorganic arsenic with respect to total arsenic for rhizomes of six plants in the Zingiberaceae family collected from four regions in Thailand (north, south, central, and northeast)^a.

Species	n	Total arsenic ^a (ng/g)		Inorganic arsenic ^a (ng/g)		% Inorganic arsenic ^b
		Wet wt	Dry wt	Wet wt	Dry wt	
<i>Alpinia galanga</i> (Khaa)	16	15.9 \pm 5.1 A (10.0–28.3)	92.4 \pm 9.2 A (74.7–107.2)	8.4 \pm 3.0 A (4.5–15.6)	48.8 \pm 7.0 C (39.3–65.1)	53.2 \pm 8.2 A (38.9–69.6)
<i>Boesenbergia rotunda</i> (Kra-chai)	16	20.8 \pm 4.6 A (11.5–27.3)	103.5 \pm 20.8 A (80.3–140.3)	13.4 \pm 3.1 B (6.4–17.6)	66.3 \pm 12.7 A (47.3–92.8)	64.4 \pm 4.9 A (56.1–74.3)
<i>Curcuma longa</i> (Khamin-chan)	16	10.3 \pm 3.8 A (5.8–21.3)	61.7 \pm 12.5 B (42.5–87.2)	4.3 \pm 1.5 A (2.6–8.2)	25.5 \pm 5.0 D (20.4–36.0)	41.8 \pm 6.2 A (30.5–53.5)
<i>Curcuma zedoaria</i> (Khamin-oi)	16	14.5 \pm 4.6 A (6.9–21.8)	89.8 \pm 17.5 A (55.0–126.8)	6.2 \pm 1.5 A (2.5–8.4)	38.7 \pm 4.7 B (24.4–44.6)	44.5 \pm 10.0 A (28.5–72.8)
<i>Zingiber cassumunar</i> (Plai)	16	17.5 \pm 6.6 A (7.9–26.8)	106.7 \pm 19.5 A (73.6–145.1)	11.7 \pm 4.5 B (5.6–20.2)	71.2 \pm 11.6 A (52.7–92.1)	67.4 \pm 7.5 A (54.6–83.2)
<i>Zingiber officinale</i> (Ginger)	16	7.9 \pm 2.2 A (4.8–13.7)	69.3 \pm 11.8 B (43.3–86.4)	4.4 \pm 1.0 A (2.6–6.0)	38.5 \pm 5.5 B (30.1–48.5)	56.9 \pm 11.8 A (42.4–82.6)

^aThere were 96 samples in total, 16 of each of the six species. Values are mean \pm SD; numbers in parentheses are ranges.

^b% inorganic arsenic = (concentration of inorganic arsenic \times 100)/concentration of total arsenic.

A, B, C, and D values in the same column followed by different letters denote significant differences ($P < 0.05$).

in rhizome samples fortified with arsenic mixture at four concentrations are shown in Table 1. The accuracy was assessed as percent recovery from the analysis of fortified arsenic mixture in the rhizome samples. The average recoveries across the four concentrations of fortified arsenic mixtures were 95.6% and 95.4% for total and inorganic arsenic, respectively. The precision of the method expressed as percentage of relative standard deviation (% RSD) was calculated with the equation % RSD = $100\text{SD}/\bar{x}$, where SD is the standard deviation and \bar{x} is the mean of arsenic concentrations recovered from the arsenic-fortified samples. The % RSD ranged from 1.7 to 5.0 for total arsenic and from 1.1 to 5.5 for inorganic arsenic.

Table 2 summarizes concentrations of total arsenic, inorganic arsenic, and percentages of inorganic arsenic with respect to total arsenic in rhizomes of six plants in Zingiberaceae family collected from four regions of eight areas

in Thailand. The highest content of inorganic arsenic was found in Plai (71.2 ± 11.6 ng/g, dry wt). The inorganic arsenic content in six dried rhizomes was expressed within the range of 20.4 to 92.8 ng/g, total arsenic levels ranged from 42.5 to 145.1 ng/g, and the percentages of inorganic arsenic with respect to total arsenic ranged from 28.5 to 83.2 ng/g.

Zhao et al. [14] determined concentrations of total arsenic in dried Ginger ($n = 3$) from China. Average concentration was 77.9 ng/g, whereas the value in this study was 69.3 ng/g. The indicated concentration may imply that the growth environments and soil condition for growing Ginger in Thailand (69.3 ± 11.8 ng/g) were safer than those in China (77.9 ± 8.7 ng/g) [14]. The study of the arsenic-affected area in India reported higher levels of arsenic in fresh cumin and turmeric powder than our study. They reported that arsenic level ranged from 47.86 to 209.75 ng/g and from 297.33 to

TABLE 3: Concentrations of total arsenic in roots or rhizomes from various countries.

Species	Location	Total arsenic concentration (ng/g)	Reference
Cumin ^a	Murshidabad district, West Bengal, India	47.86–209.75	[15]
Turmeric powder ^a	Murshidabad district, West Bengal, India	297.33–280.9	[15]
<i>Curcuma longae</i> ^b	Thailand	61.7 ± 12.5 ^c	Present study
Dry ginger ^a	Beijing, China	77.9 ± 8.7 ^c	[5]
<i>Zingiber officinale</i> ^b	Thailand	69.3 ± 11.8 ^c	Present study
<i>Isatis indigotica</i> ^b	Anguo city, Hebei, China	137.4 ± 0.0 ^c	[3]
<i>Atractylodes macrocephala</i> ^b	Anguo city, Hebei, China	371.9 ± 0.0 ^c	[3]
<i>Salvia miltiorrhiza</i> ^b	Anguo city, Hebei, China	278.9 ± 40.2 ^c	[3]
<i>Saposhnikovia divaricata</i> ^b	Anguo city, Hebei, China	175.8 ± 23.9 ^c	[3]
<i>Astragalus membranaceus</i> ^b	Anguo city, Hebei, China	140.3 ± 9.5 ^c	[3]
<i>Aster tataricus</i> ^b	Anguo city, Hebei, China	525.5 ± 103.9 ^c	[3]
<i>Anemarrhena asphodeloides</i> ^b	Anguo city, Hebei, China	216.2 ± 31.6 ^c	[3]
<i>Trichosanthes kirilowii</i> ^b	Anguo city, Hebei, China	184.4 ± 6.9 ^c	[3]
<i>Alpinia galanga</i> ^b	Thailand	92.4 ± 9.2 ^c	Present study
<i>Boesenbergia rotunda</i> ^b	Thailand	103.5 ± 20.8 ^c	Present study
<i>Curcuma zedoaria</i> ^b	Thailand	89.8 ± 17.5 ^c	Present study
<i>Zingiber cassumunar</i> ^b	Thailand	106.7 ± 19.5 ^c	Present study

^aSpecies not specified.^bThe roots or rhizomes of plants can be used as botanical products.^cValue as mean ± SD.

280.9 ng/g, respectively [22]. Our study found that the total arsenic levels in Khamin-chan (wet wt and dry wt) ranged from 5.8 to 21.3 ng/g and from 42.5 to 87.2 ng/g, respectively. These results clearly showed that the high amount of arsenic deposited in rhizomes is according to soil location.

In six plants of Zingiberaceae family in this study, the average concentrations of inorganic arsenic in the roots of *C. longa* and *C. zedoaria* were 4.3 ± 1.5 and 6.2 ± 1.5 ng/g (wet wt), respectively. The inorganic arsenic contents in two *Curcuma* species were not statistically different ($P > 0.05$) but they were significantly different ($P < 0.05$) from *Zingiber* genus. The inorganic arsenic contents in *Z. cassumunar* and *Z. officinale* were 11.7 ± 4.5 and 4.4 ± 1.0 ng/g (wet wt), respectively. The well-known species of *Alpinia* genus, *A. galangal*, showed the concentration (wet wt) of total arsenic (15.9 ± 5.1 ng/g) and inorganic arsenic (8.4 ± 3.0 ng/g). Finally, Kra-chaai in *Boesenbergia* genus contained the total arsenic level of 20.8 ± 4.6 ng/g, the inorganic arsenic level of 13.4 ± 3.1 ng/g, and the percentage of inorganic arsenic of 64.4%.

In this study, the total arsenic concentrations in fresh rhizomes of Kra-chaai, Plai, Khaa, Khamin-oi, Khamin-chan, and Ginger ranged from 11.5 to 27.3, 7.9 to 26.8, 10.0 to 28.3, 6.9 to 21.8, 5.8 to 21.3, and 4.8 to 13.7 ng/g, respectively. These values are in accordance with the fresh vegetable arsenic concentration reported that ranged from 0 to 195 ng/g. Other edible roots, such as carrot and potato were reported high levels of arsenic at 195 and 103 ng/g, respectively [29]. Muñoz et al. [25] reported that the total arsenic level for vegetable group ranged from 8 to 604 ng/g, and the total and inorganic arsenic concentrations in edible roots or rhizomes were higher than other organs of herbs.

Of the six medicinal rhizomes of Zingiberaceae family analyzed in this study, both of Kra-chaai and Plai exhibited

high mean levels (wet weight) of total arsenic (20.8 ± 4.6 and 17.5 ± 6.6 ng/g), inorganic arsenic (13.4 ± 3.1 and 11.7 ± 4.5 ng/g), and percentage of inorganic arsenic (64.4 and 67.4%), respectively. A possible explanation is that these high levels were related to their arsenic accumulative capacity in the rhizosphere soils associated with each species. In contrast, the low levels of inorganic arsenic in Khamin-chan, Khamin-oi, and Ginger (wet weight) ranged from 2.6 to 8.2, 2.5 to 8.4, and 2.6 to 6.0 ng/g, respectively, whereas the average percentages of inorganic arsenic were 41.8, 44.5, and 56.9, respectively. Form the statistical analysis, comparative contents of total arsenic (ng/g, dry wt) among six species, Khaa, Kra-chaai, Khamin-oi, and Plai were significantly different from Khamin-chan and Ginger.

From our study, the total arsenic level in ninety-six samples complied with Thai regulatory limit (2 µg/g) and national limit for arsenic in herbal medicines (4 µg/g) [30–32]. Table 3 shows a comparison of total arsenic level in some rhizomes or roots from various countries. Only a few studies were reported. Further investigations should be performed on other botanical rhizomes with incidence of high arsenic accumulation to crucially guarantee that plants are safe for dietary supplements for health and well-being.

4. Conclusion

All investigated amounts of total arsenic concentrations meet the requirements at a national level. Among six species were grown on urban and agricultural areas. We found that dried root of *Z. cassumunar* showed a higher level of inorganic arsenic than other species. Arsenic accumulation in rhizomes may be an important risk factor that needed to

be taken into account. The results will be valuable for preliminary risk assessment in raw materials of natural products. Therefore, long-term consumption of herbal products that comprised some particular Zingiberaceous rhizomes might cause adverse health effects.

Conflict of Interests

All authors declare no conflict of interests with the trademarks included in the paper.

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Research Article

Physical Stability and HPLC Analysis of Indian Kudzu (*Pueraria tuberosa* Linn.) Fortified Milk

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Functional foods provide health benefit beyond basic nutrition. Functional foods fortified with plant ingredients are well known. Ayurveda (Indian System of Medicine) has found several ways in which the medicinal benefits of herbs can be conveyed via certain foods as carriers. Milk is one such carrier which has been effectively used to deliver phytochemicals for targeted health benefits. Indian Kudzu or *Pueraria tuberosa* Linn. (Fabaceae) is an important medicinal plant of Ayurveda, and experiments suggest that it enhances the health benefits of milk when taken with milk as a carrier. Different milk combinations with *P. tuberosa* were prepared by homogenizing pasteurized toned milk with its ethanolic and hot water extracts and their stability with reference to pH and coagulation was studied over a period of 15 days. The combinations were also analyzed for puerarin, the major isoflavone C-glucoside present in *P. tuberosa*, through high-performance liquid chromatography using photo diode array detector. It was observed that there was no precipitate formation and the pH also did not change during the study period indicating their physical stability under the experimental conditions. Also there was no significant change in the content of puerarin during the study period, thereby indicating the chemical stability of the samples. These studies will be useful for developing milk nutraceuticals fortified with Indian Kudzu which has the potential to be included as an ingredient in health and functional foods.

1. Introduction

The use of botanicals in foodstuffs is well established. It includes use as vegetables, fruits, herbs, and botanical food supplements. While medicinal products are intended to prevent or treat a disease or modify the way in which the body functions, food supplements and nutraceuticals are intended to complement the diet with substances possessing health-maintenance or -promoting properties [1]. Food industries have rather high demand for the products that meet the consumer's demand for a healthy lifestyle. There are many companies already capitalizing on growing consumer acceptance of food and beverages containing herbal extracts [2]. Ayurveda (Indian System of Medicine) has found several ways in which the medicinal benefits of herbs could be conveyed via certain foods as carriers. Milk is one such carrier which has been effectively used to deliver phytochemicals for targeted health benefits in the traditional Indian system

of medical science. Milk is also one of the most widely consumed foods in the world and is an ideal vehicle for the fortification with these nutraceuticals.

Indian Kudzu or *Pueraria tuberosa* Linn. (Fabaceae) is an important medicinal plant of the Indian traditional system of medicine, that is, Ayurveda, and is mentioned in the Ayurvedic Pharmacopoeia of India under the name of Vidari. Powder of tubers of *P. tuberosa* (PT), commonly known as Indian Kudzu or Vidarikand in Hindi [3], is recommended for clinical use in the dose of 2–6 g/adult person [4]. It is used in traditional medicine as a fertility control agent and as an aphrodisiac, cardiotonic, diuretic and galactagogue. It has exhibited antihyperglycemic, anti-hyperlipidemic, antifertility in male rats, hepatoprotective, and anti-implantation activities [5–8]. It is a constituent of various formulations used as nutritive, diuretic, expectorants, and for the management of rheumatism, fever, and bronchitis [4, 9]. *P. tuberosa* tubers are rich in isoflavonoids

and the important phytoconstituents are puerarin, daidzein, genistein, puererosanol, and tuberosin [10–13]. During the past decade, interest in these isoflavonoids has increased considerably because of the beneficial effects proposed by epidemiologists, nutritionists, and food manufacturers [14]. These isoflavonoids could interact with milk proteins, namely, bovine serum albumin [15], casein micelle [16], and β -lactoglobulin [17] as has been reported in case of certain food and drug preparation containing soya isoflavonoids. *In vivo* studies further revealed that these interactions often lead to reduction in lipid oxidation and improvement in antioxidant properties which are of great significance from health point of view.

Recently, we have investigated the *in vivo* immunomodulatory and antioxidative effect of *P. tuberosa* with milk as the carrier. The results suggested that *P. tuberosa* contained active compounds that improved the therapeutic properties of milk. The milk supplemented with *P. tuberosa* extracts exhibited immunostimulatory and antioxidative properties [18]. Studies have also been carried out to determine the effect of added herb extracts on oxidative stability of ghee during accelerated oxidation condition and it was found that the ethanolic extract of vidarikand had the maximum antioxidant activity among all the herbs [19].

Since the health benefits of the nutraceuticals or functional foods containing different botanicals are due to the presence of the phytoconstituents of the added botanicals, it is important to have a biological marker and also to be able to associate that biological marker with the quality of life. Puerarin is the major constituent of *Pueraria* species. It is prescribed to treat coronary heart disease and alcoholism. It may prevent cancer, act as an antioxidant, lower serum cholesterol, and have antithrombotic and antiallergic activities [5, 20, 21]. Puerarin has exhibited antihyperglycemic effect in streptozotocin-induced diabetic rats and possesses estrogen-like biological activities. Recent studies showed that puerarin protects different cell types from damage caused by a variety of toxic stimuli. Data also suggest that puerarin might be beneficial for the treatment of Alzheimer's disease [22].

Several methods have been reported for the identification and analysis of puerarin [23]. But, to the best of our knowledge, no work on identification of *P. tuberosa* in milk nutraceuticals has been previously reported. Thus, continuing with our studies on herb-(*P. tuberosa*) milk model systems, the present work was carried out to study the stability of milk fortified with Indian Kudzu with reference to its pH and coagulation as well as to analyze Kudzu-fortified milk for puerarin, the major isoflavone C-glucoside present in *P. tuberosa*, through high-performance liquid chromatography using photo diode array detector.

2. Materials and Methods

2.1. Chemicals and Reagents. Puerarin was obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol and water were obtained from Merck (Darmstadt, Germany). Whatman (Florham Park, NJ) No. 1 filter paper was used for filtration of the samples. Other chemicals and solvents were purchased from Merck Chemicals, Mumbai, India.

2.2. Plant Material. Tubers of *P. tuberosa* were collected from the Uttarakhand region. The sample was identified and authenticated by Dr. A. K. S. Rawat, by comparison with a reference sample preserved in the Herbarium department of NBRI. They were deposited (specimen number NBR/PH/227348) in the departmental herbal drug museum of the Pharmacognosy Division, National Botanical Research Institute, Lucknow, India, for future reference.

2.3. Preparation of Ethanolic and Hot Water Extracts. The coarse air-dried, (40°–50°C), powdered tubers (500 g each) of *P. tuberosa* were extracted with ethanol by cold percolation process and with hot water by heating on a boiling water bath. The respective extracts were pooled, filtered, concentrated at reduced temperature (below 55°C) by rotary evaporation (Büchi, USA), lyophilized (Freezone 4.5; Labconco, USA) under high vacuum (133 × 104 mbar) at –40°C ± 2°C to yield the respective ethanolic (EE) and hot water (HWE) extracts, and stored at 40°C.

2.4. Procedure for Milk Fortification and Sterilization. Different milk combinations with *Pueraria tuberosa* were prepared by homogenizing pasteurized toned milk with extracts (EE and HWE) of *P. tuberosa*. For thermal treatment, fortified milk samples and milk controls were added into polypropylene tubes and these tubes were capped. All samples were thermally treated in an autoclave at 15 psi for 5 min. Once heating was completed, lids were tightened while still hot, and tubes were kept upright during observations and storage. Three separate batches of both the fortified milk samples and milk controls were prepared. The pH values of all combinations were determined for proper evaluation of any significant change in pH before and after storage.

2.5. Storage Trials, Physical Stability, and pH Tests. Fortified milk samples and milk controls were left at room temperature for 24 hr, followed by storage at 2°C to 8°C for 15 days. Stability profile was checked daily for precipitation profile and pH by bringing the samples to room temperature. Aliquots of the fortified milk samples and milk controls were observed during this period and sampled after two weeks from each of the three batches, for subsequent analyses. After sampling, the pH values of all fortified milk sample and milk controls were measured. Following the overall stability check, the analytical profiles of the 1st day and 15th day samples were compared.

2.6. Sample Preparation for HPLC Analysis. Fortified milk samples and milk controls were kept in freezer at lower temperature (–10°C) for 24 hours, frozen samples then freeze dried by FREEZONE 4.5 lyophilizer, and dried powder weighed to predict the yield from wet mass. Dry lyophilized powder was defatted three times with hexane (1:5 w/v) and finally extracted with methanol (1:3 w/v) by warming on water bath. Isolated fractions were then dried under reduced pressure and temperature. The dried ethanolic and hot water extracts (EE and HWE) of *P. tuberosa* as well as the dried methanolic extracts of the 1st day and 15th day samples

were reconstituted in methanol and working solutions of 30 mg/mL concentrations were made. They were filtered through 0.45 μm membrane filters before being subjected to HPLC analysis.

2.7. HPLC Determination of Puerarin. Analyses were performed on a liquid chromatography system (Waters, Milford, MA, USA) with 515 pumps and equipped with an online degasser, a Waters Pump Control Module (PCM), an autosampler 717, a Waters 2996 photodiode array detector (PDA), and Waters Empower software. Separation was carried out using a Supelcosil LC-8-DB column (250 \times 4.6 mm i.d.; 5 μm pore size) with a guard column (40 \times 4.6 mm i.d.) packed with the same material. The column was maintained at 25°C throughout the analysis, and detection was at 254 nm. Elution was carried out at a flow rate of 0.8 mL/min with water as solvent A and methanol as solvent B using an isocratic elution from 0–10 min with 90% of A followed by a gradient elution from 10–15 min with 90%–85% of A, 15–30 min with 85%–70% of A, 30–37 min with 70%–90% of A, and isocratic from 37 to 45 min with 90% of A. Stock solution of puerarin (1 mg/mL) was prepared in methanol and analysis was carried out under the same working conditions. Each analysis was repeated three times, and the respective retention times were averaged. Peak identification in HPLC analysis was performed by comparison of retention time with reference standard. Quantification of the compounds was achieved by use of calibration plot of the standard solution. The concentrations for the standard used for the calibration curve ranged from 1.0 μg to 5.0 μg for puerarin. Each run was repeated three times.

2.8. Validation. Validation studies were performed for determining linearity, limit of detection, limit of quantification, repeatability, and percentage recovery. Five concentration points were used to prepare the calibration curve. The calibration plot was prepared by plotting peak area against the amount of puerarin and the regression coefficient (r^2) was calculated. Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively. Repeatability was tested by analyzing the puerarin band after application of standard solution to the plate ($n = 3$) and calculating % RSD. Intraday as well as interday repeatability was estimated. Accuracy was determined using an added external standard. A sample of milk was spiked in triplicate with known quantities of puerarin and the percentage of recovery was calculated. The percentage of recovery rate was established from the experimental response values ((blank + standard) – blank) obtained according to the calibration curves and the real concentration of the standard added.

3. Results

3.1. Physical Stability and pH Tests. Stability profile with pH was checked daily for precipitation profile and pH by bringing the samples to room temperature. Aliquots of the

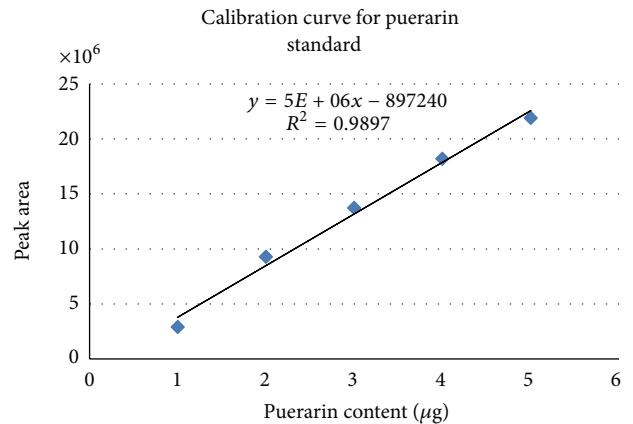


FIGURE 1: Calibration curve for standard Puerarin.

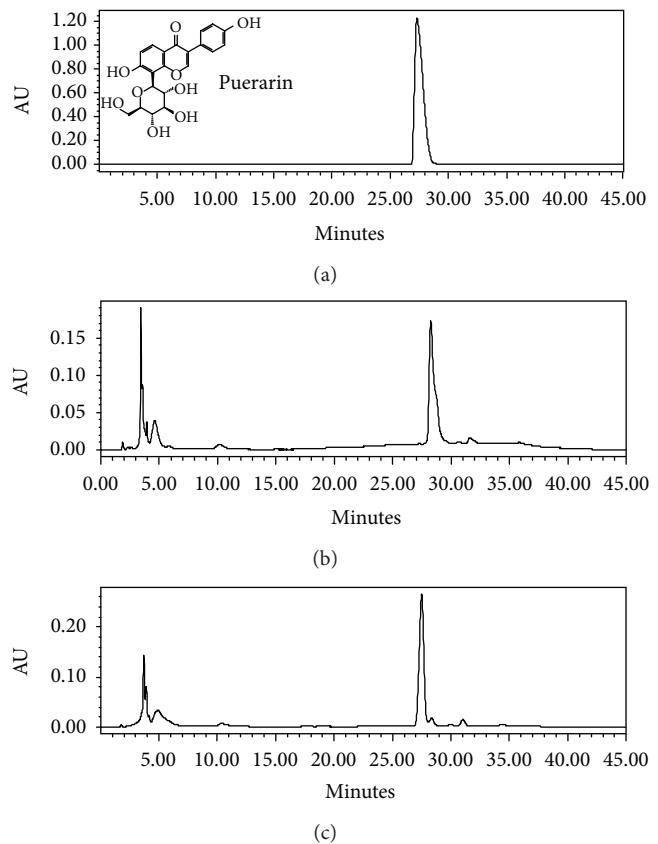


FIGURE 2: HPLC profiles of fortified milk samples (a) Puerarin standard; (b) methanolic extract of milk + 1.0% HWE of *P. tuberosa* (1st day); (c) methanolic extract of milk + 1.0% HWE of *P. tuberosa* (15th day).

fortified milk samples and milk controls were observed during this period. No precipitate formation was observed in the samples. No significant change in the pH was observed. The observations are tabulated in Table 1.

3.2. HPLC Determination of Puerarin. To determine the content of puerarin in fortified milk samples, the dried ethanolic and hot water extracts of *P. tuberosa* as well as

TABLE 1: Stability profile of milk samples.

Milk samples	Day 1 sample		Day 5 sample		Day 10 sample		Day 15 sample	
	Precipitate	pH	Precipitate	pH	Precipitate	pH	Precipitate	pH
Milk + 0.5% EE (w/v)	Absent	6.50	Absent	6.53	Absent	6.55	Absent	6.58
Milk + 1.0% EE (w/v)	Absent	6.46	Absent	6.59	Absent	6.60	Absent	6.69
Milk + 0.4% HWE (w/v)	Absent	6.42	Absent	6.50	Absent	6.59	Absent	6.63
Milk + 0.7% HWE (w/v)	Absent	6.43	Absent	6.45	Absent	6.48	Absent	6.51
Milk + 1.0% HWE (w/v)	Absent	6.42	Absent	6.50	Absent	6.55	Absent	6.59
Milk + 0.01% puerarin (w/v)	Absent	6.52	Absent	6.60	Absent	6.64	Absent	6.68
Milk (control)	Absent	6.61	Absent	6.65	Absent	6.68	Absent	6.71

EE: ethanolic extract of *P. tuberosa*.HWE: hot water extract of *P. tuberosa*.

TABLE 2: Puerarin content in Indian Kudzu fortified milk samples.

Milk sample	Day 1 sample		Day 15 sample	
	(mg/10 mL fortified milk)			
Milk + 0.5% EE (w/v)	2.39 ± 0.03			2.34 ± 0.03
Milk + 1.0% EE (w/v)	5.25 ± 0.04			5.19 ± 0.16
Milk + 0.4% HWE (w/v)	0.41 ± 0.02			0.34 ± 0.04
Milk + 0.7% HWE (w/v)	0.81 ± 0.03			0.81 ± 0.03
Milk + 1.0% HWE (w/v)	1.24 ± 0.05			1.27 ± 0.02
Milk + 0.01% puerarin (w/v)	1.03 ± 0.09			0.97 ± 0.02

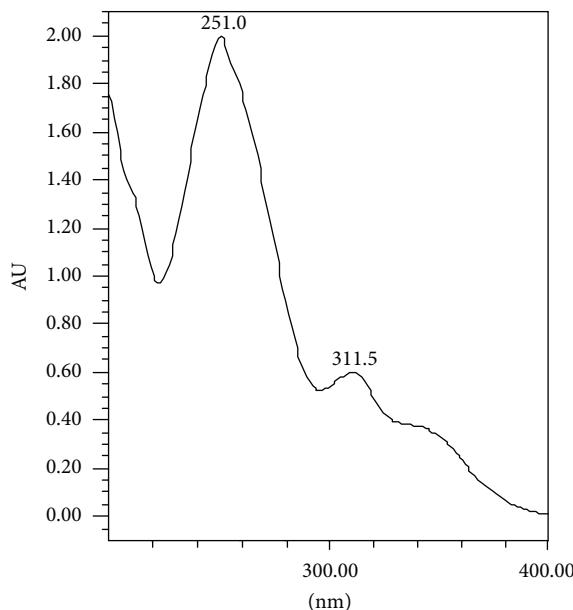
EE: ethanolic extract of *P. tuberosa*.HWE: hot water extract of *P. tuberosa*.

FIGURE 3: UV spectrum for Puerarin.

the dried methanolic extracts of the 1st day and 15th day of fortified milk samples were reconstituted in methanol and working solutions of 30 mg/mL concentrations were made for HPLC analysis. Ten μ L of each sample was injected and analysis for puerarin was carried out by using HPLC under the conditions described earlier. Puerarin was monitored at 254 nm. This compound has been of interest due to its many

potential health benefits. The calibration curve was plotted for puerarin (Figure 1) and the percentage content of puerarin in different samples was calculated. The ethanolic and hot water extracts of *P. tuberosa* were found to contain 5% and 1.13% puerarin, respectively. No peak corresponding to that of puerarin was observed in control milk samples. Figure 2 shows the HPLC chromatograms of the puerarin standard and milk sample fortified with hot water extract of *P. tuberosa* as observed on 1st and 15th day, also monitored at 254 nm. The identity of Puerarin in milk samples was confirmed by comparison of the UV spectra (Figure 3) and retention time with the authentic standard and calculated as mg Puerarin per 10 mL of fortified milk. Results of the HPLC analysis are presented in Table 2.

3.3. Validation. Linearity, limit of detection, limit of quantification, repeatability, and percentage recovery were studied. The linear range for puerarin was 1.0–5.0 μ g with a correlation coefficient (r^2) of 0.989. This correlation coefficient of >0.950 was indicative of a good linear relationship between concentration and peak area in the concentration range studied. LOD and LOQ values were 300 ng and 500 ng, respectively. Both the intra- and interday R.S.D. were less than 10% over this range. At the same concentrations, accuracy ranged from 92 to 110%. The high recovery values and a high repeatability indicated a satisfactory accuracy in the method used.

4. Discussion

In the present investigation, different herb-milk combinations were prepared by homogenizing pasteurized toned milk

with extracts (EE and HWE) of *P. tuberosa*. Storage trials for physical stability and pH monitoring of these fortified milk samples were undertaken and they were analyzed for detection and quantification of puerarin, the major isoflavone C-glucoside present in *P. tuberosa*, through high-performance liquid chromatography using photo diode array detector. A number of studies suggest that Indian Kudzu exhibits antihyperglycemic, antihyperlipidemic, hepatoprotective, antihepatotoxic, and antiimplantation activities [5–8]. Kudzu has been reported to contain high amounts of phytoestrogenic isoflavones, such as puerarin, daidzein, genistein, and their derivatives [24–26]. These compounds based on the structural similarity to internal estrogen have received much interest for the prevention of menopausal symptoms, osteoporosis, high cholesterol, heart disease, and cancer [27–32]. Also, Kudzu root powder and extract are sold in the United States, United Kingdom, and Australia as a supplement. Kudzu is often used as a single ingredient or in combination with other herbs for relieving hangover, fever, and flu; improving liver function; enhancing detoxification processes; regulating cardiac functions; and aiding weight loss [33].

Milk, being one of the most widely consumed foods in the world, is an ideal vehicle for fortification. However, it is necessary that the samples are stable and the concentration and nature of the herbs/extracts that have been added for fortification do not change on storage. It was observed that there was no precipitate formation and the pH also did not change during the study period. This indicated that the milk samples were physically stable under the experimental conditions mentioned above. Also, the biomarker used for chemical analysis was puerarin, which is the major constituent of Kudzu. HPLC analysis results showed that the puerarin content can directly be a measure of the amount of ethanolic or hot water extracts of *P. tuberosa* added for fortification. Also there was no significant change in the content of puerarin during the study period, thereby indicating the chemical stability of the samples. These studies will be useful for developing milk nutraceuticals fortified with Indian Kudzu which has the potential to be included as an ingredient in health and functional foods.

5. Conclusions

A modern lifestyle is fast paced and mostly hurried where most people battle with time poverty. As a result, it is often difficult to find the time and energy to eat correctly and supply your body with the correct type of nutrition. On top of that, an individual's health and nutrition needs do also change throughout his or her life. It is for this reason that development of functional foods and nutraceuticals with special health-promoting benefits is the need of the day. Herbal extracts in all their forms possess arguably the greatest potential for innovative functional food products. There are many companies already capitalizing on growing consumer acceptance of food and beverages containing herbal extracts [2] although the use of these extracts in milk and milk products is quite a recent development. Milk is also one of

the most widely consumed foods in the world and is an ideal vehicle for fortification with these nutraceuticals. Also, numerous nutraceutical combinations have entered the international market through exploration of ethnopharmacological claims made by different traditional practices. These foods or nutraceuticals construct a health-promoting, disease-preventing diet with protective substances. The rich nutrient food intake will provide maximum protection against not only infections, asthma, and allergies, but also against heart disease and cancer in adulthood. However, before the full market potential can be realized, the consumers need to be assured of the safety and efficacy of functional foods. Future research will focus on mechanisms by which food components such as phytochemicals positively affect health, and whether these components work independently or synergistically.

Conflict of Interests

The authors do not have any conflict of interests in this paper.

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Erratum

Erratum to “The Preventive Effect of Biochanin A on Bone Loss in Ovariectomized Rats: Involvement in Regulation of Growth and Activity of Osteoblasts and Osteoclasts”

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Since the publication of the original paper, the authors have noticed an error in the primer sequences. The correct primer sequences are as follows.

“Osterix (NM_001037632): forward: 5'-cccgctgtactttgc-cccc-3', reverse: 5'-ctgccccactgcctgcataat3' (203 bp); ALP (NM_013059): forward: 5'-tggacggtaacgggagaac-3', reverse: 5'-cagagctggccaggcaca-3' (238 bp); collagen type I (NM_053304): forward: 5'-agctggacttcctggct-3', reverse: 5'-gctcgccagggttcaccagg-3' (219 bp); Osteocalcin (NM_013414): forward: 5'-tgaggaccctctctgtc-3', reverse: 5'-accaccta-ctgcccctctg-3' (130 bp); OPG (NM_012870): forward: 5'-tgacctcagaacccggacgtc-3', reverse: 5'-gttgcactctgttacgg-3' (202 bp); RANKL (NM_057149): forward: 5'-g gtcggaggaga-tggca-3', reverse: 5'-agcggcaggaaatgaagcg-3' (119 bp); and TRAP (NM_019144): forward: 5'-cacctgtgttccctccagca-3', reverse: 5'-gccataatctgcacggtc-3' (151 bp). GAPDH (NM_017008): forward: 5'-cctgtaccac caactgttta-3', reverse: 5'-ggccatccacagtcttgag-3' (140 bp).”

In PCR amplification, “52°C for 1 min” should be corrected as “64°C for 1 min.”

In Figures 1 and 2, NADPH should be corrected to GAPDH, as shown here.

The authors apologize for this error and any inconvenience caused.

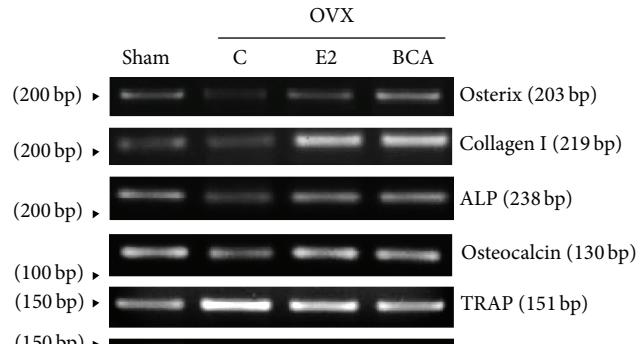


FIGURE 1

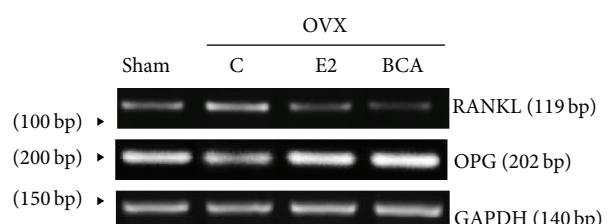


FIGURE 2

Research Article

Ursolic-Acid-Enriched Herba Cynomorii Extract Protects against Oxidant Injury in H9c2 Cells and Rat Myocardium by Increasing Mitochondrial ATP Generation Capacity and Enhancing Cellular Glutathione Redox Cycling, Possibly through Mitochondrial Uncoupling

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Mitochondrial decay is considered to be a major contributor to aging-related diseases, including neurodegenerative diseases, cardiovascular disorders, and certain metabolic diseases. Therefore, the maintenance of mitochondrial functional capacity and antioxidant status should play an essential role in preventive health. Herba Cynomorii, which is one of the most potent “Yang-invigorating” Chinese tonic herbs, was found to increase mitochondrial ATP generation capacity (ATP-GC) in rat hearts *ex vivo*. In the present study, we demonstrated that HCY2, an active fraction of Herba Cynomorii, and its major ingredient ursolic acid (UA) could protect against hypoxia/reoxygenation-induced cell apoptosis in H9c2 cells *in vitro* and also against ischemia/reperfusion-induced injury in rat hearts *ex vivo*. The cardioprotection was associated with an increase in ATP-GC and an enhancement of glutathione redox cycling. The results suggest that UA may be one of the active ingredients responsible for the cardioprotection afforded by Herba Cynomorii, and this effect may be mediated, at least in part, by enhancement of mitochondrial functional capacity and antioxidant status, possibly through the induction of mitochondrial uncoupling.

1. Introduction

Aging is defined as an inevitable degenerative process in physiological functions and metabolic processes, chronologically leading to morbidity and mortality [1]. Emerging evidence has linked mitochondrial decay to a variety of age-related diseases, including, but not limited to, neurodegenerative diseases, cardiovascular disorders, and cancer [2]. The investigation of mitochondrial function in various organs of aging animals has shown a variety of mitochondrial alterations [3, 4] that point to defects at specific sites in mitochondrial energy transfer pathways, for example, the respiratory chain [5, 6], which result in age-dependent decreases in mitochondrial ATP generation capacity (ATP-GC) [7, 8]. This age-associated deterioration of mitochondrial bioenergetics is invariably accompanied by progressive impairment in mitochondrial antioxidant status [7, 9]. Given the central

role of mitochondria in the aging process, the maintenance of mitochondrial functional capacity and antioxidant status is important for preventive health, especially for organs having a major requirement for energy in the form of ATP, such as the heart [10, 11].

Efforts to retard the adverse consequences of aging have long been a preoccupation of mankind, and traditional Chinese medicine (TCM) always emphasizes the prolongation of a healthy lifespan. In this regard, many Chinese tonic herbs have long been used for safeguarding health and delaying the onset of senility. According to TCM theory, tonic herbs, which are used for the treatment of various patterns (Yin, Yang, Qi, Blood) of deficiency in body function, are classified into four categories: Yang-invigorating, Yin-nourishing, Qi-invigorating, and blood-enriching [12]. The blood-enriching and Qi-invigorating herbs are further subgrouped under the Yin family and Yang family, respectively. Holistically,

the Yang-invigorating action involves the upregulation of cellular activities, particularly in the heart, which plays a pivotal role in fueling the vital activities in all organs. Previous studies in our laboratory have demonstrated the ability of Yang tonic herbs to increase mitochondrial ATP-GC, which is associated with an increased activity of the mitochondrial electron transport system [13]. In addition to upregulating mitochondrial functional capacity, Yang tonic herbs/formulas have also been shown to enhance cellular/mitochondrial antioxidant status [14] and also decrease mitochondrial coupling efficiency [15], thereby protecting against oxidant injury in various tissues of rats [16, 17].

Herba Cynomorii (the whole plant of *Cynomorium songaricum* Rupr., Cynomoriaceae), also known as Suo-Yang in Chinese, has been used as a herbal tonic to supplement the primordial “Yang essence” for both men and women in Chinese medicine for hundreds of years. As documented in the Chinese medicine literature, Herba Cynomorii is renowned as “Bu Lao (ageless) herb”, and this bespeaks much of its ability to sustain a youthful life. Preliminary studies in our laboratory have demonstrated that the methanol extract of Herba Cynomorii stimulates mitochondrial ATP-GC in both H9c2 cells *in vitro* and rat hearts *ex vivo*, presumably by increasing mitochondrial electron transport [13, 18]. However, the chemical components responsible for increasing mitochondrial ATP-GC remain to be identified. In addition, it is as yet unclear whether Herba Cynomorii can enhance myocardial mitochondrial antioxidant status and protect the heart against oxidant injury.

In the present study, we endeavored to examine the chemical basis as well as the biochemical mechanism underlying the “Yang-invigorating” action of Herba Cynomorii. The objectives of this study were (1) to isolate the active fraction(s) through bioactivity-guided fractionation, in which the measurement of ATP-GC in H9c2 cells was utilized as an activity monitor; (2) to identify the active principle(s) from active fraction(s); (3) to investigate whether active fraction(s) and their respective active component(s) can afford protection against hypoxia/reoxygenation-induced cell apoptosis in H9c2 cells as well as myocardial ischemia/reperfusion (I/R) injury in rats; and (4) to investigate the possible biochemical mechanism underlying the cardioprotection.

2. Materials and Methods

2.1. Drugs and Chemicals. Luciferase was obtained from Fluka (Switzerland). Ursolic acid (UA) was purchased from Wako Pure Chemical Industries, Ltd (Japan). Other chemicals were purchased from Sigma Chemical (St Louis, MO). Solvents used for HPLC were of HPLC grade.

2.2. Cell Culture. H9c2 cells, a permanent cell line derived from embryonic BDIX rat heart tissue, were purchased from American Type Culture Collection. The cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL) supplemented with 10% (v/v) fetal bovine serum. The medium contained glucose (4.5 g/L) and glutamine (4.5 mM), supplemented with NaHCO₃ (17 mM), penicillin (100 IU/mL), and streptomycin (100 µg/mL). All

cells were grown under an atmosphere of 5% CO₂ in air (v/v) at 37°C. The medium was replaced every 2-3 days. A stock of cells was grown in a 75 cm² culture flask and split before confluence at a subcultivation ratio of 1:10.

2.3. Herbal Material and Extraction. Herba Cynomorii was purchased from a local (Hong Kong-based) herbal dealer (Lee Hoong Kee, Ltd.). The herb was authenticated by the supplier and a voucher specimen (HKUSTY01001) was deposited in the Division of Life Science, the Hong Kong University of Science and Technology (HKUST). For extraction, powdered stems of Herba Cynomorii (14 kg) were extracted with 95% ethanol at an herb-to-solvent ratio of 1:6 (w/v) for 2 h under reflux at 70°C. The extraction was repeated once, and the pooled ethanol extracts were filtered and concentrated under reduced pressure using a rotavaporator. The ethanol extraction resulted in a yield of 13% (w/w) with respect to the initial amount of crude herb. The extract was stored at 4°C prior to use.

2.4. Bioassay-Guided Fractionation of the Herba Cynomorii Ethanol Extract Utilizing an ATP-GC *In Situ* Assay as Activity Monitor. The ethanol extract of Herba Cynomorii was fractionated by silica gel column chromatography, with stepwise elution using a mixture of acetone and petroleum ether (3:7, 1:1 and 7:3; two bed volumes each), which was followed by absolute ethanol. Four crude fractions, termed, A1, A2, A3, and A4, were obtained. Based on the biological activity (refer to Section 3), A1 and A2 were grouped together, and the mixture was again subjected to silica gel column chromatography, with elution by a mixture of acetone and petroleum ether (3:7, v/v) to yield three subfractions: HCY1 (57 g), HCY2 (140 g), and HCY3 (16 g). All these fractions were concentrated under reduced pressure and stored at 4°C prior to use.

For the measurement of ATP-GC *in situ*, H9c2 cells were seeded at a density of 2.0 × 10⁴ cells/well in a 24-well culture plate, and cells in each well were allowed to grow to 60–80% confluence in a humidified incubator at 37°C within 2 days prior to drug treatment. Herbal extracts (dissolved in DMSO) were added to the medium to achieve the desired final concentrations (DMSO < 0.2%, v/v). After a 4 h incubation with the herbal extract or UA, the cells were subjected to the measurement of ATP-GC, as described by Leung and Ko [19].

2.5. HPLC-QQQ-MS/MS Analysis. HPLC analysis was performed using an Agilent RRLC 1200 series system (Agilent, Waldbronn, Germany), which was equipped with a degasser, a binary pump, an autosampler, and a thermostated column compartment. HCY2 (20 µg/mL, dissolved in acetonitrile) or UA (5 µg/mL, dissolved in acetonitrile) were separated on a Waters Atlantis C18 column (5 µm id, 4.6 mm × 150 mm). The mobile phase was composed of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B), using an isocratic gradient 78% (A) for 50 min. The flow rate was 0.4 mL/min. The column temperature was 25°C. The injection volume was 5 µL. For the MS/MS analysis, an Agilent QQQ-MS/MS (6410A) equipped with an ESI ion source was operated in positive ion mode. The drying gas temperature was 325°C, with

a gas flow of 10 L/min, nebulizer pressure at 35 psig, capillary voltage at 4000 V, and delta electro multiplier voltage at 400 V. Two suitable transition pairs were chosen for acquisition in MRM mode for UA. The fragmentor voltage and collision energy values were optimized to obtain the highest abundance. Agilent Mass Hunter workstation software (version B.01.00) was used for data acquisition and processing.

2.6. HPLC-UV Analysis. For quantitative analysis, HCY2 (1.4 mg/mL, dissolved in acetone and petroleum ether (3:7)) or UA was separated using an Agilent RRLC 1200 series system (Agilent, Waldbronn, Germany) consisting of an online degasser, two binary pumps, a high-performance SL autosampler, a thermostated column compartment, and a photo-diode array UV-VIS detector. A mobile phase consisting of water (solvent A) and acetonitrile (solvent B) was applied for linear gradient elution (0 min 40% B, 50 min 100% B, 60 min 100% B) on an Agilent ZORBAX SB-Aq C18 column (5 μ m, 4.6 \times 250 mm) at a flow rate of 1.0 mL/min and a temperature of 25°C. The detection wavelength was set at 205 nm. The quantitation of UA in the HCY2 fraction was determined from a calibration curve. A 5 mg/mL standard stock solution of UA was prepared in methanol. A serial dilution with methanol was made of each stock solution to prepare standard solutions at concentrations of 0.1, 0.2, 0.5, 1, and 2 mg/mL; 10 μ L of each standard solution was used for analysis and construction of a standard calibration curve.

2.7. Measurement of Substrate-Supported Mitochondrial Respiration Rates in Digitonin-Permeabilized Cells. Respiratory activity was measured polarographically with a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK). H9c2 cells were seeded (2.0×10^6 cells) in 100 mm culture plate. After stable attachment, cells were preincubated with herbal extracts or UA at desired final concentrations for 4 h at 37°C. After the incubation, the herbal extract- or UA-containing medium was aspirated and cells were collected by trypsinization followed by centrifugation. Cells were washed with phosphate buffered saline-A (PBS-A) twice, resuspended in assay buffer (120 mM KCl, 5 mM KH₂PO₄, 2 mM EGTA, 10 mM HEPES, 0.1 mM MgCl₂, 0.5% BSA, pH 7.4), and stored at 37°C. An aliquot (1 mL) of suspended cells (1.5×10^6 cells/mL) was placed in the air-tight liquid-phase oxygen electrode chamber. The system was maintained at 30°C using a constant temperature water-jacketing system. After equilibration, a nonionic detergent, digitonin (50 μ g/mL), was added and incubated for 3 min to permeabilize the cell membrane. This was followed by the addition of pyruvate (5 μ M), malate (2.5 μ M), and ADP (60 μ M) to allow mitochondrial state 3 respiration. Mitochondrial state 4 respiration was then induced by the addition of a specific complex V inhibitor, oligomycin (1 mg/mL). A typical polarographic recording of H9c2 cells respiration rates is shown in Figure 4(a).

2.8. Measurement of Cellular GSH Levels in H9c2 Cells. H9c2 cells were seeded (3.75×10^4 cells/well) in 12-well culture plates. After stable attachment, cells were preincubated with herbal extracts or UA for increasing periods of time (2, 4, 8, 12, 16, and 24 h) at 37°C. Following incubation, reduced

glutathione (GSH) levels were determined at each time point. Cellular GSH levels were determined enzymatically using DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid) and glutathione reductase (GR), in a protocol modified from Griffith [20].

2.9. In Vitro Hypoxia/Reoxygenation-Induced Cell Apoptosis. Cells used for the experiment were seeded at 2.5×10^5 cells on a 60 mm² culture plate and allowed to grow overnight. After herbal extract or UA treatment, the cells were washed twice with Krebs-Ringer Bicarbonate buffer (KRB) containing 115 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24 mM NaHCO₃, 10 mM HEPES, and (pH 7.4). Aliquots (2.5 mL) of KRB supplemented with 0.01% bovine serum albumin (BSA) were added to the cells immediately prior to the induction of hypoxia. A Billups-Rothenberg modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) was used to produce an *in vitro* hypoxia/reoxygenation (Hypo/Reoxy) challenge. In essence, cells were placed in the sealed chamber, and the chamber was flushed with nitrogen for 15 min at flow rate of 20 mL/min. After closing all sealable connectors, the chamber was transferred to an incubator and the cells in the chamber were subjected to a 2 h period of hypoxia at 37°C. Reoxygenation was initiated by opening the chamber and replacing the KRB with fresh DMEM medium. The cells were then cultured in the incubator under an atmosphere of 5% CO₂ in air at 37°C for 16 h. The extent of apoptosis was quantified by measuring the activation of caspase 3 using a commercially available assay kit (Caspase 3 Assay Kit, Fluorimetric, SIGMA).

2.10. Animal Treatment. Adult male and female Sprague-Dawley rats (8–10 weeks; 250–300 g) were maintained under a 12 h dark/light cycle at about 22°C and allowed food and water *ad libitum*. Experimental protocols were approved by the Research Practice Committee at the Hong Kong University of Science & Technology. Animals were randomly divided into groups of 8 animals in each. In the treatment groups, male rats were intragastrically given HCY2 (dissolved/suspended in olive oil) at a daily dose of 16 or 48 mg/kg or UA (dissolved/suspended in olive oil) at a daily dose of 12 or 36 mg/kg for 14 consecutive days, respectively. Preliminary studies indicated that female rats responded to lower doses of HCY2 as assessed by the measurement of ATP-GC using isolated heart mitochondria. Female rats were given HCY2 (dissolved/suspended in olive oil) intragastrically at a daily dose of 2 or 5 mg/kg or UA (dissolved/suspended in olive oil) at a daily dose of 1.5 or 4 mg/kg for 14 consecutive days, respectively. Control animals received vehicle only. Twenty-four hours after the last dosing, animals were subjected to myocardial ischemia/reperfusion (I/R) challenge *ex vivo*.

2.11. Myocardial I/R Injury Ex Vivo. Hearts were quickly excised from phenobarbital-anesthetized rats and immediately immersed in ice-cold saline containing 50 unit/mL of heparin. The aorta was cannulated and the heart transferred to a warm, moist, perfusion chamber. The hearts were retrogradely perfused according to the Langendorff method [21]. After an initial 30 min of perfusion for equilibration, the isolated heart was subjected to a 40 min period of

“no-flow” global ischemia followed by 20 min of reperfusion. Fractions of coronary effluent, collected at 1 min intervals, were obtained both during the course of equilibration and during the subsequent reperfusion. The coronary fractions were immediately placed on ice and assayed for lactate dehydrogenase (LDH) activity. The extent of LDH leakage during the reperfusion period, an indirect index of myocardial injury, was estimated by computing the area under the curve (AUC) of a graph plotting the percentage LDH activity (with respect to the mean preischemic value measured during the equilibration period) against the reperfusion time (1–20 min), as described in [21]. The extent of damage was expressed in arbitrary units. Non-I/R hearts were perfused for 90 min. After either non-I/R or I/R procedures, heart ventricular tissue samples were obtained and subjected to biochemical analysis.

2.12. Measurement of Tissue ATP Level. Samples of perfused and I/R ventricular tissue (approximately 70 mg) were homogenized with 5% perchloric acid (PCA) (4 µL/mg tissue) at 4°C. Following centrifugation at 2150 × g for 10 min at 4°C, the supernatant was diluted 5-fold with 5% PCA. An aliquot (120 µL) of the supernatant was neutralized with 90 µL of 1.4 M KHCO₃, followed by mixing and centrifugation. The resulting supernatant was analyzed for ATP content using a bioluminescence assay (ATPlite, Perkin Elmer, Inc. USA).

2.13. Preparation of Mitochondrial Fractions. Mitochondrial fractions were prepared by differential centrifugation, as previously described in [21]. In brief, myocardial ventricular tissue was homogenized in 10 volumes of ice-cold mannitol/sucrose buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged at 600 × g for 10 min, and the pellet containing nuclear and cell debris was discarded. The supernatant was centrifuged at 9200 × g for 30 min to obtain the mitochondrial fraction. The mitochondrial pellet was suspended in ice-cold mannitol/sucrose buffer.

2.14. Biochemical Analyses in Rat Heart Mitochondria. The mitochondrial ATP-GC of rat hearts was measured *ex vivo* by a method previously described in [14]. Mitochondrial GSH level and the GSH/oxidized glutathione (GSSG) ratio were measured using an enzymatic method of Griffith [20]. Mitochondrial GR activity was measured by monitoring the oxidation of NADPH spectrophotometrically, as previously described in [22].

2.15. Protein Assay. Protein concentrations of mitochondrial fractions and cell lysates were determined using a Bio-Rad protein assay kit using bovine serum albumin as standard.

2.16. Statistical Analysis. All data were expressed as mean ± standard error of the mean (SEM). They were analyzed by one-way analysis of variance (ANOVA). Post hoc multiple comparisons were done with LSD. A difference was considered statistically significant if *P* value < 0.05.

3. Results

3.1. Bioassay-Guided Fractionation of Herba Cynomorii. The fractionation scheme of the Herba Cynomorii ethanol extract (1.82 kg) is depicted in supplementary materials (see Supplementary Materials available online at <http://dx.doi.org/10.1155/2013/924128>). As shown in Figure 1(a), preincubation with the Herba Cynomorii ethanol extract increased ATP-GC in H9c2 cells in a concentration-dependent manner, with the maximum extent of stimulation being 33% at the concentration of 300 µg/mL. Silica gel column chromatography of the Herba Cynomorii ethanol extract yielded four fractions (A1–A4), which were subjected to ATP-GC assay. Figure 1(b) shows that preincubating the cells with fractions A1 or A2 significantly enhanced ATP-GC in H9c2 cells, with the maximum extent of stimulation being 37% at 20 µg/mL and 34% at 50 µg/mL, respectively. Preincubation with fraction A4 also significantly increased ATP-GC in H9c2 cells, but to a lesser extent than that of fractions A1 or A2, the extent of stimulation being 20%. In contrast, preincubating H9c2 cells with fraction A3 produced no detectable effect on ATP-GC, when compared with the control group. Silica gel thin layer chromatography (TLC) analysis of fractions A1 and A2 revealed overlapping spots (data not shown). Fractions A1 and A2 were combined and subjected to refractionation by silica gel column chromatography. Three semipurified fractions (HCY1, HCY2 and HCY3) were obtained at yields of 3.1, 7.7, and 0.9%, respectively. As shown in Figure 1(c), preincubation with fraction HCY2 increased ATP-GC (5–25 µg/mL) in H9c2 cells in a concentration-dependent manner, with the maximum stimulation being 32% at 25 µg/mL. Preincubation with fractions HCY1 and HCY3 also maximally stimulated ATP-GC at 25 µg/mL, with the degrees of enhancement being 10% and 21%, respectively. Thus, HCY2, which was the most abundant of the three semipurified fractions, was the most effective in stimulating ATP-GC in H9c2 cells.

3.2. Identification and Quantitation of UA in HCY2 and the Effect of UA on ATP-GC. By using LC-MS/MS analysis which involved the comparison of fragmentation and retention times with those of standards in the multiple-reaction monitoring (MRM) scan mode (Figure 2(a)), the major component in HCY2 was identified as UA (see chemical structure in Figure 3(a)). The retention time of UA was 23.7 min. The MS/MS transition of UA (MW 456) monitored in the positive ion mode was *m/z* 457 [M + 1] → *m/z* 411 [M-COOH].

As shown in Figure 2(b), a baseline separation of UA was achieved by reverse phase-HPLC with UV detection. The calibration curve of UA was linear and the regression equation of the peak area (*y*) as a function of concentration (*x*) was *y* = 6609.4*x* – 271.4 (*r* = 0.999). The content of UA in HCY2 was estimated to be 75% (w/w).

Preincubation of H9c2 cells with UA increased the ATP-GC in a concentration-dependent manner, with the maximum stimulation being 25% (5 µM), when compared with the control (Figure 3(b)).

3.3. Effect of HCY2 or UA on Mitochondrial Respiration in H9c2 Cells. Figure 4(b) shows that preincubation with HCY2

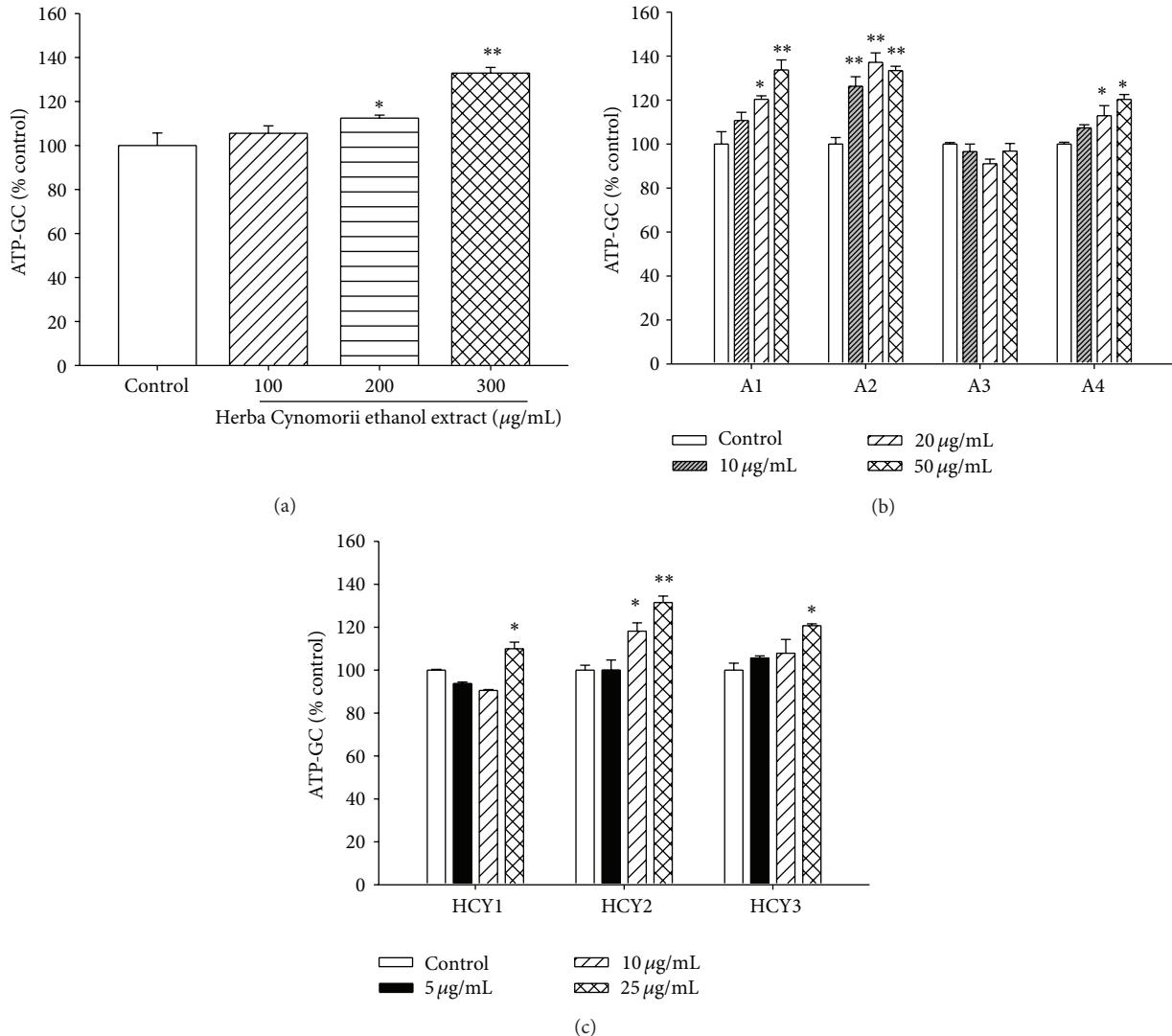


FIGURE 1: Effect of ethanol extract and different fractions of Herba Cynomorii on ATP-GC in H9c2 cells. Cells were incubated with herbal extracts at the indicated concentrations for 4 h. ATP-GC was measured as described in Section 2. Data were expressed as the percentage of nonpreincubated control values [$\text{AUC}_2 = 949 \pm 22$ (SEM)]. Values given are means \pm SEM, with $n = 6$. * $P < 0.05$ and ** $P < 0.01$, when compared with the control group without herbal extract preincubation.

or UA increased state 3 respiration in H9c2 cells in a concentration-dependent manner, with the extent of stimulation induced by HCY2 (25 $\mu\text{g/mL}$) being similar to that of UA (5 μM) (~21%). State 4 respiration was also increased by 50 and 70%, respectively, in HCY2 and UA preincubated cells (Figure 4(c)). The ratio of state 3/state 4 respiration rate, which is also referred to as the respiration control ratio (RCR) that reflects the coupling efficiency of mitochondria during respiration, was decreased by 19% (HCY2) and 29% (UA) at the highest concentration tested.

3.4. Effect of HCY2 or UA on Cellular GSH Levels in H9c2 Cells. As shown in Figure 5, exposure to HCY2 (10 or 25 $\mu\text{g/mL}$) or UA (2 or 5 μM) produced time-driven cyclic variations in cellular GSH levels in H9c2 cells, with concentration-dependent increases in the amplitude of oscillation (21–26% and 12–23%, resp.), when compared with the control.

3.5. Effect of HCY2 or UA on Hypo/Reoxy-Induced Apoptosis in H9c2 Cells. Hypoxia/reoxygenation caused apoptosis in H9c2 cells, as evidenced by a significant increase (1.2 fold) of caspase-3 activity in Hypo/Reoxy-challenged cells, when compared with unchallenged controls. Prior exposure to HCY2 (25 $\mu\text{g/mL}$) or UA (5 μM) for 4 h significantly suppressed the Hypo/Reoxy-induced increase in caspase-3 activity, with the degrees of protection being approximately 36 and 28%, respectively (Figure 6).

3.6. Effect of HCY2 or UA on Myocardial I/R Injury in Rats Ex Vivo. I/R caused tissue damage in isolated-perfused rat hearts, as evidenced by the significant increase in LDH leakage (9- to 11-fold). Hearts from male rats were more susceptible to I/R injury than those from females, as indicated by a greater extent of LDH leakage (11-fold versus 9-fold) (Figure 7). HCY2 pretreatment (16 or 48 mg/kg) dose

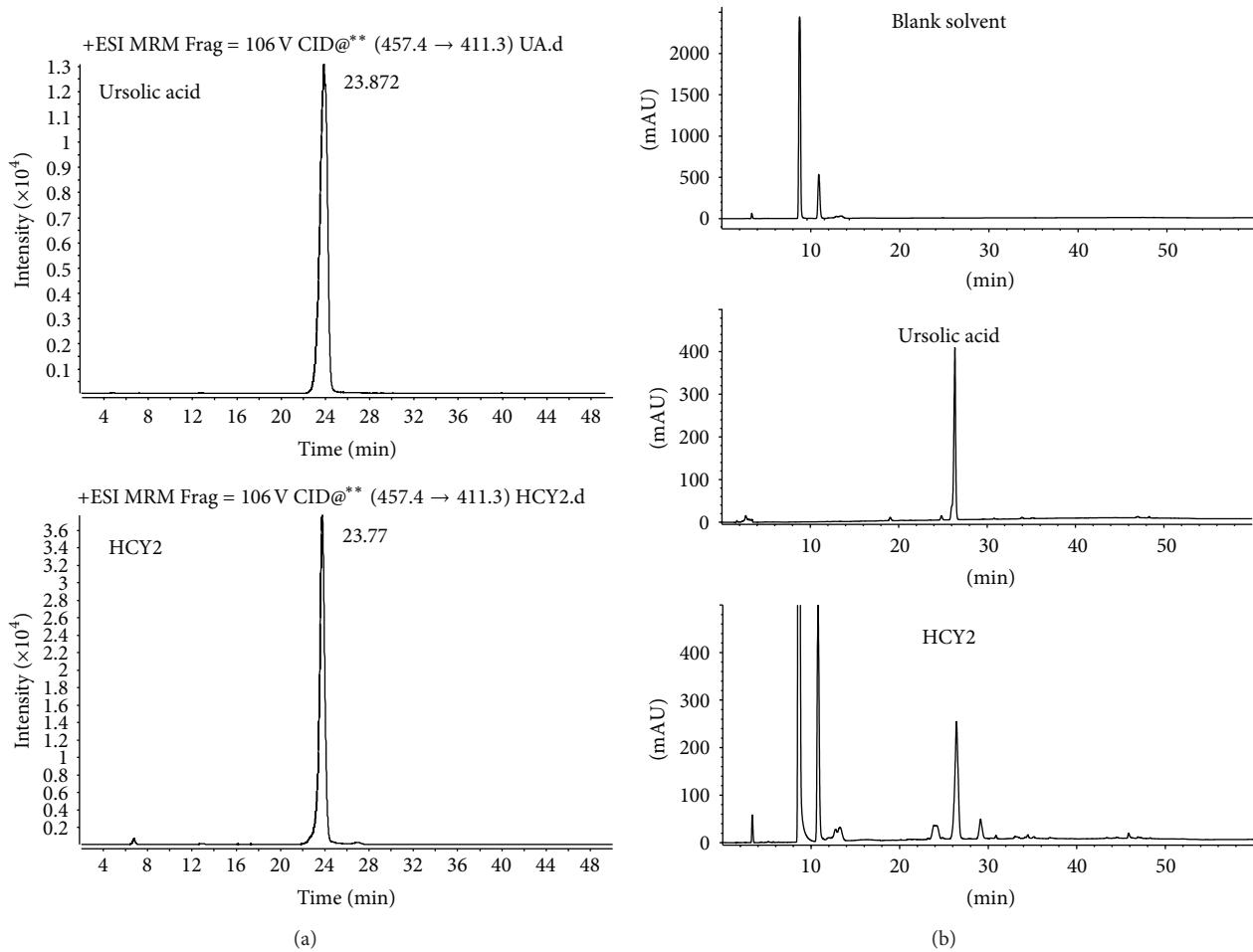


FIGURE 2: Typical HPLC-QQQ MS/MS chromatogram and HPLC-UV chromatogram of UA and HCY2. The chromatographic method is described in Section 2. (a) Selected transitions from precursor ions to product ions used for qualitative analysis of UA in HCY2 after optimization by MRM. (b) The quantitative analysis of UA was made by HPLC-UV (205 nm).

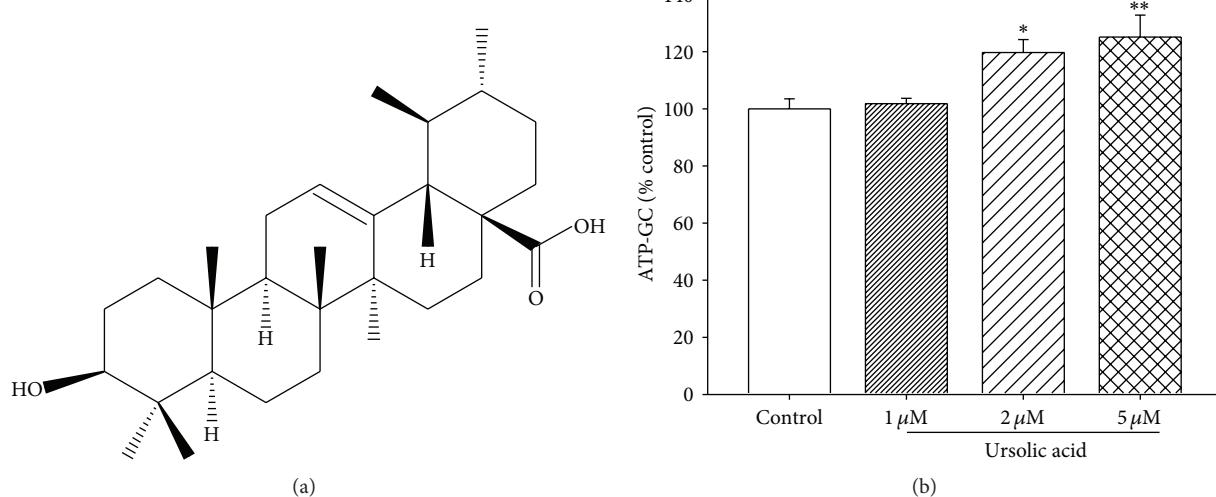


FIGURE 3: (a) Chemical structure of UA. (b) Effect of UA on ATP-GC in H9c2 cells. Cells were incubated with UA at the indicated concentrations for 4 h. ATP-GC was measured as described in Section 2. Data are expressed as the percentage of nonpreincubated control values [$AUC_2 = 750 \pm 26$ (SEM)]. Values given are means \pm SEM, with $n = 6$. * $P < 0.05$ and ** $P < 0.01$, when compared with the control group without herbal extract preincubation.

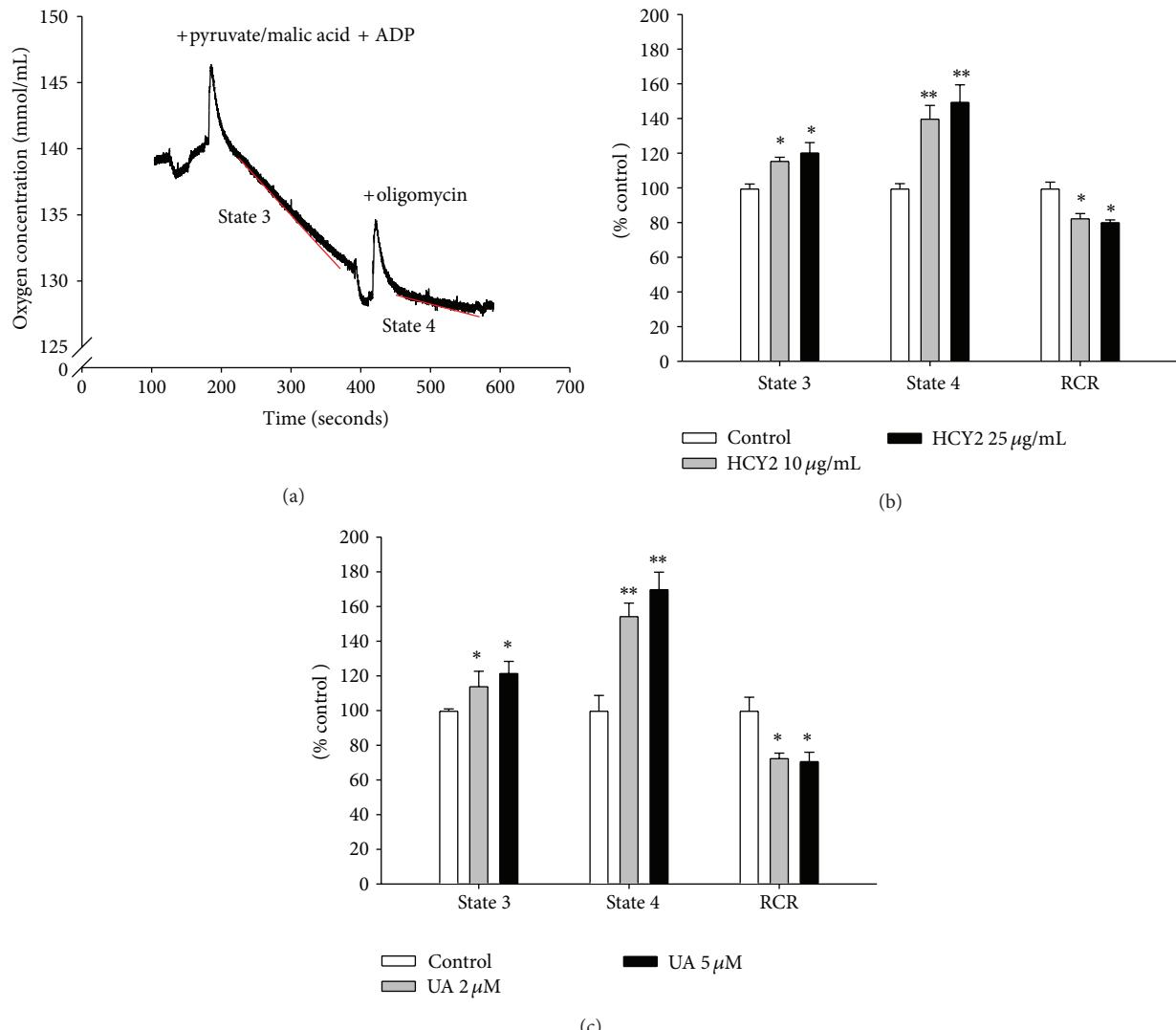


FIGURE 4: Effect of HCY2 and UA on mitochondrial respiration in H9c2 cells. Cells were preincubated with HCY2 and UA at the indicated concentrations for 4 h and subjected to the measurement of oxygen consumption. Typical oxygen-electrode traces of cellular respiration in H9c2 cells are shown in (a). The effects of HCY2 (b) or UA (c) preincubation on mitochondrial respiration were examined. Data are expressed in percent control with respect to the nonpreincubated control (state 3 respiration rate = 3.34 ± 0.06 nmol O₂/min, state 4 respiration rate = 0.78 ± 0.06 nmol O₂/min, RCR = 4.37 ± 0.28). Values given are means \pm SEM, with $n = 3$. * $P < 0.05$ and ** $P < 0.01$, when compared with the control group without drug or herbal extract preincubation.

dependently protected against myocardial I/R injury in male rats, with the degrees of protection being 27 and 37%, respectively. UA pretreatment (12 or 36 mg/kg) also protected against myocardial I/R injury in male rats, with the degrees of protection being 22 and 35%, respectively. As for female rats, although HCY2 pretreatment at a dose of 2 mg/kg did not produce any detectable effect, there was significant protection against I/R injury at a dose of 5 mg/kg, with the degree of protection being 39%. Pretreatment with UA (1.5 or 3.5 mg/kg) resulted in protection against myocardial I/R injury in female rats, with the degree of protection being 38 and 53%, respectively.

3.7. Effect of HCY2 or UA on Mitochondrial ATP-GC in Rat Hearts.

As shown in Figure 8(a), under non-I/R conditions,

HCY2 pretreatment (16 or 48 mg/kg) significantly increased mitochondrial ATP-GC, with the extent of stimulation being 24–50% in male rat hearts. HCY2 pretreatment at a dose of 5 mg/kg significantly increased mitochondrial ATP-GC by 23% in female rat hearts. UA pretreatment (12 or 36 mg/kg for males; 1.5 or 3.5 mg/kg for females) dose dependently enhanced mitochondrial ATP-GC in both male and female rat hearts under non-I/R conditions, with the extent of stimulation being 17–32%.

An increase in ATP-GC was also observed after I/R challenge in both male and female rat hearts, with the extent being 10 to 20% when compared with the non-I/R controls. Under I/R conditions, HCY2 pretreatment (16 or 48 mg/kg) further increased mitochondrial ATP-GC (21–36%) in male rats, when compared with challenged controls. HCY2

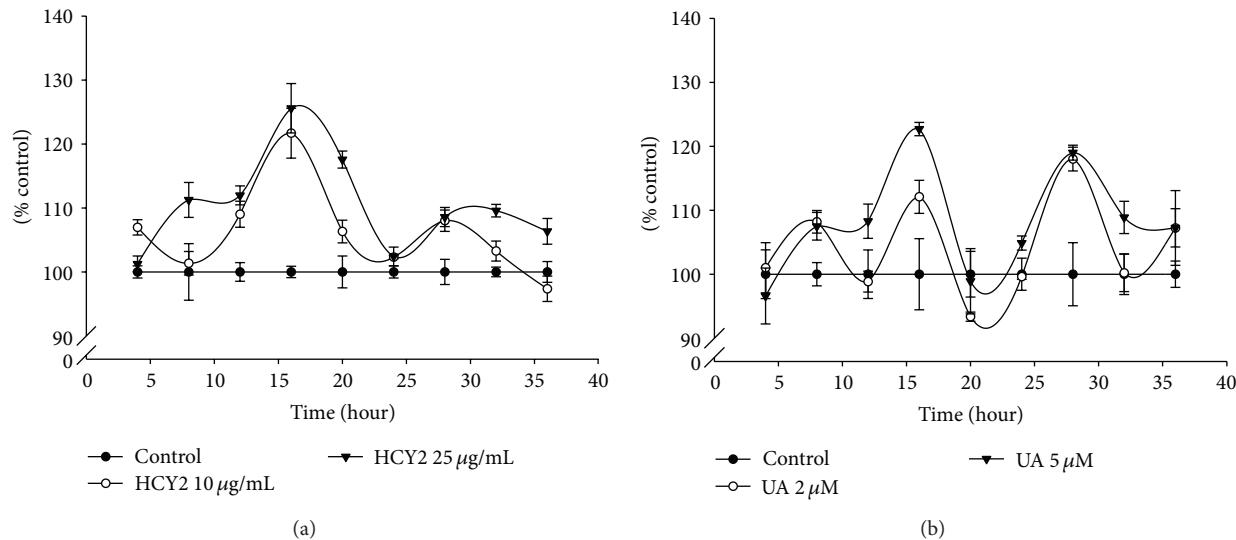


FIGURE 5: Time course of HCY2/UA-induced changes in cellular GSH level in H9c2 cells. HCY2 or UA was added at the indicated concentrations. Reduced glutathione (GSH) levels were measured as described in Section 2. Data are expressed as the percentage of non drug incubated parallel control values (initial control GSH level (a) = 30.6 ± 2.87 nmol/mg protein, (b) = 27.1 ± 1.90 nmol/mg protein) at various periods of herbal extract or drug incubation. Values given are means \pm SEM, with $n = 4$.

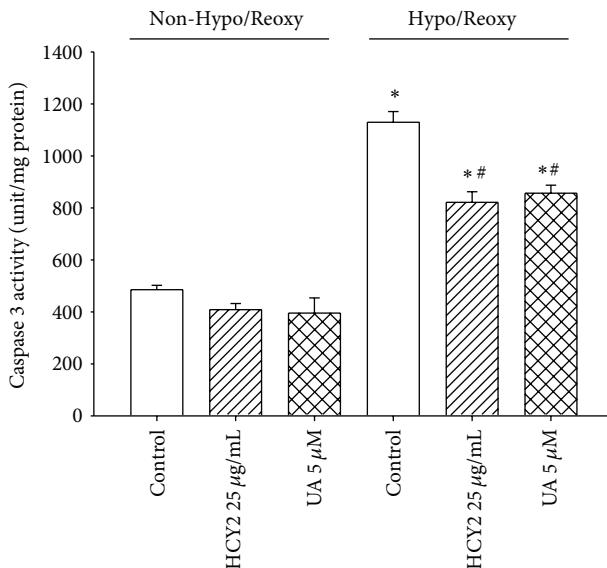


FIGURE 6: Effect of HCY2/UA preincubation on hypoxia/reoxygenation-induced apoptosis in H9c2 cells. Cells were preincubated with HCY2 (25 µg/mL) or UA (5 µM) for 4 h and then subjected to hypoxia/reoxygenation (Hypo/Reoxy) challenge, as described in Section 2. The extent of apoptotic cell death was assessed by the measurement of caspase-3 activation. Values given are means \pm SEM, with $n = 3$. “*” significantly different from the unchallenged control; “#” significantly different from Hypo/Reoxy-challenged control.

pretreatment at a dose of 5 mg/kg enhanced mitochondrial ATP-GC by 54% in female rat hearts subjected to I/R challenge, when compared with unpretreated and challenged hearts. In male rats, UA pretreatment (12 or 36 mg/kg) dose dependently increased mitochondrial ATP-GC by 13 and 22%, respectively, under I/R conditions, when compared

with I/R-challenged controls. UA pretreatment at a dose of 3.5 mg/kg also increased mitochondrial ATP-GC by 46% in female rat hearts under I/R conditions.

3.8. Effect of HCY2 and UA on Tissue ATP Level in Rat Hearts. Under non-I/R condition, HCY2 pretreatment at 48 mg/kg significantly decreased tissue ATP level by 32% when compared with the control group in male rats. In female rats, HCY2 pretreatment at a dose of 5 mg/kg significantly decreased tissue ATP level by 26%. UA (12, 36 mg/kg) dose dependently reduced tissue ATP level in male rat hearts, with the extent of reduction being 26–31%. However, UA (1.5, 3.5 mg/kg) only caused a slight decrease in tissue ATP level in female rat hearts (11 and 20%) under non-I/R condition. The I/R challenge also resulted in a dramatic decrease in tissue ATP level, with the extent of decrease being 46–70% when compared with the non-I/R control. Both HCY2 and UA partially attenuated the decrease in tissue ATP level by 10–27% (Figure 8(b)).

3.9. Effect of Pretreatment with HCY2 or UA on Mitochondrial Glutathione Redox Status in Rat Hearts. Under non-I/R conditions, HCY2 pretreatment at 48 mg/kg significantly increased the mitochondrial GSH/GSSG ratio in male rats, with the extent of enhancement being 54%. In female rats, HCY2 pretreatment at a dose of 5 mg/kg significantly increased the mitochondrial GSH/GSSG ratio (by 24%), when compared with non-challenged controls. However, UA pretreatment did not produce any detectable changes in the mitochondrial GSH/GSSG ratio in either male or female rat hearts in the absence of I/R challenge. I/R caused a significant decrease in the mitochondrial GSH/GSSG ratio (by 40–60%) in both male and female rat hearts. HCY2 pretreatment significantly reduced the change in the GSH/GSSG ratio following I/R challenge by 16% (16 mg/kg) and 30% (48 mg/kg)

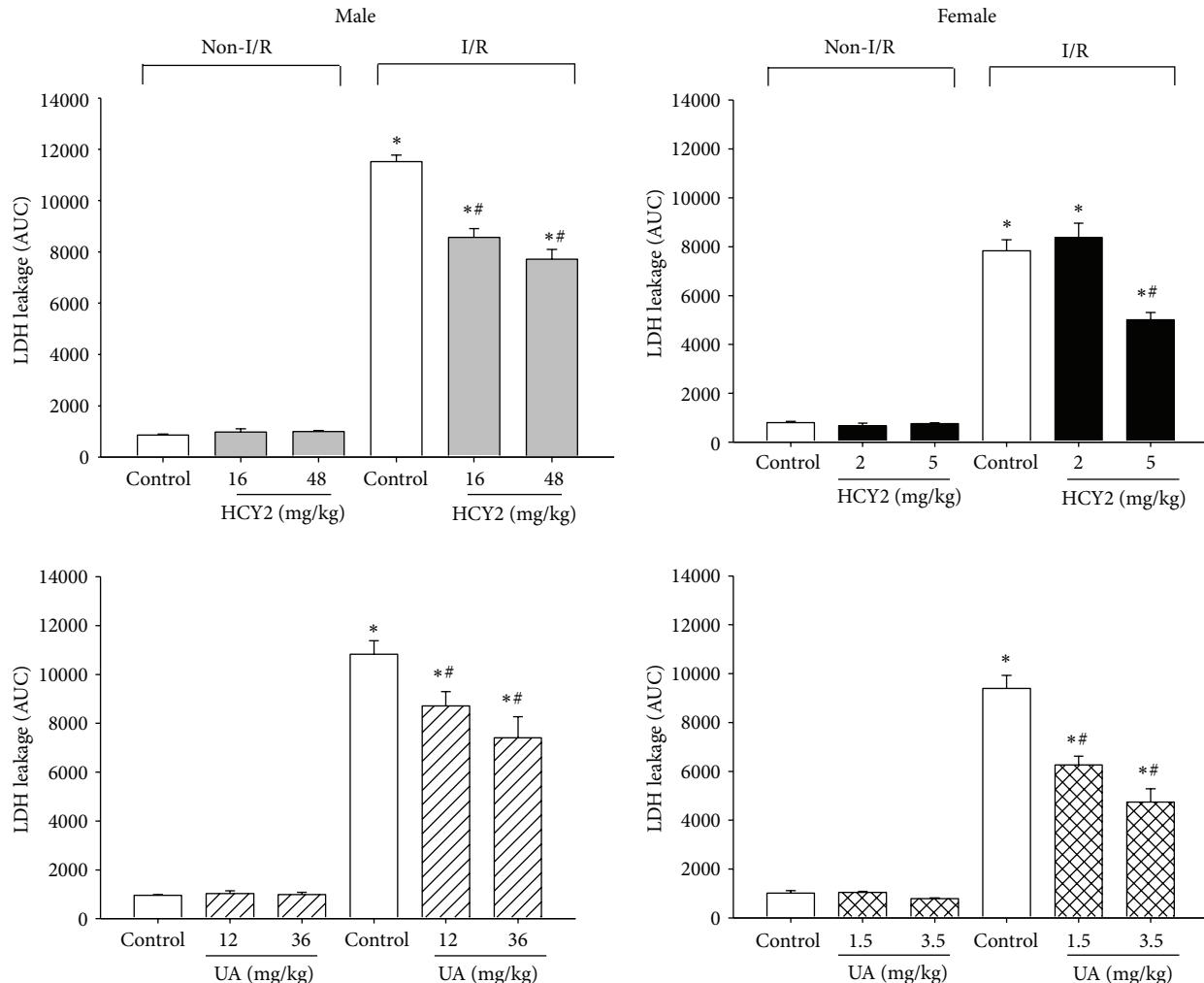


FIGURE 7: Effect of HCY2/UA pretreatment on myocardial I/R injury in male and female rats *ex vivo*. Animals were orally treated with vehicle (olive oil) or HCY2/UA (at the indicated doses) for 14 consecutive days. Isolated hearts were subjected to I/R challenge as described in Section 2. The extent of tissue injury was assessed by the measurement of LDH leakage. Each bar represents mean \pm SEM, with $n \geq 3$. “*” significantly different from the non-I/R control; “#” significantly different from I/R-challenged control.

in male rat hearts as well as 30% (5 mg/kg) in female rat hearts. UA also significantly reduced the I/R-associated change in the GSH/GSSG ratio by 32% (12 mg/kg) and 50% (36 mg/kg) in male rat hearts as well as 30% (1.5 mg/kg) and 66% (3.5 mg/kg) in female rats (Figure 9(a)).

The enhancement of glutathione redox status was accompanied by increases in mitochondrial GR activities in hearts of HCY2- and UA-pretreated animals. In male rats, HCY2 pretreatment increased the mitochondrial GR activity by 20% in the absence of I/R. In female rats, HCY2 pretreatment at a dose of 5 mg/kg caused a significant increase in mitochondrial GR activity by 11%. UA pretreatment did not produce any detectable changes in mitochondrial GR activity in either male or female rat hearts in the absence of I/R challenge. I/R caused a significant decrease in mitochondrial GR activity by 17–33%, when compared with the non-I/R controls. HCY2 pretreatment at a dose of 48 mg/kg for male rats and 5 mg/kg for female rats attenuated the I/R-induced decrease in mitochondrial GR activity by 30 and

10%, respectively. UA pretreatment also reduced the decrease in mitochondrial GR activity by 10–20% in both male and female rat hearts (Figure 9(b)).

4. Discussion

The decline in mitochondrial functional capacity can lead to a deficit in cellular energy supply, especially in organs such as the heart that has a large energy demand. An accumulated body of experimental and clinical evidence has shown that an impairment in energy metabolism resulting from mitochondrial dysfunction plays a major role in the pathogenesis of various heart diseases [23]. An impairment in myocardial oxidative phosphorylation was observed in an experimental model of heart failure, which revealed a dramatic decrease in the maximal ATP generating capacity of mitochondria [24]. Therefore, the maintenance of mitochondrial functional integrity is likely to play an important role in the preventive health of the heart.

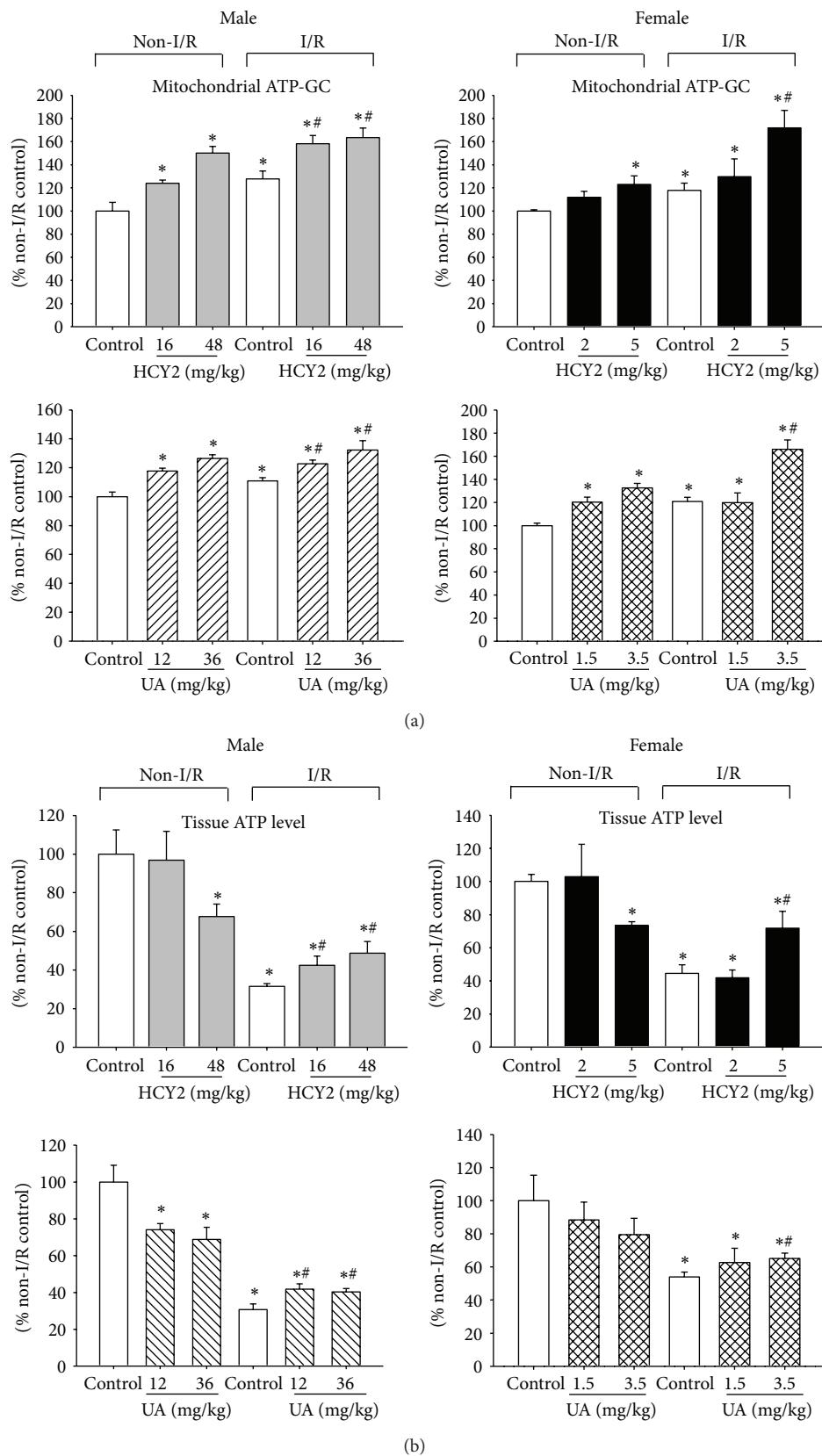


FIGURE 8: Effect of HCY2/UA pretreatment on (a) mitochondrial ATP-GC and (b) tissue ATP levels in control perfused and ischemic-reperfused hearts. Biochemical assays of ATP-GC and tissue ATP level were performed as described in Section 2. Data are expressed as the percentage of non-I/R control values. The values of ATP-GC of non I/R control were [AUC₂ = 1000 ± 24 (male HCY2), 1000 ± 36 (male UA), 1000 ± 11 (female HCY2), and 1000 ± 21 (female UA)]. The values of tissue ATP level of non-I/R control were 1365 ± 183 (male HCY2), 1024 ± 76 (male UA), 1641 ± 70 (female HCY2), and 1558 ± 240 (female UA) nmol/mg protein. Each bar represents mean ± SEM, with $n \geq 3$. “*” significantly different from the non-I/R control; “#” significantly different from I/R-challenged control.

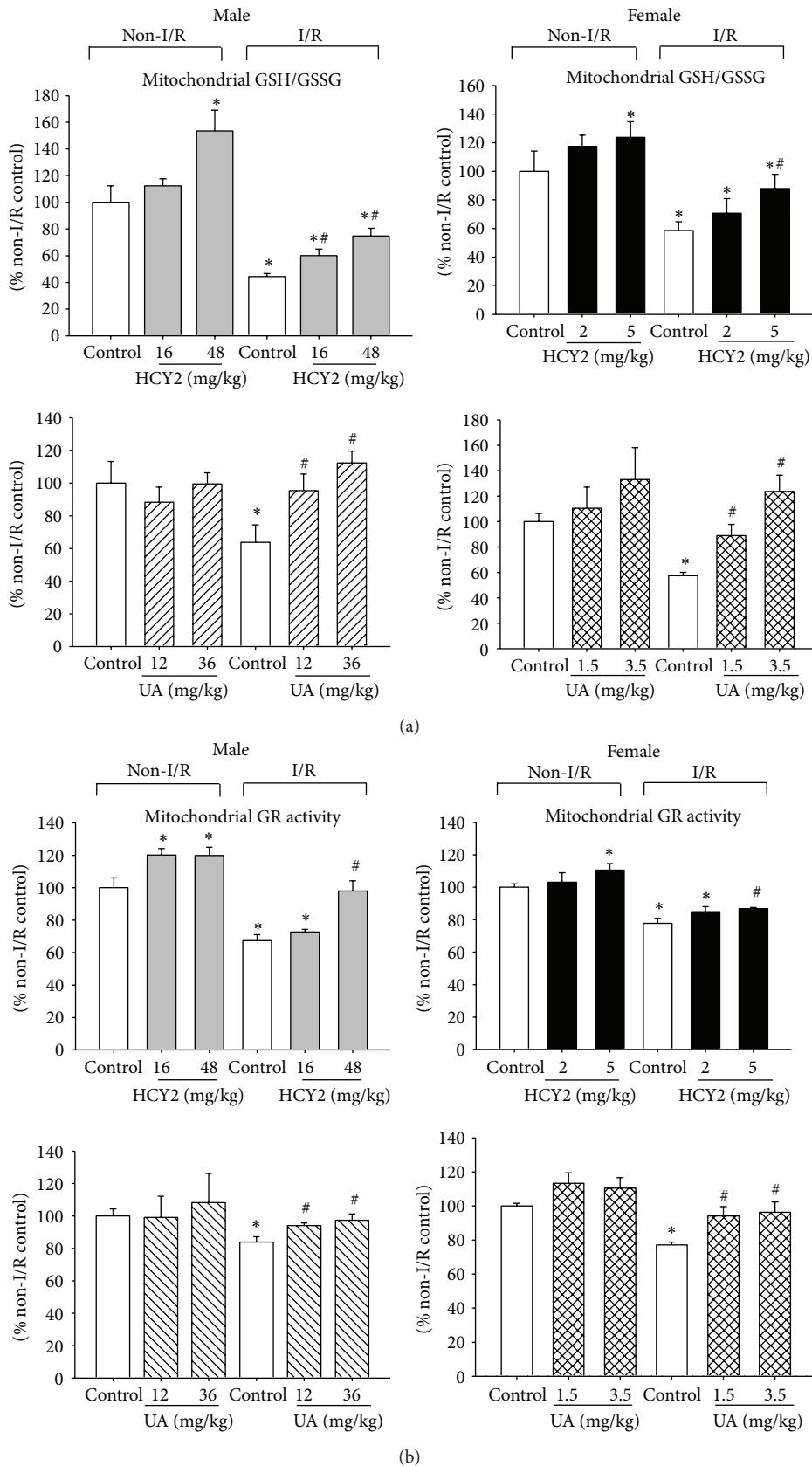


FIGURE 9: Effect of HCY2/UA pretreatment on (a) mitochondrial GSH/GSSG and (b) GR activity in control perfused and ischemic-reperfused hearts. The ratio of mitochondrial GSH/GSSG and the activity of GR were measured as described in Section 2. The values of mitochondrial GSH/GSSG of non-I/R control were 5.0 ± 0.21 (male HCY2), 4.4 ± 0.58 (male UA), 7.0 ± 0.99 (female HCY2), and 6.0 ± 0.38 (female UA). The values of mitochondrial GR activity of non-I/R control were 2.64 ± 0.11 (male HCY2), 2.38 ± 0.14 (male UA), 2.91 ± 0.06 (female HCY2), and 1.71 ± 0.03 (female UA) mU/mg protein. Each bar represents mean \pm SEM, with $n \geq 3$. “*” significantly different from the non-I/R control; “#” significantly different from I/R-challenged control.

Herba Cynomorii is one of the most commonly used “Yang-tonic” herbs for treating what is referred to as “kidney deficiency” in Chinese medicine. Previous studies have shown that administration of Herba Cynomorii enhances mitochondrial ATP-GC both *in vitro* in H9c2 cells and *ex vivo* in rat hearts, presumably through enhancing mitochondrial electron transport [13, 18]. Using an ATP-GC assay as a monitor of biological activity in the fractionation of a Herba Cynomorii ethanol extract, HCY2 was found to be the most abundant fraction with ATP-GC-stimulating activity in H9c2 cells. While Herba Cynomorii was found to contain triterpenes, steroids, flavonoids, phenolic acid, lignans, and polysaccharides [25–28], silica gel TLC analysis of HCY2 showed a major violet spot revealed after spraying with sulfuric acid in ethanol, indicative of steroid- or triterpene-like compounds (data not shown). Further chemical analysis using LC-MS/MS indicated that HCY2 contained UA as the major constituent, which amounted to 75% (w/w), as determined by HPLC analysis using authentic UA as standard. This finding is corroborated by the observation that UA (1–5 μ M) stimulated ATP-GC in H9c2 cells in a concentration-dependent manner, albeit to a lesser degree when compared with that of HCY2 at doses of 10–25 μ g/mL. However, UA at 17.5 and 45 μ M (concentrations equivalent to 10 and 25 μ g/mL HCY2, resp.) produced cytotoxicity in H9c2 cells (data not shown).

The measurement of ATP-GC using malate and glutamate as substrates is an indirect measure of state 3 mitochondrial respiration [29]. Consistently, preincubation with both HCY2 or UA significantly increased state 3 respiration rate in H9c2 cells, as assessed by oxygen consumption. HCY2 and UA preincubations also increased state 4 respiration rate in H9c2 cells, with a resultant reduction in the respiration control ratio, indicative of mitochondrial uncoupling. Mitochondrial uncoupling can be induced by chemical uncouplers as well as by activating uncoupling proteins (UCPs) [30], with resultant increases in oxygen consumption secondary to an increase in electron transport [31]. UA, the major component of HCY2, was found to induce mitochondrial uncoupling in isolated rat heart mitochondria [32]. Such an uncoupling effect produced by the UA-enriched HCY2 could stimulate electron transport in mitochondria, thereby enhancing ATP-GC, which was measured under optimal experimental conditions. In this regard, an uncoupler such as dinitrophenol was found to not only induce uncoupling, but also to stimulate ADP-stimulated respiration rate (i.e., state 3) in liver mitochondria [33]. In addition, the overexpression of UCP4 was shown to facilitate ATP production using succinate as substrate, which was also associated with an increased respiration rate, in neuroblastoma cells [34, 35].

GSH is regarded as the first line of defense in preventing cellular oxidative damage. Under the present experimental conditions, incubations with HCY2 or UA were both found to induce a time-dependent cyclic variation of cellular GSH levels in H9c2 cells, indicative of upregulation of glutathione redox cycling. As also observed in Herba Cistanches-treated H9c2 cells [36], the activation of glutathione redox cycling is presumably related to the interplay between HCY2/UA-induced reactive oxygen species (ROS) generation, an event

secondary to the increased electron transport, and the GR-catalyzed and NADPH-mediated regeneration of GSH. Given that increased formation of ROS within mitochondria can cause an adaptive response mediated by the activation of nuclear factor-erythroid 2 p45-related factor 2 [37, 38], HCY2/UA may therefore positively regulate GR, which in turn catalyzes the regeneration of GSH from GSSG, leading to the cyclic change in cellular GSH levels.

The enhancement of HCY2/UA-induced glutathione redox cycling was associated with protection against hypoxia/reoxygenation-induced apoptosis in H9c2 cells. Oxidative stress arising from an excessive production of ROS caused by Hypo/Reoxy challenge can cause cellular injury, which mimics the pathophysiological condition of I/R injury [39, 40]. It has been reported that the ethyl acetate fraction of Herba Cynomorii significantly attenuates staurosporine-induced apoptosis in SK-N-SH neuroblastoma cells [41]. UA was also found to reduce both intracellular ROS levels and the rate of H₂O₂-induced apoptosis in leukemic cells [42]. To confirm the protective effect of HCY2/UA on oxidant injury, its effects were examined in a rat model of myocardial I/R injury. In the absence of I/R, both HCY2 and UA pretreatment invariably enhanced mitochondrial functional capacity in hearts of both male and female rats. On the other hand, HCY2/UA pretreatment reduced tissue ATP levels in both male and female rat hearts, which indicated the involvement of uncoupled respiration. The observation of an increase in mitochondrial ATP-GC and a decrease in tissue ATP content would seem to be contradictory. While the mitochondrial ATP-GC reflects the ability of isolated mitochondria to generate ATP *in situ* under optimal assay conditions, the tissue ATP level indicates the steady state energy status of rat hearts under normal conditions. The enhancement of mitochondrial functional capacity was associated with an increase in mitochondrial glutathione antioxidant status, as was the case in H9c2 cells. Reperfusion of the previously ischemic myocardium triggers a burst of ROS formation, resulting in oxidative tissue damage [43]. The beneficial effects of HCY2 and UA pretreatment became more evident under conditions of I/R, where significant protection against I/R-induced tissue damage was observed. The cardioprotection afforded by HCY2 and UA pretreatments was associated with an enhancement of mitochondrial functional capacity. I/R *per se* also stimulated ATP-GC, which could reflect an adaptive response of rat heart mitochondria during reperfusion for maintaining a sufficient supply of energy for metabolism. The decreased tissue ATP content after I/R challenge was probably due at least partly to impaired oxidative phosphorylation and electron transport during postischemic reperfusion. HCY2 or UA pretreatment both ameliorated the extent of ATP depletion, presumably at least in part due to an enhancement of ATP-GC. The increase in mitochondrial functional capacity in hearts of HCY2/UA-pretreated rats was paralleled by a significant attenuation of the I/R-induced decrease in mitochondrial GSH/GSSG ratio as well as in mitochondrial GR activity, indicative of enhancement of glutathione redox cycling.

Interestingly, HCY2/UA pretreatment produced more prominent protection against myocardial I/R injury in female than in male rat hearts. While the differential susceptibility

of males and females to tissue oxidative damage, as seen in experimental and clinical observations, is likely attributed to biochemical event(s) secondary to the action of estrogens [44, 45], the gender-dependent myocardial protection afforded by HCY2 or UA pretreatment may be related to an interaction between sex hormones and HCY2/UA on mitochondrial energetics. In this regard, male sex hormones (dihydrotestosterone and testosterone) have been shown to reverse protonophore-induced weak uncoupling (i.e., recoupling) in rat liver, heart, and skeletal mitochondria, thereby increasing the mitochondrial membrane potential and lowering respiration rate [46]. In contrast, estrogen showed no such recoupling effect [46]. Given the proposed involvement of mitochondrial uncoupling in the cardioprotective effects afforded by HCY2 and UA, it is possible that male sex hormones could largely compromise the uncoupling induced by HCY2 and UA in male rats, resulting in a decrease in the extent of enhancement in ATP-GC and antioxidant response.

In conclusion, HCY2 and its major constituent, UA, increased mitochondrial functional capacity and upregulated cellular/mitochondrial antioxidant status, thereby protecting against oxidant-induced cellular/tissue injury in both *in vitro* and *ex vivo* assays. This beneficial effect produced by Herba Cynomorii is likely mediated by mitochondrial uncoupling. Even though several disparities between the effects of HCY2 and UA on mitochondrial glutathione antioxidant status were found, UA seems to be a “Yang-invigorating” ingredient of Herba Cynomorii, particularly in the HCY2 extract. Further investigation on the beneficial effects of HCY2 extract on other organs, such as the liver, kidney, and brain, would seem to be warranted.

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Research Article

Compounds from the Fruits of the Popular European Medicinal Plant *Vitex agnus-castus* in Chemoprevention via NADP(H):Quinone Oxidoreductase Type 1 Induction

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As part of our continuing efforts in the search for potential biologically active compounds from medicinal plants, we have isolated 18 compounds including two novel nitrogen containing diterpenes from extracts of the fruits of *Vitex agnus-castus*. These isolates, along with our previously obtained novel compound vitexlactam A (**1**), were evaluated for potential biological effects, including cancer chemoprevention. Chemically, the nitrogenous isolates were found to be two labdane diterpene alkaloids, each containing an α , β -unsaturated γ -lactam moiety. Structurally, they were elucidated to be 9α -hydroxy-13(14)-labden-16,15-amide (**2**) and 6β -acetoxy- 9α -hydroxy-13(14)-labden-15,16-amide (**3**), which were named vitexlactams B and C, respectively. The 15 known isolates were identified as vitexilactone (**4**), rotundifuran (**5**), 8-epi-manoyl oxide (**6**), vitetrifolin D (**7**), spathulenol (**8**), *cis*-dihydro-dehydro-diconiferylalcohol-9-O- β -D-glucoside (**9**), luteolin-7-O-glucoside (**10**), 5-hydroxy-3,6,7,4'-tetramethoxyflavone (**11**), casticin (**12**), artemetin (**13**), aucubin (**14**), agnuside (**15**), β -sitosterol (**16**), *p*-hydroxybenzoic acid (**17**), and *p*-hydroxybenzoic acid glucose ester (**18**). All compound structures were determined/identified on the basis of 1D and/or 2D NMR and mass spectrometry techniques. Compounds **6**, **8**, **9**, and **18** were reported from a *Vitex* species for the first time. The cancer chemopreventive potentials of these isolates were evaluated for NADP(H):quinone oxidoreductase type 1 (QRI) induction activity. Compound **7** demonstrated promising QRI induction effect, while the new compound vitexlactam (**3**) was only slightly active.

1. Introduction

Botanicals are widely used as either dietary supplements or herbal medicines throughout the world for the prevention and mitigation against various diseases or ailments. Among these botanicals are plants of the genus *Vitex* plants. Botanically, this genus was previously placed in the family of Verbenaceae but was recently revised as belonging to the family Lamiaceae, which itself was formerly known as the Labiateae. *Vitex* consists of about 250 species distributed worldwide, but

mainly in the tropical and temperate zones [1]. A number of species (e.g., *V. agnus-castus*, *V. trifolia*, *V. negundo*, and *V. rotundifolia*) have been used as traditional medicinal plants. To date, more than 20 *Vitex* species have been investigated for chemical and biological properties, with approximately 200 compounds, mainly flavonoids, terpenoids, steroids, iridoids, and lignans, having been isolated and characterized [2].

Vitex agnus-castus Linn., is commonly known as the chaste tree, grows to a height of 2–3 m, and is distributed in the Mediterranean Region, Central Asia, and Southern

Europe [3]. It is also cultivated in the various regions including the United States [4]. The fruits of *V. agnus-castus* are popularly used as a phytomedicine in Europe for the treatment of female hormonal disorders [5–7]. The fruit extract is also used as an alternative phytotherapeutic agent in the treatment of mastalgia [8]. There has been extensive research conducted on this phytomedicine leading to a large library of published literature on the pharmacognosy, traditional uses, chemical constituents, biology/pharmacology, and clinical studies [9]. In a previous communication we reported the isolation, structure determination, and X-ray crystallographic analysis of a novel labdane diterpene lactam from the *n*-hexane extracts of the fruits of this plant [10]. Further phytochemical studies of both of the *n*-hexane and methanol extracts resulted in the isolation of two additional new labdane diterpene lactams (**2–3**) and fifteen known compounds (**4–18**). In this paper, we describe the isolation and structure characterization of the two new metabolites and the identification of the 15 known compounds, as well as evaluating their NADP(H):quinone oxidoreductase type 1 (QR1) induction activity potentials.

2. Materials and Methods

2.1. General Experimental Procedures. All melting points were measured on an XRC-1 micromelting point apparatus and are uncorrected. 1D (one-dimensional) and 2D (two-dimensional) NMR (nuclear magnetic resonance) experiments were performed either on a Bruker AM-400 or a Bruker DRX-500 spectrometer. Unless otherwise is specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. FABMS (fast atom bombardment mass spectrometry) and HRFABMS (high resolution fast atom bombardment mass spectrometry) were taken on a VG Auto Spec-3000 or a Finnigan MAT 90 instrument. IR (infrared) spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV (ultraviolet) spectral data were obtained on a UV 210A spectrometer. Optical rotations were carried out on a HORIBA SEPA-300 High Sensitive Polarimeter or a Perkin-Elmer model 241 Polarimeter. Column chromatography was performed either on Si gel (silica gel) (200–300 mesh, Qingdao Marine Chemical Inc., China), Si gel H (10–40 μ , Qingdao Marine Chemical Inc., China), Diaion HP-20 (Shandong Lukang Pharmaceutical Co., Ltd., China), Chromatorex ODS (Fuji Silysia Chemical Corporation, Ltd., Japan), or Lichroprep Rp₁₈ gel (40–63 μ m, Merck, Darmstadt, Germany). Fractions were monitored by silica gel TLC (thin layer chromatography) [$\text{CHCl}_3\text{-Me}_2\text{CO}$ (chloroform-acetone) 9:1, 8:2, 7:3], and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH (ethanol).

2.2. Plant Material. The fruits of *V. agnus-castus* were purchased from Frontier Botanicals, Norway, IA, USA (Lot. No. 799.0116).

2.3. Extraction and Isolation. Dried fruits of *V. agnus-castus* (4077 g) were milled and sequentially extracted with *n*-hexane (3 \times 8 L) for 28 h and MeOH (methanol) (4 \times 9 L)

for 24 h. The *n*-hexane extract was filtered and concentrated *in vacuo* to dryness to afford 200 g of a residue (part I). The MeOH extract was filtered, concentrated, and diluted with water (2 L), followed by partitioning with EtOAc (ethyl acetate) (4 \times 3 L). The organic layer was evaporated *in vacuo* to dryness to give 60 g of a residue (part II). The water-soluble fraction was chromatographed on a column of Diaion HP-20 eluting with aqueous MeOH (30% \rightarrow 80% \rightarrow 100%). The 80% MeOH-H₂O fraction was concentrated *in vacuo* to yield 48 g of a dry residue (part III).

2.3.1. Isolation. Part I (200 g) was absorbed on 200 g of silica gel and chromatographed on a prepacked (500 g) silica gel column, eluting stepwise with *n*-hexane, CHCl_3 , $\text{CHCl}_3\text{-Me}_2\text{CO}/1:1$, and Me_2CO . Compound **16** (27 mg) was crystallized from the CHCl_3 fraction and compound **11** (336 mg) was crystallized from the $\text{CHCl}_3\text{-Me}_2\text{CO}/1:1$ fraction. The remaining $\text{CHCl}_3\text{-Me}_2\text{CO}/1:1$ eluate was filtered (40 g, net weight) and subjected to further chromatographic separation over a Chromatorex ODS column (eluent: 80% MeOH-H₂O as eluents) and silica gel columns (using *n*-hexane- $\text{CHCl}_3/1:2$, *n*-hexane-EtOAc/3:2, and *n*-hexane- $\text{Me}_2\text{CO}/2:1$ as eluents) to provide compounds **1** (40 mg), **2** (4 mg), **3** (11 mg), **4** (25 mg), **5** (67 mg), **6** (6 mg), **7** (14 mg), **8** (14 mg), and **13** (9 mg).

Part II (60 g) was absorbed on 100 g of silica gel and chromatographed on a prepacked (300 g) silica gel column, eluting with $\text{CHCl}_3\text{-Me}_2\text{CO}$ (1:0, 9:1, 8:2, 7:3, 0:1). Compound **12** (1.635 g) was crystallized from the $\text{CHCl}_3\text{-Me}_2\text{CO}/1:0\text{-}9:1$ fraction. Part of the $\text{CHCl}_3\text{-Me}_2\text{CO}/8:2$ fraction (0.810 g) was further chromatographed on RP₁₈ gel (100 g) with 40% aqueous MeOH as eluents to give compound **17** (125 mg).

Part III (48 g) was again chromatographed on a Chromatorex ODS column eluting with aqueous MeOH (30%) and over a silica gel column eluting with $\text{CHCl}_3\text{-MeOH}$ (3:1), $\text{CHCl}_3\text{-MeOH-H}_2\text{O}$ (4:1:0.1), and EtOAc-MeOH (12:1) to yield compounds **9** (108 mg), **10** (23 mg), **14** (55 mg), **15** (60 mg), and **18** (15 mg).

2.4. Structural Characterization of Novel Isolates

2.4.1. Vitexlactam B (2). White crystals, m.p. 162°C, $\text{C}_{20}\text{H}_{33}\text{NO}_2$; $[\alpha]_D^{23.5} + 18.75^\circ$ (c 0.2, CHCl_3); IR (KBr) ν_{max} : 3473, 3187, 3055, 2924, 2682, 1684, 1648, 1442, 1379, 1296, 1254, 1228, 1140, 1085, 1057, 1041, 1018, 972, 962, 943, 909, 832, 791, 777, 698 cm⁻¹; ¹H NMR (500 MHz, CDCl_3) δ 1.50 (1H, dd, $J = 11.0, 2.0$ Hz, H-5), 1.75 (1H, m, H-8), 1.78 (1H, m, H-11a), 1.67 (1H, m, H-11b), 2.36 (2H, br t, $J = 8.2$ Hz, H₂-12), 6.69 (1H, br s, H-14), 3.89 (2H, br s, H₂-15), 0.88 (3H, d, $J = 6.6$ Hz, H₃-17), 0.85 (3H, s, H₃-18), 0.80 (3H, s, H₃-19), 0.90 (3H, s, H₃-20), 6.61 (1H, br s, NH); ¹³C NMR data, see Table 1; EIMS (electron impact mass spectrum) m/z 319 [M]⁺ (81), 304 (7), 286 (8), 206 (7), 194 (19), 180 (100), 167 (75), 152 (11), 138 (47), 123 (17), 110 (81), 96 (86), 82 (58), 69 (72), 55 (97); HREIMS m/z found 319.2509 [M]⁺, calcd. (calculated) 319.2511.

TABLE 1: ^{13}C NMR data of compounds 1–7 (CDCl_3 , δ in ppm).

Carbon	1 ^a	2 ^b	3 ^a	4 ^a	5 ^a	6 ^a	7 ^b
C-1	33.7 t	32.5 t	33.7 t	33.8 t	33.9 t	36.5 t	25.9 t
C-2	18.8 t	18.7 t	18.6 t	18.9 t	18.7 t	20.7 t	19.4 t
C-3	43.8 t	41.7 t	43.6 t	43.9 t	43.7 t	42.3 t	39.4 t
C-4	33.9 s	33.3 s	34.0 s	34.3 s	34.8 s	32.9 s	34.6 s
C-5	47.5 d	46.2 d	47.6 d	48.0 d	47.5 d	46.3 d	132.5 s
C-6	70.6 d	21.7 t	69.9 d	70.1 d	70.3 d	21.0 t	66.2 d
C-7	36.3 t	31.4 t	36.1 t	36.4 t	36.1 t	37.9 t	72.7 d
C-8	32.1 d	36.8 d	31.9 d	32.3 d	33.6 d	74.1 s	36.4 d
C-9	76.4 s	76.8 s	76.7 s	76.8 s	76.8 s	61.2 d	42.9 s
C-10	44.0 s	43.3 s	43.8 s	44.1 s	43.7 s	38.9 s	141.5 s
C-11	32.3 t	32.0 t	32.3 t	31.9 t	31.8 t	18.6 t	29.3 t
C-12	21.7 t	22.0 t	26.5 t	25.7 t	21.5 t	45.1 t	38.6 t
C-13	140.6 s	140.8 s	163.6 s	171.3 s	125.5 s	73.6 s	73.0 s
C-14	137.1 d	136.9 d	121.2 d	115.3 d	110.8 d	146.1 d	144.5 d
C-15	46.6 t	46.4 t	175.3 s	171.3 s	142.9 d	111.1 t	112.1 t
C-16	175.3 s	175.8 s	50.5 t	73.4 t	138.5 d	27.4 q	27.8 q
C-17	16.4 q	16.6 q	16.0 q	16.3 q	16.1 q	32.0 q	11.1 q
C-18	33.6 q	33.7 q	33.6 q	33.8 q	33.6 q	33.1 q	29.3 q
C-19	23.7 q	22.1 q	23.6 q	23.9 q	23.7 q	21.3 q	28.1 q
C-20	18.9 q	16.2 q	19.0 q	19.2 q	19.0 q	24.7 q	28.0 q
OAc	170.5 s		170.3 s	170.6 s	170.7 s		170.8
(C=O)							(2C, s)
OAc	21.9 q		21.8 q	22.1 q	21.9 q		21.4 q
(CH ₃)							20.9 q

^aRecorded at 100 MHz.^bRecorded at 125 MHz.

2.4.2. Vitexlactam C (3). White crystals, m.p. 178°C, $\text{C}_{22}\text{H}_{35}\text{NO}_4$; $[\alpha]_D^{18.7} - 12.73^\circ$ (c 0.55, CHCl_3); IR (KBr) ν_{max} : 3364, 3297, 2925, 2867, 1711, 1670, 1465, 1426, 1383, 1362, 1271, 1256, 1228, 1203, 1152, 1125, 1097, 1039, 1024, 977, 953, 916, 849, 819 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 1.31 (1H, br d, $J = 13.2$ Hz, H-3a), 1.13 (1H, dt, $J = 2.7, 13.2$ Hz, H-3b), 1.58 (1H, d, $J = 2.0$ Hz, H-5), 5.35 (1H, br d, $J = 2.2$ Hz, H-6), 2.10 (1H, m, H-8), 1.90 (1H, m, H-11a), 1.72 (1H, m, H-11b), 2.43 (2H, m, H₂-12), 5.82 (1H, br s, H-14), 3.91 (2H, br s, H₂-16), 0.87 (3H, d, $J = 6.7$ Hz, H₃-17), 0.93 (3H, s, H₃-18), 0.97 (3H, s, H₃-19), 1.22 (3H, s, H₃-20), 6.92 (1H, br s, NH), 2.03 (3H, s, 6-OAc); ^{13}C NMR data, see Table 1; EIMS m/z 377 [M]⁺ (3), 317 (76), 302 (15), 284 (6), 260 (29), 242 (8), 222 (21), 202 (23), 187 (48), 167 (60), 150 (28), 133 (41), 119 (64), 110 (68), 96 (97), 83 (72), 69 (77), 55 (100); HREIMS m/z found 377.2547 [M]⁺, calcd. 377.2566.

2.5. Chemoprevention Evaluation: NAD(P)H:Quinone Oxidoreductase Type 1 (QRI) Assay. Test compounds were evaluated for their potential to induce quinone reductase type 1 (QRI) activity with Hepa 1c1c7 cells. The cells were plated in 96-well plates at a density of 2×10^4 cells/mL in 190 μL of α -MEM (minimum essential medium) containing 100 units/mL penicillin G sodium, 100 $\mu\text{g}/\text{mL}$ streptomycin sulfate, and 250 ng/mL amphotericin B supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

After preincubation for 24 h, the medium was changed, and test compounds were added to afford a final concentration range of 2 to 20 $\mu\text{g}/\text{mL}$, and then the cells were incubated for an additional 48 h. The medium was decanted, and the cells were incubated with 50 μL of 0.8% digitonin and 2 mM EDTA (ethylenediaminetetraacetic acid) solution (pH 7.8) at 37°C for 10 min. Quinone reductase activity was determined by measuring the NAD(P)H-dependent menadiol mediated reduction of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan. Cytotoxicity was determined by crystal violet staining assay. Induction of QR activity was calculated by comparing the QR specific activity of agent-treated cells with that of vehicle solvent-treated cells. 4'-Bromoflavone with a CD value of 12.9 nM was used as a positive control. CD represents the concentration of a test compound required to double QR induction in comparison with the vehicle control.

2.6. Supporting Information Available. NMR and MS data of the known compounds are available as Supplementary Material online at <http://dx.doi.org/10.1155/2013/432829>.

3. Results and Discussion

3.1. Plant Extracts and Isolation of Compounds. The purchased fruits of *V. agnus-castus* were milled and sequentially

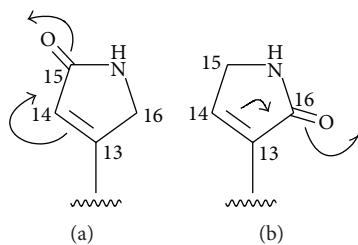


FIGURE 1: Electronic clouds movements of two different conjugated systems in compounds **3** (a) and **1** (b).

extracted with *n*-hexane and methanol. The *n*-hexane extract was successively chromatographed on silica gel and Chromatorex ODS to afford compounds **1–8**, **11**, **13**, and **16**. The methanol extract was partitioned between EtOAc and water. The EtOAc layer was chromatographed on silica gel to give compounds **12** and **17**. The water-soluble fraction was chromatographed on columns of Diaion HP-20, Chromatorex ODS, and silica gel to yield compounds **9**, **10**, **14**, **15**, and **18** (Scheme 1).

3.2. Structure Elucidation and Identification of Isolated Compounds

3.2.1. Vitexlactam B (2). Vitexlactam B (**2**) was obtained as white crystals. EI mass spectrum showed strong molecular ion peak at m/z 319 [M]⁺ (81% relative intensity), corresponding to a molecular formula of $C_{20}H_{33}NO_2$, which was confirmed by high resolution EI mass spectrum (found: m/z 319.2509, calcd. 319.2511). The existence of a nitrogen atom was supported by its odd numbered molecular weight and a positive reaction to the Dragendorff reagent.

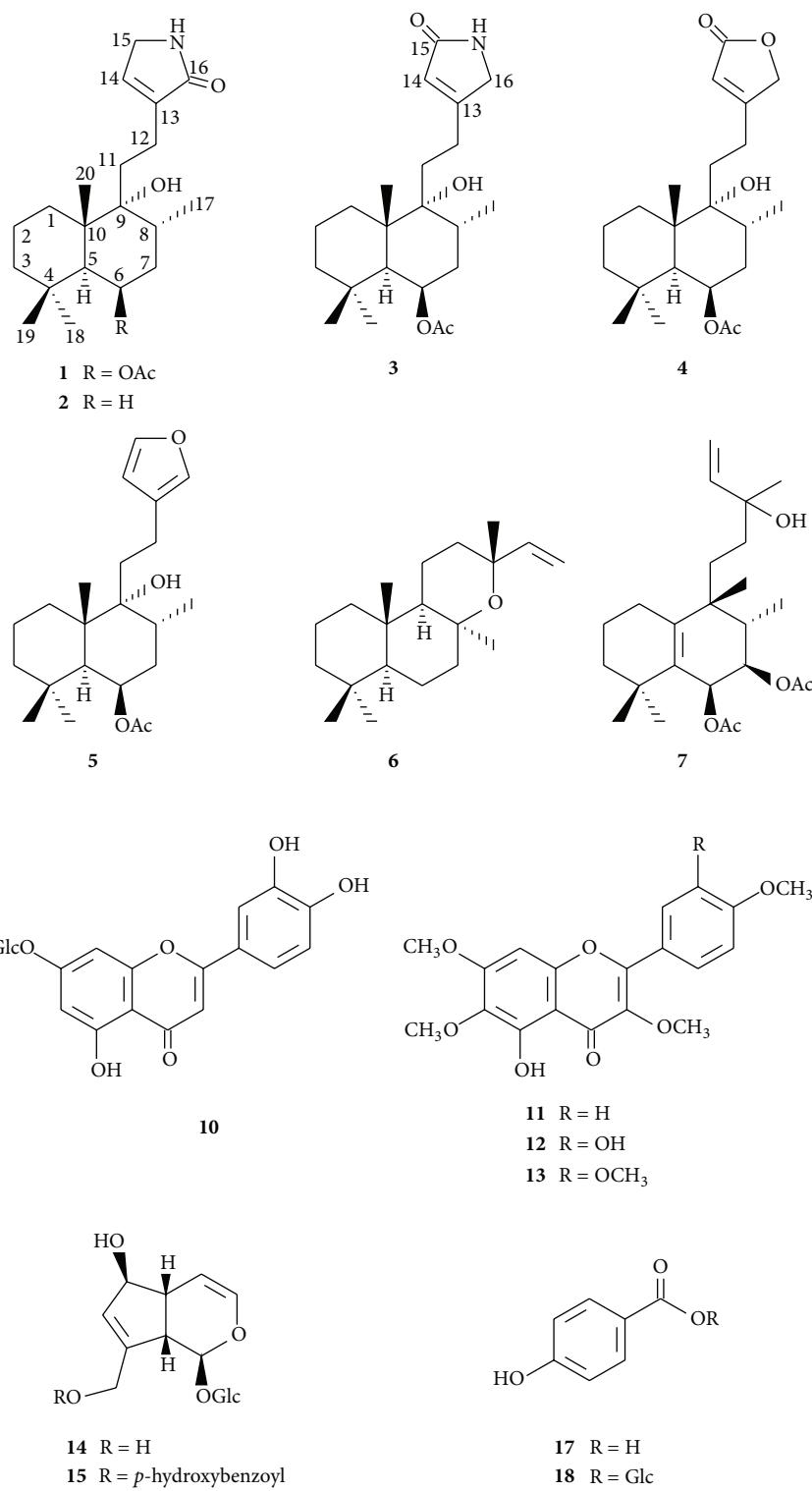
The 1H and ^{13}C NMR (Table 1) spectra of **2**, being very similar to those of **1** [10], suggested that **2** is a closely related labdane diterpene alkaloid (Table 1), with an α , β -unsaturated γ -lactam moiety at the C-9 side chain. **2** differed from **1** only by the absence of the signals for an acetyl group and the replacement of an oxygen-bearing methine at δ_C 70.6 by a methylene signal at δ_C 21.7, indicating that **2** is the 6-deacetoxy derivative of **1**. The result was further supported by the facts that **1** was 58 atomic mass units less than **2** and the lack of an acetoxy group being observed in the IR spectrum of **2**. Full assignments of **2** using 2D NMR (including 1H - 1H COSY (correlation spectroscopy), HMQC (heteronuclear multiple-quantum correlation spectroscopy), HMBC (heteronuclear multiple bond correlation spectroscopy), and ROESY (rotating-frame Overhauser spectroscopy)) techniques established the structure of **2** to be the expected 9 α -hydroxy-13(14)-labden-16,15-amide. Compound **2** was accordingly identified as the deacetoxy derivative of **1** and was given the trivial name of vitexlactam B.

3.2.2. Vitexlactam C (3). Vitexlactam C (**3**) was also isolated as white crystals. EI mass spectrum under 70 eV displayed a weak [M]⁺ ion peak at m/z 377 (3%) identical with that of **1** in both the mass charge ratio and the relative intensity [11]. In addition, a strong fragment ion peak at m/z 317 (76%)

due to $[M\text{-AcOH}]^+$ and a series of fragment ions similar to those for **1** were also observed. High resolution EI mass spectrum (found: m/z 377.2547, calcd. 377.2566) established that both compounds have the same molecular formula of $C_{22}H_{35}NO_4$. Therefore **3** was tentatively identified as an isomer of **1**. Comparison of the 1H and ^{13}C NMR (Table 1) spectra of **3** with those of **1** (Table 1) indicated that the two compounds were equivalent not only in their skeletons but also in their oxygenation patterns. NMR spectral differences between these two compounds are mainly due to the α , β -unsaturated γ -lactam moieties in their C-9 side chains. The conjugate functionality occurred in **3** was deduced to be type (a) in contrast to type (b) in **1** (Figure 1). In the former conjugating system, C-13 is in a deshielded position while C-14 and H-14 are in a shielded position. On the contrary, in the latter (type (b)), C-13 is in a shielded position while C-14 and H-14 are in a deshielded position. Accordingly, C-13 of **3** moved downfield from δ_C 140.6 (s) in **1** to δ_C 163.6 (s), and C-14/H-14 of **3** shifted upfield from $\delta_{C/H}$ 137.1 (d)/6.71 (1H, br s) in **1** to $\delta_{C/H}$ 121.2 (d)/5.82 (1H, br s). 2D NMR analysis of **3** revealed that, unlike in **1**, the 1H - 1H COSY correlation between H-14 and the nitrogen-bearing methylene at δ_H 3.91 (2H, br s) and the 1H - ^{13}C interaction (Figure 2) between H_2 -12 [δ_H 2.44 (2H, m)] and the lactam carbonyl carbon at δ_C 175.3 (s) disappeared while 1H - ^{13}C interaction between H_2 -12 and the nitrogen-occurring methylene at δ_C 50.5 (t) were observed, thus confirming the presence of a type (a) conjugate functionality in **3**. Other structural correlations, including key NOEs (nuclear Overhauser effects) (Figure 3) in **3**, were identical with those in **1**.

A detailed spectral comparison between **3** and vitexilactone (**4**) [11] was also carried out. The molecular weight of **3** is lower by 1 mass unit than that of **4**. Besides, **3** differed from **4** (Table 1) mainly by the upfield shifted H_2 -16 and C-16 signals (from $\delta_{H/C}$ 4.77 (2H, br d, J = 1.3 Hz)/73.4 (t) in **4** to $\delta_{H/C}$ 3.94 (2H, br s)/50.5 (t) in **3**) and the existence of an extra NH proton at δ_H 6.92 (1H, br s), indicating that an α , β -unsaturated γ -lactam moiety in **3** took the place of the α , β -unsaturated γ -lactone in **4**. Based on all the abovedescribed spectral features, compound **3** was consequently deduced to be 6 β -acetoxy-9 α -hydroxy-13(14)-labden-15,16-amide and was named vitexlactam C.

Considering that only mild conditions were employed and that no nitrogen containing solvents and chromatographic materials were involved in the entire extraction and separation procedures, we postulate that compounds **1–3**



SCHEME 1

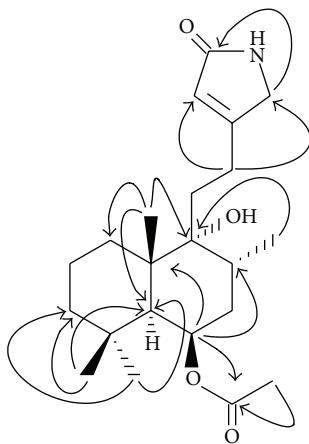


FIGURE 2: Key HMBC correlations of vitexlactam C (3).

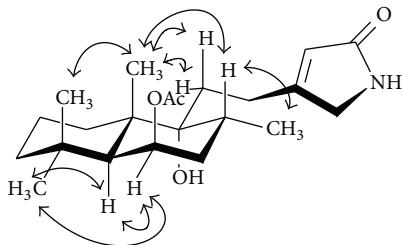


FIGURE 3: Key NOESY correlations of vitexlactam C (3).

are biogenetic amination products of their corresponding lactones (e.g., 3 was derived from 4).

3.2.3. Identification of Known Compounds. Along with the new compounds, fifteen known compounds were also isolated in the course of the current study. Through comparison of their ^1H and ^{13}C NMR and MS data with those values reported in the literature, they were identified as three labdane-type diterpenoids, vitexilactone (4) [11]; rotundifuran (5) [11], and 8-epi-manoyl oxide (6) [12] ($[\alpha]_{\text{D}}^{19.5} = 11.8^\circ$; $c = 0.55$, CHCl_3); a rearranged labdane (halimane) diterpenoid, vitetritofolin D (7) [13]; an aromadendrene-type sesquiterpenoid, spathulenol (8) [14, 15]; a lignan glucoside, *cis*-dihydro-dehydro-diconiferylalcohol-9-O- β -D-glucoside (9) [16]; four flavonoids, luteolin-7-O-glucoside (10) [17], 5-hydroxy-3,6,7,4'-tetramethoxyflavone (11) [18], casticin (12) [19], and artemetin (13) [20]; two iridoid glycosides, aucubin (14) [21] and agnuside (15) [22]; a sterol, β -sitosterol (16) (comparison with an authentic sample); and two simple phenolics, *p*-hydroxybenzoic acid (17) [22] and *p*-hydroxybenzoic acid glucose ester (18) [22]. The occurrence of compounds 7–9 and 18 in the genus *Vitex* is being reported for the first time.

3.3. Activity Evaluation of the Isolated Compounds on QR1 Induction. These compounds have been evaluated for their potential chemopreventive activity by induction of the ubiquitous flavoenzyme NADP(H):quinone oxidoreductase

type 1 (QR1) with cultured Hepa 1c1c7 cells. QR1 has been determined as an important phase II detoxification enzyme that can protect cells against the harmful effects caused by free radicals and reactive oxygen species by catalyzing the reduction of quinones to hydroquinones [23]. Hence, enhanced activity of the enzyme provides protection of cells from potential carcinogenicity. Vitetritofolin D (7) was shown to induce QR1 activity with a CD value of $23.2 \mu\text{M}$. Although vitexlactam C (3) induced QR1 by 1.5 times that of the vehicle control at a concentration of $5.3 \mu\text{M}$, it was toxic to Hepa 1c1c7 cells with 57% inhibition of the cells at $26.5 \mu\text{M}$. None of the other compounds demonstrated QR1 induction activity.

4. Conclusion

The fruits of *Vitex agnus-castus* have been popularly used as a phytomedicine in Europe, especially Germany, for the treatment of premenstrual stress syndrome. However, the evaluation of this herb or its phytochemical constituents for cancer chemoprevention activity has not been reported. Thus, we undertook a study of the 18 compounds we isolated from the fruits of this plant in a bioassay, which have been used for assessing chemoprevention potentials. The isolates, including several novel nitrogen containing labdane diterpenes, were thus evaluated for their potentials in the induction of the phase II detoxification enzyme QR1. Results showed that only the labdane compounds 3 and 7 demonstrated QR1 induction effect. We have demonstrated that compounds possessing potential chemopreventive action do exist in *V. agnus-castus* and that further phytochemical and biological investigations of this plant material coupled with structure modification studies are needed in order to discover additional/modified labdananes possessing more potent QR1 induction activity and chemopreventive potential.

Conflict of Interests

The authors have no conflict of interests with the trademarks included in the paper.

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Research Article

***Phyllanthus emblica* L. Enhances Human Umbilical Vein Endothelial Wound Healing and Sprouting**

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Endothelial dysfunction is the hallmark of impaired wound healing and increased risk of cardiovascular disease. Antioxidants from natural sources decrease oxidative stress and protect against cellular damage caused by reactive oxygen species (ROS). In this study, we examined the antioxidant constituents and capacity of *Phyllanthus emblica* L. (PE) fruit in freeze-dried powder form. The pharmacological properties of PE were investigated using human umbilical vein endothelial cells (HUVECs) in the aspects of endothelial cell proliferation, nitric oxide (NO) production, wound healing, cell migration, *in vitro* angiogenesis, and VEGF gene expression. The ASC content of PE was $1.574\% \pm 0.046\%$ (w/w) as determined by HPLC and the total phenolic content was $36.1\% \pm 0.7\%$ gallic acid equivalent when measured by Folin-Ciocalteu assay. The FRAP assay revealed a relatively high antioxidant capacity at $3,643 \pm 192.5\text{ }\mu\text{mole/mg}$. PE at 0.1 to 10 $\mu\text{g/mL}$ did not significantly influence endothelial cell proliferation, but at higher concentrations PE decreased cell survival to 62%. PE significantly promoted NO production, endothelial wound closure, endothelial sprouting, and VEGF mRNA expression. Therefore, PE is a candidate for antioxidant supplement that promotes endothelial function and restores wound healing competency.

1. Introduction

Endothelial dysfunction is the foremost step in the development of atherosclerosis and causes impairment in cell proliferation, migration, wound healing, and angiogenic function [1]. Pathologic oxidative stress conditions, such as diabetes and cardiovascular disease, potentiate endothelial dysfunction eventually leading to ischemic vascular damage and become impediment to wound healing. Current therapeutic strategies focusing on protection of endothelial oxidative damage, accelerating endothelial wound healing, or promoting angiogenesis may have a role in diabetic and oxidative related vascular disease [2].

Impaired endothelial function is mainly characterized by reduced nitric oxide (NO) production, decreased cell migration to repair the endothelial damage, and being deficient in the capacity to form new collateral vessels. Substances that enhance NO bioavailability and promote vascular endothelial growth factor (VEGF) synthesis are shown to improve wound

healing and/or angiogenesis [3, 4]. Several oxidative stress markers such as homocysteine and long-standing hypoxia are known to impair endothelial wound healing while using antioxidants have been proven to improve endothelial function and wound repair [5, 6]. Of these, natural antioxidants are the current main focus in complementary and alternative medicine research. In traditional medicine, *Phyllanthus emblica* L. (PE), an indigenous plant grown in Thailand and some parts of Asia, has long been used as a wound healing agent either in single formulation or combined components in traditional preparations. The effect of PE on wound repair has been demonstrated in rats and the mechanisms involve modulation of collagen synthesis, extracellular matrix (ECM) protein synthesis, and antioxidant status [7, 8]. However, the pharmacological action of PE exclusively on endothelial function, wound repair, and tube formation has not been reported. In this study, we investigated the properties of PE fruits in the aspects of antioxidant capacity, ascorbic acid content, total phenolics level, and vasculogenic property

that promoted endothelial NO production, wound healing, and *in vitro* angiogenesis using human umbilical cord vein endothelial cells (HUVECs).

2. Materials and Methods

2.1. Chemicals and *Phyllanthus emblica L.* Fruit Extract (PE). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or otherwise indicated. PE fruits were obtained from Nakhon Ratchasima province, Northeast of Thailand. PE fruit juice was extracted from 303 g (57 fresh fruits) using a fruit juice extractor and yielded approximately 2.54 mL/fruit. PE juice was then filtered through 0.2 µm membrane filter and underwent freeze-drying process giving 16.1% yield (w/v). The yellowish dry power of PE was kept at 4°C until use. The aqueous stock solutions at 10 mg/mL were prepared freshly at the time of use in each experiment.

2.2. Ascorbic Acid Content, Antioxidant Activity, and Total Phenolic Compounds of PE. It is well recognized that PE exerts various biological activities partly due to its antioxidant activity such as ascorbic acid (ASC), polyphenolic compounds, and flavonoids. This study evaluated ASC content which is the major antioxidant component of the extract using HPLC method (Thermo Scientific). Standard curve of ASC was established by measuring the areas under the peak after injecting a series of ASC stock solutions into reversed phase HPLC column (Luna C18, 5 µm, dimension 150 × 4.60 mm, Phenomenex, Thailand) with mobile phase (100 mM phosphate buffer 95% : methanol 5%) at the flow rate of 0.4 mL/min, isocratic elution, and detected by UV absorption at 243 nm [9].

Total antioxidant capacity of PE was determined by Ferric reducing antioxidant power (FRAP) assay. The FRAP assay determined the ability of PE to deliver one electron to Fe^{III}-TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) complex to form a color ferrous ion. Briefly, ten microliters of standard FeSO₄ or sample solutions were added to a 96-well microplate followed by adding 200 µL of FRAP reagent (acetate buffer 300 mM (pH 3.6), TPTZ 10 mM in HCl 40 mM and FeCl₃·6H₂O 20 mM) and the development of blue color was monitored at 595 nm (Synergy, BioTek, USA) [10]. The stability of antioxidant capacity was determined from the dry power of PE samples stored at 4°C for 12 months.

Total phenolic content was evaluated by Folin-Ciocalteu assay using gallic acid (GA) as an assay standard [11]. Briefly, a series of standard GA concentrations (0, 31.25, 62.5, 125, 250, 500, and 1000 µM) or samples at the volume of 1.6 mL were added to a reaction test tubes followed by 100 µL of Folin-Ciocalteu reagent and 300 µL of Na₂CO₃ solution. The mixture was incubated at 40°C for 30 min and then cooled down at room temperature for 5 min. The products of phenolic compounds reaction were measured at 756 nm (Shimadzu UV-1601, Japan). The amount of total phenolic compounds was represented as GA equivalence (GAE).

2.3. Human Umbilical Vein Endothelial Cell (HUVEC) Culture. Human umbilical cords were collected from the labor room of the university hospital and HUVECs were isolated

within 48 h as described previously [12]. Cells were cultured in M199 medium, supplemented with 20% fetal bovine serum (FBS) with antibiotic and antimycotic agents (Invitrogen, USA), in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells between passages 3 and 5 were used in the experiments and were cultured in low serum medium (1% FBS) during PE incubation or other treatments.

2.4. Cytotoxicity of PE and Ascorbic Acid (ASC). There have been reported that PE induced apoptosis in many cell types including at least 7 cancer cell lines and primary osteoclasts [13–15]. Therefore, this experiment was aimed to determine nontoxic doses of PE and its major antioxidant ASC for further experiments. HUVECs were treated with various concentrations of PE or ASC for 48 h. Cell survival was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and sulforhodamine B (SRB) cytotoxicity assay. For MTT assay, ten microliters of MTT stock solution (5 mg/mL) were added to the culture medium and incubated for 4 h or until dark blue-purple crystalline precipitates were visualized under an inverted microscope. Then, one hundred microliters of DMSO were added to dissolve the formazan products. The plate was then shacked at 200 rpm for 20 min and the relative cell viability was detected absorbance at 550 nm by Synergy plate reader (BioTek, USA).

SRB assay was performed in 96-well plate after removal of culture medium and washed once with PBS. Two hundred microliters of cold 10% trichloroacetic acid (TCA) were added to the wells to fix cells. Following 30 min incubation at 4°C, TCA was aspirated and the wells were rinsed with water 5 times. Plates were air-dried and 100 µL of SRB solution (0.4% in 1% acetic acid) was added to each well and allowed cells to be stained for 30 min. Cells were then washed with 1% acetic acid until unincorporated dye was removed (approximately 5 times). The plates were air-dried at room temperature for 30 min. The bound SRB dye was solubilized with 200 µL of 10 mM Tris (pH 10.5) for 5 minutes at room temperature. The relative cell viability was determined by absorbance at 510 nm (BioTek, USA).

2.5. Endothelial Wound Healing Assay. Scratch wound assay was used to evaluate the ability of PE in promoting endothelial wound *in vitro*. HUVECs were seeded in 6-well plate (Nunc, Thermo Fisher Scientific, USA) at 5×10^5 cells/well in culture medium and allowed the cells to grow to 90% confluence. The culture medium was then replaced with 1% FBS M199 overnight before the scratch wounds were initiated using a sterile 200 µL pipette tip. Photos of wounds were captured by a digital camera (Olympus DP20, Japan) at the same positions at 0, 24, and 48 h. The length of wound confluence was measured by Cell^B program (Olympus, Japan).

2.6. Cell Migration Assay. Migration of HUVECs toward a chemoattractant VEGF and testing substances (PE and ASC) in the cell's surrounding environment was determined by Boyden chamber in 24-well plate (Corning, USA). HUVECs were cultured and 200 µL of cells suspension (2×10^5 cells/mL

in 1% FBS) was seeded into the chamber with translucent PET membranes 8 μm pore size. Seven hundred and fifty microliters of media containing vehicle (CTRL group), 100 ng/mL VEGF, PE, or ASC were added to the lower chamber. The plate was incubated at 37°C in a CO₂ incubator for 16 hours. The inserts were then transferred to 0.25% trypsin-EDTA solution and 300 μL of 5 μM calcein-acetoxyethyl ester (CAL-AM, Sigma) was added to the lower chamber and incubated at 37°C in a CO₂ incubator for 45 minutes. Cells migrated through the micropores used enzyme esterases to hydrolyze the nonfluorescent CAL-AM to highly fluorescent product which was monitored at wavelengths 485/528 nm for excitation/emission, respectively.

2.7. Nitric Oxide (NO) Production. Endothelial NO is important to maintain endothelial health/cell survival and activation cell migration. In this study, the stable product of NO nitrite was evaluated by 2,3-diaminonaphthalene (DAN) assay which is far more sensitive than Griess reaction [16]. This fluorometric method uses the substrate DAN (nonfluorescent) to react with nitrite under acidic conditions to generate 2,3-diaminonaphthotriazole or 1-(H)-naphthotriazole (NATH), the fluorescent product. NATH fluorescent signal is further enhanced by alkalinization of medium to yield 2,3-naphthotriazole anion (NAT). NaNO₂ standard solution was freshly prepared ranging from 0.13 to 13.33 μM in DMEM before experiment. Seventy-five microliters of standard solutions or the supernatant media from PE or ASC treatments were transferred into 96-well microplate; then, 10 μL of DAN solution (50 $\mu\text{g}/\text{mL}$ in 0.62 N HCl) was added to each well for 10 min at room temperature in the dark. Finally, 5 μL of 2.8 N NaOH solution was subsequently added to each well and fluorescence was measured using a fluorescent microplate reader (Synergy HT, BioTek) with excitation/emission wavelengths of 360/460 nm. Nitrite levels of sample were calculated from NaNO₂ standard curve.

2.8. In Vitro Angiogenesis Assay. The 3D spheroid-based angiogenesis assay was performed as described by Korff [17]. Each HUVEC spheroid was composed of 500 cells distributed in 1.4% methyl cellulose (Fisher Scientific, USA). Forty-eight spheroids were embedded in 1 mL rat tail collagen (2 mg/mL) and performed in 24-well tissue culture plates. For angiogenic stimulation test, the collagen gels were overlaid with 100 μL medium containing 50 ng/mL vascular endothelial growth factor (VEGF, PeproTech, USA) or PE at various concentrations, and the gels were allowed to be stored at 37°C and 5% CO₂ for 24 h. Pictures of each spheroid were captured at 0 and 24 h with a digital camera and the cumulative sprout length was measured for at least 10 individual spheroids per treatment (Olympus, Japan).

2.9. VEGF mRNA Expression. VEGF gene expression was determined based on SYBR-Green fluorescence RT-PCR using commercial kits (BioRad, USA). Total RNA was extracted from cultured HUVECs using Trizol reagent (Invitrogen, USA). Amplification of target genes was initiated by denaturation at 70°C and cooling to 37°C prior to

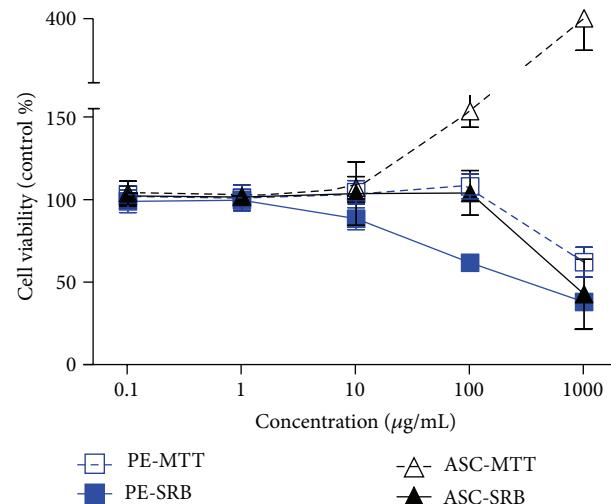


FIGURE 1: Cytotoxic doses of PE and ASC in HUVECs comparing two methods of cell viability assays. HUVECs were incubated with *Phyllanthus emblica* (PE) or ascorbic acid (ASC) at designated concentrations for 48 h. SRB and MTT assays were performed as described in Section 2.

performing reverse transcription at 37°C for 1 h as described in the manufacturer's manual. PCR amplification for β -actin and VEGF was carried out in parallel using primer pairs as follows: β -actin, forward 5'-GGACTTCGAGCAAGA GATGG-3', reverse 5'-AGCACTGTGTTGGCGTACAG-3'; VEGF; forward 5'-TATTCTTCTTGCTGCTAAAT-3', reverse 5'-AATGTTATTGGTGTCTCAC-3' (Gen Bank: NM_001025366). Reaction cycles were performed for 40 cycles with two denaturation steps (94°C, 4 min and 94°C, 4 s) followed by annealing at 58°C, 30 s, and extension at 72°C for 10 s. Amplification was terminated with 10 min extension at 72°C (Roter-Gene 6000, Corbett Life Science). Relative mRNA amounts of samples were calculated using the $2^{-\Delta\Delta CT}$ method.

3. Results

3.1. Ascorbic Acid (ASC) Content, Antioxidants Capacity, and Phenolic Content of PE. PE possessed antioxidant capacity of $3643.3 \pm 192.5 \mu\text{mole}/\text{mg}$ which was not significantly changed when kept at 4°C for 12 months ($3694.5 \pm 105.8 \mu\text{mole}/\text{mg}$). The amount of total phenolic content which is well corresponding to antioxidant capacity was calculated as $0.361 \pm 0.005 \text{ mg GAE}/\text{mg PE powder}$. HPLC analysis revealed that PE dry powder consisted of 1.57% ASC (w/w) or equivalent to 2.53 mg/mL fresh juice or approximately 6.42 mg/fruit.

3.2. Cytotoxic Effects of PE and ASC. MTT showed that only PE at 1000 $\mu\text{g}/\text{mL}$ caused cytotoxicity while SRB assay detected decreases in cell survival in a concentration-dependent manner beginning from PE at 10 $\mu\text{g}/\text{mL}$ (Figure 1). Interestingly, MTT assay showed that ASC at 100 and 1000 $\mu\text{g}/\text{mL}$ dramatically increased cell survival by 153.15% and 399.88%, respectively. But SRB assay revealed that ASC 1000 $\mu\text{g}/\text{mL}$ decreased cell survival by 42%. Cell survival

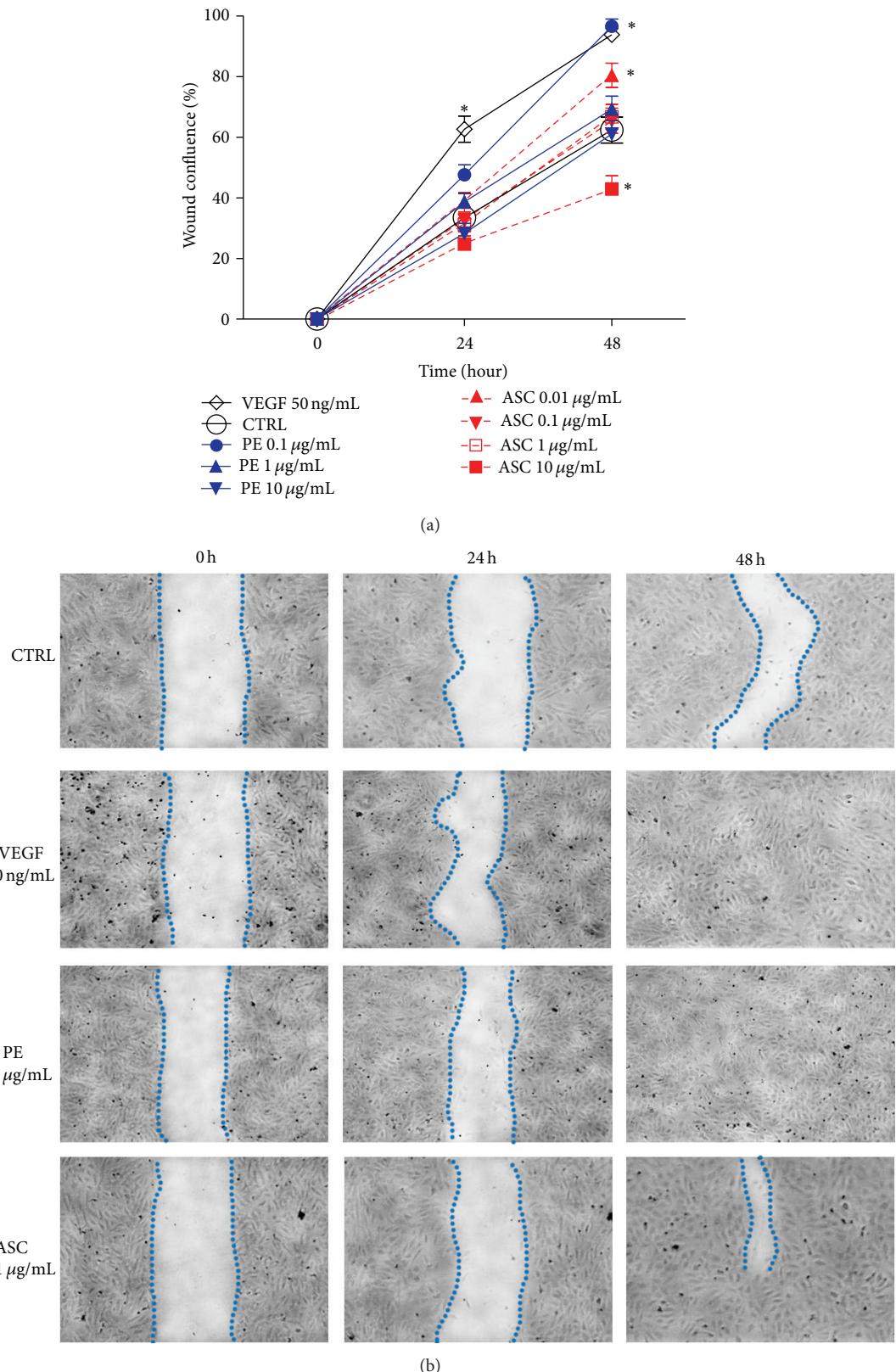


FIGURE 2: Scratch wound assay. HUVEC wounds were created using pipette tips and the widths of wound areas were measured at the same positions at time 0, 24, and 48 h, after wounds were initiated. (a) PE (0.1–100 µg/mL) or ascorbic acid (ASC, 0.01–10 µg/mL) was incubated with endothelial wounds at time 0. (b) Representative photographs of wound closure at different time points. Dashed line and the wound areas were accentuated for visual purpose. The % wound confluence was calculated compared to vehicle treated group (CTRL). * $P < 0.05$ versus CTRL.

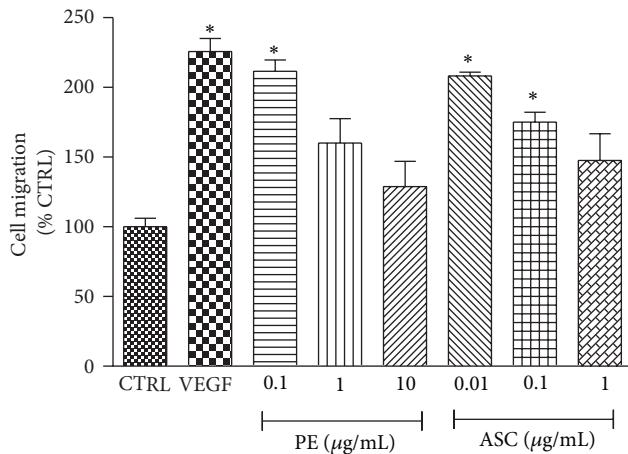


FIGURE 3: Effect of PE and ASC on cell migration. Comparison of HUVEC migration under influence of the chemotactic factor VEGF (100 ng/mL) and PE (0.1, 1, 10 μ g/mL) or ASC (0.01, 0.1, 1 μ g/mL) was determined using Boyden chamber assay as described in Section 2. * $P < 0.05$ versus control.

was then confirmed by cell morphology observed under inverted microscope (data not shown). The cell integrity and morphology of live cells were in accordance with SRB assay; thus noncytotoxic doses of PE and ASC were determined based on SRB assay.

3.3. PE and ASC Enhanced Endothelial Wound Healing. Effects of PE and ASC on endothelial wound healing were influenced by the concentrations applied to the scratch wound. At relatively lower concentrations (0.1 μ g/mL for PE and 0.01 μ g/mL for ASC) these compounds significantly promoted wound confluence only at 48 h while VEGF 50 ng/mL showed significant enhance in wound healing rate since 24 h. PE 0.1 μ g/mL completely healed endothelial scratch wound at 48 h which was comparable to wound treated with VEGF. No change was observed in the healing of HUVEC wound when treated with PE at 1, 10 μ g/mL and ASC at 0.1, 1 μ g/mL (Figure 2). Interestingly, high dose of ASC at 10 μ g/mL marked impaired endothelial wound confluence at 48 h.

3.4. PE and ASC Promoted Cell Migration. Migrated endothelial cells through modified Boyden chambers were measured by reading the fluorescent product of CAL-AM resulted from the metabolism of live cells migrated through the micropores of the upper chamber. Figure 3 demonstrated that PE (0.1, 1, and 10 μ g/mL) and ASC (0.01, 0.1, and 1 μ g/mL) inversely enhanced cell migration with respect to increasing doses. The lowest two concentrations of PE and ASC used in this experiment significantly promoted endothelial cell migration while the maximum responses were obtained at the degree comparable to the action of the chemoattractant VEGF (100 ng/mL).

3.5. Effects of PE and ASC on NO Production. It is well established that NO promotes HUVEC migration. Here we

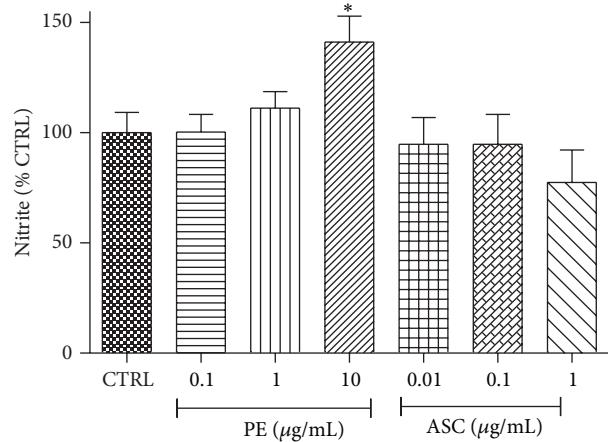


FIGURE 4: Effects of PE and ASC on nitric oxide levels. The stable product of nitric oxide, nitrite, was determined using DAN assay. Changes in the levels of nitrite in culture media were analyzed at 48 h following PE or ASC treatment as described in Section 2. * $P < 0.05$ versus CTRL.

evaluated the stable product of NO nitrite in the culture media of cells treated with PE or ASC corresponding to the doses that shown to promote endothelial migration. It appeared that significant increase in NO production was observed only in HUVEC treated with PE at 10 μ g/mL whereas PE at lower doses (0.1 and 1 μ g/mL) or all doses of ASC (0.01, 0.1, and 1 μ g/mL) did not change nitrite levels (Figure 4).

3.6. Low Doses of PE Promoted Endothelial Sprouting. PE enhanced endothelial sprouting from spheroids was observed at 0.1 and 1 μ g/mL which was similar to its effect on endothelial wound closure. Low PE doses promoted endothelial sprouting but no significant difference from CTRL group was observed at the higher concentration 10 μ g/mL (Figure 5). Effects of ASC on endothelial sprout length were inversely related to stepwise increases in concentrations used in the experiment (0.01, 0.1, and 1 μ g/mL) while ASC at 10 μ g/mL significantly suppressed *in vitro* angiogenesis.

3.7. VEGF mRNA Expression. The expression of VEGF mRNA was determined at 24 and 48 h following PE or ASC treatment (as shown in Figure 6). At 24 h after treatment, PE (1 μ g/mL) and ASC (0.1 μ g/mL) significantly enhanced VEGF mRNA expression by approximately 3.5- and 3-fold, respectively, while other concentrations did not significantly alter VEGF expression. No change was observed in VEGF gene expression when detected at 48 h.

4. Discussion

Antioxidant diets and supplements have increasingly become an important strategy to prevent or slow deterioration of vascular endothelium in well-recognized high oxidative stress conditions such as diabetes and cardiovascular disease. PE is one of the most studied natural antioxidants beneficial

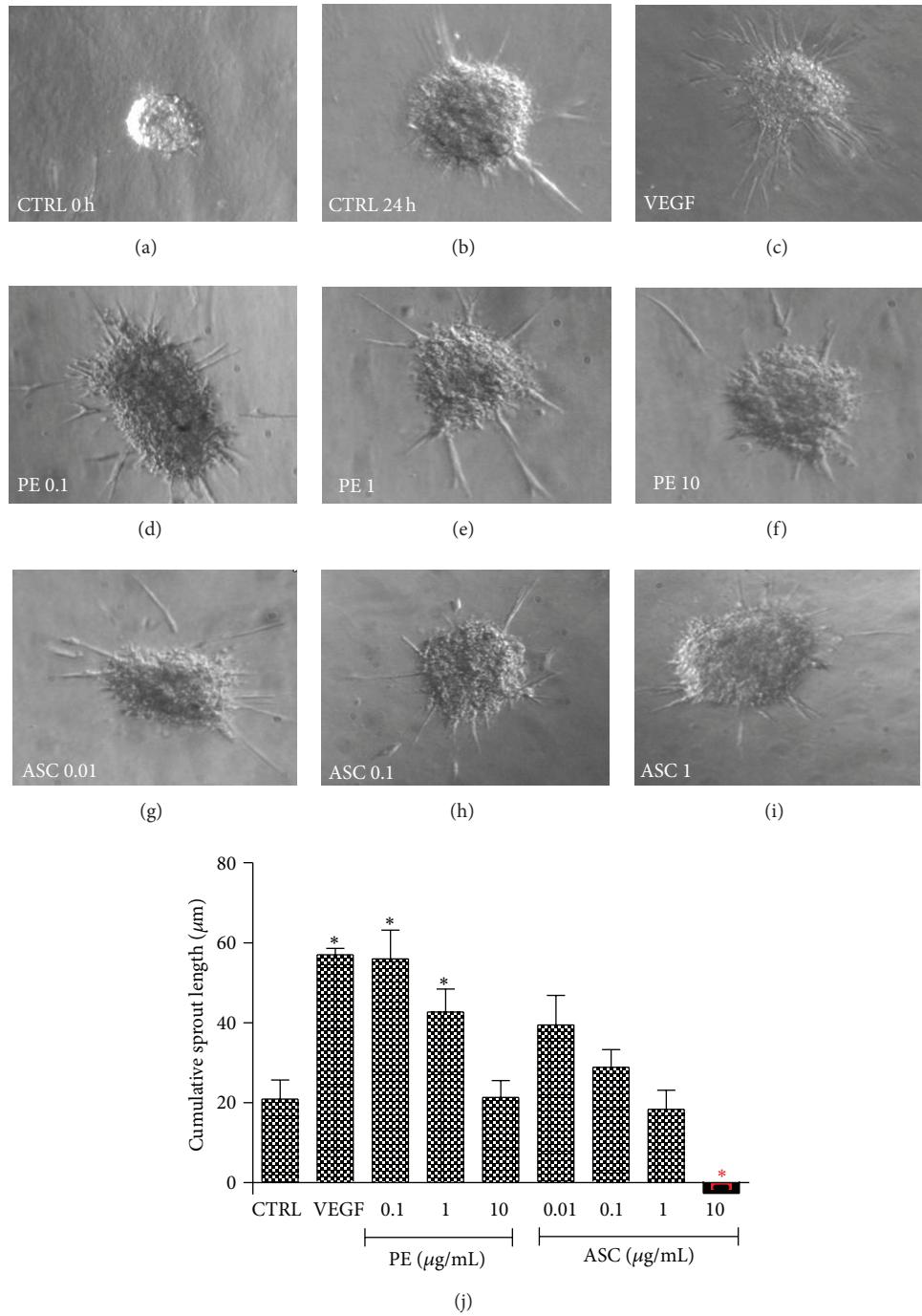


FIGURE 5: Effects of PE and ASC on endothelial sprouting. Evaluation of *in vitro* model of spheroid angiogenesis was performed 24 h after spheroids were embedded in the collagen matrix containing vehicle (CTRL), VEGF 100 ng/mL (VEGF), PE (0.1, 1, and 10 $\mu\text{g}/\text{mL}$), and ascorbic acid (ASC: 0.01, 0.1, and 1 $\mu\text{g}/\text{mL}$). Cumulative sprout length per spheroid was determined using an image program Cell^{^A}B as described in Section 2. (a) to (i) are representative photographs of spheroids with different treatments indicated in the figure; (j) bar graph demonstrates cumulative sprout length of all the treatments. * $P < 0.05$ versus control.

to endothelial health in several models of oxidative damage both *in vitro* and *in vivo* [18, 19]. Here we found that PE promoted endothelial wound healing, cell migration, nitric oxide production, and endothelial sprouting which

are crucial in endothelial cell function and restoration of endothelial integrity following oxidative damage.

PE is known to contain high antioxidant capacity among medicinal plants and most antioxidant properties found

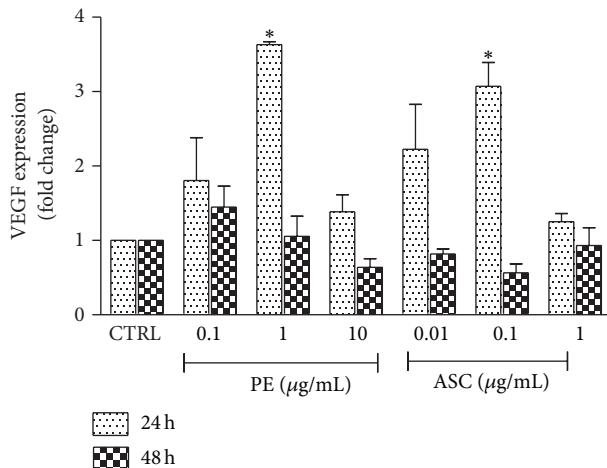


FIGURE 6: Effects of PE and ASC on VEGF mRNA expression. Changes in VEGF mRNA expression at 24 and 48 h following PE or ASC treatment were determined by real-time PCR as described in Section 2. * $P < 0.05$ versus CTRL.

are commonly contributed by ASC and polyphenolic constituents including polyphenols which are best defined in the human diet [20]. The ASC content of PE sample falls into the range of 1.1%–1.7% (w/w) reported by others [21]. Consuming nine PE fruits or 25 mL of fruit juice could receive sufficient ASC at recommended daily intake (RDI) for Thai people (60 mg). This study also revealed that PE fruit juice when kept in dry powder at 4°C retained antioxidant activity for at least 1 year as evaluated by FRAP assay and total phenolic content. This data suggest that preparing PE fruit juice in powder form may be suitable to maintain antioxidant property of the product if further used for food processing and nutrition industry.

Prior to further study for other pharmacological activities, endothelial cell survival was evaluated after being exposed to varied concentrations of PE. Several methods are available to evaluate cell survival or cell death. The two colorimetric MTT and LDH assays and the two fluorometric assays resazurin and CFDA-AM are among the most assays that used cytotoxicity assays in the field of pharmacology and ecology [22]. Selecting appropriate assay is based on detection mechanism, specificity, and sensitivity. This study first used MTT assay to evaluate the toxicity of PE in HUVEC. It appeared that there might be an interaction between MTT reagent and chemical components in PE. At high concentrations of PE and ASC, the absorbance readings did not correlate with cell viability as evaluated under inverted microscope. As a result, SRB assay was introduced to evaluate cell survival which has been proven to be more sensitive to cell death than MTT assay [23]. PE induced cytotoxicity in endothelial cell culture at concentrations above 10 µg/mL; therefore, PE concentrations used in this study were chosen to be lower than the toxic level. It is critical to choose appropriate cell viability assay for medicinal plant research since this may influence study design and hence interpretations of results from the study, especially when cell death accounts for the inhibitory effect such in wound healing or angiogenesis assay.

PE accelerated endothelial wound closure only at low concentration (0.1 µg/mL) while higher concentrations did not have any significant effect. PE inclined to cause cytotoxicity at higher concentrations observed in this study and others. For example, the plant extract induced cell death or apoptosis in many human carcinoma cell lines at the range of 50–200 µg/mL [14, 24]. Nonetheless, it also depends on the method of plant extraction and preparation. The mechanism of wound healing is complex but when exclusively considered at endothelial layer there are two main factors involved, that is, cell proliferation and cell migration. Increased cell proliferation may not contribute to the healing effect since no significant change in cell number was observed at this PE concentration. On the other hand, increased cell migration could be the mechanism underlining accelerated wound closure induced by PE where the migration effect correlated well with wound closure phenomenon. Likewise, ascorbic acid at concentrations closely related to its composition ratio in PE demonstrated similar effect on endothelial wound healing and cell migration. However, high dose of ASC (10 µg/mL) inhibited scratch wound closure and angiogenesis which is similar to a report by Mikirova et al. [25] in that high amount of ASC (3 mg/mL) inhibited *in vitro* tube formation and at higher concentrations (10–20 mg/mL) ASC inhibited cell migration 50–60% at 8 h. Thus, it is possible that PE promoted endothelial wound healing and sprouting, at least in part, through the action of ASC at low concentrations.

The main characteristic of endothelial dysfunction is the reduction of nitric oxide (NO) bioavailability. Although the dose of PE that increased NO production did not relate to its effect on endothelial wound healing or sprouting, this may have significant impact on maintaining vascular homeostasis and providing microenvironment that can partly restore wound healing deficit. Moreover, nitrate and nitrite have been shown to undergo reduction to NO in the vasculature [26]. It is possible that PE may promote endothelial function at the doses different from other effects and this effect is unlikely caused by ASC constituent but rather induced by other active constituents in PE such as gallic acid [27]. Nonetheless, determinations of changes in eNOS or iNOS expression are needed to confirm the influence of PE on NO production.

VEGF is an important growth factor for angiogenesis in promoting cell proliferations and cell migration through the signaling of VEGF receptors (VEGFR). Upon binding of VEGF to VEGFR, the protein-tyrosine kinase receptor undergoes autophosphorylation and sends signals to PI3K/AKT, resulted in increased NO production (through eNOS) and thereby enhancing cell permeability, vasodilation, and cell migration and survival. The VEGF activation cascade also includes Cd42/p38/MAPKAP-K2 pathway where it activates actin polymerization, stress fiber formation that renders the cell to migrate [28]. Signaling of $\alpha_v\beta_3$ integrin also participates in cell migration through the activation of FAK (phosphorylation at Ser732) that leads to focal adhesion turnover and hence cell migration. Our results did not show correlation between the concentrations of PE induced NO production and the doses that promoted *in vitro* angiogenesis. Thus, it is possible that PE NO alone may not be the sole mechanism that induces cell migration and endothelial

sprouting or that the kinetics of NO in the mode of paracrine effect escape sensitivity of assay. Similarly, changes in VEGF mRNA expression did not correlate well in the direction that favors the explanation of NO action. The interpretation of VEGF mRNA expression is complicated due to the intrinsic activity of test substances, experiment conditions, and times of detection. For instance, phorbol-12-myristate-13-acetate (PMA) maximally stimulated VEGF gene expression in HUVEC at 3 h and returned to baseline level within 12 h while hypoxia showed peak VEGF mRNA levels at 48 h [29]. Nonetheless, this study is the first to report the effect of PE on VEGF expression and this is substantiated by its effect on accelerated wound healing effect [7]. Interestingly, the inhibitory effect on VEGF expression is observed when either high dose of ASC was applied to endothelial cells or malignant cells and appeared to correspond to antiangiogenesis effect [30, 31]. This may be due to intracellular redox balance and the crosstalk between oxidative stress signaling and angiogenesis activation cascade [32]. Given that PE possesses high antioxidant capacity, PE might influence cellular redox status which may relate to angiogenesis in the condition that lacks influence from additional growth factors in the culture system.

One advantage using medicinal plant in crude form is the synergistic effect among different chemical constituents. In many cases, purified fractions or single chemicals demonstrate less effective than the crude extract when used at equivalent doses. For example, there are pharmacodynamic synergies among *Cinchona alkaloids* and pharmacokinetics interactions between *Artemisia annua* tea that the crude extracts decrease IC₅₀ by many folds for antimalarial effect when compared to using single compounds alone [33]. The mechanism of synergy is not fully understood but it involves the action at different pharmacological targets in the way that the operations are in parallel. In the case of PE, the concentrations that demonstrated significant changes from each experiment are incongruent in some parts. A certain set of components in PE may be responsible for the activation of NO production while another separate group of compounds work in the same pharmacological targets toward cell migration and endothelial tube formation. Accordingly, further studies to identify active ingredient(s) and the precise mechanism of PE induced endothelial migration and differentiation are warranted.

5. Conclusion

Endothelial dysfunction and endothelial damage are important risk factors associated with pathogenesis of impaired wound healing and cardiovascular disease. Consuming antioxidants and compounds that activate endothelial wound healing during oxidative stress may promote endothelial health and favor cardiovascular risk reduction. This study demonstrated that PE possessed high antioxidant capacity and enhanced endothelial wound healing and sprouting at low concentrations, but the opposite effects were observed when investigated at high concentration. These beneficial effects on endothelial cells are partly due to its antioxidant constituent ascorbic acid. Therefore, it is important to

consider this dose-related biphasic effects when designing experiments and applying information to clinical studies in order to obtain desirable pharmacological activities.

Acknowledgments

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Review Article

Research and Development for Botanical Products in Medicinals and Food Supplements Market

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Botanical products sold in the health area are generally intended as drugs, medicinal products, food supplements or substances for therapeutic use. Use of botanicals for improving or to care human health has evolved independently in different countries worldwide. Regulatory issues regarding botanical products designed for the food supplements or medicinal market and their influence on research and development are discussed. European Union (EU) and United States (US) policies regulating these products are focused with comments on the legislations delivered during the last ten years and differences existing in rules between these countries are emphasized. Research and development on botanical products nowadays strongly influenced by the product destination in the market. Addressed and differentiated research for either food supplements or medicinal markets is necessary to purchase data really useful for assessment of safe and effective use for both the categories. The main objective is to catalyze interest of academic and companies' researchers on crucial aspects to be taken into account in the research for the development of botanical products.

1. Introduction

In the scientific area, botanical products are generally intended as drugs, medicinal products, food supplements, or substances for therapeutic use derived from raw material of whole plants or parts of them. Starting from these materials, botanical substances (e.g., whole, fragmented or cut plants, algae, fungi, lichens) or botanical preparations are obtained through various processes such as extraction, distillation, purification, concentration, fermentation, and others. In many countries these products are regulated both as medicinal products and as food supplements and they are often labeled as natural foods or sport supplements [1].

Botanical products are widely available to consumers through different distribution channels. In particular, they are sold over the counter in pharmacies and can be bought also in supermarkets, herbalist's shops, or through Internet [2]. Use

of botanicals to improve or to care human health has evolved independently in the world, depending on specific cultures, current medical and nutritional practices, availability of botanical species and main policies of established companies on the territory. In consequence of this, there are different ways in which countries define medicinal plants or herbs or botanical products derived from them, and countries have adopted various approaches to licensing, dispensing, manufacturing, and trading to ensure their safety, quality, and efficacy, and due to these reasons herbal preparations vary from country to country [3]. Moreover, national legislations have also facilitated and addressed directly or indirectly the marketing of traditional botanical products as food supplements or as medicinal products [4].

For the present paper, we chose to describe shortly the most important regulatory issues being in force in EU and US, two of the largest markets for botanicals, and to highlight

the most important steps to follow in the modern research and development of health botanical products.

2. Botanicals in Herbal Medicinal Products Market

European Union (EU) policy regulating botanical products is illustrated in different legislations delivered during the last ten years. After decades of almost exclusive commercialization of herbal with medicinal properties through food supplements market, an effort to harmonize medicinal use of herbal substances through draw and delivery of the so-called Traditional Herbal Medicinal Product Directive 24/EC/2004 (THMPD) has been made. THMPD came into force in April 2011 and aimed to protect public health and at the same time secure the free movement of herbal products within the EU. This directive is the legal basis of regulation for the use of herbal medicines in phytotherapy in European countries. Once implemented, the objective of the directive is to remove in the EU the constraints that have made it difficult to grant marketing authorizations of herbal substances and preparations as traditional medicinal products under the pre-existing community legislation [5].

The Directive 2004/24/EC established that "*herbal medicines are any medicinal product exclusively containing, as active ingredients, one or more herbal substance, one or more herbal preparation or more such herbal substances in combination with one or more such herbal preparations.*" The greatest novelty contained within the directive is the amendment of the previous 2001/83/EC [6], by establishing that herbal medicinal products release in the market needs authorization as well as required for drugs. In other words, according to the 2004/24/EC, it is mandatory that botanicals authorized as medicinal products, before commercialization, have to undergo an evaluation procedure following the submission of an application for simplified registration [5].

The 2004/24/EC establishes that in European countries, for registration of botanicals as medicinal products, companies refer to one unique set of information on a herbal substance or herbal preparation purchased through the community monograph drawn by an "ad hoc" committee. The Herbal Medicinal Products Committee (HMPC) has been established at the European Medicines Agency (EMA, London), the agency which is responsible for the scientific evaluation of medicines developed by pharmaceutical companies for use in the European Union. Community herbal monographs comprise the scientific opinion of the HMPC on safety and efficacy data concerning a herbal substance and information on what are adequate herbal preparations intended for medicinal use. For any single plant, each herbal preparation is assessed individually according to the available information and it may vary from one preparation to another. The set of information included in the monograph comprises clinical (indication, posology, etc.) and safety (warnings, recommendations, and contraindications) issues [7].

The Directive 2004/24/EC introduced two subcategories of herbal products that can be commercialized as medicinal products. One of the subcategories of herbal products is that for which is possible to collect clinical data showing efficacy

for the intended use, and data demonstrating a positive risk/benefit profile. These products have been called herbal medicinal products with a "well-established use" (WEU) on the basis of the existence of body of evidence sufficient to show clinical efficacy in specific therapeutic indications. The second subcategory, comprising products derived from more numerous plants than WEU products, is represented by "traditional herbal medicinal products" (THMPs). THMPs are called botanical products for which proof of clinical efficacy is not existing, or poor or not sufficiently convincing. The Directive establishes that this subcategory can be registered as traditional medicinal products, only if the use in specific therapeutic indications (not requiring medical supervision) is plausible according to their traditional long standing utilization and if they present an acceptable safety level. Moreover, products are eligible for license as a traditional herbal medicine only if they have been used to treat a specified health complaint for at least 30 years, including a minimum of 15 years at least in one country of EU. Obviously, WEU and THMPs are held to satisfy similar safety and quality standards as pharmaceutical drugs [8].

For THMPs authorization, the 2004/24/EC contemplates a simplified registration after that application is presented to national agencies regulating drug market. Finalized (definitive) versions of community monographs have to be taken into account by the member states when examining an application for herbal medicinal products. Even though the member states are not obliged to follow the monographs, any decision not to accept the content of the monograph as it is adopted by the HMPC should be duly justified [3, 9]. HMPC has defined that at least one controlled clinical study or alternatively a well-documented clinical experience with sufficient supportive pharmacological data is needed to substantiate efficacy for a well-established use [10]. The current situation regarding the status of products registration is that in some European member states, herbal preparations have been regulated under food law, although they have pharmacological properties, and the tendency in these countries remains to place typical medicinal plants on the market as food products [8].

A "community list of herbal substances, preparations, and combinations thereof for use in traditional herbal medicinal products" has been established in Europe. It is based on proposals from HMPC and is gradually developed. When herbal substances/preparations are included in the Community list, registrations for traditional herbal medicinal products containing them have a significant advantage. The reason for this advantage is that once a traditional product is based on a herbal substance/preparation included in the Community list, the applicant will not be required to provide evidence of the safe and traditional use for its registration if the intended use and related claims in the application comply with the information contained in the list. Moreover, national competent authorities will not have the opportunity to require additional data to assess the safety and the traditional use of the product [5]. Community monographs are published by HMPC whereas list entries are published by the European Commission and have therefore a broader legal status. List entries are legally binding and competent

authorities will not request additional data to assess the safety and traditional use of the product [10].

In the United States, categorization of botanicals is based on intended use, safety, regulatory status, and degree of characterization [11]. In the field of medicinals, products can be “prescription drugs” or “over-the-counter drugs.” Authorization for this category of products requires rigorous testing including three distinct phases of clinical testing to ensure safety and efficacy and close scrutiny by the Food and Drug Administration (FDA). Today in United States, about 25% of the drugs used are based on plant-derived products [12]; however, only pure compounds isolated from plants and subjected to the same rigors as synthetic pharmaceutical can be conventional drugs [13].

The guidelines for registration of botanical drugs were released in 2004. Botanical drugs are evaluated for safety and clinical efficacy just as conventional drugs, but the process for botanical drugs can be accelerated on the basis of the empiric knowledge of safety derived from observation in human use. Botanical drugs are produced under the same strictly regulated quality conditions as conventional pharmaceuticals [11, 14].

3. Botanicals in Food Supplements Market

Use of plant products as supplements for food originates from a long tradition where the consumption of herbal infusions, digestives, juices, elixirs, and extracts had the purpose to maintain and promote health [15]. In Europe, Regulation (EC) No. 178/2002 defines “food” (or “foodstuff”) as “*any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans.*” Definition of “food” includes drink, chewing gum and any substance, water included [16]. Botanical products represent a principal ingredient, alone or in association with other substances, of food (or dietary) supplements. The food use as supplements has been ruled by the Food Supplements Directive (FSD) 2002/46/EC that established the definition of food supplements as “*foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect . . .*” [17]. With this definition is expressed the concept that food supplements have no therapeutic function, whereas health-keeping functions are emphasized.

Successively, food supplements context was further regulated through the delivery of the “*Regulation n. 1924/2006 of the European Parliament and of the Council released on 20 December 2006 on nutrition and health claims made on foods.*” It establishes the concept of “claim,” intended as “*any message or representation which states, suggests or implies that a food has particular health characteristics.*” In the same Regulation, “health claim” is defined as “*any claim that states, suggests, or implies that a relationship exists between a food category, a food or one of its constituents and health.*” Aim of the Regulation is ruling the issue regarding intended health indications for food use through the “health claims”. The Regulation 1924/2006 establishes that “nutrition claims” (for products with nutritional properties) and “health claims”

may be used in the labeling, presentation, and advertising of foods placed on the market in the EU only if they comply with the defined provisions. The use of a health claim requires an authorization by the European Commission through the Standing Committee for Food Safety and Animal Health and a scientific assessment by European Food Safety Authority (EFSA) to ensure that they are based on “*generally-accepted scientific evidence, taking into account the totality of the available scientific data, and by weighing the evidence*” [12].

On the basis of exclusive physiological function of food, the new policy points out that claims have to recall the health-keeping and nontherapeutic role of food supplements. The same regulation establishes that claims are also accepted in the case of “Reduction of disease risk claims” intended as “*any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease.*” To obtain authorization for a claim, companies have to produce an application to the Member States, which will submit it to the EFSA.

Whether or not, available data for each claim are sufficient to substantiate the claim (on the basis of accepted scientific evidence) is a scientific judgement of EFSA. This decision on the use of the claim will be taken by EFSA, after an accurate examination of the relevant scientific literature corroborating the requested claim [4].

Health claims for botanical products have become a crucial issue, since authorization of the great part of claims proposed for botanical products have been refused by EFSA. Following this decision, a criticism has been raised from companies, asking and suggesting for a more tolerating regulatory approach. In particular, it has been objected that it may be more difficult for consumers to fully understand, in the absence of health claims, the benefits, if any associated with the consumption of the product. Another objection is the idea that the current regulatory and legal situation in the EU is not adequate to lead to a harmonization that would make it possible for all European citizens to benefit from traditional botanical food supplements and medicinal products under, if not identical, at least comparable conditions [4].

Differences in botanicals regulation between Europe and United States are existing. Food supplements were previously defined in United States by the Dietary Supplement Health and Education Act (DSHEA) released in 1994 as products taken by mouth that contain a “*dietary ingredient*” intended to supplement the diet. DSHEA changed the marketing and legal climate for dietary supplements and herbs and enabled the exponential growth of product sales since that time. This Act amended previous statutes to encompass dietary supplement-specific provisions, including the definition of dietary supplements, product safety, nutritional statements and claims, ingredient and nutritional labeling, good manufacturing procedures, and the classification of “new” dietary ingredients. As defined by DSHEA, a dietary supplement is a product other than tobacco that is intended to supplement the diet and contains one of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance to supplement the diet by increasing

the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients [18].

The Nutrition Labeling and Education Act of 1990 gives the United States FDA the authority to regulate health claims on food labels [19]. These claims describe the link between specific nutrients or substances in food and a particular disease or health-related condition [20]. The "Nutrition Labeling and Education Act" permits use of health claims if there is evidence to support the claim and significant scientific agreement among qualified experts about the claim, and if the claim is not misleading. The FDA Modernization Act of 1997 permits manufacturers to use health claims based on authoritative statements by a scientific body of the US government, such as the National Institutes of Health [21]. Three categories of claims can currently be used on food and dietary supplement labels in the US: (1) health claims, (2) nutrient content claims, and (3) structure/function claims. Structure/function claims were authorized under the Dietary Supplement Health and Education Act of 1994 and describe the effect of a dietary supplement on the structure or function of the body [14]. In US, health claims are authorized by FDA only after a systematic review of scientific evidence [22]. Only studies conducted in "healthy populations" are considered, because health claims are directed to the general population or designated subgroups (e.g., elderly persons) and are intended to assist the consumer in maintaining healthful dietary practices. Health claims are limited to claims about risk reduction and cannot be about the diagnosis, cure, mitigation, or treatment of disease. The FDA exerts its oversight in determining, by means of the following Acts, which nutrient content claims may be used on a label or in labeling: (a) the Nutrition Labeling and Education Act (NLEA) of 1990, by issuing a regulation authorizing a nutrient content claim, and (b) the FDA Modernization Act of 1997, by prohibiting or modifying by regulation a nutrient content claim. The NLEA required that the FDA issue regulations for authorizing the use of a health claim about a substance/disease relationship only when the significant scientific agreement standard was met [23].

In United States, companies are responsible for the safety of their products and food supplements, including those containing botanicals, which do not need approval from Food and Drug Administration (FDA) before commercialization. Only in the case of new ingredients market introduction, legislation requires a report including safety but not efficacy data. In conclusion, botanical products are generally sold in United States as food supplements with no particular authorization needed for their release in the market, while companies have to show the truthfulness of claims [22].

4. Research and Development for Botanical Products

Until about two decades ago, scientific investigation on medicinal plants has been characterized for the most part by *in vitro* or *in vivo* scientific evidence. Most of all, they were preclinical demonstrations of one or more biological and pharmacological activities of extracts or other herbal preparations obtained from the whole plant or parts of

the plant. According to which is indicated by legislations, it is becoming clear that research supporting the use for health of botanical products has to be addressed in line with final destination in the market. Between food supplements and medicinals markets, the fundamental difference is represented by the "intended use" of each category of products. This is "health-keeping" for food supplements and "therapeutic" for medicinal products. New legislations have established that authorization needs scientific demonstration either for "health keeping" or "therapeutic" intended uses. A common aspect of scientific proof is that for both types of products it has to be obtained through studies involving human beings, generally epidemiological data or clinical studies. This will be a mandatory direction for research on botanical products since it is not more possible effective health use of botanical products in absence of clinical studies. Even if scientific preclinical experiments could seem as secondary in the new scenario, this does not mean that they are not important. Preclinical investigation keeps to be the primary and fundamental proof to address successive research for clinical evidence. For many herbal preparations contained in well-established or traditional herbal medicinal products an adequate safety profile, may be confirmed by their long-term medicinal and/or food use. However, in cases where a safety concern is recognized or suspected, non-clinical investigations may be needed. The documented experience gathered during the long-standing use generally represents the main basis of the pre-clinical assessment both for traditional and well-established herbal medicinal products [24]. However, particular attention should be paid to effects that are difficult or even impossible to detect clinically. In particular, information coming from preclinical toxicological experiments (i.e., genotoxicity, carcinogenicity, and reproductive studies) is indispensable for safety use of botanical products in humans [25]. Genotoxicity studies are designed to detect genetic damage such as gene mutations and chromosomal aberration, which may reflect teratogenic and tumorigenic potential of pharmaceuticals, including herbals [26]. Botanical drug products in the U.S., like other therapeutic agents, are required to provide genotoxicity information prior to marketing approval [27]. Recent data indicate that the European sponsors of botanical products have increasingly recognized the importance of genotoxic data and, in consequence of this, have prioritized their acquisition in drug development programs. On the basis that genotoxicity studies are highly reproducible, and have high statistical power, by purchasing comparably cost-effective data, botanicals companies should be encouraged to realize them as an early goal in their product development [28].

Carcinogenicity studies should be performed for any herbal intended for use as drug for a duration that is continuous for more than 3 months or 6 months intermittently. While for shorter term period use, carcinogenicity information is generally considered not needed. Carcinogenicity studies are generally not needed in cases where there is no suspicion for a carcinogenic potential. Furthermore, the proposed duration of treatment should also be considered [24]. A crucial issue is represented by botanical ingredients containing chemical compounds that are both genotoxic and carcinogenic. Such

compounds include, for example, the allylalkoxybenzenes estragole, methyl eugenol, elemicin, tetramethoxyalkylbenzene, safrole, myristicin, and apiole [29]. Unfortunately, in these cases, assessment of the risk to human health is complicated, and an international scientific agreement concerning the best strategy for the risk assessment of genotoxic and carcinogenic compounds is still lacking [30].

Reproductive toxicity studies are useful to support the safe use of botanicals; however, these studies are not always necessary. This is the case of botanicals which are designed for postmenopausal symptoms or for benign prostate hyperplasia. The only condition for which there is a cause for concern is for products explicitly indicated in pregnancy [24]. In general, procedures to assess reproductive toxicology should comprise the evaluation of the potential to affect fertility or early embryonic development to implantation, as well as teratology in both a rodent species and a mammalian nonrodent species, and effects on pre- and postnatal development, including maternal function [26]. Another toxicological issue is regarding carcinogenicity information. About the need to clarify toxicological issues, results from postmarketing studies or epidemiological data of adequate power or postmarketing safety studies are always auspicable.

Because of the complexity and diversity of chemicals present in botanicals, requirements of pharmacokinetic data are almost always limited. Pharmacokinetic findings that sometimes could be useful are those investigating on the inductive or inhibitory effects on P-glycoprotein drug transporters and hepatic P-450 or other drug metabolizing enzyme systems, and those predicting potential herb-drug interactions [31]. Many herbal compounds undergo metabolism *in vivo*, with a major role played by cytochrome P450s enzymes and uridine diphosphate glucuronosyltransferases playing a major role. Some herbal chemicals are substrates of intestinal, hepatic, cerebral and renal P-glycoprotein. Thus, the activities of these drug metabolizing enzymes and drug transporters are determining factors for the *in vivo* bioavailability, disposition, and distribution of herbal chemical substances. Pharmacokinetic studies of botanicals have been mainly focused on a small number of herbal medicines and purified herbal ingredients, including anthocyanins, berberine, catechins, curcumin, hypericin, hyperforin, lutein, and quercetin. For the majority of herbal remedies used in folk medicines, data on their disposition and biological fate in humans are poor or lacking [32]. About the potential herb-drug interactions, it has been suggested to consider it not to be a major issue among botanical safety concerns. The reason is that (a) a minority of herbal preparations, herb-drug interactions seem to be clinically relevant and (b) the inclusion of adequate information on such interactions into the package leaflet could be sufficient for the safe use of the products [33]. The most common botanical-drug interactions that have been described involve herbs like ginkgo biloba (*Ginkgo biloba*), gingseng (*Panax gingseng*), and St. John's wort (*Hypericum perforatum*) [30].

Finally, product destination in the market also influences both preclinical and clinical research. From now on, it will become evident that clinical design to study the effects of botanical products has to be a "dress made to measure"

according to what the researcher want to demonstrate. In this way, if the development of a botanical product is for medicinal products market, it will be necessary to show beneficial effects in the care of affected subjects (patients). In the case of botanical products for the food supplements market, aim of clinical research has to show that products are able to maintain health state, and in consequence studies will be conducted on healthy subjects. This type of clinical study, apparently easier, puts problems for clinicians normally used to design clinical study aimed to show therapeutic effects in patients. Obviously, if this is not taken in account, the risk is to conduct a study which does not reach the right objective, producing results not suitable for the intended use. On the basis of the different requests of scientific proof for food or medicinal market, also preclinical research should be addressed in the right way. So, if it is thought about a botanical product for food supplements market, research should be built with the objective to show that the product is suitable to keep health and not to care for one or more pathologies. In this case, as an example, there is a more focused experimental model showing that the intake of the product investigated reduces or abolishes the occurrence of a certain pathology, instead that experiments demonstrating that a product cares for an already present pathology. In conclusion, research and development on botanical products is today strongly influenced by the product destination in the market. For this reason, it is necessary that research is addressed and differentiated on the basis of the final market destination. Only in this way adequate safety and efficacy data can be provided for each product category.

Disclaimer

The coauthor G. Calapai is one of the coopted members of Herbal Medicinal Products Committee of European Medicines Agency (EMA London, UK). For this reason, since this paper is related to his work in EMA, according to the EMA policy on scientific publications, the following disclaimer is added: "The views expressed in this paper are the personal views of the authors and may not be understood or quoted as being made on behalf of or reflecting the position of the European Medicines Agency or one of its committees or working parties."

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Research Article

Ameliorative Effects of *Acanthopanax trifoliatus* on Cognitive and Emotional Deficits in Olfactory Bulbectomized Mice: An Animal Model of Depression and Cognitive Deficits

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Acanthopanax trifoliatus is a plant that has been traditionally used in Thailand as a vegetable and a tonic. This study investigated effects of the aqueous extract of its leaves (ATL) on cognitive and emotional deficits using an olfactory bulbectomized mouse (OBX) model. OBX mice were treated daily with ATL (250 and 500 mg/kg, p.o.) 3 days after OBX. Antidementia drug tacrine (2.5 mg/kg/day) and antidepressant drug imipramine (10 mg/kg/day) were given i.p. as reference drugs. OBX significantly impaired cognitive behavior in a novel object recognition test and a modified Y-maze test and induced depression-like behavior in a tail suspension test. ATL and tacrine treatment attenuated OBX-induced cognitive deficits, whereas ATL and imipramine improved OBX-induced depression-like behavior. Neurochemical studies conducted after completing behavioral experiments demonstrated that OBX downregulated the expression levels of cholinergic marker genes encoding choline acetyltransferase and muscarinic M₁ receptor in a manner reversed by ATL and tacrine. Moreover, ATL and tacrine administration inhibited the *ex vivo* activity of acetylcholinesterase in the brain. These findings suggest that ATL is beneficial for the treatment of cognitive and emotional deficits related to dementia with depressive symptoms and that the antidementia effect of ATL is mediated by normalizing the function of central cholinergic systems.

1. Introduction

Acanthopanax is a plant genus that possesses ginseng-like activities and thus is known as a “ginseng-like herb” [1, 2]. The roots and stem bark of *Acanthopanax* plants have been used as tonics and sedatives, as well as in the treatment of rheumatism and diabetes [3]. *Acanthopanax trifoliatus* (*A. trifoliatus*) or Phak Paem is a Thai traditional plant that belongs to this species and is used in the folk medicine of Southeast Asia [3, 4]. Indeed, the young leaves and shoots of this plant are popularly consumed as vegetables in Northern Thai traditional cuisine [4].

Our previous studies revealed that extracts from the young leaves, roots, and root bark of this plant exhibited potent *in vitro* antioxidant activities that were elucidated by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and a thiobarbituric acid reactive substances (TBARS) assay [5]. Moreover, we found that leaf extract from *A. trifoliatus* significantly exhibited acute antianxiety effects in animal models by the single oral administration at the concentration of 500 mg/kg body weight [6]. Phytochemical investigation by HPLC-MS suggested that leaf decoction extract contains phenolics and flavonoids [7]. Its ethnomedical uses as a ginseng-like herb and chemical constituents suggest that *A.*

trifoliatus may have potential for the treatment of neurodegenerative diseases and cognitive dysfunction.

Dementia including Alzheimer's disease (AD) and affective disorders such as depression are major disorders with globally increasing numbers of patients in many countries. AD is a progressive and neurodegenerative disorder that is characterized not only by memory dysfunction but also by behavioral and psychological symptoms including depression [8]. An increasing population of AD patients is a serious social and economic problem in super-aging societies; however, only a few drugs are clinically available for this disease. On the other hand, depression is an emotional disorder with estimated lifetime prevalence of about 21% of the general population [9]. Evidence indicates a close relationship between depressive disorder and cognitive deficits in human patients [10, 11]. Therefore, new drug discovery and the establishment of new therapeutic methods effective for these disorders are considered to be beneficial and pressing needs.

In this study, to obtain a better understanding of the potential availability of *A. trifoliatus* for the treatment of cognitive and emotional dysfunction, we elucidated the antidementia and antidepressive effects of *A. trifoliatus* using an animal model of olfaction deficits. We employed this model for a couple reasons. First, olfactory bulbectomy (OBX) in rodents has been used as one of the AD models since the impairment of olfactory perceptual acuity is present not only at the early stage of AD [12] and in mild cognitive disorder (MCI) patients [12] but also in a transgenic AD model of mice with overexpression of a mutant form of the human amyloid- β -precursor protein [13]. Second, OBX induces not only a loss of olfactory cue but also various behavioral and biochemical alterations such as increases in locomotor activity [14], cognitive deficits by inducing neurodegeneration of septohippocampal cholinergic innervation [8, 15], and elevation of amyloid β in the brain [16], indicating that OBX provides a beneficial animal model of AD that is independent from transgenic animal models. Moreover, OBX has also been used as an animal model because it fulfills many of the necessary criteria as a depression model, which are comparable to the features observed in patients with major depression [17]. The overall findings in this study have suggested the *A. trifoliatus* is beneficial for the treatment of cognitive and emotional deficits related to dementia.

2. Materials and Methods

2.1. Animals. The study was conducted according to the experimental protocols as described in Figure 1. Male ddY mice (Japan SLC Inc., Shizuoka, Japan) were obtained at the age of 9-week-old. The animals were habituated to the laboratory animal room for at least 1 week before surgery. Food and water were available ad libitum. Housing was thermostatically maintained at $24 \pm 1^\circ\text{C}$ with constant humidity (65%) and a 12 h light-dark cycle (lights on: 07:00–19:00). The behavioral experiments were performed during the light phase from 9:00 to 18:00. The present studies were conducted in accordance with the Guiding Principles (NIH publication no. 85–23, revised in 1985) for the Care and Use of Animals

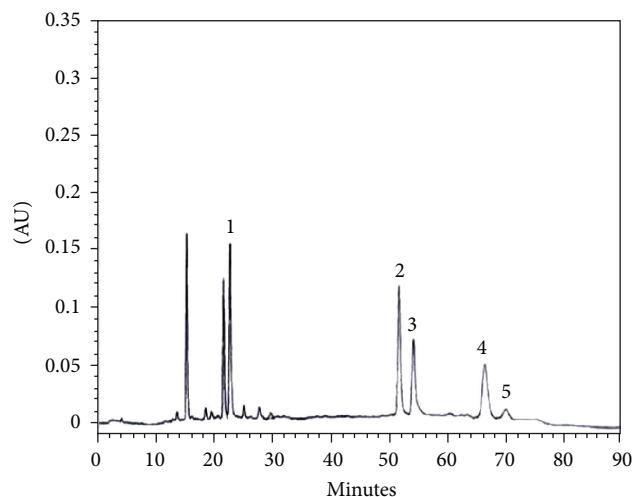


FIGURE 1: Separation of phenolics and flavonoids in *A. trifoliatus* leaf extract by HPLC; peak 1 = chlorogenic acid, peak 2 = 3,5-di-O-caffeoquinic acid, peak 3 = rutin and isoquercetin, peak 4 = 4,5-di-O-caffeoquinic acid, and peak 5 = quercitrin. Column: X-terra C₁₈. Gradient mobile phase: water/0.5% acetic acid (solvent A) and methanol (solvent B). Detector: UV detector at 310 nm.

and were approved by the Institutional Animal Use and Care Committee of the University of Toyama.

2.2. Plant Extract Preparation. The leaves of *A. trifoliatus* were collected from the Sunpathong district, Chiang Mai province, Thailand, in 2010. The plant samples were identified by Mr. Winai Supatanakul, a botanist of Thailand Institute of Scientific and Technological Research. The voucher specimens were deposited at the same place (ATI1001). The leaves were cleaned, dried in a hot air oven (60°C) and powdered with an electronic mill (20 mesh sieve), boiled with distilled water (1:10 w/v) for 3 h, and then filtered through the Whatman Filter Paper no.1. The filtrate was taken to dryness by lyophilization to yield dried leaf decoction extracts. The yield of the extract was 13.30% w/w. The extract was previously standardized using high-performance liquid chromatography (HPLC) for quantitative analysis of phenolic and flavonoid components (Figure 1) and *in vitro* antioxidant activity tests for analysis of biological activities [6, 7]. LC-MS analyses were also performed with a Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface. The ESI parameters were as follows: source voltage +4.5 kV, capillary temperature 200°C , and nebulizer gas 1.5 L/min. The mass spectrometer was operated in positive ion mode scanning from *m/z* 200 to 2000. A Waters Atlantis T3 column (2.1 mm i.d. \times 150 mm) was used, and the column temperature was maintained at 40°C . The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution, (B) acetonitrile under the following gradient conditions: 0–30 min linear gradient from 10% to 100% B, 30–40 min isocratic at 100% B. The flow rate was 0.2 mL/min. Mass spectrometry data obtained from the extract have been listed in the MassBank database [18] and stored in the Wakan-Yaku Database system (<http://wakandb.u-toyama.ac.jp/wiki/LCMS:Acanthopanax>).

trifoliatus/2012072002), Institute of Natural Medicine, University of Toyama.

2.3. Surgical Operation. OBX of mice was conducted according to previous reports [8, 15]. Briefly, the mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and fixed on stereotactic instruments (Narishige, Tokyo, Japan). The skull covering the bulbs was exposed by skin incision, 1% lidocaine solution was used as a local anesthetic, and then a 1 mm burr hole was drilled. The bilateral bulbs were aspirated through a syringe, and the cavity of the bulbs was filled with hemostatic gelatin sponge. After completing the behavioral studies, all the animals were sacrificed and the operated lesion was verified visually. The data from animals with less than 70% removal or with no intact cortex were excluded from the analysis. Sham operation was performed in a similar way without removal of the bulb. At the end of the experiments, the olfactory bulbs of sham group mice were confirmed to be intact.

2.4. Drug Administration. Except in specially stated cases, either vehicle water or test drugs were administered daily according to the experimental schedule indicated in Figure 1. On a behavioral testing day, administration was conducted 1 h before the testing. Sham group of mice and OBX control group mice were per orally administered water. Reference standard drugs, tacrine (THA; 9-amino-1,2,3,4-tetrahydro-acridine HCl) and imipramine HCl (IM) (Sigma-Aldrich Co., St. Louis, MO, USA), were dissolved in 0.9% saline and administered once daily at doses of 2.5 mg/kg (i.p.) and 10 mg/kg (i.p.), respectively. The leaf extract of ATL was dissolved in water and given per orally at daily doses of 250–500 mg/kg.

2.5. Behavioral Study

2.5.1. Modified Y-Maze Test. A modified version of the Y-maze test was conducted according to Yamada et al. [8]. The apparatus used for this test consists of black polycarbonate walls with 3 arms each 40 cm long, 12 cm wide at the top, 3 cm wide at the bottom, and 18 cm high. This test was a two-trial task with a sample phase trial and a test phase trial that were separated by an intertrial interval. In the sample phase trial, each mouse was individually placed in the maze with one of the 3 arms closed. The animals were allowed to explore the other 2 arms freely for 5 min. Thirty minutes after the sample phase trial, the animal was again placed in the maze with all 3 arms opened and was allowed to explore the arms freely. The previously closed arm that was opened in the test phase trial was defined as the new arm. The animal behavior was video-recorded for later analysis. Percent time spent in the new arm and numbers of total arm entries were analyzed using SMART system version 2.5 (PanLab, S.L., Barcelona, Spain).

2.5.2. Novel Object Recognition Test (ORT). ORT is based on the tendency of mice to discriminate a familiar from a new object. This test was conducted on the day before

the test, mice were individually habituated to an open-field box (35 × 35 × 50 cm) for 10 min, and the performance of the animals was analyzed automatically using the SMART system. The total distance exploring the arena was used to determine the locomotor activity. The ORT consists of a sample phase trial and a test phase trial. During the sample phase trial, two objects of the same material were placed in a symmetric position in the center of the chamber for 10 min. Thirty minutes after the sample phase trials, one of the objects was replaced by a novel object, and exploratory behavior was again analyzed for 5 min. After each session, objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on the object or sniffing it at a distance of less than 2 cm. Successful recognition of a previously explored object was reflected by preferential exploration of the novel object. Discrimination of spatial novelty was assessed by comparing the difference between time of exploration of the novel and familiar objects. The time spent exploring each of the two objects was analyzed using SMART system ver. 2.5 with a tri-wise module to detect the head, center mass and base-tail (PanLab, S.L., Barcelona, Spain).

2.5.3. Tail Suspension Test. Tail suspension test (TST) is a widely used model for assessing antidepressant effects [19]. Mice were subjected to the short-term inescapable stress of being suspended by the tail, which leads to the development of an immobile posture. Using another group of mice as indicated in protocol 2 (Figure 2), mice were separately suspended 50 cm above the floor in a chamber by adhesive tape placed approximately 2 cm from the tip of the tail. The animal behavior in the test was video-recorded for later analysis. Immobility was defined as a state with movement speed no more than 0.05 cm²/sec using SMART system version 2.5, and immobility time was recorded during an 8 min period.

2.6. Neurochemical Study

2.6.1. Quantitative Real-Time Polymerase Chain Reaction (PCR). To analyze changes in expression levels of choline acetyltransferase (ChAT) and muscarinic M₁ receptor mRNA in the brain as marker genes of central cholinergic systems, the animals were killed by decapitation after completing the behavioral studies. The brain was removed immediately, and the hippocampus was dissected out and kept at -80°C until use. Quantitative PCR was conducted as previously described [20, 21]. Briefly, total RNA was extracted from the hippocampus using Sepazol (Nacalai Tesque, Kyoto) according to the manufacturer's instructions. First-strand cDNA synthesis was conducted using oligo (dT) primers and M-MLV Reverse Transcriptase (Invitrogen, Rockville, MD, USA) in a total volume of 20 μL. The reaction was performed at 25°C for 10 min and heated at 37°C for 60 min and 98°C for 5 min before cooling to 4°C. DNA corresponding to the RNA was used as a template for real-time PCR. Quantitative real-time PCR was carried out using Fast SYBR Green Master Mix (Applied BioSystems,

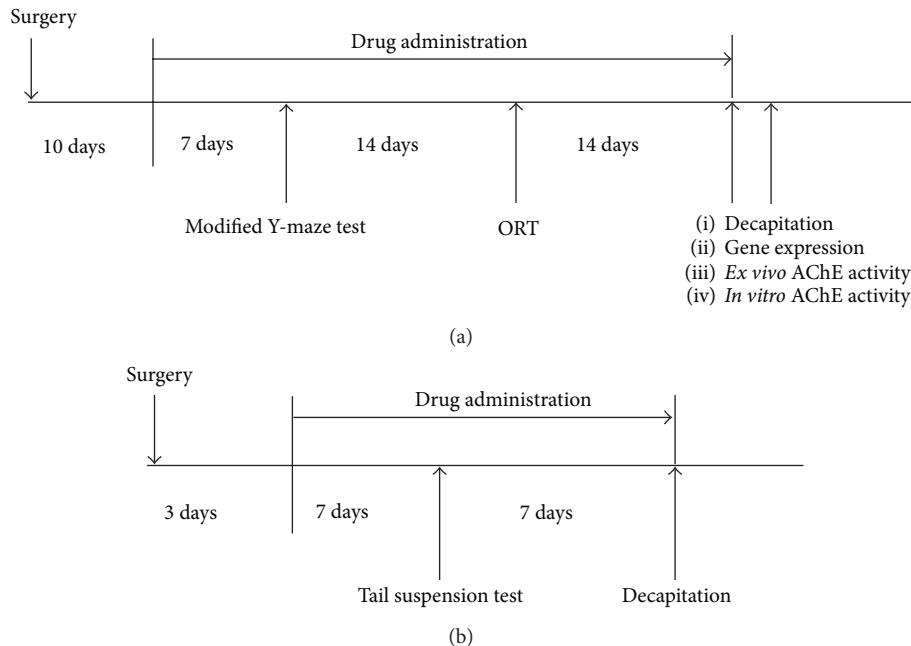


FIGURE 2: Schematic drawing of experimental schedule. Protocol 1 (a): after one week of acclimatization, the ddY mice were randomly divided into 4 groups of 10 mice. All mice (except mice in the sham group) were subjected to OBX surgery. Three days after the surgery, the drug administration was started. Modified Y-maze and object recognition tests were performed 1 and 3 weeks after starting drug administration, respectively. Quantitative real-time polymerase chain reaction and neurochemical studies were carried out after decapitation of all mice. Protocol 2 (b): after one week of acclimatization, the ddY mice were randomly divided into 4 groups of 6 mice. All mice (except mice in the sham group) were subjected to OBX surgery. Three days after the surgery, the drug administration was started. Tail suspension test was performed 7 days after starting drug administration.

Foster City, CA, USA) in a StepOne Real-time PCR System (Applied BioSystems). Melting curve analysis of each gene was performed every time after amplification was completed. Standard curves of the log concentration of each gene versus cycle threshold were plotted to prove negative linear correlations. The following primers synthesized by Nippon EGT Co. (Toyama, Japan) were used: choline acetyltransferase (ChAT, NM_009891), 5'-cctgtacaaggcttctagcttgag-3' (sense), and 5'-gttagctaaggcacaccagagatgag-3' (antisense); muscarinic M₁ receptor isoform (M16406): 5'-actgtctggcaccaggaa-3' (sense) and 5'-tgctaggccaatcatcagag-3' (antisense).

2.6.2. Ex Vivo and In Vitro Measurements of Cholinesterase Activity in the Brain. *Ex vivo* measurement: after completing the behavioral experiments, mice were decapitated and the frontal cortices were dissected out and kept at -80°C until use. Determination of cholinesterase activity was performed on the basis of the colorimetric method as previously described [8, 22, 23]. Briefly, the frozen cortex was weighed and homogenized in 10 times volume of 0.1 M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000 × g at 4°C for 20 min, the clear supernatants were collected and served as the enzyme source. Cholinesterase activity was determined in 10 μL aliquots of homogenates (run as triplicates) in a 96-well flat-bottom microplate. The reaction was started by adding 8 μL of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 16 μL of 7.5 mM

acetylthiocholine (ATCI), and 201 μL of 0.1 M sodium phosphate buffer (pH 8.0). The spectrophotometric absorption at 405 nm during a 3 min incubation period at 25°C was quantitatively measured using a microplate reader (Sunrise Classic; TECAN Japan, Kawasaki) and is expressed as nmol ACh hydrolyzed/min/mg tissue.

In vitro measurement: the assay for measuring AChE activity was modified from the assay described by Ellman et al. [22] and Ingkaninan et al. [24]. Frontal cortex supernatants were obtained from naïve ddY mice as the enzyme source. Cholinesterase activity was determined as described above.

2.7. Data Analysis. The data are expressed as the mean ± SEM. The data obtained from the behavioral tests and neurochemical experiments were analyzed by paired and unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison test (Dunnett's method) as appropriate. Differences of *P* < 0.05 were considered as significant. The analysis was conducted using SigmaStat version 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

3. Results

The oral administration of the leaf extract from *A. trifoliatus* did not produce any mortality in mice. There was no significant difference in average body weights of treated and

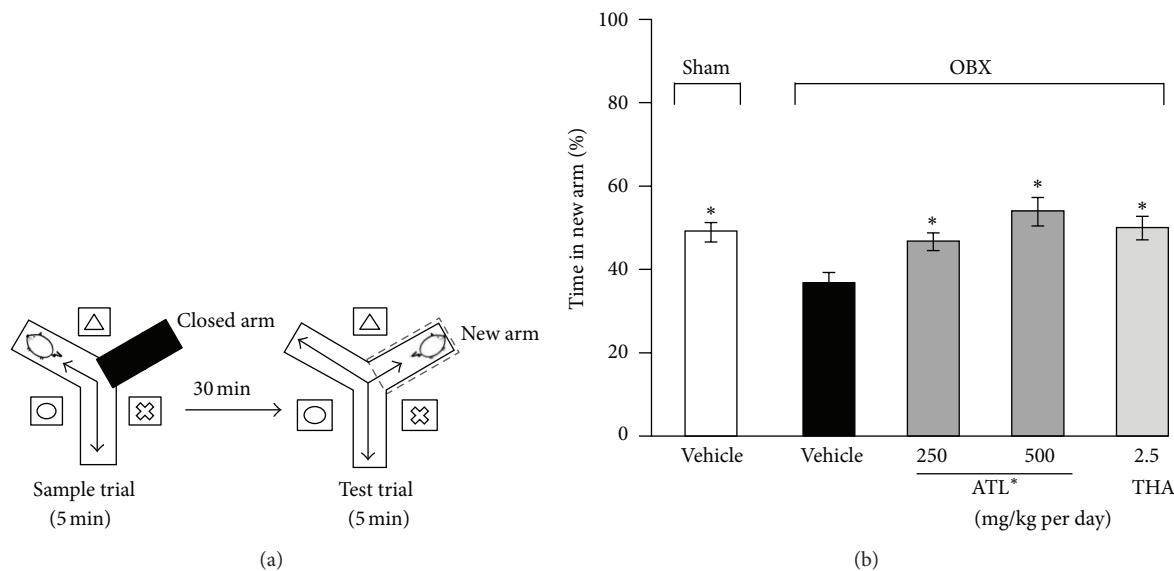


FIGURE 3: Evaluation of the modified Y-maze test of OBX-induced spatial working memory deficit mice using reference drug, tacrine or *A. trifoliatus* leaf extract (ATL). Surgical operation-naïve mice (OBX) were orally administered with distilled water 60 min before the sample phase trial while tacrine (2.5 mg/kg) and ATL (250 and 500 mg/kg) were administered by intraperitoneal injection and oral administration, respectively ($n = 10$). (a) Schematic drawings of the Y-maze and the experimental procedures. The maze was surrounded by different spatial cues. The sample trial and test trials were conducted for 5 min at a 30 min interval as described in the text. (b) The effects of ATL and THA on OBX-induced spatial working memory deficit in the modified Y-maze test. Each data column represents the mean \pm SEM. * $P < 0.05$ compared with vehicle-treated OBX group (Dunnett's test).

controlled animals (mice body weights were in the range of 40–45 g).

3.1. Modified Y-Maze Test. As shown in Figure 3, percentage time spent by the vehicle-treated sham mice visiting the new arm was significantly greater than the chance level, indicating a preference for new arms over the familiar arms. OBX mice that were administered THA (2.5 mg/kg, i.p.) or ATL (250 and 500 mg/kg, p.o.) 1 hour before the experiment spent significantly longer time exploring the new arm than the vehicle-treated OBX group.

3.2. Novel Object Recognition Test (ORT). As shown in Figure 4(a), there was no significant difference in locomotor activity determined as total distance between vehicle-treated OBX and sham mice groups. Daily administrations of THA (2.5 mg/kg, i.p.) or ATL (250 and 500 mg/kg, p.o.) for 1 week before the experiments had no effect on locomotor activity compared with those in the vehicle-treated OBX or sham mice groups. In the sample phase trial, no mouse groups showed significant differences in time spent exploring each identical object. There was also no significant difference in total time spent exploring two objects between vehicle-treated sham and OBX groups (Figure 4(b)). On the other hand, the sham group spent a significantly longer time exploring the new object than exploring the familiar one ($P < 0.05$, paired *t*-test) in the test phase trial, while the vehicle-treated OBX group showed a deficit in terms of the novel object recognition performance, as shown in Figure 4(c). THA- (2.5 mg/kg, i.p.) and ATL- (250 and 500 mg/kg, p.o.) treated

mouse groups spent significantly longer time exploring the new object than exploring the familiar one ($P < 0.01$, paired *t*-test).

3.3. Tail Suspension Test (TST). Duration of immobility in TST was measured at 10 days after OBX. The vehicle-treated OBX mice showed significantly longer immobility time than the sham-operated group (Figure 5). The OBX mice treated with daily administrations of imipramine (10 mg/kg, i.p.) and ATL (500 mg/kg, p.o.) for 1 week exhibited significantly reduced duration of immobility, while treatment of OBX mice with THA (2.5 mg/kg per day, i.p.) had no effect on the immobility.

3.4. Quantitative Real-Time Polymerase Chain Reaction (qPCR). The qPCR analysis (Figure 6) revealed that the vehicle-treated OBX group showed significantly reduced expression levels of ChAT and muscarinic M₁ receptor mRNAs in the hippocampus compared with the vehicle-treated sham-operated group. The expression levels of these genes were significantly upregulated in the hippocampus of the OBX mice treated with THA (2.5 mg/kg, i.p.) and ATL (250 and 500 mg/kg, p.o.).

3.5. Ex Vivo and In Vitro Measurement of Cholinesterase Activity in the Brain. The activity of acetylcholinesterase (AChE) in the cerebral cortex was measured in the OBX mice treated daily with ATL (500 mg/kg per day, p.o.) or THA (2.5 mg/kg per day, i.p.). As shown in Figure 7, it was found

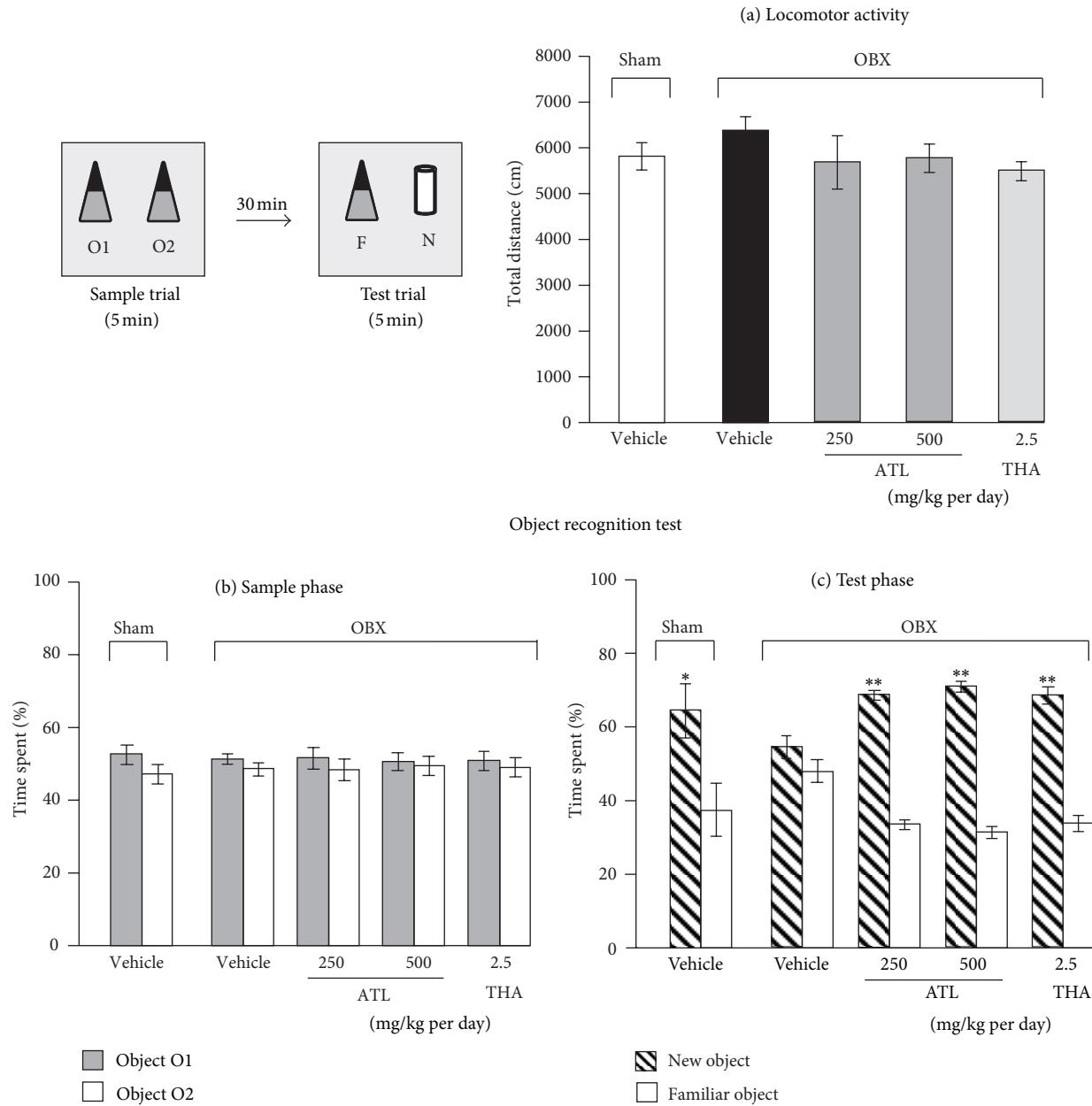


FIGURE 4: Effects of tacrine (THA) and ATL on object recognition deficits in OBX mice in the sample phase (b) and the test phase (c), while data of locomotor activities are shown in (a). Each datum represents the mean \pm SEM ($n = 10$). * $P < 0.05$ and ** $P < 0.01$ versus time spent exploring a familiar object (paired t -test).

that the activities of cortical AChE in the ATL- and THA-treated OBX groups were significantly reduced compared with the activity measured in the vehicle-treated OBX mice. No significant difference in the activity was observed between vehicle-treated sham and OBX groups. On the other hand, treatment of cortical homogenates with THA (0.05–5 μ g/mL) showed a potent inhibitory effect on the *in vitro* activity of AChE with IC₅₀ values less than 50 ng/ml, while ATL, at a concentration of 100 μ g/mL, had a negligible effect on the activity ($5.01 \pm 0.37\%$ inhibition).

4. Discussion

This study aimed to clarify the potential availability of ATL extract in the treatment of cognitive and emotional deficits using OBX mice as an animal model of AD. The present findings demonstrated that ATL administration exhibits ameliorative effects not only on cognitive deficits, like the acetylcholinesterase inhibitor THA, but also on depression-like behavior, like the antidepressant imipramine.

Working memory is one of the short-term memories that could be impaired at an early stage of AD [25]. In the present

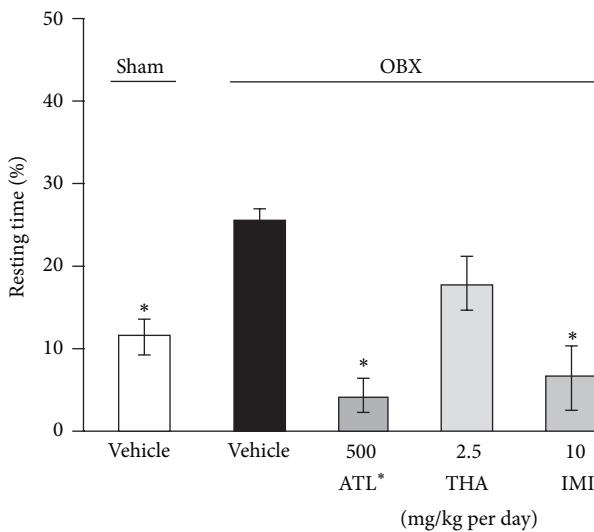


FIGURE 5: Effects of administration of tacrine (THA), imipramine (IMI), and ATL on OBX-induced depressive behavior in the TST. Each data column represents the mean \pm SEM ($n = 6$). * $P < 0.05$ versus % resting time in vehicle-treated OBX mice (Dunnett's test).

study, we employed a modified version of the Y-maze test to elucidate short-term spatial working memory as previously reported [8, 26]. Previous studies demonstrated using this test that central cholinergic and glutamatergic systems are involved in learning and memory performance since the performance is interfered with by drugs such as scopolamine and MK801 that reduce the function of these systems [8, 27] and is improved by donepezil, an acetylcholinesterase inhibitor used for AD treatment. Consistent with the data reported by Yamada et al. [8], OBX significantly impaired spatial working memory performance of mice in a manner reversible by treatment with THA, an acetylcholinesterase inhibitor. Interestingly, the present study revealed using the same task that ATL administration, as well as THA, could dose-dependently improve learning and memory deficits caused by OBX, suggesting that the ATL extract possesses an antidementia effect in an animal model of AD.

The ameliorative effect of the ATL extract on cognitive deficits was further confirmed using an object recognition test. In the sample phase trials of this test, no significant difference in total time spent exploring two identical objects was observed between sham and OBX groups, indicating no differences in ability to recognize objects between animals. In the test phase trials, the results showed that mice in the sham group spent more time exploring the new object, while vehicle-treated OBX mice showed no total time difference between familiar and new objects, indicating impairment of nonspatial object recognition memory. Administration of ATL (250 and 500 mg/kg, p.o.) and THA (2.5 mg/kg, i.p.) could significantly ameliorate OBX-induced recognition deficit against a new object. THA-induced amelioration of the impaired object recognition performance in OBX animals agrees with the observation reported in a previous study [8].

Taken together with the data obtained using a modified Y-maze test, these results suggest that ATL extract, like THA, exerts nootropic action against OBX-induced short-term memory deficits in mice and that the extract may be available for the treatment of cognitive deficits in humans.

We also examined, using the antidepressant drug imipramine as a reference standard drug and the tail suspension test as a model to detect depression-like behavior, whether ATL and THA have effects on emotional deficits that are distinctively observed in OBX animals [17, 28]. As summarized in Figure 4, the vehicle-treated OBX mice showed significantly prolonged immobility, which was reflected by an increase in percentage of the resting time during the observation period, compared with the sham-operated animals. The increased susceptibility of OBX animals to inescapable stress stimuli like tail suspension is consistent with a previous report [19]. Interestingly, daily administrations of ATL and imipramine but not THA significantly reversed the emotional deficits caused by OBX. The fact that imipramine exhibited a pharmacological profile clearly different from that of THA in the tail suspension test indicates that the susceptibility of OBX animals to inescapable stress can be used as an index to test antidepressant-like activity of test drugs. Taken together, the present findings suggest that, in addition to its antidementia effect, ATL has an antidepressant-like effect, although the molecular mechanism underlying the action is unclear. The effective dose of ATL is relevant to the dose used in our previous study where a single administration of ATL at 500 mg/kg exhibited the anti-anxiety effect in mice determined by light-dark task and hole-board test [6]. Moreover, the extract at the dose of 600 mg/kg also promoted anti-inflammatory effect in rat paw edema model [29] suggesting that the active concentration in the animal is around 500–600 mg/kg.

To understand the putative mechanism underlying the effects of ATL on impaired cognitive performance of OBX animals, we conducted quantitative real-time PCR to analyze the expression levels of central cholinergic marker genes. The results revealed that expression levels of ChAT and muscarinic M₁ receptor mRNAs in the hippocampus, a brain region responsible for spatial learning and memory performance, were significantly downregulated in the vehicle-treated OBX group, indicating OBX-induced pathological changes in central cholinergic systems. This finding agrees with previous reports [8, 15]. Interestingly, these changes were prevented in the OBX groups that had been administered ATL (250 and 500 mg/kg per day, p.o.) and THA (2.5 mg/kg per day, i.p.). There are at least a couple of mechanisms that could account for this finding. First, ATL administration is likely to elevate the expression levels of these cholinergic marker genes by increasing endogenous ACh level by acting like THA. Indeed, donepezil reportedly upregulates the expression of ChAT and other proteins such as vascular endothelial growth factor (VEFG) by increasing endogenous ACh level in the autonomic nervous system [30]. In the present study, we found that ATL extract application had no effect on the *in vitro* activity of acetylcholinesterase of brain tissues, while its administration significantly reduced *ex vivo*

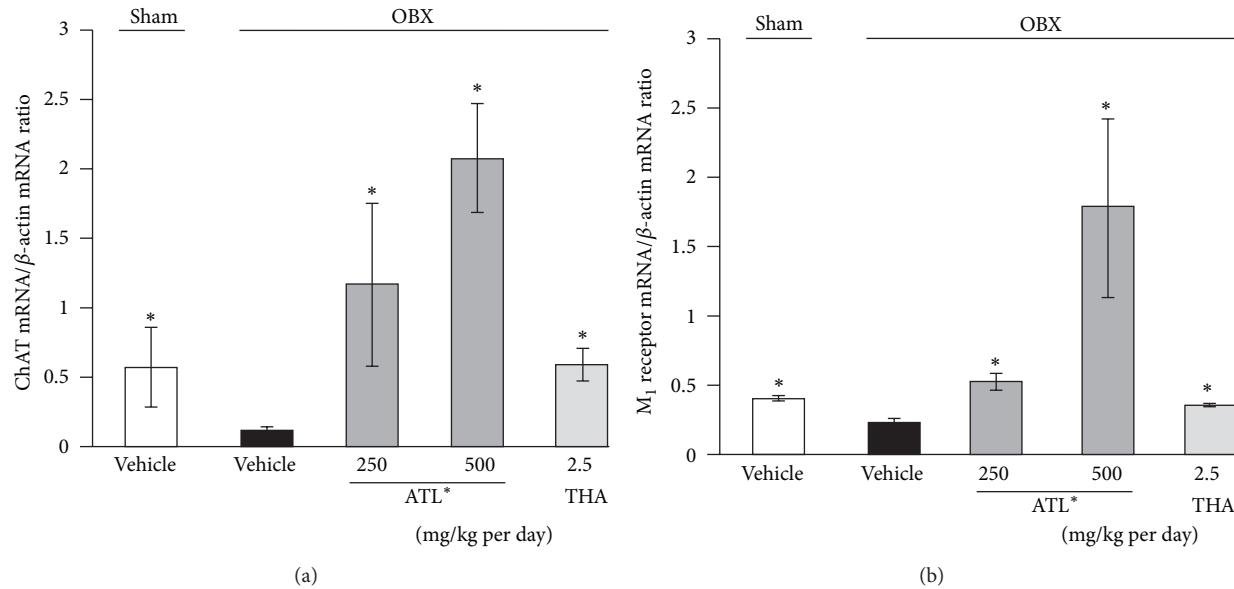


FIGURE 6: Effects of tacrine (THA) and ATL on ChAT (a) and muscarinic M₁ receptor (b) mRNA expression levels in the hippocampus of OBX mice. Each data column represents the mean \pm SEM ($n = 10$). * $P < 0.05$ versus mRNA expression levels in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

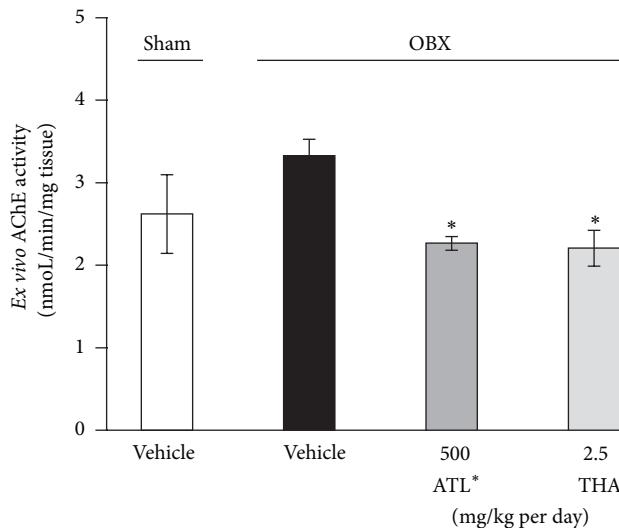


FIGURE 7: Effects of ATL and tacrine (THA) on *ex vivo* acetylcholinesterase (AChE) activity in OBX mice ($n = 10$). * $P < 0.05$ versus acetylcholinesterase activity in vehicle-treated OBX mice (Dunnett's test).

activity of the enzyme, like THA. These *in vivo* and *ex vivo* effects of ATL allow us to infer that chemical constituent(s) of ATL may be converted to the active form in the animal body and thereby interfere with acetylcholinesterase activity in the brain. Secondly, ATL administration may protect central cholinergic systems from OBX-induced cholinergic neurodegeneration. Evidence indicates that OBX causes neurodegenerative damage in central cholinergic systems, particularly in the septohippocampal cholinergic neurons and that cognitive

deficits and pathophysiological changes in central cholinergic systems become evident at least 1 week after surgery [15]. Since daily administration of ATL was started from 3 days after OBX, it is likely that the ATL administration prevented the occurrence/progression of neurodegeneration of central cholinergic systems that is caused during the 1-week period after OBX. Nevertheless, to clarify the detailed mechanism(s) underlying the ameliorative effects of ATL on cognitive deficits and downregulated expression of cholinergic marker genes in OBX animals, further investigation is required.

Considering the behavior-pharmacological and neurochemical features of ATL and THA observed in OBX animals, the present data exclude the possibility that the effect of ATL administration on central cholinergic systems is involved in the antidepressant-like action of the extract. In this context, it will also be interesting to clarify the underlying mechanism and chemical constituents that are responsible for the antidepressant-like action of ATL. Such investigations are in progress in our group.

5. Conclusion

This study demonstrated that ATL has an ameliorative effect on cognitive and emotional deficits in an animal model of OBX. The present findings suggest that ATL is beneficial for the treatment of patients with AD and affective disorder.

Acknowledgment

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Research Article

An Important Indian Traditional Drug of Ayurveda Jatamansi and Its Substitute Bhootkeshi: Chemical Profiling and Antioxidant Activity

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Nardostachys jatamansi DC. and *Selinum vaginatum* (Edgew) Cl. are two endemic high altitude Indian medicinal plants that have been traditionally known as “Jatamansi” and “Bhootkeshi,” respectively. These are used in various traditional herbal formulations and nutraceuticals, as well as to treat neurological disorders like epilepsy, hysteria, syncope, convulsions, and mental weakness. They resemble each other in their external morphological characters and characteristic odour, so their roots are often confused with each other. Since free radicals have been implicated in the pathogenesis of a considerable range of neurological disorders, including seizures and epilepsy, analysis of these two important medicinal plants was carried out based on their antioxidant activities and phenolic profiles. *N. jatamansi* expressed better antioxidant activity with both DPPH and TAC methods. Strong correlation was seen between TPC and antioxidant activities. Phenolic compounds such as chlorogenic acid, ferulic acid, protocatechuic acid, and syringic acid were analyzed qualitatively and quantitatively in the methanol extracts of *N. jatamansi* and *S. vaginatum* by HPLC. *N. jatamansi* was found to contain only protocatechuic and syringic acids while chlorogenic and ferulic acids were present only in *S. vaginatum*. The studies suggest that both of the plants exhibit distinctive properties and that their similar therapeutic uses may be dependent on synergistic effects exhibited by the different compounds present in them.

1. Introduction

Nardostachys jatamansi DC. (Valerianaceae) and *Selinum vaginatum* C.B. Clarke (Umbelliferae) are two important indigenous drugs found in Himalayan region. The roots and the rhizomes of *N. jatamansi*, as mentioned in Ayurveda, have been used in various herbal formulations including dietary supplements. This important traditional drug is also used to treat epilepsy, hysteria, syncope, convulsions, and mental weakness. The decoction of the drug is also used in neurological disorders, insomnia, and disorders of cardiovascular system. It has been reported to exhibit antidepressant, anticonvulsant and antiarrhythmic activities as well as to possess antioxidant and lipid peroxidation activities [1, 2]. The sesquiterpenes (jatamansic acid, jatamansone), lignans, and neolignans are reported to be present in the roots of this plant. *S. vaginatum* roots are also used as a nervine sedative

and considered useful in hysteria. Its oil possesses sedative, analgesic, and hypotensive properties. The roots of *S. vaginatum* have yielded several coumarins, namely selinidin, selinone, angelisin, anomalin, isopteryxin, orosenol, lomantin, and vaginidin. The roots of *N. jatamansi* (commonly known as “Jatamansi”) and *S. vaginatum* (Bhootkeshi) are often confused with each other. Due to the resemblance in between external morphological characters and characteristic odour the roots of *S. vaginatum* are being used as a substitute for *N. jatamansi* in the Indian herbal drug market [3, 4].

Neuronal hyperexcitability and excessive production of free radicals have been implicated in the pathogenesis of a considerable range of neurological disorders, including seizures and epilepsy. Epilepsy is a chronic, dynamic neurological disorder associated with ongoing neuronal damage, particularly when uncontrolled. Oxidative injury may play

a role in the initiation and progression of epilepsy. The large lipid content of myelin sheaths and the high rate of brain oxidative metabolism coupled with the low antioxidant defenses make the brain highly vulnerable to free radical damage [5, 6]. Thus, it appears that free radicals may be responsible for the development of convulsions. The increased susceptibility of the brain to oxidative damage and experimental and clinical data suggests a putative role of oxidative stress in the pathophysiology of certain seizure types. The prooxidant/antioxidant balance is not only modulated by seizures per se but also by antiepileptic drugs. The ability of antioxidants for reducing the seizure manifestations and the accompanying biochemical changes (i.e., markers of oxidative stress) further supports a role of free radicals in seizures and highlights a possible role of antioxidants as adjuncts to antiepileptic drugs for better seizure control.

Since both of these plants, that is, *N. jatamansi* and *S. vaginatum*, have been used traditionally for the treatment of epilepsy, seizures, hysteria, and so forth and because it has been well established that oxidative stress may be both an important cause and a consequence of seizures, it can be assumed that the antioxidant compounds present in these species may be responsible for their therapeutic uses. Phenolic acids are powerful antioxidants and have been reported to demonstrate several biological activities including antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions [7, 8]. To the best of our knowledge there have been no reports on the phenolic antioxidant components of both these plants. The present studies were thus carried out to evaluate their total phenolic content and antioxidant activities and to identify and quantify the major phenolic constituents present in them.

2. Materials and Methods

2.1. Standards and Reagents. Chlorogenic acid, ferulic acid, protocatechuic acid, syringic acid, rutin, and quercetin were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, water, and phosphoric acid were obtained from Merck (Darmstadt, Germany). All chemicals and solvents were purchased from Merck Chemicals (Mumbai, India). Whatman (Florham Park, NJ, USA) No. 1 filter paper was used for filtration of the samples.

2.2. Sample Collection and Preparation of Extracts. The roots of *Nardostachys jatamansi* and *Selinum vaginatum* were collected from Milam region (Uttarakhand) and Rohtang (Himachal Pradesh), India, in the months of September and October 2008, respectively. They were authenticated by Dr. A. K. S. Rawat, Head of Pharmacognosy & Ethnopharmacology Division, CSIR-NBRI, by comparison with authentic herbarium specimens and samples and deposited (Voucher specimen numbers 227331 and 227332) in the departmental herbal drug museum for future reference. A known amount of each plant material (100 g each) was kept in an oven at 40°C for drying to constant weight. Air-dried (40–50°C) roots (50 g each) were powdered and extracted with warm methanol (3 × 100 mL) by stirring on a magnetic stirrer for 30 min each

time. The extracts were combined, filtered through 45 μm filter paper, and concentrated under vacuum to obtain the dry extracts. These methanolic extracts were stored at 5°C till further use.

2.3. Determination of Total Phenolic Content. The total phenolic content (TPC) was determined using a modified colorimetric Folin-Ciocalteu method [9]. Briefly, 0.5 mL of deionized water and 0.125 mL of different extracts of known dilution (1 mg/mL) were added to reaction vial. Folin-Ciocalteu reagent (0.125 mL) was added to the solutions and allowed to react for 6 min. Then, 1.25 mL of 7% sodium carbonate solution was added to each reaction vial and the mixture was diluted to 3 mL with deionized water. The color was allowed to develop at room temperature and the final absorbance was read at 760 nm using a Thermo UV1 spectrophotometer after 90 min. A calibration curve was prepared using different concentrations of standard gallic acid solutions, each time an analysis was run. Total phenolic content in the samples was calculated from the standard calibration curve and the results were expressed as mg of gallic acid equivalents per gram (mg GAE/g) of dry extract. Each sample was measured in triplicate and the mean was taken.

2.4. DPPH Radical Scavenging Activity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of the extracts were investigated according to a previously reported method [10] with minor modifications. Briefly, to a methanolic solution of DPPH (100 mM, 2 mL), 2.0 mL of test sample dissolved in methanol was added at different concentrations (40–200 μg/mL). Equal amount of methanol was added to the control. Absorbance was recorded at 517 nm at 5, 15 and 30 min. The scavenging activity was calculated using the formula of scavenging activity (%) = [(A₅₁₇ of control – A₅₁₇ of sample)/A₅₁₇ of control] × 100. Ascorbic acid was used as a standard.

2.5. Total Antioxidant Capacity. The antioxidant capacity of the extract was measured spectrophotometrically using a phosphomolybdenum method [11], based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of specific green phosphate/Mo(V) compounds. 0.1 mL of the sample (40–300 μg) solution was combined in an eppendorf tube with 1.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 3.0 mL of reagent solution, and the appropriate volume of methanol was used for the dissolution of the samples and it was incubated under the same conditions as the rest of the samples. Ascorbic acid was used as the standard, and the total antioxidant capacity was expressed as equivalents of ascorbic acid.

2.6. HPLC Analysis. HPLC analyses were performed on a liquid chromatography system consisting of Waters (Milford, MA, USA) model 515 pumps and equipped with

TABLE 1: Total phenolic content and major phenolic acids identified in methanolic extract of *N. jatamansi* and *S. vaginatum*.

Name	TPC (gallic acid equ) mg/g	Phenolic compounds identified by HPLC (mg/100 g extract)			
		Protocatechuic acid (1)	Chlorogenic acid (2)	Syringic acid (3)	Ferulic acid (4)
<i>N. jatamansi</i>	39.544 ± 2.16	596.5 ± 1.61	—	68.5 ± 0.22	—
<i>S. vaginatum</i>	22.744 ± 1.23	—	1054.3 ± 2.86	—	61.9 ± 1.08

Values are mean ± SD ($n = 3$).

TABLE 2: Antioxidant effect on free DPPH radicals and total antioxidant capacity.

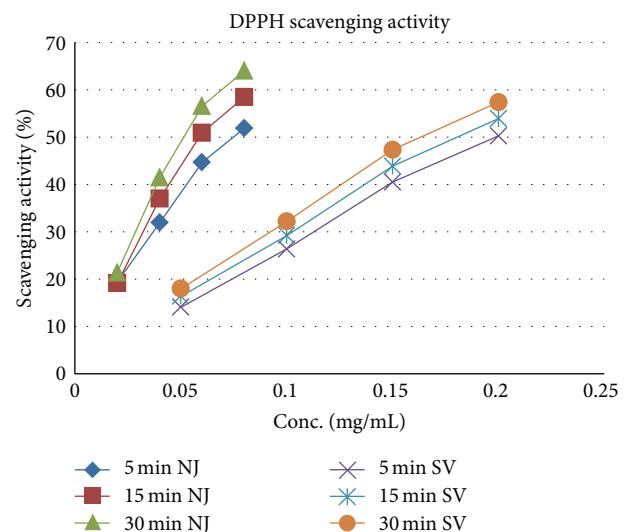
Plant name	DPPH radical scavenging ability (IC_{50} , $\mu\text{g/mL}$)	Total antioxidant capacity ($\mu\text{mol AA/g extract}$)
<i>N. jatamansi</i>	50	151
<i>S. vaginatum</i>	165	143
Ascorbic acid (AA)	2.3	—

an online degasser, a Waters PCM (Pump Control Module), a Rheodyne 7725 injection valve furnished with a $20 \mu\text{L}$ loop, a Waters 2996 photodiode array detector (PDA), and Waters Empower software. Each analysis was repeated three times, and the respective retention times were averaged. HPLC conditions: Purospherstar RP-8 column ($5 \mu\text{m}$, $4.6 \times 250 \text{ mm}$; Merck), guard column ($4.6 \times 40 \text{ mm}$) packed with the same material; solvent system: solvent A-water: phosphoric acid (99.7 : 0.3 v/v), solvent B-acetonitrile : water : phosphoric acid (79.7 : 20 : 0.3 v/v); gradient 0–5 min with 88–85% A, 5–6 min with 85–82% A, 6–9.5 min with 82–75% A, 9.5–10.5 min with 75–74% A, 10.5–12 min with 74–73% A and 12–20 min with 73–70% A, 20–30 min with 70–30% A, and isocratic from 30 to 35 min with 30% A; flow rate: 0.8 mL/min ; column temperature: 30°C ; injection volume: $10 \mu\text{L}$; standard concentration: 0.1 mg/mL ; sample concentration: 50 mg/mL ; PDA detection: 280 nm , spectra 200–600 nm.

3. Results

3.1. Determination of Total Phenolic Content. The methanolic extracts of *N. jatamansi* and *S. vaginatum* showed significant amount of phenolic content. The methanolic extracts of the roots of *N. jatamansi* contained $39.54 \text{ mg GAE g}^{-1}$ which was nearly 1.7 times the TPC of the methanolic extract of the roots of *S. vaginatum* ($22.74 \text{ mg GAE g}^{-1}$) (Table 1).

3.2. DPPH Radical Scavenging Activity. The DPPH radical scavenging activities of the methanolic extracts of *N. jatamansi* and *S. vaginatum* are given in Table 2. DPPH radicals react with suitable reducing agents losing color stoichiometrically with the number of electrons consumed which is measured spectrophotometrically at 517 nm . As shown in Figure 1, *N. jatamansi* extract strongly scavenged DPPH radicals with the IC_{50} being $52 \mu\text{g/mL}$ as compared to $165 \mu\text{g/mL}$ for *S. vaginatum*. The scavenging was found to be concentration dependent ($R^2 > 0.96$) and time dependent ($R^2 > 0.97$) (Figure 1) with the highest activity being

FIGURE 1: DPPH radical scavenging activity of *N. Jatamansi* and *S. vaginatum*.

observed at 30 mins, where at a concentration of $80 \mu\text{g/mL}$ *N. jatamansi* showed 64.1% activity as compared to 57.48% exhibited by *S. vaginatum* at a concentration of $200 \mu\text{g/mL}$. Standard ascorbic acid was found to exhibit 83.19% activity at $5 \mu\text{g/mL}$ concentration.

3.3. Total Antioxidant Capacity. The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695 nm . A direct correlation was found to exist between the concentration of the extract used and the spectrophotometrically measured phosphomolybdenum complex ($R^2 > 0.98$) (Figure 2). The total antioxidant capacity of the *N. jatamansi* and *S. vaginatum* extracts was found to be 151 and $143 \mu\text{mol ascorbic acid equivalent/g}$, respectively (Table 2).

3.4. Correlation between Antioxidant Activities and Total Phenolic Content. Correlation studies were also carried out between antioxidant activities and total phenolic content (Table 3). Based on correlation analysis, DPPH radical scavenging activities of both *N. jatamansi* and *S. vaginatum* were strongly correlated with total phenolics content of methanolic contents of the extract (*N. jatamansi*, $r = 0.9808$; *S. vaginatum*, $r = 0.9965$). Similarly the spectrophotometrically measured phosphomolybdenum complex was also strongly correlated with total phenolics content of these extracts (*N. jatamansi*, $r = 0.9909$; *S. vaginatum*, $r = 0.9897$).

TABLE 3: Correlation between bioactivities and total phenolic content.

Activities	Correlation coefficient (r)	
	<i>N. jatamansi</i>	<i>S. vaginatum</i>
DPPH radical scavenging activity (% inhibition)	0.9808	0.9965
Total antioxidant capacity		
(i) Absorption for phosphomolybdenum complex formed	0.9909	0.9897
(ii) Ascorbic acid equivalent (AAE)	0.9824	0.9897

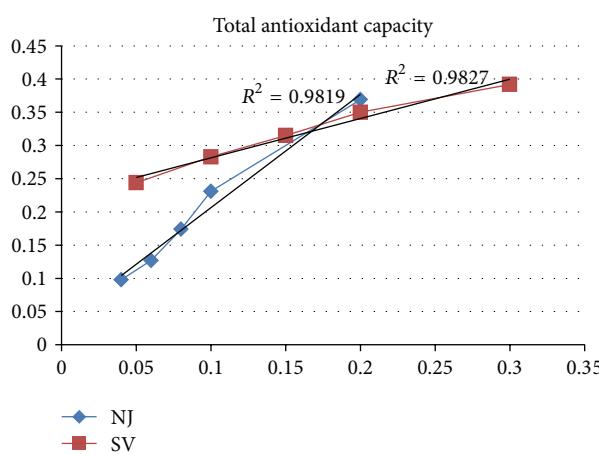
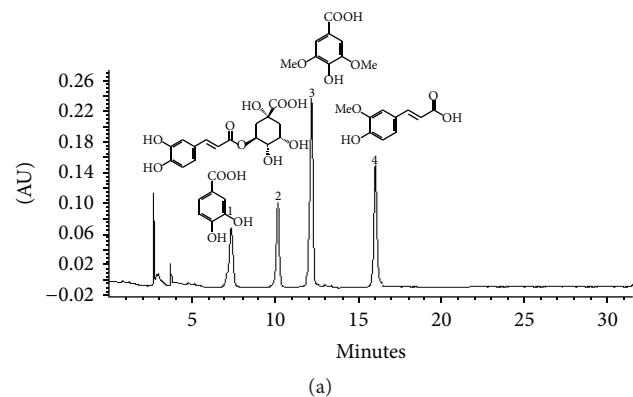
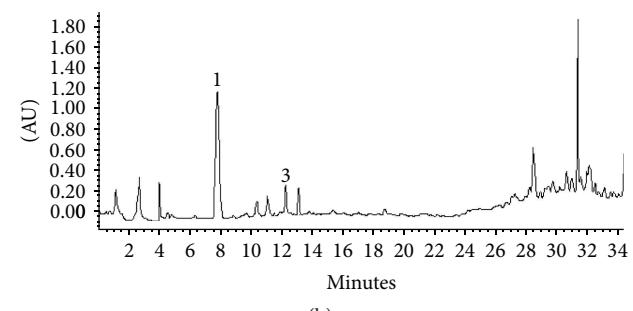


FIGURE 2: Spectrophotometrically measured phosphomolybdenum complex as a function of concentration of *N. Jatamansi* and *S. vaginatum*.

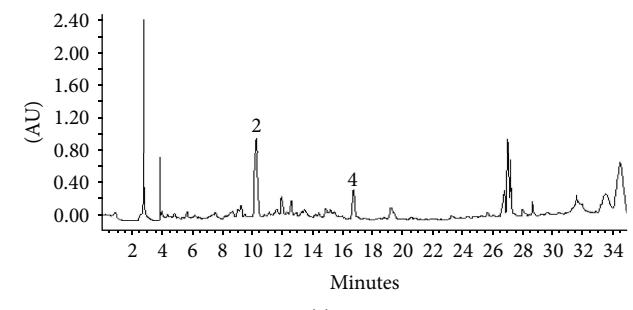
3.5. HPLC Analysis. In order to determine the phenolic acid composition of *N. jatamansi* and *S. vaginatum*, their methanolic extracts were subjected to HPLC analysis under the conditions described above. Some well-known antioxidant polyphenolics, namely, chlorogenic acid, ferulic acid, protocatechuic acid, syringic acid, rutin and quercetin, were analyzed. These compounds have been of interest due to their many potential health benefits. The HPLC studies showed that out of the compounds analysed, only the phenolic acids (chlorogenic acid, ferulic acid, protocatechuic acid, and syringic acid) were found to be present either in *N. jatamansi* or *S. vaginatum*. Rutin and quercetin were not detected. Figure 3 shows the HPLC chromatograms of the methanolic extracts of *N. jatamansi* and *S. vaginatum* monitored at 280 nm. Results of the HPLC analysis are presented in Table 1. Our observations with *N. jatamansi* and *S. vaginatum* using the four known phenolic acids indicated that there was a marked specificity to the type of phenolic acid present—*N. jatamansi* contained only benzoic acid derivatives (protocatechuic acid and syringic acid) while hydroxycinnamic acid derivatives (chlorogenic acid and ferulic acid) were present only in the methanolic extract of *S. vaginatum*. Chlorogenic acid and protocatechuic acid were present in substantial quantities in *S. vaginatum* and *N. jatamansi*, respectively.



(a)



(b)



(c)

FIGURE 3: HPLC profile of phenolic acids. (a) Standard mixture (1-protocatechuic acid, 2-chlorogenic acid, 3-syringic acid, 4-ferulic acid); (b) *N. Jatamansi* methanolic extract; (c) *S. vaginatum* methanolic extract.

4. Discussion

In the present investigation, the antioxidant properties and phenolic content of two important indigenous drugs of India, *Nardostachys jatamansi* and *Selinum vaginatum*, used in neurological disorders like epilepsy, hysteria, syncope, convulsions, and mental weakness were studied. A number of studies suggest that oxidative stress plays an important role in the etiology of epilepsy and other neurological disorders. Studies demonstrate that prolonged seizures acutely result in oxidative damage to lipids, DNA, and susceptible proteins. Such mechanisms (e.g., oxidative stress) could independently contribute to the disease progression in addition to serving as processes that underlie neuronal injury. It has been observed that compounds with antioxidant properties (superoxide dismutase (SOD) mimetics, vitamin C, spin traps, and melatonin) prevent seizure-induced pathology [12–16]. Newer

antiepileptic drugs such as zonisamide possess antioxidant properties [17]. Thus therapies aimed at reducing oxidative stress may ameliorate tissue damage and favorably alter the clinical course.

The main purpose of this study was to evaluate these two endemic high altitude Indian medicinal plants based on their phenolic profiles and antioxidant studies. Since phenolic acids are well known to be potent antioxidants, the phenolic antioxidant components present in *N. jatamansi* and *S. vaginatum* were identified. Marked difference in their phenolic profiles was observed with *N. jatamansi* containing only protocatechuic and syringic acids while chlorogenic and ferulic acids were present only in the methanolic extract of *S. vaginatum*, indicating that only benzoic acid derivatives and hydroxycinnamic acid derivatives were present in *N. jatamansi* and *S. vaginatum*, respectively. However, a more detailed study, employing many more derivatives belonging to the two groups of phenolic acids, needs to be carried out before such a conclusion is finally drawn. Secondly, both plants exhibited significant variation in their TPC and antioxidant properties. *N. jatamansi* exhibited a higher TPC as well as better antioxidant activity than *S. vaginatum*. The results demonstrated that although the roots of *N. jatamansi* (commonly known as Bhootjataa) and *S. vaginatum* (Bhootkeshi) resemble each other in their external morphological characters and characteristic odour and are often confused with each other, *S. vaginatum* roots should not be used as a substitute for *N. jatamansi*. But, since both of these plants, that is, *N. jatamansi* and *S. vaginatum*, have been used traditionally for the treatment of epilepsy, seizures, hysteria, mental weakness, and so forth and because it has been well established that oxidative stress may be both an important cause and a consequence of seizures, it can be assumed that the activity exhibited by them is dependent on synergistic effects exhibited by the different compounds present in these species that may be responsible for their therapeutic uses.

Conflict of Interests

The authors do not have any conflict of interests in this paper.

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Research Article

Apoptosis Effect of Girinimbine Isolated from *Murraya koenigii* on Lung Cancer Cells In Vitro

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Murraya koenigii Spreng has been traditionally claimed as a remedy for cancer. The current study investigated the anticancer effects of girinimbine, a carbazole alkaloid isolated from *Murraya koenigii* Spreng, on A549 lung cancer cells in relation to apoptotic mechanistic pathway. Girinimbine was isolated from *Murraya koenigii* Spreng. The antiproliferative activity was assayed using MTT and the apoptosis detection was done by annexin V and lysosomal stability assays. Multiparameter cytotoxicity assays were performed to investigate the change in mitochondrial membrane potential and cytochrome c translocation. ROS, caspase, and human apoptosis proteome profiler assays were done to investigate the apoptotic mechanism of cell death. The MTT assay revealed that the girinimbine induces cell death with an IC₅₀ of 19.01 μM. A significant induction of early phase of apoptosis was shown by annexin V and lysosomal stability assays. After 24 h treatment with 19.01 μM of girinimbine, decrease in the nuclear area and increase in mitochondrial membrane potential and plasma membrane permeability were readily visible. Moreover the translocation of cytochrome c also was observed. Girinimbine mediates its antiproliferative and apoptotic effects through up- and downregulation of apoptotic and antiapoptotic proteins. There was a significant involvement of both intrinsic and extrinsic pathways. Moreover, the upregulation of p53 as well as the cell proliferation repressor proteins, p27 and p21, and the significant role of insulin/IGF-1 signaling were also identified. Moreover the caspases 3 and 8 were found to be significantly activated. Our results taken together indicated that girinimbine may be a potential agent for anticancer drug development.

1. Introduction

Lung cancer is one of the leading causes of cancer related deaths worldwide, which has a high incidence of recurrence. It has been estimated that approximately 1.4 million are diagnosed every year and more than 1 million people die annually, 12% of which are new cases [1]. Nonsmall cell lung cancer (NSCLC) constitutes majority of lung cancers, which comprises more than 80% of total diagnoses [2]. Although

chemotherapy and radiation therapy are available to treat NSCLC, they are largely ineffective and highly toxic with a low survival profile [3]. This toxicity and resistance to the current chemotherapy made researchers focus on new drug candidates, targeting apoptosis, a programmed cell death, as physiological process that provides an effective, noninflammatory way to remove redundant or damaged cells from tissues thereby securing tissue homeostasis [4]. A multitude of signals activated by variable triggers, such

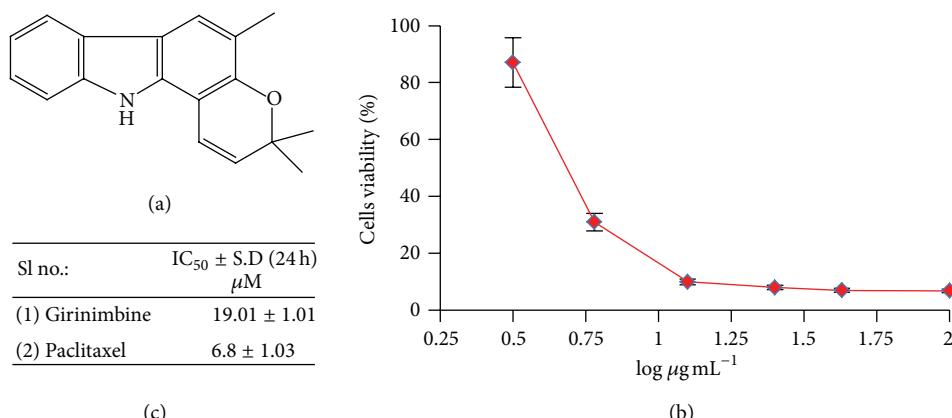


FIGURE 1: Effects of girinimbine on cell viability in A549 cells. (a) The chemical structures of girinimbine. (b) The cell viability of cells after 24 h of girinimbine treatment. Each point is the mean \pm SD of three independent experiments. (c) IC_{50} values of both girinimbine and paclitaxel (positive control).

as growth factors, cell-cell interactions, changing nutrient conditions, hypoxic conditions, and cytotoxic damage, affect the status of the apoptotic machinery [5], and it is an effective means of treating cancer, including NSCLC [6]. The literature on drug discovery published to date reveals that the knowledge of ethnopharmacology and traditional medicine has contributed a lot to development of novel clinical agents.

“Ayurveda,” an Indian system of medicine, is gaining greater attention and reputation in many regions of the world due to increased acceptance to the scientific evidence base and the essence of vigorous research. Depending on the mode of action, the Ayurvedic pharmacology system classifies medicinal plants into different groups. As per Ayurveda, plants identified as “Rasayanas” have various pharmacological properties such as being immunostimulant, anticancer, tonic, neurostimulant, antiaging, antibacterial, antiviral, and antirheumatic [7]. The use of “Rasayana” as a body resistance enhancer has been described in detail in a distinct section of Ayurveda. *Murraya koenigii*, an aromatic small tree, belonging to the citrus family, Rutaceae, that grows widely in East Asia, is one such plant which is reported as tonic and used in various disease conditions [8]. The traditional medical literature describes its potential role as a remedy for cancer [9]. Moreover, other uses such as antioxidant properties and antidiabetic, antifungal, antibacterial, antidiarrhea, and antidiarrhea effects have also been investigated [10].

The main constituents reported from the plants are sterols, aminoacids, glycosides, proteins, and flavonoids. Apart from these, many carbazole alkaloids also have been identified. It has been reported that carbazole alkaloids present in the plant have numerous biological activities such as anti-tumor, antioxidative, and anti-inflammatory activities [9]. Ramsewak et al. (1999) had reported that carbazole alkaloids obtained from *M. koenigii* show cytotoxic capacity [11]. Even though many carbazole alkaloids have been isolated and identified from the different parts of *M. koenigii*, girinimbine was the first [12]. The effect of carbazole alkaloids in cancer has been studied in detail [13]. Recently, girinimbine and some structurally similar compounds isolated from the *M.*

koenigii plant had showed that the anticancer effect of this compound involves apoptosis and free radical scavenging [14, 15]. But the involvement of proteins implicated in the intrinsic and/or extrinsic pathways including other apoptosis proteins has not been studied yet. In an effort to understand the mechanism behind the anticancer traditional pharmacological claim of this plant and to know the role of these proteins in cell death, we used A549 cells, which were shown to undergo apoptosis following exposure to girinimbine.

2. Materials and Methods

2.1. Isolation of Girinimbine. The root of *Murraya koenigii* (L.) Spreng was collected from Sik, Kedah, Malaysia, in June 2006. The experimental work on plant to obtain pure girinimbine (Figure 1) and its spectroscopic data has been reported previously [16]. Briefly, the air-dried sample (525 g) was ground and further extracted with solvents using microwave-assisted extraction (45°C , 300 W for one hour). This process gave a hexane extract (22.7 g), chloroform extract (11.7 g), and methanol extract (40.7 g). Part of the hexane extract (20.7 g) was subjected to column chromatography fractionation with a combination solvent system of hexane, ethyl acetate, and methanol in increasing polarity to give 40 fractions. Fractions 9–14 which were eluted from hexane:ethyl acetate (95:5 \geq 92:8) were combined and rechromatographed over silica gel to yield a yellow solid which was then recrystallized with hexane to obtain white crystals of girinimbine (0.77 g). The melting point of girinimbine was $171\text{--}173^\circ\text{C}$ [16]. The isolated compound (girinimbine) was characterized by infrared spectroscopy, mass spectrometry, and nuclear magnetic resonance (^1H and ^{13}C NMR). The confirmation of the compound as well as the purity was again checked using HPLC and LC-MS. Further information regarding the mass spectra and purity of girinimbine can be obtained from the supplementary information provided, available online at <http://dx.doi.org/10.1155/2013/689865>.

2.2. Cell Culture. A549, a human nonsmall cell lung cancer cell line, was purchased from ATCC (Rockville, MD, USA) and cultured in RPMI-1640 supplemented with 100 μ L/mL fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were treated with the girinimbine dissolved in DMSO, while the untreated control cultures received only the vehicle (DMSO < 1%).

2.3. Cell Viability Assay. Cells were seeded in 96-well plates at a density of 1×10^4 per well and treated with various concentrations of girinimbine for 24 h. Cells were then incubated with a medium containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for another 4 h. The viable cell number was directly proportional to formazan production which then dissolved in DMSO and was measured by spectrophotometry at 563 nm in a microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland) [17].

2.4. Microscope Examination on Cellular Morphology. Apoptosis was monitored by annexin V labeling and fluorescence microscopy [18]. Treated and untreated cells were washed with PBS and then exposed with annexin V-fluorescein (BD Pharmingen, USA) for 15 mins. After a 488 nm excitation, green fluorescence was visualized and recorded at 515 nm. Phase contrast microscopic images from the same preparations were also obtained for symptoms of apoptosis such as cell shrinkage, ruffling, and blebbing of cell membrane as well as fragmentation of cells into small apoptotic bodies using a fluorescent microscope (Nikon TE 2000U fluorescence inverted microscope, Tallahassee, Florida).

2.5. Determination of Lysosomal Membrane Stability. Cells were assessed for lysosomal stability using acridine orange (AO) uptake assay. Briefly, cells were incubated with 7 μ M AO (Sigma, USA) and 12 μ M Hoechst 33342 (Invitrogen, Carlsbad, CA, USA), diluted in culture media for 15 mins at 37°C, and immediately observed and analyzed using the ArrayScan HCS system (Cellomics, PA, USA). Acridine orange is a metachromatic fluorochrome and a weak base that exhibits red fluorescence when highly concentrated in acidic lysosomes.

2.6. Measurement of Reactive Oxygen Species Generation. The production of intracellular reactive oxygen species (ROS) was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) [19]. DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, 10 mM DCFH-DA stock solution (in methanol) was diluted 500-fold in HBSS without serum or other additives to yield a 20 μ M working solution. After 24 h of exposure to girinimbine, the cells in the 96-well black plate were washed twice with HBSS and then incubated in 100 μ L working solution of DCFH-DA at 37°C for 30 mins. Fluorescence was then determined at 485 nm excitation and 520 nm emission using a fluorescence microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland).

2.7. Multiple Cytotoxicity Assay. Cellomics Multiparameter Cytotoxicity 3 Kit was used as described in detail previously [20]. This kit enables simultaneous measurements in the same cell of six independent parameters that monitor cell health, including cell loss, nuclear size and morphological changes, mitochondrial membrane potential changes, cytochrome c release, and changes in cell permeability. Plates were analyzed using the ArrayScan HCS system (Cellomics, PA, USA).

2.8. Image Acquisition and Cytometric Analysis. Plates with stained cells were analyzed using the ArrayScan HCS system (Cellomics, PA, USA). This system is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. The ArrayScan HCS system scans multiple fields in individual wells to acquire and analyze images of single cells according to defined algorithms. In each well, 1,000 cells were analyzed. Automatic focusing was performed in the nuclear channel to ensure focusing regardless of staining intensities in the other channels. Images were acquired for each fluorescence channel, using suitable filters. Images and data regarding intensity and texture of the fluorescence within each cell, as well as the average fluorescence of the cell population within the well, were stored in a Microsoft SQL database for easy retrieval. Data were captured, extracted, and analyzed with ArrayScan II Data Acquisition and Data Viewer version 3.0 (Cellomics).

2.9. Human Apoptosis Proteome Profiler Array. To investigate the pathways by which girinimbine induces apoptosis, we performed a determination of apoptosis-related proteins using the Proteome Profiler Array (RayBio Human Apoptosis Antibody Array Kit, RayBiotech, USA), according to manufacturer's instructions. In short, the cells were treated with 19 μ M girinimbine. 300 μ g proteins from each sample and were incubated with the human apoptosis array overnight. The apoptosis array data were quantified by scanning the membrane on a Biospectrum AC ChemiHR 40 (UVP, Upland, CA) and analysis of the array image file was performed using image analysis software according to the manufacturer's instruction.

2.10. Measurement of Caspases 8, 9, and 3/7 Activities. Caspases 3/7, 8, and 9 activity was measured using luminescence-based assay, Caspase-Glo 8 Assay, Caspase-Glo 9 Assay, and Caspase-Glo 3/7 Assay (Promega). 1×10^4 cells were cultured in 96-well culture plates in 50 μ L of RPMI 1640 supplemented with 10% FBS and incubated for 24 h. Cells then were treated with different concentrations of girinimbine and incubated for 24 h. At the end of incubation, 100 μ L of assay reagent was added to be incubated for 1 h at room temperature. Luminescence was measured using a microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland).

2.11. Statistical Analysis. From several independent measurements means and standard deviations were calculated. Testing for significant differences between means were carried

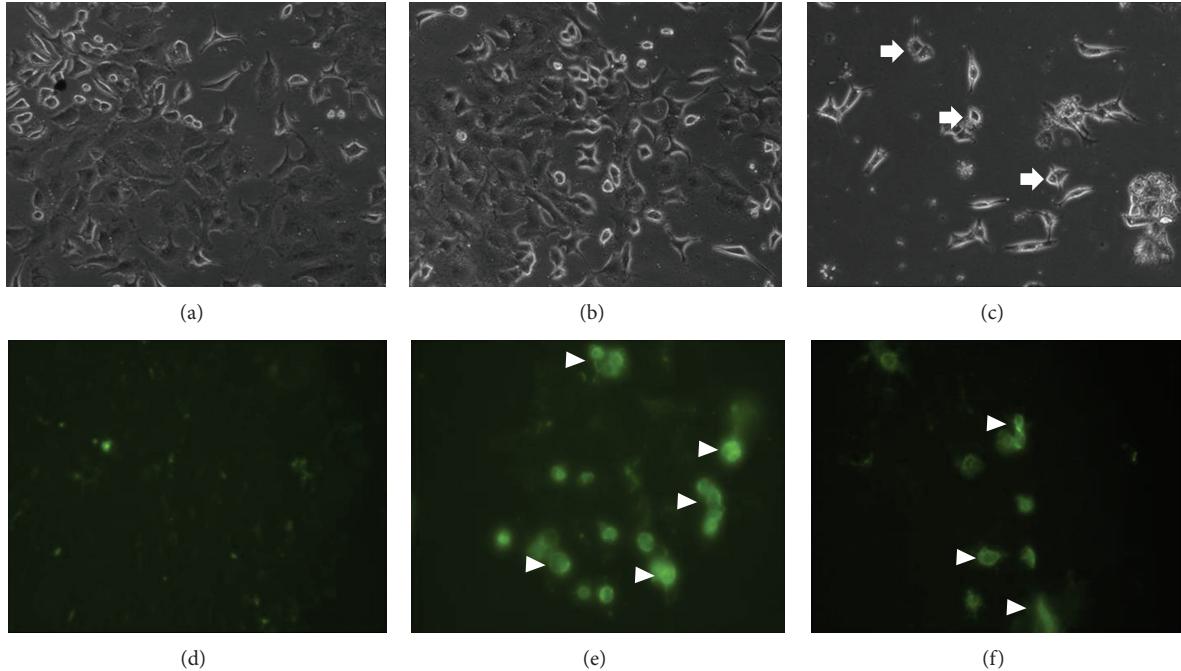


FIGURE 2: Effects of girinimbine on the morphological changes of A549 cells after 24 h treatment. Apoptosis was monitored by phase contrast ((a) control, (b) treatment 9 μ M, and (c) treatment 19 μ M) and fluorescence microscopy ((d) control, (e) treatment 9 μ M, and (f) treatment 19 μ M). Arrows indicate the cell shrinkage, ruffling, and blebbing of cell membrane while arrowheads indicate the annexin V binding to phosphatidylserine. Magnification 20X.

out using the one-way ANOVA and Dunnett's post-test at probabilities of error of 5% and 1%.

3. Results

3.1. Antiproliferative Activity. The sigmoidal dose response curves of girinimbine in the end-point assays are shown in Figure 1. Cell viability was analyzed using the MTT assay, which measures the metabolic activity of cell. In the A549 cells treated with girinimbine, metabolic activity decreased followed by 24 h treatment; meanwhile in the control plate, cell viability and metabolism were not affected. Figure 1(c) summarizes the IC₅₀ values from MTT. Meanwhile, even at 380 μ M girinimbine could not exhibit any sign of toxicity in WRL-68, a normal (data not shown) which was employed in this study to investigate the specificity of cytotoxicity.

3.2. Apoptotic Mode of Cell Death. Data obtained from fluorescence detection of A549 cells together with the phase contrast microscopic images are shown (Figure 2). Apoptosis was clearly detected by the phosphatidylserine externalization on the treated cells. Phase contrast microscopic pictures showed a clear morphological change of treated cells which were observed after 24 h of treatment with girinimbine. A549 cells were seen to have shrunk in size and there was ruffling and blebbing of cell membranes, thus suggesting that the cells were undergoing apoptosis (Figure 2(c)).

3.3. Girinimbine Initiates Lysosomal Membrane Permeabilization. Recent studies have shown that lysosomal membrane

permeabilization (LMP) is an early and perhaps initiating event in apoptosis triggered by ligation of death receptors, lysosomotropic agents, oxidative stresses, or serum withdrawal [21, 22]. To evaluate the lysosome acidification pattern, the cells were analyzed by acridine orange staining, a known pH indicator. While untreated A549 cells displayed strong granular acridine orange staining, cells treated with girinimbine presented weak lysosome staining pattern and cytoplasm acidification, indicating lysosomal membrane permeabilization (Figures 3(a)–3(c)). Girinimbine induced dose-dependent decrease of fluorescence intensity of lysosomal staining, which was well compared with chloroquine, a known inhibitor of lysosome function, was included as a positive control (Figure 3(d)).

3.4. ROS Assay. Numerous investigations have documented that oxidative stress-mediated cellular changes are frequently induced in cells exposed to cytotoxic drugs, UV, or gamma irradiation [23, 24]. We examined whether girinimbine affects the cellular levels of peroxide by measuring the changes in the fluorescence using DCF-DA. As shown in Figure 4(a), treatment with girinimbine markedly increased the DCF-DA-derived fluorescence (520 nm). This girinimbine-mediated increase in fluorescence was markedly inhibited by pretreatment with antioxidant ascorbic acid. Then, we next asked whether ROS generation induced by girinimbine is directly associated with the induction of apoptosis. Chromatin condensation was measured in the cells which were pretreated with ascorbic acid, using 12 μ M Hoechst 33342. The results shown in Figure 4(b) clearly

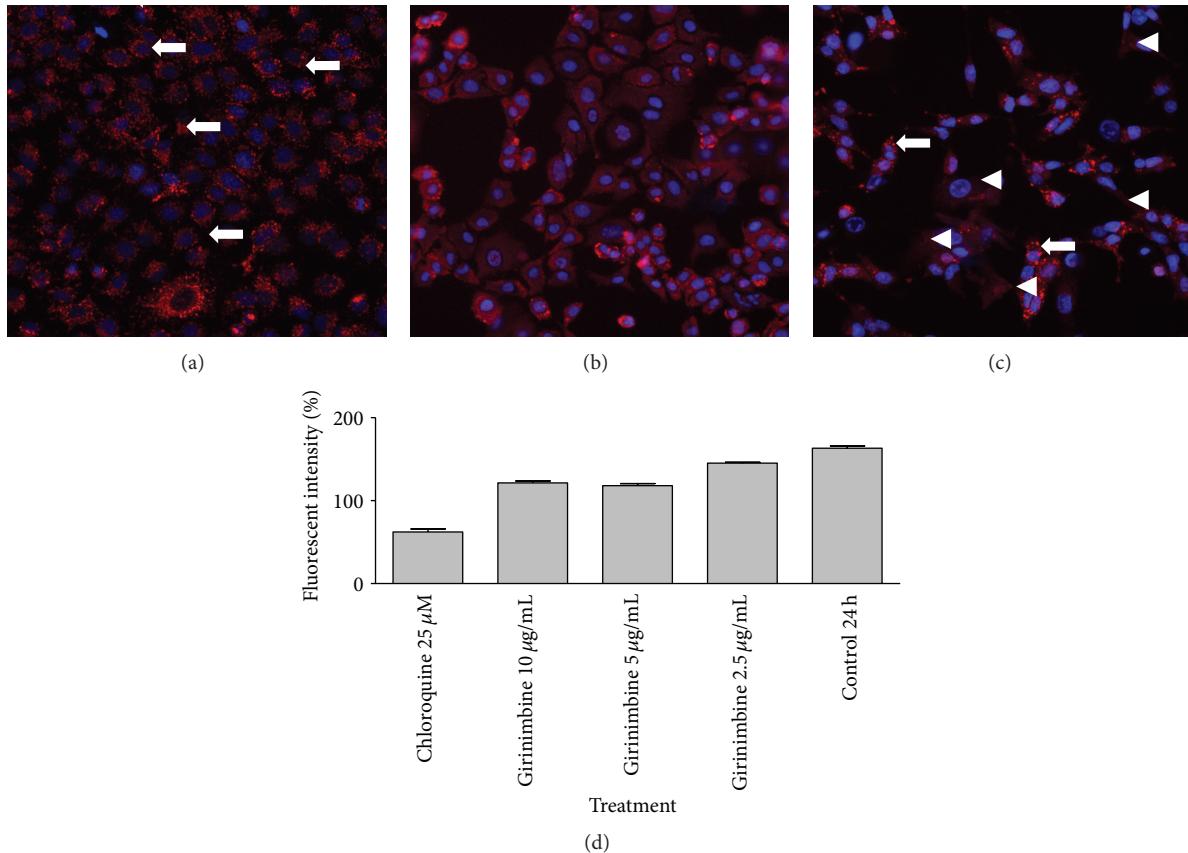


FIGURE 3: Effects of girinimbine on A549 cells lysosome acidic environment. Cells were incubated with different concentrations of girinimbine for 24 h, stained with acridine orange and analyzed by ArrayScan HCS system. Control group corresponds to untreated cells. Acridine orange is a metachromatic fluorochrome and a weak base that exhibits red fluorescence when highly concentrated in acidic lysosomes. (a) Normal lysosome acidic environment (arrow). Notice the weak lysosome staining pattern and cytoplasm acidification (arrowhead) in cells treated with chloroquine (b) and girinimbine (c), indicating lysosomal membrane permeabilization. Magnification 20X. The mean fluorescent intensity produced by the acridine orange was quantitatively measured (Figure 3(d)).

exhibited that pretreatment with ascorbic acid could not prevent the apoptosis at various time periods. Moreover, the data clearly shows the rapid decrease in the nuclear area and the increase in fragmentation, upon treatment with 19 μM girinimbine (Figure 4(c)).

3.5. Multiparameter Cytotoxicity Analysis. This assay enables simultaneous measurement of several cell-health parameters: nuclear morphology, DNA content, cell membrane permeability, and cytochrome c localization and release from mitochondria. Typical cytotoxic changes are illustrated in Figures 5 and 6. Girinimbine induced decreases in cell number; nuclear area intensity and plasma membrane permeability were significantly higher in the treated cells ($P < 0.01$) and mitochondrial membrane potential reduction was observed significantly at 19 μM ($P < 0.01$). The release of cytochrome c was observed significantly in 9 μM and 19 μM with $P < 0.05$ and $P < 0.01$, respectively. These effects occurred more rapidly and followed a dose-response pattern. Cytotoxic effects were considered to occur only when the rate of change of fluorescence was distinctly greater than for the negative controls.

3.6. Effect of Girinimbine on Apoptotic Markers. After girinimbine (19 μM) exposure, A549 cells were lysed and apoptotic markers were screened using a protein array. All major markers which are involved in both intrinsic and extrinsic pathways were induced on treatment. As shown in Figure 7, girinimbine treatment significantly increased the expression of caspase 8, suggesting the activation of the death receptor pathway. In addition, the major protein involved in the extrinsic pathway such as Fas and FasL also regulates the treatment. Moreover the involvement of mitochondria in the cell death was evident by the regulation of the Bcl-2 family of proteins such as Bad, Bax, Bcl-2, and Bim. Besides, the Bcl-2 family member Bid was found to be cleaved as well, suggesting a potential cross-talk between the death receptor and the mitochondrial pathway. The treatment also resulted in a reduction in the level of expression of the inhibitor of apoptosis XIAP as well as survivin. P53 as well as the cell proliferation repressor proteins, p27 and p21, and the heat shock proteins such as antiapoptotic HSP60, HSP70, and HSP27, which are a result of oxidative stress in the cell, were also induced. Insulin/IGF-1 signaling related protein expression has been observed on treatment. Downregulation

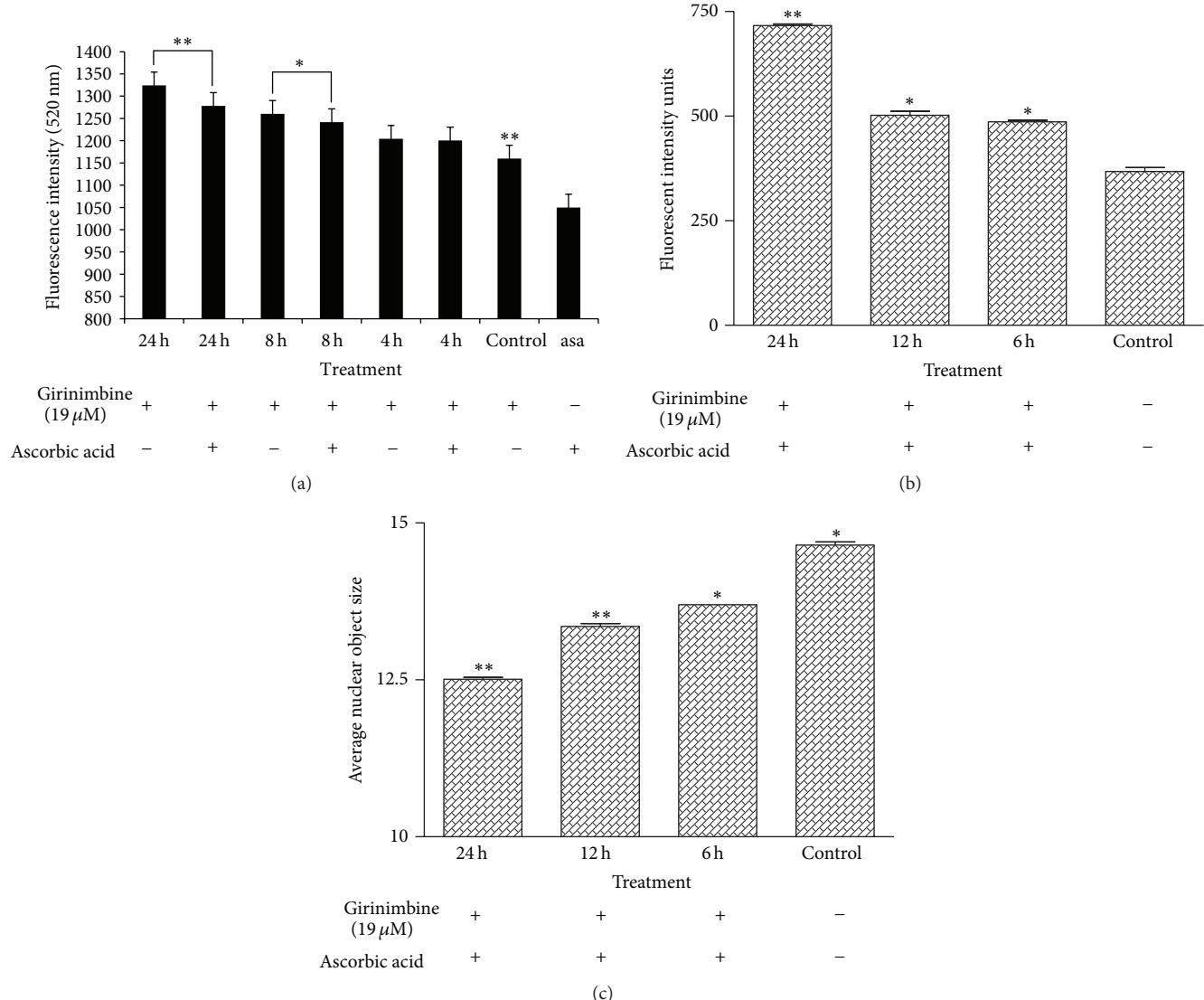


FIGURE 4: Effects of girinimbine on A549 cells ROS generation. (a) DCF-fluorescence intensity after ascorbic acid and 19 μ M of girinimbine exposure at 4, 8, and 24 h. Rate of apoptosis in terms of fluorescent intensity (b) and average nuclear object size (c) of nucleus of the girinimbine treatment was pretreated with 100 mM ascorbic acid. Values are mean \pm SD from three independent experiments. Triplicates of each treatment group were used in each independent experiment. The statistical significance is expressed as ** $P < 0.01$; * $P < 0.05$. Fluorescent intensity units directly represent the amount of ROS production.

of antiapoptosis (IGF-I, IGF-II, IGFBP1, IGFBP2) and upregulation of proapoptosis proteins (IGFBP3, IGFBP4) were observed.

3.7. Caspase Depended Apoptosis in Girinimbine Treated A549 Cells. The involvement of the caspase cascade in the girinimbine mediated cell death was confirmed. The treatment with 19 μ M was significant at $P < 0.01$ for caspase 3/7 and 9 with 8- and 5-fold increase, respectively. Meanwhile the caspase 8 showed 10-fold increase at the maximum treatment concentration (19 μ M, $P < 0.01$) (Figure 8). These results are in parallel with the protein array and indicate that the signaling cascade leading to apoptosis in

girinimbine-treated cells involves both intrinsic and extrinsic pathways.

4. Discussion

Apoptosis is a normal physiological process that plays a vital role in numerous normal functions [4]. Furthermore, it is an active physiological process causing cellular self-destruction that comprises specific morphological and biochemical changes in the nucleus and cytoplasm [25]. The involvement of an energy-dependent cascade of molecular events makes the mechanism of apoptosis very highly complex and sophisticated [26]. Apoptosis is regulated by two

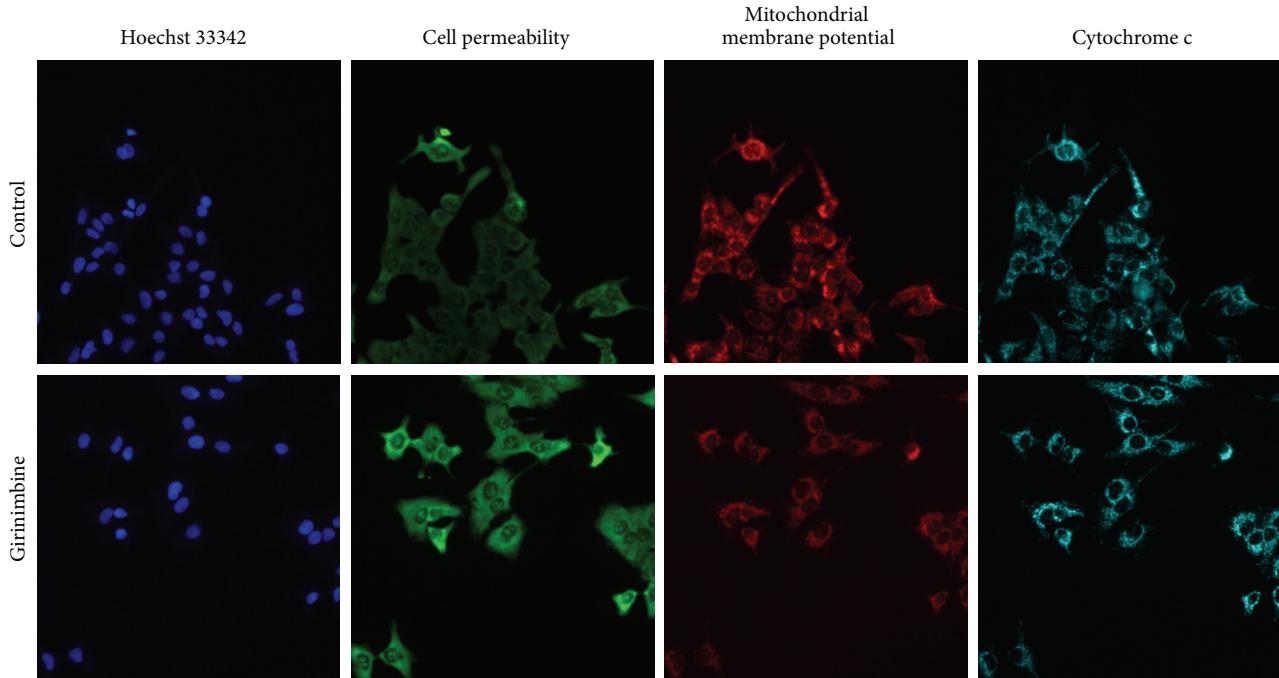


FIGURE 5: Representative images of A549 cells treated with medium alone and $19 \mu\text{M}$ of girinimbine and stained with Hoechst for nuclear, cell permeability dye, mitochondrial membrane potential dye, and cytochrome c. The images from each row are obtained from the same field of the same treatment sample. A549 produced a marked reduction in mitochondrial membrane potential and marked increases in membrane permeability and cytochrome c. Magnification 20X.

major pathways: the extrinsic and the intrinsic pathways [27]. Nevertheless, there is evidence suggesting that both the pathways are linked and that molecules involved in the pathways can influence one another [28].

The current study found that girinimbine, a carbazole alkaloid from the roots of *M. koenigii*, can inhibit cell proliferation selectively in a dose dependent manner in A549 cells. The morphological observation was conducted to explore whether the cytotoxic effect was related with the apoptotic process, and it was found that the cell death induced by girinimbine exhibited a clear morphological sign of apoptosis, as this is an important property of a candidate anticancer drug [29]. Followed by 24 h treatment, cell shrinkage, ruffling, and blebbing of cell membranes were observed. These morphological features were more evident while we analysed one of the key features of apoptosis, which is the change in plasma membrane structure by surface exposure of phosphatidylserine (PS), while the membrane integrity remains unchallenged [30]. The incorporation of annexin V in to the same treated cells showed distinctive form of binding between the externalized PS and Annexin V, while cells from test flasks showed more evidence of apoptosis than those grown under control conditions (Figure 2).

Translocation of lysosomal hydrolases from the lysosomal lumen to the rest of the cell followed by the impairment in the lysosomal membrane function is known as lysosomal membrane permeabilization (LMP) [31]. Often, these hydrolases facilitate apoptosis by inducing mitochondrial outer-membrane permeabilization and caspase activation

[32]. In our results, the main LMP consequence such as weak lysosome acidification pattern and cytoplasmic acidification was clearly evident (Figure 3). Being a weak base chloroquine, acts as lysosomotropic drug that raises intralysosomal pH. Hence we have monitored the release of liposomal content by using chloroquine as a positive control. Moreover, in the Figure 3, it is very clear that cells treated with girinimbine resented a weak lysosome acidification pattern followed by an increase in cytoplasmic acidification. Bearing in mind the significance of acidic lysosome environment for proteins and organelles degradation, it can be pointed out that; autophagy may not the main event of cell death.

Destabilization of the lysosomal membrane may be caused by increased production of ROS via massive peroxidation of membrane lipids [33]. Moreover, many studies had showed the relation between ROS and apoptosis induced in cells exposed to cytotoxic drugs [34]. Mitochondrial ROS are used as active mediators in the regulation of cell death [35]. In our study, the generation of ROS led to the upregulation of cytoprotective protein markers HSP 60, 70, and 27. Figure 4 clearly shows that girinimbine exposure leads to increase of ROS accumulation, but inhibition of ROS by ascorbic acid did not prevent girinimbine-induced apoptosis, indicating that ROS generation is not critical for the induction of apoptosis by girinimbine in A549 cells. Moreover, the treatment with girinimbine significantly decreases the cell number, nuclear area, and cell membrane permeability as shown by the multiparameter apoptosis analysis. In addition, the complex role of mitochondria in A549 cell apoptosis was investigated.

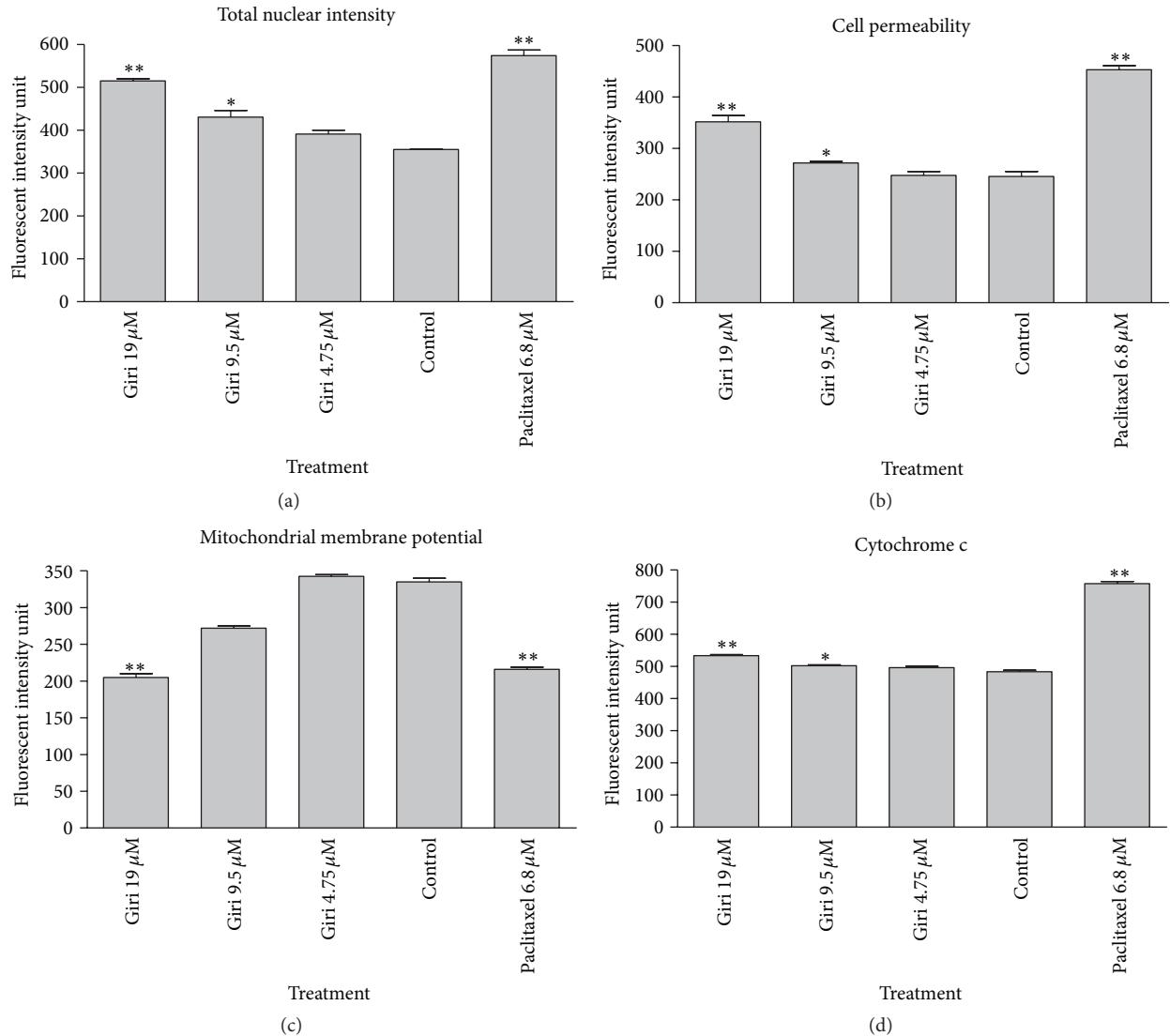
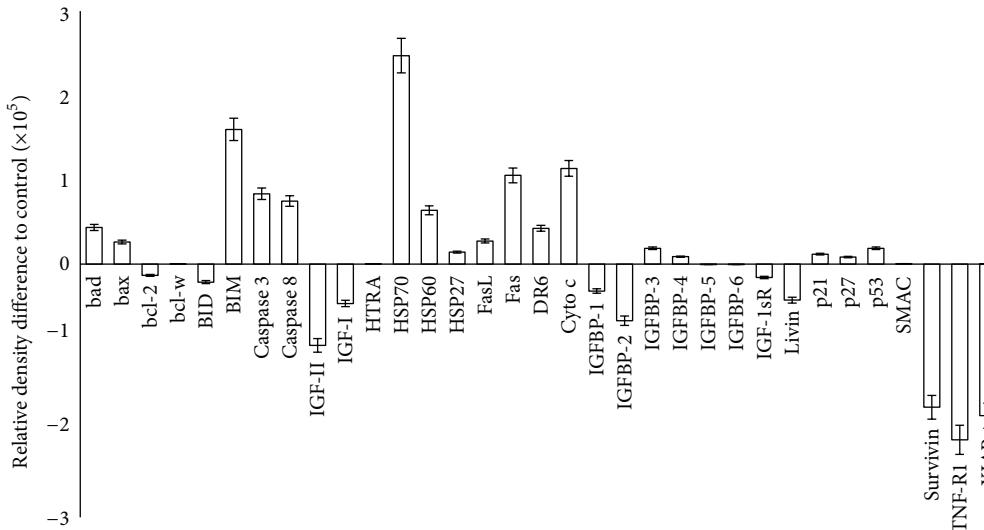


FIGURE 6: Quantitative analysis of girinimbine mediated apoptosis parameter. Changes in total nuclear intensity (a), cell permeability (b), mitochondrial membrane potential (c), and cytochrome c localization (d) were measured simultaneously in A549 cells. Following treatment with girinimbine, we saw statistically significant increase in total nuclear intensity, increased cell permeability, loss of mitochondrial membrane potential, and cytochrome c release from mitochondria. Each experiment was performed at least two times. Results are expressed as the means \pm SD. Statistical analysis was performed with one-way analysis of variance (ANOVA) using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA). Statistical significance is expressed as ** $P < 0.01$; * $P < 0.05$.

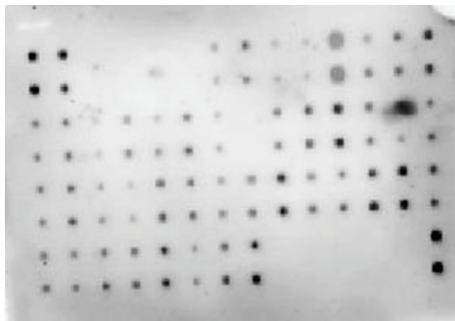
Mitochondria have been described to play a central role in the apoptotic process due to the mitochondrial proteins ability to activate cellular apoptotic programs directly [36]. Detection of changes in mitochondrial membrane potential (Ψ_m) was carried out as it is assumed that its disruption is the onset of mitochondrial transition pores (MPTP) formation. The present study revealed that girinimbine may act on mitochondria, causing loss of Ψ_m and subsequent apoptosis. This kind of Ψ_m reduction and generation of MPTP must facilitate the relocalization of apoptogenic proteins from one subcellular compartment such as mitochondria to cytoplasm to gain access to their substrates or interacting partners [37].

The multi cytotoxicity assay shows that girinimbine induced cell death could be through the classical mitochondrial

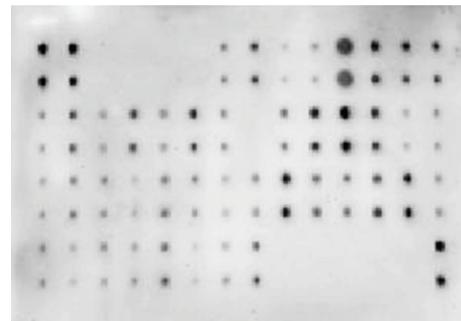
pathway with cytochrome c release and caspase-dependent apoptosis. The translocation of cytochrome c, a component of the mitochondrial electron transfer chain, was significantly increased upon girinimbine treatment. In the mitochondria mediated apoptosis, cytochrome c in turn binds to Apaf-1 to form a complex, which triggers its oligomerization to form the apoptosome. The caspase-9 holoenzyme is then formed due to the binding of procaspase-9 to the apoptosome and sequentially cleaves and activates the downstream caspases, such as caspase-3 [38]. Our findings on the upregulation of caspase 3/7, caspase 9, reduction in Ψ_m , and release of cytochrome c to the cytosol substantiate this theory and strongly support the involvement of mitochondria in the apoptosis induced by girinimbine in A549 cells.



(a)



(b)



(c)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase 3	Caspase 8
2	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase 3	Caspase 8
3	CD40	CD40L	cIAP-2	Cyto c	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	Cyto c	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP1	IGFBP2	IGFBP3	IGFBP4	IGFBP5	IGFBP6	IGF-1sR	Livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
6	IGFBP1	IGFBP2	IGFBP3	IGFBP4	IGFBP5	IGFBP6	IGF-1sR	Livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
7	sTNF-R2	TNF- α	TNF- β	TRAIL-1	TRAIL-2	TRAIL-3	TRAIL-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos
8	sTNF-R2	TNF- α	TNF- β	TRAIL-1	TRAIL-2	TRAIL-3	TRAIL-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos

(d)

FIGURE 7: Cells were lysed and protein arrays were performed. Cells was treated with $19 \mu\text{M}$ girinimbine for 24 h and the whole cell protein was extracted. Equal amount of ($300 \mu\text{g}$) of protein from each sample was used for the assay. Quantitative analysis in the arrays showed differences in the apoptotic markers (a). Representative images of the apoptotic protein array are shown for the control (b), treatment (c), and the exact protein name of each dot in the array (d).

Suppression of apoptosis may promote the cancer development by inhibiting the chemotherapy as well as other forms of inducers of cell death [39]. Regulation of apoptosis has been censoriously dependent on several genes, which have been identified earlier and includes XIAP-a member of the IAP family. It has the capacity to inhibit the activation of caspases 3, 7, and 9 [40]. In our study, we found that girinimbine treatment of A549 cells caused inhibition of XIAP, but SMAC,

an antagonist to XIAP, could be found to be slightly regulated in this treatment concentration. This is considered as a key event in the execution of cell death, which is regulated mainly by proteins of the Bcl-2 family [41, 42].

Critical issues in apoptosis include the dominance of anti-versus proapoptotic Bcl-2 members [43]. Girinimbine is found to induce apoptosis in the A549 cells, involving downregulated Bcl-2 expression. This downregulation was

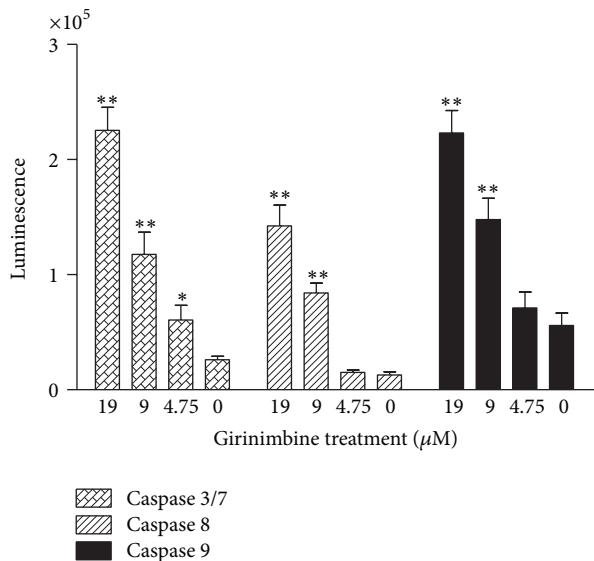


FIGURE 8: Effect of girinimbine on activation of caspases 3/7, 8, and 9. Cells were treated with girinimbine at the indicated concentrations for 24 h and activity was determined by using a luminescent enzyme assay kit. Each point represents the mean \pm SD of three different experiments. Statistical analysis was performed with one-way analysis of variance (ANOVA) using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA). Statistical significance is expressed as $^{**}P < 0.01$; $^{*}P < 0.05$.

concomitant with the upregulation of Bad, Bax, and Bim, which inhibits antiapoptotic proteins and render the cells more susceptible to apoptogenic stimuli (Figure 7). The release of cytochrome c found in our study could be due to the up regulated Bax, since Bax in association with adenine nucleotide translocator within the permeability transition pore complex will increase the mitochondrial membrane permeability, and thereby discharge a number of apoptogenic molecules into the cytosol [44]. In addition to this, upregulation of the tumor suppressor protein p53, which has a critical role in regulation of the Bcl-2, and p27, and p21, draws attention to the involvement of cell cycle arrest in the apoptosis process. Alternatively we found the involvement of extrinsic pathway proteins such as Fas and FasL upregulation, and related upregulation of caspase 8 (Figure 7). Moreover, the luminescent based caspase 8 assay had showed 10 fold differences than the control. Apart from this, we found that there is a downregulation of Bid happening too. Since mitochondrial damage in the Fas pathway of apoptosis is mediated by the caspase 8 cleavage of Bid, [45] the findings here shows the cross talk between intrinsic and extrinsic pathways.

The protein array results exhibited a significant reduction of both IGF-I and IGF-II. IGF-I has been shown to exert strong mitogenic and antiapoptotic effects in a variety of normal and cancerous cells, including lung cancer cell lines [46]. Moreover the over expression of IGFBP3 is closely associated with the downregulation of IGF-I and its mitogenic activity [47]. It is known that IGFBP-2 and IGFBP-1 are involved in the regulation of cell migration, apoptosis, and cell growth

[48]. Both are reported to be markedly overexpressed in many tumors and tumor cell lines. And it is assumed that elevation of the IGFBP-2 production is part of a mechanism to compensate for the mitogenic and antiapoptotic effects of tumor-derived IGFs [49]. Our data clearly showed the downregulation of IGFBP1 and IGFBP2 concomitant with upregulation of IGFBP3.

Our results provided a new insight into the mechanism of chemotherapeutic properties of *Murraya koenigii*. In addition, these study findings demonstrate that girinimbine mediates its apoptotic effects through both intrinsic and extrinsic pathway, which is depended on caspase mediation. Moreover, the upregulation of p53 as well as the cell proliferation repressor proteins, p27 and p21, and the significant role of insulin/IGF-1 signaling were also identified. These results indicate that girinimbine could be a candidate for a novel anticancer agent. Therefore, more in-depth *in vitro* and *in vivo* studies are currently going on in our laboratory.

Conflict of Interests

The authors declare that they do not have any conflict of interests.

Acknowledgment

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Research Article

A Phenylbutenoid Dimer, *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3'',4''-Dimethoxystyryl] Cyclohex-1-ene, Exhibits Apoptogenic Properties in T-Acute Lymphoblastic Leukemia Cells via Induction of p53-Independent Mitochondrial Signalling Pathway

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The current study was designed to evaluate the *in vitro* cytotoxicity effect of a phenylbutenoid dimer, *cis*-3-(3',4'-dimethoxyphenyl)-4-[(*E*)-3'',4''-dimethoxystyryl]cyclohex-1-ene (ZC-B11) isolated from the rhizome of *Zingiber cassumunar* on various cancer cell line, and normal human blood mononuclear cells, and to further investigate the involvement of apoptosis-related proteins that leads, to the probable pathway in which apoptosis is triggered. Cytotoxicity test using MTT assay showed selective inhibition of ZC-B11 towards T-acute lymphoblastic leukemia cells, CEMss, with an IC₅₀ value of 7.11 ± 0.240 μg/mL, which did not reveal cytotoxic effects towards normal human blood mononuclear cells (IC₅₀ > 50 μg/mL). Morphology assessments demonstrated distinctive morphological changes corresponding to a typical apoptosis. ZC-B11 also arrested cell cycle progression at S phase and causes DNA fragmentation in CEMss cells. Decline of mitochondrial membrane potential was also determined qualitatively. In the apoptosis-related protein determination, ZC-B11 was found to significantly upregulate Bax, caspase 3/7, caspase 9, cytochrome c, and SMAC and downregulate Bcl-2, HSP70, and XIAP, but did not affect caspase 8, p53, and BID. These results demonstrated for the first time the apoptogenic property of ZC-B11 on CEMss cell line, leading to the programmed cell death via intrinsic mitochondrial pathway of apoptosis induction.

1. Introduction

Plants, in particular have been used by mankind as a source of medicine since the times of yore. Evidence on plants with

healing properties exists about 5000 years ago, which had been documented since Sumerian civilization [1]. In about 3000 to 1500 years ago, curative treatments using plant-based substances had been practiced and increased widely

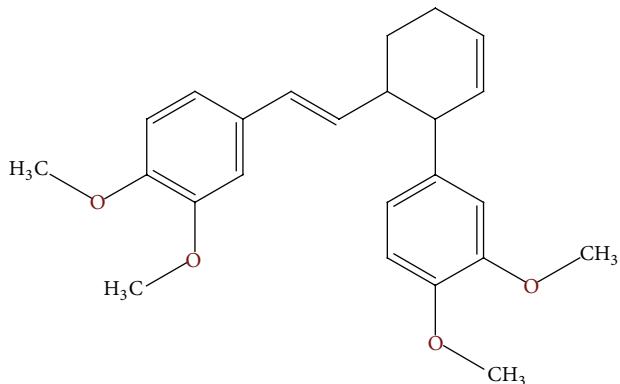


FIGURE 1: The chemical structure of ZC-B11.

in ancient Greece, followed by useful knowledge of plants having therapeutic values in China, India, and Tibet, 1000 to 2000 years ago [2]. These pieces ancient knowledge were brought down from one generation to another and with the advancement of science and technology, plant-derived drugs have made massive contributions in various clinical conditions and had provided important leads against various pharmacological targets including cancer [3].

Zingiber cassumunar (family: Zingiberaceae) is commonly known as “Plai” in Thailand and “Bonglai” in Malaysia. It has long been used in traditional medicine in Thailand, being the prime ingredient in massage oil to relieve muscle pain. In Northeast India, oral consumption of the rhizome paste of *Z. cassumunar* was reported to treat dyspepsia and stomach bloating [4, 5] whilst in Malaysia, it is used for postpartum medication.

cis-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene (ZC-B11) is a phenylbutenoid dimer isolated from the rhizomes of *Z. cassumunar* (Figure 1). To date, there have been no reported studies on ZC-B11 isolated from the rhizome of *Z. cassumunar* having antileukemic activity. Therefore, the current study was conducted to investigate the *in vitro* antileukemic properties of this compound to substantiate its anticancer activity.

2. Materials and Methods

2.1. Compound Isolation and Purification. The rhizomes of *Z. cassumunar* were collected from Jogjakarta, Indonesia, in the year 2007. Voucher specimen was deposited in Herbarium of Faculty of Pharmacy, Gajah Mada University, Jogjakarta. Briefly, the finely ground rhizomes of *Z. cassumunar* (~700 g) were soaked in petroleum ether for 72 hours at room temperature. The extraction was repeated 3 times to remove the nonpolar organic compounds, waxes, and fats. Extraction was continued with chloroform, ethyl acetate, and methanol. The solvents were removed under reduced pressure and crude extracts were obtained. Column chromatography over silica gel using a stepwise gradient elution system was utilized to fractionate the petroleum ether extract (25 g). The isolation of the crude extract yielded 64 fractions.

Fractions 12-13 showed similar pattern on TLC and were later combined. Purification of this fraction was done by column chromatography using mixture of hexane and ethyl acetate as eluent. Subfraction B11 was collected from hexane: EtOAc (8:2) and was further washed with hexane and methanol to give white solid, *cis*-3-(3',4'-dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene. The molecular weight of this compound is 380.199 g/mol with molecular formula $C_{24}H_{28}O_4$ and melting point 91°C-92°C [6]. The compound was sent for infrared (IR) and nuclear magnetic resonance (NMR) analyses at the laboratory of spectroscopic analysis, Faculty of Science, UPM. 1H NMR spectra were recorded on NMR: Bruker Avance 400 spectrometer apparatus, ^{13}C NMR spectra were reported at Ac 150 MHz instrument, and Electron Impact Mass Spectra (EI-MS) were recorded on Finnigan MAT 31 mass spectrometer with a MATSPECO data system. EI-MS analysis indicated the presence of molecular ion peak at m/z 380 which corresponded to the molecular formula of $C_{24}H_{28}O_4$. The spectral data were found to be in good agreement with the published data [6]. No previous studies have been reported on this compound except for its phytochemical structure determination and physicochemical characterization.

cis-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene: IR ν_{max} (cm^{-1} , UATR): 3017 (=C-H), 2927 (C-O), 1589 (C=C), 1509, 1458, 1233, 1138, 1019, 853, 785, 680, 614; 1H NMR (400 MHz, $CDCl_3$): δ 6.80 (1H, d, $J = 8.0$ Hz, H-5'), 6.76 (3H, br.s, H-6', H-6''', H-5'''), 6.73 (1H, s, H-2''), 6.70 (1H, s, H-2'), 6.26 (1H, d, $J = 16.0$ Hz, H-7''), 5.81 (1H, dt, $J = 10.1, 3.6$ Hz, H-1), 5.99 (1H, d, $J = 10.1$ Hz, H-2), 5.59 (1H, dd, $J = 16.0, 9.0$ Hz, H-8''), 3.86 (3H, s), 3.75 (3H, s), 3.86 (3H, s), 3.83 (3H, s), 3.51 (1H, br. s, H-3), 2.72 (1H, m, H-4), 2.22 (2H, m, H-6), 1.68 (2H, t, $J = 7.0$ Hz, H-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.9 (C-3'''-OCH₃), 148.2 (C-4'''-OCH₃), 148.0 (C-3'-OCH₃), 147.5 (C-4'-OCH₃), 133.8 (C-1'), 132.4 (C-8''), 131.0 (C-1'''), 128.0 (C-2), 128.5 (C-7''), 129.0 (C-1), 121.9 (C-6'), 118.7 (C-6'''), 113.7 (C-2'), 111.1 (C-5'''), 110.3 (C-5'), 108.9 (C-2'''), 45.8 (C-3), 42.6 (C-4), 24.8 (C-6), 24.3 (C-5); MS m/z (% intensity): 380 (M^+ , 15), 300 (2), 229 (2), 190 (100), 175 (17), 159 (80), 144 (17).

2.2. Cell Lines and Reagents. All cancer cell lines were obtained from American Type Culture Collection (ATCC). RPMI 1640 and Fetal Bovine Serum (FBS) were purchased from PAA (Germany). DMSO, penicillin, and streptomycin solution were purchased from Sigma (St. Louis, MO, USA). MTT was purchased from Amresco (USA). Quantum PBL media was purchased from PAA (Austria). Phosphate buffer saline was obtained from Invitrogen (Carlsbad, USA). All other chemicals and reagents used were of HPLC grade.

2.3. Cell Culture. Cancer cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% 100 unit/mL penicillin and 100 μ g/mL streptomycin. Cultures were maintained at 37°C in a humidified 5% CO₂ incubator. Experiments were performed at concentration of 200,000 cells/mL.

2.4. Cell Viability Assay on Cancer Cells. T-Acute lymphoblastic leukemia (CEMss), hepatocellular carcinoma (HepG2), human breast adenocarcinoma (MCF-7), human breast carcinoma (MDA-MB-231), and cervical carcinoma (HeLa) were used in this study. Cell suspension of each cell line was plated out into 96-well plates and treated with different concentrations (1.563, 3.125, 6.25, 12.5, 25, and 50 µg/mL) of ZC-BII. Control wells included vehicle-treated cells exposed to 0.1% (w/v) DMSO. After 68 h incubation, MTT (5 mg/mL) was added to each well and the plate was incubated for further 4 h. Supernatants were removed before adding 100 µL DMSO to solubilise the formazan crystals formed. Absorbance was read at wavelength of 595 nm using a microplate reader (Tecan Sunrise Basic, Groedig, Austria). Assay was performed in triplicates to calculate IC₅₀ values (concentration which inhibits 50% of cellular growth). CEMss cells were also treated with 5-fluorouracil used as positive control.

2.5. Cytotoxicity of ZC-BII on Human Blood Mononuclear Cells. The ability of ZC-BII to act selectively on cancer cells especially leukemia was evaluated by comparing the cytotoxicity of this compound towards human blood mononuclear cells. Briefly, blood was collected into the cell preparation tube containing sodium citrate (BD Vacutainer, NJ, USA). After collection, tube was stood upright for 20 min at room temperature to allow it to equilibrate and later centrifuged at 1200 × g for 20 min. Mononuclear cells and platelets underneath the plasma layer were collected using a pipette and transferred into 15 mL centrifuge tube. Cells were washed twice with PBS and cultured in complete Quantum PBL media with phytohemagglutinin (PAA, Pasching, Austria) containing 10% FBS supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂ atmosphere. Human blood mononuclear cells were treated at various concentrations of ZC-BII in triplicates and cell viability was measured using MTT assay after 72 h of incubation.

2.6. Microscopic Observation of Cellular Morphology Using Phase-Contrast-Inverted Microscopy. This investigation examines morphologically if cell death induction is implicated in ZC-BII-treated CEMss cell. CEMss cells were exposed to 7.11 µg/mL (IC₅₀) of ZC-BII for 24, 48, and 72 h. Morphological appearances of treated CEMss cells were compared with untreated control observed under normal phase contrast inverted microscope. Cells were identified as undergoing apoptosis cell death if they display condensed nuclear, fragmented nuclei, and/or blebbing.

2.7. Confocal Microscopy (Acridine Orange, AO and Propidium Iodide, and PI Double Staining). Morphological assessments of treated and untreated CEMss cells were done using a double-fluorescent dye staining method. Briefly, CEMss cells were treated with IC₅₀ concentration of ZC-BII for 24, 48, and 72 h. After the treatment period, cells were washed twice using PBS to remove the remaining media. Ten µL (10 µL) of fluorescent dyes, (AO/PI) containing AO (10 µg/mL) and PI (10 µg/mL), was added into the cellular pellet in

equal volumes. Freshly stained cell suspension was then dropped onto glass slides and covered by coverslip. Slides were observed under confocal microscope within 30 min before the fluorescence colour starts to fade. The criteria for identification are as follows: (a) green intact nucleus, viable cells; (b) dense green areas of chromatin condensation in the nucleus, early apoptosis; (c) dense orange areas of chromatin condensation, late apoptosis; and (d) orange intact nucleus, secondary necrosis [7].

2.8. Phosphatidylserine Externalisation Study. Phosphatidylserine (PS) externalisation study was done using Annexin V:FITC assay kit (AbD Serotec, USA). CEMss cells were treated with ZC-BII at IC₅₀ concentration for 24, 48, and 72 h, while untreated cells were used as negative control. After the treatment period, the supernatant was discarded and cells were washed twice using PBS. Cells were resuspended in prediluted binding buffer in 1:4 ratio (50 mL binding buffer + 150 mL distilled water); later 5 µL Annexin V:FITC was added into 195 µL of the cell suspension, mixed well, and incubated for further 10 min in the dark, at room temperature. Cells were then washed and resuspended with 190 µL of pre-diluted binding buffer followed by the addition of 10 µL of the PI solution and analysed with flow cytometer (BD FACS Canto II, USA).

2.9. Cell Cycle Distribution Analysis. A time-dependent study of cell cycle distribution of CEMss cells treated with IC₅₀ concentration ZC-BII was performed in triplicates. Untreated cells were used as negative control. After the incubation period (24, 48, and 72 h), cells were washed with PBS. To restore cell integrity, fixation of cell population for flow cytometry analysis was performed. Briefly, cell pellets were fixed with 90% cold ethanol by mixing 700 µL of 90% cold ethanol and the resulting cell suspension was kept overnight at -20°C. The cell suspension was then centrifuged at 850 rpm for 10 minutes and the supernatant containing ethanol was removed. The cell pellet was washed using 2 mL PBS and later resuspended with 600 µL of PBS + 10 mg/mL RNase + 1 mg/mL Propidium Iodide (PI). PI can bind to RNA molecule and thus, RNase enzyme was added in order to allow PI to bind directly to DNA. The cells were then incubated between 30 min to 1 h at 37°C. Finally, cell cycle kinetics was examined using flow cytometer (BD FACS Canto II, USA). Fluorescence intensity of sub-G₀/G₁ cell fraction represents apoptotic cell population.

2.10. DNA Fragmentation. DNA fragmentation was done using Suicide-Track DNA Ladder Isolation Kit (Calbiochem, Germany) according to the manufacturer's instructions. Briefly, CEMss cells were treated with ZC-BII at IC₅₀ concentration for 48 h. After treatment, DNA extraction, which involves the separation of apoptotic DNA from high molecular weight chromatin, and DNA precipitation were performed according to the manufacturer's instructions. DNA gel electrophoresis was done by preparing agarose gel (1.2%). All DNA ladder samples (21 µL each) including DNA markers (5 µL) were transferred to clean centrifuge tubes and

each sample was added with Novel Juice (GeneDirex, USA) (1-part Novel Juice with 5-part DNA sample). Novel Juice is a nonmutagenic fluorescent reagent (alternative to Ethidium Bromide) that produces instant visualization of DNA bands upon UV illumination of agarose gels. All samples were then loaded onto the gel and the gel was run at approximately 50 constant volts until the dye front is 1-2 cm from bottom of the gel. DNA was then visualized by transillumination with UV light (Biospectrum AC Chemi HR 40, UVP, Upland, CA, USA) and photographed.

2.11. Qualitative Analysis of Mitochondrial Membrane Potential. Mitochondrial membrane potential (MMP) was qualitatively analysed using Rhodamine 123 (Sigma, USA), a positive charged molecule that can accumulate in energized mitochondria, resulting in the decline of fluorescence intensity. Briefly, CEMss cells were treated with ZC-B11 compound at IC_{50} concentration for 12, 24, 48, and 72 h. Untreated cells serve as negative control. Cells in different treatment groups were adjusted to the same density, stained with 10 μ g/mL Rh123 in the dark followed by rinsing in PBS, and photographed under the fluorescent microscope (Olympus BX60F5, Japan).

2.12. Human Apoptosis Proteome Profiler Array. To determine the probable pathway of apoptosis induction mediated by ZC-B11 in CEMss cells, detection of several apoptosis-related markers was carried out using the Proteome Profiler Array (RayBio Human Apoptosis Antibody Array Kit, Raybiotech, USA) according to the manufacturer's instructions. Briefly, cells were treated with 7.11 μ g/mL of ZC-B11. Untreated cells were used as negative control. Three hundred micro gram proteins from each sample were incubated with the human apoptosis array overnight. The apoptosis array data were quantified by scanning the membrane on a Biospectrum AC ChemiHR 40 (UVP, Upland, CA, USA) and analysis of the array image was performed using image analysis software according to the manufacturer's instruction.

2.13. Bioluminescent Assay of Caspases 3/7, 8, and 9. Caspase 3/7, 8, and 9 activities of treated and untreated CEMss cells were measured using a Caspase-Glo assay kit (Promega Corp., Madison, WI, USA). Briefly, CEMss cells were seeded in a white-walled 96-well plate and treated with ZC-B11 at IC_{50} concentration for 24, 48, and 72 h. Untreated cells served as negative control. The Caspase-Glo 3/7, 8, and 9 reagents were mixed well and allowed to equilibrate at room temperature before starting the assay. The 96-well plate containing cells was removed from the incubator and allowed to equilibrate to room temperature. Then, 100 μ L of Caspase-Glo 3/7, 8, and 9 reagents were added into each well of the 96-well plate containing 100 μ L of blank (vehicle only), negative control cells or treated cells in culture medium. Contents inside the wells were gently mixed by using a plate shaker at 300–500 rpm for 30 seconds and incubated at room temperature between 30 min to 3 h. Luminescence of each sample was measured in a luminescence microplate reader (Infinite M200 PRO Tecan, Austria). Concisely, the

proluminescent substrate containing the DEVD, LEVD and LEHD (sequences are in a single-letter amino acid code) was cleaved by caspases 3/7, 8, and 9, respectively. After the caspase cleavage, a substrate for luciferase (aminoluciferin) is released, which eventually results in the luciferase reaction and the production of luminescent signal.

2.14. Western Blot. This analysis was used to investigate the expression of apoptosis-related proteins which included Bax, Bcl-2, and HSP70. CEMss cells were treated with ZC-B11 for 3, 6, 12, and 24 h. Untreated cells serve as negative control. Total proteins of cells were extracted with cell lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM PMSF), and 40 μ g of protein extract was separated by 10% SDS PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) using semidry transfer unit (Hoefer TE 70X, USA) blocked with 5% nonfat milk in TBS-Tween buffer (0.12 M Tris-base, 1.5 M NaCl, 0.1% Tween20) for 1 h at room temperature. The PVDF membrane was then incubated with appropriate primary antibody overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. The bound secondary antibody was detected using peroxidase-conjugated antirabbit antibody (1:10000) or antimouse antibody (1:10000), followed by its detection using colorimetric method. The following primary antibodies β -actin (1:10000), Bcl-2 (1:1000), Bax (1:1000), and HSP70 (1:1000) were purchased from Santa Cruz Biotechnology, Inc, CA, USA.

2.15. Statistical Analysis. Data were expressed as mean \pm SD. Statistical analysis was performed using Student's *t*-test where $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. ZC-B11 Showed Potent Antiproliferative Effect on CEMss Cells but Does Not Inhibit Human Blood Mononuclear Cells. ZC-B11 was found to exert the most potent antiproliferative effect towards CEMss cells with IC_{50} value of $7.11 \pm 0.24 \mu\text{g}/\text{mL}$ followed by HepG2, MCF-7, MDA-MB-231, and HeLa cells with IC_{50} values of $17.65 \pm 0.32 \mu\text{g}/\text{mL}$, $21.28 \pm 0.25 \mu\text{g}/\text{mL}$, $32.38 \pm 0.41 \mu\text{g}/\text{mL}$, and $>50 \mu\text{g}/\text{mL}$, respectively, after 72 h incubation (Table 1). The antiproliferative activities of ZC-B11 on CEMss, HepG2, MCF-7, and MDA-MB-231 cell lines exhibited IC_{50} values below 30 $\mu\text{g}/\text{mL}$. However, the lowest IC_{50} value of ZC-B11 on CEMss suggested preliminarily that ZC-B11 possesses high anticancer activity, which was to be suggested being useful against T-acute lymphoblastic leukemia. Thus, further experiments throughout this study were conducted using this cell line.

The crucial objective of expanding molecularly targeted drugs is to improve the efficacy and selectivity of cancer treatment by exploiting the differences between cancer cells and normal cells [8]. Hence, the ability of ZC-B11 to act selectively on cancer cells especially leukemia was evaluated by comparing the cytotoxicity of this compound on human blood mononuclear cells. ZC-B11 did not produce

TABLE 1: Effect of ZC-B11 on different cell types and the effect of 5-fluorouracil on CEMss cell line expressed as IC₅₀ values in MTT assay after 72 hours. ZC-B11 potently inhibits the growth of T-acute lymphoblastic leukemic cells.

Cell line	Tissue of human origin	Compound IC ₅₀ ($\mu\text{g}/\text{mL}$)	
		ZC-B11	5-Fluorouracil
CEMss	T-Acute lymphoblastic leukemia	7.11 ± 0.240	1.54 ± 0.035
MCF-7	Human breast adenocarcinoma	21.28 ± 0.251	—
MDA-MB-231	Human breast carcinoma	32.38 ± 0.412	—
HepG2	Hepatocellular carcinoma	17.65 ± 0.323	—
HeLa	Cervical carcinoma	>50	—
—	Human blood mononuclear cells	>50	—

TABLE 2: Flow cytometric analysis of Annexin V:FITC assay in CEMss cells treated with IC₅₀ concentration of ZC-B11 for 6, 12, 24, and 48 hours. Untreated cells serve as negative control. Data were represented as means ± SD of at least three independent experiments.

	Untreated	Number of cells (%) ± SD			
		6	12	24	48
Viable	97.30 ± 0.42	96.80 ± 0.35	95.20 ± 0.57	86.70 ± 1.06	83.20 ± 0.78
Early apoptosis	2.20 ± 0.21	3.00 ± 0.14	3.80 ± 0.42	12.80 ± 1.13*	15.60 ± 1.20*
Late apoptosis/secondary necrosis	0.50 ± 0.21	0.30 ± 0.49	0.60 ± 0.07	1.10 ± 0.21	1.20 ± 0.43

* indicates a significant difference from the control ($P < 0.05$).

any cytotoxic effect on human blood mononuclear cells up to the concentration of 50 $\mu\text{g}/\text{mL}$. 5-Fluorouracil was used as positive control and it revealed an inhibitory effect towards T-acute lymphoblastic leukemia cell line (CEMss) with an IC₅₀ value of 1.54 ± 0.035 $\mu\text{g}/\text{mL}$. In respect to this, the sensitivity of CEMss to 5-fluorouracil correlated to the MTT assay result obtained for ZC-B11.

3.2. ZC-B11 Causes Morphological Changes Related to Apoptosis. The effect of ZC-B11 on the morphology of CEMss cells was analyzed using normal phase contrast inverted microscopy at 24, 48, and 72 h incubation. Microscopic observation revealed morphological changes in CEMss cells treated with 7.11 $\mu\text{g}/\text{mL}$ of ZC-B11 in a time-dependent manner. ZC-B11-treated CEMss cells showed blebbing of the cell membrane and shrinkage of the cells. These apoptotic effects were found to be in a time dependent manner, which correlate well to the phenomenon of cell-death induction, considering that the number of blebs formation (cytoplasmic protrusion) increases as apoptosis progresses. After 24 h treatment, some cells remained healthy while some cells exhibited cytoplasmic protrusions (Figure 2(b)). The morphological changes were distinctively clear in treated CEMss cells after 48 and 72 h treatment with features of prominent growth inhibition, increased blebbing of the cell membrane, and shrinkage of cells (Figures 2(c) and 2(d)). In contrast, untreated cells showed typical nonadherent cell morphology and remained healthy and confluent throughout the treatment period (Figure 2(a)). In confocal microscopy aided with acridine orange and propidium iodide double staining, early apoptosis features such as blebbing and chromatin condensation were seen obviously in treated CEMss cells while untreated cells showed even distribution of the acridine orange stain as green intact nucleus (denotes healthy cells) with well-preserved morphology (Figure 3(a)). After 24 h exposure to ZC-B11,

blebbing of the cell membrane and dense green nucleus which indicate nuclear chromatin condensation were noticeable (Figure 3(b)). The apoptotic characteristics of CEMss became more apparent at 48 h of treatment (Figure 3(c)) and prominent at 72 h (Figure 3(d)) where most of the cells exhibited dense green nucleus and blebbing compared to untreated and 24 h treatment. Both early apoptosis features (blebbing and chromatin condensation) and late phases of apoptosis, which specify presence of intense reddish-orange colour due to acridine orange binding to denatured DNA, were observed after 48 and 72 h treatment. Apoptotic cells undergoing secondary necrosis were also detected after 72 h of treatment. This provides qualitative evidence to proof that ZC-B11 induces apoptosis in treated CEMss cells. Acridine Orange (AO) and Propidium Iodide (PI) are intercalating nucleic acid-specific fluorochromes, which emit green and orange fluorescences, respectively, when bound to DNA. Only AO can cross the plasma membrane of viable and early apoptosis cells. This criterion of cell morphology identification according to the fluorescence colour density to distinguish apoptosis was previously reported by Ciapetti et al. [7].

3.3. ZC-B11 Induces Phosphatidylserine Externalisation in CEMss Cells. In this current investigation, PS externalisation of CEMss cells undergoing apoptosis was identified using Annexin V-FITC assay according to the manufacturer's instructions. The Annexin V-FITC assay apparently showed induction of early apoptosis in CEMss cells treated with ZC-B11 in a time-dependent manner (Table 2). The exposure time chosen for this experiment was 6, 12, 24, and 48 h for the purpose of an accurate detection of early apoptotic cells. For untreated control, 97.3% of cells were viable (Annexin V/negative; PI/negative) 2.2% of cells were in early apoptosis stage (Annexin V/positive; PI/negative), while 0.5%

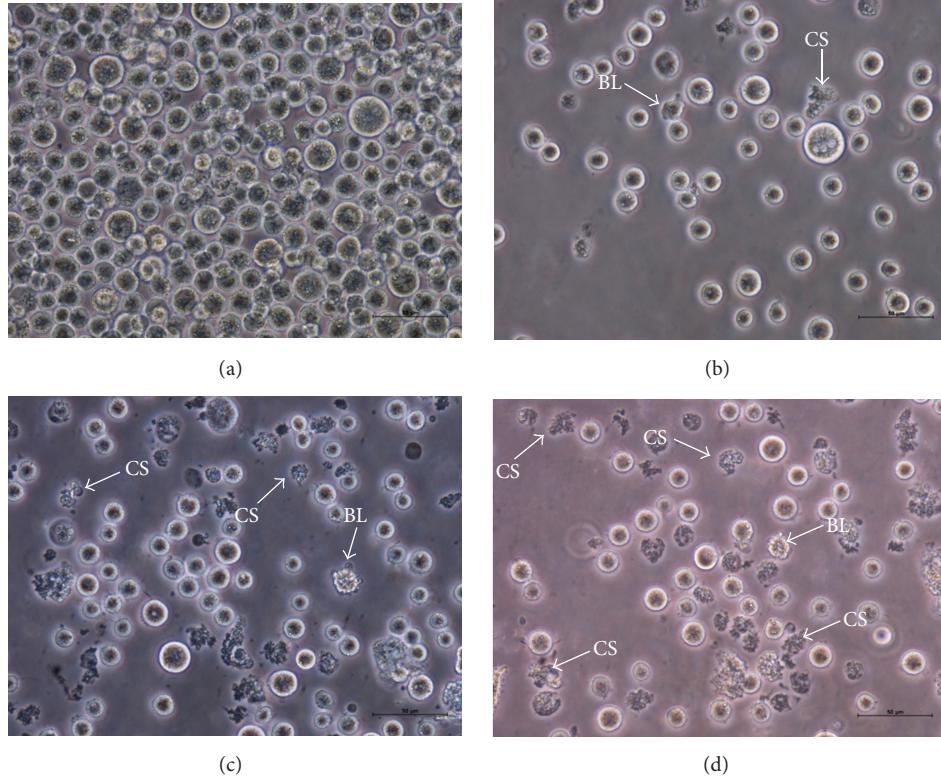


FIGURE 2: Normal phase contrast inverted micrograph of CEMss cells treated with ZC-B11 (IC_{50}) for 24, 48, and 72 h. (a) Control, (b) most of the cells exhibit normal morphology while some cells show cytoplasmic protrusions (24 h), (c) clear apoptogenic morphology such as blebbing and cell shrinkage observed (48 h), and (d) prominent growth inhibition, blebbing of the cell membrane, and shrinkage of cells observed (72 h). BL: blebbing of the cell membrane; CS: cell shrinkage (400x magnification).

were in the late apoptosis (Annexin V/positive; PI/positive) and dead stage (Annexin V/negative; PI/positive). After 6 h of treatment, the viable cells decreased to 96.8%, while early apoptosis cells were only 3%. Viable cells decreased gradually to 95.2%, 86.7%, and 83.2% after 12, 24, and 48 h of incubation, respectively. On the other hand, early apoptosis cells increased from 3% at 6 h treatment to 3.8%, 12.8%, and 15.6% at 12, 24, and 48 h respectively (Table 2). The results obtained clearly indicate that ZC-B11 is able to induce apoptosis and simultaneously exhibit clear apoptosis morphological changes attributed to the induction of apoptosis reported previously using phase contrast inverted and confocal microscopy studies.

3.4. ZC-B11 Arrests the Cell Cycle at S-Phase and Induces Apoptosis. As depicted in Figures 4 and 5, there is a significant S phase arrest in a time-dependent manner, as the number of cells increased significantly from 42.43% (untreated control) to 52.16% after 24 h of treatment, followed by 54.13% and 61.51% for 48 and 72 h of treatment, respectively. The cells in sub-G1/G0 phase also increased significantly ($P < 0.05$) from 0.01% (untreated control) to 27.16% after 48 h of treatment. These cells are considered as apoptotic cells as the “sub-G1/G0” peak in DNA histogram denotes hypodiploid DNA

content. Subsequently, the cells in the G0/G1 phase also decreased significantly from 46.78% (untreated control) to 43.25% and 32.55% after 48 and 72 h of treatment, respectively, promoting cell cycle arrest at S phase.

3.5. ZC-B11 Triggers DNA Fragmentation Which Is the Hallmark of Apoptosis. In the current study, the formation of DNA fragmentation in CEMss cells treated at IC_{50} concentration of ZC-B11 was detected on a 1.2% agarose gel electrophoresis after 48 h treatment (Figure 6). Fragmented DNA was clearly observed in treated CEMss cells, whilst the untreated control did not show evidence of ladders. Thus, it is possible that the compound, ZC-B11, triggered apoptosis in CEMss cells as the chromosomal DNA cleavage into oligonucleosomal size fragments is an integral part of apoptosis induction.

3.6. ZC-B11 Causes Decline in Mitochondrial Membrane Potential. In the current study, mitochondrial membrane potential (MMP) was assessed by the retention of Rh123, a specific fluorescent cationic dye that is readily sequestered by active mitochondria [9, 10]. Uptake of Rh123 by CEMss cells treated with ZC-B11 was observed qualitatively using fluorescent microscopy. The fluorescent intensity of the Rh123

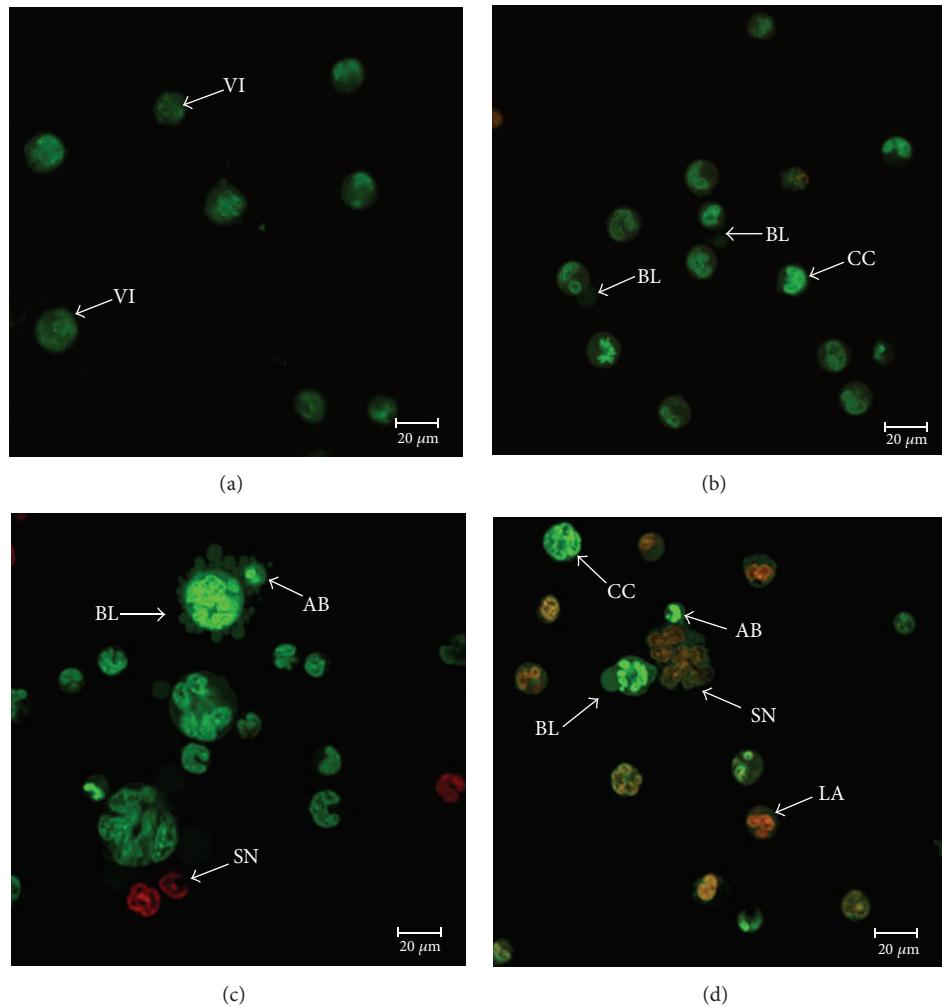


FIGURE 3: Confocal micrograph of acridine orange and propidium iodide double-stained CEMss cells after 24, 48, and 72 h treatment with ZC-B11 (IC_{50}). (a) Control, (b) cells exhibit blebbing of the cell membrane and bright green nucleus showing condensation of chromatin (24 h), (c) blebbing was observed with some orange-coloured cells which denotes late apoptosis (48 h), and (d) more blebbing and late apoptosis, orange colour represents the hallmark of late apoptosis while red color represents secondary necrosis or dead cells (72 h). VI: viable cells; BL: blebbing of the cell membrane; CC: chromatin condensation; AB: apoptotic body; SN: secondary necrosis (400x magnification).

dye decreased sequentially in a time dependent manner from 12 h to 72 h of treatment, whilst untreated cells revealed maximal dye uptake (Figures 7(a)–7(e)). The result suggested that ZC-B11 disrupts the MMP of CEMss cells after treatment.

3.7. ZC-B11 Upregulates Bax, Caspase 3, Cytochrome c, and SMAC, Downregulates Bcl-2, HSP70, and XIAP but Did Not Affect Caspase 8, p53, and BID. To further evaluate the mechanisms of apoptosis induction by ZC-B11 towards CEMss cells, screening of several proteins implicated to apoptosis induction was done using the human apoptosis proteome profiler array. Bax, caspase 3, cytochrome c, and SMAC showed significant increase ($P < 0.05$) compared to untreated control cells, whilst proteins such as Bcl-2, HSP70, and XIAP decreased significantly compared to untreated control cells (Figure 8). On the other hand, caspase 8, p53, and BID do not show significant difference from untreated control cells.

The upregulation of Bax, cytochrome c, caspase 3 and the downregulation of Bcl-2 suggest that the compound induces apoptosis in CEMss via intrinsic pathway. This is further confirmed with unchanged levels of caspase 8 and BID, which plays important role in extrinsic pathway of apoptosis. Induction of apoptosis is independent of p53 as the expression of this protein remained at basal level after treatment. Increased levels of cytochrome c and SMAC correlate well with the decline of mitochondrial membrane potential as mentioned earlier. An alteration in the permeability of mitochondrial membranes promotes translocation of the mitochondrial apoptogenic proteins which included SMAC (an inhibitor of XIAP) and cytochrome c (an activator of caspase 9) into the cytoplasm. Interestingly, there was significant decline in the level of XIAP (apoptosis inhibitor protein) suggesting possible inhibition of this protein by increased level of SMAC. Hence, the evidence gathered by the present study proposed that SMAC acts as a proapoptotic protein that

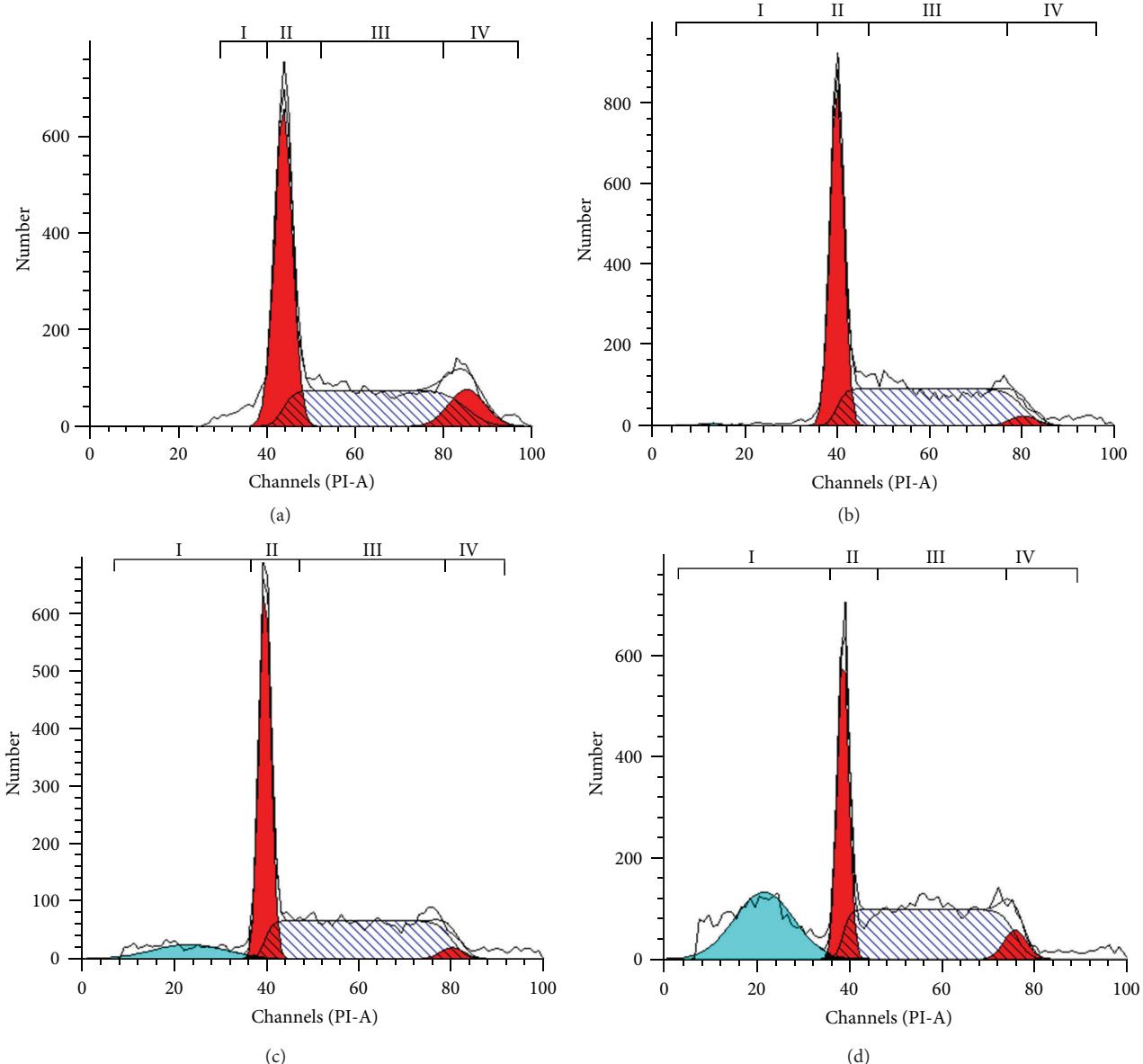


FIGURE 4: Flow cytometric analysis of cell cycle phase distribution of CEMss cells treated with ZC-B11 (IC_{50}) in a time-dependent manner. (a) Control, (b) 24 h, (c) 48 h, and (d) 72 h. Region I is “sub-G0/G1” peak denoting apoptotic cells with hypodiploid DNA content, Region II is “G0/G1” phase, Region III is S phase, and Region IV is “G2/M” phase.

binds and neutralizes the activity of XIAP. Alternatively, the level of HSP70 showed significant decrease compared to the untreated control. HSP70 is an inhibitor of apoptosis since cellular-stress response can mediate cellular protection through the expression of HSP70, which in turn can interfere with the induction of apoptotic cell death, hence resulting in tumour cells often expressing elevated levels of HSP70 [11, 12]. A previous study has found that HSP70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase3 activation [13]. Hence, in the current study, treatment of ZC-B11 towards CEMss cells decreases HSP70 protein activity, thus preventing its inhibition on cytochrome c release and caspase 3 activation. The current findings from

the apoptosis proteome profiler array suggest that ZC-B11 induces apoptosis in CEMss cells which is independent of p53 protein expression and may not involve the extrinsic pathway. Further to this, ZC-B11 may possibly activate the caspase cascades in CEMss cells, accompanied by the release of SMAC and the subsequent suppression of XIAP and HSP70 proteins.

3.8. ZC-B11 Increases the Activity of Caspases Involved in Intrinsic Pathway. CEMss cells treated with IC_{50} concentration of ZC-B11 significantly ($P < 0.05$) increased the activities of caspase 3/7 (Figures 9(a) and 9(c)) in a time

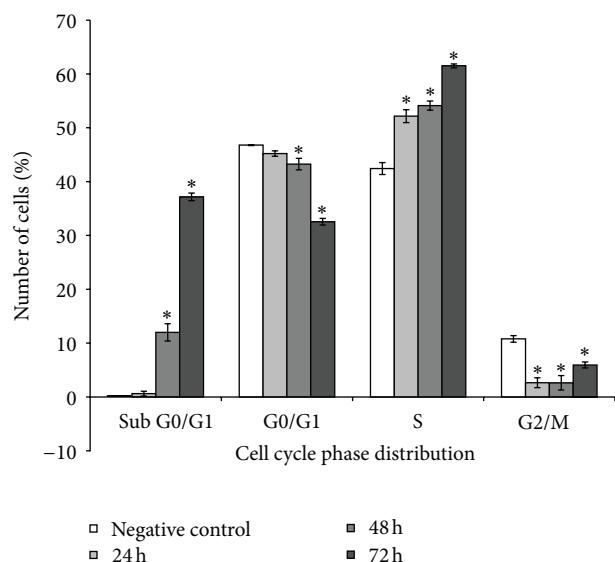


FIGURE 5: Graphical presentation of cell cycle phase distribution analysis. Induction of S phase arrest in the cell cycle progression of CEMss cells treated with ZC-B11 (IC_{50}). Results were represented as means \pm SD of three independent experiments. “*” indicates a significant difference from the control ($P < 0.05$).

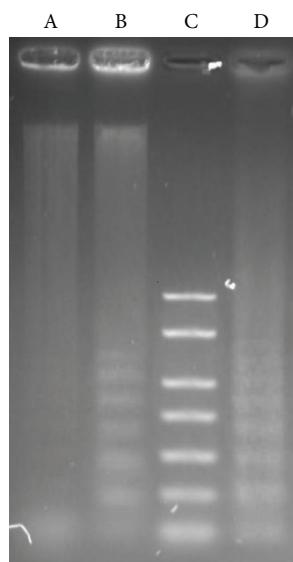


FIGURE 6: Electrophoresis separation of fragmented DNA of untreated and treated CEMss cells for 48 hours with ZC-B11 (IC_{50}). Lane A: negative control (untreated CEMss cells); Lane B: 48 hours treatment; Lane C: DNA marker; Lane D: positive control.

dependent manner whilst the activity of caspase 8 (Figure 9(b)) remained unchanged throughout the treatment period. Caspases 3/7 and 9 are caspases that are involved in the intrinsic pathway and this strongly suggests that ZC-B11 induces apoptosis in CEMss cells via intrinsic pathway. The formation of apoptosome, a catalytic multiprotein complex consisting of Apaf-1, cytochrome c, and procaspase 9 within the intrinsic apoptotic pathway, activates caspase 9 in

response to the apoptotic signals, which contributes later to the activation of caspase 3 [14, 15]. In relation to this, the current study suggests the possibility of ZC-B11 inducing the formation of apoptosome complex in treated CEMss cells, since this protein complex is implicated in activating both caspases 3 and 9 of the intrinsic pathway.

3.9. Western Blotting Confirms the upregulation of Bax and downregulation of Bcl-2, HSP70 Induced by ZC-B11. ZC-B11 increased the expression of Bax while the expression of Bcl-2 and HSP70 decreased after treatment in a time-dependent manner compared to untreated control cells. β -Actin was used as the internal control to confirm equal sample loading and protein concentration in all samples. The results obtained from the Western blot analysis confirmed that ZC-B11 induced up regulation of Bax and down regulation of Bcl-2 and HSP70 proteins in a time dependent manner (Figure 10) and these were concurrent with previous human apoptosis proteome profiler array results. As Bax and Bcl-2 are the main orchestrators of apoptosis regulation, the ability to upregulate and downregulate these proteins optimally to induce apoptosis is a crucial attribute for an anticancer agent, and ZC-B11 has demonstrated this capability when used to treat CEMss cells.

4. Discussion and Conclusion

Over the last decade, countless studies have revealed that the response to current cancer therapies crucially depends on functional cell death pathways in cancer cells. Recognition of key regulators of apoptosis in childhood cancers has provided the basis for the advancement of experimental strategies aiming at restoring intact cell death programs in cancer cells [16]. The present study elucidates the mechanism of apoptosis provoked by ZC-B11 in CEMss cells.

The antiproliferative assay used in this study is the MTT assay, an *in vitro* tetrazolium-based colorimetric assay. The method was first described by Mosmann in 1983 for detecting mammalian cell survival and proliferation [17]. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt, which is reduced to an insoluble formazan product by cleavage of the tetrazolium ring by succinate dehydrogenase enzyme within the mitochondria of metabolically active cells [18]. The amount of formazan generated is directly proportional to the viable cell number when using homogenous cell populations. This technique has since been accepted largely for its accuracy and speediness in quantifying cell survival and proliferation [19, 20]. It is well acknowledged that different cell lines demonstrate different sensitivities to cytotoxic compounds [21, 22]. The use of more than one cell line is therefore necessary in screening the antiproliferative activity of ZC-B11. The five different cancer cell lines used are from four different origins (blood, breast, liver, and cervix) that possess different morphology and tumorigenic properties [22]. The data obtained in this experiment demonstrated the antiproliferative effects of ZC-B11 on CEMss cell line selectively without affecting the normal blood mononuclear cells.

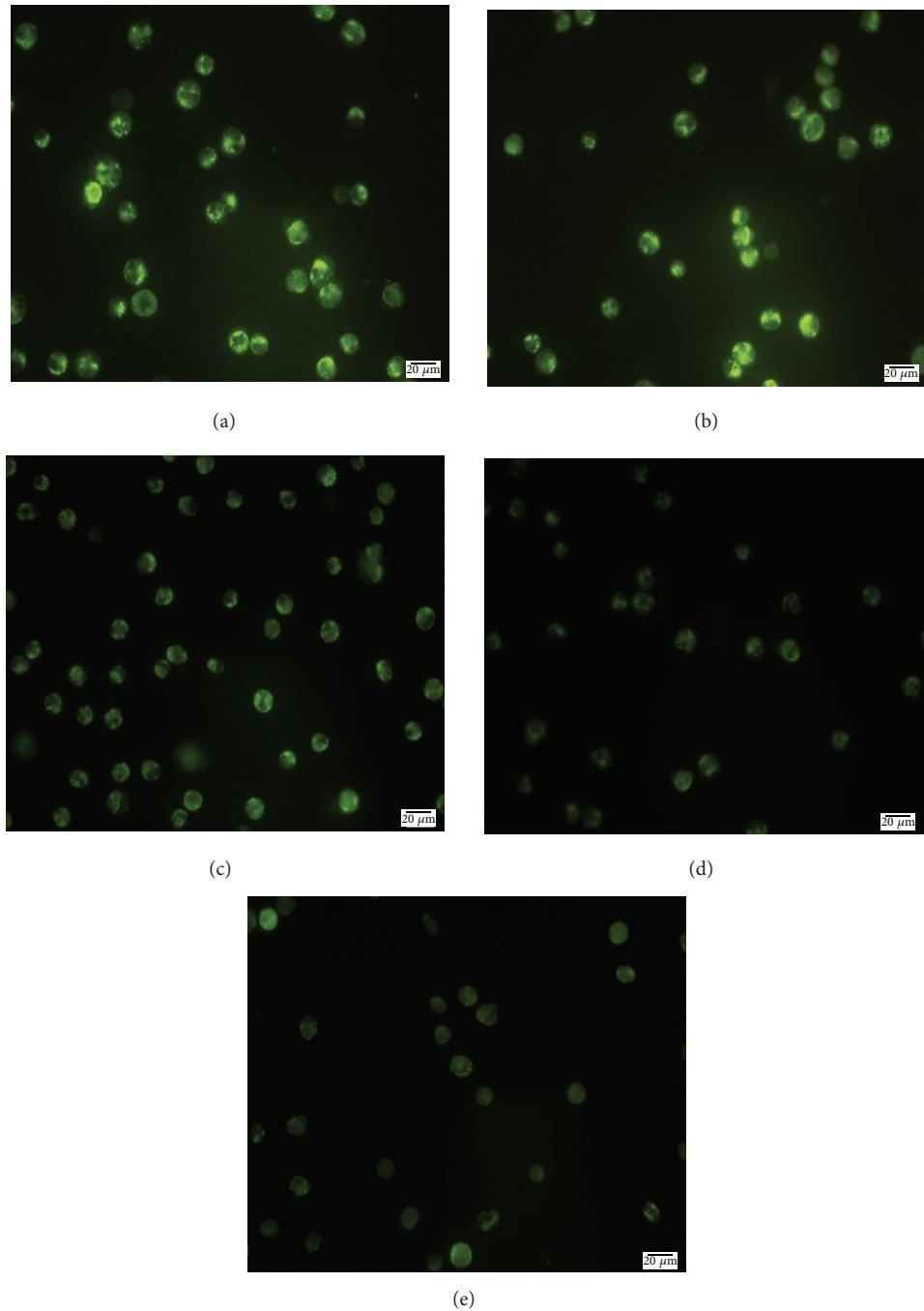


FIGURE 7: Fluorescent micrograph of CEMss cells treated with ZC-B11 (IC_{50}) for 12, 24, 48 and 72 h, stained with Rh123 dye. (a) Control, (b) 12 h, (c) 24 h, (d) 48 h, and (e) 72 h. (400x magnification).

The antileukemic activities of ZC-B11 were further established using various microscopic analyses and AO/PI staining, which showed distinctive morphological changes corresponding to typical apoptosis features such as chromatin condensation, DNA fragmentation, cell membrane blebbing, and separated apoptotic bodies. The inclusion of PS externalization study and DNA fragmentation analysis further confirmed the induction of apoptosis as the event of causing cell death towards CEMss cells. Annexin V

is a 35-kDa Ca^{2+} -binding protein initially described by Reutelingsperger et al. (1985) as a vasculature-derived protein with strong anticoagulant properties [23]. Annexin V binds preferentially to PS, which is normally absent in the outer leaflet of the plasma membrane and is only exposed on the cell surface upon induction of apoptosis. Once on the cell surface, it can be specifically detected by staining with fluorescein isothiocyanate-(FITC-) labeled annexin V (annexin V-FITC), a protein with high affinity for PS. This

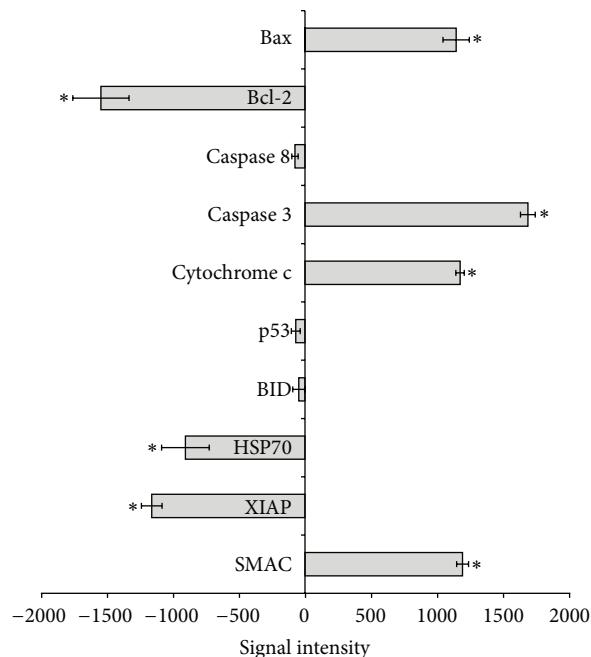


FIGURE 8: Human apoptosis proteome profiler array in CEMss cells treated with ZC-B11 (IC_{50}) for 48 hours. Graph shows the difference between treated and untreated control cells. Results were represented as means \pm SD for three independent experiments. “**” indicates a significant difference from the control ($P < 0.05$).

occurs specifically in early phases of apoptotic cell death during which the cell membrane itself remains intact [24, 25]. Fragmentation of DNA also appears to be part of an early event in apoptosis, prior to the complete digestion of DNA into multiples of nucleosomal size fragments. Demonstration of internucleosomal DNA fragmentation has been the major advance in the detection of apoptosis induction [26]. DNA fragments are detectable as a ladder pattern in gel electrophoresis of isolated DNA. Necrosis, on the other hand, is characterized by accidental DNA fragmentation which forms a “smear” on agarose gels [27].

ZC-B11 also causes DNA damage in CEMss cells by arresting the cell cycle at S phase. This is in accordance to several studies elsewhere that reported compounds isolated from natural products which arrested cell cycle at S phase similarly induce apoptosis. Triptolide, a diterpenoid obtained from *Tripterygium wilfordii* Hook. if was reported to induce S phase cell cycle arrest in human melanoma cells and apoptosis that probably is induced through the intrinsic pathway [28]. Resveratrol, a polyphenolic phytoalexin found in the skin of red grapes, various other fruits, and root extracts of the weed *Polygonum cuspidatum*, also inhibits proliferation, causes S-phase arrest, and induces apoptosis in acute myeloid leukemia and several other cancer cells [29, 30].

Mitochondria are the main producers of ATP in eukaryotic aerobic cells. In addition, mitochondria also implicated cell physiology and pathology, including involvement in ions homeostasis, regulation of the cell redox state, and transport of metabolites, including import of proteins synthesized in the cytosol, lipid and amino acid metabolism, and cell death.

These important functions are extremely dependent on the electrochemical transmembrane potential of the mitochondria [31, 32]. The disruption of mitochondrial membrane integrity is one of the early events leading towards irreversible apoptosis [33]. Mitochondrial inner membrane is negatively charged for being rich of negatively charged glycoprotein. A large migration of protons out of the inner membrane causes the transmembrane potential to decrease substantially [34]. Decline of MMP causes leakage of Rh123 from mitochondria, resulting in the decline of fluorescence intensity [35]. The rate of fluorescence decay is proportional to the decline of MMP [34]. The decline of MMP in ZC-B11-treated CEMss cells was also found to be concomitant with the upregulation of cytochrome c and SMAC, which are the key apoptotic proteins from mitochondrial intermembrane space. Therefore, one possible mechanism by which ZC-B11 induces apoptosis is through changes in the MMP, which would lead to the release of cytochrome c and SMAC from the mitochondria, leading to sequential activation of caspase 9 and caspase 3. Furthermore, regulation of several apoptosis-related proteins was also observed in the human proteome profiler array, which suggest collectively that ZC-B11 mediates cell death in CEMss via intrinsic pathway of apoptosis. These results were later confirmed by caspase bioluminescent assay and western blot analysis. HSP70 which shows decline in ZC-B11-treated CEMss cells is a protein that is abnormally expressed in different malignancies and has emerged as a promising new target for anticancer therapy [36]. The expression of p53 was found to be unaffected in ZC-B11-treated CEMss cells which suggest a probable p53-independent pathway of apoptosis. Since over 50% of human tumours contain a functionally defective p53

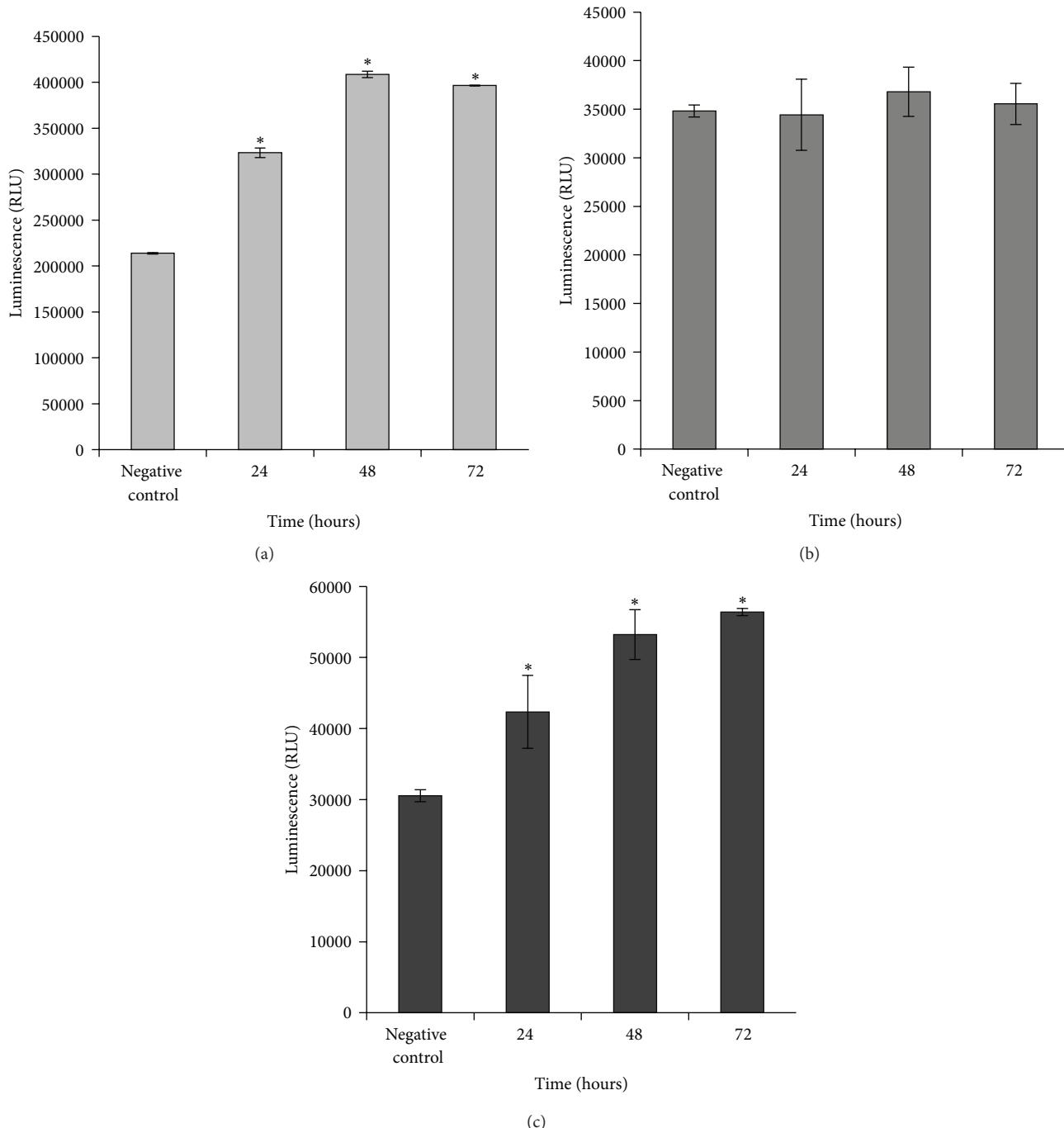


FIGURE 9: The bioluminescent assay of caspases 3/7 (a), 8 (b), and 9 (c) in CEMss cells treated with ZC-B11 (IC_{50}) after 24, 48, and 72 hours of treatment. Untreated cells serve as negative control. (a), (c) Caspase 3/7 and 9 activities increased significantly (* $P < 0.05$) compared to untreated control; (b) Caspase 8 activity remained at the basal level throughout the treatment period. Results were represented as means \pm SD for three independent experiments.

that reduces sensitivity to commonly used chemotherapeutic agents, such as etoposide and cisplatin [37], the aptitude of ZC-B11 to induce apoptosis independently of p53 may offer an advantage in anticancer therapy. Ultimately, this study has shown that ZC-B11 possesses antiproliferative properties on acute lymphoblastic leukemia and is potential to be

developed as an antileukemic and chemotherapy agent as it induces cell death via apoptosis signalling pathway.

However, *in vivo* preclinical studies and possibly further product development are needed to ascertain its ability and usefulness as a natural drug for the treatment of leukemia. Direct engagements of apoptotic pathways or combinations

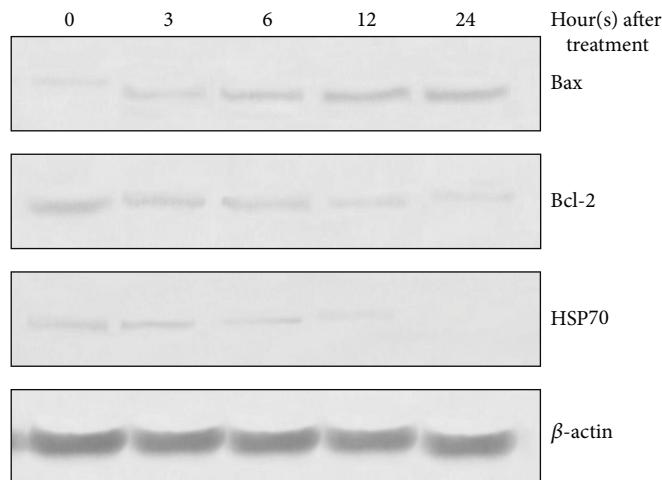


FIGURE 10: Western blot analysis of Bax, Bcl-2, and HSP70 levels in CEMss cells treated with ZC-B11 (IC_{50}) for 3, 6, 12, and 24 hours and compared with negative control (0 hours). The expression of Bax increased while Bcl-2 and HSP70 decreased after treatment in a time-dependent manner. β -actin was used as the internal control to confirm equal sample loading.

of agents that lower the threshold for apoptosis induction by conventional anticancer agents are alternatives to exploit apoptosis induction pathways for paediatric oncology such as ALL [16]. These findings will therefore help in understanding the mechanism by which apoptosis pathway is regulated in ZC-B11-treated ALL and consequently provides valuable links for developing strategies to improve the efficacy of anticancer therapy.

Abbreviations

- ALL: Acute lymphoblastic leukemia
- Apaf-1: Apoptotic protease-activating factor-1
- Bax: Bcl-2-associated X protein
- Bcl-2: B-cell lymphoma 2
- BID: BH3 interacting domain death agonist
- DMSO: Dimethyl sulfoxide
- EI-MS: Electron impact-mass spectra
- EtOAc: Ethyl acetate
- FITC: Fluorescein isothiocyanate
- HPLC: High-performance liquid chromatography
- HSP70: Heat shock protein 70
- IC_{50} : Half-maximal inhibitory concentration
- IR: Infrared
- MMP: Mitochondrial membrane potential
- MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- NMR: Nuclear magnetic resonance
- SMAC: Second mitochondria-derived activator of caspase
- XIAP: X-Linked inhibitor of apoptosis protein.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Simultaneous Determination of Crypto-Chlorogenic Acid, Isoquercetin, and Astragalin Contents in *Moringa oleifera* Leaf Extracts by TLC-Densitometric Method

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Moringa oleifera Lamarck (Moringaceae) is used as a multipurpose medicinal plant for the treatment of various diseases. Isoquercetin, astragalin, and crypto-chlorogenic acid have been previously found to be major active components in the leaves of this plant. In this study, a thin-layer-chromatography (TLC-)densitometric method was developed and validated for simultaneous quantification of these major components in the 70% ethanolic extracts of *M. oleifera* leaves collected from 12 locations. The average amounts of crypto-chlorogenic acid, isoquercetin, and astragalin were found to be 0.0473, 0.0427, and 0.0534% dry weight, respectively. The method was validated for linearity, precision, accuracy, limit of detection, limit of quantitation, and robustness. The linearity was obtained in the range of 100–500 ng/spot with a correlation coefficient (r) over 0.9961. Intraday and interday precisions demonstrated relative standard deviations of less than 5%. The accuracy of the method was confirmed by determining the recovery. The average recoveries of each component from the extracts were in the range of 98.28 to 99.65%. Additionally, the leaves from Chiang Mai province contained the highest amounts of all active components. The proposed TLC-densitometric method was simple, accurate, precise, and cost-effective for routine quality controlling of *M. oleifera* leaf extracts.

1. Introduction

Moringa oleifera Lam. (Moringaceae) is a small to medium evergreen tree widely distributed in Asia, Africa, and America. The plant is not only well known for high nutritional contents but also recognized for its therapeutic values [1]. The leaves of *M. oleifera* have been indigenously used for various medicinal purposes such as treating bronchitis, controlling glucose level, and reducing glandular swelling [1, 2]. Numerous pharmacological investigations of *M. oleifera* leaves have been reported on anti-inflammation, anti-infection, antidiabetic, antioxidant, and antihyperlipidemic activities [3–7]. Recently, isoquercetin, astragalin, and crypto-chlorogenic acid were reported to be major active components in *M. oleifera* leaves [8]. Isoquercetin is a powerful natural antioxidant which possesses several potential therapeutic effects including antiasthma and antihypertension [9–11]. Astragalin is also reported as a natural antioxidant agent exhibiting some

biological properties such as attenuation of inflammation, inhibition of dermatitis, and cellular protective effect [12–14]. Chlorogenic acid and its isomers are esters of quinic and caffeic acids that have abilities to inhibit oxidation and also promote various pharmacological activities such as antioesity, reduction of plasma and liver lipids, and inhibition of acute lung injury [15–18].

Standardization of herbal extracts is essential to ensure their quality and biological activities. Some analytical techniques including high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) were previously developed for the quantitative analysis of the *M. oleifera* leaf extract [8, 19]. However, a simple, rapid, and inexpensive method for routine analysis of major active constituents in the plant is still preferred. Thin-layer-chromatography (TLC)-densitometry is one of the suitable methods popularly used for quality control of botanical

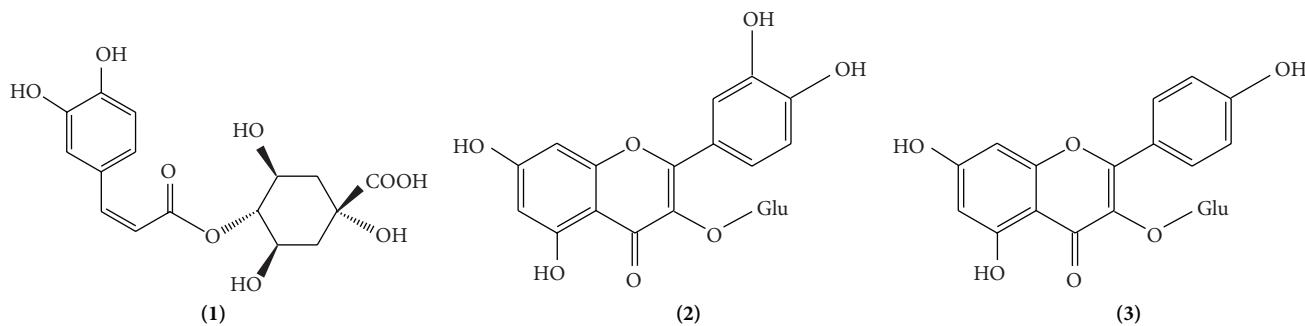


FIGURE 1: Chemical structures of crypto-chlorogenic acid (1), isoquercetin (2) and astragalin (3).

extracts because of its fast data acquisition, simplicity, and reliability [20, 21]. Moreover, there has been no report on simultaneous quantification of isoquercetin, astragalin and crypto-chlorogenic acid in *M. oleifera* leaf extracts by this method before. Thus, the objectives of this work were to develop and validate a TLC-densitometric method for quantitative analysis of these principle constituents in the extracts of *M. oleifera* leaves collected from different locations in Thailand and to find good sources of this plant's raw material for pharmaceutical and nutraceutical development.

2. Materials and Methods

2.1. General. *M. oleifera* leaves were collected during January to March 2011 from 12 different provinces in all parts of Thailand, those are, Chiang Mai, Lampang, Surin, Udonthani, Sa Kaeo, Chonburi, Ang Thong, Saraburi, Kanchanaburi, Phetchaburi, Phang Nga, and Phuket. The samples were identified by Dr. W. Gritsanapan; the voucher specimens (BVMO11001-BVMO11012) were deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Thailand. The leaves were dried in a hot air oven at 60°C for 24 hours. The dried samples were ground and passed through a sieve (20 mesh), and stored at room temperature (28 ± 2°C) protected from light till taken. All reagents and solvents were of analytical grade. TLC was carried out on precoated silica gel GF₂₅₄ sheets (Merck, Darmstadt, Germany). Pure compounds, isoquercetin, astragalin, and crypto-chlorogenic acid, isolated and identified from our previous work [8], were used as standard compounds.

2.2. Apparatus. A Linomat 5 automatic sample spotter (CAMAG, Muttenz, Switzerland) and a 100 μL syringe (Hamilton, Bonaduz, Switzerland) were used. A glass twin-trough chamber (20 × 10 × 4 cm, CAMAG), TLC scanner 3 linked to winCATS software (CAMAG), and TLC plates of 20 × 10 cm with 0.2 mm layer thickness, precoated with silica gel 60 GF₂₅₄ (Cat. No. 1.05554.0001, Merck), were used in this experiment.

2.3. Preparation of Standard and Sample Solutions. Stock solutions of isoquercetin, astragalin, and crypto-chlorogenic acid were prepared by dissolving each standard compound in 50% methanol in a volumetric flask at a concentration

of 1,000 μg/mL. The stock solution was diluted with 50% methanol to provide five working standard solutions (concentrations of 20, 40, 60, 80, and 100 μg/mL).

Each sample of *M. oleifera* powder leaves was accurately weighed (5.0 g) and extracted by maceration with 70% ethanol (1:20, w/v), which is the most suitable extraction method [22], for 72 hours at room temperature with occasional shaking. The extraction process was repeated for 5 times to provide exhaustive extraction. The extracts were combined, filtered, and dried under vacuum in a rotary evaporator. The dried extract was adjusted with 50% methanol to a 10 mL volume in a volumetric flask. The solution was filtered using a 0.45 mm nylon membrane filter before application onto the TLC plate. Each sample was prepared and analyzed in triplicate.

2.4. Validation of the Method. The analytical method was validated for linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness according to International Conference on Harmonization (ICH) guidelines [23].

2.4.1. Linearity. Linearity was determined using the standard solution in 50% methanol. Five microliters of five concentrations (20, 40, 60, 80, and 100 μg/mL) of each reference standard were individually prepared and spotted on the TLC plate to obtain the calibration range of 100–500 ng/spot. The calibration graphs were acquired by plotting the peak area versus the concentration of the standard solutions.

2.4.2. Precision. The precision was determined by analyzing 200, 300, and 400 ng/spot of each standard solution after the application by the proposed method onto a TLC plate on the same day for intraday precision and on three consecutive days for interday precision. The precision was expressed as percent relative standard deviation (RSD).

2.4.3. Accuracy. The accuracy of the analyzing method was calculated by performing recovery studies of three levels of each standard (isoquercetin, astragalin, and crypto-chlorogenic acid) added to the sample solution. The solutions were applied onto a TLC plate and analyzed by the proposed

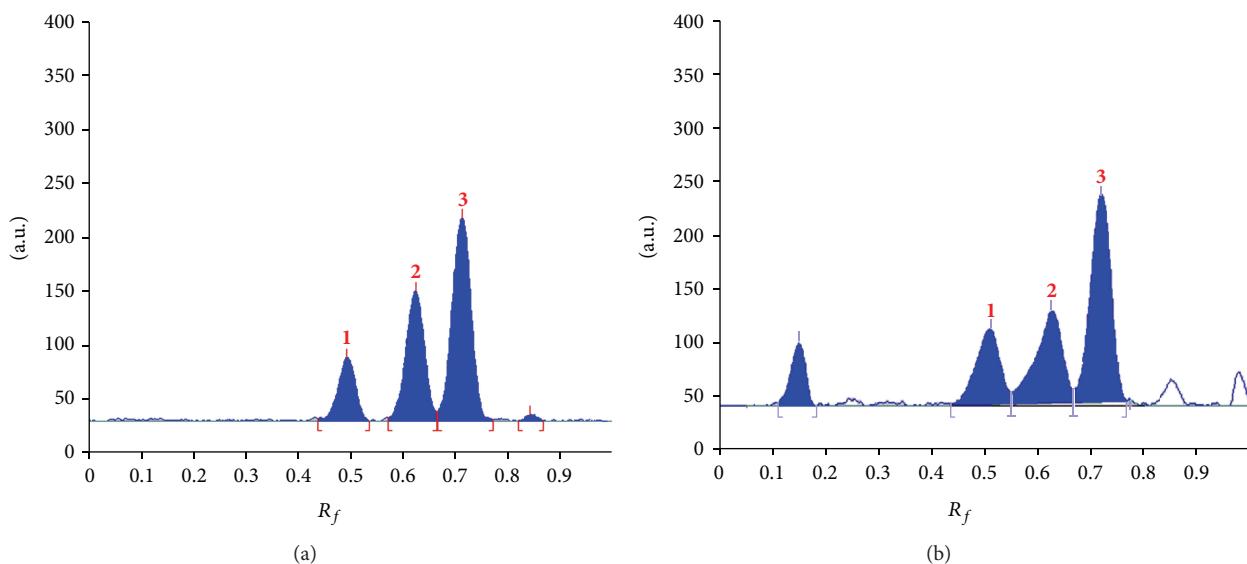


FIGURE 2: TLC densitograms of standard compounds (a) and the extract from the leaves of *M. oleifera* from Chiang Mai province (b); **1** = crypto-chlorogenic acid, **2** = isoquercetin, and **3** = astragalin.

TABLE 1: Method validation parameters for the quantitation of crypto-chlorogenic acid, isoquercetin, and astragalin by the proposed TLC-densitometric method.

Parameter	Results		
	Crypto-chlorogenic acid	Isoquercetin	Astragalin
Linear range (ng/spot)	103–505	105–510	100–500
Calibration equation	$y = -2589.922 + 15.123x$	$y = -884.153 + 16.772x$	$y = 511.081 + 20.486x$
Correlation coefficient (r)	0.9961	0.9975	0.9968
LOQ (ng)	48.93	45.99	36.13
LOD (ng)	14.68	13.80	10.84

x is the amount of each standard in ng and y is the peak area at 340 nm.

TABLE 2: Intraday and interday precisions of crypto-chlorogenic acid, isoquercetin, and astragalin.

Compounds	Concentration (ng/spot)	Intraday precision* (%)	Interday precision* (%)
Crypto-chlorogenic acid	200	3.17	4.34
	300	2.20	1.13
	400	4.00	3.59
Isoquercetin	200	3.47	4.09
	300	2.43	4.68
	400	4.47	2.47
Astragalin	200	1.56	1.99
	300	1.95	1.56
	400	2.24	2.16

*Present as percent RSD ($n = 3$).

method. Three analyses were performed for each concentration level of the standards. The average recoveries were calculated as recovery (%) = $100 \times (\text{amount found} - \text{original amount})/\text{amount added}$.

2.4.4. LOD and LOQ. LOD and LOQ were determined by preparing five different levels (20, 40, 60, 80, and 100 $\mu\text{g}/\text{mL}$) of each standard stock solution and used accordingly. Blank methanol was also spotted three times following the same method and the signal-to-noise ratio was determined. The LOD was considered as 3:1 and the LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting the known concentration of standards until the average responses were approximately 3 or 10 times of the standard deviation of the response, for three replicate determinations of LOD and LOQ, respectively.

2.4.5. Robustness. The robustness of the method was evaluated by introducing little changes in certain chromatographic parameters at each standard concentration level of 200 ng/spot. The ratio of mobile phase composition was changed slightly as 34:3.5:1.5:7.30:3:1.5:5 and 32:3.5:1.5:6, v/v/v/v, for ethyl acetate:formic acid:acetic acid:water, respectively. The length of the chromatogram on TLC plate was varied, 70 mm, 80 mm, and 90 mm. The periods of time between spotting the standards onto the TLC plate and developing the plate (5, 15, and 30 minute),

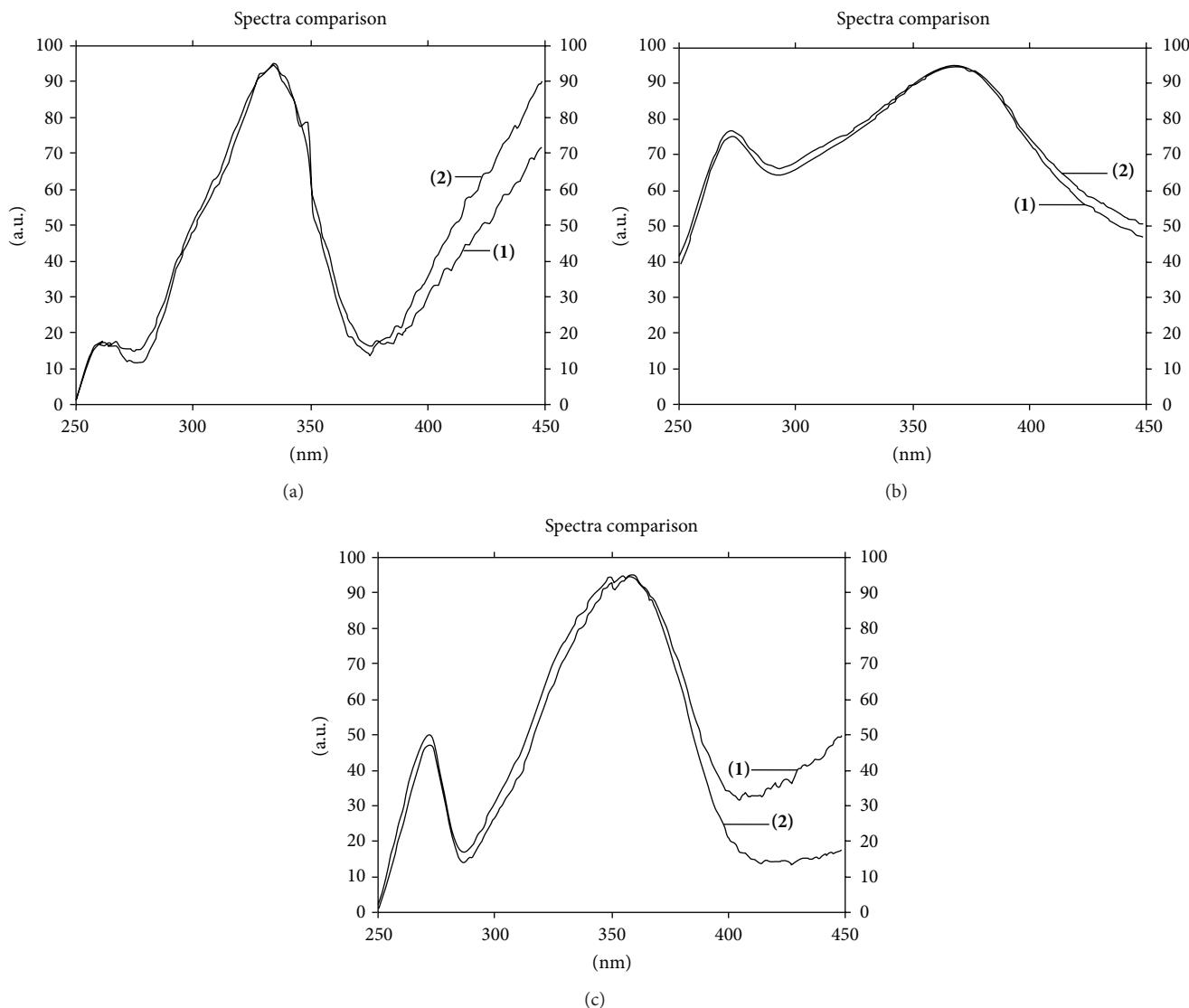


FIGURE 3: Overlay UV absorption spectra of standard compounds in the extract of *M. oleifera* leaves collected from Chiang Mai province; crypto-chlorogenic acid (a), isoquercetin (b), and astragalin (c); spectra illustrating peak purities of reference standard (1) and sample (2).

TABLE 3: Recovery studies of crypto-chlorogenic acid, isoquercetin, and astragalin.

Compound	Serial number	Theoretical value (ng)	Amount found* (ng)	Recovery* (%)
Crypto-chlorogenic acid	1	383.47	370.52 ± 8.60	98.67 ± 1.17
	2	393.06	392.69 ± 7.15	99.75 ± 2.75
	3	403.38	402.07 ± 7.89	97.79 ± 3.23
Average				98.73
Isoquercetin	1	325.82	317.49 ± 5.51	98.73 ± 1.74
	2	335.11	328.92 ± 3.92	97.68 ± 1.65
	3	343.64	341.02 ± 3.00	98.43 ± 0.89
Average				98.28
Astragalin	1	287.89	288.21 ± 4.59	100.04 ± 0.90
	2	298.45	298.68 ± 2.74	99.60 ± 1.16
	3	279.59	276.14 ± 6.82	99.32 ± 2.92
Average				99.65

*Expressed as mean \pm SD ($n = 3$).

duration of TLC tank saturation (30, 60, and 90 minute) and duration between TLC plate development and scanning (5, 30, and 60 minute) were varied. The RSD values of the peak areas of standards were calculated for all variations.

2.5. Chromatographic Conditions. The TLC plates were pre-washed with methanol and activated at 105–110°C for 15 minutes before use. The samples were spotted as 7 mm bands wide with a 100 µL Hamilton syringe at 10 mm from the bottom edge of TLC plate using a CAMAG Linomat 5 automatic sample spotter and nitrogen gas with a constant rate of 80 nL/s. Each sample solution was applied in triplicate. The mobile phase consisted of ethyl acetate : formic acid : acetic acid : water, 34 : 3.5 : 1.5 : 7, v/v/v/v. Linear ascending development was performed in a twin through glass chamber presaturated with the mobile phase for 60 minutes at room temperature. The length of the chromatogram run was 80 mm and the developing time was around 25 minutes. Densitometric scanning was performed using a CAMAG TLC 3 scanner in the reflectance-absorbance mode at 340 nm controlled by winCATs software. The slit dimension was 6.00 × 0.45 mm with a scanning speed of 20 mm/s. Five-point calibration was performed for each analysis by the proposed method. The amounts of isoquercetin, astragalin, and crypto-chlorogenic acid were calculated from peak area using linear regression from the calibration graph.

2.6. Statistical Analysis. All results were expressed as means ± standard deviations of three replicated determinations by SPSS for Windows 16.0.

3. Results and Discussion

M. oleifera leaf extract is composed of complex phenolics and flavonoids [1, 19]. These substances were reported to be biologically active compounds exhibiting several activities such as antidiabetes, antioxidation, and antihyperlipidemia [5–7]. Crypto-chlorogenic acid, isoquercetin, and astragalin (Figure 1) are major components in the leaf extracts of *M. oleifera* [8], and these compounds were employed as markers for TLC-densitometric analysis in the present study. For the chromatographic conditions, several solvents and their proportions of mobile phase were varied to optimize the chromatographic separation. Ethyl acetate : formic acid : acetic acid : water at 34 : 3.5 : 1.5 : 7, v/v/v/v demonstrated the best separation of crypto-chlorogenic acid, isoquercetin, and astragalin with R_f values of 0.51, 0.62, and 0.72, respectively. Densitograms of these three standards and other constituents in the leaf extracts of *M. oleifera* are shown in Figure 2. The identities of the chromatogram bands of these constituents in the sample were confirmed by overlaying their ultraviolet (UV) absorption spectra with those of each standard using the TLC 3 scanner (Figure 3). The method was validated for its linearity, precision, accuracy, LOD, LOQ, and robustness. The linear calibration graphs for crypto-chlorogenic acid, isoquercetin, and astragalin were within the concentration ranges of 103–505, 105–510, and 100–500 ng/spot, with correlation coefficients (r) of 0.9961, 0.9975, and 0.9968,

TABLE 4: Robustness studies of crypto-chlorogenic acid, isoquercetin, and astragalin.

Parameter	Crypto-chlorogenic acid	Isoquercetin	Astragalin	RSD (%) [*]
Mobile phase composition ratio	2.99	2.15	1.20	
Time from spotting to chromatography	1.59	1.65	0.97	
Time from chromatography to scanning	1.16	0.89	0.79	
The length of the chromatogram	1.81	1.05	1.21	
Presaturation period	2.53	0.66	1.04	

^{*}Value from six determinations.

respectively (Table 1). The interday and intraday precisions of crypto-chlorogenic acid, isoquercetin, and astragalin were illustrated in Table 2. The results demonstrated acceptable precision of the method, with RSD less than 5% (Table 2). The average recoveries at three different levels of crypto-chlorogenic acid, isoquercetin, and astragalin were 98.73, 98.28, and 99.65%, respectively (Table 3) confirming the accuracy of the method. LOD was found between 10.84 and 14.68 ng/spot and LOQ was in the range of 36.13–48.93 ng/spot (Table 1). For the robustness test, the standard deviation of peak areas was calculated for each parameter and the RSD was found to be less than 3% for all variations (Table 4). The change of mobile phase composition was the crucial deviation of the method while the others were less significant.

The validated TLC-densitometric method was used to determine the contents of crypto-chlorogenic acid, isoquercetin, and astragalin in 12 extracts of *M. oleifera* leaves collected from different locations in Thailand. The amounts of crypto-chlorogenic acid, isoquercetin, and astragalin showed a variation from nondetectable to detectable quantities and provided the averages of 0.0473 ± 0.0236 , 0.0427 ± 0.0192 , and $0.0534 \pm 0.0440\%$ dry weight, respectively (Table 5). The highest contents of these major active components were found in the sample from Chiang Mai province, where the climate is cool, while the lowest contents were in Sa Kaeo province in the warm eastern part of Thailand (Table 5). These findings confirmed the previous report of Iqbal and Bhanger that *M. oleifera* cultivated in the cold place of Pakistan contained the highest contents of total phenolic and total flavonoid compounds [24]. The developed TLC-densitometric method was accurate and precise for the qualitative and quantitative determination of crypto-chlorogenic acid, isoquercetin, and astragalin in *M. oleifera* leaves and was advantageous due to its simplicity and economy. It could be used as an alternative method for routine quantitative analysis of major compounds in the *M. oleifera* leaf extracts. These data will be also useful as guidance for standardization of *M. oleifera* leaf raw materials and for finding good sources of this plant in Thailand.

TABLE 5: Contents of crypto-chlorogenic acid, isoquercetin, and astragalin in dried powder of *M. oleifera* leaves collected from various regions of Thailand.

Location (region)	Contents of major compounds (% dry weight)		
	Crypto-chlorogenic acid	Isoquercetin	Astragalin
Chiang Mai (Northern)	0.1021 ± 0.0006	0.0733 ± 0.0023	0.1604 ± 0.0042
Lampang (Northern)	0.0693 ± 0.0004	0.0393 ± 0.0017	0.0430 ± 0.0008
Surin (Northeastern)	0.0549 ± 0.0035	0.0638 ± 0.0007	0.0370 ± 0.0004
Udonthani (Northeastern)	0.0411 ± 0.0049	0.0350 ± 0.0057	0.1264 ± 0.0049
Sa Kaeo (Eastern)	ND	ND	0.0192 ± 0.0036
Chonburi (Eastern)	0.0446 ± 0.0003	0.0707 ± 0.0005	0.0287 ± 0.0002
Ang Thong (Central)	0.0602 ± 0.0006	0.0502 ± 0.0044	0.0440 ± 0.0012
Saraburi (Central)	0.0402 ± 0.0011	0.0293 ± 0.0010	0.0219 ± 0.0014
Kanchanaburi (Western)	0.0315 ± 0.0009	0.0230 ± 0.0003	0.0548 ± 0.0010
Phetchaburi (Western)	0.0262 ± 0.0006	0.0198 ± 0.0011	0.0460 ± 0.0004
Phang Nga (Southern)	0.0178 ± 0.0028	0.0258 ± 0.0004	0.0240 ± 0.0024
Phuket (Southern)	0.0327 ± 0.0008	0.0391 ± 0.0021	0.0348 ± 0.0022
Average	0.0473 ± 0.0236	0.0427 ± 0.0192	0.0534 ± 0.0440

*Expressed as mean ± SD ($n = 3$), ND = nondetectable.

Moreover, the method can be used for quality control of raw materials, extracts, and finished products containing these phenolics and flavonoids.

4. Conclusion

The proposed TLC-densitometric method was developed and validated for the simultaneous quantitative analysis of three major active components: crypto-chlorogenic acid, isoquercetin, and astragalin in the extracts of *M. oleifera* leaves collected from 12 different locations in Thailand. The method was simple, accurate, precise and could simultaneously analyze numerous samples. These results will be valuable for further standardization of *M. oleifera* leaves and their extracts, and for indicating good sources of the leaf raw materials of this plant in Thailand. Furthermore, the method could be utilized for quality control of raw materials, extracts, and finished products containing these compounds in commercial nutraceutical and pharmaceutical products.

Conflict of Interests

All authors declare no conflict of interest with the trademarks included in the paper.

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Research Article

The Preventive Effect of Biochanin A on Bone Loss in Ovariectomized Rats: Involvement in Regulation of Growth and Activity of Osteoblasts and Osteoclasts

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Biochanin A (BCA) is a major isoflavone abundant in red clover (*Trifolium pretense*). The protective effect of BCA on bone loss in an ovariectomized (OVX) animal model has never been clarified. The objective of this study was to investigate the biological effects of BCA on bone loss in OVX rats *in vivo* and on the development of osteoblasts and osteoclasts *in vitro*. Ovariectomy resulted in a marked increase in body weight and a decrease in femoral bone mineral density and trabecular bone volume that was prevented by BCA or 17 β -estradiol (E2) treatment. However, an increase in uterine weight was observed in E2-treated OVX rats, but not in response to BCA treatment. Treatment with BCA increased the mRNA expression of osterix, collagen type I, alkaline phosphatase (ALP), and osteocalcin and decreased the mRNA expression of tartrate-resistant acid phosphatase (TRAP) and the receptor activator of nuclear factor- κ B ligand (RANKL)/osteoprotegerin (OPG) ratio in the femur of OVX rats. Treatment with BCA or E2 prevented the OVX-induced increase in urinary deoxypyridinoline (DPD) and serum tumor necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β). *In vitro*, BCA induced preosteoblasts to differentiate into osteoblasts and increased osteoblast mineralization. BCA inhibited preosteoclasts and osteoclast proliferation and decreased osteoclast bone resorption. These findings suggest that BCA treatment can effectively prevent the OVX-induced increase in bone loss and bone turnover possibly by increasing osteoblastic activities and decreasing osteoclastic activities.

1. Introduction

Osteoporosis is the most common metabolic bone disease in women. A menopausal decline in estrogen contributes significantly to an increased rate of bone remodeling and leads to an imbalance between bone resorption and formation, eventually causing bone loss and osteoporosis [1, 2]. High bone turnover, with increased bone resorption, can compromise bone strength, leading to a thinning of the bone structure, resulting in abnormal bone microarchitecture and reduced bone mineralization. This, in turn, leads to a greater propensity to fracture.

Phytoestrogens are natural plant-derived products that have structural and functional similarities to estradiol. They are considered to be a safe alternative to hormone replacement therapy (HRT) [3] and are used in phytomedicine to treat menopausal symptoms and osteoporosis. Our previous

study had demonstrated that soy isoflavone extract in combination with vitamin D3 was able to decrease bone loss during estrogen deficiency in an animal model [4], implicating that isoflavone might be an alternative to HRT. Biochanin A (BCA), a major isoflavone found in red clover (*Trifolium pretense*) and many other legumes, is commercially available as a nutraceutical and is known to exert positive health effects and may be useful in the maintenance of bone health [5]. We suggested that BCA may have benefits to osteoporosis resulted from estrogen decline.

Red clover has a higher content of BCA (5,7-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone) and a lower content of genistein (5,7,4'-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) than soy [6]. Previous studies have shown that clover-derived isoflavone supplements provide potential benefits to bone. Red clover-derived isoflavone supplements reduce the

loss of lumbar spine bone mineral content (BMC) and bone mineral density (BMD) in women [7, 8] and improve OVX-induced osteoporosis [9]. The mechanism by which these red clover phytoestrogenic isoflavones are effective at reducing bone loss induced by ovariectomy is thought to be by reducing bone turnover via inhibition of bone resorption [10]. Limited bone metabolism data have been reported for BCA. *In vitro* studies showed that BCA stimulated differentiation of osteoblastic MC3T3-E1 cell line [11] and modulated lipid metabolism [12]. However, the biological roles of BCA in the enhancement of bone formation *in vivo* and the attenuation of bone resorption *in vivo* and *in vitro* are largely unknown.

Although BCA is converted into the demethylated metabolite genistein, the biological effects of BCA observed *in vivo* are not identical to those of genistein [13]. BCA is thought to exert its beneficial effects predominantly through the estrogen receptor beta (ER β) (found in bone and blood vessels) [14, 15]; the affinity of BCA for ER β is greater than that for ER α (found in breast and uterus). Previous studies have shown that BCA has vasculoprotective effects without uterotrophic activity [14, 16]; thus, administration of ER β -selective agents might be alternative treatments to reduce the risk of cardiovascular disease and bone loss in postmenopausal women.

The aim of the present study was to evaluate the possible beneficial effect of BCA on bone loss in the ovariectomized rat model of osteoporosis. This model is the most commonly used model for the study of human postmenopausal osteoporosis [17]. In addition, plasma BCA concentrations lower than or equal to 10^{-6} M are attainable with a daily oral intake of 5–50 mg per kg of body weight in rats [18]. Indeed, the maximum plasma concentration of any isoflavone rarely exceeds 10^{-6} M following dietary intake [19]. Thus, we further examined the effectiveness and underlying molecular mechanism of BCA (at a physiologically relevant concentration of 10^{-6} M or lower) in bone health by using two major osteoporosis-related primary bone cells, osteoblasts, and osteoclasts.

2. Materials and Methods

2.1. Animals and Treatments. The animals and treatment methods used were described in a previous study [4]. Virgin female Sprague-Dawley (SD) rats aged 3 months (280–300 g) were sham-operated ($n = 10$) or bilaterally ovariectomized ($n = 30$) as an experimental animal model of estrogen depletion-induced bone loss. The OVX rats were randomly assigned to one of three treatment groups: untreated, treated for 14 weeks with E2, and treated for 14 weeks with BCA (Sigma-Aldrich, St. Louis, MO, USA). The sham-operated, OVX control and E2-treated rats received a control diet. The E2-treated rats received intraperitoneal injection of E2 (23 μ g/kg body weight per day) (Sigma-Aldrich) on 3 consecutive days per week; this dose was chosen to replace approximately 90% of the estrogen loss observed after ovariectomy. The BCA-treated group received daily oral administration of BCA (25 mg/kg body weight per day).

All rats were housed individually in metal cages. Animals were maintained at 23°C, on a 12 h light:dark cycle with standard rat food pellets. Water was provided *ad libitum*. This procedure was approved by the Kaohsiung Medical University Animal Care and Use Committee.

The rats were sacrificed by an overdose of CO₂ at the end of the study. Blood was collected in a heparinized syringe from the abdominal aorta, transferred to a glass tube for 1 h, and then centrifuged at 3000 g for 10 min at 4°C to separate the serum. The left femur was removed for histological study and for extraction of mRNA and protein. All samples were stored at -80°C until further analyzed.

2.2. Body Weight, Uterine Weight, Bone Mineral Density, Bone Mineral Content and Bone Volume. Body weight was measured on a weekly basis to monitor health and measure weight gain. At the end of the study, the rats were anesthetized and scanned under a dual energy X-ray absorptiometer (Norland XR-36; Norland, Fort Atkinson, WI, USA). The left femur was examined for BMD and BMC, and PMOD image analysis software (PMOD Technologies, Zurich, Switzerland) was used to calculate bone volume by selecting the same position of the left femur in each rat. The percentage of the trabecular bone volume in relation to the total tissue volume examined (%BV/TV) was measured. Uterine weights were determined after removal of the uterus.

2.3. Serum and Urinary Biochemical Markers. Serum calcium and inorganic phosphorous concentrations were measured using an autoanalyzer (7070; Hitachi, Tokyo, Japan). Urine samples were individually collected from rats housed in metabolic cages during the 24 hours preceding sacrifice. Urinary deoxypyridinoline (DPD) levels were measured by ELISA (DPD EIA kit, Metra Biosystems, US). Commercial kits were used to analyze serum concentrations of TNF- α and IL-1 β (R&D, Minneapolis, MN, USA).

2.4. Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was extracted from the left femur using REZol reagent (Protech, Taiwan). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described previously [20]. To synthesize complementary DNA (cDNA), 2 μ g of RNA was resuspended in 12.5 μ L of diethylpyrocarbonate-treated water, 1 μ L of oligo(dT) primer was added, and the mixture was annealed for 5 min at 70°C. The sample was then cooled to 4°C for 2 min before addition of 4 μ L of 5x reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3), 0.5 μ L of RNase inhibitor, 1 μ L of 10 mM dNTP, and 1 μ L of Maloney's murine leukemia virus reverse transcriptase (Promega, Lyon, France). The reaction mixture was heated for 60 min at 37°C to synthesize the cDNA, and the reaction was stopped by denaturing the enzyme at 94°C for 5 min. cDNA was amplified by PCR to generate the genes listed as follows: Osterix (NM_001173467): forward: 5'-ggaggcaactggctag-3', reverse: 5'-gctgccactatccccc-3' (229 bp); ALP (NM_000478): forward: 5'-ctctccgagatgggtgg-3', reverse: 5'-tggagacattctctcggtt-3' (373 bp); collagen type I (NM_000088): forward: 5'-ggctatgtgagaatcaa-3', reverse:

5'-atccaaaccactgaaacc-3' (266 bp); Osteocalcin (NM_199173): forward: 5'-tgcagagtccagcaaag-3', reverse: 5'-gctccaggggatccg-3' (125 bp); OPG (NM_002546): forward: 5'-ctgttgaggaggcatt-3', reverse: 5'-agctgtttggcggttta-3' (136 bp); RANKL (NM_033012): forward: 5'-agcatcaaaaatccaaatgtt-3', reverse: 5'-aactttaaaagccccaaag-3' (204 bp); and TRAP (NM_00111034): forward: 5'-gccattttatgtggac-3', reverse: 5'-cttgaccaggcagtgg-3' (216 bp). GAPDH (NM_001256799): forward: 5'-atggtttacatgttccaata-3', reverse: 5'-ctcgctctggaga-3' (115 bp) was amplified as a housekeeping gene. PCR amplification was performed for 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by 7 min at 72°C. The amplified PCR products were separated by gel electrophoresis in 2% agarose gel and visualized with ethidium bromide, and the intensity of each band was calculated by densitometry analysis and the results were expressed as a percentage of the density of the corresponding GAPDH band.

2.5. Bone-Cell Culture and Proliferation. To analyze the direct effects of BCA or E2 on the differentiation and function of osteoblasts and osteoclasts, rat primary osteoblast and osteoclast progenitors were isolated and cultured. The rat calvarial osteoblast culture system was as described by Udagawa et al. [21], and the osteoclast culture system using the osteoclastogenic agents 1 α ,25-dihydroxyvitamin D₃ (10 nmol/L), dexamethasone (10 nmol/L), and macrophage-colony stimulating factor (10 ng/mL) was as described by Zou and Bar-Shavit [22]. Preosteoblasts or mature osteoblasts were seeded onto 24-well plates (5×10^3 cells/well) in minimum essential medium containing 10% fetal calf serum and were allowed to attach for 24 h. Incubation was continued for another 48 h in the presence or absence of physiological concentrations of 10^{-8} – 10^{-6} M BCA. Preosteoclasts or mature osteoclasts were treated identically with the exception of inclusion of osteoclastogenic agents in the seeding medium. Crystal violet uptake by the cells was used to evaluate cell number.

2.6. Enzyme Histochemistry. Preosteoclasts or preosteoblasts were seeded onto 24-well plates (1×10^3 /well) in minimum essential medium containing 10% fetal calf serum and were allowed to attach for 24 h. Incubation was continued for 7 days with or without 10^{-8} – 10^{-6} M BCA. Fresh medium was supplied at 3-day intervals. Multinucleated cells were fixed in formalin/acetone/citric acid and checked for the presence of the osteoclast marker enzyme, TRAP, or the osteoblast marker, ALP, by assaying enzyme activity using commercially available kits (Sigma-Aldrich). Positive cells were stained brown or red. TRAP⁺-multinucleated cells and ALP⁺ osteoblasts were counted using a semiautomatic image-analyzing program.

2.7. Cellular ALP and TRAP Activity. Osteoclasts or osteoblasts (5×10^4 /well) were seeded onto a 24-well plate in minimum essential medium containing 10% fetal calf serum or osteoclastogenic agents. After incubation for 24 h, fresh medium with or without the test agents at 10 mmol/L was supplied until the cells reached confluence. The TRAP activity

of osteoclasts or the ALP activity of osteoblasts was measured using a p-nitrophenyl phosphate kit (Sigma-Aldrich).

2.8. Cell Migration Assay for Osteoblasts. Cell migration was determined following the method of Xu et al. [23]. Osteoblasts were detached and 5×10^5 cells were seeded onto Corning 24-well tissue culture plates. Incubation was continued for 48 h in fresh medium with or without 10^{-6} M BCA. The cell migration area was counted using a semiautomatic image-analyzing program. There were 24 wells per treatment group. All experimental protocols were repeated at least three times.

2.9. Mineralization Assay. The von Kossa mineralization assay was followed as described by Bellows et al. [24]. The phosphorous calcium deposition area was measured using a semiautomatic image-analyzing program (Mac Scope, Mitani, Fukui, Japan).

2.10. Bone Resorption Assay. After treatment, the osteoclasts were lysed with distilled water and the slides were stained with 5% aqueous AgNO₃, exposed to ultraviolet light for 60 min at room temperature, and then incubated in 2.5% sodium thiosulfate for 5 min. The area of the resorption pits was measured using a Zeiss Morphomat 10 (Carl Zeiss, La Pecq, France) for 100–150 pits selected at random on each slide. The pit area was counted using a semiautomatic image-analyzing program (Mac Scope). Each experiment was performed at least three times.

2.11. Statistics. The results of the *in vivo* and *in vitro* data are presented as the mean \pm standard deviation (SD). Differences among the groups (Sham, OVX, OVX+E2, and OVX+BCA) were analyzed statistically using one-way analysis of variance (ANOVA), followed by Fisher's test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Body Weight and Uterine Weight in OVX Rats. At 14 weeks after bilateral ovariectomy, serum estrogen levels had dramatically decreased from 56.3 ± 5.6 pg/mL to 3.0 ± 2.5 pg/mL. In accordance with reports that estrogen modulates lipid metabolism [25, 26], the body weight was markedly increased by $63.8 \pm 7.2\%$ compared to an increase of $33.6 \pm 5.0\%$ in the sham group. Treatment with BCA or E2 significantly decreased the body weight of OVX rats (*P* < 0.05) (Table 1). In addition, uterine weight was significantly reduced in OVX rats (*P* < 0.05). Treatment of OVX rats with E2 significantly increased uterine weight compared to OVX rats (*P* < 0.05), but uterine weight was unchanged in BCA-treated OVX rats.

3.2. Femur BMD, BMC, and BV/TV in OVX Rats. The left femur BMD and BMC were measured by dual energy X-ray absorptiometry. The results listed in Table 2 show that BMD of the OVX group was markedly reduced by 14.5% in comparison to that in the sham group (*P* < 0.05). Treatment

TABLE 1: Effect of treatments on body weight and uterine weight change.

Treatment group (<i>n</i> = 10)	Body weight		Uterine weight	
	Before treatment (g)	After treatment (g)	Increase rate (%)	(g)
Sham	290.0 ± 21.6	387.9 ± 19.6	33.6 ± 5.0 ^b	0.45 ± 2.1 ^b
OVX	298.0 ± 11.7	486.7 ± 18.4	63.8 ± 7.2 ^a	0.27 ± 2.6 ^a
OVX + E2	295.7 ± 16.2	395.7 ± 15.9	34.9 ± 4.8 ^b	0.41 ± 3.4 ^b
OVX + BCA	290.0 ± 13.1	380.0 ± 18.9	31.0 ± 3.4 ^b	0.29 ± 2.1

Values are expressed as mean ± SD. Means within a column with different superscript letters are significantly different (*P* < 0.05, ANOVA and the Fisher test).

^a*P* < 0.05, when compared with the sham groups; ^b*P* < 0.05, when compared with the OVX group.

TABLE 2: Effect of treatments on femoral bone mineral density (BMD), bone mineral content (BMC), and BV/TV of the left femur.

Treatment group (<i>n</i> = 10)	Bone mineral density (BMD) (mg/cm ²)	Bone mineral content (BMC) (g)	Bone volume (BV/TV) (%)
Sham	192.0 ± 8.0 ^b	0.48 ± 0.4 ^b	35.5 ± 5.4 ^b
OVX	164.0 ± 6.7 ^a	0.37 ± 0.3 ^a	12.6 ± 3.3 ^a
OVX + E2	185.0 ± 5.0 ^b	0.44 ± 0.5 ^b	28.7 ± 4.3 ^b
OVX + BCA	188.0 ± 8.6 ^b	0.48 ± 0.6 ^b	31.8 ± 4.5 ^b

BMD (mg/cm²) and BMC (g) were measured by Dual Energy X-Ray Absorptiometry. Values are expressed as means ± SD. Means within a column with different superscript letters are significantly different (*P* < 0.05, ANOVA and the Fisher test). ^a*P* < 0.05, when compared with the sham groups; ^b*P* < 0.05, when compared with the OVX group.

with BCA or E2 for 14 weeks maintained BMD levels similar to those of the sham group (*P* < 0.05). Bone mineral content of the OVX group was significantly lower (*P* < 0.05) than that of the sham group, but treatment with BCA or E2 effectively increased BMC in the OVX group (*P* < 0.05). Computed tomography of the distal femur showed that %BV/TV was markedly decreased by ovariectomy (Table 2), suggesting the induction of osteopenia. Treatment with BCA or E2 resulted in a significant increase in %BV/TV compared with the OVX control (*P* < 0.05).

3.3. Serum and Urinary Biochemical Markers in OVX Rats. There was no significant difference in serum calcium or phosphate levels among all groups (Table 3). The bone resorption marker, urinary DPD, was increased in the OVX group and diminished by treatment with BCA or E2 (Table 3). Serum levels of cytokines TNF- α and IL-1 β , which are responsible for enhanced osteoclastogenesis and activation of mature osteoclasts for bone resorption, were significantly increased in OVX rats and diminished by treatment with BCA or E2 (*P* < 0.05) (Table 3).

3.4. Osteogenic Marker mRNA Levels in OVX Rats. The mRNA levels of osteoblast and osteoclast marker genes in distal femur bone tissue were determined by RT-PCR. Expressions of the osteoblast osteogenic genes osterix, collagen type I, ALP, and osteocalcin were remarkably decreased in the untreated OVX group compared to those in the sham group (Figures 1(a)–1(d)). These reductions were prevented

upon treatment with BCA or E2. However, osterix and ALP expression levels in the BCA-treated groups were higher than those in the sham- and E2-treated group (*P* < 0.05). Expression of the osteoclast marker gene, TRAP, was increased in the untreated OVX group and this increase was prevented by E2 or BCA (Figure 1(e)).

3.5. Expression of RANKL/OPG during Bone Turnover in OVX Rats. Studies in normal healthy animals have revealed that OPG and RANKL play important ongoing roles in the maintenance of bone mass and in the regulation of normal bone remodeling [27]. Ovariectomy is associated with increased bone turnover and reduced BMD, volume, and strength. Ovariectomy has been shown to increase RANKL levels and decrease OPG levels in various animal models [28, 29]. The RANKL/OPG ratio is an index of osteoclastogenic stimulation and an increase RANKL/OPG ratio is observed during bone resorption in ovariectomy. Hence, we further investigated expression of RANKL and OPG *in vivo*. RANKL and OPG mRNAs were prepared from rat distal femur bone tissue and were determined by RT-PCR. The RANKL/OPG ratio was remarkably increased in the untreated OVX group compared to that in the sham group, whereas treatment with BCA or E2 significantly decreased the RANKL/OPG ratio (Figure 2). This suggested that both BCA and E2 decreased bone resorption during bone turnover in OVX rats.

3.6. Preosteoblast and Osteoblast Proliferation and Differentiation. After culture, the osteoblast precursor cells started developing into ALP⁺ preosteoblasts and osteoblasts. These cells were incubated with or without various concentrations of BCA or E2 for 2 days to examine the effects of BCA or E2 on cell proliferation, or for 7 days to examine the effects of BCA or E2 on expression of ALP, a marker of osteoblast differentiation. BCA (10⁻⁸–10⁻⁶ M) significantly increased preosteoblast cell proliferation (*P* < 0.05) and slightly enhanced osteoblast proliferation, but this was not significant compared to the control (*P* > 0.05) (Figure 3(a)). Expression and activity of ALP were significantly increased after incubation with 10⁻⁶ M BCA compared to the control (*P* < 0.05) (Figures 3(b) and 3(c)).

3.7. Preosteoclast and Osteoclast Proliferation and Differentiation. Osteoclast formation is known to be stimulated by 1 α ,25-dihydroxyvitamin D₃ [30]. In the present study, we found that osteoclastogenic agents including 10 nmol/L

TABLE 3: Bone-related parameters of serum and urine in rats after treatment.

Treatment group (n = 10)	Inorganic phosphate (mg/dL)	Calcium (mg/dL)	TNF- α (pg/mL)	IL-1 β (pg/mL)	DPD/creatinine (nM/mM)
Sham	7.2 ± 0.8	7.4 ± 1.3	20 ± 2.1 ^b	23 ± 4.9 ^b	92 ± 6.1 ^b
OVX	7.3 ± 1.2	7.7 ± 1.5	76 ± 4.3 ^a	44 ± 5.6 ^a	148 ± 8.5 ^a
OVX + E2	7.0 ± 1.1	7.3 ± 1.7	10 ± 1.9 ^b	18 ± 5.1 ^b	82 ± 4.1 ^b
OVX + BCA	7.1 ± 0.7	7.4 ± 1.2	11 ± 1.6 ^b	20 ± 2.3 ^b	79 ± 5.2 ^b

Values are expressed as means ± SD. Differences among treatment groups were evaluated using a one-way ANOVA followed by Fisher's test. ^aP < 0.05, when compared with the Sham groups; ^bP < 0.05, when compared with the OVX group. OVX: ovariectomized.

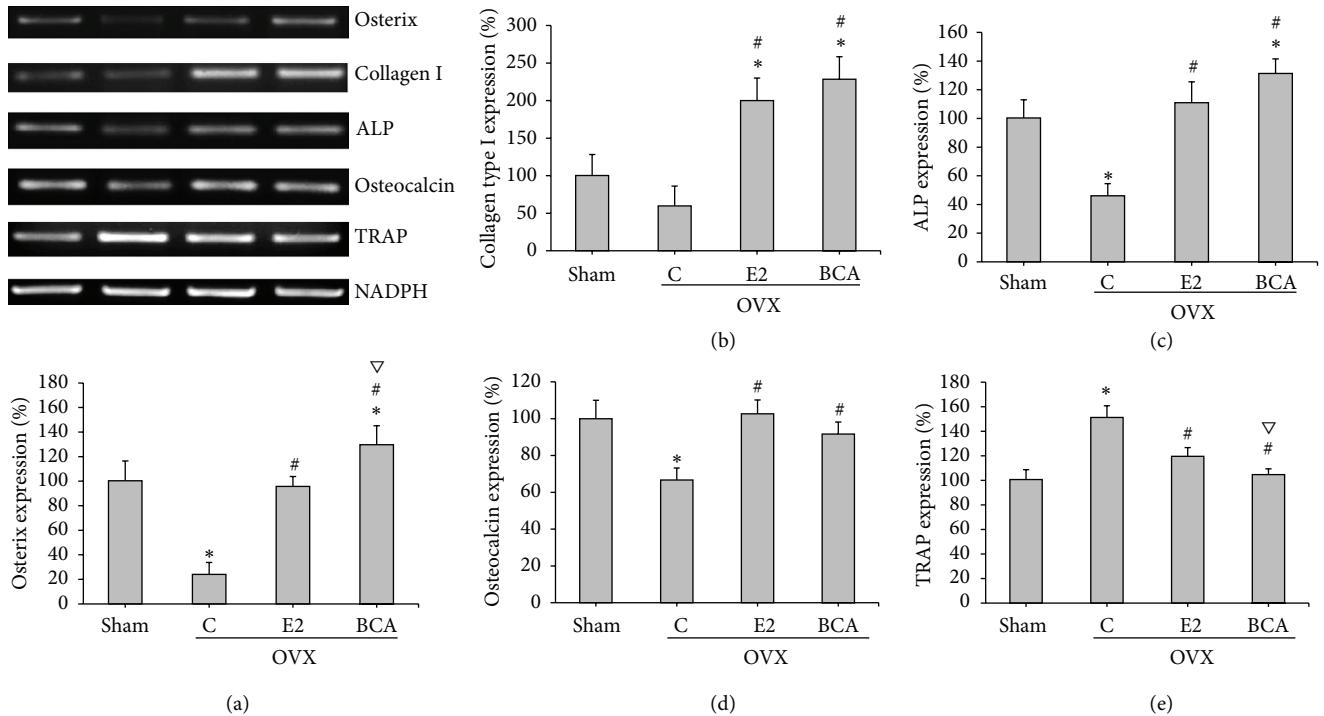


FIGURE 1: Effects of BCA and E2 on osteoblast and osteoclast marker gene expression. mRNA expression in the distal femur was determined by RT-PCR for (a) osterix, (b) collagen type I, (c) ALP, (d) osteocalcin, or (e) TRAP. Expression was normalized to that of GAPDH and expressed as a percentage of that in the sham group. The bars represent mean ± SD for ten samples. *P < 0.05 compared with the sham group; #P < 0.05 compared with the OVX C group; ▽P < 0.05 compared with the E2 group. ALP: alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase; C: control; BCA: biochanin A; E2: 17 β -estradiol; OVX: ovariectomized.

1 α ,25-dihydroxyvitamin D₃, 10 nmol/L dexamethasone, and 10 nmol/L M-CSF induced the differentiation of bone marrow cells into TRAP⁺ osteoclastic cells. BCA was added to the osteoclastogenic agent-treated cells, and these cells were cultured for an additional 2 days to examine the effects of BCA on cell proliferation. Cells were cultured for 7 days in order to analyze the effects of BCA on TRAP. The preosteoclastic cell proliferation was dose-dependent and remarkably inhibited in the presence of BCA (10^{-8} – 10^{-6} M) ($P < 0.05$) (Figure 3(d)). In addition, BCA also inhibited the proliferation of mature osteoclasts in a dose-dependent manner (Figure 3(d)). Notably, TRAP is a marker enzyme for the differentiation and formation of osteoclasts, and TRAP expression and activity were also decreased after incubation for 7 days with 10^{-6} M BCA ($P < 0.05$) (Figures 3(e)–3(f)).

3.8. Osteoblast Migration, ALP Activity, and Mineralization. A wound-healing assay was performed to evaluate the migration of mature osteoblasts. Cells were incubated in the presence or absence of 10^{-6} M BCA for 2 days. The results presented in Figure 4(a) show that 10^{-6} M BCA enhanced cell migration. Furthermore, the BCA-induced increase ALP activity was completely blocked by the protein synthesis inhibitor, cycloheximide (10^{-6} M), suggesting that the response was dependent on de novo protein synthesis (Figure 4(b)) ($P < 0.05$). Mature osteoblasts were grown in 24-well plates for 7 days in the presence or absence of BCA and then stained with von Kossa stain to examine mineralization. Figure 4(c) shows that the number of mineralized nodules (right panel) and the area of mineralization (left panel) were significantly increased by BCA.

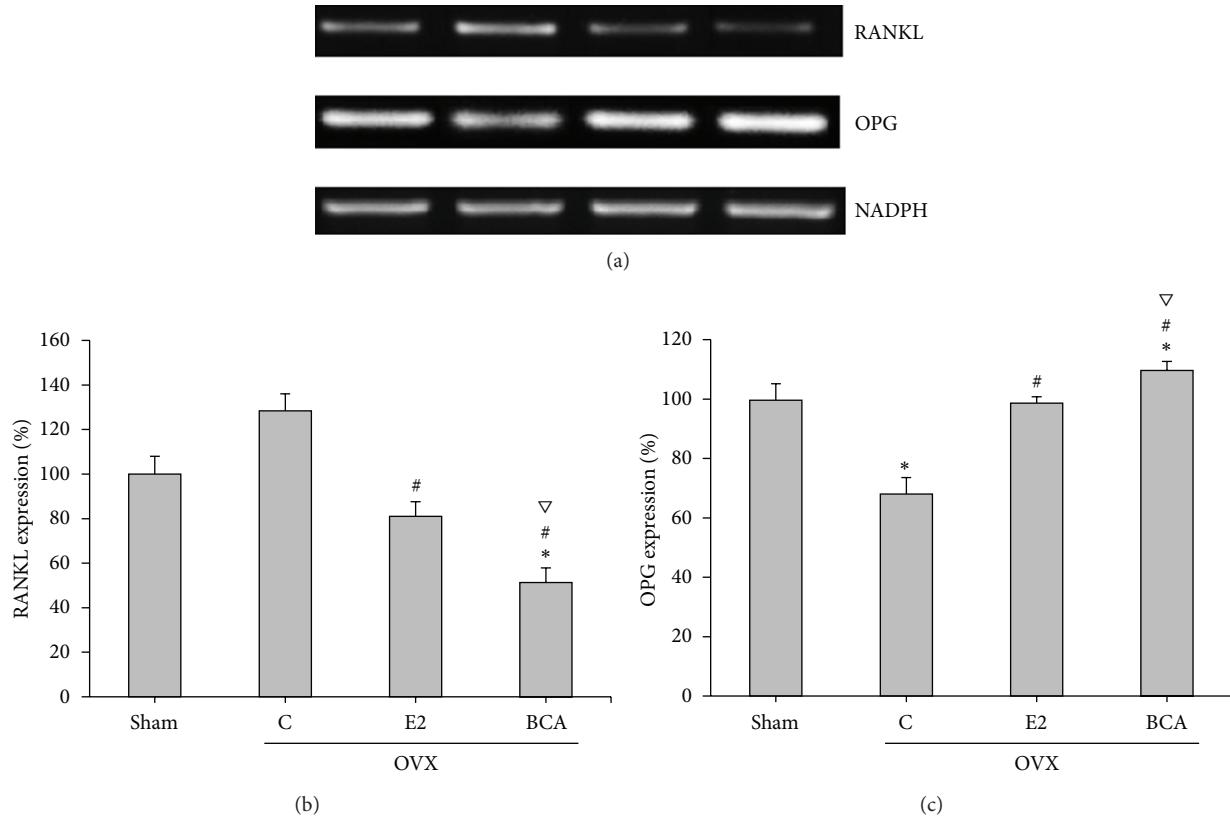


FIGURE 2: Effects of BCA and E2 on RANKL and OPG mRNA expression. The mRNA levels for RANKL (b) and OPG (c) in the distal femur were determined by RT-PCR. Expression was normalized to that of GAPDH and expressed as a percentage of that in the sham group. The bars represent the mean \pm SD for ten samples. * $P < 0.05$ compared with the sham group; # $P < 0.05$ compared with the OVX C group; ▽ $P < 0.05$ compared with the E2 group. RANKL: receptor activator of nuclear factor- κ B ligand; OPG: osteoprotegerin; C: control; BCA: biochanin A; E2: 17 β -estradiol; OVX: ovariectomized.

3.9. *Osteoclast and Bone Resorption*. Osteoclastogenic agent-induced osteoclasts were incubated with or without BCA for 7 days to detect the resorption pits. A remarkable and significant decrease in osteoclast resorption pits ($P < 0.05$) was observed (Figure 4(d)).

4. Discussion

The present study was undertaken to evaluate the effectiveness of BCA on preventing bone loss from estrogen depletion in OVX rats. Our results support previous observations pertaining to the positive effects of BCA on increasing total BMD, BMC, and BV/TV of the femur. The protective effect was manifested by the enhancement of growth and activity of osteoblasts, and repression of growth and activity of osteoclasts, and thereby reducing bone turnover. BCA at a concentration of 10^{-6} M exerted the greatest biological activity.

Bone metabolism is regulated by functions of osteoblasts and osteoclasts which are localized on bone tissues. In our previous study and this present study was shown that the isoflavone genistein and BCA molecules possess similar action in the regulation of bone metabolism [4]. In general, ER α is expressed in breast, uterus, and ovarian, whereas ER β

is expressed in bone and blood vessels. Genistein can bind to ER α to increase uterine weight [31] and bind to ER β to improve bone loss [32, 33]. However, BCA is an ER β -selective isoflavone [15]. BCA stimulates osteoblast differentiation and mineralization, whereas osteoclast differentiation and bone resorption were suppressed (Figures 3 and 4) and no proliferative effects on the uterus were observed (Table 1) [14, 16]. Thus, the data support that BCA is a selective ER β modulatory-like activity. In addition, BCA binds to ER β with considerably less affinity than genistein [34]. This may be attributed to the presence of the 4'-methoxy group on BCA. The difference in binding affinities to ERs suggests that BCA and genistein may have different biological activities.

Osterix, collagen type I, ALP, and osteocalcin are markers of the four stages of bone formation [35, 36] representing, respectively, the mesenchymal stem cell to preosteoblast stage, the osteoblastic proliferation stage, differentiation stage, and mineralization stage. The levels of mRNAs for all four genes were increased by BCA treatment. Biochemical measurements of bone turnover provide an objective assessment of disease activity and the response to treatment. The urinary excretion of DPD crosslinks is a marker of bone resorption. The urinary DPD/creatinine ratio can be used to evaluate the bone turnover in OVX rats. Ovariectomy

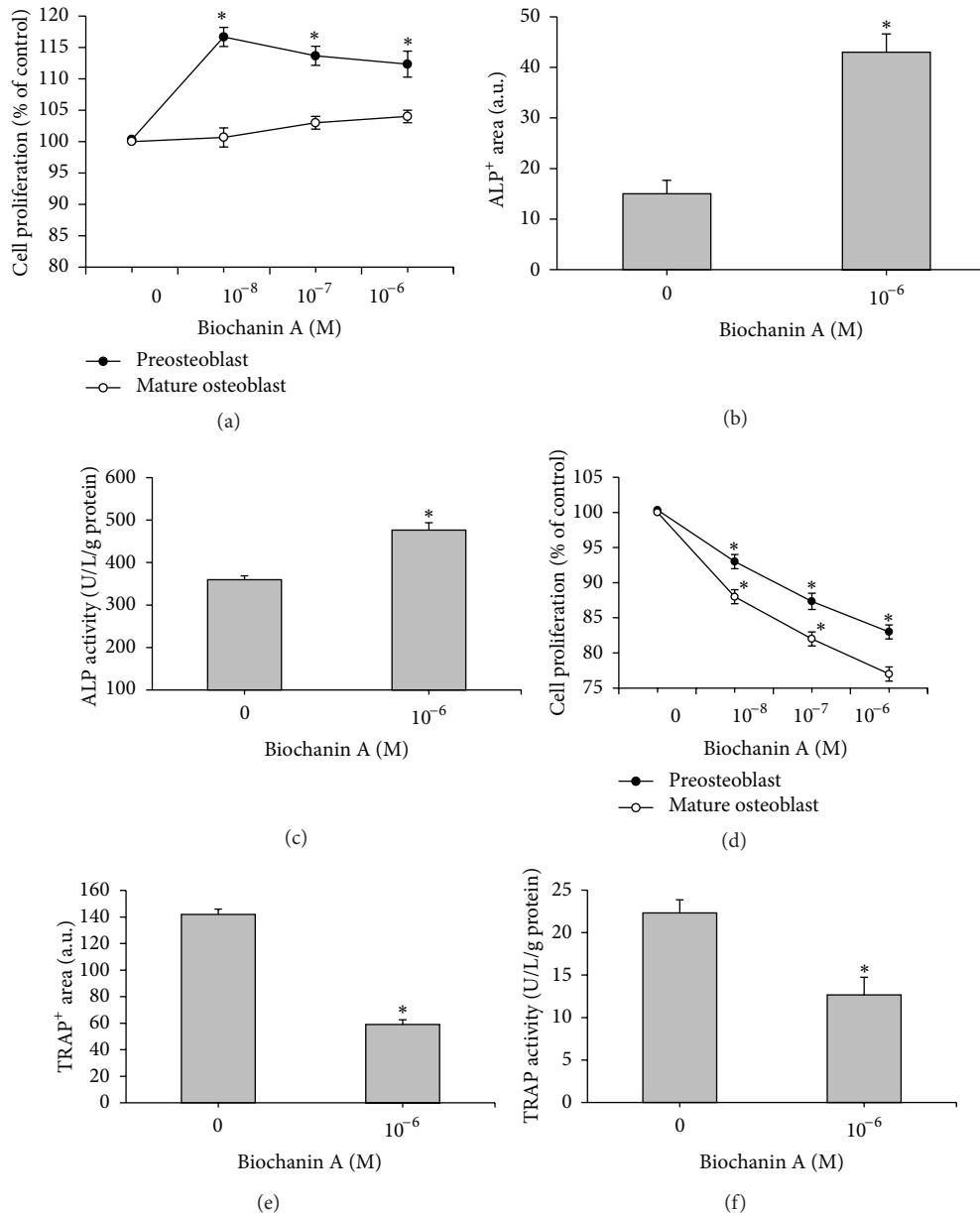


FIGURE 3: Effects of BCA on (a) the growth of preosteoblasts and mature osteoblasts, (b) preosteoblastic ALP staining, (c) mature osteoblastic ALP⁺-specific activity, (d) the growth of preosteoclasts and mature osteoclasts, (e) preosteoclastic TRAP⁺ staining, and (f) mature osteoclastic TRAP activity. All data are shown as mean \pm SD for three separate experiments. * $P < 0.05$ compared with the control. BCA: biochanin A; ALP: alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase.

significantly increased urinary DPD excretion ($P < 0.01$) compared to control values. This increase was suppressed by both E2 and BCA. These results demonstrate that BCA prevents bone loss, probably as a result of decreased bone turnover. IL-1 β and TNF- α are generally recognized as osteoresorptive factors [37]: they play a critical causal role in inducing bone loss [38] by stimulating osteoclastogenesis [39] and enhancing bone resorption via the induction of RANKL in osteoblasts and the induction of osteoclast maturation [40]. Increased bone remodeling with estrogen deficiency

is mediated by the production of cytokines such as IL-1 β and TNF- α . Therefore, the inhibitory effect of BCA on bone resorption may be associated with its anti-inflammatory effects. That is, the inhibition of IL-1 β and TNF- α may lead to the inhibition of osteoclast differentiation and related activities. Moreover, the decrease in IL-1 β and TNF- α reflected in the downregulation of bone cell expression of RANKL, and the upregulation of OPG expression resulted in decreased osteoclastic maturation. We found no change in serum inorganic phosphate and calcium levels in all groups, suggesting

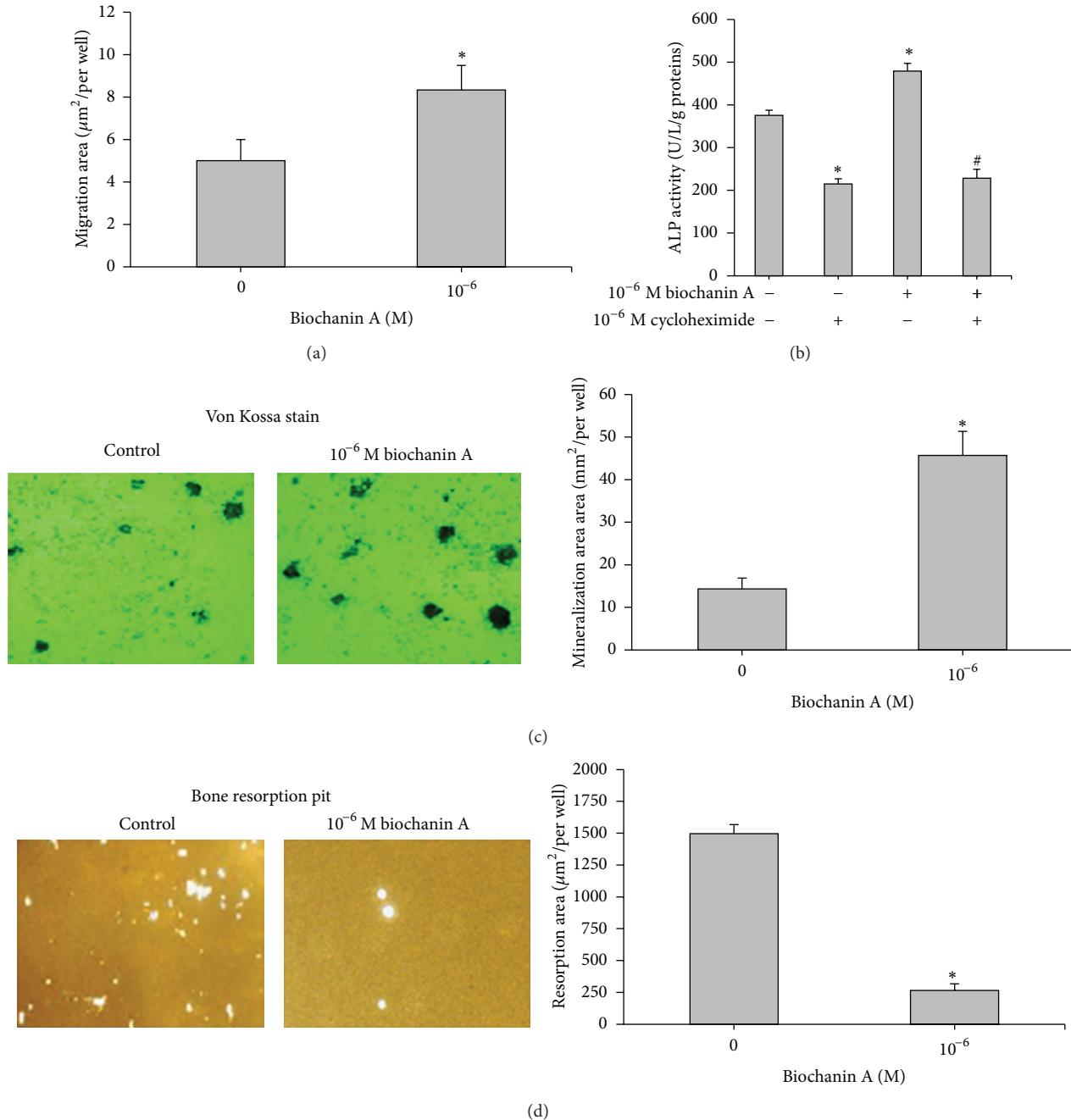


FIGURE 4: Effects of BCA on the functional activities of osteoblasts and osteoclasts. (a) Osteoblasts were incubated for 2 days with or without 10^{-6} M biochanin A, and then cell migration was evaluated as described in Section 2. (b) Osteoblasts (5×10^4 cells/well) were incubated for 7 days with 10^{-6} M BCA in the presence or absence of cycloheximide and then assayed for ALP activity. (c) Osteoblasts were cultured with or without 10^{-6} M BCA for 7 days, followed by evaluation of von Kossa staining for mineralized nodules. (d) Osteoclasts were cultured with or without 10^{-6} M BCA for 7 days, and the surface of the resorption pit area was determined. (Left) Representative photographs of stained cells. (Right) Summarized data expressed as mean \pm SD for three separate experiments. * $P < 0.05$ compared to the control; # $P < 0.05$ compared to the BCA group.

that estrogen involvement in the control of phosphate and calcium homeostasis is probably not implicated in the bone loss effects.

Bone remodeling requires a precise balance between resorption and formation. The OPG/RANK/RANKL signaling pathway is a key to regulating and maintaining the

balance between the activity of osteoblasts and osteoclasts in order to prevent bone loss and to ensure normal bone turnover [41]. The mRNA levels of OPG, which is secreted by osteoblasts and is an osteoclast-activation inhibitor that acts by binding to RANKL [42], were increased by BCA. Conversely mRNA levels for TRAP, a marker of osteoclast

differentiation [43], and RANKL, an osteoclast activating factor which induces NF κ B-p65 translocation leading to osteoclast formation [44], were decreased by BCA. The ratio of RANKL/OPG expression decreased, indicating that BCA improves bone formation, inhibits bone resorption, and decreases bone remodeling. These results show that BCA has a potent inhibitory effect at all stages of osteoclast differentiation and formation and that BCA acts on both preosteoclasts and mature osteoclasts. Furthermore, its effect appeared to be greater than that of E2. The importance of bone turnover modulation was mediated by BCA, suggesting that BCA may be a potential therapeutic drug.

Although the biochemical parameters and expression of osteogenic marker genes partly showed that BCA supplementation was more effective than E2 treatment in the present study, this might be explained by the relatively short duration of the study, or by examination of the femur tissue alone, which was not the representative of the entire physiological state. However, our results demonstrated that BCA supplementation protected against bone loss in ovariectomized rats over the time period examined in this study.

In addition, a commercial isoflavone product, BCA, offered the advantage of being relatively inexpensive, particularly compared to genistein and daidzein. We found that BCA treatment for osteoporosis was similar to that by genistein [4]; thus it may be possible to supplement with BCA tablets or isoflavone extracts manufactured from red clover.

In conclusion, BCA may provide an alternative strategy to augment bone mass, and BCA supplementation could be an alternative to HRT for the prevention of osteoporosis, especially given its range of biological effects and lack of cytotoxicity.

Acknowledgments

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Research Article

Evaluation on Anti-Inflammatory, Analgesic, Antitumor, and Antioxidant Potential of Total Saponins from *Nigella glandulifera* Seeds

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Nigella glandulifera seeds are used as a spice or remedy for the treatment of various inflammatory diseases. This study aimed to investigate analgesic (writhing test), anti-inflammatory (ear-induced edema, vascular permeability test), antioxidant, and antitumor activities of total saponins from this plant (TSN). TSN (6, 12, and 24 mg/kg) were exhibited analgesic and anti-inflammatory activities in a dose-dependent manner ($P < 0.05$). In D-galactose-induced ageing model, TSN significantly increased the plasma superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities ($P < 0.05$) and decreased the malondialdehyde (MDA) level compared to control group ($P < 0.05$). DPPH radical scavenging effect of TSN was also found. Moreover, TSN (20 mg/mL) showed 86.75% and 88.26% inhibition of the growth on Bel-7402 and Hela cells, respectively. Five compounds were further isolated and identified from TSN as Nigella A, B, C, D, and nigeglanoside, of which the content of Nigella A was 60.36 ± 1.25 g/100 g TSN by HPLC-ELSD method. Altogether, these results suggest that TSN could be considered as a potential analgesic, anti-inflammatory, antitumor, and antioxidant agent.

1. Introduction

Nigella (*Nigella sativa* L., *Nigella glandulifera* Freyn et Sint, and *Nigella damascena*, L. etc.), belonging to the buttercup family Ranunculaceae, is commonly known as black cumin (black seeds) [1]. *Nigella* seeds are widely used for medicinal purposes as a natural remedy for a number of illnesses such as hypertension, diabetes, inflammation, bronchitis, headache, and gastrointestinal disturbances [2]. In recent years, voluminous research has been carried out on the medicinal properties of the seeds as antioxidant, antimicrobial, anti-inflammatory, and anticancer agents [3–5]. These properties have been attributed to a variety of active constituents in seeds and its fixed oil [6, 7], of which saponins are mainly characteristic compounds of water-soluble extracts from *Nigella* [8]. Pharmacological effects of saponins have been reported in many references, and these compounds are considered to be beneficial to the health of mankind [9, 10].

N. glandulifera Freyn is widely distributed in Xinjiang, Yunnan, and Tibet of China and is now mainly cultivated in the Taklimakan Desert edge region in Xinjiang [11]. The seeds of *N. glandulifera* are frequently added to “naan” (a kind of crusty pancake, favorite food of the Uygur and Kazak people) as a spice, and its water decoction is used in Uighur’s traditional medicine for the treatment of numerous disorders such as diuretic, analgesic, insomnia, dizziness, tinnitus, amnesia, and bronchial asthma [12, 13]. Several classes of compounds have been isolated from water-soluble extracts of *N. glandulifera*, such as alkaloids, flavonoids, and saponins [14, 15]. Saponins with the highest content (64.5%) are mainly characteristic compound of *N. glandulifera* (TSN). However, the biological activities of saponins from this plant were rarely reported so far. Therefore, the aim of this study was to investigate anti-inflammatory, analgesic, anti-tumor, and antioxidant potential of total saponins from *N. glandulifera* by experiments *in vivo* or *in vitro*.

2. Material and Methods

2.1. Chemicals. 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Superoxide dismutase (SOD), glutathione peroxides (GSH-Pxs), and malondialdehyde (MDA) assay kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). RPMI 1640 medium was obtained from Gibco Co. (USA). HPLC-grade acetonitrile was purchased from Fisher BioReagents (NJ, USA). Yemuga tablet (YMPG) was purchased from Hepin Pharmacy Co. (Guangdong, China). The other chemicals and solvents used in this experiment were of the highest quality available.

2.2. Plant Material. *N. glandulifera* were collected from Aksu, Xinjiang, in China, in July 2011. The plant material was identified by the associate researcher Jiang He, Xinjiang Institute of Material Medica. A voucher specimen was deposited at Xinjiang Institute of Material Medica in China.

2.3. Preparation of TSN. The powdered seeds (10.0 kg) were defatted at reflux condition with petroleum ether and extracted with 30% ethanol by exhaustive maceration to yield a dark brown residue (2.2 kg). After being dissolved in water, the extract was purified by AB-8 adsorption macroporous resin to obtain total saponin extracts from *N. glandulifera* (TSN, 210 g). TSN were applied to ODS RP-18 column and eluted with mixtures of MeOH:H₂O (0:1 → 1:0) successively. Elutes were combined into five subfractions according to TLC behavior using two solvent systems CHCl₃:MeOH:H₂O (6:4:0.5) and BuOH:AcOH:H₂O (4:1:1) (spots were visualized after spraying 10% H₂SO₄). Various fractions were repeatedly purified by Sephadex LH-20 column with methanol, and five saponins were isolated from TSN, and their structures were confirmed using MS, ¹H, and ¹³C NMR (References). The purity of the saponins was determined to be more than 95% compared with the peak areas detected by HPLC-ELSD.

2.4. HPLC-ELSD Analysis of TSN. The high-performance liquid chromatography (HPLC) (LC-10A HPLC instrument, Shimadzu Co., Japan) was employed to analyze the percentage contents of Nigella A in TNS. A Cosmosil-C18 column (250 mm × 4.6 mm, 5 μm) and a Zorbax ODS C18 guard column (12.5 mm × 4.6 mm, 5 μm) were used at 40°C. A binary gradient elution system consisted of water (A) and acetonitrile (B) and separation was achieved using the following gradient program: 0–15 min, 20–40% B; 15–20 min, 40% B; 20–22 min, 40–20% B; and 22–25 min, 20% B. The flow rate was at 1.0 mL/min and the sample injection volume was 5 μL. The all tech ELSD impactor was set at ON mode, the drift tube temperature was 65°C, and the nebulizer nitrogen gas flow rate was at 2.0 L/min.

2.5. Animals. The study was conducted on Kunming mice weighting 18 ± 22 g (The Experimental Animal Center in Xinjiang, China; SCXK (Xin) 2003–2001). Animals were kept

under a 12 h/12 h light/dark cycle and allowed free access to food and water. The study protocols were approved by the Ethics Committee on Animal Experiment, Xinjiang Material Medica, China.

2.6. Cells. The human hepatoma carcinoma cell lines Bel 7402 and cervical carcinoma cell lines Hela were provided by the Institute of Materia Medica, Chinese Academy of Medical Sciences, and maintained with RPMI 1640 medium containing 10% fetal bovine serum and 100 ng/mL, each, of penicillin and streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

2.7. Acute Oral Toxicity Study in Mice. TSN were subjected to acute oral toxicity studies as per SFDA guidelines no. [Z] GPT2-1 [16]. Mice were randomly assigned to each of three groups of 30 mice (15 females and 15 males). They were fasted overnight (12 h) with free access to water prior to administration of doses (48 and 24 g/kg) of TSN dissolved in distilled water. The control group mice were p.o. administered with distilled water. The animals were observed continuously for first 72 hours and 14 days for any signs of mortality, body weight, toxicity, behavioral, and viscera changes. On day 15, mice were killed and all organs and tissues were observed macroscopically.

2.8. Cytotoxicity Assays. The human hepatoma carcinoma cell lines Bel-7402 and cervical carcinoma cell lines Hela were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in an incubator containing 5% CO₂. Cells were passaged every 2 days using Trypsin (0.25%) solution. Exponentially growing cells were used for experimentation. Cell viability assay was performed with MTT photometric analysis, as first described by Mosmann [17], with slight modification. Briefly, 4.5 × 10⁵/mL was seeded in 96-well microtiter plates at 200 μL/well. Twenty-four hours after treatment, the supernatant was removed. TNS were made into 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.156 mg/mL in cultured solution and added to 96-well microtiter plates. Forty-eight hours after treatment, 20 μL MTT reagent (5 mg/mL) was added. After 4 h incubation at 37°C, 200 μL of DMSO was added and plates were oscillated for 10 min in a balance oscillator. The extent of the MTT reduction was measured by a plate reader at a wavelength of 570 nm. The inhibitory rate of cell proliferation was calculated as follows: inhibitory rate (%) = (1 – experimental group A value/control group A value) × 100%.

2.9. Determination of Antioxidant Activity by DPPH Radical Scavenging Ability. The effects of TSN on DPPH radicals were estimated according to the method of Zhao et al. [18]. Aliquots of TSN at various concentrations were mixed with 2.0 vols of 6.5 × 10⁻⁵ M solution of DPPH. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm. The percentages of DPPH decolorization of the samples

were calculated according to the equation: radical scavenging activity (%) = [1 – (ABS sample/ABS control)] × 100. EC₅₀ value was defined as the concentration (in mg/mL) of the extract required to deplete the amount of DPPH radical by 50%. BHT was used as a positive control.

2.10. Antioxidant Effect of TSN in Ageing Mice. After 1 week of acclimatization, the mice were randomly divided into five groups (12 mice per group) and i.p. injected with 0.1 mL/10 g of 5% D-galactose once daily for 6 weeks except normal control group (i.p. injected with 0.3 mL of physiological saline) [19]. Simultaneously, normal control group mice and ageing model group mice were p.o. administered with 0.1 mL/10 g of 20% Arabic gum each, respectively; TSN treatment group mice were p.o. administered with a different dose of extract (8.8, 17.5, and 35 mg/kg). Following the 35-day treatment process and 24 h after the last administration, all 50 mice were weighed then sacrificed by the humane method of cervical dislocation. The liver was surgically excised from the animal, accurately weighed, and then homogenized immediately in ice-cold 0.9% NaCl solution (0.1 g tissue/mL solution). The suspension was centrifuged at 4000 rpm/min at 4°C for 10 min, and the supernatant was collected for further analysis. The activities of SOD, GSH-Px, and the level of MDA were measured using commercially available kits in accordance with instructions.

2.11. Analgesic Activities. The analgesic activity of TSN was investigated using acetic acid-induced writhing response in mice, and the test was carried out using the method of Muhammad et al. [20]. This method was used to preferentially evaluate possible peripheral effects of TSN as analgesic substance. Five groups of Kunming male mice (*n* = 10) were fasted overnight prior the start of the experiment, and water ad libitum. The peripheral analgesic drug, YMGP, was used as a positive control. Group 1 received the vehicle-distilled water (10 mL/kg, p.o.), and group 2 was treated with YMGP (928 mg/kg, p.o.), whereas groups 3, 4, and 5 animals were orally administered with TSN at doses of 6, 12, and 24 mg/kg. Sixty minutes after treatment, the mice were injected (i.p.) with 0.1 mL/10 g body of 0.7% acetic acid solution to induce the characteristic writhings. After 5 min, the mice were placed in an observation box, and the number of writhes in a 15 min period was counted. Antinociception (analgesia) is expressed as the reduction of the number of writhing movements between control animals (acetic-acid-treated mice) and mice pretreated with these compounds and then acetic acid.

2.12. Anti-Inflammatory Activity. The anti-inflammatory activity of TSN was investigated using the following models.

2.12.1. Xylene-Induced Ear Edema in Mice. Antiacute inflammatory activity was determined by xylene-induced mice ear edema [21]. Fifty mice were equally divided into five groups randomly including control group (distilled water), YMGP-positive control group (928 mg/kg body wt), and TSN groups (6, 12, or 24 mg/kg body wt). The vehicle and drugs

were administered orally, respectively, once per day for 3 days. One and half hour after the last administration of drugs, inflammatory response was induced on the inner and external surface of the right ear (surface: about 1 cm²) by application of 20 µL xylene. 30 min later, mice were sacrificed by cervical dislocation and a section (Ø 6 mm) of ears was removed from both the treated (right) and the untreated (left) ears. Edema rate was measured as the percentage of the weight difference between the two ear discs compared to the untreated (left) ears. The anti-inflammatory activity was expressed as the percentage of inhibition in treated mice compared to the control mice.

2.12.2. Vascular Permeability Test. The method of Koo et al. [22] was used to evaluate the effect of the extract on vascular permeability in adult albino mice of both sexes. Fifty mice were equally divided into five groups randomly including control group (distilled water), YMGP-positive control group (928 mg/kg body wt), and TSN groups (6, 12, or 24 mg/kg body wt). One and half hour after oral administration of TSN and YMGP, 0.2 mL of Evans Blue dye (0.5% in Normal saline) was intravenously administered through the tail vein. Subsequently, animals received intraperitoneal injection of 1.0 mL/100 g of acetic acid (0.6%, v/v). Treated animals were sacrificed 20 min after acetic acid injection and the peritoneal cavity washed with normal saline (6.0 mL) into heparinized tubes and centrifuged 15 min at 3000 rpm. The dye content in the supernatant was measured at 590 nm using spectrophotometer.

2.13. Statistical Analysis. The data obtained were computed using SPSS 11.5 software and later analyzed using ANOVA of variance. The Duncan test with significance level of 0.05 between means was used.

3. Results and Discussion

3.1. Phytochemical Study. Five compounds were isolated from TSN and elucidated as 3-O-[D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-28-O-[α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl] hederagenin (Nigella A, 1), 3-O-[D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-28-O-β-D-glucopyranosyl hederagenin (Nigella B, 2), 3-O-[α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-28-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl] hederagenin (Nigella C, 3), 3-O-[D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl]-28-O-[α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl] hederagenin (Nigella D, 4), and nigeglanoside (5) by extensive spectroscopic methods including 1D-¹H, ¹³C NMR experiments (Figure 1). All data were compared with those in previous literature [23–25]. Among these compounds, the content of Nigella A from TNS was far above others. Therefore, the content of Nigella A was determined by HPLC-ELSD, and its content is 60.36 ± 2.5 g/100 g in TSN (Figure 2).

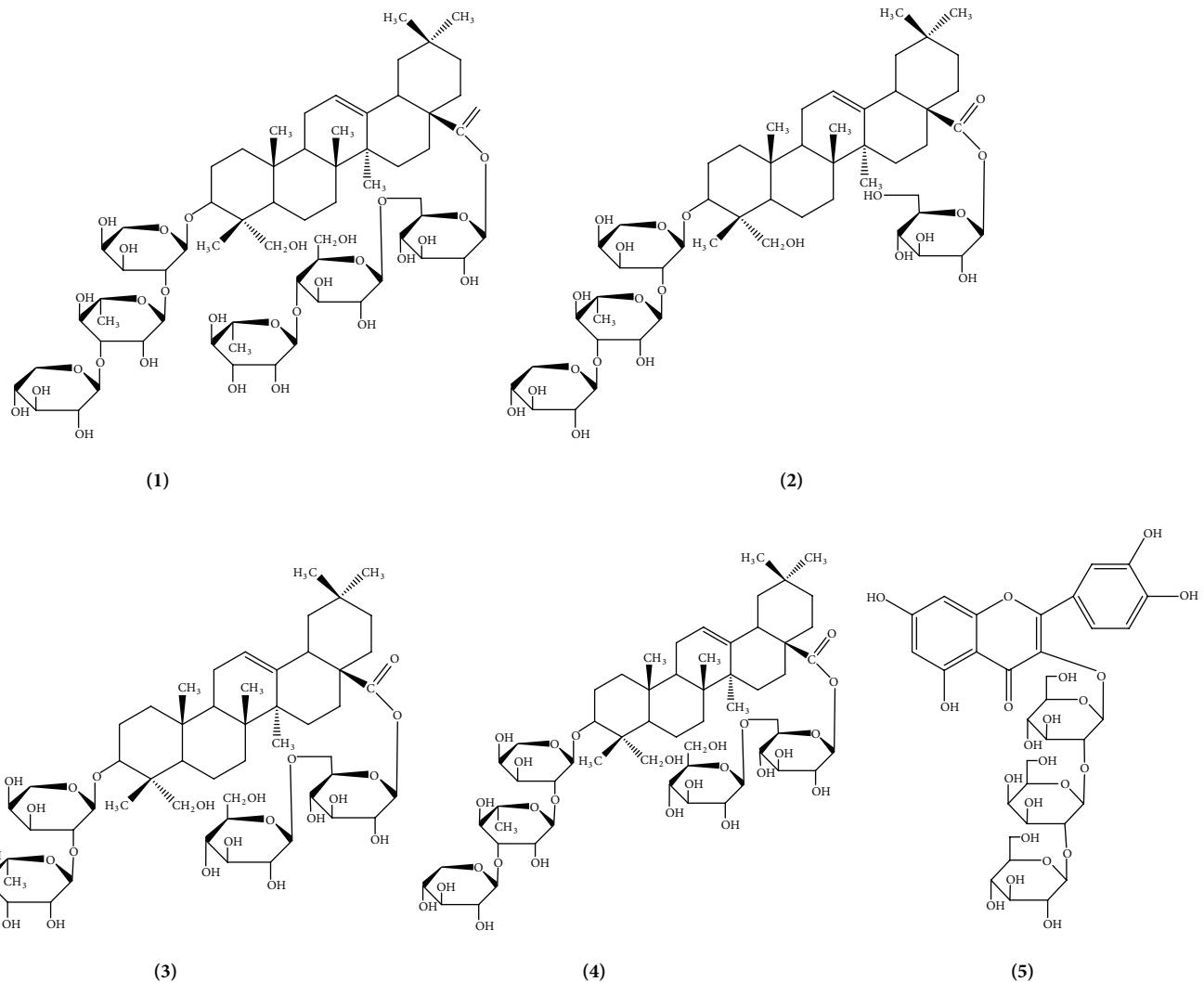


FIGURE 1: Chemical structure of compounds.

3.2. Acute Toxicity. Following administration of TSN, the mice were monitored daily for mortality, clinical signs, and gross changes in appearance and behavior. No deaths occurred in the test and control group. There were no significant differences in body weights between the TSN treated and control group in either sex (data not reported in this paper). The result indicated that treatment of TSN was safe under the maximum dose at 48 g TSN/kg body.

3.3. Cytotoxicity Activity. Two cell lines, that is, Bel-7402, and Hela cells, were used to test the effect of TSN on their cell proliferation. TSN showed significant inhibitory effects on these two cell lines, which were in a dose-dependant manner. TSN at the dose of 20 mg/mL showed 86.75% and 88.26% inhibition of the growth of Bel-7402 and Hela cells, respectively (Figure 3).

3.4. DPPH Radical Scavenging Activity. The scavenging capability of DPPH radical was determined by the decrease in

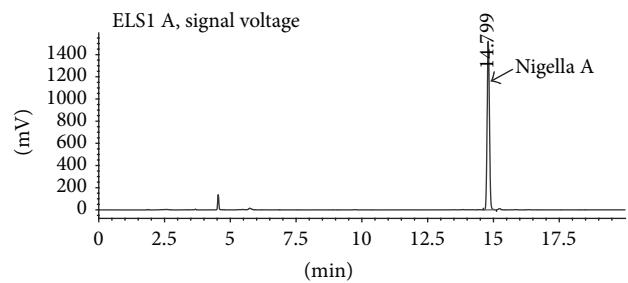


FIGURE 2: HPLC chromatogram of TSN solution.

its absorbance at 517 nm under effect of antioxidants. Due to rapid hydrogen-accepting ability, DPPH reacted with antioxidants and converted into 1,1-diphenyl-2-picrylhydrazin, shows decrease in absorbance simultaneously [26]. The degree of discoloration indicates the scavenging potential of the antioxidant extracts [27]. Figure 4 showed the DPPH free radical scavenging activities of TSN. DPPH was reduced

TABLE 1: Effect of TSN on MDA, SOD, and GSH-Px in liver in D-galactose-induced ageing mice.

Group	Dose (mg/kg·d)	MDA (nmol/mgprot)	SOD (U/mgprot)	GSH-Px (U/mgprot)
Control	—	1.181 ± 0.187	259.785 ± 34.007	577.273 ± 95.582
Model	—	1.789 ± 0.383 [#]	173.843 ± 21.939 [#]	270.775 ± 35.582 [#]
	8.8	1.296 ± 0.206 ^{**}	181.220 ± 13.194	300.344 ± 26.338 ^{**}
TSN	17.5	1.252 ± 0.398 ^{**}	194.609 ± 18.456 [*]	354.958 ± 69.549 ^{**}
	35	1.236 ± 0.315 ^{**}	203.477 ± 17.719 ^{**}	411.949 ± 57.553 ^{**}

Values are expressed as mean ± SD ($n = 12$).

[#] $P < 0.01$, compared with control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, compared with model group.

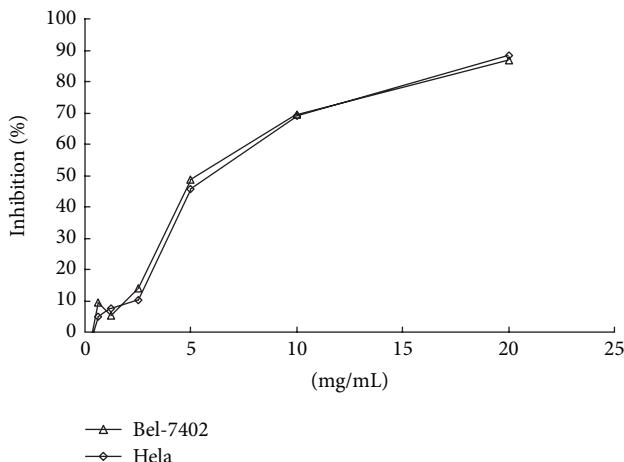


FIGURE 3: Inhibiting effect of TSN on human hepatoma carcinoma cell lines Bel 7402 and cervical carcinoma cell lines Hela growth. Results are mean ± SD ($n = 4$).

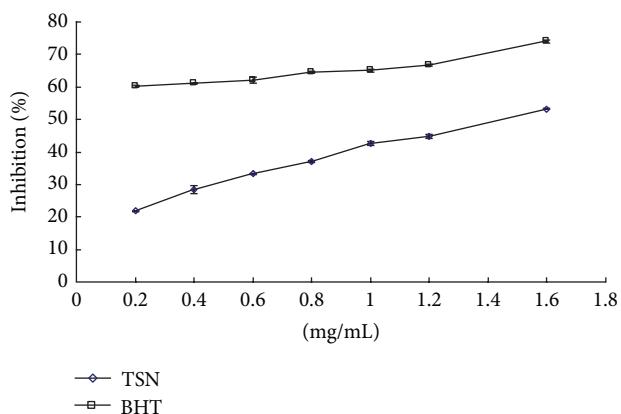


FIGURE 4: DPPH radical scavenging activity of TSN. Results are mean ± SD ($n = 3$).

by TSN in a concentration-dependent manner. Figure 4 shows the dose response curves of DPPH radical scavenging activities of TSN, at a concentration range of 0.2–1.6 mg/mL, and percent inhibition and the IC₅₀ of TSN were 21.87–53.28% and 1.58 mg/mL, respectively. BHT as a reference drug scavenged the DPPH radical by inhibitions of 60.31–74.01% and the IC₅₀ value of 58.07 μg/mL.

3.5. The Effect of TSN on the Activities of SOD, GSH-Px, and MDA in the Liver of Aged Mice. Ageing is a natural process in all living organisms, and oxidative stress is one of root causes of the ageing process [28]. The importance of antioxidant enzymes is generally emphasized in the prevention of oxidative stresses by scavenging of ROS [29, 30]. The antioxidant system comprises several enzymes such as SOD and GSH-Px [31–33]. The effect of TSN on the activities of SOD, GSH-Px, and the levels of MDA in the liver of aged mice is shown in Table 1. A marked increase in MDA and significant decrease ($P < 0.05$) of antioxidant enzymes activity (SOD, GSH-Px) were observed in the liver between the treatments of normal control group and model control group. TSN treatment significantly inhibited ($P < 0.05$) the formation of MDA in the liver and raised the activity of antioxidant enzymes in a dose-dependent manner. The administration of TSN to the D-galactose-treated mice with 8.8, 17.5, and 35 mg/kg increased both SOD and GSH-Px enzymatic antioxidant liver activity ($P < 0.01$ and $P < 0.05$).

3.6. Anti-Inflammatory and Analgesic Activities. The peripheral analgesic effect may be mediated through inhibition of cyclo-oxygenases and/or lipoxygenases (and other inflammatory mediators), while the central analgesic action may be mediated through inhibition of central pain receptors [34]. The acetic-acid-induced writhes have often been used to evaluate the peripherally and centrally acting analgesic drugs [35]. YMGP is the most popular medication for rheumatism arthritis pain, sciatica, and prosopalgia in Chinese medicine. Therefore, we select YMGP as a reference drug.

Peripheral analgesic activity was assessed by acetic-acid-induced writhing test, which showed significant ($P < 0.01$ and $P < 0.05$) suppression of writhes (Table 2). Acetic acid is known to trigger the production of noxious substances within the peritoneum, which induces the writhing response. The effect of TSN against the noxious stimulus may be an indication that it depressed the production of irritants and thereby bringing a reduction in the number of writhes in animals. Injection of acetic acid into the control mice resulted in 71 ± 19 writhes. Pretreatment with TSN at the doses of 6, 12, and 24 mg/kg reduced the number of writhes to 38 ± 8 (46.5% inhibition), 33 ± 9 (53.6% inhibition), and 29 ± 8 (59.2% inhibition) at a dose-activity dependence relationship, respectively. Interestingly, the analgesic activity of high dose was higher than that of YMGP (33 ± 8 writhes, 54.5% inhibition). It was also observed that the onset of writhing

TABLE 2: Effects of TSN on acetic-acid-induced writhing response in mice.

Treatment group	Dose (mg/kg)	Number of writhes	Inhibition (%)
Control	—	71 ± 19	—
YMGP	928	33 ± 8**	54.5
	6	38 ± 8**	46.5
TSN	12	33 ± 9*	53.6
	24	29 ± 8**	59.2

Values are expressed as mean ± SD ($n = 10$).

Control (vehicle): distilled water; YMGP: Yemugua tablet; TSN: total saponins from *Nigella glandulifera*. ** $P < 0.01$, * $P < 0.05$, compared with corresponding control.

was delayed and the duration of writhing was shortened with TSN pretreatment.

Xylene-induced mouse ear edema reflects the oedematization during the early stages of acute inflammation, which was probably related to the release and inhibition of the inflammation factors [36]. In xylene-induced ear edema, the oral administration of TSN suppressed significantly xylene-induced ear oedema in mice. The oedema inhibitory rates of casticin were 16.6%, 25.9%, and 35.5% at doses of 6, 12 and 24 mg/kg, respectively, whereas YMGP (928 mg/kg) produced 30.53% inhibitory rate compared with control (Figure 5).

The inflammatory response is a physiological characteristic of vascularized tissues also [37]. Exudation, which is a consequence of increased vascular permeability, is considered a major feature of acute inflammation. In the inflammatory reaction, increased vascular permeability leads to exudation of fluid rich in plasma proteins including immunoglobulins, coagulation factors, and cells into the injured tissues [38] (with subsequent edema at the site). Therefore, inhibition of increased vascular permeability can modulate the extent and magnitude of the inflammatory reaction [39, 40]. Chemical-induced vascular permeability causes an immediate sustained reaction that is prolonged over 24 h, and its inhibition suggests that the extract may effectively suppress the exudative phase of acute inflammation [41], and acetic-acid-induced increased vascular permeability in mouse model is a typical capillary permeability assay [42, 43]. In this study, TSN significantly reduced the increased peritoneal vascular permeability, indicating the suppression of the vascular response in the process of acute inflammation (Figure 6). Oral administration of 6, 12, and 24 mg/kg of TSN evoked a significant ($P < 0.05$) dose-related inhibition of vascular permeability induced by acetic acid in mice. At the doses of 6, 12, and 24 mg/kg, TNS produced 17.9%, 23.9%, and 29.4% inhibition of dye leakage, respectively, with dose-dependent manner, while YMGP produced 26.9% inhibition.

4. Conclusion

In summary, our results suggest that TSN possesses anti-inflammatory, analgesic, antitumor, and free radical scavenging activities and provide some scientific evidence to support

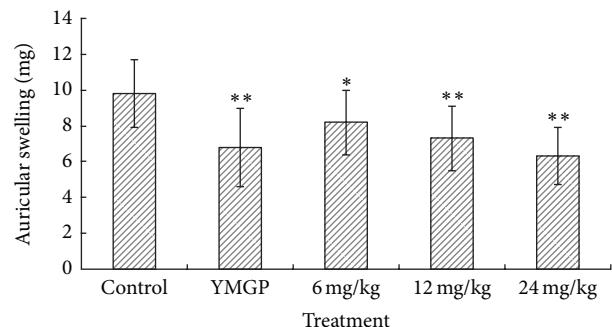


FIGURE 5: Effects of TSN and YMGP on xylene-induced ear oedema in mice. Values are mean ± SD of differences in weight between right and left ears of animals. $n = 10$. Control (vehicle): distilled water; YMGP: Yemugua tablet; TSN: total saponins from *Nigella glandulifera*. * $P < 0.05$ and ** $P < 0.01$, compared with corresponding control.

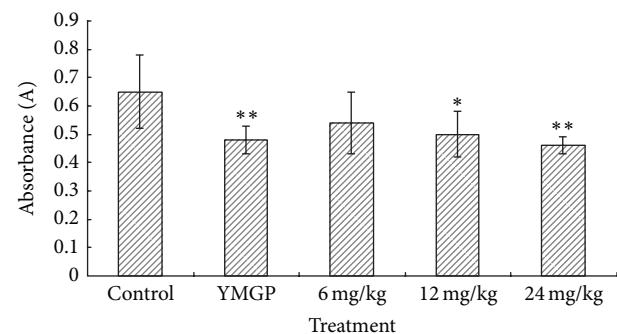


FIGURE 6: Effects of TSN and YMGP on acetic-acid-induced increased vascular permeability in mice. Values are mean ± SD, $n = 10$. Control (vehicle): distilled water; YMGP: Yemugua tablet; TSN: total saponins from *Nigella glandulifera*. * $P < 0.05$ and ** $P < 0.01$, compared with corresponding control.

the folk medicinal utilization of *N. glandulifera*. Furthermore, TSN is a good candidate for the development of new anti-inflammatory and analgesic medicine compared with efficacy of YMGP. Therefore, TSN may be worth further investigating and elucidating.

Conflict of Interests

All authors declare that they have no competing interests.

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Research Article

Use of Herbal Dietary Supplement Si-Wu-Tang and Health-Related Quality of Life in Postpartum Women: A Population-Based Correlational Study

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Objective. The aim of the study was to explore the association between women's use of herbal dietary supplement Si-Wu-Tang during the postpartum period and their health-related quality of life. **Methods.** This is a population-based correlational study. We used multistage, stratified, systematic sampling to recruit 24,200 pairs of postpartum women and newborns from the Taiwan National Birth Registry in 2005. A structured questionnaire was successfully administered to 87.8% of the sampled population. Trained interviewers performed home interviews 6 months after the women's deliveries between June 2005 and July 2006. The Medical Outcomes Study 36-item Short-Form (SF-36) was used to measure the quality of life of the women along with the frequency of Si-Wu-Tang use. **Results.** Si-Wu-Tang use after delivery improved women's score for bodily pain and also improved their score for mental health when used more than 10 times. In addition, there were increases in general health and vitality scores in the group who continuously used Si-Wu-Tang more than 10 times after using Sheng-Hua-Tang. **Conclusion.** Use of Si-Wu-Tang after delivery may be associated with women's health-related quality of life especially for those who previously used Sheng-Hua-Tang. These results are exploratory and need to be replicated.

1. Introduction

Chinese postpartum care (*zuo yuezi*) has been regarded as a crucial rite-of-passage for the woman's recovery and the transition to motherhood after childbirth [1]. Previous studies have shown the beneficial effects of *zuo yuezi* on physical and mental health [2, 3] as well as social support [4]. During the postpartum period, the use of herbal dietary

supplements is common and is frequently recommended by family members in Chinese communities [5]. The use of Sheng-Hua-Tang, which can relieve abdominal discomfort and eliminate lochia [6], is mostly common used during this period [7]. Our previous study has shown that Sheng-Hua-Tang use during the first month of the postpartum period may have a positive effect on women's health-related quality of life, especially in terms of role limitations due to physical health

and emotional problems [8]. In Taiwan, Si-Wu-Tang, which is believed to speed the recovery from delivery, is commonly used after taking Sheng-Hua-Tang [7].

Si-Wu-Tang was originally listed in the Prescriptions from the Great Peace Imperial Grace Pharmacy and has been used as a basic formula in traditional Chinese medicine for the treatment of women's illnesses since the 12th century. The formula includes 4 ingredients: Radix Angelicae Sinensis 12.0 g, Rhizoma Ligustici Chuanxiong 6.0 g, Radix Rehmanniae Praeparata 9.0 g, and Radix Paeoniae Alba 9.0 g, and is available in many forms including pill, tablet, or capsule, drink or syrup, and natural or herbal form. Si-Wu-Tang is typically used for its action as a blood-tonifying (buxie) decoction [9] and is believed to stimulate the production of blood, promote blood circulation, regulate menstruation, and relieve menstrual pain [10]. A recent Cochrane review found promising evidence for the use of various Chinese herbal medicines in reducing menstrual pain in the treatment of primary dysmenorrhoea [11]. The majority of the 39 included trials used a complicated formula with more than 5 or 6 herbs. Two of them used modified Si-Wu-Tang and found that it resulted in a reduction of menstrual pain. Two recent studies also demonstrated that Si-Wu-Tang can decrease menstrual pain for primary dysmenorrhoea [12, 13].

Although there is evidence that supports the use of Si-Wu-Tang for primary dysmenorrhoea, its potential benefits in postpartum women have not been evaluated. It has been suggested to have four potential biological effects: anti-inflammation [14–18], uterine relaxation [19], haematological responses [20–24], and psychological responses [25, 26]. These effects improve postpartum women's overall physical and mental health.

Another interesting hypothesis needs to be examined. There may be additional beneficial effects of Si-Wu-Tang use in women with previous Sheng-Hua-Tang use in the postpartum period. Sheng-Hua-Tang includes 5 ingredients: Radix Angelicae Sinensis 24.0 g, Rhizoma Ligustici Chuanxiong 9.0 g, Semen Persicae 6.0–9.0 g, Zingiberis Rhizoma 1.5 g, and Glycyrrhizae Radix 1.5 g [6]. Both Si-Wu-Tang and Sheng-Hua-Tang share two common ingredients: Radix Angelicae Sinensis and Rhizoma Ligustici Chuanxiong. These two ingredients also have three similar biological effects: anti-inflammation [27], uterine relaxation [28], and haematological response [29–31]. These combined effects enhance the effects of Si-Wu-Tang on postpartum women's health-related quality of life.

It is common that Si-Wu-Tang is taken after Sheng-Hua-Tang use during the postpartum period to speed the recovery from delivery. Our previous study in Taiwan has shown that 44.8% of postpartum women used Si-Wu-Tang following Sheng-Hua-Tang use (82.6%) after delivery [7]. Although a high percentage of postpartum women use Si-Wu-Tang after the use of Sheng-Hua-Tang, no previous studies have investigated Si-Wu-Tang's potentially beneficial effects. The aim of the study was to explore the association between women's use of Si-Wu-Tang during the postpartum period and their health-related quality of life.

2. Materials and Methods

2.1. Study Design and Participants. A population-based correlational study was based on the Taiwan Birth Cohort Study (TBCS) which is the first national birth cohort study in Taiwan. In the current study, we used a multi-stage, stratified, systematic sampling design to obtain representative samples from the Taiwan National Birth registry in 2005. We ranked a total of 369 towns in Taiwan into 12 strata according to the administrative division (four strata) and the total fertility rate (three strata). Using the principle of proportion probability to size, we randomly sampled 90 towns out of 369 in Taiwan. A total of 24,200 pairs of postpartum women and newborns were recruited from these 90 towns [7].

We conducted home interviews of 24,200 women in the postpartum period, 6 months after their delivery, from June 2005 to July 2006. We lost 2,952 women to follow-up because of refusal to participate, changes in residence, incorrect addresses, infant deaths, and other reasons. A total of 21,248 postpartum women were interviewed. The completed interview rate was 87.8% [8]. We excluded incomplete questionnaires ($N = 333$), unmarried women ($N = 586$), handicapped or severely ill women ($N = 160$), women with pre-existing conditions, including diabetes mellitus, heart disease, and hypertension ($N = 267$) and postpartum depression ($N = 139$), women admitted to the hospital during the 6 months after childbirth ($N = 269$), and women with infants who had congenital defects or severe illnesses ($N = 1,005$). A total of 18,489 women were included in this study.

2.2. Data Collection. All study participants provided informed consent approved by the Ethics Review Board of the National Taiwan College of Public Health. Before the home interview, researchers delivered a card to notify the participating women about the interview and invited them to participate in the survey. After the women agreed to participate, interviewers visited the families at their homes and asked them to give informed consent after explaining the details of the study.

Data were collected using a structured questionnaire. We requested information on age, education, family income, employment status, parity, method of delivery, breastfeeding, postpartum nursing centre, outpatient clinic visits in the past 4 weeks, primary nursery by mother, use of Si-Wu-Tang and Sheng-Hua-Tang, and health-related quality of life.

2.3. Use of Si-Wu-Tang. Information regarding the pattern of use of Si-Wu-Tang was obtained from the interview questionnaire [7]. The questions included were "Did you use Si-Wu-Tang during the postpartum period?"; "When did you use Si-Wu-Tang during the postpartum period?"; and "How many times did you use Si-Wu-Tang during the postpartum period?". We classified the frequency of Si-Wu-Tang use into three categories: 0, 1–10, and >10 times. We asked similar questions regarding the use of Sheng-Hua-Tang.

2.4. Health-Related Quality of Life Measures. The Medical Outcome Study 36-item Short-Form Health Survey (SF-36) is a generic health-related quality of life questionnaire [32]. The Taiwanese version was translated, back translated, and judged for similar meaning, and it demonstrated good reliability and validity in a healthy adult sample [33, 34]. It is identical to the original Version 1 of SF-36 and contains 36 items, which are grouped into the following eight scales: physical functioning, role limitations due to physical health problems, bodily pain, general health perceptions, vitality, energy or fatigue, social functioning, role limitations due to emotional problems, and general mental health. The score on each scale ranges from 0 to 100 with a higher score indicating better health or functioning.

2.5. Covariates. We considered potential confounders for quality of life, including maternal age (<25, 25–29, 30–34, ≥35 years), education (junior high school and below, senior high school, and university and above), family income per month (<50,000, 50,000–69,999, 70,000–99,999, ≥100,000 new Taiwan dollars, NT\$, 1 US\$ ≈ 30.5 NT\$ in 2008), employment status (yes or no), parity (1, 2, ≥3), method of delivery (normal spontaneous delivery, Caesarean section), breastfeeding (ever, never), use of postpartum nursing centre (yes or no), out-patient clinic visits during the past 4 weeks (yes or no), infant sex (male, female), low birth weight or preterm (yes or no), primary nursery by mother (no, daytime, evening or night, whole day), Sheng-Hua-Tang use (yes, no), and time periods of Sheng-Hua-Tang and Si-Wu-Tang use (no use, within first month only, within first month and later).

2.6. Data Analysis. A chi-square test was used to compare the characteristics of subjects among the three groups by frequency of Si-Wu-Tang use. We analysed the association between the frequency of use of Si-Wu-Tang and women's health-related quality of life scores by one-way ANOVA. To control for potential confounders, the differences of SF-36 scores among the groups were evaluated by analysis of covariance (ANCOVA) and a least significant difference (LSD) multiple comparison test. The statistical threshold for significance was set at 0.05. The statistical analysis was performed using SPSS for Windows, Release 16.0.

3. Results

3.1. Characteristics of Study Participants. The characteristics of study participants according to frequency of use of Si-Wu-Tang during the 6-month postpartum period are shown in Table 1. Of the 18,489 women, 10,184 (55.1%) never used Si-Wu-Tang, and 5,806 (31.4%) and 2,499 (13.5%) used Si-Wu-Tang 1–10 times and more than 10 times during the 6-month postpartum period, respectively. There were 7,354 (39.8%) and 7,964 (43.1%) women who used Si-Wu-Tang with or without previous use of Sheng-Hua-Tang, respectively. Compared with women who did not use Si-Wu-Tang, those who used it 1–10 times or more than 10 times were slightly younger and had slightly lower levels of education and family income per month ≥70,000 NT\$. In addition, these women

were more likely to be multiparous, had stayed at postpartum nursing centres, and had more visits to out-patient clinics during the past 4 weeks.

3.2. Crude SF-36 Scores by the Frequency of Si-Wu-Tang Use. Table 2 presents crude SF-36 scores by the frequency of Si-Wu-Tang use during the 6-month postpartum period. The scores for vitality ($F = 3.70, P = 0.03$) and mental health ($F = 7.52, P = 0.001$) significantly increased in the groups who used Si-Wu-Tang more than 10 times compared with those who did not use it or used it 1–10 times. However, the highest score for physical function ($F = 4.87, P = 0.01$) was present in the group that used Si-Wu-Tang 1–10 times, and the highest score for role-emotional ($F = 9.10, P < 0.001$) was present in the group that did not use Si-Wu-Tang.

3.3. Adjusted SF-36 Scores by the Frequency of Si-Wu-Tang Use Stratified by Previous Sheng-Hua-Tang Use. Figure 1 also shows adjusted SF-36 scores by the frequency of Si-Wu-Tang use during the 6-month postpartum period stratified by previous Sheng-Hua-Tang use. After controlling for potential confounders, the score for bodily pain ($F = 6.54, P = 0.001$) significantly improved in the groups who used Si-Wu-Tang either 1–10 times or more than 10 times compared with those who did not use Si-Wu-Tang. There was also a significant increase in the mental health score ($F = 7.03, P = 0.001$) in the group who used Si-Wu-Tang more than 10 times.

For women with previous Sheng-Hua-Tang use, the score for bodily pain ($F = 6.27, P = 0.002$) significantly improved in the groups who used Si-Wu-Tang either 1–10 times or more than 10 times compared with those who did not use Si-Wu-Tang. There were also significant increases in the general health ($F = 3.50, P = 0.03$), vitality ($F = 3.44, P = 0.03$), and mental health ($F = 5.62, P = 0.004$) scores in the group who used Si-Wu-Tang more than 10 times. However, no significant benefits were found in those without previous Sheng-Hua-Tang use.

4. Discussion

Our results indicate that Si-Wu-Tang use after delivery improved women's SF-36 score for bodily pain, and the use of Si-Wu-Tang more than 10 times during this period also increased their score of mental health. Additionally, the SF-36 scales displayed increases in the general health and vitality scores in the group who used Si-Wu-Tang more than 10 times after Sheng-Hua-Tang use. However, no significant benefits were found in women without previous Sheng-Hua-Tang use.

Si-Wu-Tang may benefit women's overall physical health, as shown in the bodily pain score of the SF-36 scale. It has been demonstrated to have pharmacological effects including the release of prostaglandin E2 [15], the reduction of cyclooxygenase-2 expression [18], anti-inflammatory activity [14, 15], the inhibition of histamine release [17], reducing coetaneous inflammatory diseases [16], and the inhibition of uterine contractions [19], which may be related to reduced physical pain. Si-Wu-Tang has also been shown to reduce

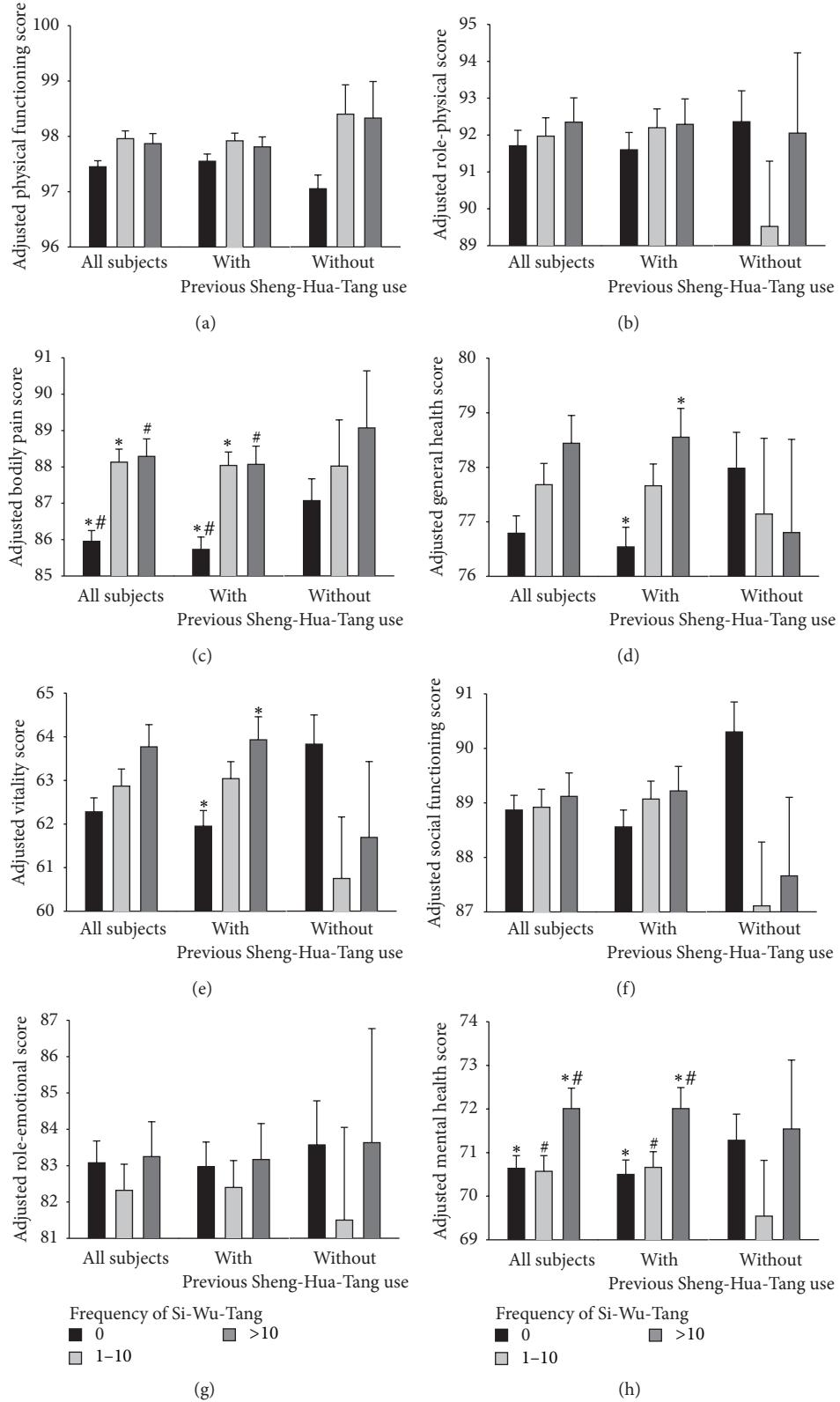


FIGURE 1: Adjusted SF-36 scores of study subjects according to the frequency of Si-Wu-Tang use during the 6-month postpartum period and stratified by previous Sheng-Hua-Tang use. Values were based on ANCOVA tests and adjusted for mother's age, education, family income per month, employment status, parity, method of delivery, breastfeeding, postpartum nursing centre, out-patient clinic visits during the past 4 weeks, infant low birth weight or preterm, primary nursery by mother, Sheng-Hua-Tang use, and duration of Sheng-Hua-Tang and Si-Wu-Tang use. *#Scores differ significantly from each other according to a least significant difference (LSD) multiple comparison test. I bars indicate standard errors.

TABLE 1: Characteristics of study subjects according to the frequency of Si-Wu-Tang use during the 6-month postpartum period.

Characteristics	Frequency of Si-Wu-Tang use during the 6-month postpartum period			χ^2	P
	0	1–10	>10		
	10184	5806	2499		
Total					
Previous Sheng-Hua-Tang use (%) ^a					
Yes	78.2	88.7	88.2	343.35	<0.001
No	21.8	11.3	11.8		
<i>Mothers' characteristics</i>					
Age (%) ^a					
<25 years	18.9	20.3	19.8		
25–29 years	35.7	37.0	40.0	44.87	<0.001
30–34 years	32.1	31.6	29.8		
≥35 years	13.3	11.1	10.4		
Educational level (%) ^a					
Junior high school or below	15.1	13.3	13.3		
Senior high school	37.4	41.5	42.9	43.44	<0.001
University or above	47.5	45.2	43.8		
Employment status (%) ^a					
Yes	57.6	59.1	58.2	3.51	0.17
No	42.4	40.9	41.8		
Family income per month (%) ^a					
<50,000 NT\$	40.8	42.2	40.7		
50,000–69,999 NT\$	25.5	27.1	26.9	19.94	0.003
70,000–99,999 NT\$	21.9	20.6	20.5		
≥100,000 NT\$	11.8	10.1	11.9		
Parity (%) ^a					
1	51.7	48.4	48.6		
2	38.4	40.0	39.1	28.46	<0.001
≥3	9.9	11.6	12.3		
Method of delivery (%) ^a					
Normal spontaneous delivery	66.3	66.4	68.4	1.10	0.58
Caesarean section	33.7	33.6	32.6		
Breastfeeding (%) ^a					
Ever	82.6	82.7	83.7	1.70	0.43
Never	17.4	17.3	16.3		
Postpartum nursing centre (%) ^a					
Yes	6.9	7.5	8.5	8.44	0.02
No	93.1	92.5	91.5		
Out-patient clinic visits during the past 4 weeks (%) ^a					
Yes	31.1	33.2	31.9	7.47	0.02
No	68.9	66.8	68.1		
<i>Infants' characteristics</i>					
Sex (%) ^a					
Male	52.3	51.5	52.2	0.95	0.62
Female	47.7	48.5	47.8		

TABLE 1: Continued.

Characteristics	Frequency of Si-Wu-Tang use during the 6-month postpartum period			χ^2	<i>P</i>
	0	1–10	>10		
Low birth weight or preterm (%) ^a					
Yes	10.6	9.9	10.4	1.71	0.43
No	89.4	90.1	89.6		
Primary nursery by mother (%) ^a					
No	14.2	13.8	14.3		
Daytime	2.1	2.1	2.8	7.56	0.27
Evening or night	8.3	8.0	7.7		
Whole day	75.4	76.1	75.2		

^aCalculated by column totals.

TABLE 2: Mean SF-36 scores and standard errors of study subjects according to frequency of Si-Wu-Tang use during the 6-month postpartum period.

SF-36 scales	Frequency of Si-Wu-Tang use during the 6-month postpartum period			<i>F</i>	<i>P</i>
	0	1–10	>10		
Physical functioning	97.54 ± 6.42*	97.85 ± 5.76*	97.74 ± 5.92	4.87	0.01
Role—physical	91.98 ± 22.42	91.66 ± 22.82	91.97 ± 22.64	0.39	0.68
Bodily pain	86.76 ± 16.53	87.17 ± 16.36	87.21 ± 16.31	1.54	0.22
General health	77.21 ± 17.44	77.15 ± 17.85	77.91 ± 17.16	1.83	0.16
Vitality	62.48 ± 17.53*	62.62 ± 17.15 [#]	63.53 ± 17.48*, [#]	3.70	0.03
Social functioning	88.93 ± 14.66	88.85 ± 14.69	89.06 ± 14.82	0.17	0.85
Role—emotional	83.76 ± 31.59*, [#]	81.55 ± 33.54*	82.26 ± 32.51 [#]	9.10	<0.001
Mental health	70.83 ± 15.83*	70.34 ± 15.96 [#]	71.78 ± 15.76*, [#]	7.25	0.001

*,[#]Scores differ significantly from each other in the row according to a least significant difference (LSD) multiple comparison test.

blood-stagnation through the inhibition of platelet aggregation [21] and induce increased levels of circulating red blood cells, haemoglobin, and haematocrit [20, 22–24]. All of these biological effects might contribute to Si-Wu-Tang's effect on women's general health and well being during the postpartum period. Antimetastatic [35] and antitumour effects of Si-Wu-Tang against endometrial carcinoma have also been found and are likely related to the suppression of oestrogen receptor-alpha mRNA expression [36]. However, we do not know whether or not there is a direct link between these effects and postpartum women's health.

Si-Wu-Tang may also benefit women's mental health, as shown in the mental health score of the SF-36 scale. We do not know the mechanisms underlying this benefit. As previously mentioned, Si-Wu-Tang likely reduces menstrual pain and increases blood circulation and production. It may also improve women's overall physical health and, in turn, enhance their mental health status. Furthermore, paeoniflorin, one of the active components of Radix Paeoniae Alba, contributes to the cognitive-enhancing effects of Si-Wu-Tang [25, 26]. More research on other psychotropic effects of Si-Wu-Tang is warranted.

However, there was no clear dose-dependent relationship between Si-Wu-Tang and the improved score for bodily pain or mental health. Because we only asked about the frequency

of Si-Wu-Tang use during the 6-month postpartum period, we do not know the exact dosage or the duration of use. In addition, the purposes for Si-Wu-Tang use vary and thus may require different dosages or durations. Dosage ratios among the 4 ingredients, additional ingredients, and preparation methods also vary for different decoction prescriptions. Thus, further randomised controlled trials are needed to answer the question.

Our previous study [8] found that the use of Sheng-Hua-Tang during the first month of the postpartum period significantly increased the SF-36 score in role limitations due to physical health and emotional problems but the use of Si-Wu-Tung in this study significantly improved the score in bodily pain and mental health. The effect of Sheng-Hua-Tang has been shown in animal models that it increases the myoelectric activity of rabbit uterine smooth muscle [37] and the contraction of the uterus when cotreated with oestrogen [38]. A recent study also showed that Sheng-Hua-Tang might increase the contractile activity and participate in the returning of the uterus to its anteverted position in postpartum women [39]. This might explain why the beneficial effects of Sheng-Hua-Tang are different from those of Si-Wu-Tang.

Additional benefits of Si-Wu-Tang use on general health and vitality based on the SF-36 scale were found in women

with previous Sheng-Hua-Tang use in the postpartum period. This finding may result from the cumulative effects of their similar ingredients or their similar pharmacological effects. For example, both Sheng-Hua-Tang and Si-Wu-Tang share two common ingredients: Radix Angelicae Sinensis and Rhizoma Ligustici Chuanxiong which have three similar biological effects: anti-inflammation [27], uterine relaxation [28], and haematological response [29–31]. On the other hand, there were no significant benefits found in women with no previous Sheng-Hua-Tang use. Although there is no obvious explanation for this discrepancy, this implies that Si-Wu-Tang taken after Sheng-Hua-Tang use during the postpartum period may speed the recovery from delivery and return to prepregnancy state.

However, the differences between the groups were very small and may not be important in clinical significance. This may be partially due to the fact that our study is based on postpartum women from the general population rather than a specific population with menstrual disorders, such as primary dysmenorrhoea. Although we excluded women with major diseases, we do not know whether our population had any other menstrual symptoms or other reasons to use Si-Wu-Tang. Si-Wu-Tang can be obtained from any Chinese herbal medicine shop without a prescription by a doctor of Chinese medicine. Thus, we cannot generalise our results to any specific menstrual disorders.

These effects may be confounded by other aspects of Chinese postpartum care (*zuoyuezi*). Chinese postpartum care may provide valuable social support [4] and reduce physical and depressive symptoms [2, 3]. However, women whose caregiver was their mother-in-law or who perceived *zuoyuezi* as unhelpful had twice the odds for postpartum depression [40]. It has been found that conflict with the mother-in-law is a significant stressor for married Chinese women. If *zuoyuezi* is included in the model, there could be over- or under-adjustment of our estimates. Thus, we only included the variable regarding postpartum nursing centres after deliveries [41].

We have also considered other major potential confounders in our model, including the mother's age, education, family income per month, employment status, parity, method of delivery, breastfeeding, out-patient clinic visits during the past 4 weeks, infant low birth weight or preterm, and primary nursery by mother [42]. However, we did not explore every dimension that could potentially be related to women's quality of life. Although we attempted to collect all this information, we are concerned that the quality may be poor in this large cohort study.

Our study has potential limitations. The retrospective measurement of Si-Wu-Tang use may be subject to recall bias. We used a structured questionnaire administered by experienced interviewers to collect information. We first asked whether they used Si-Wu-Tang and the time period of use (during or after *zuoyuezi*) and then inquired about frequency [7]. The order of questions was meant to minimise recall bias. In addition, postpartum women usually obtain Si-Wu-Tang in finished herbal products from Chinese herbal stores rather than the whole herbs. The dosage ratios among the 4 ingredients, additional ingredients, and preparation

methods may vary for decoction prescriptions. The possibility of random misclassification of the information exists, but this would not be expected to produce false-positive findings. We have also acknowledged that the cross-sectional study design may limit our conclusions. Nonetheless, the study results provide practical information for using Si-Wu-Tang in postpartum women.

5. Conclusions

Use of Si-Wu-Tang after delivery may be associated with women's health-related quality of life especially for those who previously used Sheng-Hua-Tang. Based on this and our previous studies [8], we suggested that Si-Wu-Tang taken after Sheng-Hua-Tang use during the first month of the postpartum period may benefit women's health. These results are exploratory and need to be replicated. Future studies employing a randomised, double-blind, placebo-controlled design are warranted.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Pummelo Protects Doxorubicin-Induced Cardiac Cell Death by Reducing Oxidative Stress, Modifying Glutathione Transferase Expression, and Preventing Cellular Senescence

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Citrus flavonoids have been shown to reduce cardiovascular disease (CVD) risks prominently due to their antioxidant effects. Here we investigated the protective effect of pummelo (*Citrus maxima*, CM) fruit juice in rat cardiac H9c2 cells against doxorubicin (DOX-) induced cytotoxicity. Four antioxidant compositions (ascorbic acid, hesperidin, naringin, and gallic acid) were determined by HPLC. CM significantly increased cardiac cell survival from DOX toxicity as evaluated by MTT assay. Reduction of cellular oxidative stress was monitored by the formation of DCF fluorescent product and total glutathione (GSH) levels. The changes in glutathione-S-transferase (GST) activity and expression were determined by enzyme activity assay and Western blot analysis, respectively. Influence of CM on senescence-associated β -galactosidase activity (SA- β -gal) was also determined. The mechanisms of cytoprotection involved reduction of intracellular oxidative stress, maintaining GSH availability, and enhanced GST enzyme activity and expression. DOX-induced cellular senescence was also attenuated by long-term CM treatment. Thus, CM fruit juice can be promoted as functional fruit to protect cells from oxidative cell death, enhance the phase II GSTP enzyme activity, and decrease senescence phenotype population induced by cardiotoxic agent such as DOX.

1. Introduction

Structurally belonging to anthracyclines, doxorubicin (DOX) is an anticancer drug widely used to treat many types of cancer but the dose-dependent cardiotoxic adverse effect limits its full clinical value [1]. It is well recognized that DOX-induced cardiotoxicity occurs through multiple mechanisms which involve oxidative stress generated by quinone moiety of the anthracycline structure. The redox recycling of semiquinone and its parent quinone is known to generate reactive oxygen species (ROS) leading to mitochondria dysfunction, myocyte senescence, and apoptosis, and ultimately causing cardiac remodeling and contractility impairment [2, 3]. Cardiac senescence is associated with the long-term effect of DOX where the clinical manifestation of heart failure may appear several years after DOX administration. Additionally, DOX induced oxidative stress in cardiac myocytes H9c2

represented senescence phenotype similar to characteristics of cardiac cells observed in aging rat [3].

Among several attempts initiated to decrease cardiotoxic adverse effect of this valuable drug, scavenging of ROS by natural antioxidants demonstrates favorable cardioprotective effect against DOX-induced cardiotoxicity both *in vitro* and *in vivo* [4, 5]. In many clinical studies related to natural antioxidants, citrus flavonoids and other constituents show prominent effects in reducing cardiovascular disease (CVD) risks [6]. Belonging to the Citrus family, pummelo fruits are indigenous to the oriental areas such as Thailand, China, Japan, and India. Thai pummelo fruit juices contain high antioxidants and scavenging property against free radicals [7], but their potential properties as cytoprotective nutrients against oxidative cell death, particularly DOX toxicity, has not been explored.

In addition to abrogation of oxidative stress by chemically active antioxidants, the removal of anthracycline toxic metabolites by phase II metabolizing enzyme glutathione transferases (previously glutathione-S transferases, GST) has been implicated in the protection of doxorubicin-induced cardiac cell death [8, 9]. Our previous study revealed that GST-Pi (GSTP) is the predominant GST subtype found in H9c2 and played a significant role in nuclear protection against DOX toxicity [9]. In this study, we aimed to investigate the cytoprotective effect of a natural product, pummelo fruit variety “Kao-Tang-Kwa”, on DOX-induced cardiotoxicity in cultured rat cardiomyocyte H9c2 with focus on the modifications of cellular redox stage, GST activity and expression, and cardiac senescence.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals used in this study were analytical or cell culture grade. Internal standards for HPLC analyses of ascorbic acid, hesperidin, naringin, and gallic acid were procured from Sigma-Aldrich (St. Louise, USA). Similarly, doxorubicin and assay reagents for crystal violet cell viability, total GSH levels, and GST activity, ROS determination, and senescence-associated β -galactosidase activity (SA- β -gal) assay were acquired from Sigma-Aldrich. Cell culture medium, fetal bovine serum, and supplements were supplied by Invitrogen, USA. Oligonucleotides were synthesized by Invitrogen, USA. Reagents for Western blot analysis were purchased from the sources indicated in the specific sections below.

2.2. Pummelo Fruit Extract. There are several commercially important varieties of pummelo (*Citrus maxima* (Burm.f.) Merr., CM), fruits in Thailand, including Kao-Tang-Kwa (cucumber-like white), Kao-Nam-Peung (honey-like white), Tub-Tim-Siam (Thai ruby), and so forth, of which names describe the characteristics of the inner flesh of the fruit. Each variety belongs to area-specific traditional communities across Thailand while the variety “Kao-Tang-Kwa” is indigenous to Chai-Nat province, Thailand. In this study, the pummelo fruits were harvested from a designated farm in Chai-Nat province and botanically identified by Assoc. Prof. Dr. Ampaiwan Paradornuwat, Faculty of Agriculture, Kasetsart University. The fruit juice was isolated by a fruit extractor and filtered through Whatman No. 1 filter membrane. The filtrate was then prepared in freeze-dried power yielded 8.9% (w/v) and the powder was kept at 4°C until further uses. The aqueous stock solutions of CM (10 mg/mL) were freshly prepared before use.

2.3. Analyses of Ascorbic Acid and Citrus Flavonoids Contents Using HPLC. Ascorbic acid content and 3 flavonoids commonly found in the citrus fruits including hesperidin, naringin, and gallic acid were analyzed by reverse-phase HPLC system (Thermo Separation Spectra System P4000) using Luna C18 column (5 μ m, 150 \times 4.6 mm; Fortune Scientific CO., LTD, Bangkok, Thailand). Standard curves of each reference standard were generated from a series of

dilutions 0, 2, 4, 8, 10 μ g/mL. The following systems (mobile phase, flow rate, detection wavelength) were applied for the determination of ascorbic acid (100 mM phosphate buffer pH 2.5 and methanol (95 : 5), 0.4 mL/min, 243 nm), hesperidin and naringin (12 mmol heptafluorobutyric acid in 0.05% formic acid and acetonitrile (80 : 20), 1.2, 283 nm), and gallic acid (0.17 M sodium dihydrogen phosphate and methanol (76 : 24), 252 nm) [10–12].

2.4. H9c2 Cell Culture. The cardiac cell line H9c2 derived from embryonic rat heart was acquired from The American Type Culture Collection (ATCC, CRL-1446). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with antibiotics/antimycotics and 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Culture was replaced with fresh media every 2-3 days and expanded to new culturewares when reached 80% confluence.

2.5. Cell Viability Assay. CM was added to the cell culture 30 min prior to the addition of DOX. H9c2 cells were treated with physiological relevant DOX concentration (0.1 μ M) for 48 h with or without coincubation with CM at three different concentrations (10, 100, and 1000 μ g/mL). Cell survival was evaluated using crystal violet nuclear staining assay as previously described [13]. Briefly, cells were washed with PBS, and fixed with 10% buffered formalin. Crystal violet solution (0.1% in water/MeOH, 1 : 1) was used to stain nucleus of live cells. Cells were then lysed with 50 mM sodium citrate solution in water/EtOH (1 : 1) and the percentage of cell survival relative to vehicle treatment was quantified by reading the absorbance at 595 nm.

2.6. ROS Levels. Determination of intracellular ROS levels were performed by measuring a fluorescent product formed by the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA, Sigma) and the intracellular ROS. Briefly, the culture media were removed and cells were washed with PBS. Following the addition of fresh culture media, cells were incubated with DCFHDA at the final concentration of 50 μ g/mL for 15 min at 37°C. Cells were then wash again with PBS 3 times and the relative amount of fluorescent product was monitored by a microplate reader (Synergy HT, Biotek, USA) with excitation and emission at 485 nm and 528 nm, respectively.

2.7. Cellular Glutathione Levels. Reduced glutathione (GSH) is the major antioxidant defense tool both in scavenging activity against ROS and in detoxification of drugs and xenobiotics. The free thiol group provides reducing equivalents for the glutathione peroxidase (GPx) to catalyze reduction of hydrogen peroxide resulted in oxidized glutathione (GSSG) and water. The GSSG-GSH recycle process is then introduced by glutathione reductase (GR) and NADPH. In the process of xenobiotic detoxification, glutathione-S transferase (GST) catalyzes conjugation reaction of GSH to electrophilic substrates such as DOX through the thiol group of GSH. Thus,

the availability of GSH pool is crucial for antioxidant defense in biological system.

To assess total cellular GSH (tGSH), the assay was performed according to the method described previously with some modifications [14]. The GSH in cell lysate samples were determined by the conjugation reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in assay buffer (100 mM phosphate buffer, 1 mM EDTA, pH 7.4) in the present of GR (1 Unit/ μ L) and the reaction mixture was incubated at room temperature for 5 min. Then, NADPH (0.3 mg/mL) 50 μ L was added. The formation of color product (2TNB) was then monitored at 412 nm for 3 min using kinetic mode (Synergy HT, Bioteck, USA). The amounts of total GSH (tGSH) in cell lysates were calculated from GSH standard curves and normalized to 1 mg protein.

2.8. GST Activity. The total GST activity was measured in H9c2 using total cell lysates as previously described with minor modifications [9]. Briefly, cultured cells (approximately 1×10^7 cells) were collected in assay buffer (100 mM potassium phosphate buffer with 1.0 mM EDTA and 0.1% Triton X-100, pH 6.5) using cell scraper and allowed to sit on ice for 10 min followed by centrifugation at 2000 $\times g$ for 10 min at 4°C. The supernatants were collected and assayed for protein content using BioRad protein assay kit (BioRad, USA). GST activity was measured in the presence of 0.1 mM GSH and 0.1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in assay buffer. The assay utilized CDNB as substrate for GST isozymes to form conjugated product with the thiol group of glutathione. GST enzyme solutions (0.075 to 0.15 unit/mL) were used as reference for GST activity. The rate of GS-CDNB conjugate formation was monitored for 4 min at 340 nm and GST activity was calculated as follows:

$$\text{GST activity (units/mg protein)} = \frac{\Delta A_{340}/\text{min}_{\text{Sample}} - \Delta A_{340}/\text{min}_{\text{Blank}}}{\text{Protein content (mg/mL)}} \quad (1)$$

2.9. Western Blot Analysis of GSTP. Since GSTP is the only GST subtype expressed in H9c2 as detected by immunoblotting, the effects of CM on the changes in GSTP protein expression of H9c2 were evaluated at 48 h after cells were treated with DOX. Cells were harvested using lysis buffer (20 mM Tris-HCl (pH 7.2), 130 mM NaCl, and 1% NP-40 containing 1% protease inhibitor cocktail (Sigma-Aldrich, P8340)). Cell lysates were normalized for protein content using a Bradford protein assay kit (BioRad, USA). Protein samples were separated by 7.5% SDS-PAGE under reducing conditions and then transferred to a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in TBS (10 mM Tris-HCl (pH 7.5) and 150 mM NaCl) and then incubated at 4°C overnight with anti-GSTP or anti-beta-actin antibody (Santa Cruz Laboratories, USA) in TBS containing 0.1% Tween 20. The blots were washed and then incubated with the peroxidase-conjugated secondary antibodies for 1 h at room temperature. Following several washes, the membrane was developed using the ECL chemiluminescence

detection kit (Amersham Biosciences) according to the manufacturer's instructions. The relative expression or immunological reaction bands on the membrane were quantified by band density using beta-actin bands as reference ratio expression.

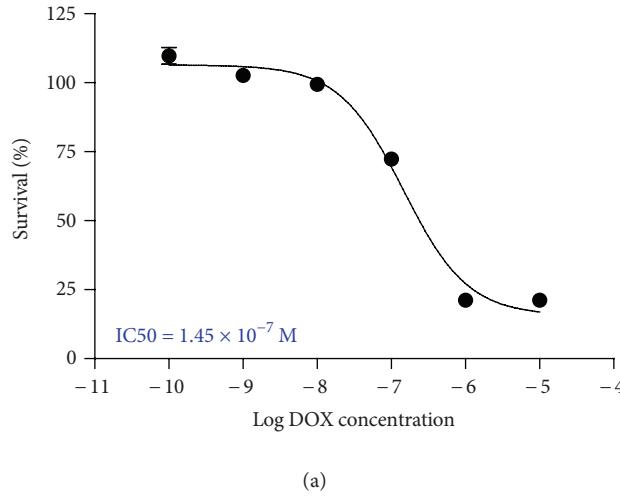
2.10. Senescence-Associated β -Galactosidase Activity (SA- β -gal) Assay. H9c2 were preincubated with CM for 7 days by replacing the media every 2-3 days with CM-containing media to final concentration of 10, 100, and 1000 μ g/mL. Forty-eight hours before the assay, DOX (0.1 μ M) was added to the culture media to induce cellular senescence. Beta-galactosidase activity was evaluated as previously described [15]. Briefly, H9c2 cells were washed with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature. Following PBS washes, fixed cells were incubated with fresh SA- β -gal stain solution (1 mg/mL 5-bromo-4-chloro-3-indyl β -D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂, 0.01% sodium deoxycholate) for 12–14 h. The development of blue color X-gal product was observed under an inverted microscope. The proportion of cells with blue staining was calculated from total cell counts for at least 300 cells.

2.11. Statistical Analysis. Data are presented as the mean \pm SEM for at least three independent experiments. Statistical analysis was performed using one-way or two-way ANOVA with Bonferroni's Multiple Comparison Test. A value of $P < 0.05$ was considered statistical significance.

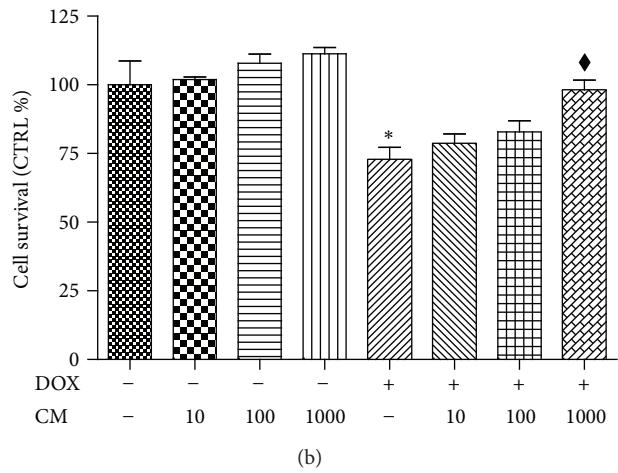
3. Results

3.1. Ascorbic Acid and Certain Flavonoids Contents in CM. HPLC analysis revealed that CM contained (% w/w) 0.52% ascorbic acid, 0.26% naringin, 0.039% gallic acid while hesperidin was not detectable. When converted to the amounts in 1 Liter CM fruit juice composed of 462 mg ascorbic acid, 231 mg naringin, and 34.6 mg gallic acid. For the purpose of further comparison, the aqueous solutions of CM at 1 mg/mL is corresponding to 29.53 microM ascorbic acid, 2.29 microM gallic acid, and 4.48 microM naringin.

3.2. CM Increased Cell Survival in DOX-Induced Cytotoxicity. Dose-response curve of DOX cytotoxicity was generated by the 48 h incubation of DOX at a range of concentrations between 10^{-10} to 10^{-5} M and the IC₅₀ was obtained at 1.45×10^{-7} M (Figure 1(a)). The concentration of DOX inducing cytotoxicity was selected at 0.1 μ M at which it significantly reduced cell survival to $72.74 \pm 4.50\%$. As demonstrated in Figure 1(b), incubation of CM alone (10, 100, and 1000 μ g/mL) did not significantly alter cardiac cell survival while cytoprotective effect of CM was observed only at 1000 μ g/mL. Cell survival was increased to $96.85 \pm 3.15\%$. In separate experiments, three pure antioxidant compounds detected in CM, including ascorbic acid, naringin, and gallic acid, were tested for cytoprotective effect at the concentrations in the range of 0.1 to 100 μ g/mL. H9c2 cell viability was



(a)



(b)

FIGURE 1: Toxicity of DOX and cytoprotective effect of CM. (a) Dose-response curve of doxorubicin (DOX). H9c2 cells were treated with DOX (0-10-5 M) for 48 h and dose-response curve was obtained with IC₅₀ of 1.45×10^{-7} M; (b) cytoprotection of pummelo in DOX-induced cytotoxicity. Cells were incubated for 48 h with DOX (0.1 µM) with or without preincubation with CM at concentrations 10, 100, and 1000 µg/mL. Cell viability was evaluated by crystal violet assay as described in material and Methods. *P < 0.05 versus vehicle treated cells (CTRL); ♦P < 0.05 versus DOX.

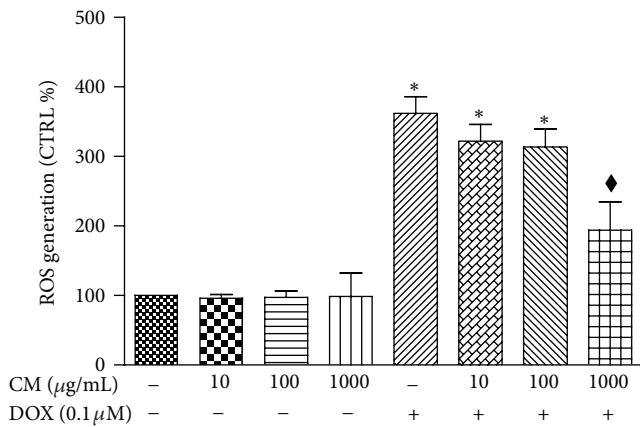


FIGURE 2: Effect of pummelo on intracellular ROS levels. Cardiac H9c2 cells were treated with DOX (0.1 µM) with or without coincubation with CM at concentrations 10, 100, or 1000 µg/mL for 48 h. Fluorescence intensity of DCF was measured and corresponding to intracellular ROS generation. Data are present as % vehicle treated cells (CTRL). *P < 0.05 versus CTRL; ♦P < 0.05 versus DOX.

not changed when each compound was incubated with the cardiac cells for 48 h. Coincubation of DOX and each pure antioxidant did not change cell survival at all concentrations of antioxidant used in the experiment (data not shown).

3.3. CM Attenuated Cellular Oxidative Stress in DOX-Treated Cells. Escalation of ROS level is commonly observed in cells undergone oxidative stress. H9c2 cells treated with DOX (0.1 µM) alone for 48 h showed more than 3-fold increase of intracellular accumulation of ROS while cells receiving CM (10, 100, or 1000 µg/mL) alone did not show significant alteration in ROS levels (Figure 2). Coincubation of CM at 10

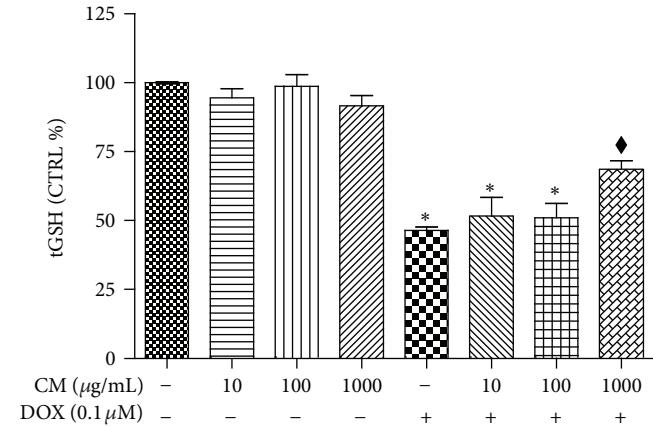


FIGURE 3: Effect of pummelo on total GSH levels. H9c2 cells were treated with doxorubicin (DOX, 0.1 µM) with or without coincubation with pummelo (CM) at three concentrations as indicated in the figure (10, 100, 1000 µg/mL). The total GSH (tGSH) levels were calculated as described in Materials and Methods. *P < 0.05 versus vehicle treated cells (CTRL); ♦P < 0.05 versus DOX.

and 100 µg/mL did not protect cardiac cells from oxidative stress induced by DOX. However, CM at high concentration (1000 µg/mL) showed significant reduction of intracellular ROS generation although it did not decrease ROS down to the level that comparable to vehicle treated cells.

3.4. Cellular tGSH Pool. It has been shown that reduction of glutathione pool impairs the cellular capacity in antioxidant defense system and likewise, increased GSH pool is associated with cytoprotection against oxidative damage. In this study as shown in Figure 3, using relative low concentration of DOX at 0.1 µM reduced GSH levels in cardiac cells approximately

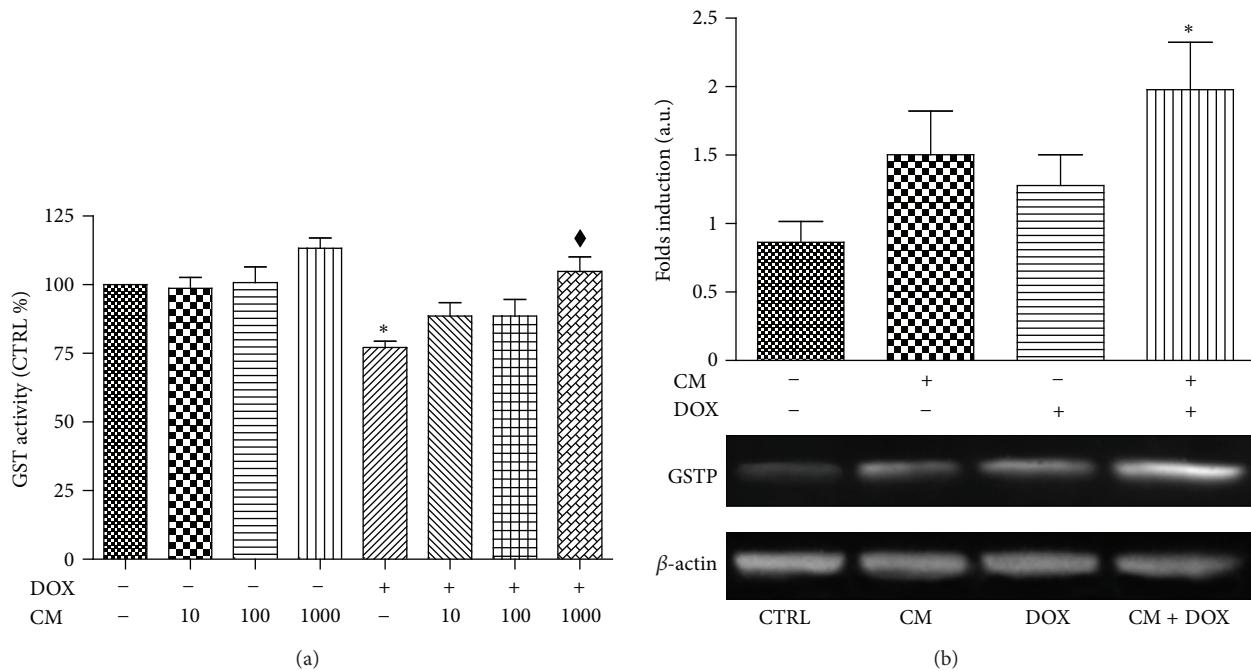


FIGURE 4: Influence of CM on GST activity and expression in H9c2 cells. Cells were treated with DOX ($0.1 \mu\text{M}$) and/or pummelo (CM) at concentrations indicated in the Figure. (a) GST activity measurements were performed using total cell lysate as described in Materials and Methods. (b) Western blot analysis of GSTP expression in H9c2 treated with vehicle (CTRL), CM, DOX, or CM and DOX (CM + DOX) for 48 h. * $P < 0.05$ versus vehicle treated cells (CTRL); ♦ $P < 0.05$ versus DOX.

17%. Treatment of cells with CM alone at all concentrations studied did not significantly influence GSH levels when compared to those of vehicle treated cells. Coincubation of CM at lower concentrations (10 or 100 $\mu\text{g}/\text{mL}$) with DOX did change GSH antioxidant pool but CM at high concentration (1000 $\mu\text{g}/\text{mL}$) significantly elevated cellular tGSH in DOX-treated cells.

3.5. CM Enhanced GST Activity and Expression in H9c2. Shown in Figure 4 is the GST activity and gene expression in cardiac H9c2 cells. DOX ($0.1 \mu\text{M}$) caused impairment in GST function approximately one-fourth of those observed in the vehicle treated cardiac cells. CM at 1000 $\mu\text{g}/\text{mL}$ significantly improved GST activity to the level comparable to control group while lower concentrations of CM (10 and 100 $\mu\text{g}/\text{mL}$) did not significantly modify GST activity (Figure 4(a)). Western blot analysis revealed that GST protein expression was significantly enhanced in cardiac cells treated with both DOX and CM (1000 $\mu\text{g}/\text{mL}$) which is consistent with GST activity observed in this group. H9c2 cells treated with DOX or CM at 1000 $\mu\text{g}/\text{mL}$ alone did not show significant alteration in GST expression (Figure 4(b)).

3.6. CM Attenuated Oxidative Stress-Induced Cellular Senescence. In immortalized cell line cultured in growth medium supplemented with growth factors cellular senescence occurs at a very low level. This study used 1% FBS in culture media to sensitize cells to undergo senescence during incubation with DOX. CM alone did not change the proportion of senescence

cells in H9c2 culture but an approximately 2-fold increase in β -gal-SA was observed in DOX treated cells. CM at all concentrations (10, 100, or 1000 $\mu\text{g}/\text{mL}$) tested in this study significantly mitigated the effect of DOX-induced senescence phenotype in H9c2 (Figure 5).

4. Discussion

ROS play an essential role in the development of cardiovascular disease associated with DOX treatment. Our study demonstrated that pummelo fruit juice (*Citrus maxima* (Burm.f.) Merr., CM) protected against DOX-induced cardiotoxicity in H9c2 via mechanisms related to the reduction of cellular oxidative stress, enhancement of GSH antioxidant pool, and increase of the detoxifying enzyme GSTP activity and expression. In addition, long-term pretreatment with CM attenuated DOX-induced cellular senescence in H9c2 cardiac cells.

It is now well recognized that increased ROS generation is the pivotal point upstream of the mechanisms associated with DOX-induced cardiotoxicity [16, 17]. Mitochondria are the primary target and the major source of ROS generation that leads to dysregulation of oxidative metabolism for ATP production [18]. However, strategy to reduce cardiotoxicity from DOX cannot rely upon reduction of ROS level alone. For example, N-acetyl cysteine (NAC) inhibited ROS formation, lipid peroxidation, and restored antioxidant enzyme activities but had modest effect on the protection of DOX-induced cardiac cell death as compared to other natural sources of antioxidants [13, 19]. It implies that alternative mechanisms

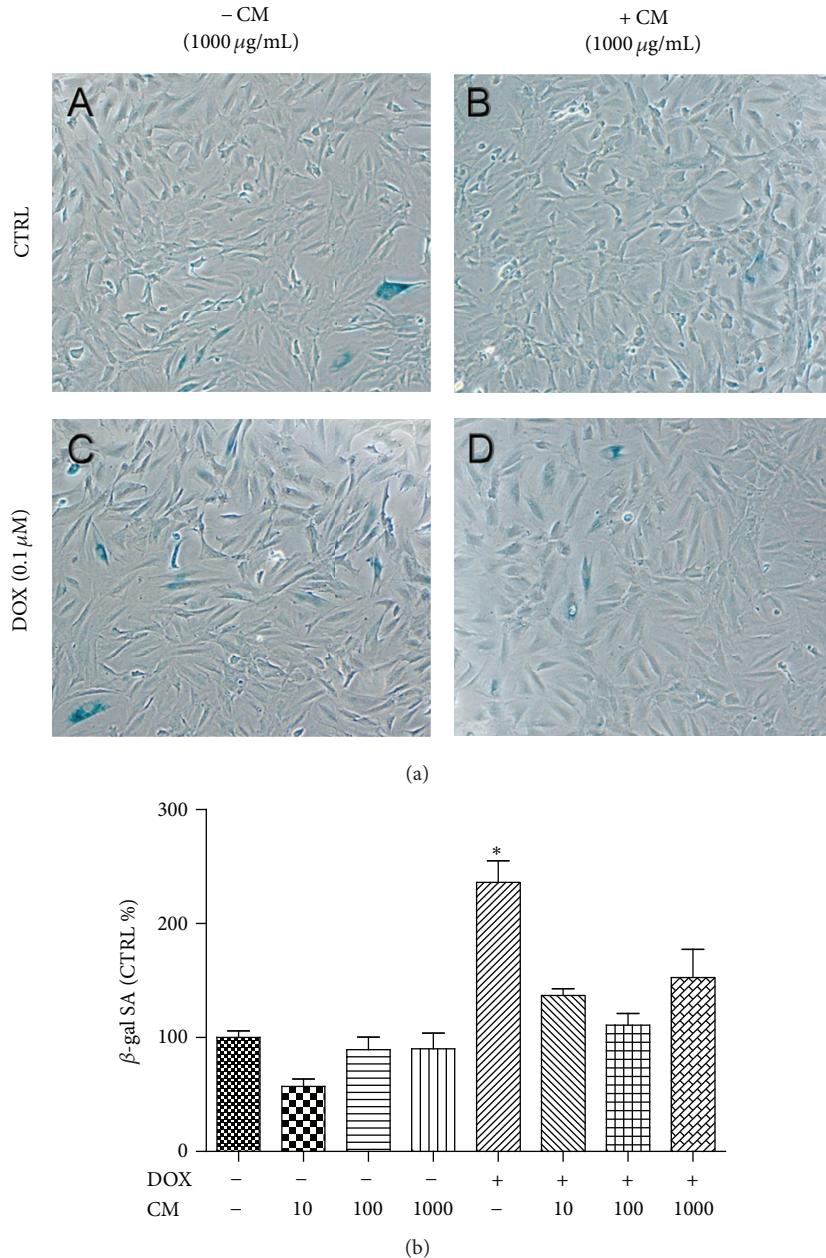


FIGURE 5: (a) β -gal staining in H9c2. Cells were preincubated with pummelo (CM) and/or DOX ($0.1 \mu\text{M}$) as indicated in the figure and photographed at 100x. (b) Positive cells for β -gal SA were counted and calculated as % vehicle treated cells (% CTRL). * $P < 0.05$ versus CTRL.

for attenuation of DOX toxicity such as an increased elimination of DOX by the modification of the phase II detoxification enzyme GSTs may play significant role in cytoprotection [8, 9, 20].

GSTs protect cellular damage against electrophiles and products of oxidative stress, particularly anticancer agents, insecticides, herbicides, and carcinogens. There are two distinct GST superfamilies, microsomal and cytosolic GSTs. While the former involves in endogenous metabolism of leukotrienes and prostaglandins, the latter is a major cytosolic enzyme in some tissues and functions as important detoxification enzyme through GSH-dependant nucleophilic

substitution, epoxide ring opening, conjugate addition, ester thiolysis, and so forth. The cytosolic GST superfamily consists of 6 subclasses, including Alpha (GSTA), Mu (GSTM), Omega (GSTO), Pi (GSTP), Theta (GSTT), and Zeta (GSTZ) [21]. GSTs can also be found in the nucleus and in membranes of the endoplasmic reticulum. The degrees of expression can be varied among tissue types and gender differences which may imply tissue's ability to manage specific forms of stress [22]. In this study, pummelo exerted its cytoprotective role in DOX toxicity by increased activity and expression of GSTP in addition to the reduction of ROS stress and maintenance of cellular GSH level. The GST activity in this cardiac cell

H9c2 is specific to GSTP subtype since our previous findings indicate that only GSTP protein expression was confirmed by immunoblotting despite mRNA expression of other GST subtypes were detected [9]. CM may partly increase cell survival from DOX toxicity via enhanced elimination of DOX using increased available GST altogether with providing its cofactor GSH. DOX alone did not change GSTP protein expression but its enzyme activity was significantly reduced possibly due to GSH depletion which is similar to cardiac tissue of rats injected with DOX [23]. It is evident that most cellular damage occurs after the depletion of GSH which sets out the onset of uncontrolled oxidative injury. For DOX detoxification, GSTP is an important cytoprotective mechanism as shown in the study that MCF-7 attributed with knockdown GSTP expression enhanced DOX-induced apoptosis [24]. Moreover, study in human lymphocytes revealed that among the enzymes involve in detoxifying gentoxicants including GSTM1, GSTT1, and GSTP1, only GSTP associates with protection against DNA damage specifically induced by DOX [25]. The significance of GSTP is extended beyond conjugation with genotoxic substances, its cytoprotective effect relates to preventive DOX accumulation through forming macromolecular complexes and disruption of JNK-mediated apoptosis pathway [24, 26]. Thus, modification of GSTP may play a crucial role in cardioprotection against DOX.

Despite cardiotoxic effect of DOX having been recognized since early 1970s, its diverse toxic consequences on the heart, acute or late onset, are still not fully understood. The cellular oxidative stress and senescence may associate with molecular mechanism of DOX-induced cardiomyopathy in the latent manifestation of toxicity years after DOX treatment [27]. Maejima et al. demonstrated that neonatal cardiac myocytes treated with low dose DOX ($0.1 \mu\text{M}$) show evidence of senescence-associated- β -galactosidase activity similar to myocytes extracted from aged rats while a higher concentration of DOX ($1 \mu\text{M}$) triggers apoptotic cell death corresponding to acute DOX toxicity [3]. Our study showed that long-term treatment with CM could significantly attenuate senescence phenotype and reduced intracellular ROS level in cardiac cells treated with low dose DOX ($0.1 \mu\text{M}$). This effect of CM may related to the reduction of telomerase activity via modification of telomere binding factors 1 and 2 (TRF1, TRF2) and dysregulation of cell cycle regulatory proteins such as checkpoint kinase Chk2 and p53-MAPK signaling which lead to chromosome aberration and delayed cell death due to mitotic catastrophe [28].

Clinical and prospective cohort studies indicated that high consumption of vegetables and fruits, especially citrus fruits enriched with antioxidants, lowers cardiovascular disease risks and prevents the development of certain types of cancer [29–31]. The major antioxidant constituents in citrus fruits consist of ascorbic acid, carotenoids, and unique “citrus flavonoids” including hesperidin, neohesperidin, naringin, narirutin, limonin, and so forth. Ascorbic content in CM fruit juice is approximately 3-fold higher than those of premium tangerine juice cultivated in Northern Thailand [32] but comparable to those of oranges grown in Italy [33] and ponkan tangerine cultivated in Brazil [34]. The HPLC analysis did

not detect hesperidine in CM (var. Kao-Tang-Kwa) which is corresponding to the previous study that it only presents in one out of seven pummelo cultivars in Thailand [7]. The flavonoid compositions vary from species and cultivars which are characteristic of species as well as determinant factor for their biological effects. Nonetheless, several studies have shown that synergy among various antioxidants in the extract reflects superior antioxidant activity than that of single component alone. For instance, a well-designed study performed by Snyder et al. [35] indicated that consumption of single flavonoid (hesperidine) had lower capacity than a mix of orange flavonoids (hesperidin, naringenin, and luteolin) at the amounts equivalent to fresh-squeezed navel orange juice in lower plasma antioxidant capacity, total phenolics, and reduction of lipoprotein oxidation.

5. Conclusion

Pummelo fruit juice is an excellent source of natural vitamin C and other antioxidant flavonoids supplements. Our study is among the first to provide a better insight into the mechanisms of pummelo in protecting against cytotoxic insults that cause oxidative cell death, specifically to the heart. Pummelo increased cardiac cell survival during DOX treatment through two main mechanisms: (1) the reduction of cellular oxidative stress and enhancement of GSH antioxidant capacity and (2) the elimination of the toxic substance from the cells by increasing the detoxifying GSTP enzyme activity. Long-term treatment with CM inhibited DOX-induced cardiac cells entering senescence-like phenotype which may implicate in the late onset of cardiomyopathy following DOX treatment. Thus, consumption of pummelo fruit may protect against DOX-induced oxidative damage associated with pathogenesis of cardiotoxicity. Defining the mechanism of different natural antioxidant action on various forms of oxidative stress is crucial for strategic design of antioxidant therapy in cardiovascular disease.

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Research Article

Citrus ichangensis Peel Extract Exhibits Anti-Metabolic Disorder Effects by the Inhibition of PPAR γ and LXR Signaling in High-Fat Diet-Induced C57BL/6 Mouse

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Obesity is a common nutritional disorder associated with type 2 diabetes, cardiovascular diseases, dyslipidemia, and certain cancers. In this study, we investigated the effects of *Citrus ichangensis* peel extract (CIE) in high-fat (HF) diet-induced obesity mice. Female C57BL/6 mice were fed a chow diet or an HF diet alone or supplemented with 1% w/w CIE for 8 weeks. We found that CIE treatment could lower blood glucose level and improve glucose tolerance. In the HF+CIE group, body weight gain, serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) levels, and liver triglyceride (TG) and TC concentrations were significantly ($P < 0.05$) decreased relative to those in the HF group. To elucidate the mechanism of CIE on the metabolism of glucose and lipid, related genes expression in liver were examined. In liver tissue, CIE significantly decreased the mRNA expression levels of peroxisome proliferator-activated receptor γ (PPAR γ) and its target genes, such as fatty acid synthase (FAS) and acyl-CoA oxidase (ACO). Moreover, CIE also decreased the expression of liver X receptor (LXR) α and β which are involved in lipid and glucose metabolism. These results suggest that CIE administration could alleviate obesity and related metabolic disorders in HF diet-induced obesity mice through the inhibition of PPAR γ and LXR signaling.

1. Introduction

According to World Health Organization (WHO) estimates, there are 1.4 billion overweight adults worldwide and more than 500 million of these are obese [1]. Obesity is one of the most notorious symptoms of metabolic disorders throughout the world. It is considered to be a major risk factor for various chronic diseases, including type 2 diabetes, major cardiovascular diseases, hypertension, dyslipidemia, and certain cancers [2]. At present, only orlistat can be used for long-term weight reduction. However, these drugs are confounded by diminishing response in long-term treatment because of side effects and limited efficacies [3, 4]. Nutritional components may play a prominent role in the

prevention and treatment of obesity and related metabolic disorders. Recently, there have been increasing efforts in research for new health-enhancing foods from natural products, and these findings also suggest that nutritional intervention could be an effective and promising strategy to inhibit obesity and obesity-related metabolic diseases [5, 6].

Citrus is one of the most important fruits in the world and is a rich source of nutrients and bioactive compounds. Citrus fruits not only provide ample vitamins, minerals, dietary fibers, and pectins but also provide an abundant of bioactive compounds, including flavonoids, coumarins, limonoids, and carotenoids. Currently, the study of bioactive compounds is one of the most active fields of food and medical science. Many epidemiological and experimental

studies have provided convincing evidence that the intake of citrus fruits is beneficial to health [7–9]. Numerous prevention and treatment properties have been attributed to citrus fruits, like antioxidant, antiinflammatory, antitumor, anticardiovascular, and antiobesity properties [10–14]. Citrus fruits are usually consumed as fresh product or juice with peels and seeds discarded. Regretfully, these wastes are abundant sources of natural bioactive compounds [15]. In China, citrus peels like *chepi* (dried peels of mature *C. reticulate*) or *qingpi* (dried peels of immature tangerine (*C. reticulate*)) have been commonly used in Chinese medicine for the treatment of a number of diseases, such as indigestion, bronchial asthma, vomiting, cough, skin inflammation, and muscle pain [16, 17]. Moreover, citrus peels have been extensively consumed as baked products, culinary seasonings, preserves, and food supplements in China for centuries. Recently, the prevention and treatment of obesity and obesity-related metabolic diseases of citrus peels have received increasing attention. Jung et al. found that *Citrus unshiu* peel extract inhibited lipid and triglyceride accumulation in 3T3-L1 adipocytes [18]. The extract from *Citrus unshiu* Mark induced the lipolysis *in vitro* [19]. A study by Bok et al. suggested that citrus peel diet reduced plasma and hepatic cholesterol in rats [20]. It has been reported that the immature *Citrus sunki* peel extract had an antiobesity effect by elevated β -oxidation and lipolysis in the adipose tissue of HF diet fed mice [21]. In addition, citrus phytochemicals, such as flavonoids, nomilin, synephrine, and auraptene, have exhibited antiobesity effects by increasing energy expenditure, improving metabolism, and enhancing lipolysis [22–25].

Emerging evidence suggests that PPARs are the targets of many citrus-derived flavonoids [26]. PPAR is the nuclear receptor transcription factor that is known to regulate carbohydrate and lipid metabolism in various tissues and cells [27]. The PPAR family includes three isoforms: PPAR α , PPAR γ , and PPAR δ/β . PPAR γ is an important regulator of adipocyte differentiation, lipogenesis, and glucose metabolism [28, 29]. Citrus flavonoids have been shown to inhibit adipogenesis and to decrease adiposity which can be explained in part by regulating the PPAR expression levels both *in vivo* and *in vitro* [30, 31]. It has been previously shown that citrus polymethoxylated flavones improve lipid and glucose homeostasis and restore insulin sensitivity through regulating the expression of PPAR α and PPAR γ [32, 33]. A recent study has suggested that *Citrus aurantium* flavonoids suppressed adipogenesis by inhibiting the expression of PPAR γ in 3T3-L1 cells [31]. Studies have also identified that LXR is a target for metabolic diseases [34]. The citrus component naringin decreases serum lipid through the increase of PPAR γ expression and inhibition of LXR expression in the liver of type 2 diabetic rats [34]. The grapefruit flavonoid naringenin has been reported to be an agonist of PPAR α and PPAR γ , and a partial agonist of LXR α [26].

Although citrus fruits are widely used in the pharmaceutical and food industries, researches on the functions of endemic citrus species remain insufficient. China has much abundant germplasm resources of the citrus fruits,

but there is underutilization of these citrus resources. *Citrus ichangensis* Swingle is a unique citrus species grown in China and is known by its unusual hardiness and contains a wide range of bioactive compounds [35]. In horticulture, *Citrus ichangensis* was mainly used as rootstock of cultivated citrus due to its stress resistance and the fruit of *Citrus ichangensis* has been used in traditional Chinese medicine for a long history. It has been shown that *Citrus ichangensis* contains the complex pattern of flavones and a large amount of nonbitter deacetylnomilin [36]. Here, we investigate whether the long-term administration of *Citrus ichangensis* peel extract (CIE) would have beneficial effects on the prevention and treatment of obesity and its related metabolic diseases. In the present study, CIE was tested for body weight gain, lipid accumulation, and gene expression involved in glucose and lipid metabolism in HF diet-induced C57BL/6 mice.

2. Material and Methods

2.1. Preparation of *Citrus ichangensis* Peel Extract (CIE). *Citrus ichangensis* Swingle was provided by the Citrus Research Institute, Chinese Academy of Agricultural Sciences, Chongqing China. Samples were prepared by adding 4 L of 95% ethanol to one kilogram of fresh citrus peel, extracting at 85°C for 2 h, cooling, and filtering the solution. The filtered solution was concentrated at 40°C with a rotary evaporator under reduced pressure, freeze-dried to a powder, and stored at –20°C until use. The frozen dried powder of CIE was added to the HF diet for the experiment.

2.2. HPLC Analysis. To determine the flavonoids content of CIE, high-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 liquid chromatograph system. The flavonoid compounds were monitored at 280 nm using a Discovery C18 HPLC Column (250 × 4.6 mm, 5 μ m). The column was operated at 30°C, and the injection volume was 10 μ L. The mobile phase consisted of 100% acetonitrile (A) and water containing 0.5% acetic acid (B) at a flow rate of 1.0 mL/min. The gradient profile was as follows: 0–12 min, 85–75% B; 12–17 min, 75% B; 17–20 min, 75–50% B; 20–30 min, 50–25% B; 30–35 min, 25–5% B; and 35–40 min, back to 85% B.

2.3. Animals and Diets. The animal study protocols were approved by the Shanghai University of Traditional Chinese Medicine. Four-week-old female C57BL/6 mice were purchased from the SLAC Laboratory (Shanghai, China). Mice were kept under 22–23°C on a 12 h light/dark cycle. After a one-week adaptation period, C57BL/6 mice were randomly separated into three groups ($n = 7$) and were fed a chow diet (10% of calories derived from fat, Research Diets; D12450B), or an HF diet (60% of calories derived from fat, New Brunswick, NJ, Research Diets; D12492), either alone or supplemented with 1% CIE (HF+CIE) diet for 8 weeks. Food intake and body weight were measured every other day. The mice were given free access to food and water.

TABLE 1: Sequences of the primers used in real-time PCR.

Gene	Forward primer	Reverse primer
β -Actin	TGTCCACCTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA
LXR α	GAGTGTGCACTTCGCAAATGC	CCTCTTCTGCCGCTTCAGT
LXR β	CAGGCTTGAGGTGGAATTTC	ATGGCGATAAGCAAGGCATACT
ABCA1	GGCAATGAGTGTGCCAGAGTTA	TAGTCACATGTGGCACCGTTTT
ABCG1	TCCCCACCTGTAAGTAATTGCA	TCGGACCCCTTATCATTCTACAGA
ApoE	GAACCGCTTCTGGGATTACCT	TCAGTGCCGTCAAGTCTTG
CYP7A1	GTGGTAGTGAGCTGTTGCATATGG	CACAGCCCAGGTATGGAATCA
SREBP1	GGCTATTCCGTGAACATCTCCTA	ATCCAAGGGCATCTGAGAACTC
LPL	ATCGGAGAACTGCTCATGATGA	CGGATCCTCTCGATGACGAA
PGC-1 β	GGGTGCGCCTCCAAGTG	TCTACAGACAGAAGATGTTATGAAACAC
PPAR γ	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC
aP2	CATGGCCAAGCCCCAACAT	CGCCCCAGTTGAAGGAAATC
ACC	GAATCTCCTGGTGACAATGCTTATT	GGTCTTGCTGAGTTGGTTAGCT
ACO	CAGCACTGGTCTCCGTATG	CTCCGGACTACCATCCAAGATG
UCP-2	GGGCACTGCAAGCATGTGTA	TCAGATTCCCTGGGCAAGTCACT
CD36	GCTTGCAACTGTCAGCACAT	GCCTTGCTGTAGCCAAGAAC
FAS	CTGAGATCCCGACACTTCTTGA	GCCTCCGAAGCCAATGAG

2.4. Intraperitoneal Glucose Tolerance Test. For intraperitoneal glucose tolerance test (ipGTT), all the mice were fasted for 12 h and a basal blood glucose levels (0 min) were determined from the tail vein. The mice were then intraperitoneally injected with glucose (1 g/kg body weight), and additional blood glucose levels were measured at 15, 30, 60, and 90 min.

2.5. Serum Chemistry Analysis. After overnight fasting, all mice were anesthetized with urethane before collecting blood samples for analysis. Blood samples were drawn from the heart into a vacuum tube and allowed to clot at room temperature for 30 min. Serum samples were separated from the blood and the serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c) were analyzed using a Hitachi 7020 Automatic Analyzer. Serum lipid parameters were measured by Clinical Reagents following the manufacturer's instructions.

2.6. Liver and Fecal Lipid Content Analysis. The liver and other tissues were rapidly collected at the end of treatment, frozen in liquid nitrogen, and stored at -80°C for further experiments. 50 mg of frozen liver tissue was minced and homogenized in 1 mL of tissue lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton) and mixed with an equal volume of chloroform. The chloroform layer was separated, dried, and resuspended in 100 μL of isopropyl alcohol to measure the lipid levels as described above. Fecal lipids were extracted and measured as described above.

2.7. Morphological Analysis of Epididymal WAT. Scanning electron microscopy was used to examine the structure of epididymal white adipose tissue (WAT) by the method

developed by Chun et al. [37]. The epididymal fat pads were fixed with 10% neutral formalin and postfixed in 1% osmium tetroxide. Samples were viewed using a Philip XL-30 scanning electron microscope with a magnifying power of $\times 200$.

2.8. Quantitative Real-Time RT-PCR. Total RNA from liver tissue was extracted with a spin column (Qiagen, Germany) according to the manufacturer's protocol. The first-strand cDNA was synthesized using the cDNA synthesis kit (Fermentas, Madison, WI, USA). The gene expression levels were analyzed by quantitative real-time RT-PCR conducted using the ABI StepOnePlus real-time PCR system (Applied Biosystems, USA). The primers involved in the experiments were shown in Table 1. The cDNA was denatured at 95°C for 10 min followed by 40 cycles of PCR (95°C , 15 s, 60°C , 60 s). All results were obtained from at least three independent experiments. The expression levels of genes were normalized using β -actin as an internal control.

2.9. Statistical Analysis. All values are expressed as the mean \pm SD unless otherwise indicated. Data analysis was performed by SPSS 12.0 software for Windows statistical program. Statistical analysis was programmed by one-way analysis of variance (ANOVA). Differences were defined as significant when $P < 0.05$.

3. Results

3.1. Flavonoid Contents in CIE. The flavonoid composition of CIE was detected through HPLC analysis. Figure 1 shows the levels of major citrus fruit flavonoids including neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, poncirin, naringenin, nobletin, and tangeretin. The major flavonoids in CIE were naringin (8.12 mg/g), hesperidin

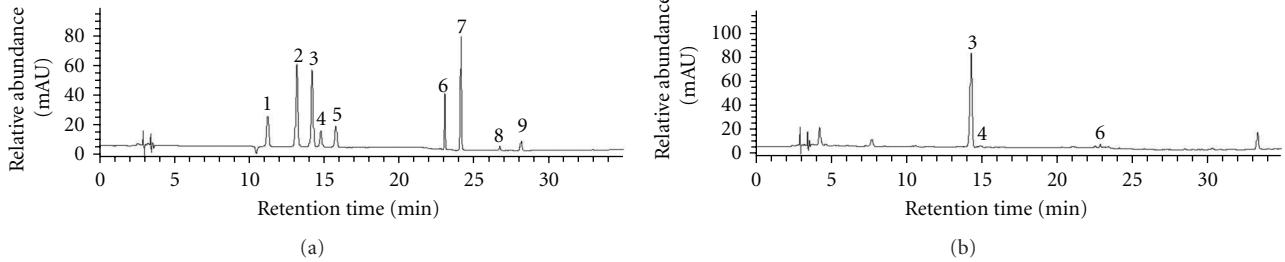


FIGURE 1: HPLC chromatograms of major flavonoids of citrus peel extract (CIE). (a) (1) Neoeriocitrin; (2) narirutin; (3) naringin; (4) hesperidin; (5) neohesperidin; (6) poncirus; (7) naringenin; (8) nobiletin; (9) tangeretin were analyzed as standards. (b) The major flavonoid components of CIE were determined to be compared to retention time of the chromatogram of standard: (3) naringin; (4) hesperidin; (6) poncirus.

(0.84 mg/g), and poncirus (1.33 mg/g). Naringin, the glycoside form of naringenin abundantly found in citrus fruits, possesses a wide range of pharmacological activities including antioxidative stress, anti-inflammatory, and anticancer effects.

3.2. CIE Blocks Body Weight Gain C57BL/6 Mouse Induced by HF Diet-Induced. To test the effects of CIE on the metabolic disorders, we fed the female C57BL/6 mice with a chow diet (Chow), or an HF diet alone or supplemented with 1% CIE (HF+CIE) diet for 8 weeks. The results showed that the mean body weight gain of the HF group was 91.9% more than those in the Chow group after 8 weeks of treatment, indicating the HF diet-induced obesity (Figure 2(a)). The body weight gain induced by HF diet was significantly suppressed by treatment with CIE from 2 weeks into the treatment period to the end of treatment (HF+CIE group). In this study, food intake in the HF+CIE group was roughly equivalent to that in the HF group (Figure 2(b)). As shown in Figures 2(e) and 2(f), the size of epididymal adipocytes was significantly elevated in the HF group compared to the Chow group after 8 weeks. Adipocyte size was markedly decreased in the HF+CIE group compared to the HF group. Furthermore, the concentrations of TG and TC in fecal material were slightly but not significantly higher in the HF+CIE diet-fed mice than the HF diet-fed mice (Figures 2(c) and 2(d)). This indicated that CIE slightly decreased lipid absorption or increased lipid excretion to antagonize diet-induced obesity. These results suggested that CIE could prevent diet-induced obesity independent of food intake inhibition and lipid absorption in the intestine.

3.3. CIE Improves Glucose Tolerance and Attenuates Dyslipidemia. To understand the effects of CIE on the metabolic disorders, we analyzed the serum biochemical contents in the mice. HF fed mice showed a significant increase in fasting blood glucose levels compared to the Chow group mice ($P < 0.05$). In contrast, the CIE groups showed a statistically significant ($P < 0.05$) decrease in fasting blood glucose levels compared to the HF group (Figure 3(a)).

We further tested ipGTT of the mice. As shown in Figure 3(b), the glucose levels were significantly increased in the HF group mice at 15, 30, 60, and 90 min following

the injection of glucose, whereas the blood glucose levels in CIE-treated mice significantly decreased at 15, 30, and 90 min compared to the HF group. The total area under the curve of blood glucose levels between 0 to 90 min was 11.6 ± 1.7 mmol/L/min for the HF group and 9.8 ± 1.2 mmol/L/min for the HF+CIE group ($P < 0.05$). The results indicate that CIE treatment improved the glucose tolerance induced by an HF diet in the mouse.

The fasting serum TG, TC, and LDL-c concentrations of HF group were increased by 44.4%, 20.3%, and 91.1%, respectively, compared to the Chow group (Figures 3(c)–2(e)), whereas the level of HDL-c decreased by 8.7% when compared to the Chow group (Figure 3(f)). The levels of TC and LDL-c were markedly reduced by CIE treatment in HF diet-induced mice. However, a slight increase was observed in HDL-c in the HF+CIE groups when compared to the HF group. We did not observe the differences of the TG levels between the HF and HF+CIE groups (Figure 3(f)). These results indicate that CIE is effective in attenuating high-fat diet-induced risk for dyslipidemia *in vivo*.

3.4. CIE Prevents Hepatic Lipid Accumulation. The liver, one of the insulin-sensitive tissues, plays a pivotal role in the processes of hyperglycemia and dyslipidemia [38, 39]. Therefore, we examined the lipid contents in the liver of the mice. As shown in Figure 4, the levels of TG and TC in liver of HF group were increased 2.1 and 2.2 times, respectively, compared to the Chow group ($P < 0.05$). Supplementation with CIE significantly reduced TG and TC accumulation in liver compared to the HF group ($P < 0.05$). This finding suggests that CIE could block the HF diet-induced lipid accumulation in the liver.

3.5. CIE Inhibits the Transactivities of PPAR γ , LXR α , and LXR β . To determine the mechanism of CIE ameliorates disorders of glucose and lipid metabolism, related genes' expressions in the liver were examined, as shown in Figures 5(a) and 5(b). PPAR γ , one of the most important nuclear receptor transcription factors, regulates the expression of a group of genes involved in lipid and glucose metabolism. Compared with the HF group, CIE treatment notably decreased the mRNA expression of PPAR γ in the mouse liver. Moreover, the mRNA levels of PPAR γ target genes, including fatty acid

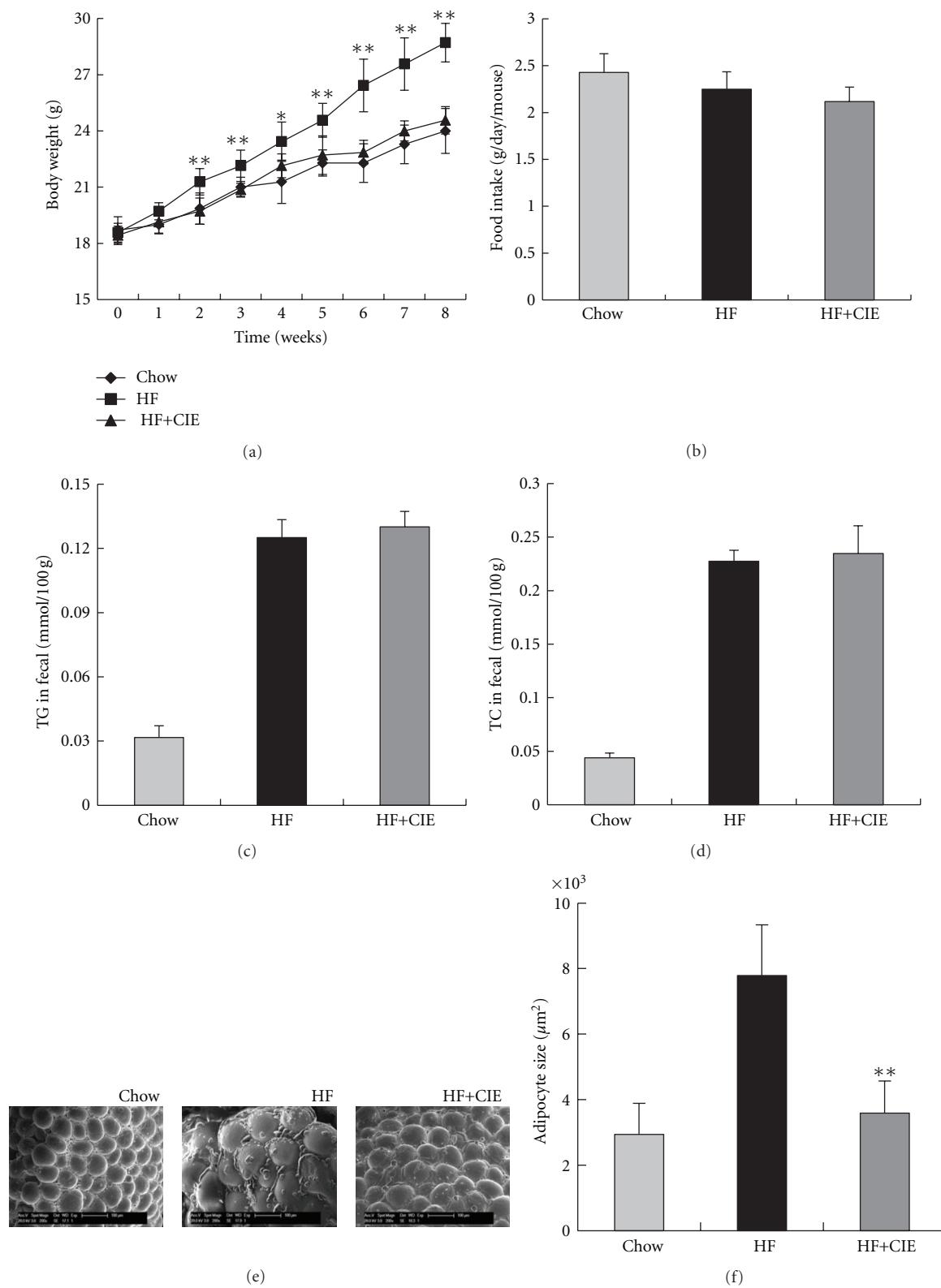


FIGURE 2: CIE prevents HF diet-induced weight gain. C57BL/6 mice were fed a chow diet (Chow) or an HF diet alone (HF) or supplemented with 1% of CIE (HF+CIE) for 8 weeks. (a) Body weight change; (b) food intake expressed as g/day/mouse; (c) TG concentrations in fecal material; (d) TC concentrations in fecal material; (e) epididymal WAT morphology are shown at 200x; (f) size of adipocytes. Values are expressed as means \pm S.E. ($n = 7$; * $P < 0.05$, ** $P < 0.01$ versus the HF group).

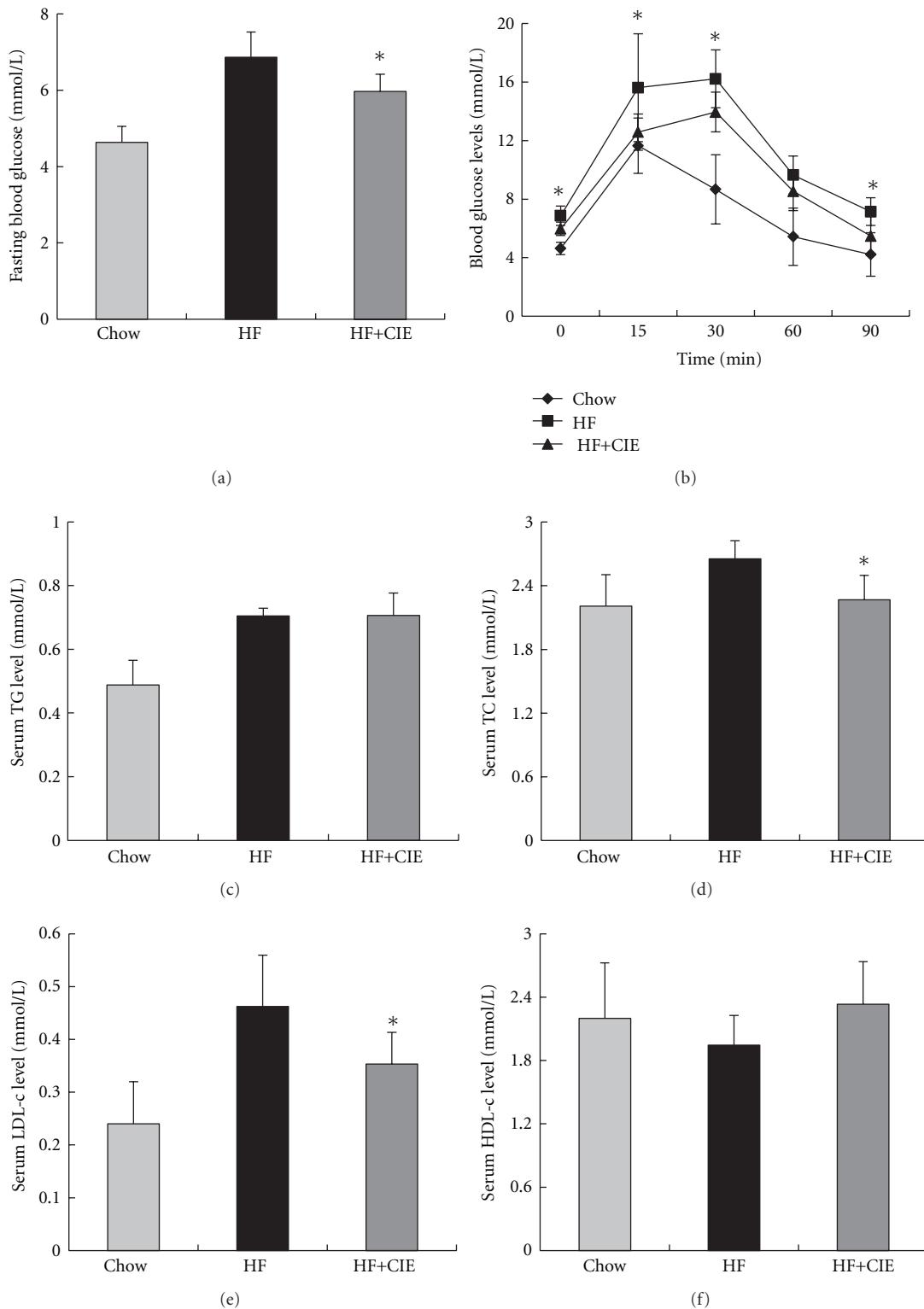


FIGURE 3: CIE decreases the blood glucose and serum lipid levels in HF diet-fed mice. Mice were fed a chow diet (Chow) or an HF diet alone (HF) or supplemented with 1% of CIE (HF+CIE) for 8 weeks. (a) Fasting blood glucose concentration. The mice were fasted for 12 hours and the tail vein blood was used to test the glucose level. (b) Glucose tolerance test was performed by intraperitoneal injection of glucose (1 g/kg body weight) into mice and blood glucoses were measured at 0, 15, 30, 60, and 90 min. ((c)–(f)) TG, TC, LDL-c and HDL-c concentrations in serum from fasted mice. Values are expressed as means \pm S.E. ($n = 7$; * $P < 0.05$, ** $P < 0.01$ versus the HF group).

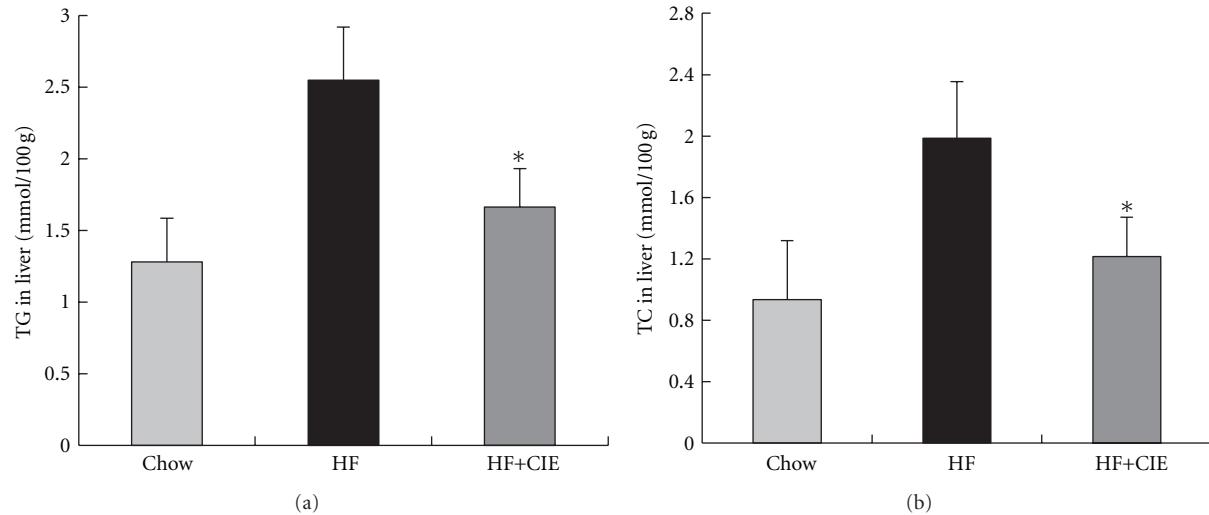


FIGURE 4: Effects of CIE on hepatic lipid levels in HF diet-fed mice. (a) TG concentrations in liver; (b) TC concentrations in liver. Values are expressed as means \pm S.E. ($n = 7$; * $P < 0.05$, versus the HF group).

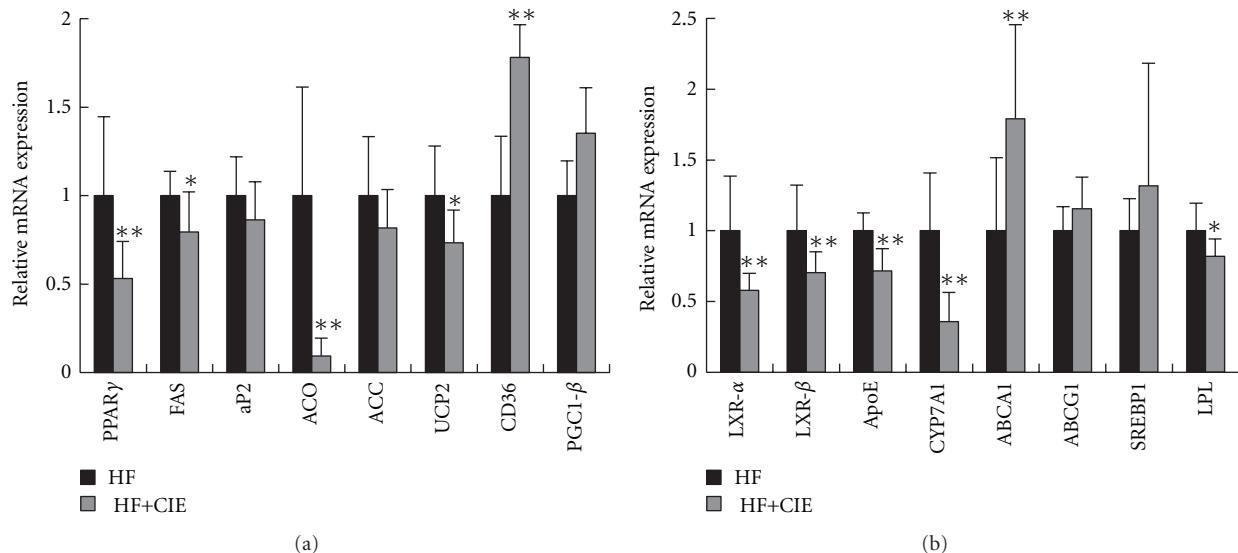


FIGURE 5: Effects of CIE on the relative mRNA expression in liver tissue. (a) PPAR γ and its target genes. (b) LXR and its target genes. Beta-actin was used as an internal control. Values are expressed as means \pm S.E. ($n = 7$; * $P < 0.05$, ** $P < 0.01$, versus the HF group).

synthase (FAS), acyl-CoA oxidase (ACO), and uncoupling protein 2 (UCP2), were also significantly decreased in the livers of HF+CIE group compared with the HF group. However, the expression of adipose fatty acid-binding protein (ap2), acetyl-CoA carboxylase (ACC), and PPAR coactivator1- β (PGC1 β) were not changed significantly by CIE treatment. CIE increased the levels of CD36 mRNA, which is involved in low-density lipoprotein oxidation. These results indicate that CIE plays a role in the regulation of lipid and glucose homeostasis by regulating the expression of PPAR γ and its target genes.

Next, we analyzed the mRNA abundance of LXR and its target genes that regulate fatty acids, cholesterol synthesis,

and glucose metabolism, such as apolipoprotein E (ApoE), cytochrome P450 7A1 (CYP7A1), lipoprotein lipase (LPL), ATP-binding cassette subfamily G member 1 (ABCG1), ATP-binding cassette transporter A1 (ABCA1), and sterol regulatory element-binding transcription factor 1 (SREBP1), in the liver tissue of CIE-treated and untreated mice. As shown in Figure 5(b), the expression levels of ApoE, CYP7A1, LPL, LXR α , and LXR β were significantly decreased in CIE-treated mice. There was no significant difference in the mRNA expression levels of ABCG1 and SREBP1 in the liver of the HF group compared with the HF+CIE group. These results indicate that CIE plays a role in metabolic disorders partly through LXR signaling.

4. Discussion

Citrus peels are rich in flavonoids that have various biological activities. We found that the major flavonoids in CIE were naringin, hesperidin, and poncirin, of which the highest was naringin. This is different from the findings from *Citrus unshiu* peel extract, which showed that the flavonoid compositions were hesperidin, narirutin, and naringin, with hesperidin being the highest [18]. Both hesperidin and naringin exhibit various biological and pharmacological effects, including antitumor, antiinflammatory, and antioxidant activities, and the potential to improve hyperglycemia, dyslipidemia, and hepatic steatosis in Type 2 diabetes [21, 40–42]. Hesperidin also exhibited hypoglycemic activity in STZ-induced diabetic rats [43]. In a clinical trial, it was demonstrated in hypercholesterolemia patients that naringin supplementation reduces LDL-c by 17% and TC by 14% [7]. Yoon et al. suggested that poncirin promotes osteoblast differentiation and prevents adipogenesis in mesenchymal stem cells [44]. Our results suggest that CIE may be used in the prevention and treatment of the metabolic disorders.

Our animal study showed that CIE could lower blood glucose levels and improve glucose tolerance. In the HF group, body weight gain, serum TC and LDL-c levels, and liver TG and TC levels were significantly increased relative to those in the Chow group and were improved by CIE supplementation. Similar to *Citrus unshiu* peel extract, the CIE also significantly reduced hepatic lipid content as well as blood glucose level. Furthermore, dietary intake of CIE effectively reduced body weight gain and epididymal WAT size in the experimental mice. These positive effects were due to the citrus flavanones such as naringin and hesperidin. We noticed that the food intake was not changed between the groups of HF and HF mixed with CIE, suggesting that the weight-reducing effects of CIE are not caused by suppressing appetite. Also, the fecal lipids were not altered in the CIE-treated mice, suggesting that the body weight reducing effect of CIE is not caused by the inhibition of lipid absorption in the intestine. These results provide convincing evidence that the extract of *Citrus ichangensis* has prevented HF diet-induced obesity and related metabolic disorders.

Citrus aurantium flavonoids show antiadipogenesis activity by downregulating the expression of PPAR γ in 3T3-L1 cells. In the present study, CIE treatment decreased the gene expression of major glucose and lipid metabolism regulators, including PPAR γ and LXR s . The active element of *Citrus ichangensis* probably is naringin, that is, the richest flavonoid in the extract. PPAR γ is a major nuclear receptor transcription factor for adipogenesis and lipogenesis. It regulates the expression of a group of genes, including CD36, ACC, ACO, UCP2, FAS, and aP2, which are related to fatty acid synthesis, oxidation, and adipogenesis. It has been shown that PPAR γ antagonists can prevent and treat HF diet-induced obesity [45]. Gong et al. reported that suppressing PPAR γ -activity can inhibit adipocyte differentiation *in vitro* [46]. Our *in vivo* studies have showed that CIE efficiently suppressed the gene expression of PPAR γ in the liver tissue. The expressions of FAS, aP2, ACO, and UCP2 were also

significantly decreased in the liver of mice treated with CIE. Moreover, the lipid levels of the liver were significantly lower in the HF+CIE group than in the HF group. These results indicate that CIE may reduce fat weight through the regulation of PPAR γ signaling.

To detect other prospective molecular targets through which CIE exhibits anti-metabolic disorders effects, we examined the transactivity of LXR in liver tissue. LXR is known to play a major role in regulation of cholesterol, fatty acid, and glucose homeostasis metabolism [47]. It has been demonstrated that naringenin may decrease serum lipid levels by inhibiting the activation of LXR α [26], indicating that the inhibition of LXR also has a therapeutic role. In this study, both LXR α and LXR β transactivities were inhibited by CIE. The mRNA expression of LXR target genes such as ApoE, CYP7A1, ABCA1, ABCG1, SREBP1, and LPL were further confirmed. The expression levels of ApoE, CYP7A1, and LPL were significantly decreased in the CIE+HF group in comparison to the HF group. However, ABCG1 mRNA expression was not changed significantly by CIF treatment. These results demonstrate that CIE regulates cholesterol and glucose metabolism partly through LXR antagonism in high-fat diet-induced obese mice.

In conclusion, we found that CIE prevents the development of obesity induced by a HF diet and lowers hyperlipidemia and hyperglycemia, while protecting against the lipid accumulation in the liver. These effects may involve multi-molecular targets, including the inhibition of PPAR γ and LXR s , in the liver tissue. Our results suggest that *Citrus ichangensis* could be used as a dietary supplement for antiobesity and hyperlipidemia-lowering therapy. However, the mechanism needs to be further investigated.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgment

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