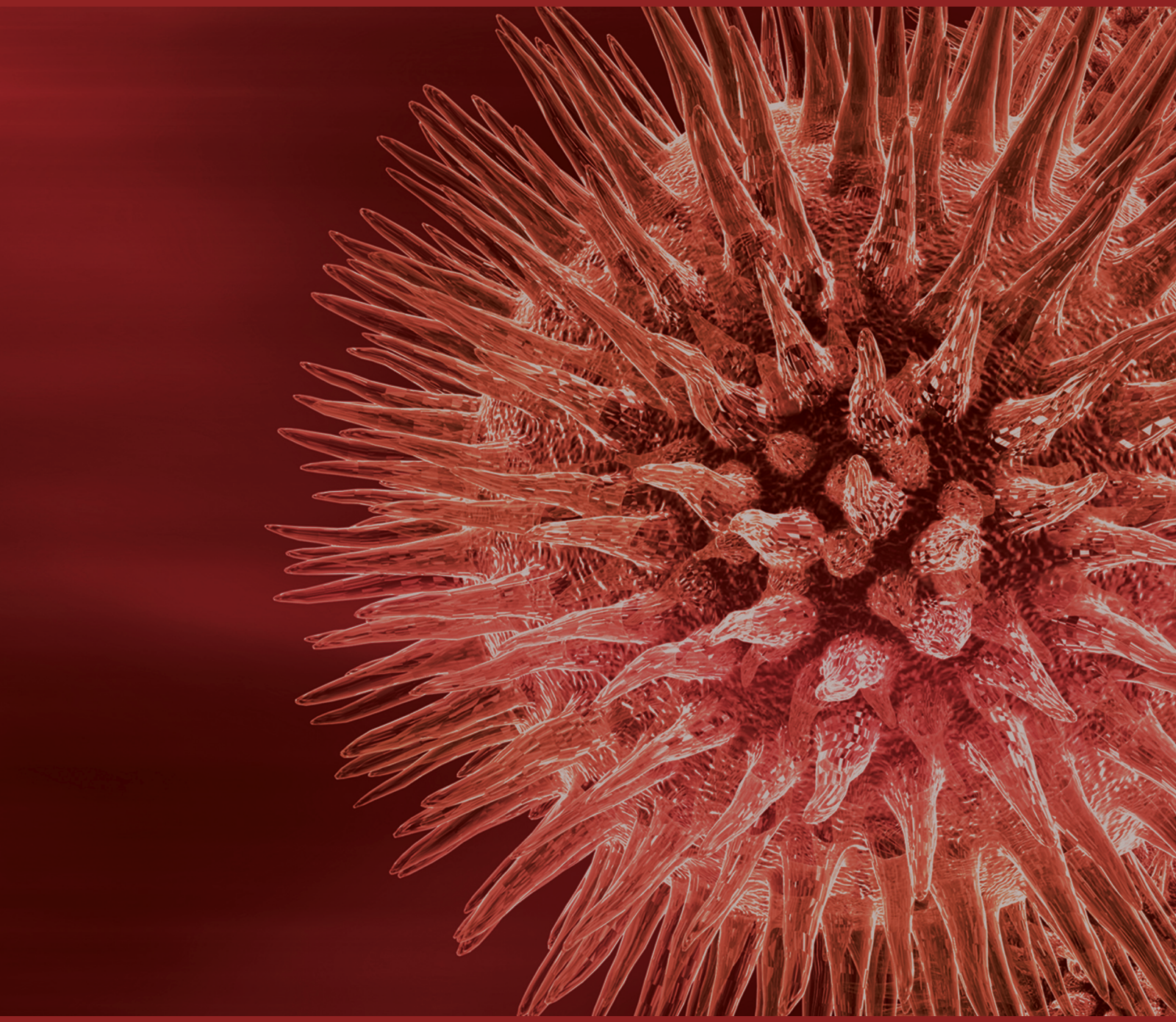
BioMed Research International

Biologic Activity and Biotechnological Development of Natural Products

Guest Editors: José Carlos Tavares Carvalho, Fabio Ferreira Perazzo, Leandro Machado, and Didier Bereau



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Editorial

Biologic Activity and Biotechnological Development of Natural Products

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Flora and Fauna diversity is an essential element of our environment. For millennia, human beings have used it for food as well as nonfood purposes such as medicine, dye-based tincture, or cosmetics, in direct application or after processing. Certain species, in view of their characteristics, have been domesticated, improved for outreaching the stadium of harvest or of hunting for family purposes, to be cultivated and fostered in large scale, entering market economy. They have thus become a source of wealth and economic development by establishing product branches and sophisticated exploitation.

However, it seems that, especially in tropical surroundings, this biodiversity is far from being valued adequately. Even though being fertile and abundant, little is known about it, in endemic and in cultivated wildlife species. Yet, knowing this biodiversity would allow exploiting it at its best while protecting it, which presents a major challenge.

In addition, currently there is a strong consumer demand; therefore there is a strong commercial pressure on

(i) improving the quality of consumer products,

(ii) allocation of extracts, means purified substances, having quantified biological activities,

(iii) a high level of safety for consumers.

Additionally, there are high expectations regarding environment-friendly exploitation of the natural resources, both for production and processing, as well as a fair return to the population having ceded their knowhow.

From 37 submissions, 24 papers are published in this special issue. Each paper was reviewed by at least two reviewers and revised according to reviews comment. The works presented in this special issue, related to different scientific fields, highlight the imperial need to characterize the natural substances physically and chemically and to evaluate their numerous biological activities.

Even if trace metals are important for living organisms in order to stabilize protein structures, to facilitate electron transfer reactions, and to catalyse enzymatic reactions, they can also lead to toxicity, mainly for oxidative stress reason. Fungi may be the alternative solution to resins because of their ability to tolerate and detoxify metals. In N. M. Abdel-Monem et al.'s paper *"Pretreatment hepatoprotective effect of the marine fungus derived from sponge on hepatic toxicity induced by heavy metals in rats,"* the authors prove a pretreatment hepatoprotective effect of the extract of marine-derived fungus *Trichurus spiralis* Hasselbr. isolated from *Hippospongia communis* sponge on the hepatotoxicity, following heavy-metal mixture's administration (Cd, Co, Hg, Ni chloride, and

Pb acetate 0.25 mg) investigated in rats for a period of 7 days. They demonstrated that marine-derived fungus extract (T.S) possesses a hepatoprotective property due to its proven antioxidant and free radical scavenging properties.

Nowadays, reduction in release of methane into the environment is required for all countries. Lovastatin, which is a natural polyketide synthesized by *Aspergillus terreus* and *Pleurotus ostreatus* (oyster mushroom), may play this important role in rumens by inhibiting HMG-CoA reductase activity, a key enzyme involved in the formation of isoprenoid building blocks that are essential for cell membrane synthesis in methanogenic Archaea. Because of its expensive cost for large scale use in agriculture, an alternative could be the study done in M. F. Jahromi et al.'s paper *"Lovastatin in Aspergillus terreus: fermented rice straw extracts interferes with methane production and gene expression in Methanobrevibacter smithii."* The authors described a series of experiments designed to test the hypothesis that unpurified lovastatin secreted by *Aspergillus terreus* in fermented rice straw extracts (FRSE) inhibits growth and CH4 production in a representative methanogen—*Methanobrevibacter smithii* (DSM 861). FRSE stronger effect when compared to commercial lovastatin was demonstrated. Thus, it is feasible to use *A. terreus*-fermented rice straw (an industrial byproduct) as feed additive in ruminants.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA) is a ubiquitous phenolic acid in the plant kingdom. It plays an important role, like other flavonoids, in health owing to its potential biological activities. However, studies revealing its adverse effects are increasing. In C. C. Peng et al.'s paper *"Cytotoxicity of ferulic acid on T24 cell line differentiated by different microenvironments,"* the authors tried to understand the biological behaviors and cytotoxicity involved in 3D-cells culture (native three-dimensional structure) compared to 2D-monolayers when FA was occurring. FA (2 mM) in the 3D-culture revealed significantly higher cytotoxicity than the 2D-culture suggesting that biological significance may be implicated by using either 3D- or 2D-culture model.

Inflammatory which is responsible for morbidity and mortality in the world can be treated either with nonsteroidal anti-inflammatory drugs (NSAIDs) or steroidal drugs. Both have proven to be effective but can have negative side effects. In a way to find new molecules with anti-inflammatory activity but with fewer side effects, in C. P. González et al.'s paper *"Anti-inflammatory activity and composition of Senecio salignus Kunth,"* the authors investigated the anti-inflammatory activity of *Seneco salignus*, a plant issued from traditional medicine, by induced edemas and the composition of its active fraction by GC-MS. An active fraction was obtained from chloroform extract in both anti-inflammatory tests in which a total of 185 compounds were isolated.

An endemic plant of the Brazilian Atlantic Forest and widely spread in the sandbanks of "Restinga de Jurubatiba" National Park, *Neomitranthes obscura* (DC.), was studied in order to evaluate chemical composition of essential oils obtained from specimens with different fruit color by GC and GC/MS analysis. In R. R. Amaral et al.'s paper *"Essential oils from fruits with different colors and leaves of Neomitranthes obscura (DC.) N. Silveira: an endemic species from Brazilian Atlantic Forest,"* the authors demonstrated the predominance of sesquiterpenes (in leaves and yellow fruits essential oils), meanwhile monoterpenes were found in black fruits oils. All these results were suggesting the occurrence of unless two different varieties for this species.

In hypertension and cardiovascular diseases which affect developed countries, Angiotensin I-converting enzyme (ACE) plays important physiological role in blood pressure regulation. The consumption of ACE inhibitory peptides may be useful. In J. C. Wu et al.'s paper *"Preparation of ACE inhibitory peptides from Mytilus coruscus hydrolysate using uniform design,"* the authors investigated the ACE inhibitory potential of peptides isolated from mussel, *Mytilus coruscus*, according to variable factors such as protease concentration, hydrolysis time, PH, and temperature. Uniform Design, a new statistical experimental method, was operated. A high percentage of lysine, leucine, glycine, aspartic acid, and glutamic acids were found suggesting nutraceuticals and pharmaceuticals purposes.

Rubiaceae family is well known for the presence of secondary metabolites with pharmacological potential among them antibacterial properties. In D. Martins et al.'s paper *"Triterpenes and the antimycobacterial activity of Duroia macrophylla Huber (Rubiaceae),"* an occurring species in the Amazon Forest, *Duroia macrophylla*, was studied in order to evaluate the antimycobacterial activity of its extracts and isolate and identify the substances present. The authors found major biological activity against *Mycobacterium tuberculosis* in leaves dichloromethane extract. Molecules belonging to terpenes, alkaloids, and flavonoids were also isolated; oleanic and ursolic acids were reported for the first time in *Duroia* genus.

Flavonoids, and especially anthocyanins, are well known for their biological activities: antiproliferative, hypoglycemic, antioxidant, and antiobesity effects. Anti-inflammatory properties are also well described in several biochemical pathways. In N. M. A. Hassimotto et al.'s paper *"Inhibition of carrageenan-induced acute inflammation in mice by oral administration of anthocyanin mixture from wild mulberry and cyanidin-3-glucoside,"* attention has been paid to an anthocyanin-enriched (AG) fraction from mulberry and cyanidin-3-glucoside (C3G) to evaluate their potential against two acute inflammations induced by carrageenan in mice (peritonitis and paw oedemas). Positive results have been demonstrated suggering the use of AG and C3G for prophylactic or therapeutic purposes.

Interest in natural products and especially in bee products as honey, royal jelly, pollen, and propolis is increasing. This latter showed various biological properties, among them, immunomodulatory, antibacterial, fungicidal, anti-inflammatory, healing, anesthetic, and anticarcinogenic effects. In M. M. Possamai et al.'s paper *"Brazilian propolis: a natural product that improved the fungicidal activity by blood phagocytes,"* the authors focused on the propolis of a Brazilian stingless bee (*Meliponae* subfamily) in order to evaluate the potential effects of its adsorbed form on polyethylene glycol (PEG) on the activity of human phagocytes against *Candida albicans*. Immunostimulatory effects were demonstrated

suggesting this adsorbed form of propolis for many other therapeutic uses.

Ampelozizyphus amazonicus Ducke (belonging to Rhamnaceae family) is a popular plant from Amazonian folks and is described by ethnobotanists as useful in the treatment and prevention of malaria. As no direct action on *Plasmodium* blood stage forms was demonstrated, in L. M. T. Peçanha et al.'s paper *"Immunobiologic and antiinflammatory properties of a bark extract from Ampelozizyphus amazonicus Ducke,"* the authors paid attention to the immunological response of *Plasmodium chabaudi*-infected mice, treated by an aqueous extract of this plant. Immunomodulatory and anti-inflammatory properties were found, maybe due to the presence of a complex dammarane-type saponin.

Mushrooms are well implanted in Asian, therapies, specifically for their medicinal uses. *Sparassis crispa*, known as cauliflower mushroom, is getting more and more popular thanks to not only its taste but also its therapeutic applications. In T. Kimura's paper *"Natural products and biological activity of the pharmacologically active cauliflower mushroom Sparassis crispa,"* the author presents an overview of the pharmacological properties of this mushroom and the mechanisms of action of its bioactive components. Specifically, the immunomodulatory mechanisms of beta-glucan, an important present molecule, are well described.

In response to an increasing interest of consumers for functional foods, studies using probiotic organisms are more frequent. In J. E. Park et al.'s paper *"Lactobacillus plantarum Lg42 isolated from Gajami Sik-Hae inhibits adipogenesis in 3T-L1 adipocyte,"* the authors investigated a popular Korean fermented fish product (Gajami Sik-Hae), paying special attention to the beneficial influence of the herein *Lactobacillus plantarum* lactic acid bacteria (GLAB) on lipid accumulation in adipocytes by regulating the expression of adipogenesis-related genes in differentiated 3 T3-L1 cells. Inhibitory effects of the GLAB by modulating the expression of adipogenic transcription factors were demonstrated, suggesting the antiobesity property of this fermented seafood.

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder resulting in a defect in the muscle membrane protein called dystrophin. Its consequences may be a loss of ability to walk and cardiac function and respiratory muscles strength reductions. In D. Feder et al.'s paper *"Hormonal receptors in skeletal muscles of dystrophic Mdx mice,"* the authors, for the first time, investigated the gene expression of hormone receptors in different muscles (dystrophic and healthy) in mdx and C57BL6 mice. Dystrophic muscles have some significant differences in hormone receptor expression when compared to normal mice suggesting more studies to be enhanced on this path in a way to understand the events related to this pathophysiology.

Atherosclerosis is a cardiovascular disease resulting from the rupture of an atherosclerotic plaque. Its stabilization and the inhibition of its progression with macrophages apoptosis are of paramount importance. In F. Wang et al.'s paper *"The sonodynamic effect of curcumin on THP-1 cell-derived macrophages,"* the authors chose a new method with ultrasound using curcumin as a sonosensitizer on THP-1 derived macrophages: the sonodynamic therapy (SDT). They concluded that curcumine by SDT decreases macrophages viability and induces their apoptosis or necrosis. Both loss of mitochondrial membrane potential and morphological changes of cytoskeleton were apparent. So, curcumin could be a new sonosensitizer in a promising treatment for atherosclerosis.

Tandem repeats of proteins and peptides are known to have many stabilizing functions. Thymosin alpha 1 (Tα1), which is composed of 28 amino acids, has immunemodulatory and antitumor properties. In X. C. Xue et al.'s paper *"Construction, expression, and characterization of thymosin alpha 1 tandem repeats in Escherichia coli,"* the authors created, in *E. coli* TOP10, the molecule Tα1③, which is composed of three repeated copies of Tα1 and is fuse-expressed with thioredoxin. Its biological activity on T lymphocyte proliferation and IL-2R expression was significant. This work is an efficient tool for a large scale production of this active protein.

2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) is a known environmental contaminant involving the formation of CYP1A1, a carcinogen-activating enzyme. Harmine and harmaline, β-carboline alkaloids, were already described in the literature as molecules with pharmacological activities. In a previous work, M. A. M. El Gendy et al. had already demonstrated the inhibitory effects of these alkaloids on CYP1A1 in vitro. In the paper *"Harmine and harmaline downregulate TCDD-induced Cyp1a1 in the livers and lungs of C57bl/6 mice"* by M. A. M. El Gendy and A. O. S. El-Kadi, this work is enhanced in in vivo conditions on mice livers and lungs. Promising results were found so that harmine and harmaline could be purposed as good candidates in the strategy against carcinogenesis.

Female reproductive ability can be affected by several parameters: production and transport of gametes, endocrine system, sexual behavior, gestation, parturition, lactation, and alterations in other functions. *Pradosia huberi*, a well known Sapotaceae plant from the Brazilian folk medicine, can present potential toxic effects next to its anti-inflammatory properties. In A. O. B. Rocha et al.'s paper *"Evaluation of the toxicity of Pradosia huberi extract during the preimplantation in Wistar rats,"* the authors paid attention to the effects of hydroalcoholic extracts of *P. huberi* during the uterine implantation time of Wistar rats. Compromising results were found on the reproductive ability during the embryonic preimplantation phase, suggesting a possible toxic effect upon the reproductive system of Wistar rats.

Manilkara subsericea is a widely spread species on the sandbanks of eastern Brazil with food and construction local uses. Potential biological activities and chemical compositions (including triterpenes, saponins, and flavonoids) were already reported in the literature for the genus *Manilkara*. In C. P. Fernandes et al.'s paper *"Triterpene esters and biological activities from edible fruits of Manilkara subsericea (Mart.) Dubard, Sapotaceae,"* the authors focused on antibacterial and cytotoxicity of extracts from *Manilkara subsericea* and chemically characterized the hexanic extract from its edible fruits. Beta- and alpha-amyrin caproates and caprylates were reported for the first time for this species. Antimicrobial

activity against *Staphylococcus aureus* was relevant. And weak toxicity against Vero cells was also demonstrated.

Given the lack of knowledge concerning the nutrient contents of fish and shellfish species in Malaysia, in N. Abd Aziz et al.'s paper *"Quantitative determination of fatty acids in marine fish and shellfish from warm water of Straits of Malacca for nutraceutical purposes,"* the authors investigated through qualitative and quantitative nutritional analysis 20 species issued from Straits of Malacca. Fatty acids composition and amount of each fatty acid were determined. Most samples contained fairly high amounts of polyunsaturated fatty acids (PUFAs), meanwhile some species showed significantly high amounts of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), alpha-linolenic acid (ALA), and omega-3 fatty acids. The polyunsaturated-fatty-acids/saturated-fatty-acids (P/S) ratios for most samples were high. These promising results could be useful for nutraceutical purposes.

Allium hirtifolium Boiss, known as Persian shallot, is an Iranian native spice with traditional uses, specifically in food uses. The numerous biological activities attributed to both famous plants issued from the same genus (garlic and onion) were relevant enough so studies be investigated on this species. In S. Ismail et al.'s paper *"Chemical composition and antibacterial and cytotoxic activities of Allium hirtifolium Boiss,"* the authors paid attention to the chemical composition, antibacterial and cytotoxic effects of the spice. Hydromethanolic extract, through GC/MS analysis, revealed the main presence of 9-hexadecenoic acid, 11,14-eicosadienoic acid, and n-hexadecanoic acid. And persian shallot, because of its efficiency against 10 different species of pathogenic bacteria, could be considered as a safe and strong antibacterial agent.

Because of the increasing bacteria antibiotic resistance, plants could play an alternative role. *Amburana cearensis* A. C. Smith and *Anadenanthera macrocarpa* (Benth.) Brenan are well known from folks of South America. In F. G. Figueredo et al.'s paper *"Modulation of the antibiotic activity by extracts from Amburana cearensis A. C. Smith and Anadenanthera macrocarpa (Benth.) Brenan,"* the authors investigated the phytochemical composition and the antibacterial and modifying antibiotic activities of these plants. Their ethanolic extracts demonstrated antibacterial action due to the presence of several antibacteria responsible for modulatory effects. The use of these natural products combined with aminoglycosides in order to increase their antimicrobial potential against multiresistant microorganisms could be a serious alternative.

Pothomorphe umbellata L. (*Piperaceae*) is well know in Brazil for its different pharmacological activities (anti-inflammatory, analgesic, antiulcer, gastroprotective, antimalarial, and antioxidant) mainly due to a phenolic compound located in the vegetable roots and leaves: the 4-nerolidylcathecol. In A. P. Lopes et al.'s paper *"Antioxidant and cytotoxic effects of crude extract, fractions and 4-nerolidylcathecol from aerial parts of Pothomorphe umbellata L. (Piperaceae),"* the authors paid attention to the crude ethanolic and aqueous-ethanolic extracts, sterol fraction, and 4-nerolidylcathecol of the plant in order to evaluate its antioxidant activity and its cytotoxic effect in HL-60 cells. Significant antioxidant potential and low toxicity were found to be corroborating the safe and effective use of *P. umbellata* by folklore medicine.

Breast cancer causes the highest percentage of the cancer deaths in women worldwide (in both developing and developed countries). Next to the existing options of treatment (mostly correlated to serious side effects), bioactive components of algae can be a potential alternative. In S. E. Nigjeh et al.'s paper *"Cytotoxic effect of ethanol extract of microalga, Chaetoceros calcitrans, and its mechanisms in inducing apoptosis in human breast cancer cell line,"* the authors investigated the cytotoxic effect and apoptosis mechanism of crude ethanol extracts of an indigenous microalga from Malaysia, *Chaetoceros calcitrans,* on human breast cell lines. Exposure of MCF-7 and MCF-10A cells to crude ethanol extracts of *C. calcitrans* (EEC) resulted in cell number decrease through induction of apoptosis or modulation of gene expression related to cell cycle. *Chaetoceros calcitrans* could be a chemopreventive agent for breast cancer treatment.

Diabetes is a worldwide epidemic which is characterized by a disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Treatment may be operated by several oral antihyperglycemic agents, but side effects occur. In D. Cheng et al.'s paper *"Antihyperglycemic effect of Ginkgo biloba extract in streptozotocin-induced diabetes in rats,"* the authors evaluated the antihyperglycemic effects of a *Ginkgo biloba* extract (GBE), a beneficial plant in Chinese medicine, on streptozotocin (STZ) induced diabetes in rats. Increase in body weight and antioxidant ability and decrease in blood glucose, lipid profile, and lipid peroxidation were noticed, suggesting GBE as supplement or adjunct treatment for diabetics.

These papers are real state of the art which emphasize potential uses for natural products. Originated from temperate or tropical climate, natural substances are applied in various domains like food, pharmaceutics, or cosmetics purposes. They are key elements for the future development of the countries they originate from. We hope that this special issue would attract a major attention of the peers.

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José Carlos Tavares Carvalho
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Research Article

Cytotoxicity of Ferulic Acid on T24 Cell Line Differentiated by Different Microenvironments

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Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA) is a ubiquitous health beneficial phenolic acid. Although FA has shown a diversity of biological activities including anti-inflammatory, antihypercholesterolemic and anticancer bioactivities, studies revealing its adverse effects are accumulating. Recently, 3D-cultures are shown to exhibit uniquely biological behaviors different from that of 2D cultures. To understand whether the cytotoxicity of FA against the T24 cell line (a bladder cancer cell line) in 2D-culture could consistently retain similar bioactivity if cultured in the 3D-systems, we conducted this experiment with 2 mM FA. Much higher cytotoxicity was found for 3D- than 2D-culture, showing (2D vs. 3D): apoptotic rates, 64% and 76%; cell killing rates, 3.00×10^5 cells $mmol^{-1} \cdot h^{-1}$ and 2.63×10^6 cells $mmol^{-1} \cdot h^{-1}$, attaining a 8.77-fold. FA upregulated the activities at 72 h (2D vs. 3D in folds that of control): SOD, 1.73-folds ($P < 0.05$) versus 3.18 folds ($P < 0.001$); and catalase, 2.58 versus 1.33-folds. Comparing to the control (without FA), Bcl-2 was prominently downregulated while Bax, caspase-3 and cleaved caspase-9 were more upregulated in 3D-cultures ($P < 0.05$). Conclusively, different microenvironments could elicit different biological significance which in part can be ascribed to different mass transport rate.

1. Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA), an effective component of many Chinese medicinal herbs like *Angelica sinensis, Cimicifuga heracleifolia,* and *Lignsticum chuangxiong*, is a ubiquitous phenolic acid in the plant kingdom [1]. FA exhibits many physiological functions including antioxidant, antimicrobial, anti-inflammatory, antithrombosis, antihypercholesterolemic, anticancer activities, and spermatozoa activating bioactivity [1]. Interest in the role of flavonoids to act as health benefits is emerging owing to their potential biological activities. However, to date, epidemiologic studies exploring the role of flavonoids in human health have been inconclusive [2]. Some studies supported the protective effect of flavonoids on cardiovascular disease and cancer, others demonstrated no effect [2], and interestingly a few suggested them to be potentially harmful [3]. More recently, we demonstrated FA to be nephrodamaging when used for a long-term treatment for chronic kidney disease (CKD) [4].

Conventional adherent tissue culture involves growing cells on solid flat surfaces as two-dimensional (2D) monolayers. Although such practices are routine and suitable for transformed or immortalized cell lines, dedifferentiation and loss of specialized functions occur when primary cells are removed from their host tissue and grown as 2D monolayers.

This is generally believed to be a result of the dissociation of primary cells from their native three-dimensional structure *in vivo* to their two-dimensional propagation on flat impermeable substrates *in vitro* [5–7]. As such, there is a continuing need to develop tissue culture systems which can either promote redifferentiation of laboratory cell lines or prevent primary cell lines from dedifferentiating.

The reason(s) eliciting different biological outcomes by different microenvironments is still unclear. With an aim to understand more about the cellular physiology and conversely the different cytotoxicity of a given flavonoid like FA that may occur in different microenvironments as specified by the 2D and 3D cultures, we carried out this present study. We compared the cell viability, the cellular morphology, the oxidative stress defensive markers, and the apoptotic and antiapoptotic signals between the 2D and 3D cultures in the T24 cell line (a balder cancer cell line). For interpretation we developed a diagrammatic model to emphasize the mass transport in part to be an important role affecting such an outcome.

2. Materials and Methods

2.1. Chemicals and Kits. Ferulic acid (FA) was supplied by Sigma Aldrich (Saint Louis, MO, USA). The medium McCoy's 5A was provided by (GIBCO, USA), which was supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (GIBCO, USA). The pH of 2D culture was unadjusted after incubation, while that of 3D cultures was controlled through CO_2 atmosphere. The fixing solution was prepared by dissolving glutaraldehyde (2.5 g) and paraformaldehyde (2 g) in 100 mL of 0.2 M of sodium cacodylate (CaCo). The washing buffer was prepared by dissolving 7 g of sucrose in 100 mL of 0.1 M CaCO. Ferulic acid (FA) stock solution was prepared by dissolving authentic ferulic acid in DMSO to make a 2 M solution (stock solution). The required experimental solutions were prepared by diluting the stock solution with appropriate amount of medium McCoy's 5A to the experimental concentrations as indicated. Antibodies Bcl-2 (1 : 1000), Bax (1 : 1000), cleaved caspase-3 (1 : 1000), cleaved caspase-9 (1 : 1000), and β-actin (1 : 1000) were purchased from Bioscience Co. (United Kingdom).

2.2. Cells. The human urinary bladder cancer cell line, T24 (HTB-4, ATCC), was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). T24 cells were derived from an invasive bladder tumor of grade 3, having *p53* nonsense mutation at codon 126 (TAC to TAG) [8].

2.3. Cell Culture

2.3.1. D Culture of T24 Cell Line. According to the method of [9], T24 cells at a density of 2×10^4 cells/mL were seeded onto a 6-well plate in medium McCoy's 5A containing 2 mM FA. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 24 h. The cultivation of T24 cells was maintained within 20 passages. These cells were further used for cultivation in RWV.

2.3.2. 3D Culture of T24 Cell Line. The T24 cells were harvested from the 2D plate culture. By following the manufacturer's instructions, the cell count was enumerated and inoculated at 2×10^5 cells/mL to the 50 mL spinner vessel (Techne) of the Rotary Cell Culture System (RCCS) (Synthecon Co., Houston, TX, USA), which has been always referred to as the three-dimensional rotating-wall vessel (RWV) [10]. CultiSpher-G was prepared according to instructions and the amount used was either 2 g/L (Vero) or 1 g/L (GMK). Medium McCoy's 5A was used to fill up the entire vessel to get rid of the air. The RWV containing the medium and cells was incubated at 37°C at an agitation speed 45 rpm. The incubation was continued and the medium was replaced every 2 days together with 25 mL of sterilized FA (4.0 mM) solution to sustain the FA concentration at 2 mM. On day 3, the cells were harvested and transferred into a centrifuge tube and centrifuged at 10000 ×g for 10 min. The supernatant was decanted. The cell cluster was rinsed thrice with sterilized PBS, each time with 20 mL.

2.4. SEM Examination of Morphological Changes. The cells were diverged in the fixing fluid for 2 h and then centrifuged. The fixing fluid was decanted off. The residual cells were rinsed with washing buffer thrice, each time for 10 min. The rinsed cells were remained in the rinsing solution until SEM scanning. 1 μL of the sample was measured from the sampling port of RCCS. Cryofixation of the suspension was finished in HPM 010 high Pressure Freezing Machine (TESCAN, USA). Graphene support films for electron microscopy (Electron Microscopy Sciences) were used. The specimens were uniformly coated with one layer gold powder using EMS 150R boast (Electron Microscopy Sciences). EM image was taken by the transmission electron microscope (DELONG TEM LVEM5) operated at an accelerating voltage 100 kV. The aperture was set at "1" with a motorized JSM-840A (Deben) to fit the TEM console. The electron gun was made of lanthanum hexaboron operated at 1500 K.

2.5. Cell Viability Assay

2.5.1. MTT Assay for 2D Culture. According to the method described by [9], T24 cells were seeded at a density of 5×10^3 cells/well onto a 24-well plate, treated with FA (DMSO vehicle, 2 and 4 mM), and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 24, 48, and 72 h. To each well 0.2 mL of MTT was added and the cultivation was continued for additional 3.5 h. The MTT solution was removed by sucking off, and 0.5 mL DMSO was added to dissolve the blue formazan precipitate. After 10 min, the optical density was read at 570 nm and the cell viability was evaluated.

2.5.2. Cell Enumeration for 3D Culture. Following the manufacturer's instructions, duplicate samples of 0.5 mL were taken from the spinner. After sedimentation of the beads, 0.3 mL supernatant was withdrawn and 0.8 mL dispase

(5 mg/mL in PBS) was added. Beads were completely dissolved after 30 min at 37°C. Cells were collected by centrifugation at 12000 ×g and 1.0 mL of citric acid (0.1 M) containing Triton X-100 (1%, w/v) and crystal violet (0.01%, w/v) added. Stained nuclei were counted in a hematocytometer (Percell Biolytica Application Note 115).

2.6. ELISA for Serum Superoxide Dismutase and Catalase. The levels of superoxide dismutase (SOD) and catalase were measured by the SOD and catalase ELISA Kits provided by PeproTech Inc. (NJ, USA). All ELISA protocols were performed according to manufacturer's instructions. The readings were conducted with the SYSMEX K-1000 Reader, a product of San-Tong Instrument Co. (Taipei, Taiwan).

2.7. Western Blotting. The cells (approximately 100 mg) obtained in the above were homogenized with the homogenizer (T10 basic, The IKA Company, Germany) in 1 mL of ProPREP lysis buffer (pH 7.2). The homogenate was centrifuged at 12000 ×g for 20 min at 4°C, and the supernatant was collected as cell lysate sample. The cell lysate was heated at 100°C for 10 min before loading and separated on precasted 7.5% SDS-PAGE. The protein content was analyzed before loading according to the manufacturer's instructions. Aliquots of the treated lysates containing protein 50 μg/μL were electrotransferred onto the PVDF membrane in transfer buffer for 1 hr. The nonspecific binding to the membrane was blocked for 1 hr at room temperature with 5% nonfat milk in TBS buffer. The membranes were then incubated for 16 hr at 4°C with various primary antibodies. After extensive washing in TBS buffer, the membranes were then incubated with secondary antibody in blocking buffer containing 5% nonfat milk for 1 hr at room temperature. Membranes were then washed with TBS buffer, and the signals were visualized using the Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Levels of Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, and β-actin were assayed, respectively, by immunoassay according to the manufacturers' instructions. β-actin was used as the reference protein.

2.8. Statistical Analysis. Data obtained in the same group were analyzed by Student's t-test with computer statistical software SPSS 10.0 (SPSS, Chicago, IL, USA). ANOVA software statistical system was used with Tukey's testing to analyze the variances and significances of difference between paired means. Significance of difference was judged by a confidence level of $P < 0.05$.

3. Results and Discussions

3.1. SEM Scanning Revealed Ferulic Acid and 3D Microenvironment Induced Cell Elongation. In the absence of FA, T24 cells proliferated equally well despite 2D (Figure 1(a)) or 3D (Figure 1(c)) cultures. The presence of FA (2 mM) slightly elongated the cell shape, and in parallel the cell number was largely reduced (Figure 1(b)). The majority of the cells died after being cultivated for 72 h at 37°C in both 2D and 3D cultures. The dead cells on the 3D matrix appeared puffy, elongated, and not well shaped (Figure 1(d)).

3.2. Cell Viability Affected by Ferulic Acid and Microenvironment. The viability of T24 cell line was seen inhibited in time-responsive manner in the presence of 2 mM FA. In 2D culture, the cell viability was reduced to 72, 53, and 36%, respectively, compared to the control (Figure 2(a)). The corresponding values reached 63, 32, and 24%, respectively (Figure 2(b)). The killing rate in 2D culture was found to be 3×10^5 cells mmol^{-1}·h^{-1}. More severe killing rate in the 3D cultures reached 2.63×10^6 cells mmol^{-1}·h^{-1}, giving a difference of 8.77-fold.

Cherng et al. indicated that FA (4 mM) effectively suppressed the proliferation of J82 cells, another bladder cancer cell line, to a viability 48.79% [9]. In contrast to our data, 64% of T24 cells in the 2D and 76% in the 3D cultures were killed by ferulic acid (2 mM) after being incubated for 72 h ($P < 0.01$) (Figures 1 and 2), evidently implicating the astonishing effect caused by different microenvironments. Alternatively, different cell lines responded differently to the same phytochemical in equal strength even in the 2D culture [10]. Thus, the outcome of chemicobiological interaction depends not only on the microenvironmental factor but also on the cell genotypes.

Rhee demonstrated nonrandom genetic and phenotypic changes in prostate epithelial cells. The occurrence of such permanent changes may be highly contact dependent and appears to be driven by specific microenvironmental factors surrounding tumor cell epithelium grown as 3D prostate organoids [11].

Why could 3D culture exhibit a higher killing rate? This part will be discussed in Section 3.6.

3.3. Effect of Ferulic Acid on the Superoxide Dismutase and Catalase Activities. The activity of SOD was upregulated by FA in 2D and 3D cultures, increasing approximately to 1.73-fold ($P < 0.050$) and 3.18-fold ($P < 0.001$), respectively (Figure 3(a)). Conversely the catalase activity was highly induced by 2D but only slightly significant by 3D cultures. The increase reached 2.58-fold for 2D and 1.33-fold for 3D cultures (Figure 3(b)).

Superoxide is one of the main reactive oxygen species (ROS) in the cells. Approximately 0.4–4% of all oxygen consumed during normal respiration is converted into superoxide within the mitochondrion [12], the chief source of reactive oxygen species (ROS) within the cell. Superoxide dismutases (SOD, EC1.15.1.1) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen [13]. It is simply stated that SOD outcompetes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity [14]. Superoxide is known to denature enzymes, oxidize lipids, and fragment DNA. SODs catalyze the production of O_2 and H_2O_2 from superoxide ($\bullet O_2^-$), which results in less harmful reactants. When acclimating to increased levels of oxidative stress, SOD concentrations typically increase with the degree of stress conditions [15].

Catalase is one of the most potent catalysts known. The reactions it catalyses are crucial to life. Catalase catalyses

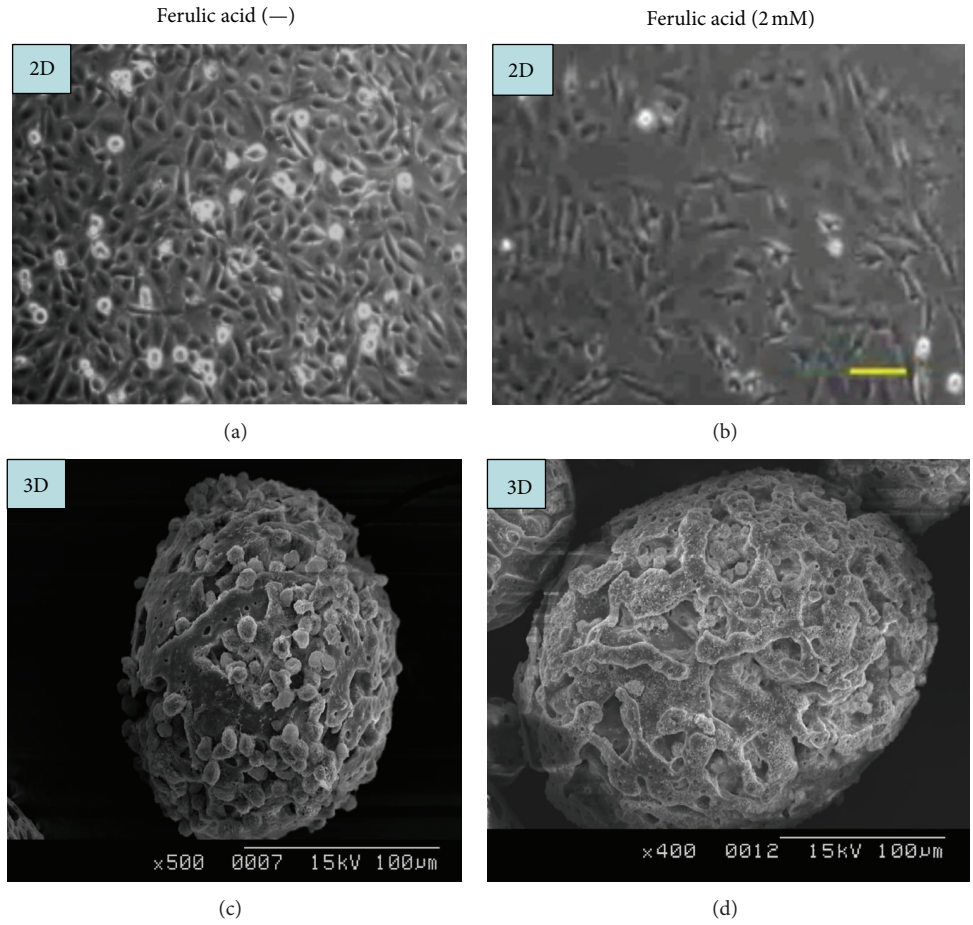

Figure 1: SEM scanning of the T24 cell morphology affected in the absence and presence of 2 mM ferulic acid in 2D and 3D cultures. 2D culture: control (a); 2D + ferulic acid 2 mM (b) (magnification ×500, scale bar = 0.1 mm). 3D culture: control (c) (magnification ×500); 3D + ferulic acid 2 mM (d) (magnification ×400). Cultivation time was 72 h at 37°C for 2D and 3D cultures, respectively.

conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen. Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage to cells and tissues, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules [16]. Catalase also uses hydrogen peroxide to oxidize toxins including phenols and alcohols [17]. Catalase is essential to protect the stability of ferrous ion-requiring enzymes both *in vitro* and *in vivo* systems [18]. Hydrogen peroxide has recently been shown to inactivate the enzyme by oxidation of crucial cysteines [19].

Alternatively, space microenvironment could play an important role in balancing these antioxidative enzymes. Speculatively, the synergistic interaction of SOD and catalase was effectively operating in the T24 cells and obviously there might have been much higher ROS occurring in the 2D culture, as evidenced by the highly raised catalase activity (Figure 3(b)). The major part of superoxide anions produced could have been consumed through the NO pathway by the huge amount of NO otherwise produced (not shown); hence the apparent level of SOD was highly suppressed in the 2D cultures (Figure 3(a)). Such a case was not seen in the 3D culture. In 3D culture, the T24 cells were freely rotating with the matrix and had much larger space for cell proliferation. Consequently, the ROS produced could rapidly diffuse out the cells, immediately diluted by the bulk fluid around the cells. The lower catalase activity found for the 3D culture may give a strong support to this (Figure 3(b)).

3.4. Western Blot Indicated 3D Culture Showed Stronger Apoptotic Effect than 2D Culture. As seen, the antiapoptotic cytokine Bcl-2 was suppressed by 3D to 0.5-fold compared with 0.80-fold by the 2D and 1.00-fold in the control. Conversely, the proapoptotic cytokine Bax was similarly upregulated by 3D and 2D, reaching 1.45- and 1.40-fold, respectively, compared with the control, 1.00-fold. Alternatively, the cleaved caspase-9 and cleaved caspase-3 were all substantially upregulated by 3D and 2D cultures. However it is worth noting that cleaved caspase-3 was more evidently induced by 3D than 2D cultures (Figure 4). Results implicated that 3D culture exhibited higher cytotoxicity than 2D identity.

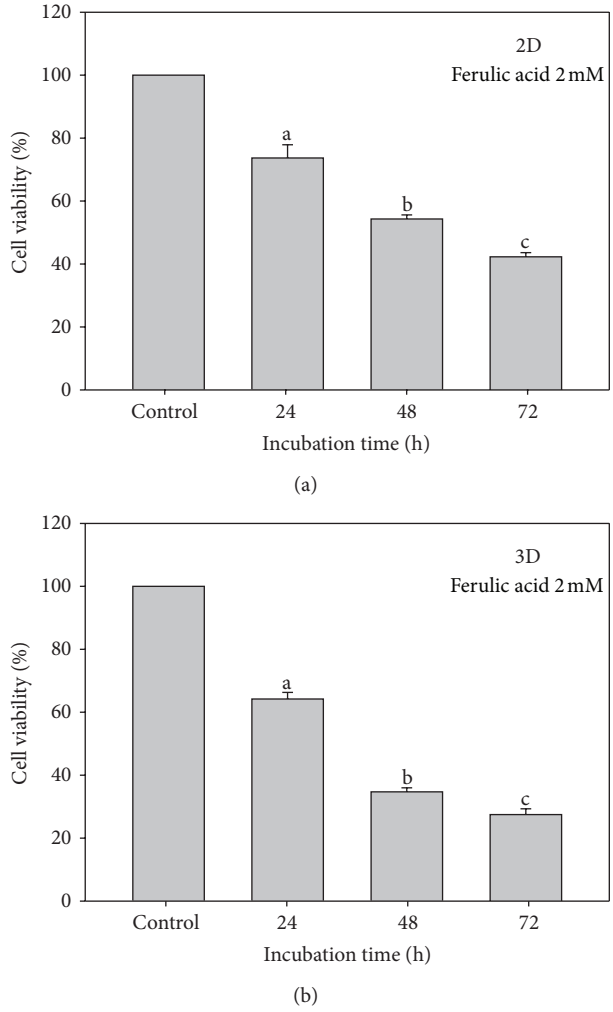

Figure 2: Comparison of cell viability of T24 cell lines in the presence of ferulic acid. 2D culture (a) and 3D culture (b). The concentration of ferulic acid used was 2 mM. The cultivation was conducted at 37°C for 24, 48, and 72 h, respectively. Data was expressed in mean ± SD from triplicate experiments ($P < 0.05$).

3.5. Intrinsic Mitochondrial Pathway Was Involved in Apoptosis. Western blotting revealed the Bcl-2 level was more prominently downregulated by 3D ($P < 0.05$); conversely, the levels of Bax, cleaved caspase-3, and cleaved caspase-9 were all significantly upregulated in 2D and 3D cultures ($P < 0.05$), comparatively, upregulated slightly higher in 3D culture (Figure 4), an implication in the higher extent of apoptosis occurring in 3D culture (Figure 2).

3.6. Difference in Mass Transport in Part Can Impart the Causality of Cell Death. To interpret the difference of the microenvironmental factor, we established the diagrammatic figure to show the difference of mass transport in these two cultures (Figure 5). The mass transport in 2D culture is limited by two barriers: "the stagnant region" and "the cell membrane" [20] (Figures 5(a) and 5(b)). The so-called "stagnant region" always occurs near the junction of a membrane or the surface of catalysts. Diagrammatically, the bulk

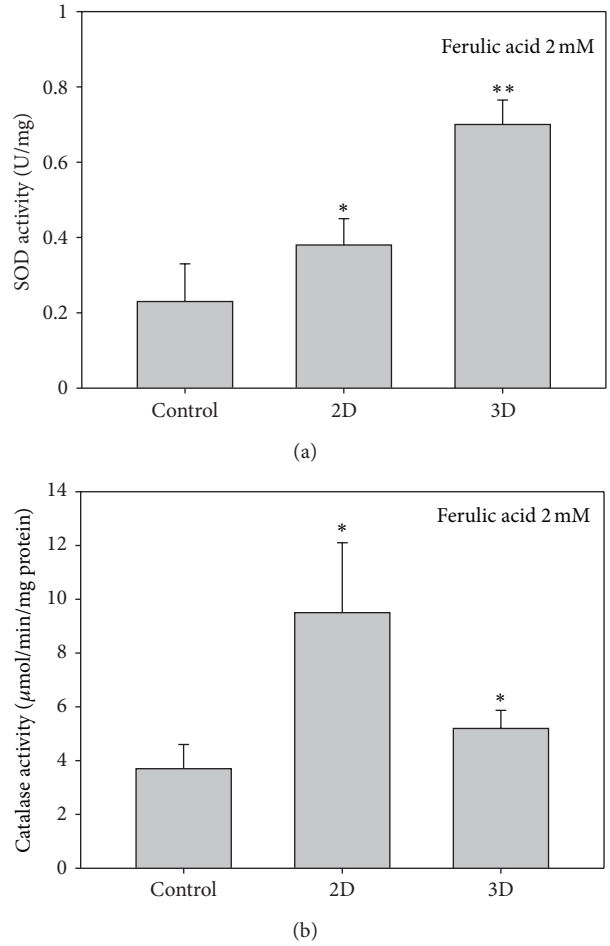

Figure 3: Variation of superoxide dismutase and catalase activities in T24 cell lines caused by 2D and 3D cultures. Superoxide dismutase (a) and catalase (b). Since the 2D without ferulic acid showed the lowest levels of SOD and catalase, we used the 2D without FA as the controls.

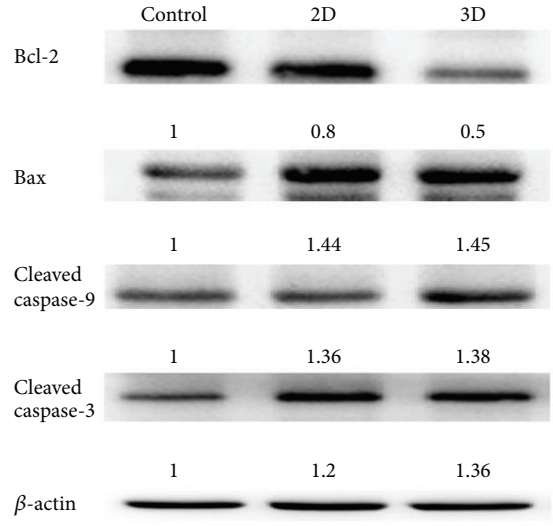

Figure 4: Western blot of Bcl-2, Bax, Bad, cleaved caspase-3, and cleaved caspase-9.

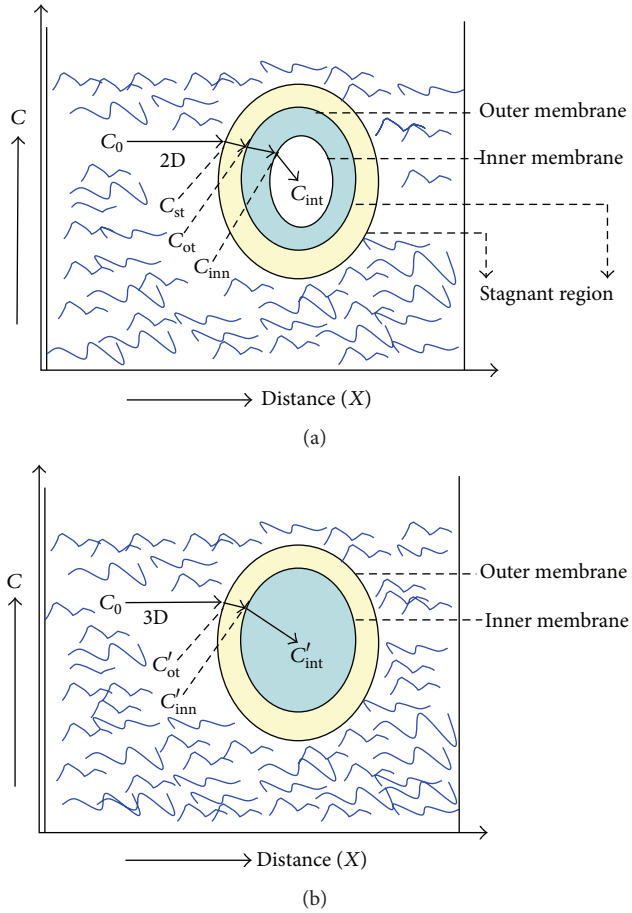

FIGURE 5: Diagrammatic model showing the difference of mass transports between the 3D (a) and 2D (b) cultures. In this model, due to the constantly free rolling of cells with the matrix in the medium for the 3D mass transport (a), the stagnant region could not be created. As contrast, the 2D mass transport (b) encounters two barriers: the stagnant region and the cell membrane. The stagnant region is a spontaneous barrier for mass transport adjacent to any membrane or catalyst surface. There would occur a concentration drop no sooner than the cells start to consume the medium.

concentration of ferulic acid in 2D-culture medium (C_o) is first transported to the surface of stagnant region (denoted by C_{st}, the concentration at the surface of stagnant region), dropping to C_{ot} (the concentration at the outer membrane) (1) and then to C_{inn} (the concentration at the junction of inner membrane; the subscript mem denotes "membrane") (2), and eventually is assumed by the cells, and hence the concentration drops to C_{int} (the intracellular concentration) (3), (4) (Figure 5(a)) (1)–(4):

$$C_{ot} = C_o - \int_{t1}^{t2} \left(\frac{\delta C}{\delta x}\right)_{st} \left(\frac{\delta x}{\delta t}\right)_{st}, \quad (1)$$

$$C_{inn} = \left(\frac{C_{ot}}{\delta x}\right)\left(\frac{\delta x}{\delta t}\right)_{mem}, \quad (2)$$

$$\left(\frac{\delta C}{\delta t}\right)_{int} = k_{2D}\,[C]\,. \quad (3)$$

Integration of (3) yields

$$\int_{C_{inn}}^{C_{int}} \frac{dC}{C} = k_{2D} \int_{t1}^{t2} dt, \quad (4)$$

where K_{2D} is the consuming rate coefficient in 2D culture.

In contrast, due to the constantly free rolling of the cells with matrix in the medium, the stagnant region may disappear or be neglected in the 3D culture, where the bulk concentration (C_o) directly drops from the concentration at the outer membrane (C'_{ot}) to the concentration at the inner membrane (C'_{inn}) (5) and then degraded to the intracellular concentration (C_{int}) (6), (7) (Figure 5(b)) (5)–(7):

$$C_{inn} = \left(\frac{C'_{ot}}{\delta x}\right)\left(\frac{\delta x}{\delta t}\right)_{mem}, \quad (5)$$

$$\left(\frac{\delta C}{\delta t}\right)_{int} = k_{3D}\,[C]\,. \quad (6)$$

Integration of (6) yields

$$\int_{C'_{inn}}^{C'_{int}} \frac{dC}{C} = k_{3D} \int_{t1}^{t2} dt, \quad (7)$$

where K_{3D} is the consuming rate coefficient in 3D culture.

Obviously without the presence of stagnant region in 3D culture, the mass transport will be less hindered. Hence, the cells in 3D culture would frequently "feel" or encounter higher toxicant concentration than the 2D culture. Moreover, the elongation of cells in 3D culture would provide a larger surface area for mass transport; as a consequence, the transport would fulfill the conditions $C'_{inn} \gg C_{inn}$ and $C'_{int} \gg C_{int}$, and the 3D cultures could be affected by ferulic acid at higher concentration. Thus although 3D culture may have many advantages: (i) largely reducing shear and turbulence generated by conventional stirred bioreactors, (ii) minimizing mechanical cell damage, and (iii) continuously free falling, promoting the assembly of 3D cellular aggregates which allow a microenvironment for more efficient cell-to-cell interactions and exchange of growth factors [7], we suggest that the 3D RWV culture cannot be as real as the *in vivo* 3D growth.

To summarize, experiment with the T24 cell lines-ferulic acid (2 mM) model reveled that FA showed different cytotoxicity on T24 cell line in the 2D and 3D culture systems. The cell shape was more elongated in the 3D culture. The SOD activity was higher, and conversely, the catalase activity was lower in the 3D culture. The antiapoptotic signal Bcl-2 was downregulated, while all the apoptotic signals Bax, cleaved caspase-3, and cleaved caspase-9 were upregulated by FA, despite in the 2D or the 3D cultures. The overall apoptotic rate was higher for the 3D culture. To extend, more complicated pharmacokinetic and pharmacodynamic events could be expected in the *in vivo* 3D tissues.

4. Conclusions

Apparently, 3D culture has shown more powerful cytotoxicity than the 2D analogue. In cancer therapy, we encourage that

the outcome of 2D culture must be corrected for the results to be applied to the *in vivo* cancer treatment.

Disclosure

The authors do not have any conflict of interests, and the authors have already disclosed any actual or potential conflict of interests including any financial, personal, or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence (bias) their work and related potential conflict of interests including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Potential conflict of interests should be disclosed at the earliest possible stage.

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Research Article

Lovastatin in *Aspergillus terreus*: Fermented Rice Straw Extracts Interferes with Methane Production and Gene Expression in *Methanobrevibacter smithii*

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Lovastatin, a natural byproduct of some fungi, is able to inhibit HMG-CoA (3-hydroxy-3methyl glutaryl CoA) reductase. This is a key enzyme involved in isoprenoid synthesis and essential for cell membrane formation in methanogenic Archaea. In this paper, experiments were designed to test the hypothesis that lovastatin secreted by *Aspergillus terreus* in fermented rice straw extracts (FRSE) can inhibit growth and CH_4 production in *Methanobrevibacter smithii* (a test methanogen). By HPLC analysis, 75% of the total lovastatin in FRSE was in the active hydroxyacid form, and *in vitro* studies confirmed that this had a stronger effect in reducing both growth and CH_4 production in *M. smithii* compared to commercial lovastatin. Transmission electron micrographs revealed distorted morphological divisions of lovastatin- and FRSE-treated *M. smithii* cells, supporting its role in blocking normal cell membrane synthesis. Real-time PCR confirmed that both commercial lovastatin and FRSE increased ($P < 0.01$) the expression of HMG-CoA reductase gene (*hmg*). In addition, expressions of other gene transcripts in *M. smithii* with a key involvement in methanogenesis were also affected. Experimental confirmation that CH_4 production is inhibited by lovastatin in *A. terreus*-fermented rice straw paves the way for its evaluation as a feed additive for mitigating CH_4 production in ruminants.

1. Introduction

The formation of isoprenoid chains is a key component of membrane phospholipid synthesis in Archaea. This pathway requires the production of mevalonic acid from 3-hydroxy-3methyl glutaryl CoA catalyzed by the enzyme HMG-CoA reductase, a critical rate-limiting step shared in common with cholesterol biosynthesis in humans (Figure 1). Lovastatin is a natural polyketide synthesized by *Aspergillus terreus* and *Pleurotus ostreatus* (oyster mushroom), where it may occur at concentrations as high as 2.8% dry weight [1]. Lovastatin prescribed at a dosage of 80 mg daily can dramatically reduce cholesterol levels by 40% simply through the inhibition of HMG-CoA reductase activity. Through interference with membrane synthesis (Figure 1), lovastatin can inhibit the growth of methanogenic Archaea in the rumen without adverse effects on other cellulolytic bacteria [2] and, in this way, mediates reduction in methane (CH_4) release into the environment. However, the high cost of lovastatin preempts its use as a feed additive in the mitigation of ruminal CH_4 production. Another approach that may be economically viable is to incorporate *A. terreus* as a feed supplement and inhibitor of methanogenic Archaea that produces methane in the process of methanogenesis (Figure 2). Furthermore, since

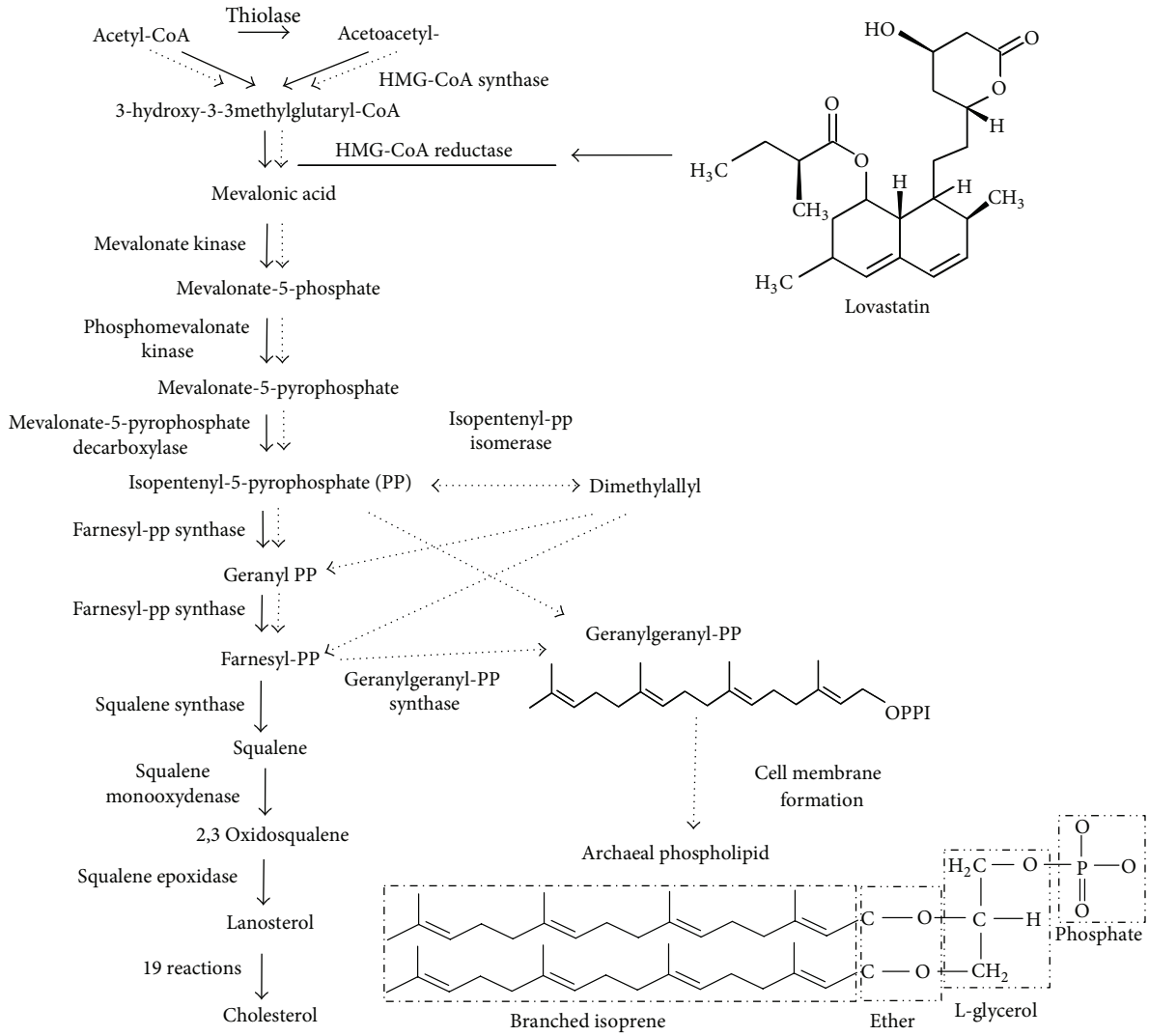


Figure 1: Biosynthesis pathway of cholesterol production in humans (solid-line markers) and phospholipids production in Archaea (dotted-line markers). HMG-CoA reductase is the common enzyme converting HMG-CoA to mevalonic acid in the two pathways. Lovastatin is an inhibitor of HMG-CoA reductase and thus reduces the production of mevalonic acid in both pathways (modified from http://ourbiochemistry.blogspot.com/2008/08/29-cholesterol-synthesis.html).

A. terreus is a known producer of cellulolytic enzymes [3–5], it complements the degradation of lignocellulose components in the rumen enhance feed conversion efficiency. This paper describes a series of experiments to test the hypothesis that lovastatin generated by *A. terreus* fermentation of rice straw (fungal treated rice straw extracts or FRSE) inhibits the growth and methanogenesis by *Methanobrevibacter smithii* (DSM 861), a gastrointestinal methanogen similar to the dominant species in the rumen. The molecular mechanism for this effect was also elucidated by real-time PCR.

2. Materials and Methods

2.1. Substrate, Microorganism, and Spore Suspension. Rice straw (RS) was collected from the local rice fields in the state of Selangor, Malaysia. The material was dried and ground to uniform size (No. 6 mesh) and stored in plastic bags at 4°C for later use as a substrate.

A. terreus ATCC 74135, obtained from American Type Culture Collection (ATCC), was maintained on potato dextrose agar (PDA) slants at 25°C for 7 days, stored at 4°C, and subcultured every two weeks. Spore suspension was prepared in 0.1% Tween-80 solution in approximately 10^7 spores/mL concentration.

2.2. Solid-State Fermentation. Solid state fermentation of RS was carried out in 2 L Erlenmeyer flasks. About 200 grams of RS, 200 mL distilled water (containing 1% urea) were added to give moisture content of approximately 50%. The flasks were plugged with cotton-wool and autoclaved at 121°C for 15 min prior to inoculate with 40 mL of an *A. terreus* spore suspension (containing 10^7 spores/mL). A sample was

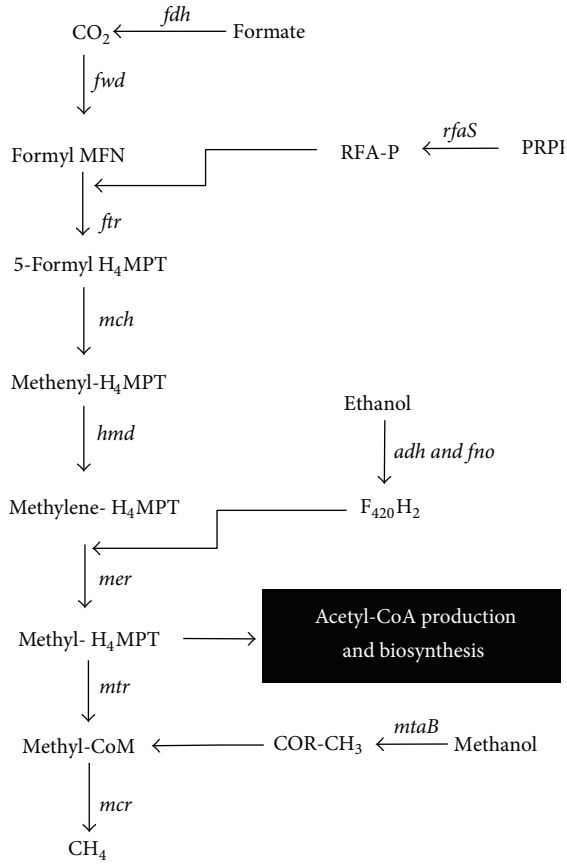


Figure 2: Methanogenesis pathway in *M. smithii*. *M. smithii* is able to use the Methyl H_4MPT in the middle step of methanogenesis pathway for production of acetyl-CoA and biosynthesis reactions. Enzymes and genes encode: formate dehydrogenase (*fdh*); formyl-MF dehydrogenase (*fwd*); MFN, methanofuran; ribofuranosylaminobenzene 5_-phosphate (RFA-P); ribofuranosylaminobenzene 5_-phosphate synthase (*rfaS*); 5-phospho-a-D-ribosyl-1-pyrophosphate (PRPP); formyl-MF: H_4MPT formyltransferase (*ftr*); tetrahydromethanopterin (H_4MPT); methenyl-H_4MPT cyclohydrolase (*mch*); methylene-H_4MPT dehydrogenase (*hmd*); methylene-H_4MPT reductase (mer); methyl-H_4MPT: HS-CoM methyltransferase (*mtr*); alcohol dehydrogenase (Adh); F420-dependent NADP oxidoreductase (*fno*); methanol : cobalamin methyltransferase (*mtaB*); methyl CoM Reductase (*mcr*) (modified from Hendrickson et al. [14]).

fermented at 25°C for 8 days, conditions which had previously been found to be optimal [6]. At the end of fermentation, the sample was dried at 60°C for 48 h.

2.3. Preparation of FRSE. For preparation of FRSE, 200 g of the fermented rice straw was mixed with 1.5 L of methanol and shaken for 2 h at room temperature. The solid samples were removed from the suspension by vacuum filtration (0.45 μm pore size, Pall Corporation, Ann Arbor, MI). Methanol was removed by rotary evaporation at 45°C (Eppendorf, USA), and the solid residual or FRSE was used in the further experiments.

2.4. Lovastatin Quantification by HPLC. The concentration of lovastatin in the FRSE was quantified using HPLC (Waters, USA, 2690) and an ODS column of Agilent (250 × 4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile and water (70 : 30 by volume) containing 0.5% acetic acid. The UV photo diode array (PDA) detection range was set from 210 to 400 nm, and lovastatin was detected at 237 nm. The sample injection volume was 20 μL, and the running time was 15 min. Different concentrations of lovastatin (mevinolin, 98%, HPLC grade, sigma, M2147) were used as standard.

2.5. Microorganism and Anaerobic Microbial Culture. Methanobrevibacter smithii DSM 861 used in this study was obtained from the German Resource Centre for Biological Material (DSMZ, Germany). The Balch medium 1 was used for the growth of *M. smithii* with some modification on it containing 0.45 g/L of K_2HPO_4, 0.45 g/L of KH_2PO_4, 0.45 g/L of $(NH_4)_2SO_4$, 0.9 g/L of NaCl, 0.12 g/L of $CaCl_2{\cdot}2H_2O$, 0.19 g/L of $MgSO_4{\cdot}7H_2O$, 2.5 g/L of $NaHCO_3$, 2.0 g/L of Trypticase, 2.0 g/L of yeast extract, 2.5 g/L of sodium acetate, 2.5 g/L of sodium formate, 4.9×10^{-5} g/L of coenzyme M (sodium 2-mercaptoethane-sulfonate), 0.5 g/L of cysteine·HCl, 0.5 g/L of $Na_2S{\cdot}9H_2O$, and 0.001 g/L resazurin (pH 6.9). Vitamin and trace mineral solutions were added according to Balch et al. [7], and a VFA mixture was added according to Lovley et al. [8]. The mixture was flushed with CO_2, and approximately 10 mL of medium was transferred into 50 mL serum bottles under anaerobic conditions. The bottles were

Table 1: Primers used in gene expression study.

Official symbol	Descriptor		Primer sequence (5′ → 3′)	Amplification size (bp)
Met	16S rRNA	Forward Reverse	GCTCAGTAACACGTGG CGGTGTGTGCAAGGAG	343
mcrA	Methyl coenzyme-M reductase, subunit A	Forward Reverse	TTCGGTGGATCDCARAGRGC GBARGTCGWAWCCGTAGAATCC	140
fno	F420-dependent NADP reductase	Forward Reverse	GGGTTCAGCAGCAGAAAGG CACATTCAATTGGGTCTGGA	118
mta	Methanol : cobalamin methyltransferase	Forward Reverse	ATGTGGTGCAAAAGGACCTC CAGAGTGTGCACAAACAGCA	112
adh	Alcohol dehydrogenase	Forward Reverse	AAGAAGTCCCGGAATGTGG TCCGATAGCTCCTTCCCATA	102
hmd	Methylene-H_4MPT dehydrogenase	Forward Reverse	ACCCAGGTGCTGTACCTGAAAT TGTGAATGCAGATCCTCTTGCT	119
mtr	Methyl-H_4MPT : coenzyme M methyltransferase	Forward Reverse	AACAAAGCGGCTTCTGGTGAA CGACACAAGATCCCATTGCAAT	127
hmg	HMG-CoA reductase	Forward Reverse	GGCTGTGAATTACCGCATATGG TAACGGTCCGGCTACACCTACA	117

closed by rubber stoppers and aluminum seals and autoclaved in 121°C for 15 min. Lovastatin in final concentrations of 1, 10, and 50 μg/mL and FRSE in final concentration of 10, 100, and 500 μg/mL were filter-sterilized using 0.2 μm sterile syringe filters (Pall/Gelman, East Hills, NY, USA) and added into the medium after autoclaving. The samples were inoculated with 5% of a 72 h culture of *M. smithii*. The gas phase in each bottle was exchanged with an 80% H_2-20% CO_2 gas mixture at 100 kPa. The bottles were incubated at 39°C for 72 h. Growth was monitored from the optical density at 620 nm.

2.6. Methane Determination. The concentration of CH_4 in the headspace gas phase was determined with an Agilent 6890 Series Gas Chromatograph (Wilmington, DE, USA). Separation of the gases was achieved using an HP-Plot Q column (30 m × 0.53 mm × 40 μm) (Agilent Technologies, Wilmington, DE, USA) with N_2 as the carrier gas with a flow rate of 3.5 mL/min (MOX, Kuala Lumpur, Malaysia). The isothermal oven temperature was 50°C, and separated gases were detected using a thermal conductivity detector. Methane was eluted in 4 min. Calibration used standard gas prepared by Scott Specialty Gases (Supelco, Bellefonte, PA, USA), which contain 1% of CH_4, CO, CO_2, O_2, and H_2 in N_2.

2.7. RNA Extraction and Gene Expression. Cells from two milliliters of culture were harvested by centrifugation at 10,000 rpm for 2 min at 4°C and directly used for RNA extraction. RNA was extracted using the RiboPure Bacteria RNA Isolation kit (AMBION, AM1925, Austin, TX, USA) according to the manufacturer's protocol and reverse transcribed into cDNA using First Strand cDNA synthesis Kit according to the manufacturer's instructions (Maxime RT-PCR Kit, iNtRON, Germany). In the next step, Real-time PCR was performed with the BioRad CFX96 Touch (Bio-Rad, USA) using optical grade plates. The PCR reaction was performed on a total volume of 25 μL using the iQSYBR Green Supermix (BioRad, USA). Each reaction included 12.5 μL SYBR Green Supermix, 1 μL of each Primer, 1 μL of cDNA samples, and 9.5 μL H_2O. All real-time PCRs were performed in duplicate. Primers used in this study are shown in Table 1. 16S rRNA was used as reference gene [9]. The $2^{-\Delta\Delta CT}$ method was used for determination of relative gene expression [10]. Results of the real-time PCR data were represented as CT values of the threshold cycle number at which amplified product was first detected. ΔCT is difference in CT value of the target gene from the CT value of the reference gene (16S rRNA). ΔΔCT is ΔCT of treatment samples (lovastatin and FRSE) minus ΔCT of the untreated control. Data is presented as fold change expression in the target gene of a treatment sample compared to the normal sample.

2.8. Transmission Electron Microscopy (TEM). The procedure of sample preparation of Hayat [11] with minor modified [12] by the Electron Microscopy Unit, Institute of Bioscience, Universiti Putra, Malaysia, was used for the TEM study. A Hitachi H-7100 (Japan) transmission electron microscope was used.

2.9. Statistical Analysis. All of the experiments were performed in triplicate. Data were analyzed as a completely randomized design (CRD) using the general linear model (GLM) procedure of SAS 9.2 [13]. All multiple comparisons among means were performed using Duncan's new multiple range test ($\alpha = 0.05$).

3. Results

3.1. Purity of Lovastatin. The purity of lovastatin in FRSE was compared with the commercially available form by HPLC. Figure 3 shows that commercial lovastatin was >98% in the lactone form. In contrast, the yield of lovastatin was 97 mg/g dry matter in FRSE, and approximately 75% of this was in the bioactive hydroxyacid form (73 mg/g DM). More information about production of lovastatin by *A. terreus* in

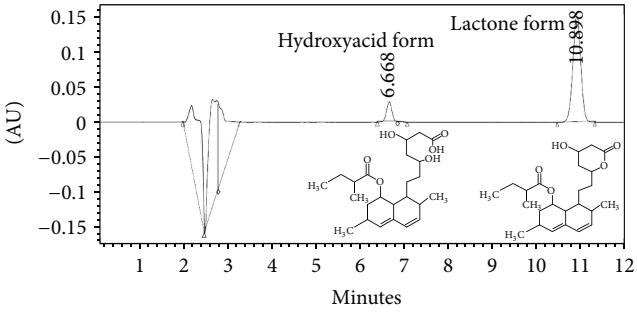


Figure 3: Molecular structure and HPLC chromatogram of lovastatin.

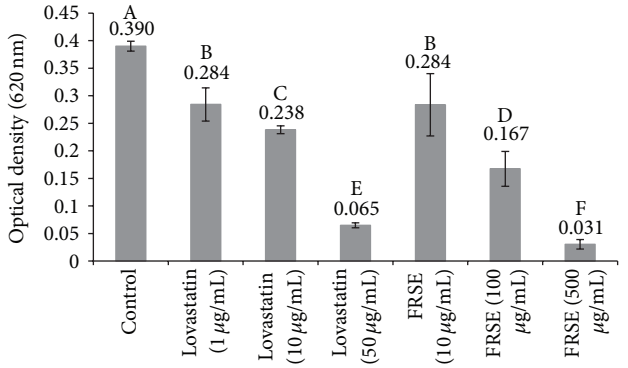


Figure 4: The effect of the addition of commercial lovastatin and fermented rice straw extract (FRSE) in the broth culture of *M. smithi* on growth rate after 72 h of incubation. The error bar represents one standard deviation. A, B, C, D, E and F indicating differences between means ($P < 0.01$).

solid state fermentation was published in our previous paper [6]. To evaluate the effectiveness of the commercial and FRES lovastatin, 3 dilutions representing 1, 10, and 50 μg/mL of commercial lovastatin and 10, 100, and 500 μg/mL of FRSE (contain lovastatin) were used to investigate the biological activity of lovastatin on growth morphology, methane production, and gene transcript activity.

3.2. Microbial Growth and CH_4 Production. Treatment with commercial and FRSE lovastatin significantly ($P < 0.01$) inhibited the growth of *M. smithii* (Figure 4). Inhibition by commercial lovastatin at 10 and 50 μg/mL was similar to that of 100 and 500 μg/mL FRSE, respectively. At the same concentration of total lovastatin, the growth inhibitory effect of FRSE on *M. smithi* was much stronger than when commercial lovastatin was used alone.

Commercial lovastatin and FRSE also inhibited CH_4 production after 72 h of incubation (Figure 5). At the same concentration of total lovastatin, CH_4 production in the FRSE treatments was lower than treatments containing commercial lovastatin. There was no significant difference in CH_4 production by 10 μg/mL commercial lovastatin and 10 μg/mL FRSE (equivalent to 1 μg/mL total lovastatin) while CH_4 was not detected in cultures containing 500 μg/mL FRSE (Figure 5).

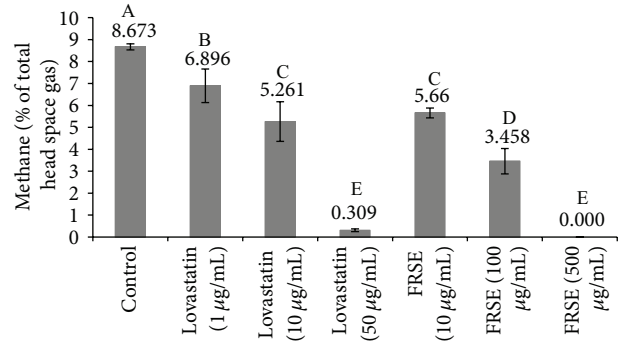


Figure 5: The effect of the addition of commercial lovastatin and fermented rice straw extract (FRSE) in the broth culture of *M. smithi* on methane production (as % of total headspace gas) after 72 h of incubation. The error bar represents one standard deviation. A, B, C, D, and E indicate differences between means ($P < 0.01$).

3.3. Microbial Morphology. Following growth with commercial lovastatin or FRSE, the morphology of *M. smithii* was greatly altered (Figure 6). The lines of cell division in *M. smithii* for the control samples (Figures 6(a) and 6(b)) displayed symmetrical cell division, while mitotic figures in treated samples were off-centered resulting in aberrant division figures.

3.4. Gene Expression. To obtain some insight into the mechanism of decreased CH_4 production in *M. smithii,* real-time PCR was used to analyse the effect of commercial lovastatin and FRSE on expression of some of the key genes involved in the methanogenic pathway (Figure 2). Since little growth and CH_4 are produced by *M. smithii* in the high concentration of lovastatin and FRSE and it is not possible to extract sufficient quantity of RNA in these samples, RNA was extracted only from control and two lower levels of treatments. Both commercial lovastatin and FRSE significantly increased the expression of HMG-CoA reductase gene (*hmg*) ($P < 0.05$). Fold change in expression of this gene in FRSE treated cells was higher than those treated with commercial lovastatin (Figure 7(a)), and the maximal change caused by the 50 μg/mL FRSE was a 9-fold increase.

The FRSE treatments, but not commercial lovastatin, also had a significant effect on expression of methylene-H_4MPT dehydrogenase gene (*hmd*) that encodes the enzyme for conversion the methenyl-H_4MPT into methylene-H_4MPT (Figure 2). Similarly, with commercial lovastatin and FRSE reduced the expression of alcohol dehydrogenase gene (*adh*), this reduction was not significant ($P > 0.05$) (Figure 7(c)). Treatments containing 10 μg/mL Lovastatin, 10 μg/mL and 50 μg/mL FRSE significantly ($P < 0.01$) reduced the expression of F420-dependent NADP reductase gene (*fno*) in *M. smithii* (Figure 7(d)). L-lovastatin and FRSE increased the expression of methyl-H_4MPT : coenzyme M methyltransferase gene (*mtr*) (Figure 7(e)). This gene produces the enzyme for the transfer of methyl group from methyl-H_4MPT to HS-COM [15]. Methyl coenzyme-M reductase (*mcr*) is the last enzyme in the methanogenesis pathway.

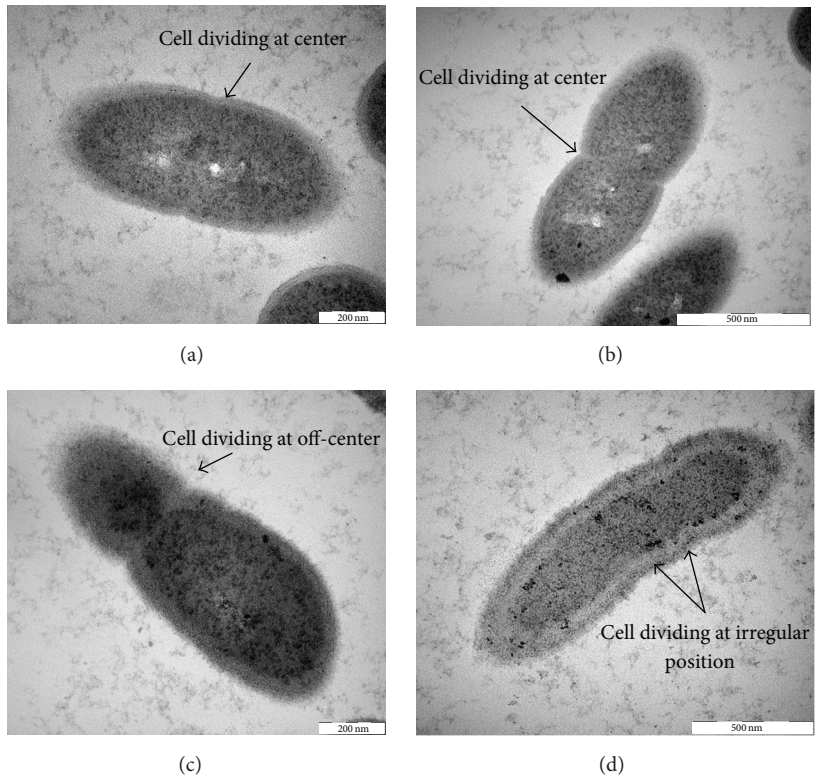


Figure 6: Transmission electron micrograph of *M. smithii* as affected by commercial lovastatin (c) and fermented rice straw extract (FRSE) (d). Division of normal *M. smithii* cells (a) and (b) occurred at the middle forming two equal cells. In cultures treated with commercial lovastatin (c) and FRSE (d), cell division was off-center and irregular. Pictures were obtained using a Hitachi H-7100 (Japan) transmission electron microscope (TEM).

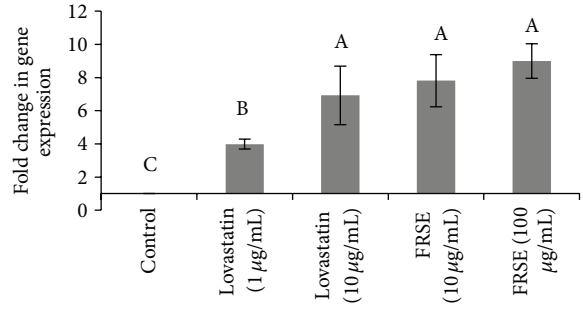
The effect of lovastatin and FRSE on expression of this gene is shown in Figure 7(f). The result shows that lovastatin has no effect on the expression of this gene, but FRSE at both levels increased the expression of this gene in *M. smithii* ($P < 0.01$). Methanol : cobalamin methyltransferase gene (*mta*) is the gene for encoding of methanol : cobalamin methyltransferase that catalyses the conversion of methanol into COR-CH_3 and production of CH_4 in the process of methanogenesis. Both lovastatin and FRSE significantly increase the expression of this gene in *M. smithii* ($P < 0.01$) (Figure 7(g)). The enhancement effect of FRSE on expression of this gene was higher than L-lovastatin.

4. Discussion

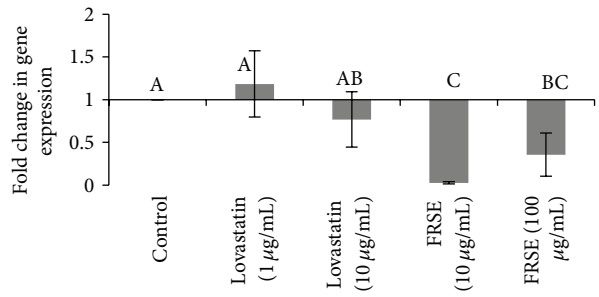
Lovastatin is an effective therapy in the treatment of hypercholesterolemia because of its ability to inhibit HMG-CoA reductase activity, a key enzyme involved in cholesterol synthesis [16]. Because of this, it is easy to ignore the fact that generic fungal statins have evolved to allow producer strains to gain a competitive survival advantage in complex ecological communities by interfering with the assembly of isoprenoid chains required for membrane phospholipid synthesis [17]. In this way, statin-producing fungi can arrest the growth rates of susceptible strains [18] by interfering with cell wall formation and arresting cellular division [19]. To test whether such a strategy could be used for the reduction of methane production by ruminants, it was necessary to show firstly that rice straws fermented with a representative statin-producing fungal strain of *Aspergillus terreus*, was capable of synthesizing biologically active lovastatin. HPLC confirmed that while commercial lovastatin existed primarily in a biologically inactive lactone (L) form, the biologically active hydroxyacid or H-form predominated in FRSE (Figure 3).

In the second stage, it was necessary to demonstrate that the lovastatin in FRSE was able to exert a biological impact on growth and cell membrane assembly in the target experimental methanogen—*M. smithii.* As shown in Figure 4, growth rates of *M. smithii* were inhibited by both commercial lovastatin and FRSE. At the same time, electron micrographs of *M. smithii* showed abnormal formation of cell membranes in mitosis, presumably caused by interference in the synthesis of isoprenoid building blocks. Although both treatments significantly ($P < 0.01$) inhibited the growth of *M. smithii,* the growth inhibitory effect of FRSE on *M. smithi* was much stronger than when control L-lovastatin was used alone. It is likely that this could have been the consequence of having to convert the lactone form to the hydroxy form of lovastatin before the inhibition of HMG-CoA reductase can occur in *M. smithii*.

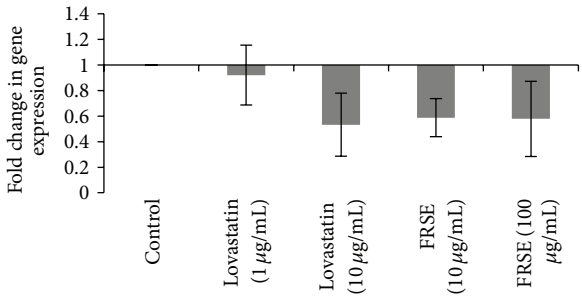
Commercial lovastatin contains 2% of the active H-form of lovastatin, so their titrations of 1, 10, and 50 ug/mL



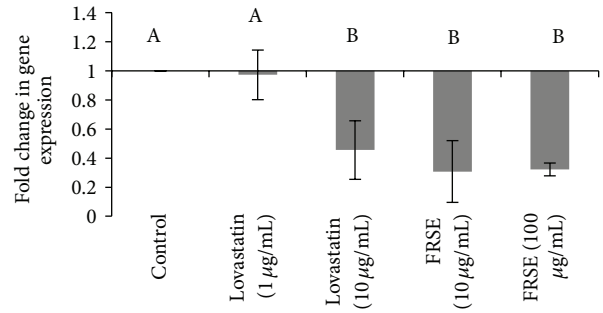
(a) Effects of treatments on expression of HMG-CoA reductase gene, *hmg* ($P < 0.01$)



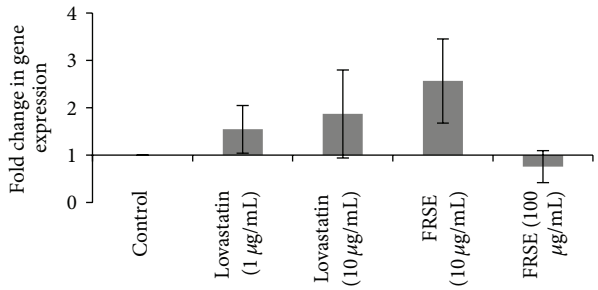
(b) Effect of treatments on expression of methylene-H_4MPT dehydrogenase gene, *hmd* ($P < 0.01$)



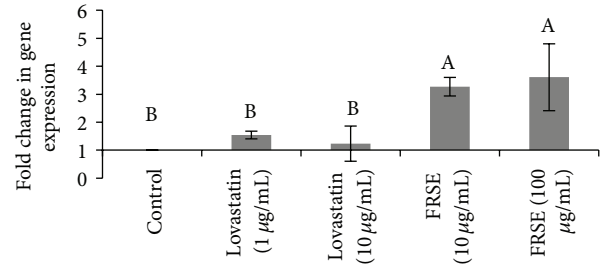
(c) Expression of alcohol dehydrogenase gene, *adh* ($P > 0.05$)



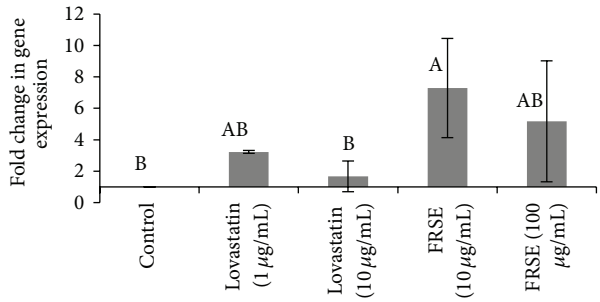
(d) Effects of treatments on expression of F420-dependent NADP reductase gene, *fno* ($P < 0.01$)



(e) Effects of treatments on expression of methyl-H_4MPT : coenzyme M methyltransferase gene, *mtr* ($P > 0.05$)



(f) Expression of methyl coenzyme-M reductase gene subunit A, *mcrA* ($P < 0.01$)



(g) Effects of treatments on expression of methanol:cobalamin methyltransferase gene, *mta* ($P < 0.05$)

FIGURE 7: Effect of lovastatin and FRSE on genes expression in *M. smithii*. 16S rRNA was used as reference gene. Data were normalized by control and reference genes. Error bar represent standard deviation. Letters on the columns indicating differences between means ($P < 0.05$).

represent 0.02, 0.20, and 1 ug H-form per mL. The FRSE contains 7.3% H-form of lovastatin and 10, 100, and 500 ug DM/mL FRSE are actually 0.73, 7.3, and 36.5 ug/mL H-lovastatin. Thus, their lowest concentration of FRSE is similar in H-form content with their highest commercial lovastatin concentration, which was much more inhibitory in the experiments shown in both Figures 4 and 5. Similarly, in Figure 4, 1 ug/mL of the commercial form appears to be roughly

equivalent to 10 ug/mL of FRSE. In Figure 5, 10 ug/mL of the commercial form appears to be roughly equivalent to 10 ug/mL of FRSE. This would seem to suggest that on a total (H + L-form) lovastatin basis, the FRSE appears to be roughly equivalent to possibly 10-fold more active.

It is important to note that this activity of lovastatin against a methanogen operates differently from its antiproliferative activity against eukaryote cells. Damage of the human cell division by lovastatin has been reported in a previous study [20]. Van de Donk et al. [21] showed that lovastatin negatively affected membrane structure in the myeloma plasma cells and reduced the plasma cell viability, but this was due to the induction of apoptosis and inhibition of proliferation and probably a pleiotropic effect of statins on nuclear receptors in eukaryote cells [22]. Lovastatin interference with nuclear receptors can act synergistically with its ability to inhibit polyisoprenylation and subsequent downstream distortion of intracellular matrix reorganization during cell division [21, 23, 24]. The antiproliferative activity of statins had found increasing use as anticancer drugs in cancer therapy [25, 26].

Microbial diversity in the rumen enables ruminants to convert lignocellulosic materials into useful nutrients such as VFA and microbial protein for the host animal. This is complemented by another group of microorganisms, the methanogenic Archaea which coexists within the rumen ecosystem by converting H_2 and CO_2 into CH_4, a greenhouse gas which has been a serious contender for global warming and climate change. Mitigation of rumen CH_4 production has two advantages: reduction of dietary energy loss, thus improving the efficiency of nutrient utilization by the host animal, and mitigation of enteric CH_4 production. Both commercial lovastatin and FRSE significantly inhibited CH_4 production, growth, and cell division of *M. smithii*. Other strategies for mitigation of CH_4 production in the rumen ecosystem have been extensively researched but with limited success. The idea of applying lovastatin to suppress methanogenesis in methanogenic Archaea [2] has been tested previously. These authors reported that commercial lovastatin inhibited growth of methanogenic Archaea without adversely affecting other cellulolytic bacteria. In practical terms, it is simply uneconomical to use lovastatin as a feed additive for reduction of methanogenesis in ruminants under farm conditions.

A major difference between Archaea and other microorganisms lies in the structure of their cell membrane. The lipid arm of phospholipids in Archaea is made up of branched isoprenoid, but in other microorganisms, it is fatty acid [27]. The process of isoprenoid and phospholipid biosynthesis in Archaea (Figure 1 in dotted-line markers) share similarities with cholesterol biosynthesis in eukaryotic cells (Figure 1 in solid-line markers) with HMG-CoA reductase as a key enzyme in both pathways, primarily to convert 3-hydroxy-3-3methylglutaryl-CoA (HMG-CoA) to mevalonic acid. Figure 7(a) showed a significant increase in HMG-CoA reductase gene (*hmg*) transcripts in *M. smithii* following lovastatin exposure, a result consistent with increased cellular need for more HMG-CoA reductase enzyme to process a buildup of HMG-CoA because of lovastatin-mediated competitive inactivation of HMG-CoA reductase. It is also evident from Figure 7(a) that H-lovastatin in FRSE was more effective than commercial L-lovastatin in generating a buildup of *hmg* gene transcripts. This is the first report of statins on expression of HMG-CoA reductase gene in Archaea and complements other *in vitro* and *in-vivo* experiments showing increases (seven fold after atorvastatin treatment) and decreases (two-fold after simvastatin treatment) in HMG-CoA reductase gene activation [28] in eukaryote cells. As well, enhancement of the relative expression of *hmg* genes and protein production involved in cholestrol biosynthesis by lovastatin have also been reported for many other systems [28–32]. Essentially, the mode of action of FRSE lovastatin on HMG-CoA reductase in Archaea is similar to its effect in animal cells.

While the experimental evidence so far supports the working hypothesis that interference of the isoprenoid synthetic pathway by lovastatin inhibition of HMG CoA synthetase is primarily responsible for reduced cell growth and decreased methane production, it is likely that the pleiotropic consequences of lovastatin on eukaryote cells in terms of its anti-proliferative and antimetabolic activity may have similar effects on other metabolic pathways in Archaea. For instance, quantitative proteomic analysis has revealed that lovastatin induced perturbation in multiple cellular pathways in HL-60 cells [33]. In the case of Archaea, methane production could also be affected by lovastatin by interference in its synthetic pathway. To assess this possibility, the biosynthetic pathway of methanogenesis is summarized in Figure 2. Methyl H_4MPT is a pivotal component in this pathway because its synthesis is vital for the production of acetyl-CoA, a key element in cellular processes and metabolism [14]. We selected 3 genes—*hmd*, *adh,* and *fno* that are involved in the synthesis of Methyl H_4MPT and 3 others responsible for methane synthesis—*mtr, mcr,* and *mta* for real-time PCR assays using the same RNA message transcripts from cultures sampled for *hmg* analysis. The results in Figure 7 show that *hmd* (B), *adh* (C), and *fno* (D) gene transcripts were all depressed in *M. smithii* following exposure to lovastatin. We propose that lovastatin interference of isoprenyl precursor synthesis has caused an increased buildup of acetyl-CoA in the metabolic pool resulting in feedback suppression of genes engaged in the synthesis of Methyl H_4MPT. Surprisingly, despite a drop in overall CH_4 production in the cultures, there was an increased expression in the three genes (*mtr, mcr,* and *mta*) responsible for CH_4 synthesis. We propose that this anomaly is not spurious but represents the transcriptome of *M. smithii* cells that are not dividing because they have been adversely affected by lovastatin. In these cells, *mtr, mcr,* and *mta* gene transcripts (Figures 7(e), 7(f), and 7(g), resp.) are working in cohort to dissipate intracellular pools of Methyl-H_4MPT.

In conclusion, we have shown that sufficient levels of biologically active H-lovastatin are produced in rice straws fermented by *A. terreus,* and fermented rice straw extracts are able to disrupt cell wall formation in the chosen rumen test methanogen *M. smithii*. This disruption is associated with decreased CH_4 production driven in part by interference with isoprenyl synthesis and also through pleiotropic interference of lovastatin in other metabolic pathways. The incorporation of FRSE as a feed additive for ruminants appears to be an

economically viable and environmentally sustainable strategy to mitigate CH_4 production.

Acknowledgment

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Research Article

Anti-Inflammatory Activity and Composition of *Senecio salignus* Kunth

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We investigated the anti-inflammatory activity of *Senecio salignus*. This medicinal plant is often used in Mexico for the treatment of fever and rheumatism. Chloroform and methanol extracts of the plant were tested on 12-O-tetradecanoylphorbol-13-acetate-(TPA-) induced edema in mice ears. The methanol extract of the plant inhibited edema by 36 ± 4.4% compared with the control, while the chloroform extract exhibited an even greater level of inhibition (64.1%). The chloroform extract was then fractionated, and the composition of the active fraction was determined by GC-MS. The anti-inflammatory activity of this fraction was then tested on TPA-induced ear edema in mice, and we found that the active fraction could inhibit edema by 46.9%. The anti-inflammatory effect of the fraction was also tested on carrageenan-induced paw edema in rats at doses of 100 mg/kg; a 58.9 ± 2.8% reduction of the edema was observed 4 h after administration of carrageenan, and the effect was maintained for 5 h.

1. Introduction

Inflammatory diseases are a major cause of morbidity and mortality in the world. These diseases are mainly treated with nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs, which have proven effective but can have negative side effects. For instance, NSAIDs may induce gastric and intestinal ulcers, anaemia, platelet inhibition in uterine motility, and, in some reported cases, an increased risk of myocardial infarction [1]. Steroidal anti-inflammatory drugs prevent or suppress inflammation but do not attack the root cause of the disease, and the prolonged use of these compounds can inhibit the synthesis of the inducible isoform of nitric oxide synthase enzyme and cause pituitary-adrenal suppression, hyperglycaemia, glycosuria, and an increased susceptibility to infections and peptic ulcers [2]. Therefore, searching for new molecules with anti-inflammatory activity but with fewer side effects is vital, and plants may represent a potential source of such compounds.

Ancient Mexican culture is rich in information on the plants used in traditional medicine, one of which is *Senecio salignus*. This plant is used to treat intermittent fevers and rheumatism, and in the state of Chiapas, it is used as an insecticide in corn stores and also as an ornamental plant [3, 4].

S. salignus is a leafy shrub with many branches; its leaves are sessile and are generally very narrow (up to 1.5 cm wide) and pointed, with numerous dense inflorescences and cones. Each inflorescence bears 5 to 6 bright yellow flowers. The plants grow in areas of desert scrub at altitudes less than 2870 m [5].

From the aerial parts of *S. salignus*, some pyrrolizidine alkaloids, lactones, furoeremophilanes, sesquiterpenes, and other compounds have been isolated [6]. However, there are no reports of anti-inflammatory activity for this plant.

In the present study, we investigated the anti-inflammatory activity of *S. salignus* and the composition of the active fraction of the plant.

2. Materials and Methods

2.1. Biological Material. *Senecio salignus* Kunth (Compositae) was collected around Tenancingo in the state of Mexico in July 2010. Dr. Abigail Aguilar Contreras authenticated the species, and a voucher was deposited in the herbarium of the Instituto Mexicano del Seguro Social (IMSSM 15,546). The aerial parts of the plant were dried in the shade at room temperature.

2.2. Experimental Animals. The present study used male Wistar rats (180 to 200 g) and CD-1 male mice (20 to 25 g) provided by the Unidad de Producción y Experimentación de Animales de Laboratorio (UPEAL) at the Universidad Autónoma Metropolitana Xochimilco. The animals were provided with food and water *ad libitum* and housed in a facility with light and dark periods of 12 hours.

All experiments were carried out according to the guidelines of laboratory animal care of the Guide for the Care and Use of Laboratory Animals [7].

2.3. Extract Preparation. A mixture of 500 g of dried, ground leaves and 3.5 L of chloroform or methanol was placed into a 5 L flask with a reflux condenser. The mixture was heated for 4 h at boiling temperature and then cooled and filtered; the solvent was then evaporated in a rotary evaporator to dryness under reduced pressure. The yield was 0.6% and 1.2%, respectively.

2.4. Chloroform Extract Fractionation. The chloroform extract was separated by column chromatography; the column was packed with silica gel (Kieselgel 60, 70–230 mesh ASTM), which was prepared using hexane as the mobile phase, and then the polarity was increased with ethyl acetate. Fractions of 100 mL were collected and compared by thin-layer chromatography; fractions with the same chromatographic pattern were then pooled. The resulting fractions were tested on ear edema in mice induced by TPA, and the composition of the fractions with the highest activity was then determined.

2.5. Active Fraction Analysis (AF). The analysis was performed on a gas chromatograph coupled to a mass spectrometer (Agilent Technology, model 6890N); this was coupled to a mass selective detector (model 5973) with a DB-5HT capillary column (15 m in length, 0.25 mm internal diameter, and 0.10 μm film thickness). We used a temperature program starting at 100°C for 3 min with a heating rate of 10°C per min up to 320°C; this temperature was maintained for 5 min. The splitless injection was performed at a ratio of 1 : 100, and the injector temperature was 320°C. The spectra were determined at 70 eV, and the mass range analysed was from 33 to 800 m/z. The compounds were identified from the mass spectra and by comparing the spectra to the spectra reported in the NIST database (Wiley09/NIST11).

2.6. Anti-Inflammatory Activity

2.6.1. 12-O-Tetradecanoylphorbol-13-Acetate- (TPA-) Induced Mouse Ear Edema. The model for TPA-induced edema in mouse ears has been described previously [8]. A solution containing 2.5 μg of TPA in 25 μL of acetone was applied topically to the inner and outer surfaces of the right ears in a group of eight male CD1 mice. Thirty minutes later, 2.0 mg of the chloroform extract or AF or indomethacin dissolved in acetone was topically applied to the right ear, and acetone was applied to the left ear. Six hours later, the animals were sacrificed, and 6 mm plugs of the central portion of both ears were weighed. The percentage inhibition of edema was determined.

2.6.2. Carrageenan-Induced Rat Paw Edema. The model for carrageenan-induced edema in the rat paw has been described previously [9]. Paw edema was induced by intradermal injection of 0.1 mL of a 1% carrageenan suspension in the left hind footpad. One hour prior to carrageenan injection, groups of eight rats each were treated with 50, 100, 200, or 400 mg/kg CESS or AF, while another group received 8 mg/kg indomethacin. The control group received the vehicle alone (polyvinyl pyrrolidone (PVP)). The paw volume was measured by the volume displacement method using a plethysmometer (Ugo Basile) at 1, 2, 3, 4, and 5 h after carrageenan administration, and the percentage of edema inhibition was determined [10].

2.6.3. Acute Toxicity. AF was orally administered as a single dose at different concentrations (312–5000 mg/kg) to groups of mice ($n = 5$). After administration, the animals were observed under open-field conditions for a 72 h period. The number of animal deaths and signs of clinical toxicity were recorded [11].

2.7. Statistical Analysis. Data are expressed as the mean ± S.E.M. The statistical analysis was performed using Student's t-test ($P < 0.05$), and ANOVA followed by Dunnett's test ($P < 0.05$) was used to determine significance.

3. Results and Discussion

The methanol extract of *S. salignus* inhibited TPA-induced ear edema by 36.4 ± 4.4%, while the chloroform extract (CESS) diminished the ear edema by 64.1±3.9%; the effect was higher than that obtained with indomethacin (41.5 ± 4.3%). The study was then continued with the CESS, which was separated by column chromatography to give 12 fractions. Fraction 5 (AF) (hexane: AcOEt 7 : 3) showed the highest inhibition of TPA-induced ear edema (46.9 ± 5.3%).

The inflammation induced by TPA is mediated by protein kinase C, which stimulates phospholipase A2 [12] and cyclooxygenase, resulting in the release of arachidonic acid and prostaglandin E2 [13]. The AF displayed good activity, which suggested that at least one of the compounds present in *S. salignus* may inhibit the production of protein kinase C, resulting in the observed effect.

Table 1: Anti-inflammatory effect of the chloroform extract of *Senecio salignus* on rat paw edema induced by carrageenan.

Time	Doses				
	Indomethacin	Doses of *Senecio salignus*			
	8 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
1 h	37.1 ± 3.9*	31.2 ± 4.8	35.7 ± 7.3*	32.4 ± 4.1*	48.1 ± 2*
2 h	62 ± 4.1*	50.8 ± 5.7*	56.1 ± 5.2*	55.4 ± 4.7*	56.8 ± 7*
3 h	63 ± 4.2*	56.7 ± 6.7*	54 ± 4*	60.6 ± 4.9*	64.1 ± 4*
4 h	57.9 ± 3.5*	40.8 ± 4.3*	51.1 ± 5*	60.1 ± 2.3*	59.7 ± 6.2*
5 h	66.8 ± 3.6*	38.5 ± 8.8	45.3 ± 4.2*	63.7 ± 5.6*	55.5 ± 5.9*

Results are expressed as percentage of inhibition and mean of eight determinations ± SE. One-way ANOVA, Dunnett test $^*P < 0.05$ ($P = 0.04$) for the comparison of *S. salignus* with indomethacin-treated groups.

Table 2: Anti-inflammatory effect of AF on paw edema induced by carrageenan.

Time (H)	% Inhibition				
	Indomethacin	*Senecio salignus*			
	8 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
1	15.1 ± 2.8*	10.3 ± 4.02*	15.03 ± 3.1*	17.5 ± 2.3*	27.5 ± 3.8
2	28.3 ± 3.8*	21.3 ± 2.4*	27.8 ± 3.1*	29.1 ± 3.1*	34.5 ± 2.2*
3	43.4 ± 3.2*	26.6 ± 2.1	47.5 ± 4.02*	44.6 ± 3.8*	57.3 ± 2.6
4	61.1 ± 3.8*	37.2 ± 4.6	58.9 ± 2.8*	63.5 ± 2.2*	70.3 ± 4.5
5	62.2 ± 3.9*	38.9 ± 3.3	60.1 ± 4.7*	62.9 ± 2.04*	80.1 ± 4.5

Results are expressed as percentage of inhibition and mean of eight determinations ± SE. One-way ANOVA, Dunnett test $^*P < 0.05$ for comparison of *S. salignus* with indomethacin-treated groups.

CESS and AF were also tested on carrageenan-induced paw edema in rats, and the results are shown in Tables 1 and 2.

The activity of CESS at doses of 50, 100, 200, and 400 mg/kg 3 h after carrageenan administration was similar to that presented with indomethacin. After 5 h, the activity of the extract diminished at doses of 50 and 100 mg/kg, whereas at 200 and 400 mg/kg (63.7 ± 5.6% and 55.5 ± 5.9%, resp.), the inhibition was similar to that by indomethacin (66.8 ± 3.6%).

In contrast, AF (Table 2) at a dose of 50 mg/kg inhibited the edema by only 38.9 ± 3.3% 5 h after carrageenan administration; however, at doses of 100, 200, and 400 mg/kg, the effect (60.1 ± 4.7, 62.9 ± 2.0, and 80.1 ± 4.5%, resp.) was similar to that obtained with indomethacin (62.2 ± 3.9%). The best activity was observed at doses of 400 mg/kg, but at this dose, AF has some toxic effects.

Leukocyte migration to injured tissue is an important aspect of the inflammatory process. The release of several mediators of the phlogistic response, including histamine and serotonin, is responsible for the immediate inflammation response [14], whereas kinins and prostaglandins mediate the prolonged response [15]. In contrast, some plant ingredients significantly inhibit the biosynthetic pathways of inflammation mediators [12]. AF showed an effect in the animal model, suggesting that its anti-inflammatory activity could inhibit the production of these mediators.

AF was analysed by GC-MS, and its composition is shown in Table 3. We found a total of 185 compounds, of which 50 were identified. In Table 3, only those compounds whose concentration was higher than 0.2% have been shown; the major compounds were hexadecanoic acid (3.76%), (Z, Z)-octadecadienoic acid (7.5%), (Z, Z, Z)-9,12,15-octadecatrienoic acid (5%), squalene (5.17%), and nonacosane (10.11%).

Nonacosane is also found in the essential oil of *Artemisia annua*, a plant whose anti-inflammatory activity on carrageenan-induced edema has been attributed to this compound [16].

Reports in the literature also indicate that (Z, Z, Z)-9,12,15-octadecatrienoic acid prevents inflammatory problems as a precursor of prostaglandins. Simopoulos [17, 18] found that this compound and (Z, Z)-9,12-octadecadienoic acid could be used in the treatment of health problems such as type 2 diabetes, some types of cancer, ulcerative colitis, psoriasis, and rheumatoid arthritis.

These facts suggested that (Z, Z, Z)-9,12,15-octadecatrienoic acid, (Z, Z)-9,12-octadecadienoic acid, and nonacosane might be responsible for the observed anti-inflammatory activity.

AF produced a slight change in the normal colour of the kidney at doses of 625 mg/kg. However, at doses of 312.5 mg/kg, no damage was observed in any of the animals' organs. This dose is higher than the active dose of AF (100 mg/kg). These results represent the first step in the process of obtaining a standardised extract and developing a phytomedicine.

4. Conclusions

The active fraction of *S. salignus* was separated from the chloroform extract. The composition was characterised by

Table 3: *Senecio salignus* composition of the active fraction.

Retention time (min)	Compound	% Relative
4.670	Isocaryophyllene	0.61
4.833	α-cadinene	1.0
7.524	Tetradecanoic acid	0.57
9.701	Hexadecanoic acid	3.76
10.163	3-(6-Methoxy-3-methyl-2-benzofuranyl) ethyl butyrate	0.41
11.286	(Z, Z)-9.12-Octadecadienoic acid	7.5
11.337	(Z, Z, Z)-9,12,15-Octadecatrienoic	5.0
11.569	Octadecanoic acid	1.24
13.283	Eicosanoic acid	1.53
14.860	Docosanoic acid	0.88
15.597	Tricosanoic acid	0.24
16.111	Heptacosane	0.84
16.325	Tetracosanoic acid	0.54
16.822	Octacosanol	0.46
16.976	Squalene	5.17
17.576	Nonacosane	10.11
17.713	Hexacosanoic acid	0.63
18.151	Triacontane	1.45
18.990	Octacosanoic acid	0.53
19.393	Dotriacontane	0.32
19.993	Tritriacontane	1.15
20.199	Triacontanoic acid	0.56
20.807	Acetate(3α-21α)-α-neogammacer-22(29)-en-3-ol	1.56
21.313	Dotriacontanoic acid	0.45

GC-MS. AF exhibited anti-inflammatory activity in the inflammation models used in this work when administered topically and orally.

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Review Article

Natural Products and Biological Activity of the Pharmacologically Active Cauliflower Mushroom *Sparassis crispa*

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Sparassis crispa, also known as cauliflower mushroom, is an edible mushroom with medicinal properties. Its cultivation became popular in Japan about 10 years ago, a phenomenon that has been attributed not only to the quality of its taste, but also to its potential for therapeutic applications. Herein, I present a comprehensive summary of the pharmacological activities and mechanisms of action of its bioactive components, such as beta-glucan, and other physiologically active substances. In particular, the immunomodulatory mechanisms of the beta-glucan components are presented herein in detail.

1. Introduction

Medicinal mushrooms have an established history of use in traditional Asian therapies. Over the past 2 to 3 decades, scientific and medical research in Japan, China, and Korea, and more recently in the United States, has increasingly demonstrated the potent and unique properties of compounds extracted from mushrooms for the prevention and treatment of cancer and other chronic diseases. Various important pharmaceutical products with proven medicinal applications have been derived from mushrooms [1].

Sparassis crispa Wulf.:Fr. (Figure 1), also known as cauliflower mushroom, is an edible mushroom with various medicinal properties whose cultivation has recently become popular in Japan. The taxonomy of *S. crispa* is as follows: kingdom, Fungi; phylum, Basidiomycota; class, Agaricomycetes; order, Polyporales; family, Sparassidaceae; genus, *Sparassis*; and species, *crispa*. It is a brown-rot fungus that primarily grows on the stumps of coniferous trees and is widely distributed throughout the North Temperate Zone. *S. crispa* has been reported to have many biological activities, which are detailed below.

2. Chemical Constituents and Bioactive Components of *S. crispa*

Scientific investigation has led to the isolation of many compounds from *S. crispa* that have been shown to have health-promoting activities. The fruiting bodies of *S. crispa* contain approximately 90% water, protein, lipid, carbohydrate, ash, and dietary fiber (Table 1) [3]. Furthermore, the content of vitamin D_2, which aids intestinal calcium absorption, was shown to be 0.17 mg per 100 g of dry weight, a concentration that is higher than that observed in other mushrooms [4]. Also *S. crispa* contained a relatively large amount of glucosyl ceramide (approximately 0.2%), which is a glycoside of ceramide. It was demonstrated that the moiety of sphingoid base was characterized by the unique structure [5]. Though *S. crispa* has a scent of its own, the results of headspace analyses showed that 3-octanone, DL-3-octanol, and 1-octen-3-ol contributed mutually to the particular aroma of this mushroom [6]. It is noteworthy that the beta-glucan content of *S. crispa* is more than 40% of the dry weight of the fruiting bodies, as measured by the enzyme method of the Japan Food Research Laboratories (Tokyo) [3].

Figure 1: *Sparassis crispa* Wulf.:Fr. [2].

Table 1: Approximate composition of *Sparassis crispa* (per 100 g dry sample).

Components	Amount (g)
Protein	13.4
Fat	2.0
Ash	1.8
Carbohydrate	21.5
Dietary fiber (DF)	61.2
Beta-glucan from DF	43.5

2.1. Polysaccharide (Beta-Glucan)

2.1.1. Primary Structure. Using chemical, enzymatic, and NMR analyses, it was shown that the primary structure of a purified beta-glucan (designated SCG), obtained from cultured fruiting bodies of *S. crispa* is a 6-branched 1,3-beta-glucan, with one branch in approximately every 3 main chain units (Figure 2) [7–9].

2.1.2. Biological Activities. Tumor size in cancerous (Sarcoma 180) ICR mice was dose-dependently decreased after 5 weeks of oral administration of *S. crispa* (10 or 100 mg/kg) in comparison to a control group. Furthermore, the survival rate of these model mice was higher when similarly treated with *S. crispa* [2]. Since SCG content in dry powder of *S. crispa* was measured to be more than 40%, SCG was likely be responsible for this antitumor effect.

Ohno et al. prepared polysaccharide fractions from the fruiting bodies of cultured *S. crispa* and showed their antitumor activity against the solid form of Sarcoma 180 in ICR mice with strong vascular dilation and hemorrhage reactions [7]. Furthermore, intraperitoneal and oral SCG over a wide range of concentrations enhanced hematopoietic responses in mice with leukopenia induced by cyclophosphamide (CY, a DNA-alkylating agent) [10, 11]. This effect was augmented in combination with isoflavone aglycone [12]. SCG was also shown to stimulate leukocytes to produce cytokines such as IL-8 in whole-cell cultures of human peripheral blood [13] and in mouse splenocytes [14].

Yamamoto et. al reported antiangiogenic and antimetastatic effects of SCG on neoplasm by using different animal models [8]. Oral administration of SCG suppressed B16-F10 cell-induced angiogenesis in a dorsal air sac assay using ICR mice and suppressed vascular endothelial growth factor induced neovascularization in a Matrigel plug assay using C57BL/6J mice. Furthermore, it suppressed the growth and number of metastatic tumor foci in the lung, along with primary tumor growth in a C57BL/6J mice model of spontaneous metastasis. From these findings, it is apparent that the oral administration of SCG exerts a suppressive effect on tumor growth and metastasis in the lung through the inhibition of tumor-induced angiogenesis.

Taken together, these results demonstrate that SCG exhibits various biological activities, including antitumor effects, enhancement of the hematopoietic response, and induction of cytokine production *in vivo* and *in vitro*.

2.1.3. Mechanisms. Harada et al. reported strain-specific differences of the reactivity of mice to SCG, with DBA/1 and DBA/2 mice being highly sensitive to SCG. In splenocytes derived from various inbred strains of mice, interferon-γ (IFN-γ) production was not induced by SCG. However, splenocytes from naïve DBA/1 and DBA/2 mice strongly react with SCG to produce IFN-γ [14]. Furthermore, in addition to IFN-γ, cytokines induced by SCG were screened for and found to include tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-12 (IL-12p70) [15]. Since the sera of naïve DBA/1 and DBA/2 mice contain significantly higher titers of antibody against SCG than other strains of mice [16], it seems likely that these mice strains are sensitive to SCG. Thus, DBA/1 and DBA/2 mice would be useful models for future studies of SCG.

Harada et al. further demonstrated that GM-CSF was one of the key factors in reactivity to SCG in DBA/2 mice [15, 17]. Neutralizing GM-CSF using an anti-GM-CSF monoclonal antibody significantly inhibited IFN-γ, TNF-α, and IL-12p70 elicited by SCG. The splenocytes in various strains of mice showed similar patterns of cytokine production in response to SCG cotreatment in the presence of recombinant murine GM-CSF. The high sensitivity to SGG shown by DBA/1 and DBA/2 mice may be attributable to differences of their regulation of GM-CSF compared with that in other mice.

Harada and Ohno also proposed an interesting model for the mechanism of cytokine induction by SCG in DBA/2 mice [18]. Broadly speaking, SCG directly induces adherent cells to produce TNF-α and IL-12p70, whereas cell-cell contact mediated by the association of $CD4^+$ T cells expressing LFA-1 and antigen-presenting cells such as dendritic cells expressing ICAM-1 is required for the induction of IFN-γ and GM-CSF by SCG.

Neutrophils, macrophages, and dendritic cells express several receptors capable of recognizing beta-glucan in its

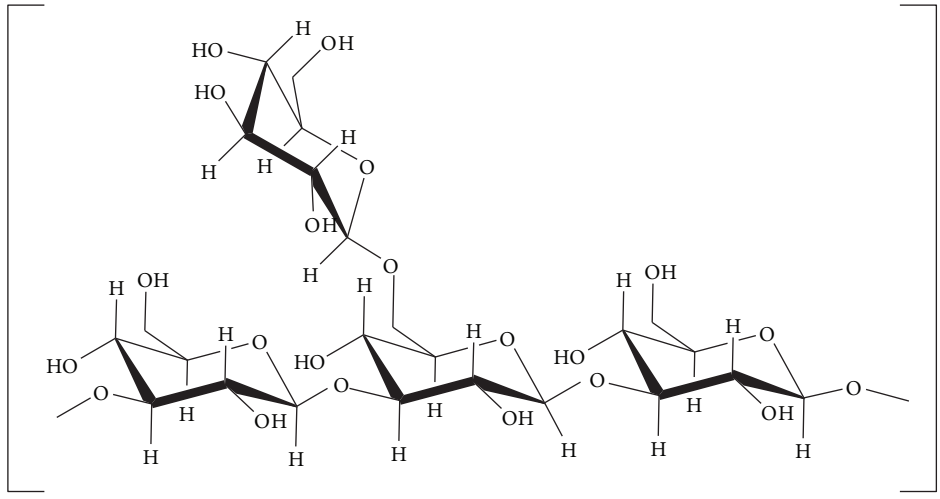

Figure 2: Chemical structure of SCG [9].

various forms. Dectin-1, complement receptor 3, lactosylceramide, and scavenger and Toll-like receptors are all candidates that have been reported thus far [19–23]. Among these, dectin-1, which is a C-type lectin, is an archetypical non-Toll-like pattern recognition receptor expressed predominantly by myeloid cells. Dectin-1 can induce its own intracellular signaling and can mediate a variety of cellular responses, such as cytokine production [24].

The magnitude of cytokine induction from bone-marrow-derived dendritic cells (BMDCs) by SCG and the expression level of dectin-1 on BMDCs in DBA/2 mice are both higher than that of other strains of mice. Furthermore, blocking dectin-1 significantly inhibits the induction of TNF-α production by SCG. These results suggest that the BMDCs from DBA/2 mice are highly sensitive to SCG-induced cytokine production *in vitro*, and that this sensitivity is related to the expression level of dectin-1 [25].

The molecular mechanism of the enhanced hematopoietic response has been investigated in CY-treated mice (both ICR and C57BL/6 strains) [26]. According to this report, the levels of IFN-γ, TNF-α, GM-CSF, IL-6, and IL-12p70 were all shown to be significantly increased in SCG-treated splenocytes of CY-treated mice. GM-CSF production in the splenocytes of CY-treated mice was reportedly higher than that in normal mice regardless of SCG stimulation. Neutralizing GM-CSF significantly inhibited the induction of IFN-γ, TNF-α, and IL-12p70 by SCG. The level of cytokine induction by SCG was modulated by the amount of endogenous GM-CSF produced in response to CY treatment in a dose-dependent manner. The expression of beta-glucan receptors, such as CR3 and dectin-1, was upregulated by CY treatment. Blocking dectin-1 significantly inhibited the induction of TNF-α and IL-12p70 production by SCG. Taken together, these results suggest that the key factors in cytokine induction in CY-treated mice are the enhanced levels of both endogenous GM-CSF production and dectin-1 expression. It is very interesting that agents that modulate GM-CSF production and dectin-1 expression, such as CY, can control reactivity to SCG and the expression of various cytokines.

Shibata et al. found that both GM-CSF and TNF-α synthesis in DBA/2 mouse splenocytes stimulated with SCG, but not with lipopolysaccharide, were significantly enhanced in the presence of cytochalasin D (CytD), an inhibitor of actin polymerization [27]. On the other hand, Kim et al. pointed out the importance of the role of Toll-like receptor 4 (TLR4) [28]. They examined the effect of SCG on adherent monocytes, such as macrophages and dendritic cells (DCs), and nonadherent lymphocytes, such as T and B cells, and demonstrated that SCG mainly activated DCs and macrophages, but not T and B cells. The role of TLR4 as a membrane receptor of SCG was shown by the impairment of maturation of DCs generated from bone marrow cells of $tlr4^{-}/^{-}$ knockout mice and TLR4-mutated C3H/HeJ mice, and by using an anti-MD-2/TLR4 neutralizing antibody. SCG increased the phosphorylation of ERK, p38, and JNK and enhanced nuclear translocation of NF-κB p50/p65 in DCs. These results indicate that SCG activates DCs via MAPK and NF-κB signaling pathways, which are signaling molecules downstream of TLR4.

2.2. Low-Molecular-Weight Compounds. S. crispa possesses a wide range of bioactive metabolites which are products of secondary metabolism (Figure 3).

2.2.1. Antimicrobial Compounds. It has been reported that *S. crispa* produces antibiotic substances. For example, suppression of *Bacillus subtilis* growth on agar media is known to be due to sparassol (methyl-2-hydroxy-4-methoxy-6-methylbenzoate) (1) [29]. Woodward et al. reported that *S. crispa* produced 3 antifungal compounds when submerged in culture in a 2% malt broth. The compounds included

sparassol, and two other antifungal compounds, methyl-2,4-dihydroxy-6-methylbenzoate (2) and methyl-dihydroxy-methoxy-methylbenzoate (the positions of substituents were unclear), both of which showed higher antifungal activity than sparassol against *Cladosporium cucumerinum* [30].

A novel compound (4) and a previously known one (3) were isolated from *S. crispa* [31]. Both compounds were shown to inhibit both melanin synthesis and methicillin-resistant *Staphylococcus aureus* (MRSA) growth. The minimum inhibitory concentration (MIC) values of compounds 3 and 4 in the anti-MRSA assay were 0.5 and 1.0 mM, respectively. IC_{50} values of compounds (3) and (4) in the melanin production inhibition assay were 33 μM and 12 μM, respectively. Since the IC_{50} value of an existing whitening agent, arbutin, is reported to be 1.32 mM, these compounds have potential as constituents of cosmetic products.

In the course of screening for compounds that inhibit MRSA growth, Kodani et al. discovered 2 known chalcones, xanthoangelol (5) and 4-hydroxyderricin (6), in the extract of *S. crispa*, which have been previously isolated from the plant *Angelica keiskei*. These compounds showed anti-MRSA activity, and their MICs were 2 and 0.25 mM, respectively. This was the first report of the isolation of chalcones from a representative of the Fungi kingdom [32].

2.2.2. Other Bioactive Compounds. A new sesquiterpenoid was also isolated from *S. crispa* [33]. Its structure was determined to be (3R*, 3aS*, 4S*, 8aR*)-3-(1′-hydroxy-1′-methylethyl)-5,8a-dimethyldecahydroazulen-4-ol (7) by a combination of NMR and ESI-MS analyses. This was the first isolation of an isodaucane-type sesquiterpenoid from a fungus, including mushrooms.

Yoshikawa et al. isolated three novel phthalides, designated hanabiratakelide A (8), B (9), and C (10) in addition to three known phthalides, from the *S. crispa* fruiting body [34]. The 6 isolated compounds were tested for their antioxidant activity. The *in vitro* superoxide dismutase-like activity of the three hanabiratakelides was stronger than that of vitamin C. These compounds also inhibited lipopolysaccharide-stimulated nitric oxide and prostaglandin E2 production by a murine macrophage cell line, RAW264. In addition, the growth of the colon cancer cell lines Caco-2 and colon-26 was significantly inhibited by treatment with all 3 of the hanabiratakelides.

3. Pharmacological Aspects of *S. crispa*

3.1. Antiviral Activity. Reverse transcriptase (RT) is one of the key enzymes in human immunodeficiency virus (HIV) replication. HIV replication is interfered with when the enzyme is inhibited. Thus, RT inhibitors can be used to treat AIDS. Hot water extracts from the fruiting bodies of 16 species of mushroom, including *S. crispa*, were screened for HIV-1 RT inhibitory activity. The extract of *S. crispa* elicited 70.3% inhibition when tested at a concentration of 1 mg/mL. However, the active component remains unclear [35].

3.2. Antihypertensive Effects. One of the main causes of stroke is hypertension. Therefore, it is important to avoid high blood pressure as a preventative measure. Yoshitomi et al. investigated not only the preventive effects of *S. crispa* against stroke and hypertension in stroke-prone spontaneously hypertensive rats (SHRSP), but also the mechanism involved by studying the cerebral cortex [36]. SHRSP rats given feed containing 1.5% *S. crispa* had a delayed incidence of stroke and death, significantly decreased blood pressure, and increased blood flow. Moreover, the urinary nitrate/nitrite excretion and the nitrate/nitrite concentration in cerebral tissue were higher than those of control SHRSP rats. In the cerebral cortex, phosphor-eNOS (Ser1177) and phosphor-Akt (Ser473) in *S. crispa*-treated SHRSP rats were increased compared with those of control SHRSP rats. In conclusion, *S. crispa* can ameliorate cerebrovascular endothelial dysfunction by promoting recovery of Akt-dependent eNOS phosphorylation and increasing nitric oxide (NO) production in the cerebral cortex.

In addition, Lee et al. indicated that SCG was able to stimulate NO production as well as enhance the expression of inducible NO synthase (iNOS) from macrophage-like RAW264.7 cells [37]. Since NO production is strongly suppressed by mitogen-activated protein kinase (MAPK) inhibitors, it is likely that SGG-induced NO release is mediated by MAPK.

3.3. Antidiabetic Activity. It has been shown that dietary *S. crispa* improves the symptoms of both type 1 and type 2 diabetes. The consumption of a diet containing more than 0.5% *S. crispa* results in significant improvement in diabetes symptoms (body weight loss and increased blood glucose) in ICR mice with STZ-induced diabetes [38]. Furthermore, Yamamoto and Kimura examined the effect of dietary *S. crispa* on KK-Ay mice, an animal model of type 2 diabetes mellitus [39]. The group that was fed 5.0% *S. crispa* diet showed not only a significant decrease of blood glucose and insulin levels, but also a pronounced increase in plasma levels of adiponectin in comparison with a control group. Although the *S. crispa* diet had no effect on body and adipose tissue weights in KK-Ay mice, the size of the mesenteric adipose cells of mice in the *S. crispa* group tended to be smaller than the control group. Thus, the *S. crispa* diet may decrease the adipose cell size in order to increase plasma adiponectin levels. Considering the physiological significance of adiponectin, these findings imply that dietary *S. crispa* has the potential to ameliorate type 2 diabetes.

GPR40 is one of the G protein-coupled receptors, which has 7 transmembrane spanning helical bundles. GPR40 distributes in pancreas and central nervous system. It can be bound by medium- and long-chain fatty acid and activate the intracellular signal pathways, which in turn regulates the function of cells. In pancreatic beta-cell, intracellular calcium concentration elevates when GPR40 is binding to fatty acid, thereby promoting the release of insulin [40]. Yoshikawa et al. demonstrated that a couple of unsaturated fatty acids in *S. crispa* were the agonist of GPR40, which might be used for preventing and treating the diabetes [41].

The normal healing process in healthy individuals takes place at an optimal rate, but it is usually delayed, or even

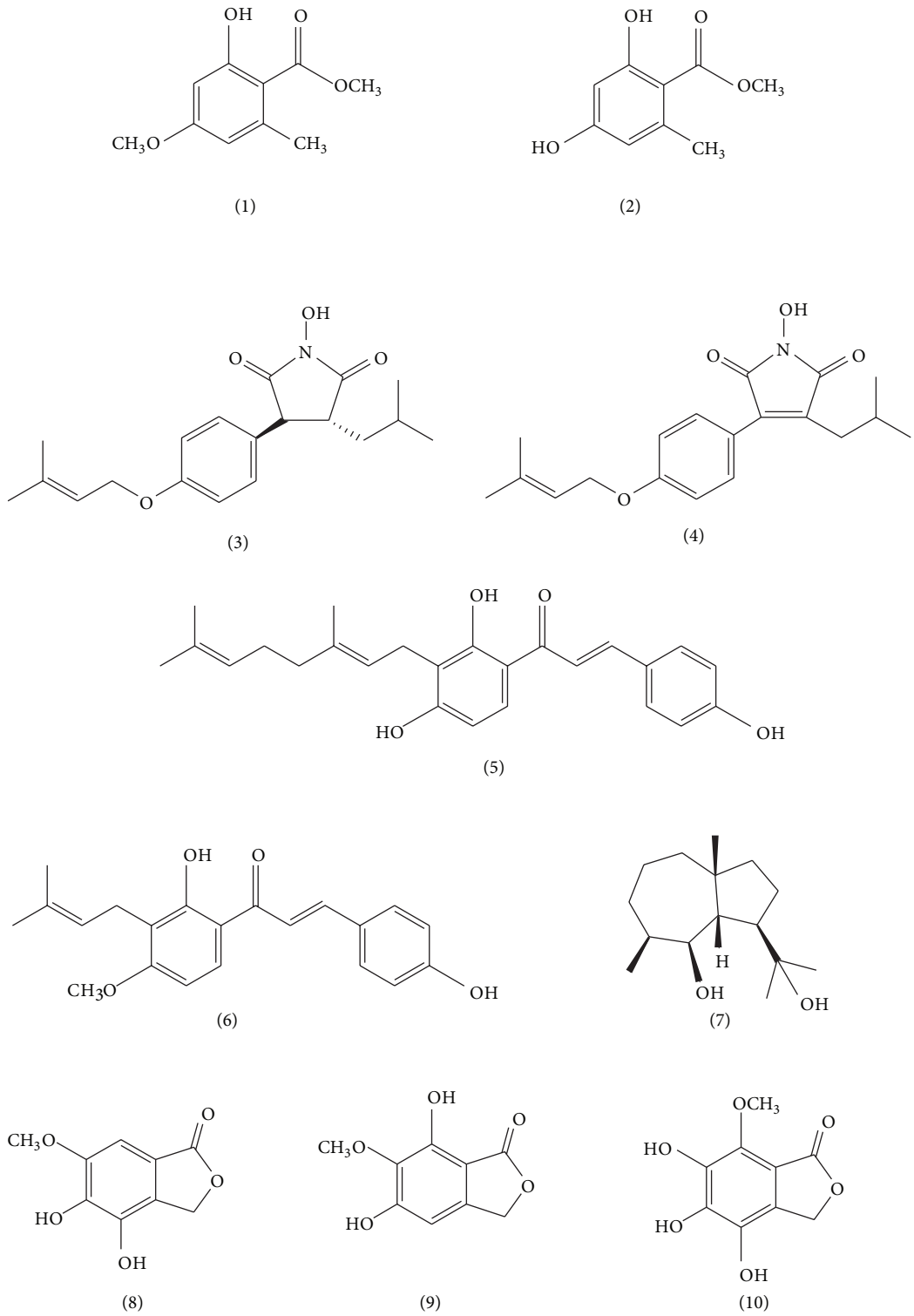

Figure 3: Chemical structure of the low-molecular-weight compounds found in *S. crispa* [29–34].

completely impaired in patients with diabetes. Thus, the impaired wound healing that occurs in diabetes mellitus is a major clinical problem. It is also generally accepted that wound repair is an immune-mediated physiologic mechanism. Oral administration of 1,000 mg/kg body weight per day of *S. crispa* for 4 weeks was shown to significantly accelerate wound healing in rats with streptozotocin- (STZ-) induced diabetes, which is an insulin-dependent model of diabetes mellitus (type 1) [42]. Furthermore, in *S. crispa*-treated wounds there were significant increase in macrophage and fibroblast migration, collagen regeneration, and epithelialization compared with a control group. Therefore, the use

of *S. crispa* may be extended to the clinical setting, and it may effectively promote wound healing in patients with diabetes.

Yamamoto and Kimura investigated whether oral and topical administration of *S. crispa* could restore effective wound healing in ICR mice with STZ-induced diabetes [38]. Mice consuming a diet containing more than 0.5% *S. crispa* showed significantly improved wound healing. Notably, the rate of wound healing in mice fed a diet containing 2.5% *S. crispa* was almost the same as that in mice treated with topical trafermin (basic fibroblast growth factor formulation). Moreover, topically administered SCG significantly promoted wound healing in mice with diabetes, resulting in a wound contraction ratio of 37% after treatment for 9 days, a result that was superior to that of trafermin.

3.4. Antitumor and Anticarcinogenic Activity (except for SCG). Yamamoto et al. investigated the antitumor effects of a low-molecular-weight (below approximately 8 kDa) fraction (FHL) containing no beta-glucan isolated from a hot water extract of *S. crispa* [43]. The oral administration of FHL (30 mg/kg) to tumor- (sarcoma 180) bearing ICR mice was observed to suppress tumor growth. Furthermore, the IFN-γ level in the culture supernatant of splenic lymphocytes from FHL-fed tumor-bearing mice was significantly increased compared to a control group. Tumor-induced angiogenesis in the dorsal air sac (DAS) system was also suppressed by FHL administration. These results suggest that the oral administration of FHL induces antitumor activity through the enhancement of the Th1-response in tumor-bearing mice. Additionally, the antiangiogenic activity of FHL may contribute to its antitumor activity.

Yoshikawa et al. investigated the possible preventive effects of *S. crispa* on azoxymethane-induced colon aberrant crypt foci (ACF) in F344/N rats. *S. crispa* feeding dose-dependently suppressed the malignant changes of ACF by 54% (0.3% group), 64% (1.0% group), and 75% (3.0% group). They concluded that the anticancer-related activity may originate from the aforementioned hanabiratakelides [34].

3.5. Antiallergic Activity. Allergic inflammatory diseases, such as food allergy, asthma, hay fever, and atopic dermatitis, are increasing worldwide. Some recent reports have demonstrated antiallergic activities of *S. crispa*. Atopic dermatitis (AD) is a common inflammatory skin disease for which few effective treatments are available. Oral administration of 250 mg/kg body weight per day of *S. crispa* decreased both blood immunoglobulin E (IgE) level and scratching index in Nc/Nga mice with dermatitis induced by a continuous application of 2,4,6-trinitrochlorobenzene [2]. In addition, the antirhinitis properties of *S. crispa* were also investigated in mice [44]. To determine the immunomodulatory activity of oral *S. crispa*, splenocytes obtained from ovalbumin-sensitized BALB/c mice fed 0.25% *S. crispa* were restimulated *in vitro* with the same antigen. The oral *S. crispa* induced IFN-γ secretion, but inhibited IL-4 and IL-5 secretion, and suppressed ovalbumin-specific IgE secretion by the splenocytes. The effects of *S. crispa* were further investigated by using an allergic rhinitis model in BALB/c mice. Nasal symptoms, sneezing, and nasal rubbing induced by ovalbumin challenges were inhibited by oral administration of *S. crispa* (36 or 120 mg/kg) in a dose-dependent manner. Furthermore, ovalbumin-specific serum IgE levels were diminished by *S. crispa* treatment in this model. These results demonstrate that *S. crispa* may be effective in suppressing symptoms of allergic rhinitis through suppression of the Th2-type immune response.

Kim et al. reported the effect of a water extract of *S. crispa* (WESC) on mast-cell-mediated allergic inflammation and the possible mechanisms of action using *in vivo* and *in vitro* models [45]. WESC inhibited compound 48/80-induced systemic anaphylaxis and serum histamine release in mice. WESC decreased IgE-mediated passive cutaneous anaphylaxis. Additionally, WESC reduced histamine release and intracellular calcium in human mast cells activated by both phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187. Since intracellular calcium plays an important role in the release of histamine and the expression of cytokines, the decreased intracellular calcium levels may be involved in the inhibitory effect of WESC on histamine release. WESC decreased PMA and A23187-stimulated expression of proinflammatory cytokines, such as TNF-α, interleukin- (IL-6), and IL-1β. The inhibitory effect of WESC on proinflammatory cytokines was shown to be dependent on nuclear factor-κB, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase. Since the beta-glucan content in WESC was measured to be 39.3%, beta-glucan may be responsible for its antiallergic effects.

4. Human Clinical Evaluation

In a study where healthy men were given *S. crispa* powder orally at 300 mg per day for 8 weeks, NK cell cytotoxicity was significantly enhanced without increasing the number of NK cells when compared to preadministration [2]. In addition, Kimura investigated whether dietary *S. crispa* influenced human skin condition [46]. Oral administration of *S. crispa* powder (320 mg/day) for 28 consecutive days dramatically reduced transepidermal water loss, an indicator of the skin barrier condition, while that of a placebo group was unchanged during the testing period. These observations imply that oral administration of *S. crispa* has a positive effect on the skin barrier.

A clinical trial of *S. crispa* used an orally delivered powder (300 mg/day) in patients with several different types of cancer (lung, stomach, colon, breast, ovarian, uterine, prostate, pancreas, and liver cancers) after the patients had received a single course of lymphocyte transfer immunotherapy [47]. Patient assessment of 14 cases after a several month follow-up period (mean: 15 months) revealed that the performance status in 9 cases showed improvement in quality of life, and so forth.

5. Conclusions and Future Prospects

S. crispa has been described in the literature as a mushroom with great potential for therapeutic applications. The

medicinal value of this mushroom is mainly attributable to its abundant 6-branched 1,3-beta-glucan (SCG). By chemical analysis, we found that the primary structure of a purified beta-glucan obtained from liquid cultured mycelium of *S. crispa* was a 6-branched 1,3-beta-glucan, having one branch approximately every 6 residues, with a degree of branching that is relatively less than that of SCG. The effect on tumor-(sarcoma 180) bearing ICR mice was much weaker than that of SCG given by oral administration (data not shown). Furthermore, using 1D- and 2D-NMR spectroscopy, Tada et al. elucidated the fine primary structure of SCG and compared it with sonifilan (SPG) from *Schizophyllum commune*, which is a 6-branched 1,3-beta-glucan and has been used clinically for cancer therapy in Japan, examining differences in the biological effects between these beta-glucans [9]. Though both major structural units are the same beta-(1–3)-glucan backbone with single beta-(1–6)-glucosyl side branching units every 3 residues, the production of IL-6 and TNF-alpha from BMDCs was significantly increased by SCG, whereas these effects were not observed with SPG treatment. These findings may indicate that the biological activities of beta-glucan are attributable not only to its primary structure but also to its conformation.

Though it has been suggested that dietary *S. crispa* is useful for cancer immunotherapy in combination with lymphocyte transplantation [47], the study described in this previous report did not include a randomized control group. Further clinical trials are needed to confirm the pharmacological activity of dietary *S. crispa*. There is still little scientific evidence to explain the differences in responsiveness to beta-glucan in humans. The studies of differences in reactivity to SCG in different animal strains [14, 15, 17] are important from this viewpoint. Furthermore, it is interesting that agents such as CY can control reactivity to SCG, as well as the expression of various cytokines [26]. Further research on reactivity to SCG could provide clues for developing more effective cancer immunotherapies using SCG.

As mentioned above, dectin-1 and TLR4 have been proposed as SCG receptors. It is noteworthy that either treatment with a blocking antibody against dectin-1 [25, 26] or genetic deletion of TLR4 [28] completely prevents SCG-induced DC maturation. These observations might indicate that one signaling pathway did not compensate for the other in SCG-treated DCs, suggesting that both receptors are required for SCG action. However, further analysis of the role of these receptor candidates, which contain complement receptor 3, lactosylceramide, and scavenger-like receptors, in response to SCG would be needed in order to clarify the details of its mechanism of action in DCs [48].

The question has been raised as to how orally administered beta-glucan exerts its effects. The evidence presented in this review clearly indicates that dietary SCG has immunomodulatory actions. Therefore, it must be assumed that orally ingested SCG interacts with either intestinal epithelial cells and/or intestinal DCs, ultimately resulting in the priming or activation of other immune cells.

The antitumor mechanisms of SCG, except for its immunomodulatory actions, have not been well studied. Hence, we tried to elucidate the possible mechanisms of its antiangiogenic effects. As a result, it was demonstrated that SCG has both antiangiogenic functions and antimetastatic effects on neoplasm using different animal models [8]. The antitumor effects of SCG may be partially attributable to its antiangiogenic actions. Numerous reports concerning the antitumor activity of edible mushrooms have taken particular notice of beta-glucan. However, few studies have focused on antitumor components other than beta-glucan. It is worth mentioning that *S. crispa* has been shown to produce some low-molecular-weight constituents with antitumor activity, such as hanabiratakelide [34] and FHL [43].

Recently, Park et al. reported a novel process for nanoparticle extraction of beta-D-glucan from *S. crispa* using insoluble tungsten carbide [49]. This nanoknife method results in high yields of SCG (70.2%) with an average particle size of 150 and 390 nm. The extracted SCG showed a remarkably high water solubility of 90% at room temperature. This nanoknife method could be a potent technology to produce SCG for food, cosmetics, and pharmaceutical industries.

The extract of *S. crispa* might be applied to produce health products such as food, beverage, and antineoplastic drug. Actually, *S. crispa* extractions, resveratrol, and collagen peptide were claimed as antiaging agents and food supplements [50]. Formulation examples of granules and health drinks were disclosed.

Many people in Japan consume *S. crispa*, and to date, no reports of adverse events due to *S. crispa* consumption have been reported. Therefore, dietary treatment with *S. crispa* may prove to be a safe therapy for cancer and other chronic diseases.

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Research Article

Lactobacillus plantarum LG42 Isolated from Gajami Sik-Hae Inhibits Adipogenesis in 3T3-L1 Adipocyte

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We investigated whether lactic acid bacteria isolated from gajami sik-hae (GLAB) are capable of reducing the intracellular lipid accumulation by downregulating the expression of adipogenesis-related genes in differentiated 3T3-L1 cells. The GLAB, *Lactobacillus plantarum* LG42, significantly decreased the intracellular triglyceride storage and the glycerol-3-phosphate dehydrogenase (GPDH) activity in a dose-dependent manner. mRNA expression of transcription factors like peroxisome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer-binding protein (C/EBP) α involved in adipogenesis was markedly decreased by the GLAB treatment. Moreover, the GLAB also decreased the expression level of adipogenic markers like adipocyte fatty acid binding protein (aP2), leptin, GPDH, and fatty acid translocase (CD36) significantly. These results suggest that the GLAB inhibits lipid accumulation in the differentiated adipocyte through downregulating the expression of adipogenic transcription factors and other specific genes involved in lipid metabolism.

1. Introduction

Gajami sik-hae is a fermented fish product popular in the northeastern coastal area of Korea. Sik-hae is a traditional Korean fermented seafood and has long been used for seasoning and is the generic name for a class of Korean lactic acid fermented fish products [1]. It is prepared through blending of various kinds of seafood, including cooked rice, red pepper, radish, garlic, ginger, malt meal, and salt, that becomes palatable through the subsequent preservation and fermentation [2]. The unique taste of sik-hae is due to the presence of the aforementioned ingredients and also due to the fermenting action of various microorganisms during the fermentation period [3]. Lactic acid bacteria (LAB) are the most predominant microorganisms involved in sik-hae fermentation. Some LABs were isolated from fish fermented food in Korea [4]. LAB is a viable bacteria that beneficially influence the health of the host. Recently, LAB has been revaluated for their nutritional, physiological, and pharmacological aspects, which have raised attention towards the functional effect of Korean traditional foods including kimchi and joet-gal [5]. Various nutritional and therapeutic effects ascribed to LAB in the human body are the metabolic stimulation of vitamin synthesis and enzyme production, antimutation, anticancer, gastric secretomotor, and immune function [6].

Nowadays, the consumer pays a lot of attention in looking for the relation between food and its health benefit. As a consequence, the market for foods with health-promoting properties, the so-called functional foods, has shown a remarkable growth over the past few years [7]. The development of food, which utilizes the functionality and effectiveness of probiotics, has been officially recognized as an important field of study. Consequently, numerous studies for using probiotic organisms as a functional food have been investigated. Earlier studies on probiotics have been brought into effect on the stabilization of gastrointestinal microflora, the reduction of saprogenic products, the prevention of degenerative disease, activation of the immune system, anticancer activities, antiobesity, lowering of cholesterol, and the suppression and prevention of constipation [8–12].

γ-Aminobutyric acid (GABA) is a four-carbon nonprotein amino acid conserved from bacteria to plants and

vertebrates. It was discovered in plants more than half a century ago, but the interest in GABA shifted to animals when it was revealed that GABA occurs at high levels in the brain, playing a major role in neurotransmission [13]. The pathway for GABA synthesis is composed of the cytosolic enzyme glutamate decarboxylase (GAD) and the mitochondrial enzymes GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) [14]. The consumption of GABA-enriched foods such as milk, soybean, and gabaron tea has been reported to suppress the elevation of systolic blood pressure in spontaneously hypertensive rats (SHRs) [15–17]. In this study, isolated LAB with GABA producing ability from traditional Korean fermented foods such as kimchi [18] and sik-hae (S.H. Oh). The isolated LABs were *Lactobacillus brevis* OPK-3, *Lactobacillus sakei* OPK2-59, and *Lactobacillus plantarum* LG42. Therefore, it was our interest to find whether the isolated LAB can be a novel nutraceuticals. This study was carried out with the objective of testing the antiobesity property of *Lactobacillus plantarum* LG42 (GLAB) with GABA producing ability. Our finding reveals the inhibitory effect of the GLAB on adipocyte differentiation through modulating the expression of adipogenic transcription factors and other adipogenesis specific genes, suggesting the antiobesity property of GLAB.

2. Materials and Methods

2.1. Cell Culture. 3T3-L1 cells (American Type Culture Collection (ATCC)) were cultured in DMEM containing high glucose supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in 6 well culture plates. Two days after confluence cells were cultured in the adipocyte differentiation cocktail media containing 5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM dexamethasone (Sigma, USA), and insulin (10 mg/mL) in DMEM supplemented with 10% fetal bovine serum (FBS) for 2 days. The differentiation was complete after 6 days.

2.2. GLAB Sample and Treatment. L. plantarum LG42, a lactic acid bacteria having GABA producing capacity, isolated from gajami sik-hae (GLAB), was supplied by Woosuk University, genetic engineering laboratory, Korea. For TLC (thin layer chromatography) analysis, silica gel 60 F254 (Merck, Germany), standard GABA (Sigma, USA), solvent mixture (butanol: acetic acid: dichloromethanol: water in 5 : 3 : 3 : 3 ratio) were used. The cultured medium, cell-free supernatant, and cytoplasmic fraction samples (0.3 μL each) were spotted 3 times. The GLAB was incubated at 37°C for 16~18 hr in MRS agar plates (Difco, Detroit, USA). All purified strains were kept at −70°C until use. After culturing the GLAB, all strains were harvested in a refrigerated centrifuge (1,100 ×g for 3 min at 4°C) and washed three times with distilled water for the removal of MRS broth. The washed GLAB was freeze-dried and resuspended in distilled water at a concentration of 10 mg/mL and homogenized for 50 sec followed by 1 min rest (repeated 3 times) using a sonicator (Fisher Scientific Co., Toronto, ON, USA). The suspension was centrifuged at 1,100 ×g for 15 min at 4°C. The 3T3-L1 cells were treated with five different concentrations of the supernatant, that is, 0 (control), 10, 20, 30, and 40 μg/mL.

2.3. Oil Red O Staining of 3T3-L1 Adipocyte. Intracellular lipid accumulation was measured using oil red O (Sigma, St. Louis, MO). 3T3-L1 cells were fixed with 3.7% formaldehyde/PBS and stained with oil red O. Quantification of lipid accumulation was achieved by oil red O from stained cells with isopropyl alcohol and measured spectrophotometrically at 510 nm. The oil red O stained material was expressed on a per cell basis using the cell number determined from similar plates. The percentage of oil red O stained material relative to control wells containing cell culture medium without compounds was calculated as $A_{510\,\mathrm{nm}}$ (GLAB)/$A_{510\,\mathrm{nm}}$ (control) × 100.

2.4. Triglyceride Content. Triglyceride content was determined using a commercial triglyceride assay kit (Zen-bio, Research Triangle Park, NC), according to the manufacturer's protocol. The protein concentration was determined by using a Bradford reagent (Sigma, St. Louis, MO).

2.5. Glycerol-3-Phosphate Dehydrogenase Activity. GPDH was measured by following the disappearance of NADH during enzyme-catalysed dihydroxyacetone phosphate reduction using the GPDH activity assay kit (TAKARA BIO INC., Japan). GPDH activity was spectrophotometrically determined at 340 nm. One unit was defined as the amount of enzyme required for the consumption of 1 μmol of NADH for one minute at 30°C. The enzyme activity was calculated with the following formula: GPDH activity (units/mL) = ($\Delta\mathrm{OD}_{340} \times A$ (mL) × dilution ratio of the test sample)/(6.22 ×B (mL) × C (cm)).

$\Delta\mathrm{OD}_{340}$: decrease in the absorbance at 340 nm per minute.

A (mL): total reaction volume.

B (mL): the volume of enzyme solution (diluted sample) added.

C (cm): optical path length of the cell used*.

6.22: millimolar absorption coefficient of NADH molecules.

2.6. Quantitative Real-Time PCR Analysis. Total RNA was extracted from 3T3-L1 cells at various times after adipogenic induction using Trizol reagent (Invitrogen Life Technologies; Carlsbad, CA, USA) and the concentration measured spectrophotometrically. The extracted RNA was reverse transcribed into complementary DNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Then the RNA expression level was quantified by a quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Woolston, Warrington, UK) and the 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacture's protocol. The Sequences of primers used for quantitative real-time PCR are as follows: fatty acid binding protein (aP2)

F: 5′-AGTGAAAACTTCGATGATTACATGAA-3′ and R: 5′-GCCTGCCACTTTCCTTGTG-3′; fatty acid translocase (CD36) F: 5′-TTGTACCTATACTGTGGCTAAATGAGA-3′ and R: 5′-CTTGTGTTTTGAACATTTCTGCTT-3′; CCAAT/enhancer-binding protein α (C/EBPα) F: 5′-AGCAACGAGTACCGGGTACG-3′ and R: 5′-TGTTTGG-CTTTATCTCGGCTC-3′; peroxisome proliferator-activated receptor γ (PPARγ) F: 5′-CAAGAATACCAAAGTGCG-ATCAA-3′ and R: 5′-GAGCTGGGTCTTTTCAGAAT-AATAAG-3′; (leptin) F: 5′-CCGCCAAGCAGAGGGT-CAC-3′ and R: 5′-GCATTCAGGGCTAACATCCAACT-3′; glycerol-3-phosphate dehydrogenase(GPDH) F: 5′-CTCT-TCTTGCCGCTTCAGTTT-3′ and R: 5′-CATGTAGGCCA-TGAGGTCCACCAC-3′; β-actin F: 5′-ATGGATGACGA-TATCGCT-3′ and R: 5′-ATGAGGTAGTCTGTCAGGT-3′. Relative quantification of gene expression with real-time PCR data was calculated relative to β-actin.

2.7. Analysis of GABA Production. GABA formation in cultured medium and cytoplasmic fraction of *L. plantarum* LG42 cells were analyzed by TLC as described (25). In order to produce GABA in the culture medium, 1% MSG was added to MRS broth and then cultured for 48 h at 30°C, and verified the existence or nonexistence of GABA in the bacterial cytoplasmic fraction by using TLC.

2.8. Statistical Analysis. All values are expressed as mean ± SD. The data were analyzed by one-way ANOVA using SPSS version 16.0. The differences among groups were assessed using Duncan's multiple range test. Statistical significance was considered at P value < 0.05.

3. Results

3.1. Oil Red O Staining and Intracellular Triglyceride. The effects of GLAB on oil red O stained 3T3-L1 adipocyte are shown in Figure 1. Cells treated with 10 μg and 20 μg concentrations did not show any significant effects compared with untreated cells. Differentiated cells treated with 30 μg of GLAB accumulated about a 30% decrease in intracellular lipid compared to control. Also, the treatment with 40 μg GLAB resulted in further reduction to approximately 58% of the lipid accumulation of the control.

The effect of GLAB on the inhibition of intracellular triglyceride in 3T3-L1 adipocyte is shown in Figure 2. The results demonstrated that GLAB caused an inhibition on intracellular triglyceride accumulation. Especially, 40 μg of GLAB treatment, which was most effective compared with 10 μg treated and untreated cells (Figure 2).

3.2. Measurement of GPDH Activity. The effects of GLAB on GPDH activity in 3T3-L1 adipocyte are shown in Figure 3. GPDH activity, which indicates the late phase of adipocyte differentiation, was also determined. The effect of GLAB on adipogenesis was clearly dose dependent. The high dose, 30 and 40 μg, of GLAB significantly decreased GPDH activity (Figure 3).

3.3. mRNA Expression of Lipid Metabolism-Related Gene in Differentiated 3T3-L1 Adipocyte. PPARγ and C/EBPα mRNA levels in differentiated 3T3-L1 adipocyte were significantly decreased in cells treated with GLAB compared with untreated cell, especially 40 μg treated cell was most effective (Figure 4). In addition, the expression of PPARγ target gene, aP2 was significantly decreased in GLAB treated cells, and CD 36 was significantly decreased in 20, 30, and 40 μg treated cells compared with untreated cell during adipocyte differentiation (Figure 4). Cells treated with GLAB resulted in a significant decrease in the mRNA levels of leptin compared to cell untreated with GLAB. The GPDH mRNA level was significantly decreased in 30 and 40 μg/mL treated cells compared with 10 μg/mL treated and untreated cells.

3.4. GABA Production by L. plantarum LG42. GABA production by *L. plantarum* LG42 strain was studied in MRS broth containing 1% MSG. Based on the TLC analysis data, the cultured medium and cell-free culture supernatant of *L. plantarum* LG42 cells contained GABA, but not the cytoplasmic fraction (Figure 5). This data indicated that *L. plantarum* LG42 cells have GABA producing ability and the GABA was excreted mainly in the cell culture medium.

4. Discussion

Obesity threatens to become the 21st century's leading metabolic disease in the world [19]. Complications associated with obesity are responsible for the most obesity related morbidity and mortality. Obesity increases circulating cholesterol and triglyceride levels and is closely associated with hypertension, cardiovascular disease, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis [20]. Obesity is a condition in which adipocytes not only accumulate a large amount of fat, but also become enlarged. At a cellular level, it is characterized by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in the adipose tissue [21]. The severity of obesity is correlated with the degree of differentiation of preadipocytes to adipocytes and the enlargement of adipocytes in adipose tissues [22]. PPARγ and C/EBPα are transcriptional activators, which play a major role in coordinating the adipocyte gene expression during adipocyte differentiation [23]. Therefore, we investigated the effects of lactic acid bacteria isolated gajami sik-hae (GLAB) on the differentiation of 3T3-L1 cells to clarify its antiobesity mechanism. GLAB significantly suppressed lipid accumulation in 3T3-L1 cells in a nontoxic concentration. GPDH activity was decreased in 3T3-L1 cells treated with GLAB compared to control cells incubated in the differentiation medium (Figure 3) ($P < 0.05$). GPDH is a key enzyme important for triacylglycerol synthesis [24], the present result indicates that GLAB suppresses adipocyte differentiation resulted in reducing GAPDH level.

It is noteworthy that PPARγ and C/EBPα levels were downregulated in 3T3-L1 cells treated with GLAB (Figure 4). PPARγ and C/EBPα play vital roles in the early stage of adipose differentiation [25]. PPARγ and C/EBPα regulate the expression of adipogenic genes such as CD36, leptin,

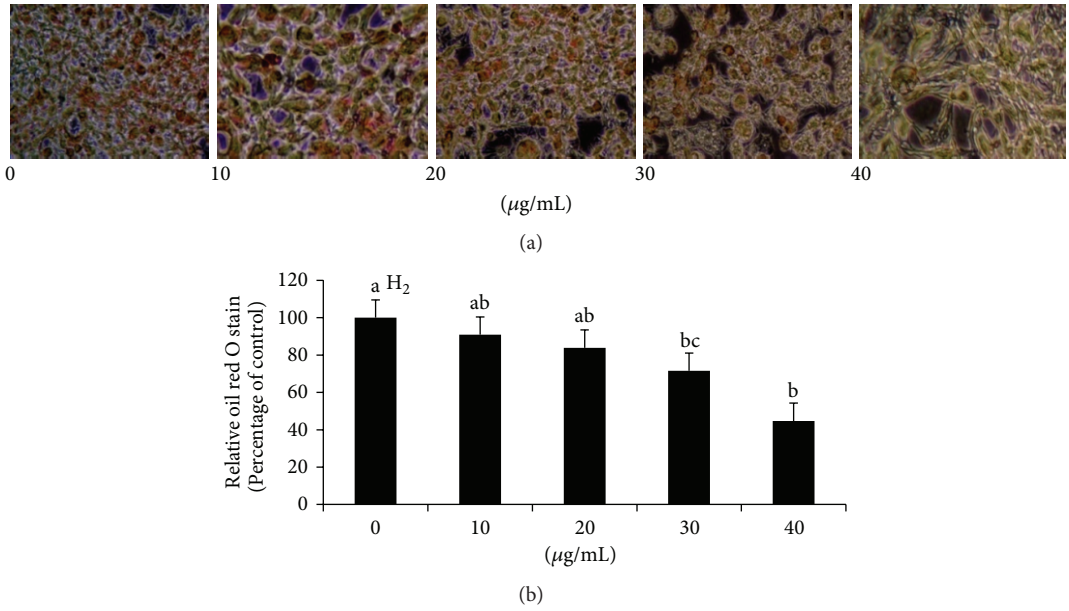

Figure 1: The effect of lactic acid bacteria isolated gajami sik-hae on oil red O stained in 3T3-L1 adipocyte (a) Photograph of oil red O staining. (b) Quantification of oil red O staining. Values with different superscripts are significantly different by ANOVA with Duncan's multiple range tests at $P < 0.05$.

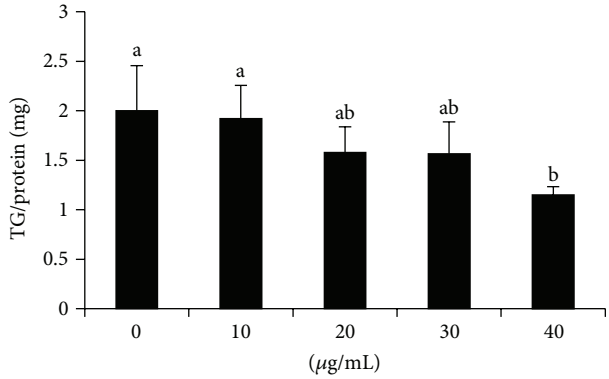

Figure 2: The effect of lactic acid bacteria isolated gajami sik-hae on triglyceride accumulation in 3T3-L1 adipocyte. Values with different superscripts are significantly different by ANOVA with Duncan's multiple range tests at $P < 0.05$.

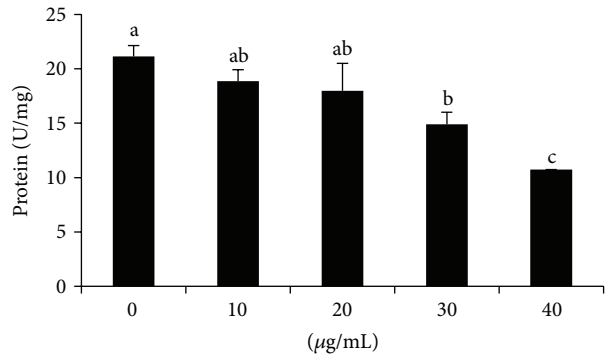

Figure 3: The effect of lactic acid bacteria isolated gajami sik-hae on GPDH activity in 3T3-L1 adipocyte. Values with different superscripts are significantly different by ANOVA with Duncan's multiple range tests at $P < 0.05$.

GPDH, and aP2 triggering the accumulation of fat in the cells [26, 27]. In this study, the expression of PPARγ and C/EBPα was inhibited by GLAB together resulting in reduced adipogenesis indirectly confirmed by measuring oil red stain and GPDH activity. Therefore, it appears that GLAB inhibits adipogenesis by reducing or suppressing the expression of PPARγ and C/EBPα levels. It is also reasonable to articulate that GLAB acts directly on PPARγ and C/EBPα. Kim et al. (2008) showed that treating 3T3-L1 cells with milk fermented with lactic acid bacteria isolated from kimchi decreased the levels of PPARγ and C/EBP-α expression [28]. Thus, it could be possible that lactic acid bacteria isolated from gajami sik-hae affects the downstream genes of PPARγ and C/EBPα, thus inhibiting adipogenesis in 3T3-L1 cells.

aP2 gene is central to the pathway that links obesity to insulin resistance, possibly by linking fatty acid metabolism to the expression of (tumor necrosis factor-α) TNF-α [29, 30]. CD36 mRNA expression is activated during 3T3-L1 adipocyte differentiation, and CD36 protein levels are positively correlated with PPARγ and C/EBPα [31]. CD36 is a long chain fatty acid transporter present on the plasma membrane, as well as in intracellular pools of the skeletal muscle [32]. High level CD36 might result in lipid accumulation, which is supported by studies using CD36 null mice [33]. Leptin, a hormone and the product of the *ob* gene, is primarily secreted by adipose tissue. It is involved in the regulation of energy expenditure and food intake [34]. The expression of leptin is regulated by several substances like insulin, glucocorticoids, and TNF-α. Here in this study, we found that aP2, CD36, and

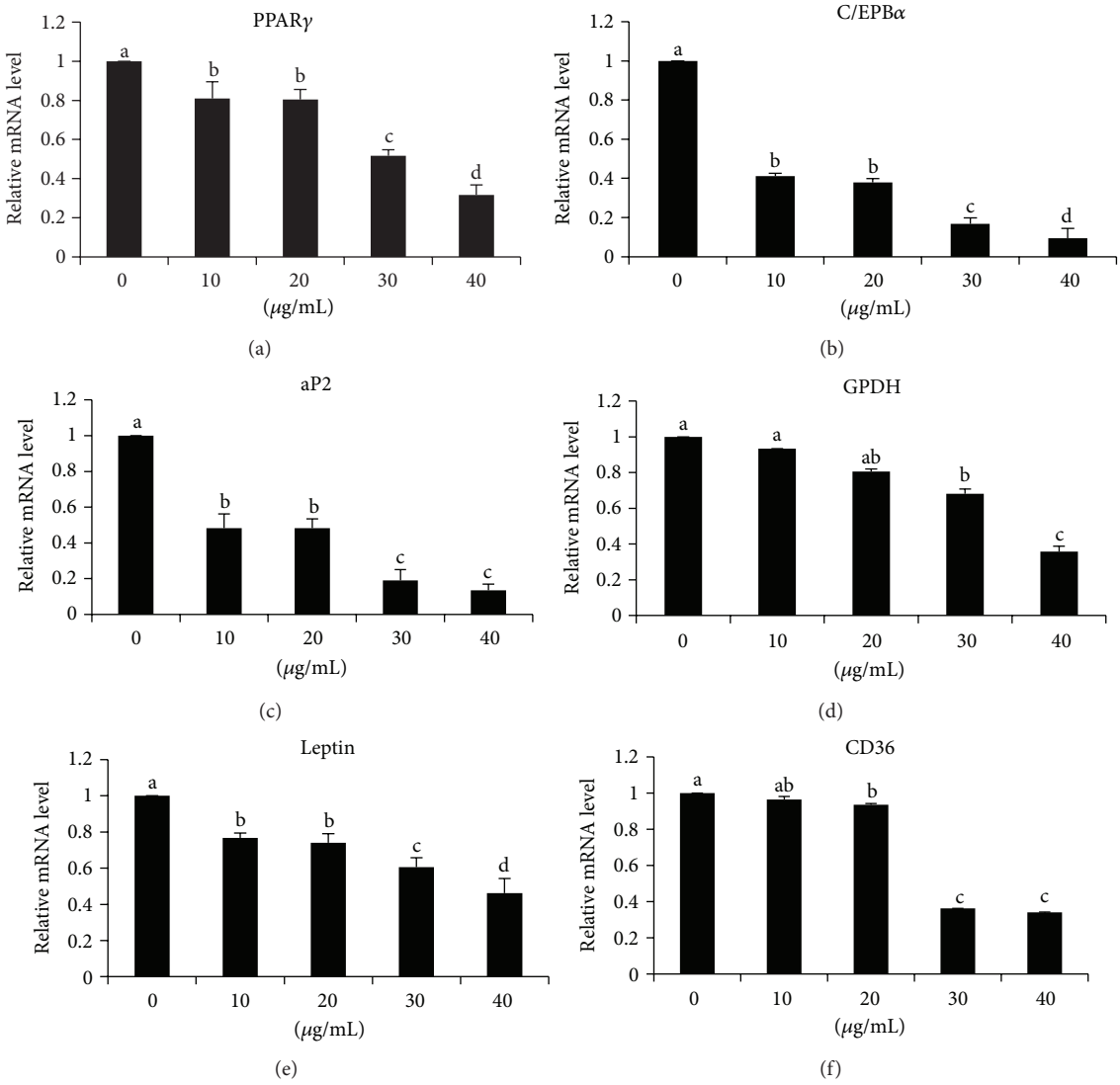

Figure 4: The effect of lactic acid bacteria isolated gajami sik-hae on mRNA levels of PPARγ, C/EBPα, aP2, GPDH, leptin, and CD36 in 3T3-L1 cells. Values with different superscripts are significantly different by ANOVA with Duncan's multiple range tests at $P < 0.05$. Peroxisome proliferator-activated receptor γ (PPARγ); CCAAT/enhancer-binding protein α (C/EBPα); fatty acid binding protein (aP2); glycerol-3-phosphate dehydrogenase(GPDH); fatty acid translocase (CD36).

leptin mRNA expressions were inhibited by GLAB (Figure 4). aP2, CD36, and leptin are the target genes of C/EBPα and PPARγ and are regulated by these transcription factors. Thus, our results suggest that the inhibition of adipogenic gene expression induced by GLAB may be mediated by the inhibition of C/EBPα and PPARγ expression.

Gajami sik-hae is a fermented food containing many kinds of microorganisms, with *Lactobacillus* and *Streptococcaceae* as the dominant species [35]. *Lactobacillus* and *Leuconostoc* species among lactobacillus produces various materials by proliferation, fermentation, and metabolism. Functional organic acids like lactic acid and citric acids are the major products of fermentation by these bacteria. Functional materials such as acetylcholine, dextran, bacteriocin, and γ-aminobutyric acid are also produced by these microbes depending on the origin of the fermenting materials. The fermenting materials containing carotenoids, ascorbic acids, phenolic compounds, and amino acids play a major role in this process [18, 36]. Among the various beneficial health effects of the probiotics [37, 38], their biological impact on obesity has generated a considerable interest. Regarding the anti-obesity property of probiotics, some reports demonstrated that dairy products fermented with lactic acid bacteria exert anti-obesity effects [28, 39, 40]. Previously, it has been shown that cytoplasmic fractions of useful LAB have some beneficial effects for the improvement of health related symptoms [41, 42]. Among various compounds from LAB contributing to anti-obesity effects and improving lipid profiles are conjugated linoleic acid (CLA), ornithine, GABA, hydroxy methyl glutaric acid, orotic acid, and so forth [18, 43–46]. Although GABA can exert an anti-obesity effect and the GLAB has GABA producing capacity

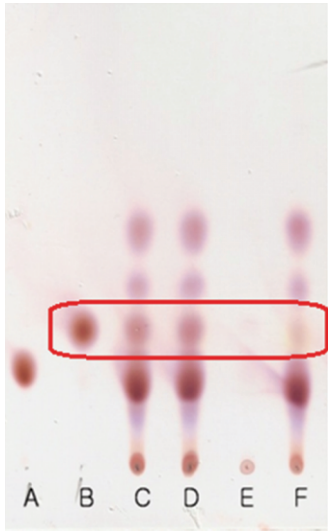

Figure 5: TLC analysis of GABA in the culture medium and cytoplasmic fraction of *L. plantarum* LG42 cells. A: spot of standard MSG; B: spot of standard GABA; C–E: spots of cell culture medium (C), cell-free supernatant (D), and cytoplasmic fraction of cells (E) cultured in MRS broth with 1% MSG; F: spot of cell culture medium cultured without added MSG.

and since the cytoplasmic fraction used in this study contains a negligible amount of GABA (Figure 5), it is not clear what compounds of GLAB worked as the main principles of the anti-obesity effects. Therefore, GLAB having GABA producing ability can be used as a useful material not only for the production of fermented foods such as sik-hae with an enhanced level of GABA, but also for the investigation of unknown compounds except GABA for the anti-obesity effects. Further studies are required to identify the active compounds of GLAB that have specific effects on obesity.

In conclusion, the inhibitory effects of GLAB on 3T3-L1 adipocyte, as indicated by a decrease in intracellular triglyceride content and GPDH activity have been elucidated. It appears to be mediated through downregulating the expression of adipogenic transcription factor, PPARγ and C/EBPα, and adipocyte-specific gene such as aP2, leptin, GPDH, and CD36. These results indicate that GLAB may play a role in the control of adipogenesis and might have further implication for *in vivo* antiobesity effect.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Construction, Expression, and Characterization of Thymosin Alpha 1 Tandem Repeats in *Escherichia coli*

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Thymosin alpha 1 (Tα1), which is composed of 28 amino acids, has been commercialized worldwide for its immune-modulatory and antitumor effects. Tα1 can stimulate T cell proliferation and differentiation from bone marrow stem cells, augment cell-mediated immune responses, and regulate homeostasis of immune system. In this study, we developed a novel strategy to produce Tα1 concatemer (Tα1③) in *Escherichia coli* and compared its activity with chemically synthesized Tα1. Results showed that Tα1③ can more effectively stimulate T cell proliferation and significantly upregulate IL-2 receptor expression. We concluded that the expression system for Tα1 concatemer was constructed successfully, which could serve as an efficient tool for the production of large quantities of the active protein.

1. Introduction

The tandem repeats of proteins and peptides are studied widely and formidable progress has been made in this field. It was reported that tandem amino acid repeats have many functions of stabilizing proteins [1], maintaining conformation [2], elevating activity, and increasing half-life of proteins in blood or tissues. Frasch and colleagues suggested that tandem repeats present in *Trypanosoma cruzi* transsialidase stabilized the catalytic activity. In addition, repeats present on *T. cruzi* shed proteins increased trans-sialidase half-life in blood from 7 to almost 35 h [3]. Some proteins that contain tandemly repeated sequences play important roles in cell membrane skeleton system [4, 5].

Thymosin alpha1 (Tα1) is a heat-stable, acidic polypeptide composed of 28 amino acid residues blocked at the N-terminus by an acetyl group [6, 7]. It is an immune modifier that has been shown to trigger lymphocytes maturation, augment T cell function, induce T-cell differentiation, and promote reconstitution of immune defects. All these findings showed that Tα1 could be a useful restorative therapeutic agent in the treatment of immunodeficiency diseases and immunosuppressed conditions [8–10].

In this study, Tα1③ which was composed of three repeated copies of Tα1 was fuse-expressed with thioredoxin (trx) in *E. coli* TOP10 strain and purified by heat treatment and Q-Sepharose Fast Flow ion-exchange chromatography. Then, Tα1③ was released by treatment with 0.5 M Cyanogen bromide (CNBr) and purified by SP-Sepharose Fast Flow chromatography. In our strategy, trx acts as a chaperon to help Tα1③ folding and CNBr treatment removed any exogenous amino acid (such as Met at the N-terminus for translation start) from Tα1③ molecule. So we can get the "natural" Tα1③. Finally, the biological activity of Tα1③ on T lymphocyte proliferation and IL-2R expression was assessed.

2. Materials and Methods

2.1. Materials. Restriction enzymes, Taq DNA polymerase, and T4 DNA ligase were purchased from TaKaRa. Expression vector pThioHisA and *E. coli* strain TOP10 (F-mcrAΔ(mrr-hsd RMS-mcrBC) φ80 lacZΔM15 ΔlacX74 recA1 araΔ139Δ (ara-leu)7697 galU galK rpsL (Strr)endA1 nupG) were from Invitrogen. DNA fragments were synthesized in BIOASIA. Synthetic Tα1 (ZADAXIN) was from Sciclone Pharmaceuticals, USA. The anti-Tα1 antibody (ab55635) was purchased

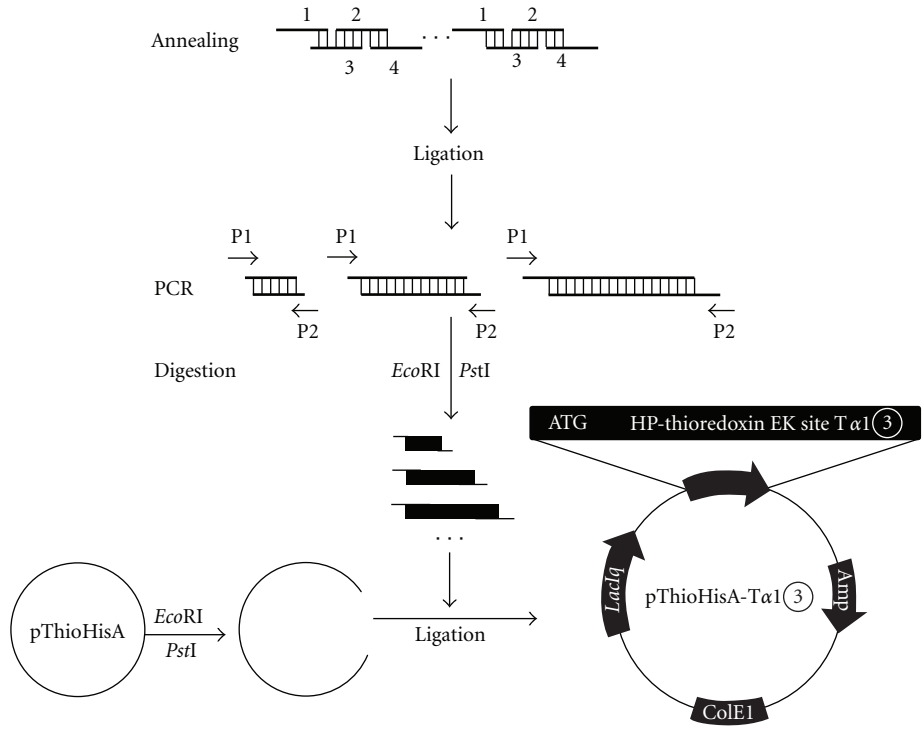

Figure 1: Schematic diagram shows the strategy for constructing Tα1 concatemers. The Arabic numerals 1 to 4 are sequences 1 to 4 split from Tα1 described previously for concatemers assembly. P1 and P2 are forward and reverse primers for PCR.

from Abcam and FITC-anti-IL-2Rβ (18344D 554452) was from BD Pharmingen.

2.2. Tα1③ Gene Amplification. Tα1③ gene was cloned by gene synthesis and PCR (Figure 1). The forward primer (with an introduced *Eco*R I site) was p1: 5′-GGAATTCATGTCTGATGCAGCCGTGGACACCAGCAGCG-3′ and the reverse primer (with an introduced *Pst* I site) was p2: 5′-GCACTGCAGTCAGTTCTGGGCCTCCTCCACCACCT-3′. The template for PCR was annealing products of 4 synthesized fragments listed in Table 1. PCR product was cloned into pGEM-3Zf plasmid to obtain vector pGEM-Tα1③ for identification.

2.3. Construction of Expression Vector pThioHisA-Tα1③. The vector pGEM-Tα1③ was digested with *Eco*R I and *Pst* I and cloned into expression vector pThioHisA digested with the same enzymes. The candidate plasmid pThioHisA-Tα1③ was then confirmed by restriction enzymes digestion and DNA sequencing.

2.4. Expression of the Fusion Protein. The plasmid pThioHisA-Tα1③ was transformed into *E. coli* TOP10 strain. A single colony was inoculated into 10 mL Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/mL) and grown at 200 rpm and 37°C overnight. Then it was inoculated into 300 mL fresh LB medium in a 500 mL shake flask and cultured until the OD600 reached 0.5. Trx-Tα1③ expression was induced by 1 mM IPTG (final concentration) for 4 h. Large scale fed-batch culture was performed in a 5-L fermentor as previously described [11].

Table 1: Nucleotide sequence of DNA fragments split from Tα1 for concatemers assembly.

Number	nucleotide sequence
1	GACACCAGCAGCGAGATCACCACCAAGGACC GGAAGGAGAAGAAGGAGGTGGTG
2	GAGGAGGCCGAGAACAGCGACGCCGCCGTG
3	CTTGGTGGTGATCTCGCTGCTGGTGTCCACGG CGGCGTCGCT
4	GTTCTCGGCCTCCTCCACCACCTCCTTCTTCTCC TTCAGGTC

2.5. Purification of Tα1③. Cell pellet was suspended in 20 mM Tris/HCl buffer (pH 8.0) in proportion of 200 g/L and disrupted by sonication. Then, the lysate was incubated at 80°C for 10 min (shaken once every 2-3 min) and cooled quickly. Samples were centrifuged at 12 000 g for 20 min and the supernatant was loaded onto a Q-Sepharose Fast Flow chromatography column and eluted with linear NaCl gradient. The purified Trx-Tα1③ was then cleaved by CNBr (0.5 M) in 70% formic acid for 24 h. The cleavage reaction was stopped by addition of ten times amount of H_2O [12] and Tα1③ was purified by SP-Sepharose Fast Flow chromatography. Purified Tα1③ was dialyzed against PBS for later use.

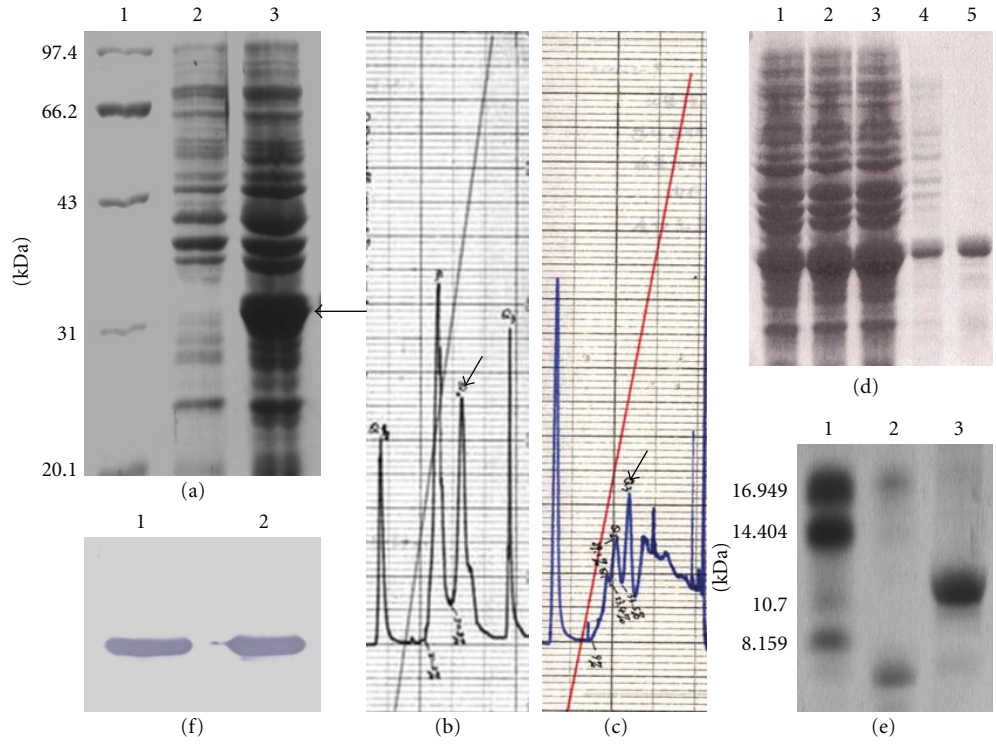

Figure 2: Expression, purification, and identification of Tα1③. (a) Expression of trx-Tα1③ in TOP10. Lane 1: protein marker; lane 2: total bacterial proteins of pThioHisA-Tα1③/TOP10 without induction; lane 3: total bacterial protein with IPTG induction. (b) Chromatogram of Q-Sepharose Fast Flow chromatography for purification of trx-Tα1③. The arrow indicated trx-Tα1③. (c) Chromatogram of SP-Sepharose Fast Flow chromatography for purification of Tα1③. The arrow indicated Tα1③. (d) SDS-PAGE analysis of trx-Tα1③ purification. Lane 1–3: total proteins of pThioHisA-Tα1③/TOP10 after IPTG induction (1 h, 3 h, 5 h); lane 4: supernatant of lysate heated at 80°C for 10 min; lane 5: purified trx-Tα1③. (e) Tricine-SDS-PAGE analysis of Tα1③ purification. Lane 1: standard peptide marker; lane 2: cleavage products without Tα1③; lane 3: purified Tα1③. (f) Western-blot analysis of trx-Tα1③. Lane 1: total bacterial proteins after IPTG induction; lane 2: purified trx-Tα1③.

2.6. Western-Blot Analysis. Proteins were transferred to nitrocellulose membranes (0.22 μm, Invitrogen) after SDS-PAGE using a Bio-Rad Semi-Dry electrophoretic cell. Western blot analyses were carried out using a Tα1 specific antibody and followed by a phosphatase-conjugated goat anti-mouse IgG (Boster, China). Western Blue Stabilized Substrate (Promega) for alkaline phosphatase was used for visualization.

2.7. Biological Activity Assay. The proliferation response of splenocytes was determined by MTT assay. Spleens from C57BL6 mice were dispersed through nylon mesh to generate a single-cell suspension. Then lymphocytes were separated by EZ-Sep 1× Lymphocyte Separation Medium (DKW33-R0100, Dakewe Biotech Company, China) and suspended at 4×10^6/mL in RPMI 1640 media. For proliferation assay, cells were seeded in 96-well plates (4×10^5/well) and cultured in the presence of 2.5 μg/mL concanavalin A (ConA) at 37°C in 5% CO_2 in humid air. Six h later, 90 μL of Tα1③ diluted with RPMI 1640 media was added to all but the control wells. The synthetic Tα1 and media were used as positive and negative controls. After 66 h incubation, 20 μL of MTT (0.5 mg/mL) solution was added and the plates were centrifuged (2000 rpm, 25°C, 10 min) 4 h later. Supernatants were discarded, and 100 μL of DMSO was added. After incubated at room temperature for 10 min, the solubilized reduced MTT was measured at 570 nm using a Bio-Rad plate reader and the optical densities were used for calculate growth rate with the formula

$$\text{Growth rate } (\%) = \frac{\text{OD sample}}{\text{OD control}} \times 100\%. \quad (1)$$

To evaluate the effect of Tα1③ on the expression level of IL-2R on T lymphocytes, cells were isolated as before and cultured in the presence of ConA and Tα1③. The synthetic Tα1 and a recombinant Tα1 monomer prepared in our lab were used as positive controls. Cells were collected and stained 48 h later according to standard protocol. In brief, 5×10^5 cells were washed with PBS and stained in "FACS buffer" (PBS with 0.1% sodium azide, 2% FBS, and 1 μM EDTA) with FITC-anti-mIL-2Rβ for 10 min at room temperature. After washing, cells were fixed for 30 minutes on ice with 4% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

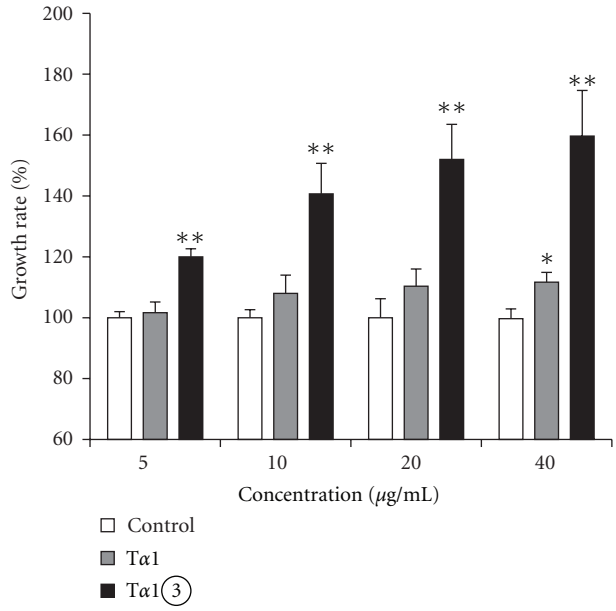

FIGURE 3: Tα1③ stimulated proliferation of mouse spleen lymphocytes. T lymphocytes from B6 mice spleen were treated with ConA (control) or ConA plus Tα1③ or synthetic Tα1. Cell proliferation was determined by the MTT viability assay. The assays were repeated in triplicate. ($^{*}P < 0.05$ compared with control group. $^{**}P < 0.05$ compared with Tα1 group.)

3. Results and Discussion

3.1. Tα1③ Gene Cloning. Although synthetic Tα1 has been successfully applied in clinical trials for immunodeficiency diseases therapy, the high costs is still a hard nut to crack. Fortunately, molecular biology techniques allowed us to produce recombinant Tα1 in *E. coli*. Considering that Tα1 is too small to be directly expressed in *E. coli*, it was usually assembled as concatemers. But some exogenous amino acid residues such as His6 tag or methionine (Met) introduced by the initiation codon AUG usually affects the effect of concatemers [13].

In order to produce the real "natural" concatemers of Tα1, we put forward a new strategy as showed in Figure 1. By this strategy, we obtained a series of Tα1 concatemers in which Tα1③ that was assembled by three repeated copies of Tα1 gene owned highest proportion. After cloning into pGEM-3Zf vector, the gene was proven by enzyme digestion and DNA sequencing. The sequence of Tα1③ gene was consistent with our design as follows: 5′-atgagcgacgccgccgtggacaccagc-agcgagatcaccaccaaggaccggaaggagaagaaggaggtggtggaggag-gccgagaacagcgacgccgccgtggacaccagcagcgagatcaccaccaaggac-cggaaggagaagaaggaggtggtggaggaggccgagaacagcgacgccgcc-gtggacaccagcagcgagatcaccaccaaggaccggaaggagaagaaggaggtg-gtggaggaggccgagaactga-3′.

3.2. Expression of Recombinant Fusion Protein. Both SDS-PAGE (Figure 2(a)) and Western blot (Figure 2(f)) analyses of the induced supernatant from pThioHisA-Tα1③/TOP10 showed that a new 31 kDa protein which can be specifically recognized by Tα1 antibody was produced. It suggested that trx-Tα1③ was successfully expressed. Trx was used as a chaperon to guarantee the correct folding of Tα1③ and trx-Tα1③ was expressed as a soluble fusion protein.

3.3. Purification of Tα1③. Both trx and Tα1 are heat-stable proteins, so trx-Tα1③ was easily purified by one-step Q-Sepharose Fast Flow chromatography after the lysate of recombinant bacterial cells was heated at 80°C for 10 min (Figures 2(b) and 2(d)). Then, the purified trx-Tα1③ was cleaved by CNBr, and Tα1③ was purified by SP-Sepharose Fast Flow chromatography (Figures 2(c) and 2(e)). 2L-Tricine-SDS-PAGE [14] and HPLC analyses were used to identify the purity of Tα1③. CNBr treatment was utilized here to remove the redundant Met from the N-terminus of Tα1③ to obtain the real "natural" molecule.

3.4. Biological Activity of Tα1③. We expected that the tandem repeats could obtain stronger activity through elongating the half-life of Tα1 and simulating polymerization of monomer molecules and thereafter triggering the polymerization and activation of receptors which was usually used by molecules to gain function.

To examine the effect of Tα1③ on stimulating the proliferation of splenic lymphocytes, we compared the proliferation ratio of mice lymphocytes treated with synthetic Tα1 ZADAXIN and Tα1③. MTT assay results showed that 40 μg/mL synthetic Tα1 could induce significant proliferation of lymphocytes compared to the control ($P < 0.05$), whereas 5 μg/mL Tα1③ could induce significant proliferation ($P < 0.05$). Furthermore, the effect of 10 μg/mL Tα1③ was stronger than that of 40 μg/mL synthetic Tα1 (Figure 3).

In addition, the upregulation of IL-2R on lymphocytes by ZADAXIN purified recombinant Tα1 monomer and Tα1③ was compared. Results showed that when costimulated with

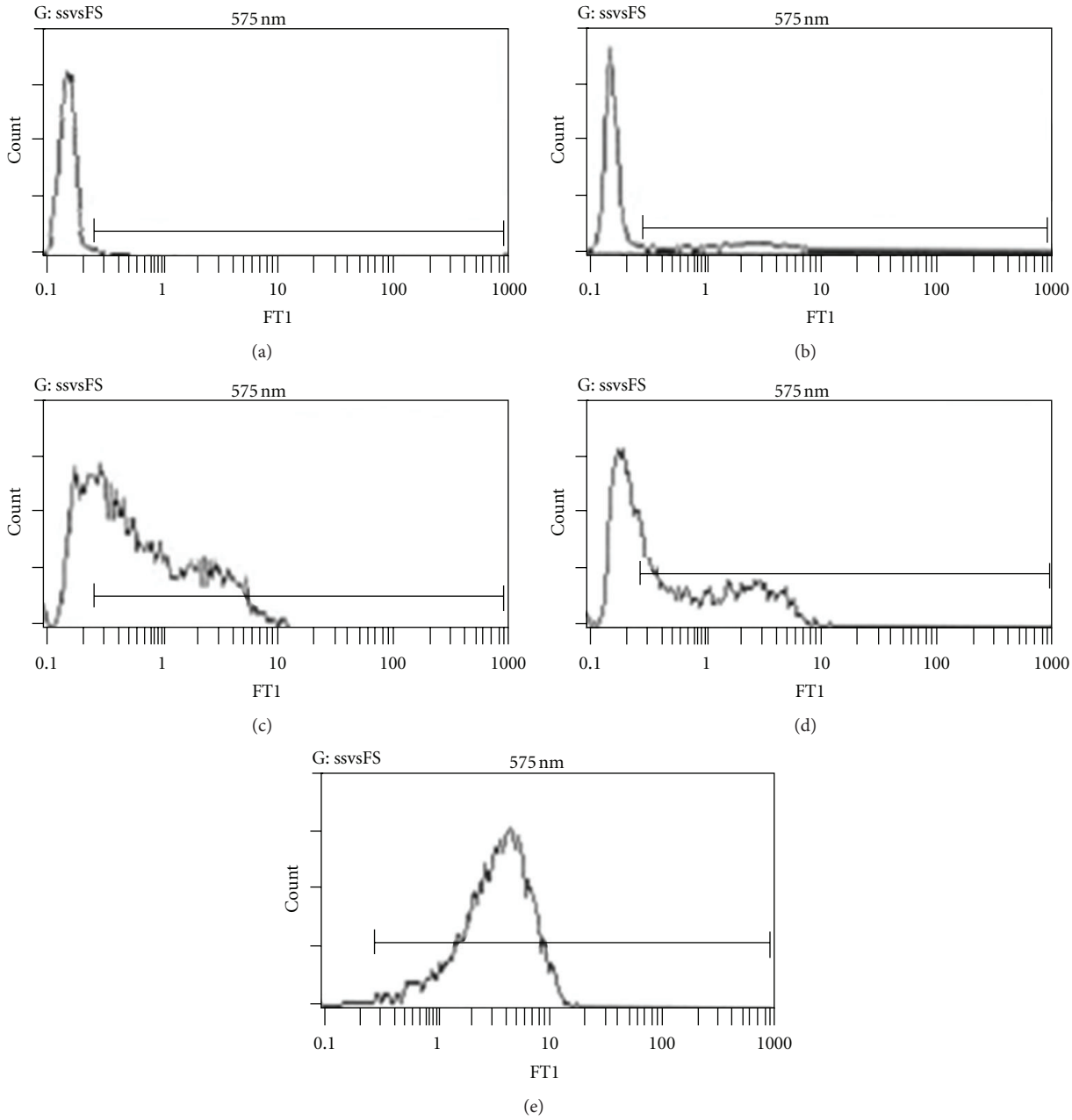

Figure 4: Effect on upregulation of IL-2R expression level on T cell surface of Tα1③. T lymphocytes from B6 mice spleen were cultured in the presence of ConA (b) or ConA plus standard synthesized Tα1 (c), recombinant Tα1 (d), and recombinant Tα1③ (e), respectively. (a) The unstrained control. Data are representative of three experiments.

ConA, IL-2R expression level on T cell was upregulated by all these three molecules and Tα1③ obtained strongest effect (Figure 4).

4. Conclusions

Trx-Tα1③ was expressed in *E. coli* as a soluble form and the real "natural" Tα1③ was conveniently purified by heat treatment and ion-exchange chromatography. As expected, the bioactivity of Tα1③ was stronger than that of synthetic Tα1. Lower dose (5 μg/mL) of Tα1③ apparently stimulated the proliferation of T lymphocytes compared with that of ZADAXIN (40 μg/mL). In addition, Tα1③ significantly upregulated IL-2R on T cell, which is very important for T cell activation and proliferation *in vivo*. The detailed mechanism for stronger effect of Tα1③ and the pharmacokinetics of different tandem repeats are still under investigation.

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Research Article

Immunobiologic and Antiinflammatory Properties of a Bark Extract from *Ampelozizyphus amazonicus* Ducke

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Ampelozizyphus amazonicus is used in the treatment and prevention of malaria. The effect of an aqueous extract from this plant (SART) on the immune response was investigated by measuring immunoglobulin production induced by immunization with the antigen TNP-Ficoll in *Plasmodium chabaudi*-infected mice. SART treatment increased antigen-specific IgM and IgG levels in TNP-Ficoll-immunized mice. The B cell response during malarial infection was also modified by SART. There was an increase in total serum IgM and IgG and a decrease in the percentage of splenic plasma cells (CD138+ cells) in *P. chabaudi*-infected, SART-treated animals. SART (1, 3 or 10 mg/kg, p.o.) and the reference drug dexamethasone (5 mg/kg) were also tested in carrageenan-induced leukocyte migration to the subcutaneous air pouch (SAP). All SART doses significantly reduced leukocyte migration into the SAP. The protein concentration resulting from extravasation into the peritoneum was also significantly reduced. Our data indicate that SART possesses immunomodulatory properties, inducing an *in vivo* modification of the B lymphocyte response and anti-inflammatory properties, which are partly due to a reduction in cell migration and are most likely due to an inhibition of the production of inflammatory mediators. Preliminary HPLC-ESI-MS/MS analysis of SART shows a complex saponin profile with deprotonated molecule $[M\text{-}H]^-$ ions in the range of m/z 800–1000.

1. Introduction

Ampelozizyphus amazonicus Ducke (Rhamnaceae) is an Amazonian medicinal plant popularly known as "saracura-mirá" that is found in the Amazon forest territories of Brazil, Venezuela, Colombia, Peru, and Ecuador. In Brazil, it is restricted to the states of Amazonas, Pará, and Roraima and grows mainly in the "terra firme" forests near waterfalls or "igarapés" [1]. An aqueous drink can be prepared from the bark and roots of *A. amazonicus*. This drink has a very bitter taste and forms abundant foam when shaken, similar to beer, which also gives rise to its other popular names: "cervejinha," "cervejeira," "cerveja-do-mato," "cerveja-de-índio," and "cerveja-de-preto" [2–5]. These properties can

be explained by the high saponin content in the species. Indeed, Silva et al. [6] showed the presence of approximately 48% saponins in an aqueous extract from the roots of the plant. So far, only three saponins with a dammarane-type aglycone have been described in the literature [7, 8], as well as the presence of free triterpenes such as melaleucic acid, 3β,27α-dihydroxylup-20(29)-en-28β-oic acid, betulinic acid, betulin, lupeol, and phytosteroids [7–9].

Several ethnobotanical studies have shown that *A. amazonicus* is useful in the treatment and prevention of malaria [2, 3, 10–12]. Previous investigations of the antimalarial properties of an extract from this plant have shown that it does not have a direct action on *Plasmodium* blood stage forms, either *in vivo* or in red blood cell cultures [13]. However, this natural product could be effective in controlling infection induced by sporozoite forms [13]. Based on the findings that *A. amazonicus* does not have a direct effect upon blood stage forms of the protozoan, it might be possible to suggest that the control of the infection induced by this plant could be obtained by an overall augmentation of the immunological response. In fact, ethnopharmacological studies indicate both stimulatory and energetic properties for *A. amazonicus* [5, 12]. In an ethnopharmacological study conducted by our group in the "quilombola" communities of Oriximiná, State of Para, Brazil, the plant is used in the treatment of liver disorders, gastritis, inflammation of the prostate, and "inflammation of a woman" and as a fortifying tonic and aphrodisiac, among other uses [12, 14]. In the Rio Negro valley and Jaú National Park in the state of Amazonas, *A. amazonicus* is used against rheumatism and other types of pain and for the general treatment of inflammation [5, 11].

Based on the reported properties of this plant and its uses in folk medicine, our group suggests that *A. amazonicus* could act as an adaptogen by enhancing immune system function and could alleviate the inflammatory disorders caused by malaria. In the present work, we aimed to investigate the toxicity of *A. amazonicus* and its effects on the immune response, as well as its anti-inflammatory properties.

2. Material and Methods

2.1. Plant Material and Preparation of Extracts. *Ampelozizyphus amazonicus* Ducke was collected in August 2008, in the Brazilian Amazon region of Oriximiná (Para state), at the Pancada community (S 01°04′09.4″ and W 056°02′40.9″). Plants were collected as a part of a bioprospecting project in "quilombola" communities from Oriximiná that received authorization by the Brazilian Directing Council of Genetic Heritage (Conselho de Gestão do Patrimônio Genético), through Resolution number 213 (6.12.2007), published in the Federal Official Gazette of Brazil on December 27, 2007. Plants were identified by Mr. Jose Ramos (parataxonomist). A voucher specimen was deposited at the Instituto Nacional de Pesquisas da Amazônia INPA herbarium (Manaus, AM, Brazil) under the registration INPA 224161.

Dried and ground bark (250 g) of *A. amazonicus* was used for the preparation of the extracts. The bark was submitted to extraction with boiling water (5% w/v) for 15 minutes and filtered. A second extraction was performed with boiling water (2.5%, w/v, 30 minutes). The extracts were mixed and infused into a spray-dryer nozzle unit of Büchi Mini Spray Dryer B-290 (Büchi Laboratorius-Technik AG, Switzerland). The conditions of the spray-drying process were as follows: nozzle diameter 0.3 mm, aspirator pressure 80%, flow rate 6 mL/min, inlet temperature 190 ± 3°C, and outlet temperature 88 ± 1.5°C. The atomized powder was collected by a cyclone and is designated as SART throughout the text.

2.2. Estimation of Daily Dose for Animal Assays. The daily oral dose of *A. amazonicus* (SART) used was determined based on its traditional use. A drink was prepared according to the "quilombola" traditional method, as described by Oliveira et al. [14]. Briefly one tablespoon of ground bark (8.3980 g) was added to 200 mL of cold water and "shaken" seven times. The foam produced after each shaken was discarded. The extract was filtered, and a total solid yield of 0.21% (w/v) was obtained [15]. Considering that the prophylactic use of the drink (to prevent from malaria) is 0.630 g/day or 300 mL/day of extract at 0.21% (w/v) of total solid yield, the daily oral dose for an adult weighing 70 kg would be 9 mg/Kg. Therefore, all biological assays were standardized to a 10 mg/kg oral dose of SART as obtained by spray dryer.

2.3. HPLC-DAD Profile of SART. HPLC analysis of SART (aqueous atomized extract of *A. amazonicus*, 2 mg/mL in acetonitrile : water, 95 : 5) was performed on a VWR-Hitachi Elite LaChrom system consisting of an L-2130 quaternary pump with online degasser, a Rheodyne injector (20 μL loop), L-2455 diode array detector (DAD), equipped with a Zic-Hilic PEEK column (SeQuant-Merck, 250 × 4.6 mm i.d., particle size 5 μm, 200 A), coupled with a security guard column (4 × 3.00 mm i.d. C-18 cartridge). The elution solvents used were ammonium acetate 10 mM, pH 5.8 corrected with acetic acid (solvent A) and acetonitrile (solvent B), and the flow rate was 0.5 mL/min, detection: DAD (210 nm). The elution was as follows: 0–10 min, isocratic 4% (solvent A): 96% (solvent B), 10.1–65 min, linear gradient where solvent B decreased from 96% to 50%. Betulinic acid from SIGMA (0.125 mg/mL in acetonitrile : water, 95 : 5) was used as standard.

2.4. HPLC-DAD-ESI-MS Preliminary Profile of SART. An HPLC system, including a Surveyor Autosampler, a Surveyor LC pump, and an LCQ Advantage ion-trap mass spectrometer (Thermo Finningan, San Jose, CA, USA), equipped with an electrospray ion source, was used for the preliminary analysis of SART. Chromatographic conditions were the same reported above. MS data were acquired in negative ionization mode, and the spectra were recorded in full scan (m/z 250–1500) and tandem mass (collision energy of 40% of the instrument maximum) scanning modes. Instrumental parameters were optimized using a purified saponin mixture isolated from SART by countercurrent chromatography (data not shown). Saponin mixture was dissolved (14 $\mu g \cdot mL^{-1}$) in ACN : H_2O (1 : 1, v/v) and infused in the ESI source at the flow rate of 5 $\mu g \cdot min^{-1}$ by a syringe pump.

2.5. Animals. Experiments were performed with male Swiss mice (20–25 g) obtained from Instituto Vital Brazil or BALB/c mice with ages ranging from 6 to 8 weeks supplied by the Central Animal Facility from Instituto de Microbiologia from Federal University of Rio de Janeiro. Animals were maintained in a room with controlled temperature (22 ± 2°C) with a 12 h light/dark cycle and free access to food and water. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA). They were approved by the Ethical Committee for Animal Research (Biomedical Science Institute/UFRJ) and received the numbers ICBDFBC-015 and IMIPPG-012.

2.6. Drugs and Extract Administration. Carrageenan and dexamethasone were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved in phosphate buffer saline (PBS) just before use. SART was dissolved in sterile water and administered by oral gavage at doses of 1, 3, and 10 mg/kg in a final volume of 0.1 mL. Dexamethasone (5 mg/kg) was used as reference drug and was administered via oral gavage as well. The negative control group was composed of mice given the vehicle (sterile water); significant effects due to the water per se were not observed throughout the study. The dose of dexamethasone was chosen based on previous experiments done by our group.

2.7. Determination of SART Toxicity In Vitro. Cells from the cell line A20 (3×10^4) were plated in flat bottom 96-well tissue culture plates. Cultures were performed in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco-BRL, Grand Island, NY, USA), L-glutamine (2 mM), 2-ME (50 μM), and gentamicin (50 μg/mL). Reagents were obtained from SIGMA Chemical Co. (St Louis, MO, USA). Some cultures received different doses of SART. An incubation of 48 hours was performed at 37°C in CO_2 Incubator (Forma Scientific, Marietta, OH, USA). Afterwards, cell viability was determined by the XTT-based viability assay [16]. Plates were read in a microplate reader (model 550 reader, Bio-Rad, Boston, MA, USA) to determine A_{450}.

2.8. Anti-TNP-Ficoll Immunoglobulin Production. TNP-Ficoll was obtained from Bioresearch Technologie, Inc. (Novato, CA, USA). TNP-Ficoll was diluted in PBS and administered by the intravenous injection of 50 μg of the polysaccharide per animal. Oral treatment with SART was begun ten days before immunization, and each animal was treated daily with a 10 mg/kg dose of SART. The animals were bled through the tail vein weekly, and serum samples were prepared. Serum anti-TNP-Ficoll IgM and IgG titers were measured by ELISA as previously described [17]. Briefly, flexible polyvinyl chloride round bottom microtest plates (BD Falcon, BD Labware, Franklin Lakes, NJ, USA) were covered with a solution of TNP-Ficoll diluted in 1 M pH 8.3 Tris buffer. Serially diluted samples were added to the plates and were performed in duplicate. Secondary alkaline phosphatase-labeled anti-IgM or IgG antibodies (SIGMA Chemical Co. St Louis, MO, USA) were used to reveal antigen-specific antibody binding. Plates were read using a microplate reader to determine A_{405} (model 550 plate reader; Bio-Rad, Boston, MA, USA). The titer of each sample was the serum dilution giving an A_{405} reading midway on the linear portion of the titration curve.

2.9. Malarial Infection and Immunoglobulin Measurement. *P. chabaudi* infection was established by the intraperitoneal injection of 10^6 *P. chabaudi*-infected red blood cells. Serum samples were obtained as described above and were used for measuring total IgM and IgG circulating concentrations. This measurement was performed by a sandwich ELISA as previously described [18]. Unlabeled goat anti-IgM or anti-IgG antibodies (SIGMA Chemical Co., St Louis, MO, USA) were used as primary antibodies. Standard curves were set up using purified mouse IgM or IgG from ICN Biomedicals (Irvine, CA, USA). Secondary alkaline phosphatase-labeled antibodies were also obtained from SIGMA Chemical Co. The reaction was quantified by the addition of *p*-nitrophenolphosphate (SIGMA Chemical Co.), and absorbance was measured at 405 nm with a model 550 plate reader (BioRad, Boston, MA, USA).

2.10. Determination of Antibody-Producing Cells by Flow Cytometry. A splenic cell suspension was obtained from individual animals and cells were diluted in RPMI 1640 medium supplemented with 5% FCS. Samples of 10^6 cells were labeled with FITC-conjugated anti-B220 and PE-conjugated anti-CD138 antibodies (BD Biosciences, San Jose, CA, USA). The samples were washed, fixed with PBS/1% formaldehyde, and were analyzed by flow cytometry using a FACSCalibur, and data were analyzed using the CellQuest software (both are from BD Biosciences, San Jose, CA, USA).

2.11. Formalin Test. This procedure was similar to the method described by Gomes et al. [19]. Mice received an injection of 20 μL of formalin (2.5% v/v) into the dorsal surface of the left hind paw. The time that the animal spent licking the injected paw was immediately recorded. The nociceptive and inflammatory responses consists of the following two phases: the first phase lasts until 5 min after the formalin injection (first-phase, neurogenic pain response), and the second phase occurs 15–30 min after the formalin injection (second-phase, inflammatory pain response). The animals were pretreated with oral doses of SART or vehicle for 60 min before the administration of formalin.

2.12. Subcutaneous Air Pouch (SAP). The method was adapted from Raymundo et al. [20]. Air pouches were produced by subcutaneous injection of 10 mL of sterile air into the intrascapular area of the backs of the mice. After 3 days, another 10 mL of air were injected to maintain the pouches. The animals received an injection of 0.5 mL of a sterile carrageenan suspension (1%) into the SAP three days after the last injection. The animals were pretreated with oral doses SART (1, 3, and 10 mg/kg) 60 min before carrageenan injection. The animals were killed 24 h after carrageenan injection and the cavity was washed with 2 mL of sterile PBS.

The liquid in the SAP was collected and quantified. Total cell count was done in an automatic cell counter (pocH-100iV Diff, Sysmex). The exudates were centrifuged at 170 ×g for 10 min at 4°C, and the supernatants were collected and stored at −20°C until dosages. The protein content of each supernatant was determined using the BCA method (BCA Protein Assay Kit, Pierce).

2.13. Reduction of Spontaneous Activity. Spontaneous activity was evaluated as described by Barros et al. [21] and Figueiredo et al. [22]. Mice received 10 mg/kg of SART by oral administration and were immediately placed individually in an observation chamber with a floor that was divided into 50 squares (5 cm × 5 cm). The total number of squares that the mouse walked through during a 5 min period was counted. The effect of SART on locomotor performance was also tested on the rotarod apparatus as described previously [22, 23]. All animals were trained on the rotarod (3.7 cm in diameter, 8 rpm) twenty-four hours before the experiments until they could remain in the apparatus for 60 s without falling. On the day of the experiment, mice were treated with SART (at 10 mg/kg, p.o.) and tested on the rotarod from 0.5 to 3.5 h after SART administration. The number of falls from the apparatus was recorded with a stopwatch for up to 240 s.

2.14. Acute Toxicity. The parameters were determined as described by Lorke [24]. A single oral dose of SART (10 mg/kg) was administered to a group of ten mice (five males and five females). We observed various behaviour parameters including convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, and food and water intake over a period of 15 days. After this period, the animals were euthanized by cervical dislocation, their stomachs removed, an incision along the greater curvature was made, and the number of ulcers (single or multiple erosion, ulcer, or perforation) was counted. Hyperaemia was also evaluated.

2.15. Statistical Analysis. For *in vivo* assays all experimental groups consisted of 6–10 mice. For the *in vitro* assays, all studies were done in triplicate and each protocol was repeated at least 4 times. The results are presented as mean ± S.D. Statistical significance between groups was performed by applying analyses of variance (ANOVA) followed by Bonferroni's test or Student's t-test for independent samples. P values less than 0.05 ($P < 0.05$) were considered significant.

3. Results and Discussion

3.1. HPLC-DAD Profile of SART. In a previous study from our group [14], we demonstrated that an aqueous extract from *A. amazonicus* prepared by the traditional quilombola method contained free betulinic acid. After acid hydrolysis followed by gas chromatography-mass spectrometry analysis, the presence of a dammarane-type triterpene skeleton was also characterized in that extract [14]. In the present study, a different methodology was used for extraction, which used hot water instead of tap water, to raise the extraction yields. Because we have suggested that some of the use indications of this plant, for instance, as a tonic and for treating malaria, might be related to its properties as an adaptogen and to the immunostimulatory properties of the saponins and betulinic acid in the drink, we investigated the presence of betulinic acid in SART. The HPLC profile of SART was obtained in a Zic-Hilic column in which two different zones can be found: the first refers to triterpenic and nonpolar compounds, while the second refers to saponins and polar compounds (Figure 1(a)), thus showing the efficiency of this zwitterionic column in the separation of different compounds and polarities. The presence of betulinic acid in SART (t_R 8,387 min) was confirmed by standard injection.

3.2. HPLC-DAD-ESI-MS/MS Profile of SART. To more closely investigate the chemical composition of SART, we performed a preliminary HPLC-ESI-MS/MS analysis of the extract. The resulting (−)-MS chromatogram (Figure 1(b)) and its expansion (30–41 min) show a complex saponin profile with deprotonated molecule $[M\text{-}H]^-$ ions in the range of *m/z* 800–1000. Analysis of the product ion mass spectra of $[M\text{-}H]^-$ ions implied that the compounds belong to the class of triterpenoidal saponins.

The peaks at 35.0 and 32.0 min ($[M\text{-}H]^-$ at *m/z* 897 and 983) showed a fragmentation pattern that suggested the presence of triglycosides of jujubogenin. The peak at 35.0 min yielded product ions at *m/z* 765, 735, 603, and 471 corresponding to [M-H-pentose]$^-$, [M-H-hexose]$^-$, [M-H-pentose-hexose]$^-$ and [M-H-2 × pentose-hexose]$^-$. The diagnostic signals of the peaks at 32.0 min, observed in the MS^n spectra at *m/z* 941 ($[M\text{-}H\text{-}42]^-$), 923 ($[M\text{-}H\text{-}CH_3COOH]^-$), 821 ([M-H-hexose]$^-$), 633 ([M-H-42-hexose-deoxyhexose]$^-$), and 471 ([M-H-42-2 × hexose-deoxyhexose]$^-$), indicated the structure of an acetylated jujubogenin triglycoside.

The peak at 36.0 min ($[M\text{-}H]^-$ at *m/z* 959) showed in the MS^n spectra a product ion at *m/z* 817 ($[M\text{-}H\text{-}C_8H_{14}O_2]^-$) due to a McLafferty rearrangement that is characteristic of the keto-dammarane-type triterpene saponins [25]. Other product ions at *m/z* 655 ($[M\text{-}H\text{-}C_8H_{14}O_2$-hexose$]^-$), 509 ($[M\text{-}H\text{-}C_8H_{14}O_2$-hexose-deoxyhexose$]^-$), and 347 ($[M\text{-}H\text{-}C_8H_{14}O_2$-2 × hexose-deoxyhexose$]^-$) confirmed the presence of a trisaccharide chain. The peak at 35.7 min ($[M\text{-}H]^-$ at *m/z* 973) displayed MS^n spectra very similar to those of the previous peak: product ions at *m/z* 817 ($[M\text{-}H\text{-}C_9H_{16}O_2]^-$), 655 ($[M\text{-}H\text{-}C_9H_{16}O_2$-hexose$]^-$), 509 ($[M\text{-}H\text{-}C_9H_{16}O_2$-hexose-deoxyhexose$]^-$) and 347 ($[M\text{-}H\text{-}C_9H_{16}O_2$-2 × hexose-deoxyhexose$]^-$). The difference in the *m/z* values of the $[M\text{-}H]^-$ ions of these two compounds was equivalent to 14 (corresponding to a CH_2 unit), indicating that the structural difference is in the alkyl chain of the keto-dammarane skeleton. An elucidation of the structures of these compounds is currently in progress.

3.3. Immunobiologic Properties of A. amazonicus. To evaluate the hypothesis that *A. amazonicus* could act as an adaptogen by enhancing immune system function, its immunobiological properties were examined in different *in vitro* and *in vivo* models. The daily oral dose of *A. amazonicus* used in all biological experiments was estimated based on its traditional use

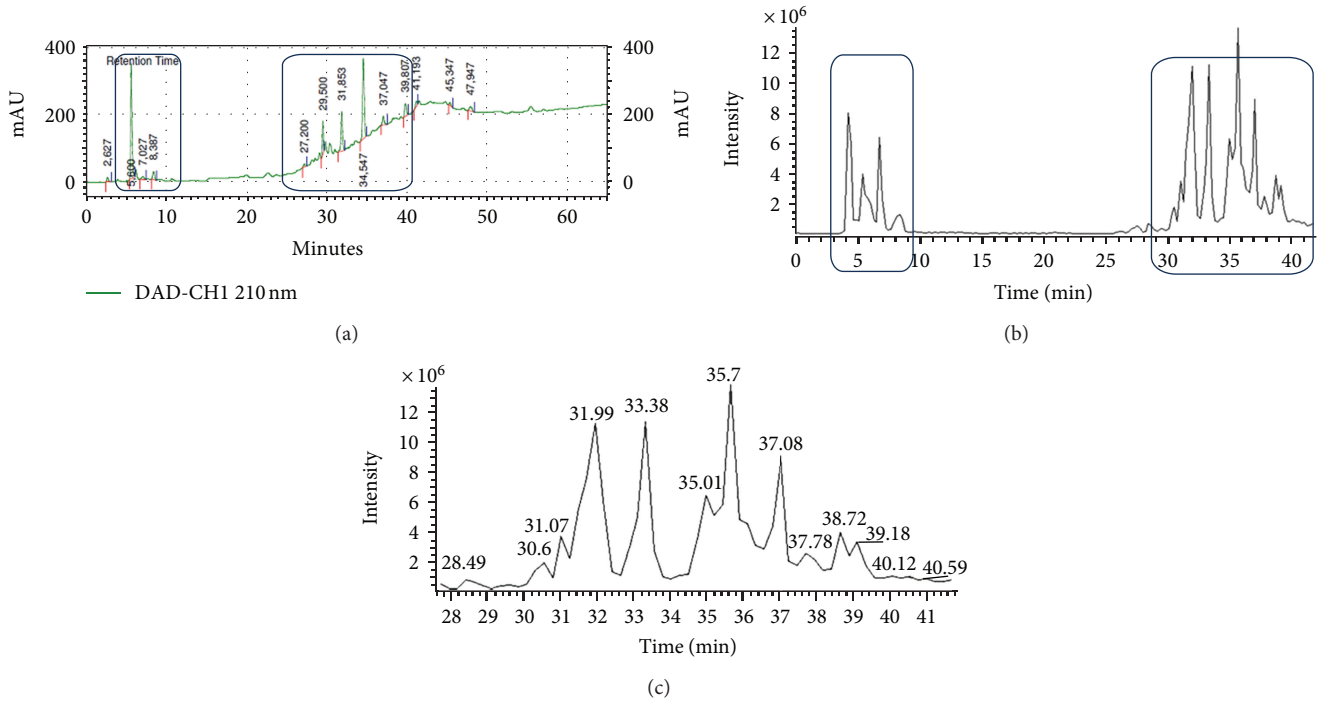

FIGURE 1: (a) HPLC-DAD chromatogram of SART (detection 210 nm) and betulinic acid at Rt = 8.387 min. (b) HPLC-DAD-ESI-MS (negative mode) chromatogram of SART. Triterpene zone between 5 and 10 min and saponin zone between 30 and 40 min (c) HPLC-DAD-ESI-MS chromatogram of SART: expansion of the saponin zone between 30 and 44 min.

by the preparation of a drink according to the "quilombola" traditional method.

3.3.1. Determination of SART Toxicity and Its Effect on B Cells In Vitro. We initially measured the toxic effects of different doses of SART on A20 lymphoma cell cultures. SART did not modify cell viability in cultures incubated with this extract for either 48 h (Figure 2) or 72 h (data not shown). We also tested whether SART would modify the B lymphocyte response in culture. We employed in this investigation cultures of purified B cells stimulated with LPS and measured the stimulation of two parameters associated with B cell activation by LPS: immunoglobulin secretion and the membrane expression of the protein CD86. We did not observe any significant modification of either immunoglobulin M (IgM) secretion or CD86 cell surface expression in B cell cultures incubated with different doses of SART (data not shown).

3.3.2. Anti-TNP-Ficoll Immunoglobulin Production. We next investigated the effect of SART on the *in vivo* immune response. We used immunization with the T-independent type 2 antigen TNP-Ficoll, which induces antigen-specific immunoglobulin production, as a model. This class of antigen stimulates B cells in the absence of T cell help and induces the production of both IgM and IgG [26]. Mice were immunized by intravenous injection of TNP-Ficoll, and antigen-specific immunoglobulin was measured weekly after immunization. As observed in Figure 3, oral treatment with SART induced an increase in both IgM (Figure 3(a)) and IgG (Figure 3(b)) anti-TNP-Ficoll antibody titers.

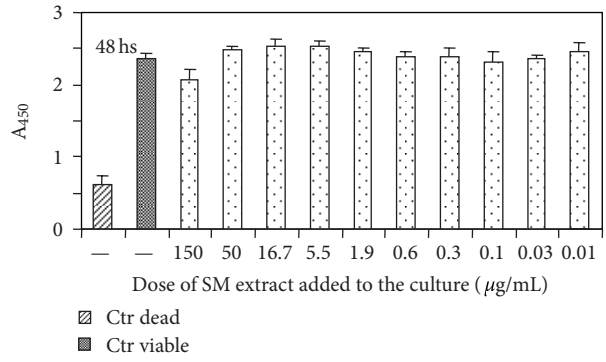

FIGURE 2: Evaluation of the toxicity of SART *in vitro* in cultured cells. The cell line A20 (3×10^4 cells/well) was cultured in the presence of the indicated concentrations of SART. Control cultures were established without the addition of SART. Cultures were incubated for 48 h at 37°C in a 7% CO_2 atmosphere. Cell viability was measured by the XTT reduction measurement. Ctr viable indicates untreated cultures incubated with medium alone. Ctr dead indicates control cultures to which 1 μL of Triton X-100 was added prior to the addition of XTT. Mean values ± SD of absorbance (A_{450}) are shown.

The finding that SART treatment could amplify the response of murine B cells to a T-independent type 2 antigen suggests that components of the plant extract might have immunopotentiating effects. Saponins are the major components of the extract obtained from SART. Several studies have shown that these compounds contain adjuvant properties [27], including the ability to enhance immunoglobulin

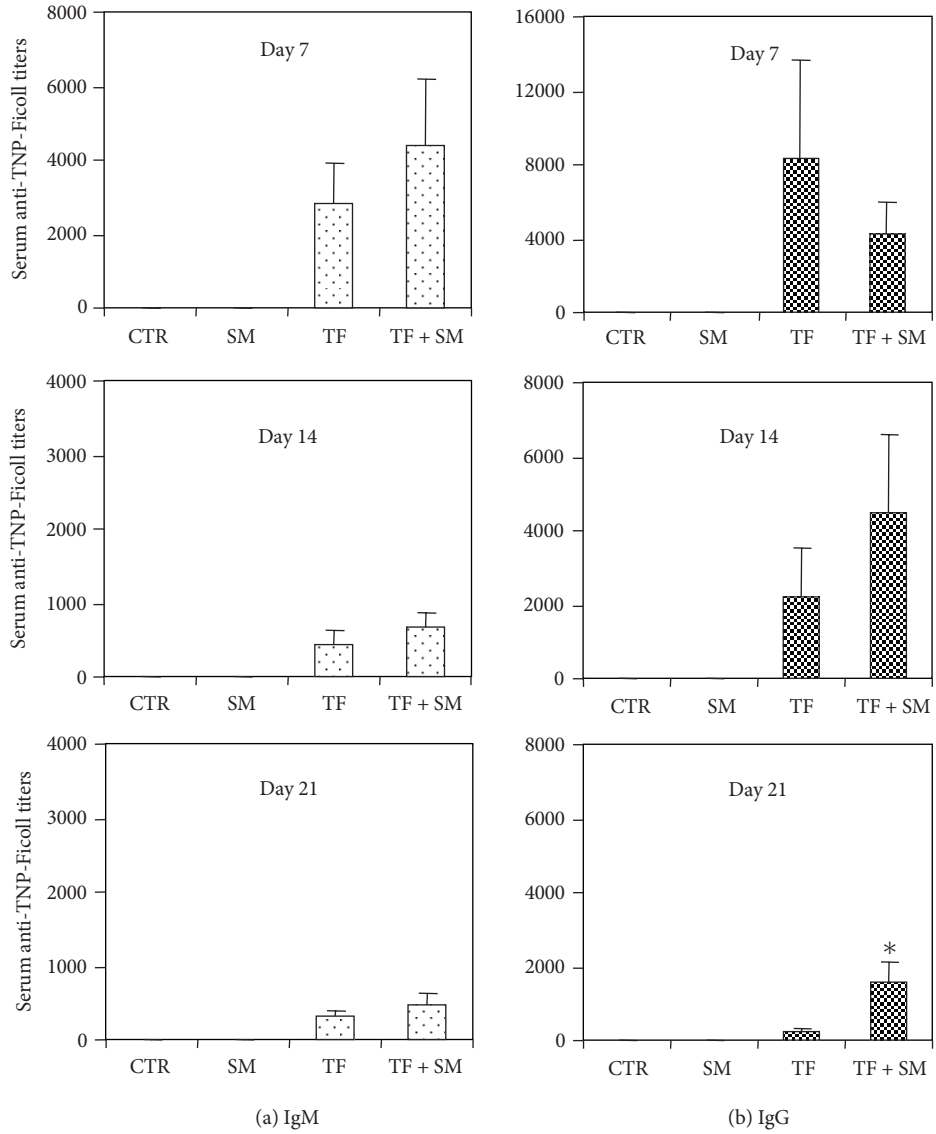

Figure 3: Antigen-specific immunoglobulin production in animals treated with SART. The four indicated groups were established: CTR (PBS-treated mice), SART (mice treated daily with an oral dose of SART), TF (mice immunized with TNP-Ficoll), and TF + SART (mice immunized with TNP-Ficoll and treated daily with SART). Oral treatment with SART was onset 10 days before immunization. Serum was obtained from individual mice at days 7, 14, and 21 after immunization, as indicated in the figure. Anti-TNP-Ficoll serum titers were determined by ELISA. (a) show data on IgM antibodies and (b) the one on IgG levels. Data indicate mean values ± SD of 6 animals per group. Data are representative of two independent experiments. $^{*}P \leq 0,05$ when compared to the TF group.

production [28] and also to stimulate the release of immune mediators and the proliferation of immune cells *in vitro* [29].

3.3.3. Malaria Infection: Measurement of Serum Immunoglobulin Levels and Numbers of Splenic Antibody-Producing Cells. As described above, SART treatment is popularly used to control malaria infection. The role of B cells in this infection was previously investigated, and it was shown that mice deficient in mature B cells had reduced primary acute infections but were unable to eliminate parasites, indicating that B cells are required for final parasite clearance [30]. It was also described that *Plasmodium*-infected animals showed an increase in circulating antibody levels when compared to normal animals [31]. Therefore, we next investigated the effects of SART treatment on the course of the B cell response in *P. chabaudi*-infected mice. We examined whether SART treatment could induce a modification in immunoglobulin production during infection. Treatment with SART induced an increase in the levels of circulating total IgM and IgG in *P. chabaudi*-infected mice (Figure 4). We next investigated the levels of antibody-secreting cells in the spleen of *P. chabaudi*-infected SART-treated mice. To perform this study, we measured the percentage of cells expressing the surface protein CD138, a marker for antibody-secreting cells [32]. Despite the increase in circulating immunoglobulin levels, there was a decrease in the number of antibody-producing

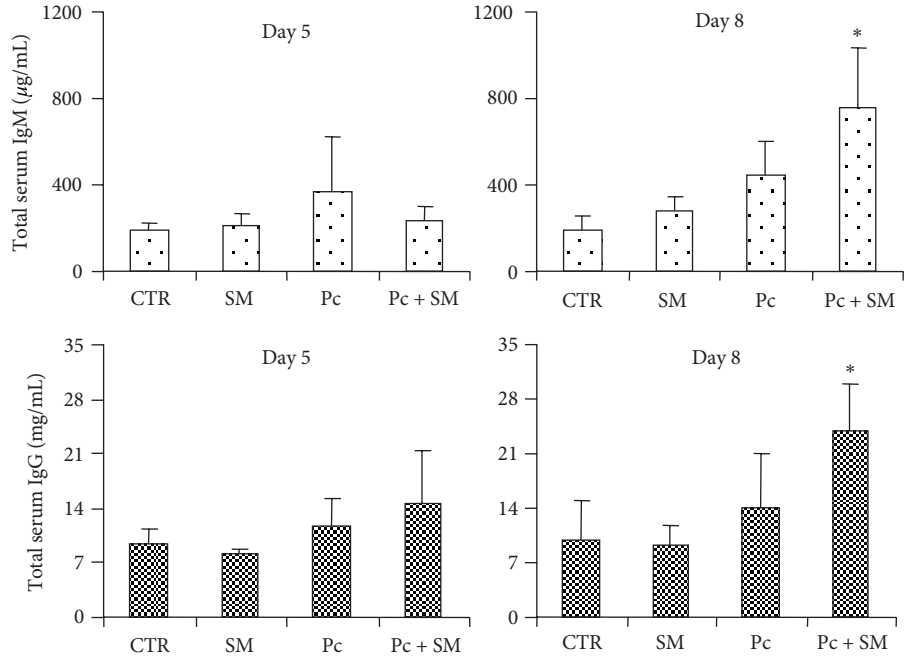

Figure 4: Serum total IgM and IgG levels in mice infected with *P. chabaudi* and treated with SART. The four indicated groups were set up: CTR (vehicle-treated animals), SART (mice treated with a daily oral dose of SART), Pc (animals injected with 10^6 *P. chabaudi*-infected red blood cells), and Pc + SART (*P. chabaudi*-infected SART-treated mice). Groups of 8 animals were used and two independent experiments were performed. Serum was obtained at the indicated periods after infection and total IgM (top panels) and IgG (bottom panels) were determined by ELISA. Data indicate mean immunoglobulin levels ± SD. $^*P \leq 0,05$ when compared to the Pc group.

plasma cells in the spleens of *P. chabaudi*-infected, SART-treated mice 8 days after infection when compared to infected untreated animals (Figure 5).

Taken together, our data indicate that SART modulates malaria infection because SART increases immunoglobulin production during infection and regulates the appearance of antibody-secreting cells. The relevance of this phenomenon on resistance and the progression of infection need to be further analyzed.

Due to the effect of SART on immunoglobulin production, the saponins may have antimalarial properties that are potentiated through other mechanisms. Dammarane-type saponins have shown potent hepatoprotective effects in different models [33–35], and these effects could justify the use of this plant for the treatment of liver disorders and malaria infection.

3.4. Evaluation of the Anti-Inflammatory Activity of SART. Based on the ethnopharmacological information that *A. amazonicus* is indicated to treat "inflammation of the prostate," "inflammation of a woman," and liver disorders that could be related to inflammatory processes, we decided to evaluate the possible anti-inflammatory activity of this plant in different models.

3.4.1. Formalin Test. The injection of formalin (2.5%) induces a biphasic licking response in the injected paw of mice. The first phase occurs until 5 min after injection, and the second phase occurs between 15 and 30 min after formalin injection. Pretreatment of mice with 1, 3, or 10 mg/kg SART did not reduce the time that the animal spent licking the formalin-injected paw in the first or second phase (data not shown).

3.4.2. Subcutaneous Air Pouch (SAP). To evaluate the possible anti-inflammatory activity of SART, the carrageenan-induced inflammation model in the SAP was used. This model involves synovial inflammation caused by carrageenan injection into the air pouch that forms in the back of mice. This procedure induces the proliferation of cells that stratify on the surface. The injection of carrageenan drastically increased the exudate volume into the pouch up to twice the level of mice that received PBS in the SAP. In the absence of SART, the numbers of leukocytes in the carrageenan-injected air pouch exudates were markedly increased up to 57-fold higher than control levels Figure 6(a); ($1.25 \pm 1.1 \times 10^6$ cells/mL versus $72 \pm 12.4 \times 10^6$ cells/mL). These numbers were markedly reduced by approximately 56%, 24%, 77.6%, and 47% by pretreatment with increasing doses of SART. The pretreatment of mice with SART significantly reduced the volume of exudate recovered from the air pouches. Carrageenan treatment also caused a 14-fold increase in the exudate protein concentration, and the pretreatment of mice with SART significantly inhibited carrageenan-induced protein leakage with all doses tested Figure 6(b). Using an *in vivo* air pouch inflammation model, we showed that carrageenan increased both the exudate volume and the exudate protein concentration, which indicates vascular leakage of the serum contents. Carrageenan is also an important chemotactic agent because it induces

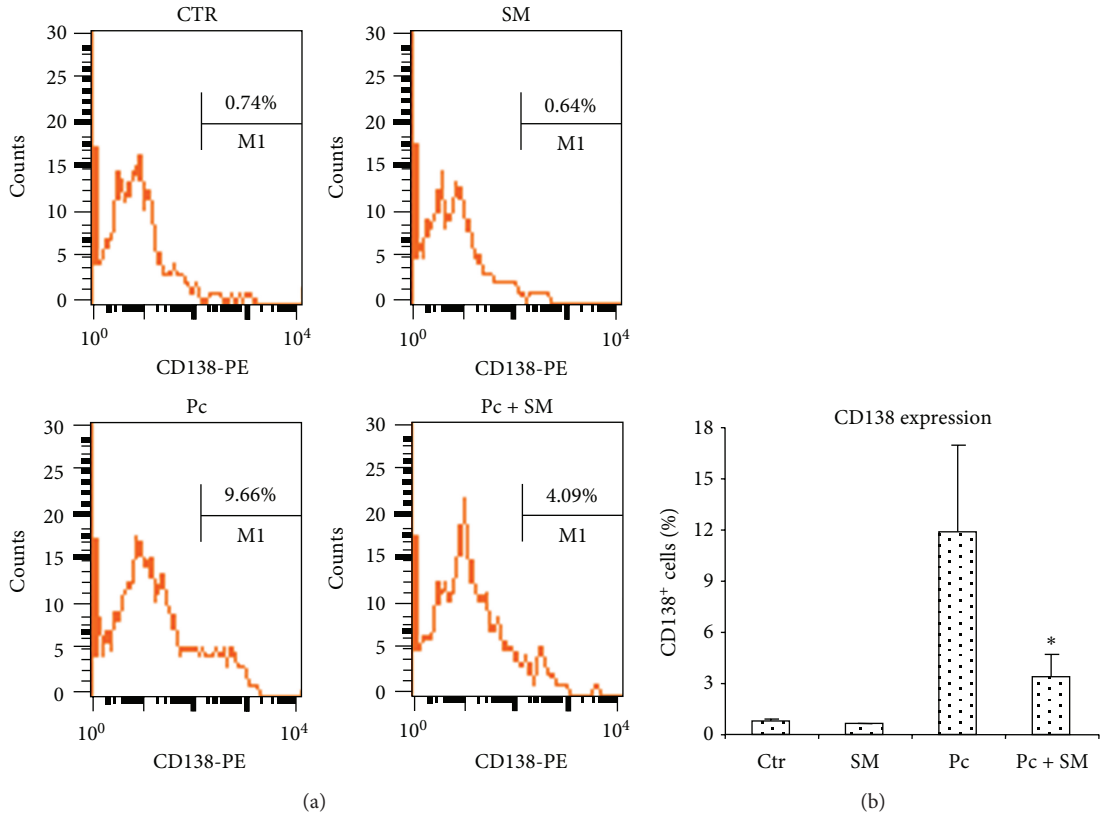

FIGURE 5: Levels of antibody-producing ($CD138^+$) cells in the spleen of *P. chabaudi*-infected animals treated with SART. Four different groups: CTR, SART, Pc, and Pc + SART were established as detailed in Figure 4. Splenic cells were obtained from individual animals and 10^6 cells were labeled with anti-B220-FITC and anti-CD138-PE antibodies. Samples were run in a FACScalibur Flow Cytometer. Data shown in (a) indicate a typical profile of $CD138^+$ cells in the $B220^+$ lymphoid population in each experimental group. The percentage of $CD138^+$ cells is included in each graphic. Data in (b) indicate the mean value ± SD of the percentage of $CD138^+$ cells from each group. Six animals were used per group and data are representative of two independent experiments. $^*P \leq 0,05$ when compared to the Pc group.

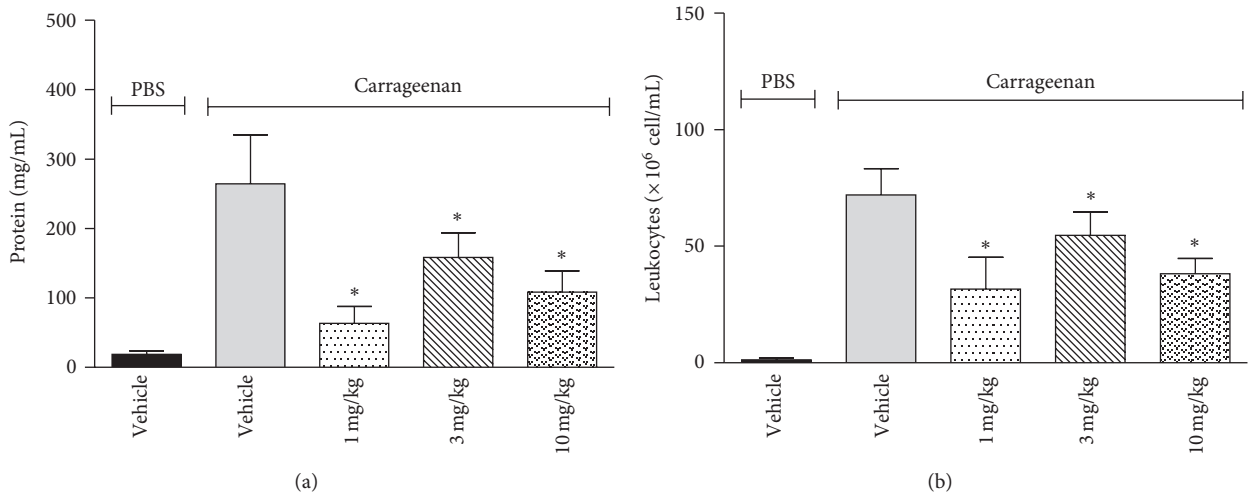

FIGURE 6: Effect of SART on leukocyte migration (a) and protein extravasation (b) into the subcutaneous air pouch. Animals were pretreated with oral administration of different doses of SART 1 h prior to carrageenan (1%) injection into the SAP. The results are presented as mean ± S.D. ($n = 6-10$) of number of leukocyte ($\times 10^6$ cell/mL) (a) or protein (mg/mL) (b). Statistical significance was calculated by ANOVA followed by Bonferroni's test. $^*P < 0.05$ when comparing latex-treated mice to the vehicle-treated group; $^\#P < 0.05$ when comparing vehicle-treated mice to the PBS-treated group.

the migration of inflammatory cells such as neutrophils and macrophages [36]. In this study, SART reduced leukocyte migration, exudate volume, and protein extravasation, even at a dose of 1 mg/kg, suggesting a suppression of vascular leakage. These results show an anti-inflammatory effect for SART, which was demonstrated by reduced cell migration and liquid and protein extravasation. Acute inflammation, such as carrageenan-induced cell migration, involves the synthesis or release of mediators at the injured site. These mediators include prostaglandins, particularly those of the E series, histamine, bradykinins, leukotrienes, and serotonin, all of which also cause pain and fever [37]. Inhibiting these mediators to prevent them from reaching the injured site and exerting their pharmacological effects will normally ameliorate inflammation and other symptoms. This study has shown that SART possesses the ability to significantly affect cell migration and protein leakage induced by carrageenan, suggesting a possible modulation of some events related to inflammatory process. Because the carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents that act on mediators of acute inflammation [38, 39], these results are an indication that SART may be effective in treating acute inflammatory disorders. Moreover, this effect could explain the reduction in the inflammatory processes that accompany malaria, which have already been discussed for other plant species used in the treatment of this disease [40]. Triterpene saponins have demonstrated anti-inflammatory properties through the inhibition of iNOS and proinflammatory cytokine expression [41]. These compounds exhibited significant xanthine oxidase inhibitory activity, preventing the overaccumulation and deposition of uric acid in the joints, which leads to painful inflammation [42].

More assays, such as assays that determine the dosing of some inflammatory mediators, are underway to reveal the exact mechanisms of the anti-inflammatory action of SART.

3.5. Acute Toxicity and Reduction of Spontaneous Activity. The oral administration of a 10 mg/kg dose of SART did not induce any toxic effects. No behavioral alterations, lesions, or gastric bleeding was observed. Additionally, no signs of intoxication, including convulsion, death, or gastric ulcer, were observed, even after 5 days of a single dose (data not shown). It is noteworthy that the oral administration of SART did not show any gastric disorders because the irritation of the gastric mucosa is an important side effect that is expected with the oral use of saponin-containing herbs such as Horse Chestnut, *Ruscus* [43].

4. Conclusions

HPLC-ESI-MS/MS analysis of SART showed a complex dammarane-type saponin profile with molecular weights in the range of *m/z* 800–1000. The biological results shown here indicate that SART possesses immunomodulatory properties that induce an *in vivo* modification of the B lymphocyte response, as well as anti-inflammatory properties, partly due to a reduction in cell migration and most likely due to an inhibition of inflammatory mediator production. Therefore, the results presented in this work support our hypothesis that *A. amazonicus* could act as an adaptogen by enhancing immune system function and could mitigate the inflammatory disorders caused by malaria. Future studies on the chemical structures of the saponins isolated from SART and their roles in the B cell response, resistance to malaria infection, and the anti-inflammatory activity of the extract will be important.

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Research Article

Triterpenes and the Antimycobacterial Activity of *Duroia macrophylla* Huber (Rubiaceae)

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Duroia macrophylla popularly known as "cabeça-de-urubú," "apuruí," or "puruí-grande-da-mata" occurs in the Amazon Forest. Its leaves and branches were collected twice and extracted with dichloromethane and methanol. All extracts were subjected to phytochemical investigation and terpenes and flavonoids were found in all dichloromethane and methanol extracts, respectively. Methanol extracts from both branches (1st collection) and leaves (2nd collection) presented hydrolyzed tannins, yet alkaloids were only detected in the dichloromethane and methanol extracts from branches at the 2nd collection. Phenol compounds were found in both dichloromethane extracts' collections. The action of every extract was assayed against *Mycobacterium tuberculosis* (RMPr, H37Rv, and INHr strains), showing that the dichloromethane extract from leaves (1st collection) has the major biological activity, with a MIC of 6.25 μg/mL for the INHr strain, 25.0 μg/mL for the RMPr strain, and $\leq$6.25 μg/mL for the H37Rv strain. The chromatographic fractioning of the dichloromethane extract from leaves (1st collection) yielded the isolation of two triterpenes: oleanolic and ursolic acids, which were identified by NMR analysis and reported for the first time in the *Duroia* genus.

1. Introduction

Rubiaceae is the largest family in the Magnoliopsida class, encompassing around 550 genera and 9,000 species being used in several ethnomedicinal practices [1]. The family is characterized by the production of several classes of secondary metabolites with a great pharmacological potential, mainly alkaloids, terpenes, quinovic acid glycosides, flavonoids, and coumarins with antibacterial properties [2]. Rubiaceae plants' secondary metabolites have been investigated scientifically for antimicrobial activities and a large number of plant products have shown to inhibit the growth of pathogenic microorganisms [3–6]. A literature review article pertaining to Rubiaceae species reveals that 48 out of 611 genera showed a wide range of antibacterial [3] and antimycobacterial activities [4].

The *Duroia* genus, belonging to Gardenieae tribe and Ixoroideae subfamily, holds about 30 species but few studies have been carried out on this genus. *D. hirsuta*, which is used as folk healing medicine, is one of these species which has undergone investigation and showed antimycobacterial activity against *Mycobacterium phlei* [7] and antiviral activity against *Herpes simplex viruses* (HSV) on *in vitro* studies [8]. One flavone, one lactone iridoid, one flavonol [9] and one tetracyclic iridoid [10] were isolated from its root petroleum ether and $CHCl_3$ extract. There is still a large number of species with no chemical or biological study.

Duroia macrophylla Huber, popularly known as "cabeça-de-urubú," "apuruí," or "puruí-grande-da-mata," occurs in the Amazon Forest [11]. To the best of our knowledge, no chemical or biological investigations other than ours [12, 13] have been carried out on this species as yet. Hence this

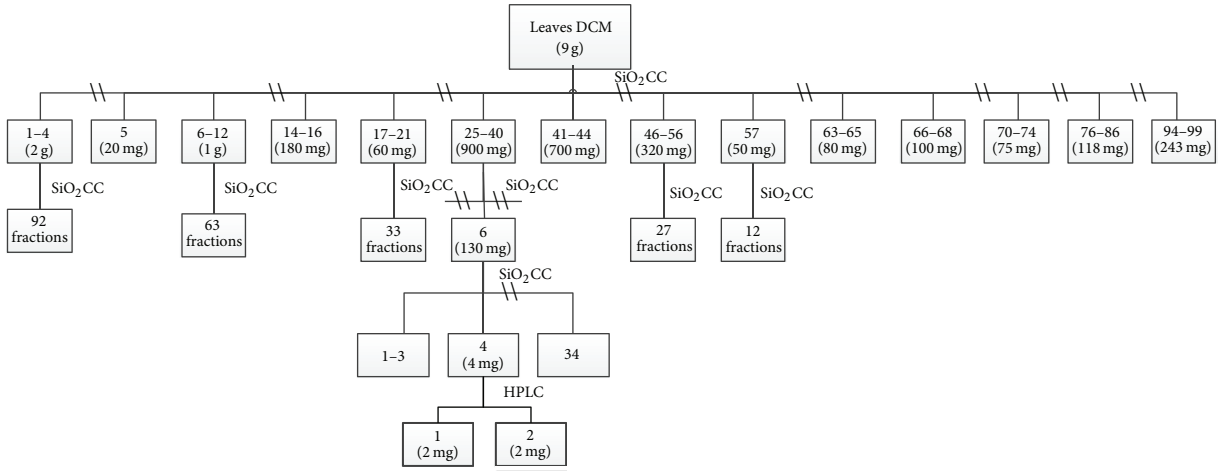

Figure 1: Schematic representation of *D. macrophylla* dichloromethane extract of leaves (1st collection) fractionation. DCM: dichloromethane; CC: chromatographic column; HPLC: high-performance liquid chromatography.

work aims to evaluate the antimycobacterial activity of their extracts and isolate and identify the substances present in *D. macrophylla* active extracts.

2. Material and Methods

2.1. Plant Material. Two collections were performed, the first one, at the "A. Ducke" Forest Reserve, 26 km from Manaus, was carried out on December 5th, 2008, and a voucher specimen (222383) was deposited at the Herbarium of the Botanical Research Coordination of the National Research Institute of Amazonia (INPA). The second one at the Natural Heritage Private Reserve, locally known as "Cachoeira da Onça," in "Presidente Figueiredo" County, AM, was carried out on May 18th, 2011. A voucher specimen (222501) was deposited at the same Herbarium.

2.2. Extracts Preparation. Plant material (leaves and branches) was dried in an oven at 50°C and powdered. Each plant part was extracted three times separately, first with dichloromethane (DCM) followed by methanol (MeOH), in a sonic bath for 20 minutes. After filtration, DCM and MeOH extracts were concentrated under reduced pressure.

2.3. Phytochemical Investigation. The extracts were analyzed following the methodology described by Matos [14], as well as by thin layer chromatography (TLC) (Merck) using silica with UV_{254} fluorescence detector on aluminum support, eluted with appropriated systems, and revealed with UV light (λ = 254 and 365 nm), sulfuric *p*-anisaldehyde, $Ce(SO_4)_2$, 2,2-diphenyl-1-picrylhydrazyl (DPPH), $FeCl_3$, and Dragendorff. Chemical extract profile was identified through ^{1}H-NMR on an Anasazi NMR spectrometer operating at 1.4 Tesla (60 MHz).

2.4. Extract Fractionation. Dichloromethane extract from leaves (1st collection) (9 g) was submitted to a chromatographic column (CC) fractionation on silica gel (332 g), eluted with gradients of hexane/ethyl acetate and ethyl acetate/methanol, yielding 99 fractions with 50 mL each. Fraction 25–40 (900 mg) was fractionated on silica gel (90 g) CC and eluted with hexane/ethyl acetate and ethyl acetate/methanol gradients, yielding 42 fractions with 20 mL each. Fraction 25–40.6 (130 mg) was fractionated on silica gel (17 g) CC and eluted with hexane/ethyl acetate and ethyl acetate/methanol gradients, yielding 19 fractions with 10 mL each. Afterwards, fraction 25–40.6.4 (4 mg) was submitted to high-performance liquid chromatography (HPLC) analysis. HPLC was performed with a Shimadzu system SCL-10AVP, processing software programs CLASS VP, dual LC-6AD pumps, 10AF autosampler, SPD-M20 diode-array detector, cyanopropyl column (250 × 10 mm, 4 μm particle sizes, Luna-Phenomenex), with acetonitrile : water (90 : 10) as the isocratic mobile phase, at a 5 mL/min flow rate. The injection volume was 35 μL. The resolved peaks retention times were 11.5 and 12 min, identified by NMR analyses as oleanolic acid (**1**) and ursolic acid (**2**), respectively (Figure 1).

All fractions were evaluated by TLC analysis, eluted with appropriated systems, and revealed under UV light exposure (λ = 254 and 365 nm), sulfuric *p*-anisaldehyde, $Ce(SO_4)_2$, 2,2-diphenyl-1-picrylhydrazyl (DPPH), $FeCl_3$, and Dragendorff reagents.

2.5. NMR Data. The NMR data was obtained at 295 K on a Bruker AVANCE 400 NMR spectrometer operating at 9.4 Tesla, observing ^{1}H and ^{13}C at 400 and 100 MHz, respectively. The spectrometer was equipped with a 5 mm multinuclear direct detection probe, with z-gradient. One-bond (HSQC) and long-range (HMBC) ^{1}H-^{13}C NMR correlation experiments were optimized for coupling constants $^1J_{H,C}$ and $^{LR}J_{H,C}$ of 140 and 8 Hz, respectively. All NMR chemical shifts were expressed in ppm related to TMS signal at 0.00 ppm as internal reference, and samples were dissolved in $CDCl_3$.

2.6. Antimycobacterial Activity. Resazurin microtiter assay (REMA) was used to evaluate the antimycobacterial activity. This method uses resazurin as an oxidoreduction indicator to evaluate the bacterial viability and contamination, in addition to analyzing the antimicrobial activity [15].

2.6.1. Microorganisms. The extracts activity was evaluated against three *Mycobacterium tuberculosis* strains: one pansensible (H37Rv, ATCC 27294), one isoniazid monoresistant (INH, ATCC 35822) with mutation in *kat*G, codon S315T (AGC-ACC), and other rifampicin monoresistant (RMP, ATCC 35338), with mutation in *rpo*B, codon H526T (CAC-TAC). The strains were cultivated in Ogawa-Kudoh's medium at 37°C for nearly 14 days. The bacterial suspension of each strain was prepared in a sterile tube with glass pearls and turbidity adjusted with distillated water, according to Mc Farland scale's number 1 tube, which corresponds to approximately 3×10^8 CFU/mL. Then, Middlebrook 7H9 medium was added to bacterial suspension in 1 : 20 ratio [15].

2.6.2. Assay Procedure. Samples were first evaluated in 96-well microplates at a 200 μg/mL concentration against the three *M. tuberculosis* strains. The assay started adding 75 μL of Middlebrook 7H9 medium enriched with 10% of OADC (oleic acid, albumin, dextrose, and catalase) for *M. tuberculosis*, 75 μL of each extract, and 75 μL of inoculum. Then, 200 μL of sterile water were added to each peripheral well, so as to avoid medium liquid evaporation when heater-incubated. Finally, the plate was incubated at 37°C for seven days.

2.6.3. Minimum Inhibitory Concentration Determination. The extracts presenting an antimycobacterial activity at the 200 μg/mL concentration screening were chosen to evaluate their minimum inhibitory concentration (MIC) value [16]. This value was determined by adding 100 μL of medium, 100 μL of extract (starting at 200 μg/mL concentration on the first well and performing a 1 : 2 microdilution), and 100 μL of bacterial inoculum in each well. Also, 200 μL of sterile water was added to each peripheral well, in order to avoid medium liquid evaporation when heater-incubated. Then, the plate was incubated at 37°C for seven days.

2.6.4. Bacterial Viability. Following the incubation period, 30 μL of resazurin (0.02%) was added in each well and incubated for two days at 37°C. The biological activity was based on the color change, from blue to pink when an oxidoreduction reaction of the reagent occurs due to bacterial growth [15].

3. Results and Discussion

3.1. Phytochemical Investigation. All *Duroia macrophylla* extracts were analyzed in order to evaluate the chemical profile [17, 18]. Dichloromethane extracts from branches and leaves in both collections showed to be rich in terpenes. Regarding methanolic extracts, only those from branches (1st collection) and leaves (2nd collection) showed the presence of both terpenes and hydrolyzed tannins. Alkaloids were only detected on dichloromethane and methanolic extracts from branches (2nd collection). All methanolic extracts showed the presence of flavonoids. All dichloromethane extracts from branches showed the presence of phenolic compounds. ^{1}H-NMR spectra analysis showed the presence of aromatic substances in the methanolic extract of branches (1st collection), with several signals between 6.50 and 7.80 ppm.

3.2. Substances Isolation and Identification. Following crude extracts chemical and biological analysis, the dichloromethane extract from leaves (1st collection) was chosen to be fractionated, since it showed to be the most active against the three *Mycobacterium tuberculosis* strains (RMPr, H37Rv and INHr) (Table 2).

Fraction 25–40.6 ^{1}H-NMR data showed the presence of several signals in the shielded region between δ_H 0.7 and 1.2 (s), characteristic of methyl hydrogens; two signals at δ_H 5.31 (dd, J = 3.6; 3.5 Hz) and 5.27 (dd, J = 3.6; 3.5 Hz) characteristic of olefinic hydrogen, and also two signals at 3.23 (dd, J = 10.7; 4.7 Hz) and 3.22 (dd, J = 10.8; 4.9 Hz) which agree with carbinolic hydrogens. All this data suggests the mixture of two triterpenes.

HPLC fractionation of this mixture was performed in order to isolate them, and yielded two fractions, **1** and **2**, with retention times of 11.5 and 12.0 min. The ^{1}H-NMR spectrum from fraction 25–40.6.4.1 showed the signal at δ_H 5.31 (dd, J = 3.6; 3.5 Hz) and from fraction 25–40.6.4.2, the signal at δ_H 5.27 (dd, J = 3.6; 3.5 Hz).

The substance **1** ^{1}H-^{13}C NMR (HSQC) correlation map showed the hydrogen at 5.31 ppm with the carbon at 122.8, which were identified as the vinilic C-12 carbon of oleanolic acid [7, 19] (Table 1). The signal in δ_C 180.0 was assigned to the carboxyl group (C-28).

The ^{1}H-NMR spectrum from fraction 25–40.6.4.2 showed several signals at the shielded region, between δ_H 0.79 and δ_H 1.72 characteristic of methyl hydrogens, moreover two signals were observed at 3.22 (dd, J = 10.8 and 4.9 Hz) and δ_H 5.27 (dd, J = 3.6 and 3.5 Hz) characteristic of olefinic hydrogen, which were assigned to H-12 in triterpenes, characterizing the ursanic skeleton of substance **2**.

When analyzing the ^{13}C-NMR spectral data one can find seven methyl carbons (CH_3), nine methylene carbons (CH_2), seven methine carbons (CH) and seven non-hydrogenated carbons (C), resulting in thirty carbons characteristic of pentacyclic triterpenes. δ_C 179.6 from the carboxylic acid carbon (not hydrogenated), δ_C 137.9 characteristic of unhydrogenated olefinic carbon (sp^2) and δ_C 125.9 of hydrogenated olefinic carbon are the major signals characteristic of a ursanic skeleton. These signals represent, carbons C-28, C-13 and C-12 in ursolic acid triterpene, respectively.

On the other hand, the ^{1}H-^{13}C NMR (HSQC) correlation map showed correlation of the hydrogen at 5.27 ppm with the carbon at 125.9, which were identified as the vinilic C-12 carbon and the multiplicity of the signals corresponding to

Table 1: ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) data of oleanolic and ursolic acids (400 MHz, $CDCl_3$).

Position	Oleanolic acid			Ursolic acid		
	δ_C	δ_H (multiplicity)	δ_C Literature [7]	δ_C	δ_H (multiplicity)	δ_C Literature [7]
1	38.5	1.63 (m)	39.0	38.6	1.72 (m)	39.2
2	28.1	1.60 (m)	28.1	28.2	1.60 (m)	28.2
3	79.1	3.23 (dd; J = 10.7; 4.7 Hz)	78.2	78.7	3.22 (dd; J = 10.8; 4.9 Hz)	78.2
4	38.8	—	39.4	38.5	—	39.6
5	55.3	0.74 (m)	55.9	55.2	1.34 (m)	55.9
6	18.8	1.54 (m)	18.8	18.3	1.60 (m)	18.8
7	32.7	1.49 (m)	33.4	32.9	1.72 (m)	33.7
8	39.3	—	39.8	39.5	—	40.1
9	47.6	1.54 (m)	48.2	47.3	1.60 (m)	48.1
10	37.0	—	37.4	37.0	—	37.5
11	23.8	0.94 (m)	23.8	23.7	1.91 (m)	23.7
12	122.8	5.31 (dd; J = 3.6; 3.5 Hz)	122.6	125.9	5.27 (dd; J = 3.6; 3.5 Hz)	125.7
13	143.5	—	144.8	137.9	—	139.3
14	41.5	—	42.2	42.0	—	42.6
15	27.7	1.60 (m)	28.4	28.1	1.60 (m)	28.8
16	23.7	0.94 (m)	23.8	25.0	1.34 (m)	25.0
17	46.7	—	46.7	48.1	—	48.1
18	42.1	2.82 (m)	42.1	53.8	2.2 (m)	53.6
19	46.0	2.87 (m)	46.6	38.5	1.00 (m)	39.5
20	31.0	—	31.0	38.5	0.95 (m)	39.4
21	33.9	1.62 (m)	34.3	30.3	1.27 (m)	31.1
22	33.2	1.30 (m)	33.2	37.4	1.72 (m)	37.4
23	28.0	1.00 (s)	28.8	28.9	1.00 (s)	28.8
24	16.8	0.79 (s)	16.5	15.6	0.79 (s)	16.5
25	15.3	0.93 (s)	15.6	15.4	0.94 (s)	15.7
26	17.1	0.79 (s)	17.5	17.1	0.82 (s)	17.5
27	26.0	1.16 (s)	26.2	23.5	1.10 (s)	24.0
28	180.0	—	180.0	179.6	—	179.7
29	33.1	0.92 (s)	33.4	17.0	0.87 (d; J = 6.4 Hz)	17.5
30	23.7	0.94 (s)	23.8	21.4	0.97 (d; J = 6.3 Hz)	21.4

H-18 and related CH_3-29 and CH_3-30 determined the ursolic acid.

In the two-dimensional ^{1}H-^{1}H NMR (COSY) correlation map, the following correlations are observed: hydrogen H-11 (δ 1.91) with H-12 (δ 5.27).

It is common to isolate the ursolic acid with oleanolic acid mixture due to molecule similarity, yet a few differences between them enable telling them apart through NMR, due to the difference between the H-18, C-18, C-12, C-13 and C-29 [20] chemical shifts, and mainly on account of H-29 being a doublet for ursolic acid, and a singlet for oleanolic acid.

^{1}H-NMR spectra and HSQC and HMBC NMR correlation maps overall analysis as well as comparison with literature data [7] enabled the complete structure to be determined as the triterpenes oleanoic and ursolic acids (Table 1) (Figure 2).

The mass spectra analysis of each triterpene isolated showed the molecular ion peak at m/z = 456 u, and showed the common fragmentation pattern of triterpenes, described in the literature [21]. All these data together confirmed to be the triterpenes, ursolic acid and oleanolic acid. To the best of our knowledge, this is the first report of these two triterpenes in *Duroia* genus.

3.3. Antimycobacterial Activity. All extracts showed activity against *M. tuberculosis* at least for one strain, except for the methanol extract of branches (1st collection) (Table 2). The dichloromethane extract of leaves (1st collection) showed the highest activity, with a MIC of 6.25 μg/mL for INHr strain, 25.0 μg/mL for RMPr strain and ≤6.25 μg/mL for H37Rv strain. Triterpenes oleanoic and ursolic acids were isolated from this extract. The methanolic extract of leaves (2nd collection) that showed a MIC of 12.5 μg/mL for INHr strain was the second most active one (Table 2).

The wide variety of natural products chemical structures plays a major role on the development of new antimycobacterial drugs generations, as shown in the extensive literature

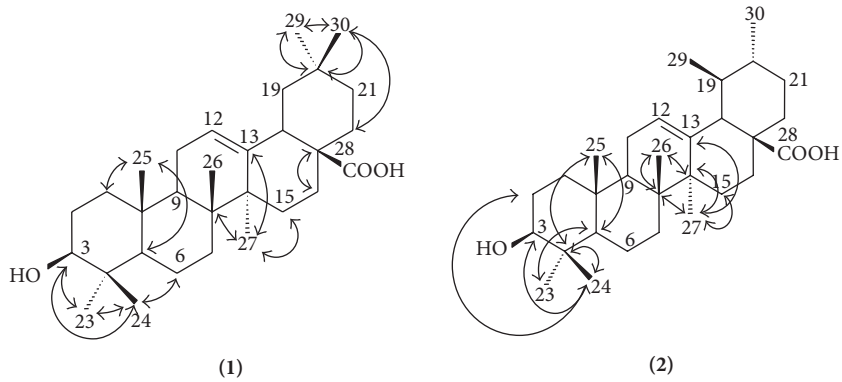

Figure 2: Structures of oleanolic and ursolic acids and their ^{1}H-^{13}C long-range correlations.

Table 2: Minimum inhibitory concentration (MIC) of *D. macrophylla* extracts against *M. tuberculosis* strains.

Extracts	*M. tuberculosis*					
	H37Rv	(μg/mL)	INHr	(μg/mL)	RMPr	(μg/mL)
1st Collection						
Leaves DCM	S	6.25	S	25	S	≤6.25
Leaves MeOH	R	>200	R	>200	S	200
Branches DCM	S	100	S	100	S	100
Branches MeOH	R	>200	R	>200	R	>200
2nd Collection						
Leaves DCM	S	200	S	50	R	>200
Leaves MeOH	S	100	S	12.5	S	100
Branches DCM	S	25	S	50	R	>200
Branches MeOH	S	100	S	100	S	100

DCM: dichloromethane, MeOH: methanol, R: resistant, S: sensible, H37Rv: sensible strain, INHr: isoniazid resistant strain, RMPr: rifampicin resistant strain. Extract with MIC > 200 μg/mL were considered inactive.

revision made by Copp [22], from 1990 to 2002, which uncovered 352 substances isolated from natural products (terrestrial and marine) presenting an antimycobacterial activity and a MIC ≤ 64 μg/mL.

The highest activity of the dichloromethane extract from leaves (1st collection) in this work could be attributed to the presence of terpenes. Several studies, such as those performed by Newton et al. [23], Cantrell et al. [24], Copp [22], Seidel and Taylor [25], Aguiar et al. [26], and Higuchi et al. [27], showed terpenes to be responsible for the antimycobacterial activity.

Extracts and compounds from other Rubiaceae species, such as *Duroia hirsuta* and *Psychotria vellosiana*, showed activity against *Mycobacterium phlei* [6] and *M. tuberculosis* and *M. kansasii*, respectively [28]. According to some authors, the antimycobacterial activity can also be related to the presence of alkaloids, normally found in Rubiaceae species [29, 30].

Out of the 27 assayed fractions present in this work, only fraction 63-65 was as active against *M. tuberculosis* INHr strain as the dichloromethane extract of leaves (1st collection) (MIC of 25 μg/mL). Among the others, 15 fractions were active against *M. tuberculosis* H37Rv strain (MIC between 50 and 200 μg/mL), 17 fractions were active against INHr strain (MIC between 25 and 200 μg/mL), and 16 fractions were active against RMPr strain (MIC between 50 and 200 μg/mL) (Table 3). Fraction 25-40.6 was active against the three strains, with a MIC of 200 μg/mL and their fractionation yielded the substances **1** and **2** corresponding to the triterpenes oleanoic and ursolic acids, respectively.

Studies conducted by Higuchi et al. [27] reported the oleanolic and ursolic acids' mixture MIC to be 62.5 μg/mL. Other studies showed the growth inhibitory activity against *Mycobacterium tuberculosis* with a MIC of 16 μg/mL for oleanolic acid and 50 μg/mL for ursolic acid [24].

Ge et al. [31] also obtained a better MIC value for oleanolic acid (MIC of 28.7 μg/mL) than that for ursolic acid (MIC of 41.9 μg/mL). These latter examples showed each isolated compound to exhibit a better MIC than the mixture's.

The high lipophilicity of terpenes is probably the main factor that allows their penetration through the mycobacterial cell wall [27].

Other studies showed these substances inhibited 99% the growth of *M. tuberculosis* H37Rv [32]. The literature data reported that oleanolic acid has a synergistic effect when combined with isoniazid, rifampicin, or ethambutol (first line antitubercular drugs) [32].

According to Pauli et al. [33], a crude extract MIC may or not be a reliable antimycobacterial activity indicator since such extracts could hold active compound antagonist substances decreasing the MIC. Otherwise, a crude extract could hold compound agonists with increasing effects on MIC, the so called synergism effect. According to the author an extract with high activity (lower MIC) could present large amounts of compounds with moderated antimycobacterial activity. In other scenario, crude extracts with moderated MIC could hold small amounts of chemically active compounds. When the extract loses its activity during the fractionation, it could be due to a synergism effect between the substances on it. Therefore, the combined action of two or more substances

Table 3: Minimum inhibitory concentration (MIC) of dichloromethane fractions from the leaves of *D. macrophylla* (1st collection) against *M. tuberculosis* strains.

Fraction	*M. tuberculosis*					
	H37Rv (μg/mL)		INHr (μg/mL)		RMPr (μg/mL)	
Fr 1–4	R	>200	R	>200	R	>200
Fr 1–4.17–20	R	>200	R	>200	R	>200
Fr 5	R	>200	R	>200	R	>200
Fr 6–12	S	50	S	100	S	100
Fr 6–12.30	R	>200	R	>200	R	>200
Fr 6–12.33–35	R	>200	R	>200	R	>200
Fr 6–12.38–63	R	>200	R	>200	R	>200
Fr 14–16	S	100	S	50	S	100
Fr 17–21	R	>200	R	>200	R	>200
Fr 17.21.1–5	R	>200	R	>200	R	>200
Fr 25–40	S	200	S	200	S	200
Fr 25–40.2	R	>200	S	200	S	200
Fr 25–40.6	S	200	S	200	S	200
Fr 25–40.6.32	S	100	S	100	S	100
Fr 41–44	S	100	S	50	S	100
Fr 46–56	S	200	S	200	S	100
Fr 46–56.5	S	200	S	200	S	200
Fr 46–56.8-10	S	50	S	50	S	50
Fr 46–56.13–17	R	>200	R	>200	R	>200
Fr 57	S	100	S	200	S	200
Fr 57.6–12	S	100	R	>200	R	>200
Fr 63–65	S	100	S	25	S	100
Fr 66–68	S	200	S	100	S	200
Fr 70–74	R	>200	S	200	R	>200
Fr 76–86	R	>200	S	200	S	200
Fr 87–92	S	200	S	50	S	100
Fr 94–99	S	200	S	200	S	100

Fr: fraction, R: resistant, S: sensible, H37Rv: sensible strain, INHr: isoniazid resistant strain, RMPr: rifampicin resistant strain. Fractions with MIC > 200 μg/mL were considered inactive.

can result on a biological effect higher than any single one's. Hence, more thorough studies are necessary to find which substances should be mixed in order to attain the desirable antimycobacterial activity.

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Research Article

Inhibition of Carrageenan-Induced Acute Inflammation in Mice by Oral Administration of Anthocyanin Mixture from Wild Mulberry and Cyanidin-3-Glucoside

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Anthocyanins are flavonoids which demonstrated biological activities in *in vivo* and *in vitro* models. Here in the anti-inflammatory properties of an anthocyanin-enriched fraction (AF) extracted from wild mulberry and the cyanidin-3-glucoside (C3G), the most abundant anthocyanin in diet, were studied in two acute inflammation experimental models, in the peritonitis and in the paw oedema assays, both of which were induced by carrageenan (cg) in mice. In each trial, AF and C3G (4 mg/100 g/animal) were orally administered in two distinct protocols: 30 min before and 1 h after cg stimulus. The administration of both AF and C3G suppresses the paw oedema in both administration times ($P < 0.05$). In the peritonitis, AF and C3G reduced the polymorphonuclear leukocytes (PMN) influx in the peritoneal exudates when administered 1 h after cg injection. AF was more efficient reducing the PMN when administered 30 min before cg. Both AF and C3G were found to suppress mRNA as well as protein levels of COX-2 upregulated by cg in both protocols, but the inhibitory effect on PGE_2 production in the peritoneal exudates was observed when administered 30 min before cg ($P < 0.05$). Our findings suggest that AF and C3G minimize acute inflammation and they present positive contributions as dietary supplements.

1. Introduction

Anthocyanins, glycosylated polyphydroxy, and polymethoxy derivates of flavilium salt are natural colorants belonging to the flavonoid family and largely intaken from vegetable foods [1]. These pigments are responsible for the pink, red, violet, and blue colours in the flowers, fruits, and vegetables. There is a great variety of anthocyanins spread in the nature but only six are the most common: cyanidin, pelargonidin, malvidin, peonidin, petunidin, and delfinidin [2]. Interest in biological effects of anthocyanins has increased during the last decade because of increasing evidence demonstrating their potential therapeutic effects. Some anthocyanins have demonstrated to inhibit the growth of cancerous cells [3–5], to decrease hyperglycemic levels [6] and to promote antiobesity effects [7, 8]. Furthermore, anthocyanins possess antioxidant [9, 10] and anti-inflammatory [11–13] properties. This group of compounds has been demonstrated to modulate inflammation process dependent on the COX-2 pathway *in vitro* experimental protocols [14–18] and through the inhibition of nitric oxide biosynthesis [10].

Wild black mulberry (*Morus nigra* L.) extracts contains high level of anthocyanins. The identified anthocyanins are mainly cyanidin-3-glucoside (C3G), and in minor level cyanidin-3-rutinoside and pelargonidin derivate [19]. We previously reported that the anthocyanin-enriched extract (AF) obtained from wild black mulberry increased the plasma antioxidant capacity and the plasma catalase activity after oral intake in human [20]. Also, AF demonstrated inhibitory effect on the migration and invasion of a human lung cancer cell [5]. However, there are few studies that use *in vivo* experimental protocols, in order to demonstrate if oral intake of anthocyanins could affect inflammation. Since the anthocyanin is commonly intake daily from vegetable foods, it is important to establish evidence for the effect of anthocyanin consumption on health.

Inflammatory responses are a series of well-coordinated events that depend on the increase in vascular permeability and sequential release of inflammatory mediators, leading to oedema and arrival of inflammatory leukocytes to the site of inflammation, where neutrophils and macrophages are known to recruit and play pivotal roles in acute and chronic inflammation, respectively [21]. Cyclooxygenases (COXs) are the key enzymes in the synthesis of lipid mediators called prostaglandins observed in inflammation events. COXs convert free arachidonic acid, following its release from membrane phospholipids by phospholipases A_2, to prostaglandin H_2, the common precursor for all prostanoids. Nowadays, there are three COX isoforms named COX-1, COX-2, and COX-3 [22, 23]. COX-1 is a housekeeping enzyme, constitutively expressed in most mammalian tissues, and it is responsible for maintaining normal cellular physiologic functions. COX-2 is also present at a basal level in certain tissues, but its expression is induced in inflammatory cells and tissues in response to cellular activation by endotoxin, cytokines, mitogens, and other stimulus [24, 25]. COX-2 is the main enzyme providing a mechanism for the generation of proinflammatory prostanoids, such as prostaglandin E_2 (PGE_2), a potent vasodilator, which enhances oedema formation [26, 27]. COX-3, in turn, has been cloned [28, 29], but its function have yet to be well studied.

Therefore, in this study, we have examined, in mice, the anti-inflammatory activity of oral administration of an anthocyanin-enriched extract obtained from mulberry and its major component, the C3G, in the acute inflammation, peritonitis and paw oedema assays, induced by carrageenan, mainly on COX-2 mRNA and protein expression and PGE_2 production.

2. Material and Methods

2.1. Mulberry Anthocyanin Preparations. The anthocyanin-enriched fraction (AF) was prepared from wild black mulberry according to the previously published method [19]. Briefly, the sample (approximately 5 g) was extracted three times with 100 mL of methanol:water:acetic acid (70 : 30 : 5, v : v : v) (Brinkmann homogeniser, Polytron-Kinematica GmbH, Kriens-Luzern, Sweden) in an ice bath. The homogenate was filtered under reduced pressure through filter paper (Whatman number 06). The methanol extract obtained was concentrated, under vacuum until methanol content elimination, using a rotary evaporator (Rotavapor RE 120, Buchi, Flawil, Sweden) and made up to 50 mL with distilled water. The extract (25 mL) was passed through polyamide (CC-6, Macherey-Nagel, Germany) column (10 g/60 mL) previously conditioned with 50 mL of methanol and 100 mL of distilled water. Impurities were washed out with distilled water and retained flavonoids were eluted with 120 mL of methanol acidified with 0.1% HCl. The flow rate through the columns was controlled by means of a vacuum manifold Visiprep 24DL (Supelco, Bellefonte, PA). The eluate was evaporated to dryness under reduced pressure at 40°C and dissolved in distilled water prior administration. This fraction corresponds to AF. C3G was further purified from AF according to Chen et al. [5] by passing it through a Bio-Gel P-2 column (40 cm × 2.5 cm) (Bio-Rad Laboratories, Hercules, CG), eluting it with aqueous acetic acid, pH 2.5, and monitoring it by spectrophotometer at 520 nm (Hitachi L-4000 UV-vis detector). The fraction corresponding to C3G, which was confirmed by HPLC-DAD, was collected and lyophilized. C3G was dissolved in distilled water prior to administration.

2.2. Anthocyanin Quantification. For anthocyanin quantification, aliquots of AF and C3G were diluted with methanol: acetic acid (99 : 5, v : v) and filtered through a 0.45 μm PTFE filter (Milipore Ltd., Bedford MA) prior to quantification by HPLC-DAD [19]. The column used was a Prodigy 5 μm ODS3 (250 mm × 4.6 mm i.d., Phenomenex Ltd.) and elution solvents were (A) water : THF : TFA (98 : 2 : 0.1, v : v : v) and (B) acetonitrile. Solvent gradient consisted of 8% B at the beginning, 10% at 5 min, 17% at 10 min, 25% at 15 min, 50% at 25 min, 90% at 30 min, 50% at 32 min, and 8% at 35 min (run time, 35 min). Eluates were monitored at 270 and 525 nm. Flow rate was 1 mL/min; column temperature was 30°C. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards and the library spectra. Cochromatography was used when necessary. C3G, C3R, and pelargonidin (Plg) (Extrasynthese, Genay, France) were used as standard. The total anthocyanin content of AF was expressed as C3G equivalent. The anthocyanin composition of AF is 85% C3G, 12% C3R, and 3% Plg derivate and they were previously identified by LC-MS [19]. The anthocyanin profile of AF and the purity of C3G are shown in Figure 1.

2.3. Animals. Male Swiss mice, weighing 18–20 g (approximately four weeks old), were acclimated to housing for at least 1 week prior to investigation. The night before the experiment, food was withdrawn from the cages but water was given *ad libitum*. Animals were randomly assigned to each treatment group and all testing was performed between 8:00 and 9:00 a.m. All animals were handled and experiments were conducted in accordance to the Guidelines for Animal Experimentation of the University of São Paulo, Brazil, after approval by the Ethics Committee of the Pharmacy Faculty of the University of São Paulo (Protocol number 53, FCF-USP).

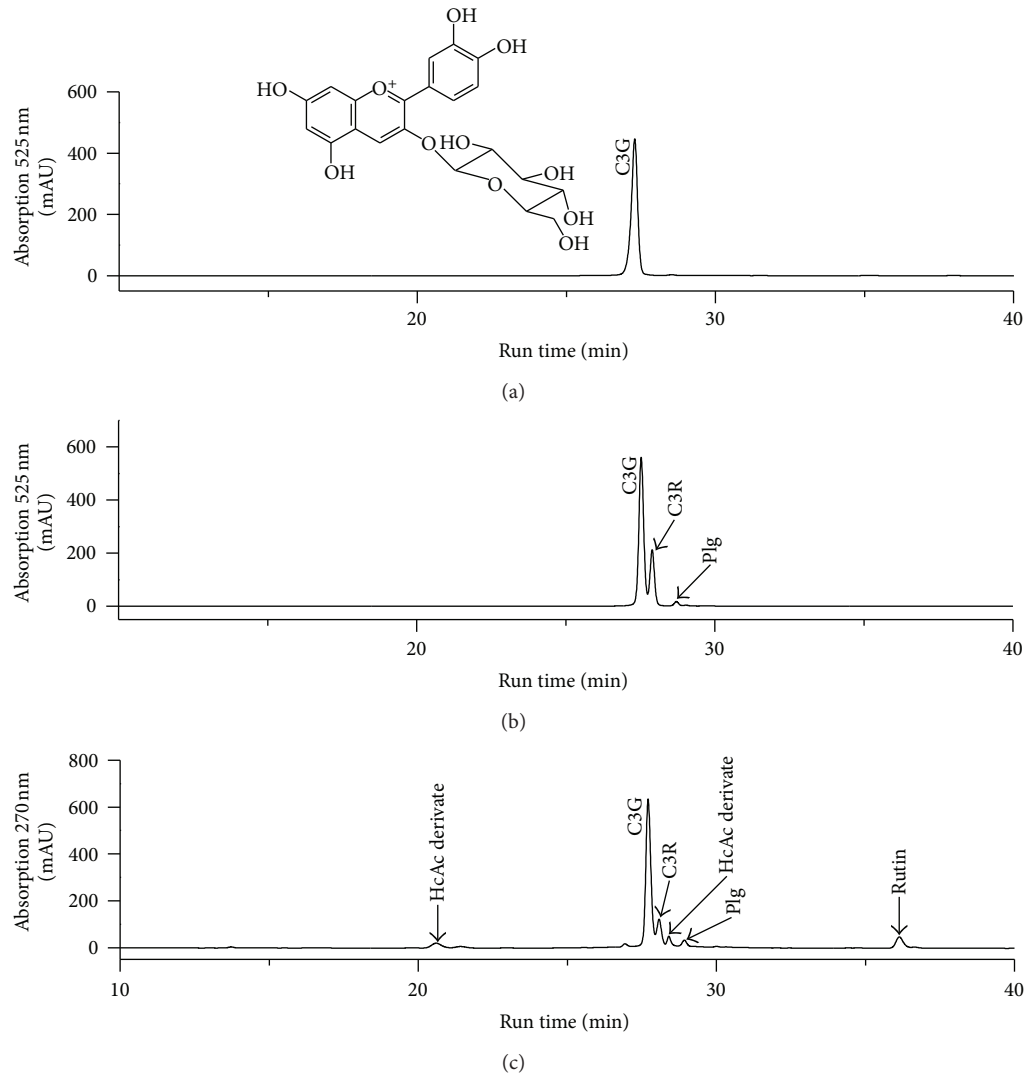

Figure 1: HPLC-DAD of cyanidin-3-glucoside (C3G) at 525 nm (a) and anthocyanin profile of AF at 525 nm (b) and 270 nm (c). Peaks were identified by MS/MS as C3G (structure showed), cyanidin-3-rutinoside (C3R), and rutin. Abbreviations: Hydroxycinnamic acid derivate (HcAc derivate) and pelargonidin (Plg).

2.4. Carrageenan-Induced Paw Oedema in Mice. To assess the effects of the AF and C3G on acute inflammation, the animals were deprived of food overnight and orally administered with an aqueous solution using an intragastric tube as described below.

AF Group: 200 μL of the AF (4 mg C3G equiv/100 g body weight) were administered 30 min before (n = 8) and 1 h after (n = 8) intraplantar (i.pl.) injection of 50 μL cg in saline (0.5% m/v) into the left hind paw.

C3G Group: 200 μL of C3G (4 mg/100 g body weight) were administered 30 min before (n = 8) and 1 h after (n = 8) i.pl. injection of 50 μL carrageenan (cg) in saline (0.5% m/v) into the left hind paw.

Control Group: 200 μL of saline were administered 30 min before (n = 8) and 1 h after (n = 8) i.pl. injection of 50 μL cg in saline (0.5% m/v) into the left hind paw.

Indomethacin Group: indomethacin (1 mg/kg, i.v) was administered 30 min before (n = 8) and 1 h after (n = 8) injection of 50 μL cg in saline (0.5% m/v) into the left hind paw.

The contralateral paw was injected with 50 μL of saline solution and used as a control. The volumes of both hind paws were measured by plethysmometry (model 7140 plethysmometer, Ugo Basile, Italy) 1, 2, 3, 4, and 5 h after the injection of cg. The results were reported as the percent inhibition of the volume increase to be compared with the preinjection paw volume. Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods.

2.5. Carrageenan-Induced Peritonitis in Mice. The animals were deprived of food overnight and orally administered with one of the following solutions.

AF Group: 200 μL of the AF (4 mg C3G equiv/100 g body weight) were administered 30 min before ($n = 8$) and 1 h after ($n = 8$) intraperitoneal (i.p.) injection of 1 mL of cg in sterile saline (0.3%, m/v).

C3G Group: 200 μL of the C3G (4 mg/100 g body weight) were administered 30 min before ($n = 8$) and 1 h after ($n = 8$) i.p. injection of 1 mL of cg in sterile saline (0.3%, m/v).

Carrageenan Control Group: 200 μL of saline solution were administered 30 min before ($n = 8$) and 1 h after ($n = 8$) i.p. injection of 1 mL of cg in sterile saline (0.3%, m/v).

Indomethacin Group: 200 μL indomethacin (4 mg/100 g body weight) were administered 30 min before ($n = 8$) and 1 h after ($n = 8$) i.p. injection of 1 mL of cg in sterile saline (0.3%, m/v).

Saline Control Group: 200 μL saline solution were administered 30 min before ($n = 8$) and 1 h after ($n = 8$) i.p. injection of 1 mL of sterile saline solution.

Three hours after cg injections, the animals were killed by overexposure to CO_2 and the peritoneal exudate was withdrawn after washing the peritoneal cavity with 2 mL of saline solution. Aliquots of the washes were used to determine total cell counts. An aliquot of the 1×10^6 and 3×10^6 cells was centrifuged at 800 g/6 min/22°C and used for COX-2 expression analysis by western blotting and RT-PCR, respectively. The supernatant was used for PGE_2 quantification.

2.6. Leukocyte Harvesting and Counting. Aliquots of the peritoneal washes were used to determine total cell counts in a Newbauer chamber after dilution (1 : 20, v : v) in Turk's solution (0.2% crystal violet dye in 30% acetic acid). For differential cell counts, cytospin preparations were stained with Hema^3 stain. Differential cell counts were performed by counting at least 100 cells, which were classified as either polymorphonuclear or mononuclear cells, based on conventional morphological criteria.

2.7. Western Blotting. The precipitate of cells (1×10^6) was lisate with 100 μL of sample buffer [30] and heated for 10 min/100°C. An aliquot of 14 μL of the lisate was separated on SDS-polyacrylamide gels (10%) at 150 V and electrophoretically transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk in Tris buffered saline with 0.05% Tween 20 and incubated 1 h at room temperature with the antibody against COX-2 (1 : 1500) (Cayman Chemicals, Ann Arbor, MI) followed by incubation in the same buffer with the appropriate anti-rabbit horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK) for 1 h at room temperature (1 : 1500). Further, the membrane was also incubated with the antibody against β-actin (1 : 2000) (Sigma, St. Louis, USA) followed by incubation with the anti-mouse secondary horseradish peroxidase-conjugate (1 : 2000) (GE Healthcare, Buckinghamshire, UK). Immunoreactive bands were detected using ECL kit (GE Healthcare, Buckinghamshire, UK). Densities of the bands were determined by a GS 700 Densitometer (Bio-Rad Laboratories, Richmond, CG) using the image analysis software from Molecular Analyst (Bio-Rad Laboratories, Richmond, CG).

2.8. RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Cells (3×10^6) were washed once with sterile saline and mixed with 500 μL of Trizol reagent (Invitrogen, Rockville, MD, EUA) and the RNA was extracted according to the manufacturer's instructions. Complementary DNA was synthesized using an Improm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions and conducted at a thermocycler Gene AMp (PCR System 2400, Applied Biosystems). PCR was performed by denaturing at 94°C for 60 s, annealing at 57°C (COX-2) and 60°C (β-actin) for 1 min and by extension at 72°C for 60 s. Thirty additional cycles for COX-2 and 25 cycles for β-actin were used for amplification. The primer pairs used for analysis were 5′-TTTGTTGAGTCGTTCGCCGGACGGA-3′ and 5′-CGGTATTGAGGAGAAGAGATGGGATT-3′ for sense and antisense primers of the COX-2 gene, respectively [31]; 5′-TGGAATCCTGTGGCGTCCGTGAAAC-3′ and 5′-TAAAACGCGGCTCGGTAACGGTCCG-3′ for sense and antisense primers of the β-actin gene, respectively [32], used as an inner control.

2.9. PGE_2 Quantification. Concentrations of PGE_2 were determined by a specific enzyme immunoassay [33] using a commercial kit (Cayman Chemical Company, Ann Arbor, MI). The extraction of PGE_2 was performed on Sep Pak C18 columns (Waters Corporation, Milford, MA) and eluted with ethanol. In brief, 50 μL aliquots of each extracted sample were incubated with the PGE_2 conjugated with acetylcholinesterase and the specific rabbit antiserum in 96-well plates, coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbance of the samples was recorded at 405 nm in a microplate reader (Labsystem Multiscan), and concentrations of eicosanoids were estimated from standard curves.

2.10. Statistical Analysis. Results were presented as mean ± EPM. The statistical analyses were performed by one way analysis of variance (ANOVA) and *Tukey posthoc test* for comparison, using the Statistic software package version 5.0 (StatSoft, Inc.). Results were considered statistically significant for P values <0.05.

3. Results and Discussion

3.1. Effect of C3G and AF on Carrageenan-Induced Paw Oedema. The oral dose of both extracts and the two protocols applied in this study (30 min before or 1 h after inflammation stimulus) were chosen in order to provide high concentration of C3G in the plasma based in its rapid absorption and excretion [20].

The inflammatory response to subplantar oedema induced by cg in mice was significantly reduced by prior and after oral administration of AF and C3G. Figures 2(a)

and 2(b) show the time course of the paw oedema after i.pl. injection of cg (0.5% m/v). Carrageenan caused progressive increase in the paw oedema 1 h after the injection, presenting the maximum peak at 4 h, decreasing to basal level after 5 h. Before and after treatment of animals with indomethacin significantly reduced cg-induced paw oedema as expected, in comparison with the respective controls (saline). C3G (4 mg/100 g body weight), administered by gavage either 30 min before or 1 h after the cg stimulus significantly decreased ($P < 0.05$) the paw oedema (around 40% and up to 80%, resp.) at the fourth hour after cg injection when compared with the control group (Figures 2(a) and 2(b)). Also, the oral administration of AF decreased the paw oedema approximately 40% in both administration times.

The dose of AF and C3G used in the present study is ten-times lower than that necessary of the anthocyanin mix from tart cherry to suppress the 25% complete Freund's adjuvant and cg-induced paw oedema [13] but closer than ginkgo biloba extract concentration necessary to inhibit the paw oedema induced by cg in rats [34]. This fact suggested that C3G is one of the anthocyanins that presented high anti-inflammatory activity.

It has been established that the paw oedema induced by the subplantar injection of cg is biphasic; the early phase involves the release of the mediators serotonin, histamine, and kinins, while the late phase is characterized by the infiltration of leukocytes and mediated only by prostaglandins [35]. These results suggest that the inhibitory effect of AF or C3G on oedema formation is due to the inhibition of the synthesis and/or release of these mediators, in the early phase of inflammatory effect of cg, especially by inhibiting probably cyclooxygenase products. To support this observation, the data indicate that C3G promoted similar effectiveness in suppressing oedema, when compared to the inhibitory profile of indomethacin, a COX activity inhibitor, on cg-induced inflammation.

3.2. Effect of C3G and AF on Carrageenan-Induced Cellular Influx into Peritoneal Cavity. Intraperitoneal administration of cg produces a sustained increase in postcapillary *venule* permeability, thereby leading to increased cellular infiltration, particularly of neutrophils [36]. The recruitment of leukocytes from the circulation to sites of inflammation is enhanced by a series of proinflammatory mediators, such as IL-8 and vasoactive amines, ICAM and VCAM, that are produced and released into the tissue by mast cells, macrophages, and activated endothelial cells, as well as transmigrated leukocytes [36].

Figure 3 presents the total leukocyte influx and differential cell into the peritoneal cavities after oral administration of C3G or AF (4 mg/100 g body weight) or indomethacin (4 mg/100 g body weight) or saline (control) 30 min before and 1 h after i.p. injection of cg (0.3% w/v) or saline solution (without stimulus).

The oral administration of AF 30 min before the i.p. injection of cg caused a significant decrease ($P < 0.05$) in the number of total leukocytes (29% decrease) (Figure 3(a)), but not when administered 1 h after the stimulus. No reduction of total leukocytes in peritoneal exudate was observed when indomethacin was injected 30 min before cg. On the other hand, the C3G decreases the number of total leukocytes when administered 1 h after the cg stimulus (38% decrease) (Figure 3(d)). Similar effects were obtained with indomethacin administration, which promoted reduction of leukocytes (55% decrease) when administered 1 h after i.p. injection of cg.

Differential cell counts showed that leukocytes present in the peritoneal cavity, after i.p. injection of cg, were predominantly polymorphonuclears (PMN), mainly neutrophils, when compared with the group that received saline (without stimulus). The mean values of PMN were $74 \pm 4 \times 10^5$ cells/mL, and $51 \pm 1 \times 10^5$ cells/mL, in the groups that received saline by gavage 30 min before and 1 h after cg injection, respectively (Figures 3(b) and 3(e)). On the other hand, in the group that received saline instead of cg (without stimulus), in both administration times, the mononuclear leukocytes (MN) were predominant ($13 \pm 1 \times 10^5$ cells/mL). In addition, our results showed that cg injection caused a decrease in the number of MN in the peritoneal cavity ($7.1 \pm 0.1 \times 10^5$ cells/mL) (Figures 3(c) and 3(f)).

Like what occurred with the total leukocytes, the number of PMN in peritoneal fluid in mice was significantly reduced when treated with C3G (39% decrease) or indomethacin (40% decrease) 1 h after the i.p cg stimulus, when compared to the control group that received saline orally (Figure 3(e)). On the other hand, AF administered 30 min before cg, promoted a significant decrease in the recruited PMN (24% decrease), compared to the control group (Figure 3(b)).

These results were different from those observed in other tissues, such as air pounch cg inflammation in mice and acute lung inflammation in rats where a decrease in the influx of cell was observed when C3G was previously administered before the cg stimulus [14, 17].

In relation to MN influx, C3G or AF or indomethacin administrated 30 min before cg injection did not change the decrease counts of MN promoted by cg injection (Figures 3(c) and 3(f)), when compared with the group without cg stimulus.

Since C3G was detected intact and in low concentration in plasma of rats after mulberry juice intake [20], the oral intake performed 1 h after cg stimulus probably could provide an ideal concentration of C3G in plasma, resulting in the observed effect. However, this experimental protocol showed that AF is more effective than C3G as a preventive compound against leukocyte migration, suggesting that the complex mixtures of anthocyanins in AF may provide antileukocyte influx effect mainly through a combination of additive and/or synergistic effects.

3.3. Effect of C3G and AF on Carrageenan-Induced Cyclooxygenase-2 Expression in Peritonitis. The effect of C3G or AF (4 mg/100 g body weight) on cg-induced COX-2 transcription was measured in peritoneal leukocytes by RT-PCR. As shown in Figures 4(a) and 4(c), the i.p. injection of cg (0.3% w/v) drastically increased COX-2 mRNA and protein expression. On the other hand, the oral administration of

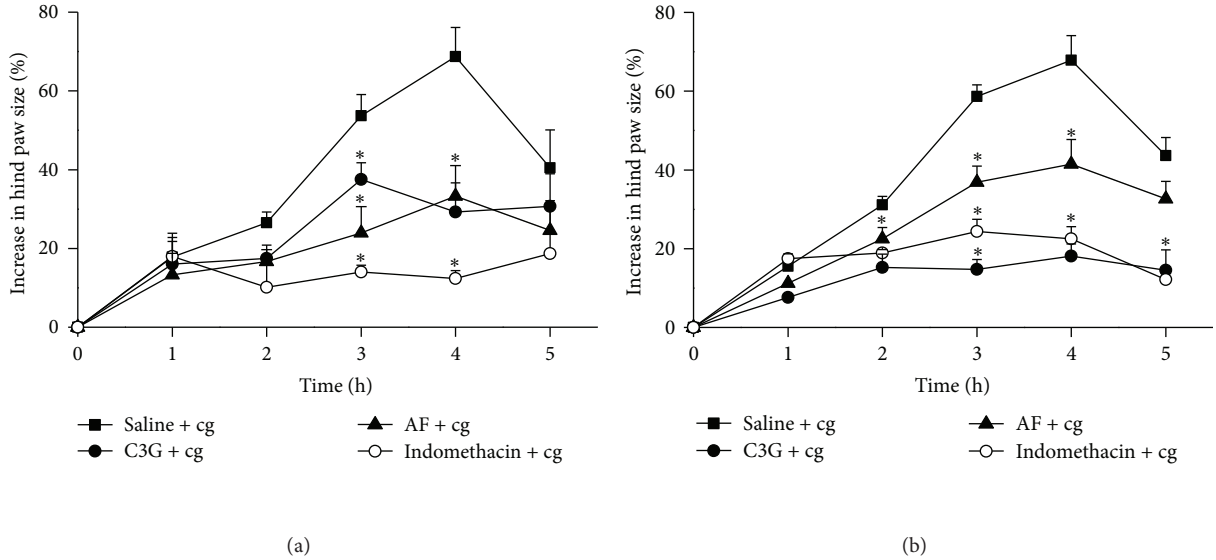

Figure 2: Effect of C3G and AF on carrageenan-induced paw oedema. Footpad oedema was induced by injection of cg (0.5% w/v in saline, i.pl.) and was evaluated by plethysmometry. C3G or AF (4 mg/100 g body weight) or indomethacin (1 mg/kg, i.v.) or saline (control oedema) was orally administered in two different times: 30 min before (a) and 1 h after (b) i.pl. injection of cg. The increase paw size was measured 1, 2, 3, 4, and 5 h after cg injection. The time zero corresponds to cg injection. The results were expressed as mean ± EPM of 8 mice. Statistically significant difference regarding saline (control group) and C3G and AF and Indomethacin groups is expressed as $^{*}P < 0.05$.

C3G and AF, either 30 min before and 1 h after cg i.p. injection, clearly downregulated COX-2 mRNA expression (50% reduction) and decreased the levels of COX-2 protein expression, when compared with the control group.

Although some studies have documented that anthocyanins inhibit COX-2 expression in human keratinocyte cell line [15] and cultured macrophages [37, 38] and in asthma model [16], our study provides the first evidence that an anthocyanin mixture or C3G can inhibit, both preventively and therapeutically, the expression of COX-2 protein with a single oral dose. Several lines of evidence clearly established, in *in vitro* models, that the inhibition of some inflammatory cytokines [12, 16] and inhibition of activation of nuclear factor pathway, such as NF-κB [10, 15], could explain the mechanisms of action of anthocyanins on the inhibition of COX-2 expression.

Also, some sources of anthocyanins, such as black soybean anthocyanin and anthocyanins from sweet purple have showed inhibition the COX-2 expression through NF-κB inhibition when administered before the stimulus in inflammation models [11, 12].

3.4. Effect of C3G and AF on Carrageenan-Released PGE_2 in Peritonitis. Further, this study investigated the effect of C3G and AF (4 mg/100 g body weight) on PGE_2 production, the main inflammatory prostaglandin produced by COX activity, in peritoneal exudates from mice induced by cg. Figures 5(a) and 5(b) showed that i.p. administration of cg induced more than a 25-fold (14.5 ± 2.5 ng/mL) increase in PGE_2 generation compared with the groups without the cg stimulus (0.50 ± 0.05 ng/mL). The PGE_2 concentration was significantly decreased by the oral pretreatment with C3G, AF, and indomethacin, 30 min before cg injection (4.5 ± 1.0 ng/mL, 5.0 ± 2.0 ng/mL and 2.1 ± 0.1 ng/mL, resp.). In this administration time, the AF and C3G promoted approximately 70% reduction in PGE2 production by cg (Figure 5(a)). On the other hand, the oral treatment of AF or C3G, 1 h after i.p. injection of cg, did not induce any modification in the high levels of PGE_2 release by cg (Figure 5(b)). However, in such experimental condition, the indomethacin suppressed the PGE_2 production by cg stimulus.

Prostaglandin E_2 is a product generated by cyclooxygenases from arachidonic acid, and it is an important mediator in the inflammatory process. In this study, it was observed that after 3 h of administration, cg produced an increase in PGE_2 levels into peritoneal cavity. In parallel, the results showed that C3G produced significant inhibition of PGE_2 production when injected 30 min before cg. However, C3G did not produce such equivalent effectiveness towards cg-induced PGE_2 release when administered 1 h after cg injection. These results are curious because in both administration times used in the present study it was possible to observe that the oral intake of C3G was effective in inhibiting COX-2 expression. Therefore, this suggests that although COX-2 mRNA and protein expression were detected at 3 h after cg injection, this isoform of COX did not present catalytic activity in this period of time. In fact, studies have demonstrated that cg-induced PGE_2 are produced by COX-1 in the first phase, while COX-2-derived PGE_2 turned to be involved in the second phase induced by cg injection [35]. In parallel, our data demonstrated that indomethacin was effective to inhibit PGE_2 production in both administration

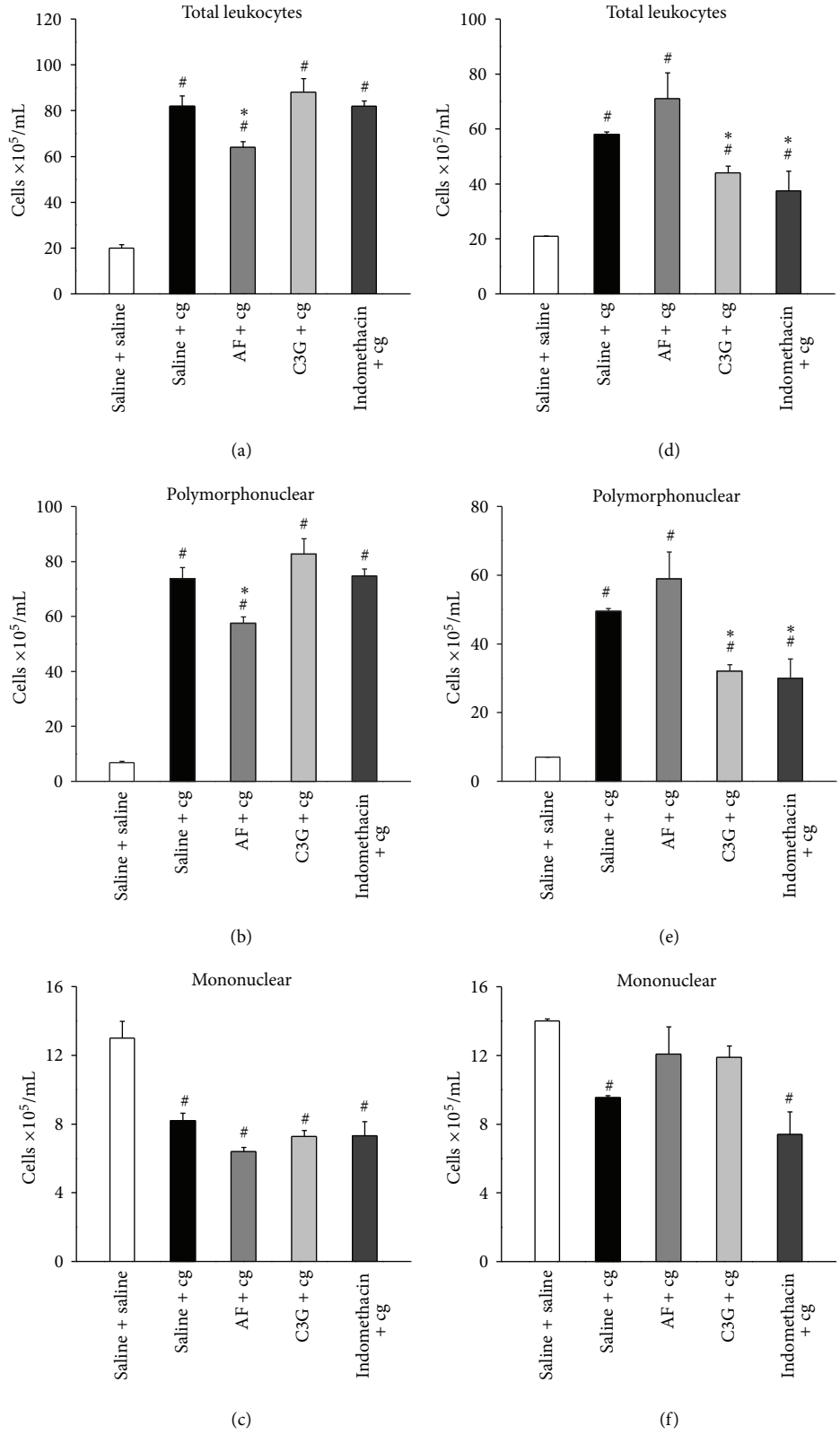

Figure 3: Effect of C3G and AF on carrageenan-induced leukocyte influx into peritoneal cavity. Groups of mice received C3G or AF (4 mg/100 g body weight) or indomethacin (4 mg/100 g body weight) or saline (control) by gavage in two different times: 30 min before (a, b, and c) and 1 h after (d, e, and f) cg or saline injection into the peritoneal cavity. Total leukocyte (a, d), PMN (b, e) and MN (c, f) cell counts were determined in peritoneal washes collected 3 h after cg or saline i.p. injection, as described in Section 2. Values are mean ± EPM of 8 animals. $^{\#}P < 0.05$ when compared with the corresponding group without cg stimulus (saline + saline). $^{*}P < 0.05$ when compared with the corresponding control group (saline + cg).

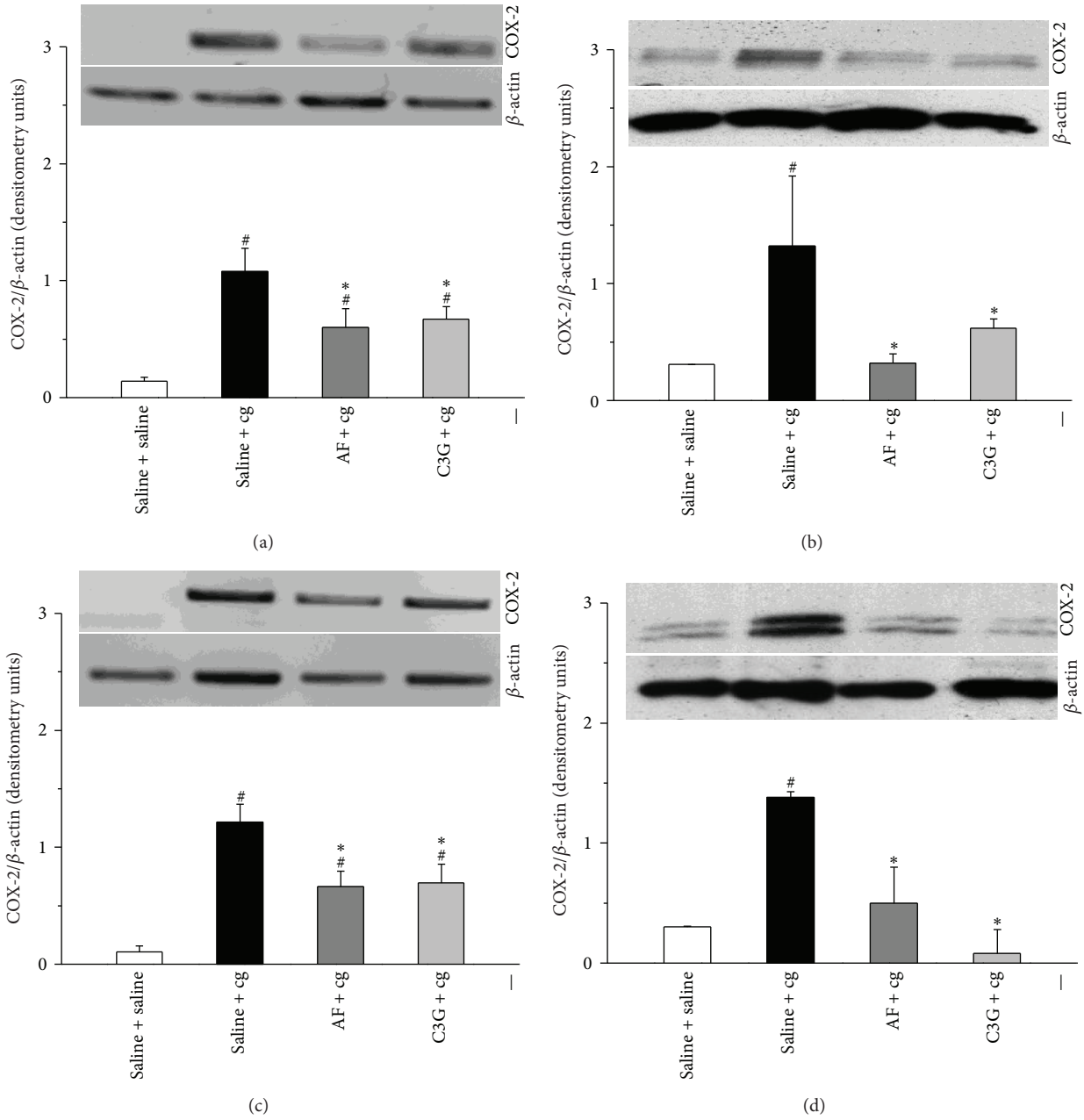

Figure 4: Effect of C3G and AF on carrageenan-induced cyclooxygenase-2 expression in peritoneal leukocytes. Groups of mice received C3G or AF (4 mg/100 g body weight) or saline by gavage in two different times: 30 min before or 1 h after cg (0.3% w/v) or saline injection into the peritoneal cavity. Peritoneal leukocytes were collected 3 h after i.p. administration of either cg or saline and whole cells were analyzed for COX-2 expression by RT-PCR and western blotting performed, as described in Section 2. (a and c) RT-PCR of COX-2, and β-actin (loading control); Bar graph shows densitometric analysis of mRNA COX-2. (b and d) Western blotting of COX-2, and β-actin (loading control) of leukocytes present in the inflammatory exudates; bar graph shows densitometric analysis of protein COX-2. The densities (in densitometry units) were normalized with those of β-actin. Results were expressed as mean ± EPM from 8 mice. $^{\#}P < 0.05$ when compared with the corresponding group without cg stimulus (saline + saline). $^{*}P < 0.05$ when compared with the corresponding control group (saline + cg).

times. Although it is generally accepted that nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin are inhibitors of activity of both isoforms of COXs, it is known that these compounds inhibit COX-1 activity more potently than COX-2 in broken cells and in intact cells of mice [39, 40]. In addition, the absence of PGE_2 inhibition when C3G was administered 1 h after cg stimulus compared to the preventive effect obtained by C3G when administered 30 min before the

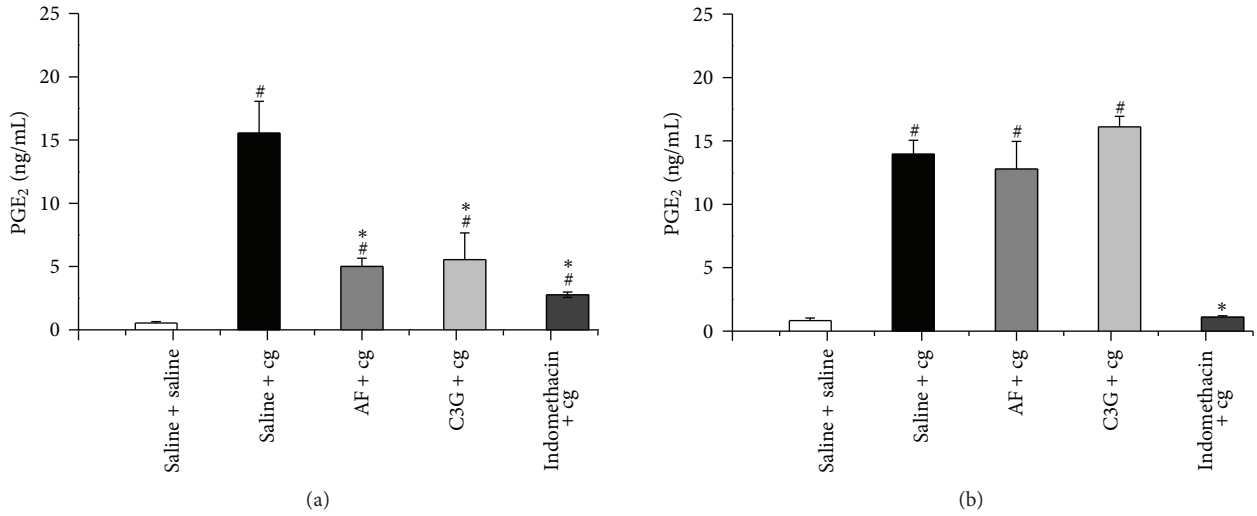

Figure 5: Effect of C3G and AF on carrageenan-released PGE_2 in peritonitis. Groups of mice received C3G or AF (4 mg/100 g body weight) or indomethacin (4 mg/100 g body weight) or saline (control) by gavage in two different times: 30 min before (a) or 1 h after (b) cg or saline (control) injection into the peritoneal cavity. PGE_2 was quantified in peritoneal exudates collected after 3 h of cg or saline administration. Values are mean ± EPM of 8 mice. $^{\#}P < 0.05$ when compared with the corresponding group without cg stimulus (saline + saline). $^{*}P < 0.05$ when compared with the corresponding control group (saline + cg).

stimulus may be a reflection of the plasma concentrations of this anthocyanin in each administration time.

4. Conclusions

In the present study, AF and C3G have been found to be prophylactic or therapeutically efficient on suppressing cg-induced acute inflammation in mice, like oedema and peritonitis, demonstrating to be an anti-inflammatory component from *Morus nigra*. The results suggest that the anti-inflammatory properties of AF and its major component, the C3G, might be correlated to inhibition of the PMN influx, to downregulation of COX-2 expression, and to inhibition of PGE_2 production.

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Research Article

Pretreatment Hepatoprotective Effect of the Marine Fungus Derived from Sponge on Hepatic Toxicity Induced by Heavy Metals in Rats

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The aim of this study was to evaluate the pretreatment hepatoprotective effect of the extract of marine-derived fungus *Trichurus spiralis* Hasselbr (TS) isolated from *Hippospongia communis* sponge on hepatotoxicity. Twenty-eight male Sprague-Dawley rats were divided into four groups ($n = 7$). Group I served as −ve control, group II served as the induced group receiving subcutaneously for seven days 0.25 mg heavy metal mixtures, group III received (i.p.) TS extract of dose 40 mg for seven days, and group IV served as the protected group pretreated with TS extract for seven days as a protection dose, and then treated with the heavy metal-mixture. The main pathological changes within the liver after heavy-metal mixtures administrations marked hepatic damage evidenced by foci of lobular necrosis with neutrophilic infiltration, adjacent to dysplastic hepatocytes. ALT and AST measurements show a significant increase in group II by 46.20% and 45.12%, respectively. Total protein, elevated by about 38.9% in induction group compared to the −ve control group, in contrast to albumin, decreased as a consequence of metal administration with significant elevation on bilirubin level. The results prove that TS extract possesses a hepatoprotective property due to its proven antioxidant and free-radical scavenging properties.

1. Introduction

1.1. Heavy Metals as Major Toxicological Problems. A number of trace metals are used by living organisms to stabilize protein structures, facilitate electron transfer reactions, and catalyze enzymatic reactions [1]. For example, copper (Cu), zinc (Zn), and iron (Fe) are essential as constituents of the catalytic sites of several enzymes [2]. However, other metals, such as lead (Pb), mercury (Hg), and cadmium (Cd) may displace or substitute for essential trace metals and interfere with proper functioning of enzymes and associated cofactors. Metals are usually present at low (or very low) concentrations in the oceans [1]. In coastal waters, metals can occur at much higher concentrations, probably due to inputs from river systems [3]. Close to urban centers, metal pollution has been associated with sewage outlets [4, 5].

Metal-induced toxicity is very well reported in the literature [6, 7]. One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress. A growing amount of data provide evidence that metals are capable of interacting with nuclear proteins and DNA, causing oxidative deterioration of biological macromolecules [6, 7]. One of the best evidence supporting this hypothesis is provided by the wide spectrum of nucleobase products, typical for the oxygen attack on DNA in cultured cells and animals [7, 8].

Cadmium (Cd) is listed by the US Environmental Protection Agency as one of 126 priority pollutants. The most dangerous characteristic of cadmium is that it accumulates

throughout a lifetime. Cadmium accumulates mostly in the liver and kidney, and has a long biological half-life time of 17 to 30 years in humans [9, 10].

Lead is known to induce a broad range of physiological, biochemical, and behavioral dysfunctions in laboratory for animals and humans [11, 12], including central and peripheral nervous systems [13], hemopoietic system [14], cardiovascular system [15], kidneys [16], liver [17], male and female reproductive systems [18, 19].

Mercury is a transition metal. It promotes the formation of reactive oxygen species (ROS) such as hydrogen peroxides. These ROS enhance the peroxides and reactive hydroxyl radicals [20, 21]. Mercuric chloride is an inorganic compound that is used in agriculture as a fungicide in medicine as topical antiseptic and disinfectant and in chemistry as an intermediate in the production of other mercury compounds [22]. Poisoning from environmental sources usually arises from contaminated drinking water as well as plant and animal sourced food products. The metal has been reported to be highly prone to bioaccumulation, leading to biomagnification along the food chain [23]. The absorption, distribution, metabolism, excretion, and toxic dynamics of mercury have been reported to depend on the form and oxidation states [23]. The forms of mercury which are important from a toxicological point of view are elemental (vapor), inorganic salts, and organic salts of mercury. Ingestion of inorganic mercury salts such as mercuric chloride had been reported to cause mainly severe gastrointestinal irritation and renal failure [24]. The toxic effects of an organic and elemental mercury have also been widely reported [25]. Several epidemiologic studies had been conducted on the exposure of humans to mercury through fish and marine mammals' consumption in different geographical areas [26].

Cobalt and nickel are essential trace metals in the human diet. Also, they are major components of the alloys employed in the plate and screw used for connecting bones in orthopedic surgery and in the manufacture of artificial organs [27].

Cobalt is also used as coloring agents for pottery, ceramics, and glass. However, excessive amounts of these transitional metal ions are toxic. For example, cobalt and nickel salts have been reported to induce convulsions [28]; and to cause DNA strand breaks [29]; and to be organ toxic [30]. Cobalt salts are thought to promote the oxidation of reduced glutathione [31] to produce the reduction on a number of hepatic hem proteins such as cytochrome P450, and to interfere with heme metabolism by accelerating its breakdown and inhibiting its synthesis [32]. In addition, numerous authors have studied the impact of nickel on health. Nickel can cause dermatitis to certain persons [33]. Particles of nickel may cause some morphological transformations in numerous cellular systems and chromosomal aberrations [34]. Cobalt was also found obviously harmful on the prenatal development of mice, rats, and rabbits [35].

Nickel breaks down the immunity by affecting the T-cell system and suppresses the activity of natural killer cells in rats and mice [36, 37]. Nickel has been shown to interact with a number of trace elements that include iron, zinc, copper, manganese, sodium, and potassium [38, 39]. Nickel mobilizes and promotes the excretion of copper, zinc, and manganese from organs and promotes storage of chromium in organs [40].

The salts of nickel as particles of nickel can be allergens and carcinogens in man while forming the oxygenated radicals [41]. This cytotoxicity was investigated in numerous microorganisms [42]. Nickel was also found to be responsible for many sexual disorders [43].

1.2. Marine Natural Product as Potent Detoxifier Agent. Over the last forty years, sponge (phylum Porifera) has been identified as an excellent source of unique marine natural products, having a high incidence of biologically active compounds than any single marine phylum [44]. The exploration of microorganisms living inside invertebrates is one of the most exciting strategies to solve the pressing supply issue inherent to marine drug discovery. Marine microorganisms, including fungi, have shown to be the potential source of pharmacologically active metabolites, because of their capability to adapt and survive in the marine environment, and to produce unique secondary metabolites [45].

Fungi are known to tolerate and detoxify metals by several mechanisms, including valence transformation, extra- and intracellular precipitation, and active uptake [46, 47]. The high surface to the volume ratio of microorganisms and their ability to detoxify metals are among the reasons that they are considered as a potential alternative to synthetic resins for remediation of dilute solutions of metals and solid wastes [48, 49].

Metal resistance is defined as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to metal species concerned. Biological mechanisms implicated in fungal survival include extracellular precipitation, complexion and crystallization, transformation of metals, biosorption to cell wall and pigments, decreased transport or impermeability, efflux, intracellular compartmentation, and sequestration [47, 50–54].

2. Materials and Methods

2.1. Materials

2.1.1. Sampling. Samples of honeycomb sponge (*Hippospongia communis*) were collected from the Egyptian western region of the Mediterranean Sea (from Sidi-Krir to El-Salloum) by dragging ships.

2.1.2. Isolation of Sponge-Derived Fungi. To get rid of nonspecific fungal propagules from seawater column on sponge and jellyfish surfaces, animal tissues were rinsed three times with sterile seawater. The surface of the sample was disinfected with 70% ethanol for 2 minutes. The inner tissue was taken out with a scalpel and forceps and then cut into small cubes approx. 0.5 cm^3. A total of 15–20 cubes of each sample were placed on isolation media.

All isolation and culture maintaining media for marine taxa were prepared by sea water (SW) and isolation media basically were supplemented with Rose bengal (1/15,000) and chloramphenicol (50 ppm) for suppression of bacterial growth. Five media were adopted for isolation after Atlas

(2004) they were: Sea Water Rose bengal Chloramphenicol Agar (SWRCA), Sea Water Czapeks Yeast Extract Agar (SWCYA), Sea Water Oatmeal agar (SWOA), Sea Water Agar (SWA), and Sea Water Potato Dextrose Agar (SWPDA).

For maintaining cultures and for proper identification, pure cultures of isolated fungi were grown on standard media such as Vegetable Agar (V8), Oatmeal Agar (OA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), and Potato Carrot Agar (PCA).

2.1.3. Identification of Isolates. Taxonomic identification using morphology characteristics of fungal isolates down to the species level on standard media was mainly based on the following identification keys: Raper and Thom [55], Pitt [56] for *Penicillium* (on Czapek Yeast Extract Agar (CYA) and Malt-Extract Agar (MEA)); Raper and Fennell [57] for *Aspergillus* (on Czapek Agar (CZ)); Ellis [58, 59] for dematiaceous hyphomycetes (Potato Carrot Agar (PCA)); Booth [60] for *Fusarium* (Potato Dextrose Agar (PDA)); von Arx [61], Domsch et al. [62], Watanabe [63] for miscellaneous fungi (on MEA, PDA, CYA); von Arx et al. [64] and Cannon [65] for *Chaetomium* (Oat Meal Agar + Lupin Stem (OA + LUP)). The systematic arrangement follows the latest system of classification appearing in the 10th edition of Ainsworth and Bisby's Dictionary of the fungi [66] and Species Fungorum website (http://www.speciesfungorum.org/Names/Names.asp).

2.2. Preparation of Marine Fungus Extract. Regarding fungal diversity of *Hippospongia communis* a total number of 18 taxa were encountered which only one ascosporic species were recorded. *Trichurus spiralis* Hasselbr. has been selected as a promising taxon for *in vitro* and *in vivo* biochemical assays. Preparative-scale production (0.5 L) was carried out in 1 L Erlenmeyer flasks contained potato dextrose extract (Difco) for 2 weeks at 28°C in a shaking incubator at 102 rpm. Pellets were homogenized and centrifuged by using cooling centrifuge at 8000 rpm for 2 min at 4°C. Resultant mixtures were extracted with ethyl acetate (1 × 50 mL), the organic fractions were combined, and the solvent removed at reduced pressure and 35°C. Residues were redissolved in DMSO for further bioassay. The steps of isolation and extraction of fungi and secondary metabolites are shown in Figure 1.

2.3. Animals and Treatment. A total number of 28 adults white male, Sprague Dawley rats, weighing from 100 to 120 g, were obtained from the animal house of the Faculty of Veterinary Medicine, Assiut University. They were kept in plastic cages, each cage containing five animals. They were maintained under standard laboratory conditions of temperature (about 33 ± 3°C), humidity (20 ± 2%), and duration of light (7:00 a.m. to 7:00 p.m.)/dark (7:00 p.m. to 7:00 a.m.) cycles and were fed on standard rodent chow, with water provided ad libitum. After 1 week of acclimatization to the laboratory environment, the rats were divided into the following groups:

Group I: received saline solution subcutaneously for one week and served as the negative (–ve) control group.

Group II: received subcutaneously for one week; 0.25 mg /100 gm body weight/day of the heavy metal mixture (Ni, Cd, Co and Hg chloride and Pb acetate) and served as an induced toxicity group.

Group III: received intraperitoneal (i.p); 40 mg/100 g/body weight/day, of Trichurus spiralis extract (the most effective fungal extract) dose for one week and served as a positive (+ve) control group.

Group IV: received i.p dose of Trichurus spiralis extract as in group III (40 mg/100 g/body weight/day), for one week as a protection dose before administration of heavy metal mixture (Ni, Cd, Co and Hg chloride and Pb acetate) dose as mentioned in group III. This group served as a protective group.

2.4. Biochemical Profiling for Fungus Extract

2.4.1. Elemental Analysis of Trichurus spiralis Extract. The fungal extract subjected to elemental analysis instruments to determine hydrogen, carbon, nitrogen, and sulfur ratio (Elemental analysis CHNS elementary, Vario EL III, Germany).

2.4.2. Determination of Total Phenolic Content in (T.S) Fungus Extract. Total phenolic compounds in the fungal extract were determined by the method of [67].

2.4.3. Determination of Total Flavonoid Content in (T.S) Fungus Extract. Total flavonoid content was determined by a colorimetric method of [68].

2.4.4. Diphenyle-α-Picrylhydrazyl (DPPH) Radical Scavenging Effect of (T.S) Fungus Extract. DPPH radical scavenging assay of the total extract was performed by using the previously established modified methodology of [69, 70]:

$$\%\ \text{scavenging} = \frac{\left[A_{\text{control}} - A_{\text{sample}}\right]}{A\text{control} \times 100}. \quad (1)$$

2.4.5. Determination of Thiobarbituric Acid Reactive Substance Method Using TBARS Assay for (T.S) Fungus Extract. The method used was adapted from [71] and modified by K.M. Fisch:

$$\%\ \text{scavenging} = \frac{\left[A_{\text{control}} - A_{\text{sample}}\right]}{A\ \text{control} \times 100}. \quad (2)$$

2.5. Biochemical Assay in Serum. The appropriate kits (Bio diagnostic kits) were used for the determination of serum total protein according to [72], aminotransferase activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) according to [73]. Determination of albumin level was determined according to the method of [74]. Determination of total bilirubin was determined by the method described by [75].

2.6. Histopathology. The fixed liver tissues in formalin were dehydrated in ascending grades of alcohol, then cleaned by immersing the tissues in xylene for 1 h (three times), followed

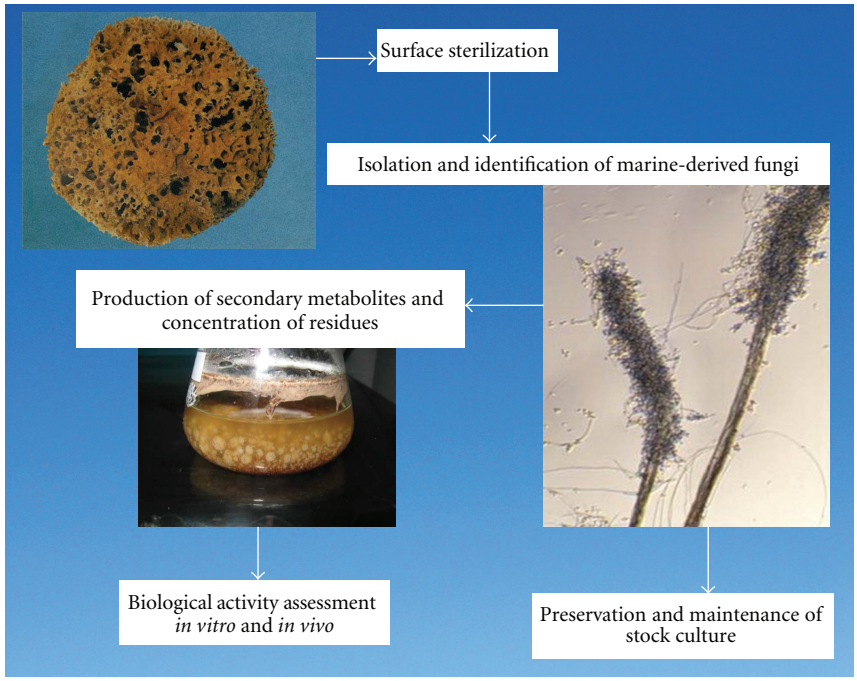


FIGURE 1: Schematic overview on important steps involved in the isolation of fungi from marine sponge and in preparation of their secondary metabolites.

by impregnation in melted paraffin, in wax, then in oven at 60°C for 1 h. The specimens were embedded in paraffin and were left to solidify at RT. Using a rotatory microtone, sections of 5 μm thick were cut and mounted on clean glass slides. Sections were stained with hematoxylin and eosin (H&E) and examined for any histopathological changes [76].

2.7. Statistical Analysis. The data were given as individual values and as means (X) ± standard deviation (SD) for 7 animals in each group. Comparisons between the means of various treatment groups were analyzed using least significant difference (LSD) test. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using the statistical software SPSS, version 11.5.

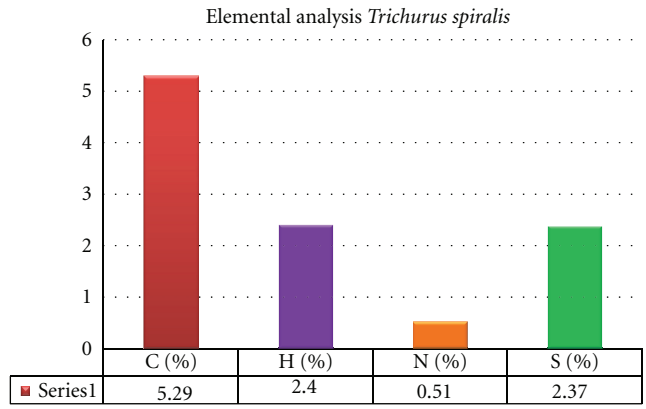


FIGURE 2: The elemental analysis of *Trichurus spiralis* extract as percentages.

3. Result

The biochemical profile for *Trichurus spiralis* fungus extract show the higher ratio of sulfur content in the elemental analysis as shown in Figure 2, where the flavnoids content in fungal extract is higher than phenolic as shown in Figure 3. The antioxidant capacity using DPPH assay and inhibition of lipid peroxidation using TABRS *in vitro* show higher antioxidant capacity by 85% and 78.80%, respectively, as shown in Figure 4.

3.1. Mortality Rate. The courses of mortality rate for each group are shown in Figure 5. In the group II (induction group), four of 12 animals died by day 6: two of them died within the first 48 h, and the two others died by 72 and 144 h following induction of heavy metal mixtures. In the group IV (protection group), two rats died in first 72 h (after marine fungal extract and heavy metal administration), where the third died by 120 h. although, there were no differences at the other two groups, group I (−ve control) and group III (+ve control).

3.1.1. Histological Findings. The results of rats liver histopathological studies are shown in Figures 6, 7(a)–7(h), 8 and 9 for the induced toxicity group (Group II) compared to other groups (I, III, and IV). The figures showed that group I (−ve control), and group III showed normal liver with no remarkable pathological changes in (Figures 6 GI and 8 GIII). The rat's liver of individual induced toxicity

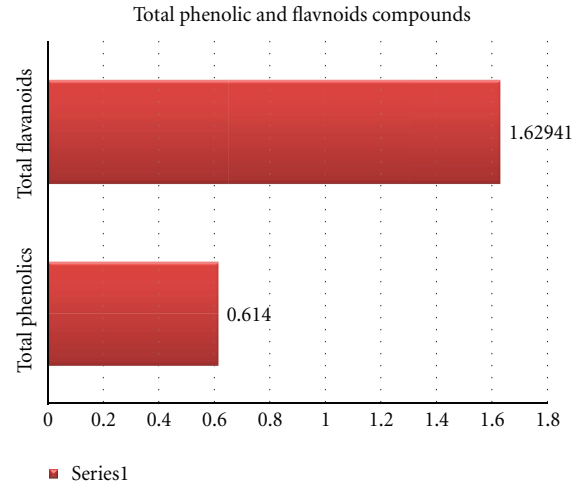
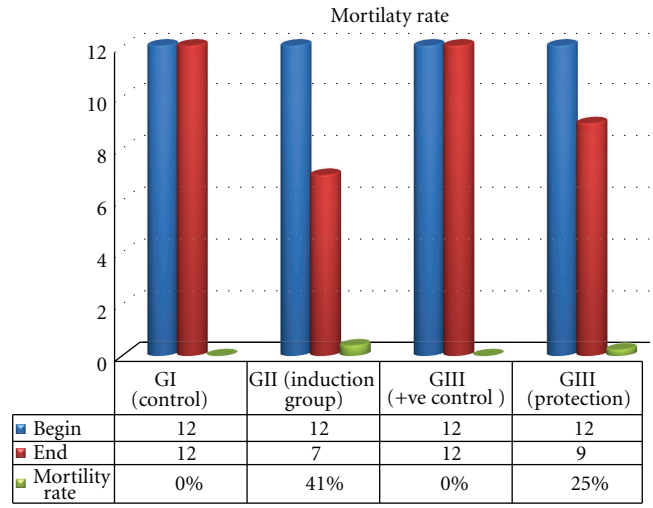
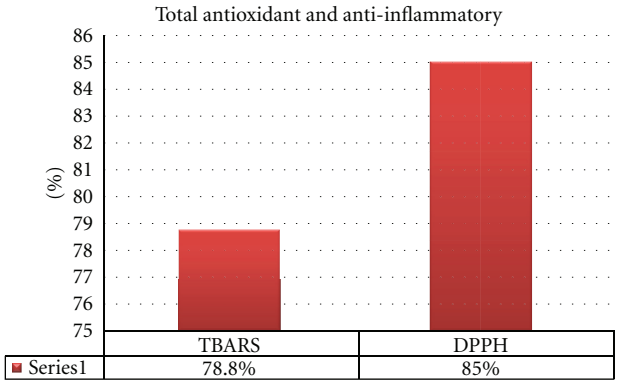


Figure 3: Total phenolic and flavnoids content of *Trichurus spiralis* extract.



	GI (control)	GII (induction group)	GIII (+ve control)	GIII (protection)
Begin	12	12	12	12
End	12	7	12	9
Mortility rate	0%	41%	0%	25%

Figure 5: Mortality rat for each group during the study course.



	TBARS	DPPH
Series1	78.8%	85%

Figure 4: Total antioxidant capacity of *Trichurus spiralis* extract (6 mg/mL). (The percent of *Trichurus spiralis* inhibition to word oxidative stress and lipid peroxidation *in vitro*).

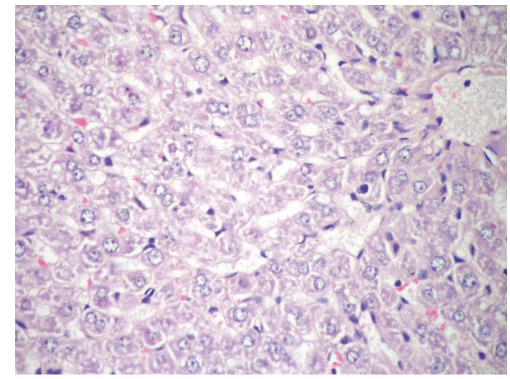


Figure 6: The normal liver showing hepatic architecture formed of cords of hepatocytes separated by hepatic sinusoids (H&E 400x).

group, group II (treated with the heavy metal mixture, 0.25 mg/100 gm b.wt/7 day), showed different proportional to histopathological changes appeared as an extensive loss of hepatic architecture and large amount of damage as shown in Figures 7(a)–7(h) and summarized in Table 1.

Some animals with the group IV, protected group (treated with fungal extract prior to their treatment by the heavy metal mixture), showed normal hepatic architecture, while others showed preserved hepatic architecture with mild portal inflammatory infiltrate and frequent apoptotic (Figure 9 G IV).

The Effect of Trichurus Spiralis Extract on the Activities of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) in the Serum of Rats, Treated with Heavy Metal Mixture (Induced Toxicity). Levels of both ALT and AST in the group IV showed nonsignificantly increase and /or decrease when compared to their corresponding values either of group, I or group III (positive control group), as shown in Table 2 and Figures 10 and 11, respectively.

In rats treated with the heavy metal mixture in group II, the activity of serum ALT (119.28 ± 26.58 U/L, $P < 0.001$) and AST (117.79 ± 26.58 U/L, $P = 0.003$) were significantly increased than that of –ve control group (group I) rats (85.32 ± 8.16), respectively. In contrast, the fungus extract pretreated group (group IV) at 40 mg/100 g b.wt/day for 7 days had a significantly lower ALT (65.45 ± 3.85) and AST (63.37 ± 20.54), when compared to group II, at $P < 0.003$.

The Effect of Trichurus Spiralis Extract on the Levels of Total Protein, Albumin and Bilirubin. The levels of total protein and total bilirubin are found to be significantly increased in the heavy metal mixture treated group (group II) comparing to their corresponding values of –ve control group (group I) by about 38.9% and 20 times, respectively. The albumin value in group II showed a nonsignificant decrease by 14.16% compared to group I (–ve control group).

Administration of fungus extract prior to heavy metal mixture injection (group IV) showed a significant decrease in total bilirubin, compared to group II by 87.3% (0.12 ± 0.05

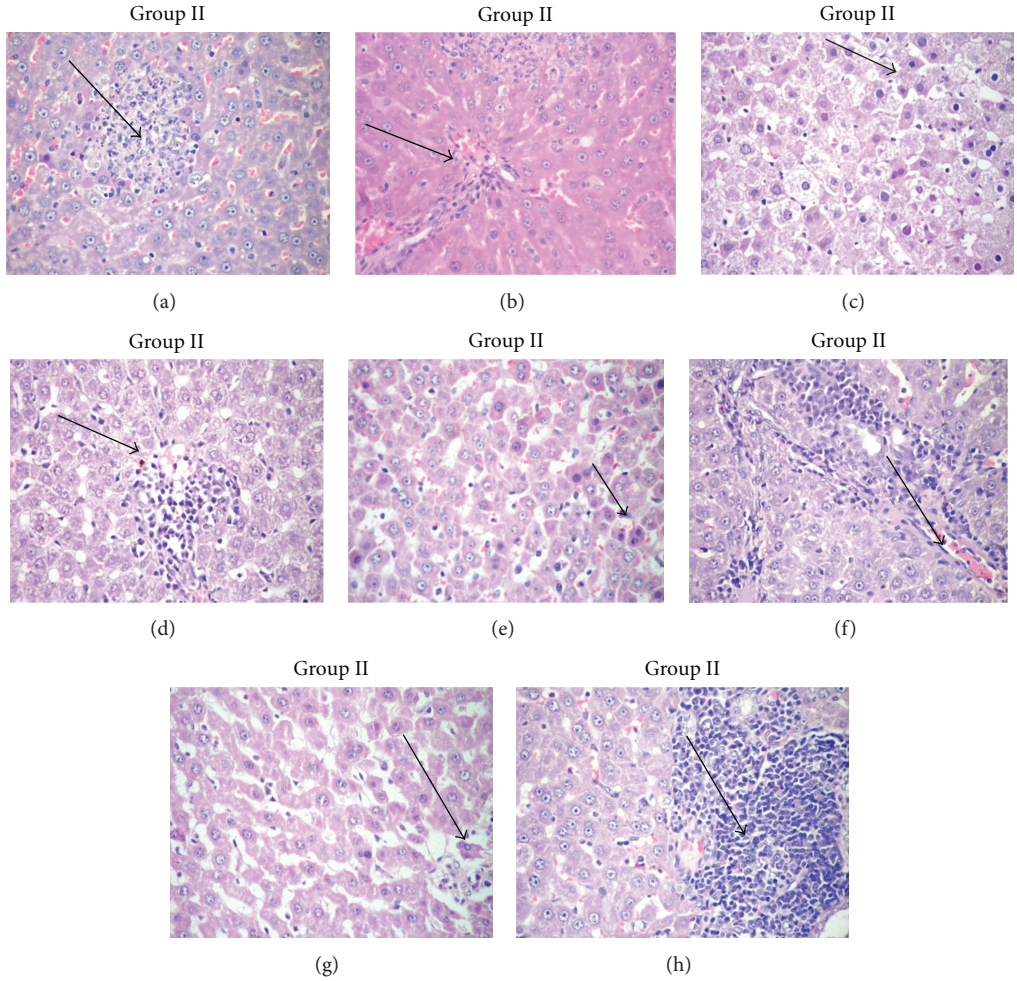


Figure 7: ((a) and (b)) Light microscopic observations on the histological liver structures of two individual rats from induced toxicity group (group II (a) and (b)). ((c) and (d)) Light microscopic observations on the histological liver structures of two individual rats from induced toxicity group (group II (c) and (d)). ((e) and (f)) Light microscopic observations on the histological liver structures of two individual rats from induced toxicity group (group II (e) and (f)). ((g) and (h)) Light microscopic observations on the histological liver structures of two individual rats from induced toxicity group (group II (g) and (h)).

Table 1: Livers of rats in the −ve control group showed normal histopathological appearance, where livers of rats treated with heavy metal mixtures that showed many histopathological changes are listed in the following table.

Figure	Histopathological change
(a)	Marked hepatic damage evidenced by foci of lobular necrosis with neutrophilic infiltration, adjacent to dysplastic hepatocytes, congested sinusoids, and frequent apoptotic nuclei
(b)	Marked large cell dysplasia of hepatocytes with focal necrosis and mild portal inflammatory infiltrate
(c)	Liver showing hydro pic changes in hepatocytes, and moderate portal lymphoplasmacytic infiltrate
(d)	Liver showing marked parenchymal hydro pic changes with apoptosis and adjacent regenerative hepatocytes with binucleated cells
(e)	Liver showing degenerative changes with frequent apoptotic cells at the same time evidence of beginning regeneration is seen with the appearance of binucleated cells
(f)	Section in the liver showing moderate portal lymphoplasmacytic infiltration with mild interface hepatitis. (H&E 400x)
(g)	Section in the liver showing intense heavy portal lymphoplasmacytic inflammatory infiltrate and dyspalstic changes of hepatocytes. (H&E 400x)
(h)	Liver showing coagulative necrosis and karyopickosis of the hepatocytes together with evidence of regeneration, multinucleated

Table 2: Effect of *Trichurus spiralis* extract on the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Group	ALT (U/mL) Mean ± SD	P_1	AST (U/mL) Mean ± SD	P_1
I (−ve control group)	80.91 ± 8.35		85.32 ± 8.16	
II (induced group)	119.28 ± 26.58	<0.001*	117.79 ± 26.56	0.003*
III (+ve control group)	75.18 ± 8.04	0.471	78.05 ± 13.54	0.470
IV (protected group)	65.45 ± 3.85	0.060	63.37 ± 20.54	0.037*

Number of rats for each group = 7, P_1: P value of LSD test between −ve control group and other groups.

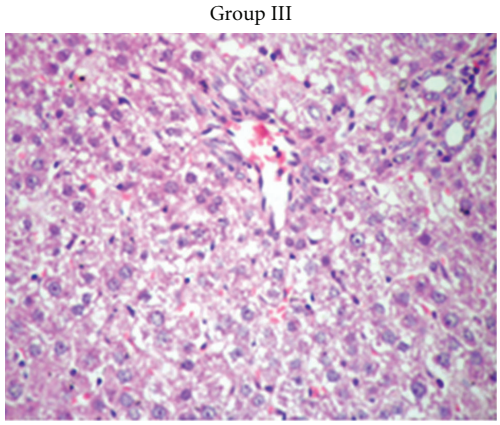


Figure 8: The liver showing normal hepatic architecture (H&E 400x).

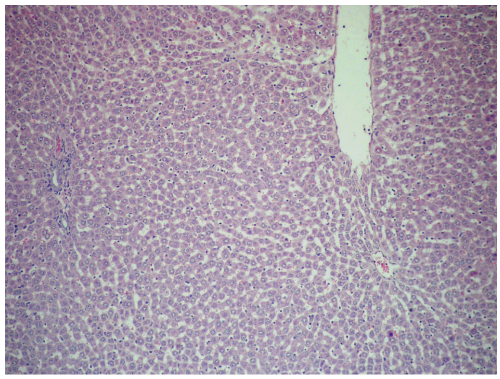


Figure 9: The liver showing preserved hepatic architecture with, mild portal inflammatory infiltrate and frequent apoptotic figures, group IV. (H&E 200x).

versus 0.95 ± 0.5 g/dL, $P < 0.001$), while the level of total protein shows a non-significant decrease compared to −ve control group. Also it showed a nonsignificant increase in albumin level between group IV and group I.

The levels of total protein, total bilirubin and albumin in group I, III, and IV showed a non-significant increase and/or decrease, when compared to each other, at $P < 0.05$, as shown in Table 3 and Figures 12, 13, and 14, respectively.

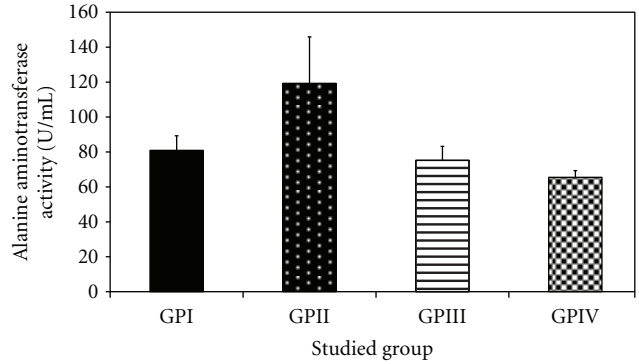


Figure 10: Effect of *Trichurus spiralis* extract on the activity of alanine aminotransferase (ALT, mean ± SD) in rat sera of induced toxicity group comparing to other groups.

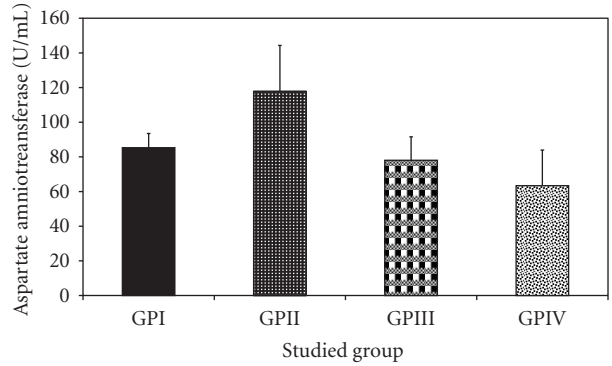


Figure 11: Effect of *Trichurus spiralis* extract on the activity of aspartate aminotransferase (AST, mean ± SD) in rat sera of induced toxicity group comparing to other groups.

4. Discussion

Liver damage mainly occurs due to excessive alcohol consumption, viral infections; and as a consequence of drug adverse effects. Nowadays, liver diseases constitute a major medical problem of worldwide proportions [77, 78].

There are approximately 35 heavy metals in our environment. Heavy metals become toxic when they are not metabolized, which allows them to accumulate in several organs leading to tissue damage due to their toxicity [79, 80]. According to ASTDR (2005–2007), the most known pollutants in our environment are Cd, Co, Hg, Ni, and Pb. On the other hand, liver tissues are the factory of biological

Table 3: Effect of *Trichurus spiralis* extract on the serum levels of total protein, albumin, and total bilirubin of induced toxicity groups of rats.

Mean ± SD Groups	I (−ve control group)	II (Induced group)	III (+ve control group)	IV (Protected group)
Total protein (g/dL)	4.60 ± 1.67	6.39 ± 1.06	5.65 ± 1.08	5.53 ± 0.56
P_1		**0.008***	0.101	0.146
Albumin (g/dL)	3.46 ± 0.30	2.97 ± 0.40	5.92 ± 1.87	3.89 ± 0.50
P_1		**0.369**	<0.001*	0.436
Total biluribin (g/dL)	0.04 ± 0.04	0.95 ± 0.50	0.04 ± 0.02	0.12 ± 0.05
P_1		<0.001*	0.992	0.571

Number of rats for each group = 7, P_1: P value of LSD test between control group and other groups.

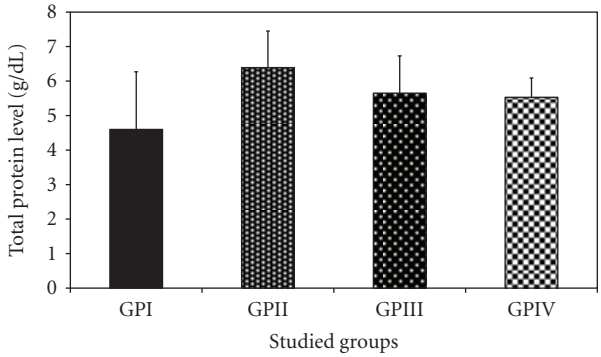


Figure 12: Effect of *Trichurus spiralis* extract on the level of total protein (mean ± SD) in rat sera of induced toxicity group compared to other groups.

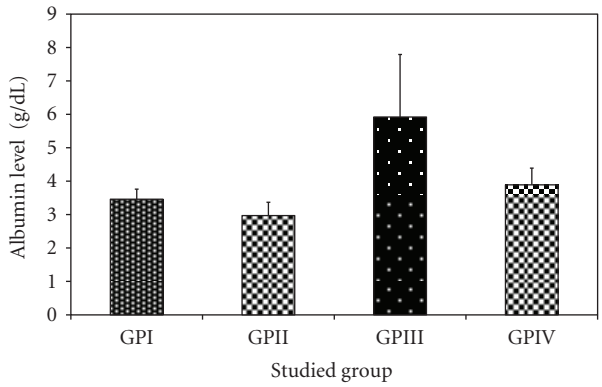


Figure 13: Effect of *Trichurus spiralis* extract on the level of albumin (mean ± SD) in rat sera of induced toxicity group compared to other groups.

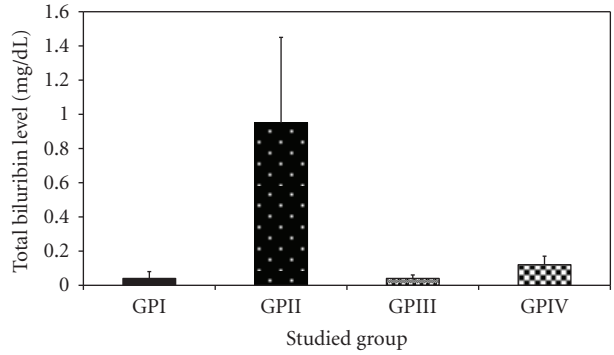


Figure 14: Effect of *Trichurus spiralis* extract on the level of total bilirubin (mean ± SD) in rat sera of induced toxicity group compared to other groups.

metabolism in mammals and act as the master player in the detoxification process. Liver tissues are also a victim for heavy metal toxicity. So, our *in vivo* study are designed to investigate the hepatoprotective effect of *trichuris* extract isolated from the marine sponge, against the heavy-metal mixture of Cd, Co, Hg, Ni chloride, and Pb acetate.

Hepatic system is the major organ system involved in metabolism, detoxification, and excretion of various endogenous and exogenously administered/ingested substances, like xenobiotics, pollutants, and so forth [81]. The physiological activity in the liver results is due to to the generation of highly reactive free radicals; which covalently bonds with membrane lipids, causing lipid preoxidation. Lipid preoxidation alters the membrane permeability and causes tissue damage [81]. Since the liver is involved in various biochemical reactions; it is prone to be attacked by the free radicals and cell necrosis resulted. However, inbuilt antioxidant systems like superoxide dismutase (SOD), reduced glutathione (GSH), and so forth protect the tissue from free radical attack [12]. Excessive release of ROS powers over this system resulted in organ damage. Strengthening of inbuilt protective mechanisms or exogenous administration of antioxidants may be useful in the protection of the organs from ROS damage. In spite of phenomenal growth of the allopathic system of medicine, synthetic antioxidant/organs protectants are not available. Hence, researchers worldwide are engaged in searching for organ protective, such as hepatoprotectants drugs from natural origin [82–84].

Natural products are of considerable importance for the discovery of new therapeutic agents [85]. Apart from plants, bacteria and fungi are the most important producers of such compounds [86]. For a long time neglected as a group of producers of natural products, marine microorganisms have more recently been isolated from a variety of marine habitats such as sea water, sediments, algae, and different animals, to discover new natural products [87, 88]. In particular, sponges which are filter feeders and accumulate high numbers of

microorganisms have attracted attention [89, 90]. Consistently, fungi isolated from sponges account for the highest number (28%) of novel compounds reported from marine isolates of fungi [45]. Marine isolates of fungi evidently are a rich source of chemically diverse natural products, which have not been consequently exploited so far [91].

Fungi, like all living organisms, have evolved a set of mechanisms that control and respond to the uptake and accumulation of heavy metals. Possible interactions between toxic metals and fungi include: (a) production and secretion of organic acids, polysaccharides, melanin, or proteins and subsequent binding/complexion and/or precipitation of metal ions [47, 92–94], (b) metal binding to cell walls [95], (c) transport of metal cations [95–97], (d) chemical transformation of metals [47], (e) organelle compartmentation [95–97], and (f) synthesis of thiol-containing compounds, such as the non-proteinacious glutathione, phytochelatins, and the metallothioneins proteins of families 8–13 (fungi I–VI MTs), which can sequester metal ions [47, 98–101]. Among microorganisms, fungi biomass is known to possess excellent metal-binding properties which offer the advantages of having the high percentage of cell wall material [102].

Metal-induced toxicity is very well reported in literatures [6, 12]. These metals generate reactive species, which in turn may cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals [8, 103]. The aminotransferase are intracellular enzymes, which are active in the operating the reversible exchange of amino acids between alpha-amino and alph-keto acids. As all the naturally occurring amino acids can undergo aminotransferase reaction, this class of intracellular enzyme (aminotransferases) represent an important link between protein and carbohydrate's metabolism. It is now well authorized that the liver has an important function through the regulation of trace element metabolism [104, 105]. Further trace elements serve as cofactors for many enzymes in numerous metabolic pathways, therefore, changes in the distribution of these essential and toxicological consequences with regard to the metabolism of other metals [105, 106]. Those metals which are essential for maintenance of the structural and functional integrality of the living organisms are found in all living systems and are conserved within strict concentration limits in the systems [107].

However, imbalance in the supply of any of these essential elements in the body can have both nutritional and toxicological consequences with regard to the metabolism of other metals. They can further be responsible for the development of clinical signs of trace elements deficiencies or can modify the susceptibility to metal toxicity [108]. It will insinuate that metals which have similar chemical, and physical properties, would often interact biologically and antagonize or embellish each other's function [109]. That is also confirmed in our study on the activity of aminotransferases, total protein, albumin, and bilirubin, which is confirmed by histopathology of liver as shown in Tables 2 and 3 and Figures 10, 11, 12, 13, and 14.

ALT and AST in this study show a significant increase as the consequence of heavy-metal administration in group II (induction group) by 46.20% and 45.12%, respectively. This result in agreement with previous studied, which carried on exposure to mercury chloride [110–114], and Ni [115]. Furthermore, this agrees with several investigators which reported that Cd and Pd administration increase aminotransferase, especially ALT, as a result of the necrotic lesion in the liver [114, 116, 117]. As seen in Figure 7(b), on the other hand, lead overloads stimulated oxidative damage in the liver tissue by causing oxidation of lipid. These enzymes cause liver injury [118]; this was confirmed in this study by the histopatholgical result of liver tissue induced by the heavy metal mixture alone (group II) as seen in Figures 7(a)–7(h).

Total protein, is elevated by about 38.9% in blood serum induction group compared to control group (−ve group). This observation may be as a result of the injury inflicted on the liver; thereby making the proteins synthesized in the liver and spill out into the blood [116]. Also, this result is compatible with [119, 120]. Possible explanation for protein elevation is due to toxic insult of mercury that leads to induce a number of stress proteins [119, 120]. These large groups of proteins include heat shock proteins (HSPs) and glucose regulated proteins (GRPs). As reported in [120], an enhanced *de novo* synthesis of several stress proteins when chick embryos, were exposed to mercury.

In contrast, albumin and protein are predominately produced within the liver, decreased as a consequence of metal elevation. This suggests that the heavy metals, like cadmium and lead, occurs when present in toxic concentrations in the system, impair the protein synthesis in liver [116].

Bilirubin is also regarded as a member of an antioxidant family even if it is known to have toxic effects at high concentration [121, 122]. Bilirubin has been regarded for many years as cytotoxic, mainly because of its associations with neonate jaundice at high concentrations [123]. It is only since the early 1990s that a physiological role for bilirubin as potent antioxidant has emerged. Reference [124] noted that bilirubin possesses strong antioxidant potential against peroxyl radicals. However, high level bilirubin may exacerbate oxidative stress [122]. Reference [125] showed that the increase of bilirubin formation due to activation of HO-2 (constitutive isoform of HO) protects against hydrogen-peroxide-induced neurotoxicity. It has been also demonstrated that intracellular bilirubin concentrations can be locally and temporarily increased by induction of HO-1 (inducible isoform of HO) or rapid activation of HO-2, so as to resist short- and long-lasting oxidative stress [123].

It has been proposed that the specific induction of HO-1 by various forms of oxidative stress, for example, different heavy metals, CCL4, and aminoacetophenone, was part of the defensive mechanism mounted by cells against stress injury, to decrease the levels of potential pro-oxidants and to increase the concentrations of active bile pigments that can act as antioxidants [126, 127]. HO-1 upregulation is followed by an increased bilirubin production, altogether determining the adaptive response of cells to oxidative stress [126].

Some natural antioxidant products have been shown to protect cells from oxidative injury [128], the high antioxidant capacity of fungal extract as shown in Figure 4, due to its high total phenolic and flavonoid content shown in Figure 3, which is confirmed *in vitro*. From the *in vitro* results, the *Trichurus* extract has high flavonoid content, where flavonoids are best

known for their antioxidant properties and may act *in vitro* as reducing agents, hydrogen donors, free radical quenchers, and metal ion chelators [129].

It has been demonstrated previously that fungi, as well as algae are potentially biosorbent, for heavy metals [130–132]. This fact has also been confirmed in the present study.

According to [133], this general chelating ability of phenolic compounds is probably related to the high nucleophilic character of the aromatic rings, rather than to be specific chelating groups within the molecule. This agrees with our results, as the extract shows high total polyphenol content (phenolic and flavonoid content) as shown in Figure 3. There is another mechanism underlying their antioxidant ability. Metal ions decompose lipid hydroperoxide (LOOH) by the hemolytic cleavage at the O–O bond and give lipid alkoxyl radicals, which initiate free radical chain oxidation. Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical; and that was confirmed by our results, as the fungal extract showed a high antioxidant activity and a high inhibition ratio to lipid peroxidation *in vitro*, by a percentage of 70.80% and 85%, respectively, as shown in Figure 4. This activity depends on the structure of the molecules, the number, and position within the hydroxyl group in the molecules [134]. Many flavonoids have also been found to possess hepatoprotective activity [135].

Reference [136] show that phenolic (especially flavonoids) is able to alter peroxidation kinetics, by modifying the lipid packing order. They stabilize membranes by decreasing the membrane fluidity (in a concentration-dependent manner) and hinder the diffusion of free radicals and restrict per oxidative reaction [136, 137]. According to [138], in addition to the known protein-binding capacity of flavanols and procyanidins, they can interact with membrane phospholipids through hydrogen bonding, to the polar head groups of phospholipids. As a consequence, these compounds can be accumulated at the membranes' surface, both outside and inside the cells.

For this activity, polyphenols possess an ideal structural chemistry and have been shown to be more effective *in vitro* than vitamins E and C on the molar basis [139]. Many beneficial pharmacological properties have been attributed to flavonoids, including antioxidant, anti-inflammatory, anticarcinogenic, chemo preventive, and cytochrome-P450-inhibitory activities [140, 141].

In addition to high total phenolic and flavonoid content for fungal extract, the fungal extract shows high sulfur content, where sulfur is an essential component in normal physiological function and is incorporated into amino acids, proteins, enzymes, and micronutrients [142]. Humans satisfy their nutritional needs of sulfur by consuming plants and animals [143]. The high content of sulfur due to marine chemodiversty is also heightened due to the composition of sea water, which has itself a concentration of halides in sea water of 1900 mg/L Cl^-, 65 mg/L Br^-, 5×10^{-4}, and $I/IO_3{}^-$, which are reflected by the number of compounds incorporating these elements and sulfated compounds that can account for by the relatively high concentration of sulfur, 2700 mg/L seawater. That is confirmed in our study as shown in Figure 2 [144].

Marine natural product in general, and especially marine fungi, can be good hepatoprotective candidates in using the heavy metal as a toxicology model. AS in the bioassay-directed searching for the hepato-protective agents from natural sources, employing the closely relevant model system to human liver toxicosis, could be an effective way to identify therapeutically applicable agents [145].

5. Conclusion

In conclusion, there is a beneficial influence of the investigated fungus extract against heavy-metal mixtures-intoxicated rats. We could confirm that this extract possesses hepatoprotective property due to its proven antioxidant and free radical scavenging properties, in addition to its high sulfur content. However, other possible mechanisms such as inhibition of antioxidant enzymes, induction of oxidative stress, and the influence on different signal pathways in liver cells should not be neglected. Further investigations of these matters are warranted, particularly that of fungus extract, as well as elucidation of compounds that are responsible for such activities and their effect on liver antioxidant capacity, which should be carried out.

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Research Article

The Sonodynamic Effect of Curcumin on THP-1 Cell-Derived Macrophages

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Curcumin is extracted from the rhizomes of the traditional Chinese herb *Curcuma longa* and has been proposed to function as a photosensitizer. The potential use of curcumin as a sonosensitizer for sonodynamic therapy (SDT) requires further exploration. This study investigated the sonodynamic effect of curcumin on macrophages, the pivotal inflammatory cells in atherosclerotic plaque. THP-1-derived macrophages were incubated with curcumin at a concentration of 40.7 μmol/L for 2 h and then exposed to pulse ultrasound irradiation (2 W/cm^2 with 0.86 MHz) for 5–15 min. Six hours later, cell viability was decreased in cells that had been treated with ultrasound for 10 and 15 min. After ultrasound irradiation for 15 min, the ratio of apoptotic and necrotic cells in SDT group was higher than that in ultrasound group, and the ratio of apoptotic cells was higher than that of necrotic cells. Both loss of mitochondrial membrane potential and morphological changes of cytoskeleton were apparent 2 h after treatment with curcumin SDT. These findings support that curcumin had sonodynamic effect on THP-1-derived macrophages and that curcumin SDT could be a promising treatment for atherosclerosis.

1. Introduction

Atherosclerosis poses a severe threat to human health. Most acute cardiovascular events result from the rupture of an atherosclerotic plaque, and macrophages play a crucial role in the progression [1–3]. Decreasing the infiltration of an atherosclerotic plaque by macrophages could stabilize the plaque and inhibit its progression. Photodynamic therapy (PDT) for atherosclerosis is a new treatment modality that has been proven to induce plaque regression in animal atherosclerosis models [4, 5]. The mechanism may involve macrophage apoptosis induced by PDT [6]. However, PDT has two recognized drawbacks: (i) it can only be applied to superficial lesions because of the limited penetration of light into tissues, even though atherosclerotic lesions may exist deep in the human body. (ii) PDT-treated patients tend to suffer long-lasting skin sensitivity due to the retention of photosensitizers in skin, and they may need to spend several weeks in the dark after such treatment.

To resolve the problem of tissue penetration, another method called sonodynamic therapy (SDT) has been investigated. Ultrasound has an appropriate tissue attenuation coefficient, allowing it to penetrate into tissues and reach non-superficial objects while maintaining the ability to focus energy into small volumes and activate sonosensitizers. Among noninvasive treatment options, this advantage is unique compared to the use of laser light for photodynamic therapy. The basis of the therapy is to administer a small amount of sonosensitizer, which is selectively taken up by target cells, and then expose the target lesion to ultrasound to activate sonosensitizer [7]. Until now, there have been extensive investigations of the effects of SDT on tumors [8].

However, this technique has not been applied to atherosclerosis.

The sonosensitizer is crucial during SDT in order to enhance the cytotoxicity of the ultrasound. Photochemically active hematoporphyrin derivatives (HPDs), including hematoporphyrin, photofrin II, ATX-70, and ATXS10, have been demonstrated to induce cell death when activated by ultrasound irradiation, indicating that these chemicals, which were originally generated for PDT, are applicable as sonosensitizers [9]. However, HPDs are likely to cause photodermatitis and are not generally used in clinical practice. To avoid photodermatitis, development of a new sonosensitizer that can be widely used is necessary.

Curcumin is the major constituent of *turmeric* powder, which is extracted from the rhizomes of the plant *Curcuma longa*. As a powder, *turmeric* is widely used as a coloring and flavoring spice in foods as well as in folk medicine for the management of various inflammatory disorders and wound healing [10]. Curcumin has been described as having antioxidant, anti-inflammatory, and anticarcinogenic properties [11–13]. It can also protect against lipid-induced damage in the inflammatory cells of the vascular system by the upregulation of FOXO3a activity [14]. To our knowledge, there has been no report of photodermatitis caused by curcumin. In recent years, curcumin derivatives have been demonstrated to reduce aortic fatty streak formation and to protect animal models against atherosclerosis [15–17]. It has been reported that the use of curcumin as a photosensitizer has induced apoptosis of tumor cells through activation of caspase pathways [18]. However, whether curcumin could be used as a sonosensitizer is still unknown.

We hypothesized that curcumin would have a sonodynamic effect on macrophages, which would enable curcumin SDT to potentially be used as a treatment for atherosclerosis. In this study, we used curcumin to mediate the effects of SDT on macrophages in order to determine whether it can induce cell apoptosis.

2. Materials and Methods

2.1. Chemicals. Curcumin was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The reagent was a commercial product of analytical grade with purity ≥ 98%. It was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma, USA) and stored at −20°C in dark. The stock solutions were diluted 10- to 10^3-fold in the final experimental conditions. The final concentration of DMSO in cells was 0.1%. RPMI Medium (1640) and bovine fetal serum were obtained from HyClone Chemical Co. (HyClone, Logan, UT, USA). Phorbol-12-myristate-13-acetate (PMA) was obtained from EMD Biosciences, Inc. (La Jolla, USA). Hoechst 33342 and propidium iodide (PI) were from Sigma. JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) probe was purchased from Beyotime (China). A rabbit polyclonal antibody against vimentin (ab8545) was purchased from Abcam (Hong Kong) Ltd. A rabbit polyclonal antibody against β-tubulin (H-235) and a goat polyclonal antibody against α-actin (C-11) were purchased from Santa Cruz Biotechnology, Inc (USA). All other reagents were obtained from Sigma Chemical Co. Ltd.

2.2. Measurement of Absorption and Fluorescence Spectra of Curcumin. The absorption spectrum of curcumin was measured with a spectrophotometer (USB 2000, Ocean Optics Incorporated, FL, USA) under a 40 W wolfram lamp. The fluorescence spectrum of curcumin was measured with a spectrophotometer at a wavelength of 405 nm.

2.3. Cell Culture. Human THP-1 cells [19] (ATCC, USA) were cultured in RPMI Medium (1640) supplemented with fetal bovine serum (FBS) and Penicillin-Streptomycin (56.1 μmol/L penicillin and 27.4 μmol/L streptomycin). The cells were maintained at 37°C, with 5% CO_2/95% air in a humidified incubator, and they were harvested for passage when they reached confluence. For experiments, cells were plated into microculture plates at 1×10^5 cells/mL (Costar, Corning Incorporated, USA) in their usual medium plus PMA at a concentration of 16.2 μmol/L (a final concentration of 162 nmol/L) for 72 h. Then the medium was removed and replaced with fresh medium without PMA.

2.4. Detection of Intracellular Uptake of Curcumin. Cells were grown to confluence in 24-well culture plates in standard culture conditions. Curcumin was added at a final concentration of 13.6–81.4 μmol/L to the wells, which had been seeded with 1×10^4 cells/mL. After 2 h, the cells were washed twice with PBS, and the curcumin that was taken up by the cells was examined by a fluorescence microscope (IX71, OLYMPUS, Japan) using a filter with an excitation wavelength of 420–480 nm and an emission wavelength of 480–550 nm.

2.5. MTT Assay after SDT. The cells were seeded into flat plates with a diameter of 3.5 cm and incubated with 40.7 μmol/L curcumin for 2 h in the dark. They were then exposed to pulse ultrasound (Sheng Xiang Technology, 838A-H-O-S multifunctional ultrasonic therapeutic device, China) at a power of 2 W/cm^2 with 0.86 MHz for 5–15 min [20]. Control plates were sham-exposed to ultrasound. After SDT, each flat plate was incubated for 6 h. The survival rate of the cells was measured by MTT assay. All experiments were repeated three times independently.

2.6. Hoechst/PI Staining after SDT. The cells were divided into four groups including control (cells alone), curcumin treated (40.7 μmol/L), ultrasound-treated (2 W/cm^2 for 15 min), and SDT (ultrasound 2 W/cm^2 for 15 min and curcumin 40.7 μmol/L). Six hours after SDT, the cells were stained with Hoechst 33342 at 8.1 μmol/L for 5 min, and they were then stained with PI at 15.0 μmol/L for 5 min. The cell monolayer was washed twice with PBS and then examined under a fluorescence microscope with an excitation wavelength of 330–385 nm and emission wavelength of 420–480 nm. The percentages of apoptotic and necrotic cells were calculated from the total cell numbers. All cells from ten random microscopic fields at 40x magnification were counted. Experiments were repeated three times independently.

2.7. Cytoskeletal Protein Immunofluorescent Staining after SDT. Two hours after SDT, the cells were fixed with paraformaldehyde (PFA). Then the cells were perforated with a detergent such as Triton X-100 to allow exposure of the antibodies to the structures inside the cells. To avoid nonspecific binding of the second antibody, the cells were blocked with 1% BSA at room temperature for 1 h. Primary antibodies without fluorophore were added at 37°C for 1 h. Then the secondary antibody, which was conjugated with FITC (fluorescein isothiocyanate), was added at 37°C for 2 h. DAPI was added at room temperature for 2 min. The cell monolayer was washed twice with PBS and then examined under a fluorescence microscope with an excitation wavelength of 330–385 nm and emission wavelength of 420–480 nm, as well as an excitation wavelength of 420–480 nm and an emission wavelength of 480–550 nm. The status of cytoskeletal protein polymerization was quantitated by randomly choosing 10 microscopic fields at 40x magnification and counting cells in the field as either having cytoskeletal filaments that were intact or disturbed. The proportion of cells with disturbed cytoskeletal filaments was expressed as "the number of cells with disturbed cytoskeletal filaments/the total number of cells."

2.8. Mitochondrial Membrane Potential (MMP, $\Delta\psi m$) Assay after SDT. The loss of mitochondrial membrane potential ($\Delta\psi m$) was quantitatively determined by flow cytometry using the lipophilic cationic probe JC-1. When the cell is in a normal state, MMP is high and JC-1 predominantly appears as red fluorescence. When the cell is in an apoptotic or necrotic state, the MMP is reduced, and JC-1 appears as a monomer indicated by green fluorescence [21]. A change in the florescence from red to green indicates a decrease in the MMP. Tow hours after SDT, the cells were then washed with PBS and incubated with JC-1 working solution for 20 min at 37°C in the dark. Cells were washed with PBS and resuspended in 500 μL PBS. The stained cells were analyzed by flow cytometry to determine the change in the florescence from red to green.

2.9. Statistical Analysis. All values are expressed as the means ± standard deviation. The Dunnett-T and SNK tests were used to assess the effects of varying curcumin concentration without irradiation on cell viability. The LSD and SNK tests were used to assess the effects of sonoactivated curcumin on cell viability. P value < 0.05 was considered to be significant.

3. Results

3.1. Intracellular Accumulation of Curcumin. As shown in Figure 1, the absorption wavelength of curcumin was less than 520 nm, and the fluorescence emission wavelengths of curcumin ranged from 470 nm to 700 nm. The dye appeared to be distributed throughout cells, and in some cells, it was distributed in the cytoplasm only.

3.2. Cell Viability after Curcumin SDT. As shown in Figure 2, curcumin SDT decreased cell viability more significantly [from 78.46 ± 8.22% (10 min) to 51.69 ± 9.39% (15 min)] than treatment with ultrasound alone [from 90.50 ± 4.74% (10 min) to 73.51 ± 9.42% (15 min)]. Cell viability was not significantly affected in cells treated for 5 min ($P > 0.05$). Treatment with curcumin alone did not affect cell viability compared to control ($P > 0.05$). DMSO at a concentration of 0.1% showed no effect on cell viability after ultrasound irradiation (data not shown).

3.3. The Apoptosis and Necrosis of Cells. As shown in Figure 3(A), the ratio of apoptotic cells in the SDT and ultrasound-treated groups was higher than that of control (34.90 ± 4.01% versus 4.41% ± 2.98%, $P < 0.01$; 25.02 ± 7.45% versus 4.41% ± 2.98%, $P < 0.01$). The ratio of apoptotic cells in the SDT group was higher than that in the ultrasound group (34.90 ± 4.01% versus 25.02 ± 7.45%, $P < 0.01$). The ratio of necrotic cells in the SDT group and the ultrasound group was higher than that of control (16.91 ± 5.01% versus 2.26% ± 1.10%, $P < 0.01$; 4.97 ± 2.31% versus 2.26%± 1.10%, $P < 0.05$). The ratio of necrotic cells in the SDT group was higher than that in the ultrasound group (16.91 ± 5.01% versus 4.97 ± 2.31%, $P < 0.01$). There was no difference apoptotic and necrotic cells between the curcumin-treated group and the controls. As shown in Figure 3(B), normal cells showed uniform blue fluorescence; apoptotic cells were seen as bright blue fluorescence spots, and necrotic nuclei were identified by the presence of staining with PI, which was evident as pink fluorescence.

3.4. Morphological Changes of the Cytoskeleton. As shown in Figure 4(A), control cells showed a regular cytoskeletal network (green fluorescence), and the nucleus showed uniform blue fluorescence. The fluorescence signal of the cytoskeletal protein was slightly attenuated 2 h after treatment in some cells, as shown in Figure 4(A)-a3, b3, and c3. In the case of cells treated with curcumin SDT, α-actin, β-tubulin, and vimentin filaments diffused obviously, formed clusters, and the plasma membrane lost its normal structure shown in Figure 4(A)-a4, b4, and c4. As shown in Figure 4(B), the percentage of cells with disturbed cytoskeletal filaments in the SDT group and the ultrasound group was higher than that in the control group (α-actin: 53.41 ± 9.48% versus 6.72% ± 2.54%, $P < 0.01$; 23.42 ± 5.43% versus 6.72% ± 2.54%, $P < 0.01$. β-tubulin: 49.89 ± 9.13% versus 6.06 ± 2.61%, $P < 0.01$; 21.94 ± 6.72% versus 6.06 ± 2.61%, $P < 0.01$. Vimentin: 44.48 ± 12.48% versus 4.95% ± 3.44%, $P < 0.01$; 18.83 ± 2.25% versus 4.95% ± 3.44%, $P < 0.01$). The percentage of cells with disturbed cytoskeletal filaments in the SDT group was higher than that in ultrasound group (α-actin: 53.41 ± 9.48% versus 23.42 ± 5.43%, $P < 0.01$. β-tubulin: 49.89 ± 9.13% versus 21.94 ± 6.72%, $P < 0.01$. Vimentin: 44.48 ± 12.48% versus 18.83 ± 2.25%, $P < 0.01$). There was no difference between the curcumin-treated group and the controls.

3.5. The Changes of the Mitochondrial Membrane Potential after SDT. As shown in Figure 5, the relative green signals of normal macrophages (Figure 5(a)) and cells with curcumin alone (Figure 5(b)) were 16.40 ± 2.44% and 17.14 ± 2.17% (versus control, $P > 0.05$), respectively. Those of the

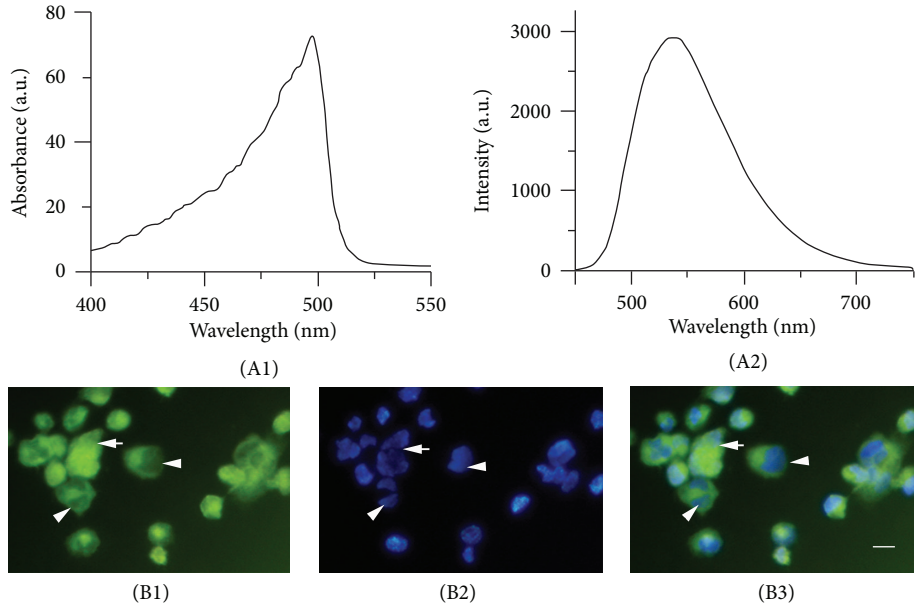

Figure 1: The spectrum of curcumin and the intracellular accumulation of curcumin (×400). Panel A1 is the absorption spectrum of curcumin under a wolfram lamp. panel A2 is the fluorescence emission spectrum of curcumin at 405 nm. Panel B1 is curcumin fluorescence in unfixed macrophages. Panel B2 is nuclei of the same cells after staining with Hoechst 33342. Panel B3 is a merged image of B1 and B2. Scale bar: 20 μm. The dye appeared to be distributed throughout the cells (arrows), and in some cells, it was distributed in the cytoplasm only (arrowhead).

macrophages exposed to ultrasound alone (Figure 5(c)) and the macrophages subjected to curcumin SDT (Figure 5(d)) were 37.78 ± 4.17% (versus control, $P < 0.01$) and 68.23 ± 3.80% (versus control and ultrasound alone group, $P < 0.01$), respectively. There is a substantial shift in fluorescence emission, indicating a decrease in MMP in the SDT group (Figure 5(e)) compared to other groups ($P < 0.01$).

4. Discussion

PDT is based on the principle of energy transfer from light to a photosensitizer to tissue. When an excited photosensitizer returns to the ground state and interacts with molecular oxygen, reactive oxygen species (ROS) are formed [22]. ROS promote photoinduced damage to biological molecules including lipids, proteins, and DNA [23]. Consequently, cell death occurs. The mechanism of the cytotoxicity in SDT seems to be theoretically similar to that in PDT. In SDT, the activation of HPDs through acoustic cavitation by ultrasound is attributed to the generation of active oxygen [24]. When a sonosensitizer is exposed to sonoluminescent light, it is activated from its ground state into an excited state; as the activated sonosensitizer returns to the ground state, the energy is released. Functional groups are required to accomplish this energy transition. The more conjugated macro-π bonds inside sensitizer structures, the longer the absorption wavelength of the sensitizers. The number of macro-π bonds inside curcumin is less than that of HPDs, so the absorption wavelength of curcumin is shorter than that of HPDs. Through measurement of the absorption spectrum, it was discovered that curcumin absorbed light at wavelength of

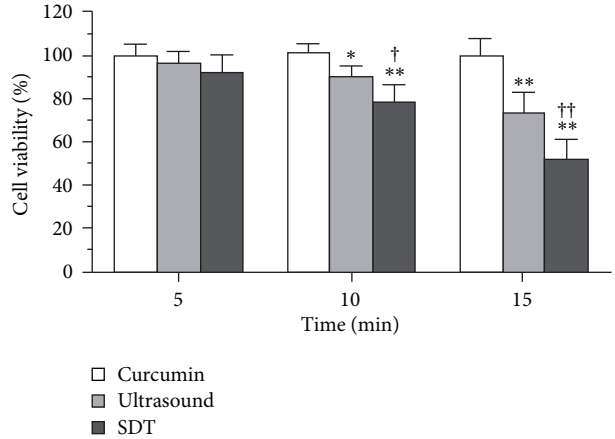

Figure 2: Cell viability after curcumin SDT. The effect of curcumin SDT on cell viability was assessed by MTT assay. Our results showed that the cell viability decreased with increasing time of ultrasound exposure. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus ultrasound alone; $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

less than 520 nm. The penetration depth of light depends on the wavelength. For example, wavelengths of 600–1000 nm can penetrate around 8–10 mm [25]. To the issue resolve penetration, ultrasound was used to activate curcumin in this study.

During SDT, accumulation of the sensitizer in the target lesion is vital. The sensitizer targets and accumulates in metabolically active inflammatory cells, such as macrophages in an atheromatous plaque [2]. In this study, the uptake

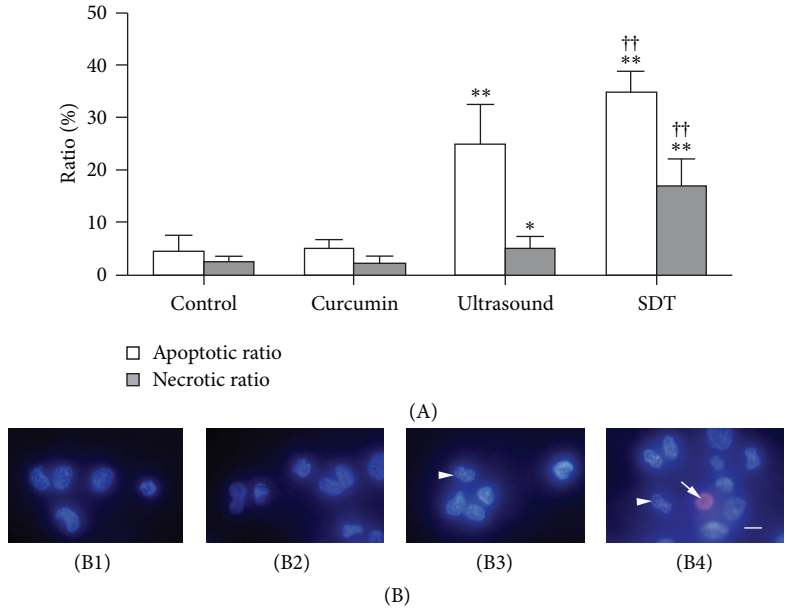

Figure 3: Apoptosis and necrosis of macrophages induced by curcumin SDT (×400). Panel A is the ratio of apoptotic and necrotic macrophages 6 h after curcumin SDT. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control; $^{\dagger\dagger}P < 0.01$ versus ultrasound alone. B1 is control; B2 is curcumin alone; B3 is ultrasound irradiation alone; B4 is curcumin SDT. Scale bar: 20 μm. Control and curcumin-treated cells showed uniform blue fluorescence in B1 and B2. Apoptotic cells were seen as bright blue fluorescent spots and are shown in B3 and B4 (arrowheads). Necrotic nuclei showed pink fluorescence in B4 (arrow).

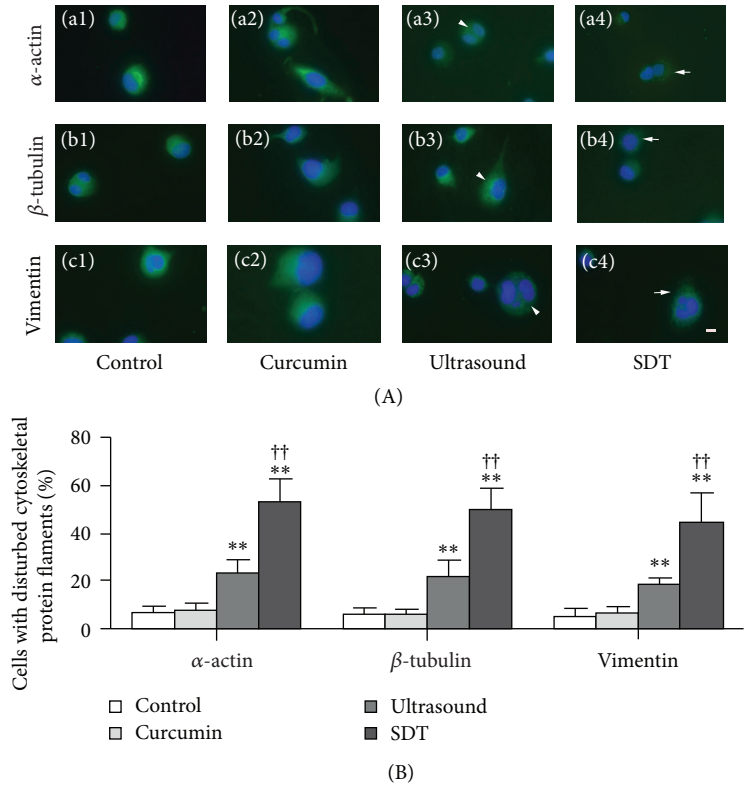

Figure 4: Changes in the cytoskeleton induced by curcumin SDT (×400). (A)-a1, b1, and c1 and (A)-a2, b2, and c2 are control and curcumin-treated cells showed a regular cytoskeletal network. (A)-a3, b3, and c3 are the fluorescence signal of cytoskeletal protein was slightly attenuated, as shown by arrowhead. (A)-a4, b4, and c4 are in some cells, cytoskeletal protein filaments diffused obviously, formed clusters and the plasma membrane lost its normal structure, as shown by arrow. Scale bar: 20 μm. (B) is the percentage of cells with disturbed cytoskeletal filaments in each group. $^{**}P < 0.01$ versus control, $^{\dagger\dagger}P < 0.01$ versus ultrasound group.

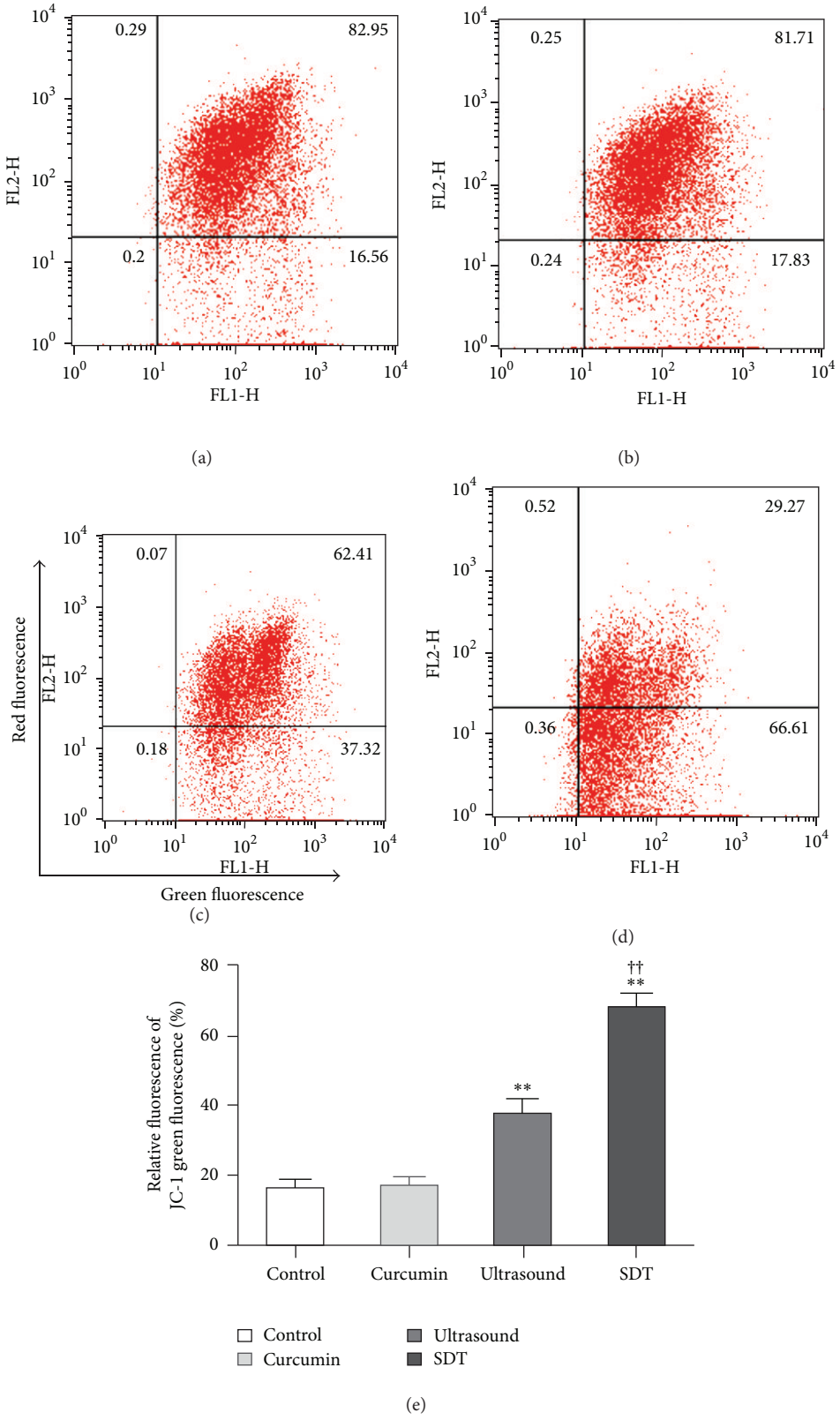

Figure 5: The change in mitochondrial membrane potential by curcumin SDT. (a) is control; (b) is curcumin alone; (c) is ultrasound irradiation alone; (d) is curcumin SDT; (e) is The representative dot plots from a single analysis are shown with the percentage of the cells in the lower right (LR) quadrant that emits only green fluorescence indicating the depolarized mitochondrial membrane. $^{\dagger\dagger}P < 0.01$ versus ultrasound alone; $^{**}P < 0.01$ versus control.

of curcumin by macrophages was detected. It was shown the accumulation of curcumin in macrophages increased in accordance with its concentration. The cytotoxicity curcumin also depended on its concentration. The results indicated that curcumin concentration over 81.4 μmol/L would kill macrophages; on the contrary, curcumin concentrations below 13.6 μmol/L did not exhibit intracellular drug fluorescence (data not shown). Liposoluble sensitizers likely enter cells through LDL-R [26], and because curcumin is liposoluble, it probably enters macrophages through this receptor.

The cell survival rate in the curcumin SDT group was much lower than that in the ultrasound alone group under the same exposure conditions, while curcumin alone had little effect (Figure 2). Cell viability decreased gradually as the amount of ultrasound irradiation increased. This indicated that curcumin SDT can effectively kill macrophages in vitro. Moreover, cell viability in curcumin SDT was not altered when cells contained only intracellular curcumin. The MTT test provided some information concerning the function of mitochondria; however, it did not assess the late, irreversible changes that would indicate the mode of cell death. The Hoechst-PI assay was therefore more informative than the MTT assay. Apoptotic nuclei presented bright blue fluorescence spots accompanied by nucleus deformation while necrotic nuclei presented pink fluorescence (Figure 3(B4)). Because of resistance to fluorescence dye, the nuclei of live cells presented uniform blue fluorescence (Figure 3(B1)). In this study, ultrasound exposure alone could induce cell death, which became obvious when the amount of irradiation was augmented. Furthermore, this effect was highly enhanced when curcumin was added to the cells. The ratios of both apoptotic and necrotic cells increased. There was a synergistic relationship between curcumin and ultrasound. Therefore, curcumin may be a promising natural sonosensitizer when used at the proper concentration combined with the appropriate amount of ultrasound irradiation for treatment of atherosclerosis. Hematoporphyrin-SDT induced apoptosis of tumor cells through a mechanism that involved the mitochondria-caspase signaling pathway [27]. The mechanism of macrophage apoptosis induced by curcumin SDT may also involve activation of the mitochondria-caspase signaling pathway.

Oxidative stress induced by PDT can affect several types of biomacromolecules including proteins, lipids, and DNA. Deleterious effects of PDT on the cytoskeletal proteins have been documented [28, 29]. SDT may also affect the cytoskeleton through a mechanism similar to that of PDT. Cytoskeletal F-actin might represent an important target for the SDT treatment [30]. In this study, α-actin, β-tubulin, and vimentin were detected. The fluorescence signal of cytoskeletal proteins in the cells treated with ultrasound alone was partially attenuated, and this attenuation was greatly enhanced by adding curcumin. Cytoskeletal filaments were cleaved and formed clusters. The plasma membrane lost its normal structure and became deformed as blebs. No obvious deformation of the cytoskeleton was observed in cells treated with curcumin alone or controls. It is possible that the disruption of the cytoskeleton was one of the causes of cell death induced by curcumin SDT.

Mitochondria-mediated cell death plays a crucial role in the pathophysiology of atherosclerosis. The loss of mitochondrial membrane potential was the upstream event for apoptosis [31]. Opening of the mitochondrial permeability transition pore (mPTP) induces swelling of mitochondria, leading to rupture of the mitochondrial outer membrane (MOM), and rupture of the MOM results in release of cytochrome c into the cytosol, triggering apoptosome formation [32]. The voltage-dependent anion channel (VDAC) lies in the outer mitochondrial membrane (OMM) and forms a common pathway for the exchange of metabolites between the mitochondria and the cytosol, thus playing a crucial role in the regulation of metabolic and energetic functions of mitochondria. VDAC appears to be a convergence point for a variety of cell survival and cell death signals, mediated by its association with various ligands and proteins [33, 34]. It was also proposed that VDAC and tubulin form a supercomplex with MtCK, which is structurally and functionally coupled to the ATP synthasome [35]. Actin-VDAC interactions are not a species-specific oddity and may be a more general phenomenon, the role of which ought to be further investigated [36]. Thus, VDAC interactions with actin and tubulin may have broader implications for various mitochondrial processes, including interactions between mitochondria and the cytoskeleton, in turn affecting mitochondrial dynamics [33]. In the present study, we have shown that ultrasound with or without curcumin results in the loss of mitochondrial membrane potential, and this loss was greatly enhanced by adding curcumin. The precise mechanism of how curcumin SDT linked to these mitochondrial events remains to be determined.

Currently, many possible sonosensitizers have been investigated, but few are approved for clinical use. Curcumin is widely used as a coloring and flavoring spice. Our results suggest that curcumin SDT may therefore be a useful clinical treatment for atherosclerosis. Whether curcumin SDT can induce atherosclerotic plague regression will require further study in animal models.

5. Conclusions

Curcumin had sonodynamic effect on THP-1-derived macrophages. Curcumin SDT decreased macrophages viability obviously and induced apoptosis or necrosis of macrophages. Both loss of mitochondrial membrane potential and morphological changes of cytoskeleton were apparent after treatment with curcumin SDT. In conclusion, curcumin is a new sonosensitizer, and curcumin SDT could be a promising treatment for atherosclerosis.

Acknowledgments

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Research Article

Cytotoxic Effect of Ethanol Extract of Microalga, *Chaetoceros calcitrans*, and Its Mechanisms in Inducing Apoptosis in Human Breast Cancer Cell Line

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Marine microalgae have been prominently featured in cancer research. Here, we examined cytotoxic effect and apoptosis mechanism of crude ethanol extracts of an indigenous microalga, *Chaetoceros calcitrans* (UPMAAHU10) on human breast cell lines. MCF-7 was more sensitive than MCF-10A with IC50 value of 3.00 ± 0.65, whilst the IC50 value of Tamoxifen against MCF-7 was 12.00 ± 0.52 μg/mL after 24 hour incubation. Based on Annexin V/Propidium iodide and cell cycle flow cytometry analysis, it was found that inhibition of cell growth by EEC on MCF-7 cells was through the induction of apoptosis without cell cycle arrest. The apoptotic cells at subG0/G1 phase in treated MCF-7 cells at 48 and 72 hours showed 34 and 16 folds increased compared to extract treated MCF-10A cells which showed only 6 and 7 folds increased at the same time points, respectively. Based on GeXP study, EEC induced apoptosis on MCF-7 cells via modulation of CDK2, MDM2, p21Cip1, Cyclin A2, Bax and Bcl-2. The EEC treated MCF-7 cells also showed an increase in Bax/Bcl-2 ratio that in turn activated the caspase-dependent pathways by activating caspase 7. Thus, marine microalga, *Chaetoceros calcitrans* may be considered a good candidate to be developed as a new anti-breast cancer drug.

1. Introduction

Breast cancer is the most prevalent cancer in the world (22% of all the cases) and causes the highest percentage of the cancer deaths (14% of all cancer deaths) in women worldwide. In fact, it is the most common female cancer in both developing and developed countries [1]. There are different options of treatment for breast cancer like surgery, radiation therapy, hormone therapy, chemotherapy, and targeted therapy but these therapies are also associated with some serious side effects. Algae have been widely used by millions of humans and animals around the world as nutritional or pharmaceutical ingredient. Many studies have shown that some algae contain various biologically active substances with potential therapeutic applications in humans [2].Due to the diverse structural forms and biological activities of marine microalgae, they can be used as a valuable source of molecules for new drug development, including novel anticancer compounds [3]. Indigenous microalgae from Malaysia such as *Chaetoceros calcitrans* have been shown to be potential sources of high-value chemicals including polyunsaturated fatty acids, carotenoids, phycocyanin, and phycoerythrin. All of these active ingredients indicate that such microalgae are a potential source of natural antioxidant and may show anti-inflammatory and cytotoxic effects [4]. This study was aimed to illustrate the cytotoxic and apoptosis effects of ethanol crude extract of an indigenous microalga, *Chaetoceros calcitrans*, (EEC) in human breast cell line, MCF-7. We have further measured the changes of apoptotic related genes such as Bax, Bcl-2, BCL2L1, TNF, P53, Fas, Casp3,

Casp7, and Casp9 and cell cycle related genes such as P21, Cyclin A2, CDK2, and MDM2 using GeXP assay. Based on the result in this study, microalgae possessed the potential as sources of anticancer agent.

2. Materials and Methods

2.1. Microalgae Strain and Culturing of Chaetoceros calcitrans. A pure culture of an indigenous microalga *Chaetoceros calcitrans* (UPMAAHU10) was cultured in 250 to 500 mL of Conway media incubated at ambient temperature of $24 \pm 2^{\circ}$C under constant light of 120 μmol photons m^2 sec^{-1} in an automatic oscillating shaker at 110 rpm. Meanwhile, cultures of 5 litters were maintained in flasks at similar condition with aeration.

2.2. Preparation of Microalgae Extract. Microalga culture reaching stationary phase (6-7 days) was harvested by centrifugation at 3500 rpm for 8 min. Resulting pellets were washed with 0.5 M ammonium formate (Sigma-Aldrich, USA) to remove excess salt. Concentrated microalgae cells were collected and dried in 40°C incubator for 3 days. The cells were homogenized with 100% ethanol (Sigma-Aldrich, USA) for 4 days. After that, the supernatant was filtered using filter cotton and 0.2 μm filtration unit (Millipore, Bedford, Japan). Supernatant was then rotary evaporated under reduced pressure at 30°C (Buchi Rotavapor R 200; Buchi Labortechnik, Flawil, Switzerland) to remove all the ethanol, as described by Lee et al. [5]. The extracts were weighted and mixed with DMSO and kept in −20°c until further use.

2.3. Cell Cultures. The MCF-7 cells were cultured in DMEM medium with L-glutamine (Gibco, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) and Penicillin (100 U/mL)/Streptomycin (100 U/mL), (Gibco, USA). The MCF-10A cells were maintained with DMEM-F12 medium, (Gibco, USA), supplemented with 10% FBS, HEPS, Glutamine, and Penicillin (100 U/mL)/Streptomycin (100 U/mL), (Gibco, USA). For the preparation of peripheral blood mononuclear cells (PBMC), 10 mL of whole blood from healthy individual were taken by using a 10 mL single-use syringe (Omnifix, Germany), mixed, and diluted with the same volume of phosphate buffer saline (PBS). Samples were centrifuged on Ficoll-Paque Plus (Amersham Biosciences, USA) at 400 ×g for 40 minutes. Interface containing lymphocytes were collected, washed, and pelleted down with PBS. Finally, the supernatant was removed and the pellet was mixed with 2 mL RPMI-1640 media with 10% fetal bovine serum (FBS). The trypan blue dye (Sigma-Aldrich, USA) exclusion assay was performed to determine cell number and viability.

2.4. Cytotoxic Assay (MTT Assay). Cytotoxicity effect of EEC was determined by MTT assay, as previously described by Mosmann [6]. Briefly, MTT solution (Sigma-Aldrich, USA) was dissolved in PBS at 5 mg/mL. Twenty μL of 5 μg/mL MTT solutions was added directly to all appropriate wells. Cells were plated in 96-well plates at an initial density of 1×10^5 cells/mL. After incubation for 24 h at 37°C, cells were treated with various concentrations of EEC and incubated for 24, and 72 hours. MTT solution was added to each well and further incubated for 4 h at 37°C. The optical density was read with an ELISA reader (Bio-Tek Instruments, USA) at 570 nm. Each concentration of the algal extract was assayed in triplicate. IC50 values were determined by plotting a linear regression curve. The percent cell viability was calculated as follows:

$$\text{cell viability } (\%) = \frac{\text{OD of treatment}}{\text{OD of control}} \times 100. \quad (1)$$

2.5. Morphological Study. MCF-7 and MCF-10A cells were treated with IC50 concentration (3.00 μg/mL) of EEC and observed under light microscope after 24 and 72 hours of exposure to the EEC.

2.6. Apoptosis Study-Annexin V/Propidium Iodide. The Annexin V/Propidium Iodide assay was performed according to the manufacturer's recommendation (BD Pharmingen FITC Annexin V Apoptosis Detection Kit). Briefly, MCF-7 cells were plated into a 6-well plates (Nunc, Denmark) and incubated for 12 and 24 hours with ECC at IC50 (3.00 μg/mL) and higher IC50 (3.50 μg/mL) concentrations. The cells at a density of 2.5×10^5 cells/mL were transferred to 1.5 mL centrifuge tube and spun down at 400 × g for 5 minutes at room temperature. The supernatant was discarded and the pellet was incubated in 25 μL of Solution A (trypsin buffer) for 10 minutes at room temperature. Then, 200 μL of Solution B (trypsin inhibitor and RNase buffer) was added to each tube and incubated for 10 minutes at room temperature. After that 200 μL of cold Solution C (propidium iodide stain solution) was added to each tube and gently mixed by tapping the tube by hand, incubated for 10 minutes in the dark at 4°C. After filtration the samples through 50 μL nylon mesh were analyzed by flow cytometry (BD FACSCalibur, USA). Each sample was tested in triplicate and untreated MCF-7 cells were used as controls.

2.7. Cell Cycle Analysis. MCF-7 and MCF-10A cells were incubated with EEC at IC50 concentration for 24, 48, and 72 hours. The cells were then harvested by trypsinization, washed with PBS, and subjected to flow cytometry analysis according to the manufacturer's recommendation (BD CycleTEST PLUS DNA Reagent Kit). Briefly, cell pellets were fixed with 500 μL iced cold 80% ethanol and kept at −20°C for 2 hours. The cells were washed twice with PBS, dissolved, and stained in 1 mL PBS buffer containing 0.1% triton X-100, 10 mM EDTA, 50 μg/mL RNase, and 2 μg/mL propidium iodide. The cells were then incubated for 30 minutes at 4°C and were analyzed with flow cytometer (BD FACSCalibur, USA) within 24 hours. Each sample was tested in triplicates and untreated MCF-7 and MCF-10A cells were used as controls.

2.8. RNA Extraction. RNA was extracted from the treated and untreated cell lines by using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions.

Table 1: Selected apoptotic and cell cycle related genes used in GeXP assay.

Gene	Accession number	Product size	Left sequence*	Right Sequence**
BCL2	M14745	157	ACCACTAATTGCCAAGCACC	TTTTCCATCCGTCTGCTCTT
Fas	NM_000043	165	CTCCAAGGGATTGGAATTGA	TGCAGTCCCTAGCTTTCCTT
TNF alpha	NM_000594	171	CTATCTGGGAGGGGTCTTCC	ATGTTCGTCCTCCTCACAGG
Caspase 3	NM_004346	182	GAACTGGACTGTGGCATTGA	ACCAGGAGCCATCCTTTGA
p21Cip1	NM_000389	202	TGTGGACCTGTCACTGTCTTG	TAGGGCTTCCTCTTGGAGAA
Cyclin A2	NM_001237	212	TATTGCTGGAGCTGCCTTTC	CTTTTCTCTTATTGACTGTTGTGCAT
BCL2L1	NM_001191	232	CCACAGCAGCAGTTTGGAT	GGGATTGTTCCCATAGAGTTCCACAA
MDM2	NM_002392	239	GGTGGGAGTGATCAAAAGGA	ACCAGGCTTTCATCAAAGGAA
Caspase 7	NM_033340	247	CAGACCGGTCCTCGTTTGTA	ACCTCGGCATCTTTGTCTGTT
CDK2	NM_052827	285	TGGTGGCGCTTAAGAAAATC	ACAGCTGGAACAGATAGCTCTTGA
ACTB[a]	NM_001101	295	CTGGCACCACACCTTCTACA	AAGGGCATACCCCTCGTAGAT
Bax	BC014175	316	CCCTTTTGCTTCAGGGTTTC	ACAAAGTAGAAAAGGGCGACAA
KAN[b]	Kan(r)	325	ATCATCAGCATTGCATTCGATTCCTGTTTG	AATTCCGACTCGTCCAACATC
Caspase 9	NM_001229	332	GGGCTCACTCTGAAGACCTG	ATCTGGAAGCTGCTAAGAGCC
P53	NM_000546	340	TTTTGGGTTTTGGGTCTTTG	ATTCAACATGAGGGACAGCTT

*Forward universal primer sequence (AGGTGACACTATAGAATA).
**Reverse universal primer sequence (GTACGACTCACTATAGGGA).
[a]Gene used for normalization.
[b]Internal control.

2.9. Primer Designing. Primers for a total of 13 target genes, 1 internal control and 2 house-keeping genes were designed using GenomeLab eXpress Profiler software (Table 1). Fragment sizes ranged from 150 to 350 nt with a 7-nt minimum separation size between each PCR product were considered in this study. Kanamycin gene (KAN^r) was used as an internal control gene, whilst a house-keeping gene, β-actin (ACTB) was used as the normalization gene.

2.10. cDNA Synthesis and PCR Amplification. The reverse transcription reactions and PCR amplification were performed according to the GenomeLab GeXP Start Kit using the manufacturer's protocols (Beckman Coulter, USA).

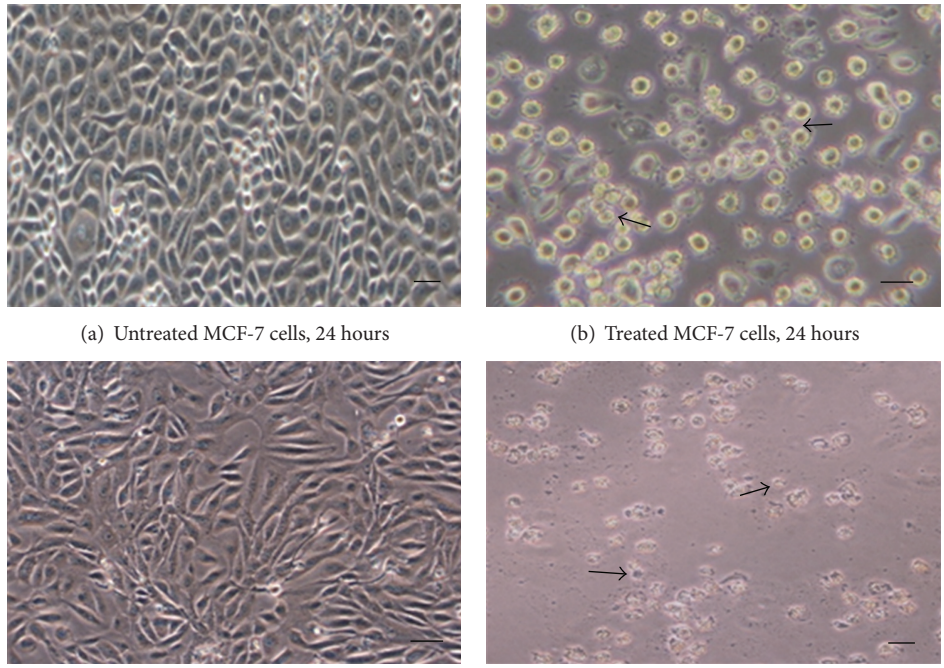
2.11. GeXP Multiplex Data Analysis. The GeXP system was used to separate PCR products based on size by capillary gel electrophoresis and to measure their dye signal strength in arbitrary units of optical fluorescence, defined as the fluorescent signal minus background. PCR product sizes were determined using GenomeLab GeXP software and were compared to the expected PCR product size to identify each transcript. The data were imported into the analysis module of eXpress Profiler software. The housekeeping gene ACTB was used to normalize the results from each gene. The gene expression data were normalized by dividing the peak area of each gene by the peak area of the ACTB gene and the fold change of expression of each gene was calculated using the following formula: fold change = normalized data of the gene from treated samples/normalized data of the gene from untreated samples. The data for each gene and technical replicate were averaged and calculated.

2.12. Statistical Analysis. All data were expressed as means ± SEM of at least three independent experiments. Data were analyzed by ANOVA and Duncan grouping was performed using the SAS system program version 16.0 to identify significant differences between samples. Differences were considered to be significant when $P < 0.05$. Dose response curves were plotted and the IC50 values (concentrations at which cellular effects are inhibited by 50%) were calculated using a linear regression analysis.

3. Results

3.1. Cell Viability Study (MTT Assay). Cytotoxic effect of ethanol extract from *Chaetoceros calcitrans* (EEC) was tested against MCF-7 and MCF-10A cells using colorimetric method MTT assay. Tamoxifen was used as a standard to compare with the EEC tested against MCF-7 cells. Furthermore, the effect of EEC was tested on PBMC to determine the cytotoxicity effect of EEC on human normal peripheral blood mononuclear cells. Bioactivity of EEC was determined based on the concentration that induced 50% inhibition on the growth of the treated cells as compared to the controls in triplicate. MCF-7 and MCF-10A cells were exposed to various concentrations of EEC (0 to 30 μg/mL) for 24 and 72 hours. The IC50 values of EEC on MCF-7 and MCF-10A cells were 3.00 ± 0.65 and 12.00 ± 0.59 μg/mL for 24-hour exposure, respectively. A further reduction in IC50 values of EEC was observed after treatment at 72 hour on MCF-7 and MCF-10A cell lines, 2.69 ± 0.24 and 3.30 ± 0.36 μg/mL, respectively. The IC50 values of Tamoxifen on MCF-7 cells at 24 and 72 hours were 12.00 ± 0.52 and 9.00 ± 0.40 μg/mL, respectively (Table 2). Furthermore, EEC did not show cytotoxic effect on PBMC at different concentrations (Table 2).

3.2. Morphological Study. The EEC treated MCF-7 cells became rounded up, shrunken in size, and detached from the monolayer surface of the wells (Figure 1). Number of cells was also found decreased when compared to the control and some EEC treated cells showed membrane blebbing and



(a) Untreated MCF-7 cells, 24 hours

(b) Treated MCF-7 cells, 24 hours

(c) Untreated MCF-7 cells, 72 hours

(d) Treated MCF-7 cells, 72 hours

Figure 1: Morphology changes of MCF-7 cells after EEC treatment for 24 (a) and 72 (c) hours. Treated cells were rounded up and cell to cell adhesion was lost. Arrows show membrane blebbing and rounded cells at 40x magnification. The black bar in each image represents 10 μm.

Table 2: IC50 of EEC and Tamoxifen on MCF-7 cells.

	IC50 (μg/mL)	
	24 hour	72 hour
EEC treated MCF-7	3.00 ± 0.65	2.69 ± 0.24
EEC treated MCF10A	12.00 ± 0.59	3.30 ± 0.36
EEC treated PBMC	>30	>30
Tamoxifen treated MCF-7	12.00 ± 0.52	9.00 ± 0.40

Values are expressed as mean ± S.E. from triplicate.

formation of apoptotic bodies which appeared to be round or oval masses of cytoplasm smaller than the original cell.

3.3. Annexin V/Propidium Iodide Study. A significant difference ($P < 0.001$) in viability, early apoptosis, and late apoptosis of MCF-7 cells were detected after treatment with EEC for 12 hours at IC50 concentration (3.00 μg/mL) as compared to the control (Figure 2). Incubation of MCF-7 with IC50 concentration of EEC after 12 hour reduced the cell viability to 26.45±0.41% with 31.92±0.48% and 23.98±0.45% of early apoptosis and late apoptosis, respectively. When the EEC concentration was increased to IC75 (4 μg/mL), a further reduction in cell viability to 23.99±0.33% with 30.82± 0.31 and 20.23 ± 0.32% of early apoptosis and late apoptosis in MCF-7 cells, respectively, was detected.

A similar reduction in cell viability and increase in apoptotic cells were detected in MCF-7 cells after EEC incubation for 24 hours. The control untreated cells showed 86.62 ± 0.23%, 8.62 ± 0.19%, and 1.28 ± 0.02% of viability, early apoptosis, and late apoptosis, respectively. However, incubation of MCF-7 with IC50 concentration of EEC after 24 hours reduced the cell viability to 31.61 ± 0.24% with 49.84 ± 0.47% and 12.63 ± 0.24% of early apoptosis and late apoptosis, respectively (Figure 2). In comparison, the higher doses (4 μg/mL) of EEC showed 14.05±0.22%, 50.25± 0.55%, and 30.05 ± 0.50% of viability, early apoptosis, and late apoptosis, respectively, in MCF-7 cells after 24-hour incubation. Only the percentages of viable and late apoptotic between the two different concentrations were significantly different, indicating that increasing the concentration of EEC reduced the viable cells, and increased the number of cells that underwent late apoptosis at 24 hour incubation time. Furthermore, when the proportion of normal and apoptotic cells was scored as a percentage of the total cell population, viable cells decreased from 91% before treatment to less than 26% after 12 hours and 32% after 24 hours. Meanwhile the apoptotic cells increased from 0.3% before treatment to 24% and 13% after 12 and 24 hours of incubation, respectively.

3.4. Cell Cycle Analysis. In cells treated with EEC, a subpopulation of cells appears before the G1 peak is referred to as the subG1 peak. Results indicated that the subG1 population, which indicated apoptotic cells [7], increased from 0.39% in the control (untreated cells) to 2.31% after exposure to 3.00 μg/mL of the EEC for 24 hours. The subG1 population increased further from 1.62% for the control cells to 55.75% after exposure to EEC for 48 hours and from 4.53% for the control cells to 72.75% after exposure to EEC for 72 hour (Table 3). EEC treated MCF-10A cells also showed similar patterns with a less significant effect where the subG0-G1 population increased from 4.10% in the control to 17.52% after exposure to 3.00 μg/mL of EEC for 24 hours.

Table 3: Cell cycle progression of EEC treated and untreated MCF-7 (a) and MCF-10A cells (b) after 24, 48, and 72 hours incubation.

(a)

	MCF-7 cells Cell cycle phases (Percentage)			
	G0/G1	G2/M	S	SubG0/G1
Control*				
24 hours	61.42 ± 0.07	17.12 ± 0.24	22.48 ± 0.19	0.39 ± 0.008
48 hours	63.38 ± 0.43	14.19 ± 0.21	14.67 ± 0.40	1.62 ± 0.11
72 hours	87.17 ± 0.13	1.63 ± 0.04	6.07 ± 0.03	4.53 ± 0.12
IC50-3 μg/mL				
24 hours	64.82 ± 0.32	12.93 ± 0.56	21.24 ± 0.30	2.31 ± 0.13
48 hours	44.6 ± 1.37[a]	0.01 ± 0.005[a]	0.46 ± 0.02[a]	55.75 ± 1.25[a]
72 hours	27.85 ± 1.26[a]	0.02 ± 0.01	0.35 ± 0.02[a]	72.75 ± 1.23[a]

Values are expressed as mean ± S.E. (Standard Error), μg/mL. ($P < 0.001$, ANOVA).
*Untreated cells were used as a control. [a]Significant difference with untreated group ($P < 0.05$).

(b)

	MCF-10A Cell cycle phases (Percentage)			
	G0/G1	G2/M	S	Sub G0/G1
Control*				
24 hours	63.45 ± 1.70	14.16 ± 1.01	17.20 ± 0.75	4.10 ± 0.14
48 hours	62.25 ± 1.39	12.68 ± 0.51	16.10 ± 0.20	4.50 ± 0.08
72 hours	58.12 ± 1.01	11.38 ± 0.29	20.45 ± 1.22	5.00 ± 0.18
IC50-3 μg/mL				
24 hours	60.49 ± 1.06	3.84 ± 0.12	16.37 ± 0.22	17.52 ± 0.57
48 hours	53.39 ± 0.69	6.4 ± 0.008	12.25 ± 0.29	27.24 ± 0.40
72 hours	44.76 ± 0.77	5.91 ± 0.21	10.63 ± 0.34	37.18 ± 0.53

Values are expressed as mean ± S.E. (Standard Error), μg/mL. ($P < 0.001$, ANOVA).

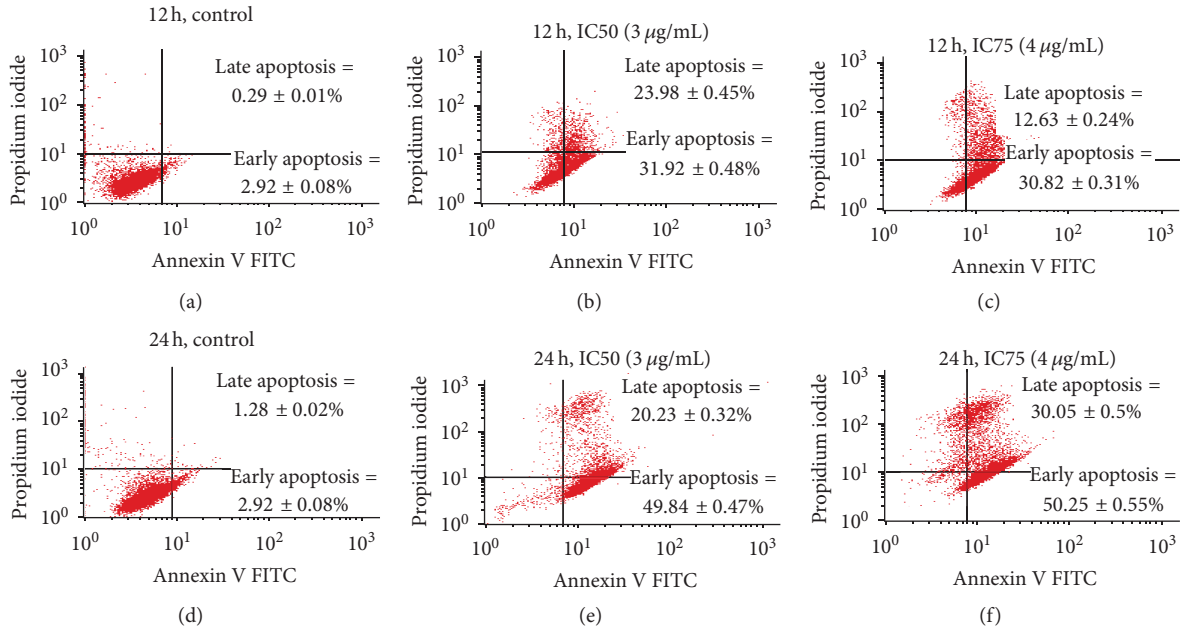


Figure 2: Annexin V/PI study on MCF-7 cell treated with IC50 and IC75 of EEC for 12 and 24 hours.

Similarly, the subG0-G1 population increased from 4.50% in the control cells to 27.24% after exposure to EEC for 48 hours and also increased from 5% in the control cells to 37.18% after exposure to EEC for 72 hours (Table 3).

3.5. Gene Expression Study. The differential expressions of genes in MCF-7 and MCF-10A cells after treatment were compared with the controls (untreated MCF-7 and MCF-10A cells) and expressed as fold change. As shown in Figure 3, the fold changes of MDM2 and Cyclin A2 in MCF-7 cells decreased from 1.8 and 3.5 at 6 hours to 1.4 and 1.5 at 24 hours, respectively. Meanwhile, the fold change of p21Cip1 increased from 0.8 at 6 hours to 1.9 at 24 hours. An increase in fold change of proapoptotic gene, Bax, from 1.1 at 6 hours to 1.9 at 24 hours was also detected from the treated MCF-7 cells. In contrast, the fold change of antiapoptotic gene, Bcl-2, decreased from 2.5 at 6 hours to 1.1 at 24 hours. The EEC treated MCF-7 cells also showed a decrease in fold changes of effector caspase, where caspase 3 and caspase 7 in treated MCF-7 decreased from 1.5 and 2.1 at 6 hour to 1.3 and 2.0 at 24 hours, respectively.

As shown in Figure 4, the fold changes of all the genes in MCF-10A cells were not more than 1.0 except for p53 and MDM2 at both time points 6 and 24 hours as well as for p21Cip1, BCL2l1, caspase 3, caspase 7 and caspase 9 at 24 hours. The fold changes of Bax, Bcl-2, p53, Cyclin A2, and CDK2 decreased after 24-hour incubation time compared to 6 hour samples. Meanwhile, the fold changes for other genes such as p21Cip1, Fas, TNF alpha, MDM2, BCL2L1, caspase 3, 7, and 9 increased at 24-hour incubation time compared to 6 hour samples.

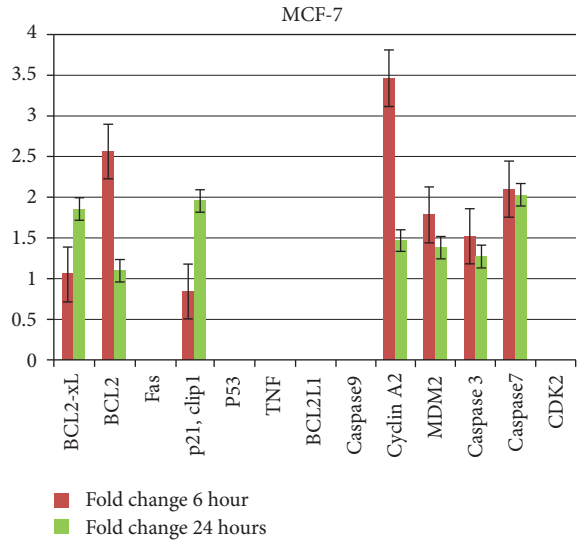


Figure 3: Fold change analysis of gene expressions in MCF-7 cells after 6- and 24-hour treatment with EEC. The lowest fold change 0.8 in p21Cip1 and the highest fold change 3.4 in Cyclin A2 at 6 hours of treatment.

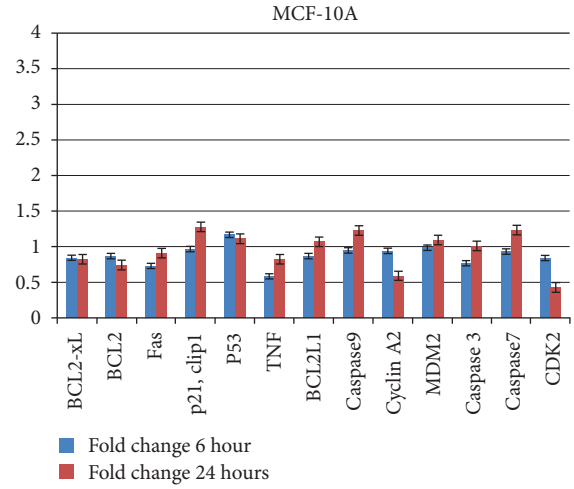


Figure 4: Fold change analysis of gene expressions in MCF-10A cells after 6- and 24-hour treatments with EEC. The lowest fold change 0.4 in CDK2 and the highest fold change 1.3 in p21Cip1 at 24 hours of treatment.

4. Discussion

In this study, the ethanol extract from *Chaetoceros calcitrans* (EEC) was extracted and tested on human breast cancer cell lines. In general, EEC showed different IC50 values on the tested cell lines, MCF-7 and MCF-10A at different time points. However, MCF-7 was more responsive to the EEC than MCF-10A with IC50 value of 3.00 ± 0.65. Based on MTT assay, EEC can be considered as potential cytotoxic agent because it showed four fold cytotoxic effect on MCF-7 compared to MCF-10A, with no significant effects on PBMC. This result confirmed the earlier study that reported on the potential antiproliferative effect of five *Dunaliella salina* ethanol extract on AML cell lines [8].

Apoptosis, or programmed cell death, is characterized by a number of well-defined features which include condensation and fragmentation of the chromatin, internucleosomal DNA cleavage, membrane blebbing, caspase activation, and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane [9]. Hence, induction of apoptosis is one of the useful approaches in cancer therapies [10]. Based on Annexin V/PI and cell cycle flow cytometry analysis, we found that inhibition of cell growth by EEC on MCF-7 cells is through the induction of apoptosis without cell cycle arrest. In a previous study, the ethanolic extracts of *Corallina pilulifera* was reported to induce apoptosis in HeLa cells without cell cycle arrest [11, 12]. Even though the EEC treated MCF-10A cells undergo apoptosis, the percentages of apoptotic cells are lower compared to MCF-7 cells. The apoptotic cells at subG0/G1 phase in treated MCF-7 cells at 48 and 72 hours showed 34- and 16-fold increase compared to EEC treated MCF-10A cells which showed only 6- and 7-fold increase at the same time points, respectively. Other study demonstrated that the methanolic extracts of *Plocamium telfairiae* induce apoptosis in HT-29 human colon carcinoma cells [13].

MCF-10A is a nontumorigenic mammary epithelial cell line [14]. The expression profiles of apoptotic genes in human breast cancer lines including the normal human breast cell

line, MCF-10A cells, have been studied by several investigators [15, 16]. In this study, we analyzed the expression of 13 apoptotic and cell cycle related genes following treatment with EEC on MCF-7 and MCF-10A cells. The expression levels of all the genes in MCF-10A were detected and the majority of them did not show significant variation in gene expression where the lowest and the highest fold changes were 0.4 and 1.3 for CDK2 and p21Cip1, respectively. However, the fold change of MDM2 in EEC treated MCF-7 cells was 1.8 and 1.4 at 6 and 24 hours, respectively (Figure 3). Hence, the result supported an earlier study indicating that there is a direct link between MDM2 expression and programmed cell death [17]. Moreover, the fold change of Cyclin A2 expression in MCF-7 cells decreased from 3.5 after 6 hours of exposure to 1.5 after 24 hours. Besides that, the fold changes of p21Cip1 in treated MCF-7 cells were 0.8 and 1.9 after 6 and 24 hours of exposure to EEC, respectively (Figure 3). The function of CDKs is tightly regulated by cell cycle inhibitors like p21Cip1 and p27Kip1 [18] where uncontrolled CDK activity is usually the reason of cancer. p21Cip1 protein binding inhibits the activity of cyclin-CDK2 complexes. This protein was reported to be specifically cleaved by caspase 3 like caspases which cause the activation of CDK2 and may be instrumental in the execution of apoptosis following activation. Other studies have shown that the growth inhibitory effect of astaxanthin-rich *H. pluvialis* extract on HCT-116 colon cancer cells was associated with an increase in p21Cip1 expression, cell cycle arrest, and the induction of apoptosis [17].

Besides that, the fold change of proapoptotic gene, Bax, expression in treated MCF-7 cells for 6 and 24 hour exposed samples was 1.1 and 1.9, respectively (Figure 3). Bax gene is a member of the Bcl-2 family, an apoptosis promoter that regulates the release of cytochrome *c* from mitochondria, and its expression is identified to lead to the activation of caspases and programmed cell death [19]. Besides that, an earlier study has shown that the ethanol extract of *Dunaliella salina* induced apoptosis of A549 human lung cancer cell line by elevating Bax expression [20]. Meanwhile, acetone extract of *Lethariella zahlbruckneri* increased the expression of the proapoptotic protein, Bax, and decreased the expression of the antiapoptotic protein, Bcl-2 [21]. As shown in Figure 3, the fold changes of Bcl-2 in MCF-7 cells were 2.5 and 1.1 at 6 and 24 hours, respectively. More importantly, increasing the incubation time from 6 to 24 hours led to the increase of Bax/Bcl-2 ratio, which is an important apoptosis inducer indicator in cancer cells. Our results supported a previous study that demonstrated the inducing of apoptosis of astaxanthin-rich *Haematococcus pluvialis* extract on HCT-116 colon cancer cells by the increasing of the ratio of Bax/Bcl-2 expression [22]. Overexpressions of Bcl-2 and Bcl-xl have been demonstrated in a large variety of human malignancies, including breast, prostate, colorectal, and lung cancers [23]. The ability of tumour cells to undergo apoptosis by chemotherapeutic agents is controlled by the ratio of Bax/Bcl-2 in the mitochondria [24]. The Bax activation might have involved in the release of cytochrome c from the mitochondria and clustered with APAF-1, an apoptotic protease activating factor 1 and resulted in activation of caspase 9 which then cleaved the downstream caspease 3, 6, and 7 that led to apoptosis [25]. Figure 3 showed upregulation of effectors caspase, caspase 3, and caspase 7 in treated MCF-7 at 6 hour (1.5 and 2.1) and 24 hours (1.3 and 2.0), respectively. Results from this study concur with a previous study that demonstrated chloroform extract of *Physalis minima* produced a significant growth inhibition and induced apoptosis against human MCF-7 by activation of caspase 3 [14]. Thus, activation of caspase is recognized to be the most specific indication of apoptosis [23].

Based on the results obtained from this study, it postulates that EEC could induce apoptosis through a caspase-dependent pathway by activating caspase 3 and 7 in MCF-7 cells. Hence, Bax activation might have involved in the release of cytochrome c from the mitochondria and probably clustered with APAF-1 and resulted in activation of caspase 9 which then cleaved the downstream caspase 3, 6, and 7 that led to apoptosis. Thus, this study concluded that the crude ethanol extracts of *C. calcitrans* has the potential to be used as therapeutic and chemopreventive agents for breast cancer treatment.

Acknowledgment

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Research Article

Brazilian Propolis: A Natural Product That Improved the Fungicidal Activity by Blood Phagocytes

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Natural product incorporation into microcarriers increases the bioavailability of these compounds, consequently improving their therapeutic properties. Natural products, particularly those from bees such as propolis, are widely used in popular medicine. Propolis is a powerful treatment for several diseases. In this context, the present study evaluated the effect of propolis *Scaptotrigona* sp. and its fractions, alone or adsorbed to polyethylene glycol (PEG) microspheres, on the activity of human phagocytes against *Candida albicans*. The results show that propolis exerts a stimulatory effect on these cells to assist in combating the fungus, especially as the crude extract is compared with the fractions. However, when incorporated into microspheres, these properties were significantly potentiated. These results suggest that propolis adsorbed onto PEG microspheres has immunostimulatory effects on phagocytes in human blood. Therefore, propolis may potentially be an additional natural product that can be used for a variety of therapies.

1. Introduction

The current focus on natural products and alternative medicines has renewed interest in bee products such as honey, royal jelly, pollen, and propolis [1, 2]. Propolis is a sticky dark-coloured material collected by worker bees from the leaf buds or exuded from numerous tree species. Once collected, this material is enriched with salivary and enzymatic secretions and used in the construction, adaptation, and protection of their nests [3, 4]. In this way propolis chemical composition is a direct reflex of the vegetable flora and bee species [5, 6].

In Brazil, besides wide variety of flora there are also several bees species, among which stand out those belonging to Apidae family and Meliponinae subfamily, better known as indigenous stingless bees, and which produce propolis from the resinous material of plants with wax and soil. In this group are found bees of the *Scaptotrigona* genus with twenty four species described and eight of which already identified in Brazil [4, 7–9].

In particular, the propolis shows potential because of its therapeutic properties and possible applications in the pharmaceutical industry [10–12]. A range of biological activities have been attributed to propolis, including immunomodulatory [13, 14], antibacterial [15], fungicidal [16, 17], anti-inflammatory, healing [18], anesthetic [19], and anticarcinogenic effects [20].

On the other hand, many natural products are not therapeutically effective when used without modification. In many cases, this failure is attributed to low concentrations at the therapeutic targets. One factor that influences the bioavailability of natural products is the extensive metabolism that they undergo in vivo by commensals or probiotics during their passage through the intestine and liver, significantly changing the exact species that is found in systemic circulation [21]. Susceptibility to chemical

hydrolysis at physiological pH has been demonstrated for natural substances with high biological activity [22].

One alternative to these problems is the use of polymeric microparticle systems, which have shown promise for the adsorption of phytopharmaceuticals. These systems also promote the controlled release of drugs or biologically active hydrophilic or hydrophobic substances [23, 24]. Among the polymers used in the preparation of microcarriers, polyethylene glycol (PEG) has excellent properties such as solubility in both water and organic solvents and the absence of toxicity and antigenicity, which are essential for biomedical applications. There have been multiple studies of the effect of pairing natural or synthetic drugs with PEG microparticle systems [25–33]. These combinations present numerous advantages, such as prolonging residence in the body, decreasing metabolic degradation by enzymes, and reducing or eliminating the immunogenicity of proteins [27]. It is likely that the development of drugs that incorporate natural materials will be able to reduce side effects, decrease costs, and maximize the benefits of natural product formulations to avoid the aforementioned problems.

In this context, the aim of this study was to evaluate the immunomodulatory and fungicidal effects of propolis adsorbed to PEG microspheres on human phagocytes in the blood.

2. Materials and Methods

2.1. Propolis. Propolis samples were collected in February 2011, directly from beehives of *Scaptotrigona* sp. in a meliponary of Barra do Garças city ($15^{\circ}52'19.4''$S and $52^{\circ}10'27.03''$W), eastern region of Mato Grosso state, Brazil, where predominates the Cerrado stricto sensu. The mean annual temperature is 25.5°C, with two well-defined seasons: rainy (October to April) and dry (May to September) seasons. The ratio of annual rainfall is 1750 mm [34].

Propolis samples had resinous aspect, balsamic, with dark brown color, typical vegetable odor, and solid impurities free. After collecting was promptly stored in lidded container for transport.

2.2. Preparation of Crude Extract from Propolis. The production of an ethanol extract of propolis was adapted from Miorin [4], where 30 grams of propolis, fragmented into small pieces, was placed in a container with a lid, and 100 mL of absolute ethyl alcohol was added. The solution was left for 7 days at room temperature with periodic agitation. Subsequently, the solution was filtered through Whatman no. 3 filter paper and placed in petri dishes (preweighed). An oven 40°C was used to evaporate the ethanol, resulting in 19.3 g of crude extract with a dark brown, molasses-like appearance.

2.3. Chemical Screening of Crude Extract of Propolis. Qualitative chemical screening of hydroalcoholic extract of propolis was performed to verify the presence of cyanogenic glycosides, phenols, tannins, anthocyanidins, anthocyanins, flavonoids, leucoanthocyanidins, catechins, flavanones, flavonols, xanthones, steroids, triterpenoids, saponins, and alkaloids according to Harbone [35]. The following reagents and chemicals were used: alkaloids with Dragendorff's reagent, flavonoids with metallic magnesium and HCl, saponins with the ability to produce foam, reducing sugars with Fehling's reagent, glycosides with Liebermann's test, tannins with ferric chloride, and polysaccharides with iodine solution [24].

2.4. Fractionation of Extract of Propolis. Fractions of the propolis extract were obtained by the modification of the classical liquid chromatography method described by Santos et al. [36]. Silica with a 60~230 mesh was used as the stationary phase and organic solvents (hexane, dichloromethane, ethyl acetate, and methanol) were used as the mobile phase. The stationary phase of the column was prepared by filling the column with silica suspended in hexane. Next, 5 grams of propolis was dissolved in hexane and added to the stationary phase. Three hundred milliliters of each mobile phase was eluted through the column after the sample was applied, except that 450 mL of methanol was applied as the final column wash. The fractions eluted with hexane, dichloromethane, ethyl acetate, and methanol were pooled based on related spectrophotometric profiles and then placed in a drying oven at 40°C for three days to remove the solvents. After drying, the samples were weighed so that the solutions of known concentrations could be prepared.

2.5. Poly(ethylene glycol) (PEG) Microsphere Preparation. Microspheres were produced in accordance with the method described by Scott et al. [32] and modified by Scherer et al. [33]. Briefly, 20 g of PEG 6000 (synth) was suspended in 100 mL of phosphate-buffered saline (PBS) and then mixed (v/v) with a 2% sodium sulfate solution in PBS and incubated at 37°C for 45 minutes. After incubation, the mixture was diluted 3 : 1 in PBS. The formation of microspheres was thermally induced by heating the solution to 95°C for 5 minutes. A solution (v/v) of the crude extract or fractions and microspheres was incubated for 30 minutes at 37°C for adsorption.

Microspheres of PEG with or without the crude extract or fractions adsorbed were stained with a solution of DyLight fluorochrome-488 (10 μg/mL, Pierce) overnight at room temperature in dimethylformamide at a 100 : 1 molar ratio of PEG: DyLight and subsequently analyzed by fluorescence microscopy.

2.6. Blood Samples. A sample of 15 mL of blood was collected from 200 clinically healthy male volunteers aged between 18 and 35. All volunteers signed an informed consent form that was approved by the local Ethics Committee before entering the study.

2.7. Separation of Blood Cells. Blood samples were collected into heparinized (25 U/mL) tubes. The cells were separated with a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) to produce the preparations of mononuclear cells with 98% purity as analyzed by light microscopy. The purified

macrophages were resuspended independently in 199 serum-free medium, to a final concentration of 2×10^6 cells/mL.

2.8. Culture of Candida albicans. The standard *Candida albicans* strain ATCC 10231 was used in the study. Twenty-four hours prior to testing, the fungal samples were suspended in brain heart infusion broth (BHI) and incubated at 37°C for 24 hours. After growth, the fungi were washed two times in PBS, and the concentration was adjusted to 2.0×10^7 yeast cells/mL [37].

2.9. Determination of Concentration-Response Curve. To determine the concentration-response curve, the effects of doses of 0 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL of propolis and propolis fractions were determined by the release of superoxide anions by phagocytes as described in Section 2.9. All experiments were performed in duplicate or triplicate.

2.10. Cytotoxic Analysis. The cytotoxic test was conducted using the acridine orange method [38] as described in Section 2.10. In this assay, phagocyte viability was evaluated when treated with propolis or propolis fractions. All experiments were performed in duplicate or triplicate.

2.11. Release of Superoxide Anion. One of the methods used for functional evaluation of the phagocytes treated with propolis or propolis fractions, either alone or adsorbed to PEG microspheres, was the analysis of oxidative metabolism as measured by superoxide anion release testing in the presence or absence of *Candida albicans*. The cytochrome C reduction method described by Pick and Mizel [39] and adapted by Honorio-França et al. [40] was utilized. Briefly, mononuclear phagocytes in the presence or absence of the fungus were treated with propolis or propolis fractions, either alone or adsorbed to PEG microspheres, for 30 minutes at 37°C. As a control, PBS-treated mononuclear phagocytes were used. After treatment, the cells were washed and resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 mg/mL cytochrome C. The suspensions (100 μL) were then incubated for 60 minutes at 37°C on culture plates. The reduction of cytochrome C was measured in a microplate spectrophotometer at 550 nm. The superoxide anion concentration was calculated according to the following relationship: O_2^- concentration (nmol) × 100 = optical density/6.3 and the results were expressed as nmol/O_2^-. All experiments were performed in duplicate or triplicate.

2.12. Phagocytosis and Fungicide Activity. In addition to the method described above, phagocytosis and fungicidal activity tests to evaluate the function of cells treated with propolis or propolis fractions, either alone or adsorbed to PEG microspheres, were performed. The acridine orange method proposed by Bellinati-Pires et al. [38] was used for this purpose. Briefly, equal volumes of mononuclear phagocytes and fungal suspensions were treated with propolis or propolis fractions, either alone or adsorbed to PEG microspheres, and incubated for 30 minutes at 37°C with continuous shaking. PBS-treated mononuclear phagocytes and the fungal suspension were used as the control. After incubation, phagocytosis was stopped by incubation on ice. To remove the extracellular fungal cells, the suspensions were washed and centrifuged twice (160 ×g, 10 min, 4°C). Then, the cells were resuspended in serum-free medium 199 and centrifuged. The supernatant was discarded and the pellet was stained with 200 μL of acridine orange (14.4 g/L of PBS) for 2 minutes. The pellet was then resuspended in cold culture 199 medium, washed twice, and observed under a fluorescence microscope at 400x and 1000x magnification. The phagocytosis index was calculated by counting the number of cells ingesting at least 3 yeast cells within a pool of 100 cells. To determine the fungicide ratio, 100 phagocytes that had ingested yeast cells were counted, taking into account the number of live yeast (green) and dead yeast (orange) × 100 [41]. All experiments were performed in duplicate or triplicate.

2.13. Statistical Analysis. Analysis of variance (ANOVA) was used to evaluate the viability, superoxide release, phagocytosis, and the phagocytes' fungicide activity. Statistical significance was defined by a P value lower than 0.05 ($P < 0.05$).

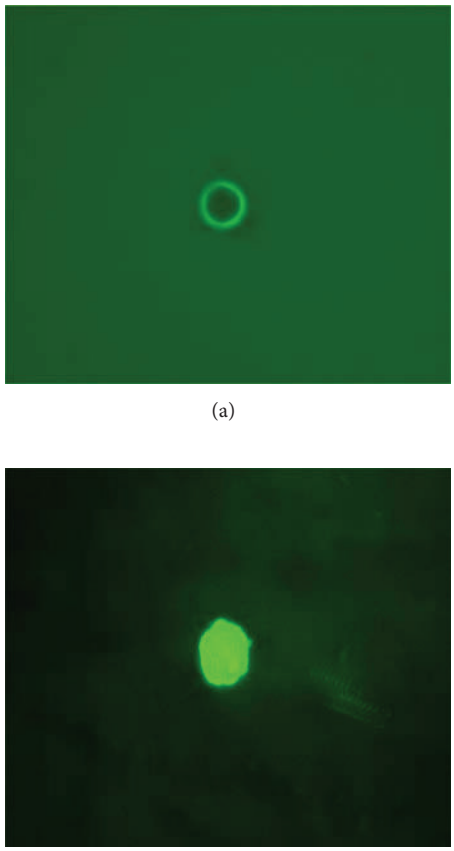
3. Results

3.1. Chemical Screening of Crude Extract from Propolis. Chemical screening showed the presence of tannins, phenols, flavones, flavonoids, and xanthones. The chemical composition of the mixture also contained to a lesser degree, flavanones, and resins (Table 1).

3.2. Characterization of PEG Microspheres. Fluorescence microscopy was used to analyze the morphology of the PEG microspheres. All PEG microspheres showed similar geometric shapes and sizes with a smooth surface as presented in Figure 1(a). Figure 1(b) shows the adsorption of propolis in a heterogeneous manner along the microsphere surface. The adsorption is observed as the presence of small regions on the hollow microspheres.

3.3. Concentration-Response Curves for Propolis and Propolis Fractions. To determine the concentration-response relationship, four different doses of propolis and each propolis fraction were examined (0 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL). Superoxide release by phagocytes upon exposure to propolis and propolis fractions was evaluated, and a correlation between concentration and response was observed, with superoxide release increasing with the size of the dose. Based on the results, we used a test concentration of 50 μg/mL (Figure 2).

3.4. Cytotoxic Effect of Propolis and Propolis Fractions on Mononuclear Phagocytes. Neither the PEG microspheres nor propolis affected the viability of the phagocytes. The dichloromethane fraction, when incubated with phagocytes, had a viability index of less than 90% and was not tested in any



(a)

(b)

Figure 1: (a) Polyethylene glycol microspheres with a homogeneous surface. (b) Polyethylene glycol microsphere with adsorbed propolis. Heterogeneous adsorption on the microsphere surface.

Table 1: Chemical screening for identification and indication of main chemical constituents of crude extract from propolis.

Analysis	Propolis extract
Cyanogenic heterosides	Absent
Phenols and tannins	Present
Anthocyanins and anthocyanidins	Absent
Flavones, flavonols, and xanthones	Present
Chalcones and aurones	Absent
Leucoanthocyanidins	Absent
Catechins	Absent
Flavanones	Present
Flavonols, flavanones, and/or xanthones	Present
Steroids and triterpenoids	Absent
Saponins	Absent
Strong fixed acids	Absent
Resins	Present
Alkaloids	Absent
Quaternary compounds	Absent
Quinones	Absent
Flavonoids aglycones	Absent
Steroid aglycone triterpenoids	Absent

Notes: present, positive reaction; absent, negative reaction.

Table 2: Blood mononuclear phagocyte (MN) viability index in the presence of propolis and its fractions. The results represent the means (±SD) of ten experiments with cells from different individuals (ANOVA $P > 0.05$).

Experimental group	MN phagocytes viability (%)
Control	98,2 ± 0,83
Propolis	90,4 ± 1,14
Hexane	94 ± 1,58
Dichloromethane	88,4 ± 1,14
Ethyl acetate	91,6 ± 2,07
Methanol	91 ± 1,58

further assays of functional cell activity. The other fractions did not affect cell viability and were tested for functional activation (Table 2).

3.5. Effect of Propolis and Propolis Fractions, Alone or Adsorbed to PEG Microspheres, on Superoxide Anion Release. The PEG microspheres did not alter the superoxide release by phagocytes when compared to spontaneous release. In the presence of the fungus, phagocytes showed O_2-release at rates similar to spontaneous release. The cells treated with propolis or propolis fractions alone also presented rates similar to that of a spontaneous superoxide release.

Phagocytes treated with the propolis or propolis ethyl acetate fraction adsorbed to PEG showed a significant increase in superoxide release, when comparing release in the presence of the fungus to spontaneous superoxide release ($P < 0.05$).

When incubated with propolis adsorbed onto the microspheres, the cells showed an increase in superoxide release compared with phagocytes exposed only to propolis that was not on microspheres. A similar effect was observed when the phagocytes were incubated with the propolis ethyl acetate fraction adsorbed to the microspheres (Table 3).

3.6. Effect of Propolis and Propolis Fractions, Alone or Adsorbed to PEG Microspheres, on Phagocytosis. Phagocytes exhibit a basal phagocytic activity in response to *Candida albicans*. This activity did not increase in the presence of PEG microspheres. When phagocytes were stimulated with propolis or propolis fractions, an increase in the phagocytic index was observed, except in the case of the hexane fraction, which significantly reduced the phagocytic activity. The addition of propolis adsorbed onto PEG microspheres resulted in an increased phagocytic activity compared to the treatment with only PEG microspheres or propolis alone (Figure 3).

3.7. Effect of Propolis and Propolis Fractions, Alone or Adsorbed to PEG Microspheres, on the Microbicidal Activity of Phagocytes. Phagocytes present low fungicidal activity against *Candida albicans*. Increased fungicidal activity was observed when the cells were incubated with propolis and the methanol fraction of propolis. The hexane fraction showed a significant decrease in antifungal activity. An increase in the

Table 3: Superoxide anion release of phagocytes in the presence or absence of *Candida albicans*, stimulated by propolis and its fractions, alone or adsorbed to PEG. ($^*P < 0.05$).

MN phagocytes	*Candida albicans*	Superoxide anion release (nmol)
Control	Absence	3,67 ± 0,89
	Presence	4,16 ± 2,71
Propolis	Absence	4,12 ± 1,05
	Presence	4,72 ± 2,30
Hexane	Absence	4,21 ± 1,38
	Presence	5,00 ± 2,04
Ethyl acetate	Absence	4,31 ± 0,94
	Presence	4,00 ± 1,46
Methanol	Absence	4,30 ± 0,86
	Presence	3,62 ± 1,58
PEG	Absence	3,30 ± 0,83
	Presence	3,03 ± 0,49
Propolis + PEG	Absence	19,41 ± 0,50*
	Presence	17,44 ± 1,13*
Hexane + PEG	Absence	4,63 ± 1,42
	Presence	4,85 ± 1,63
Ethyl acetate + PEG	Absence	15,77 ± 0,57*
	Presence	15,48 ± 0,60*
Methanol + PEG	Absence	4,51 ± 1,00
	Presence	4,59 ± 1,62

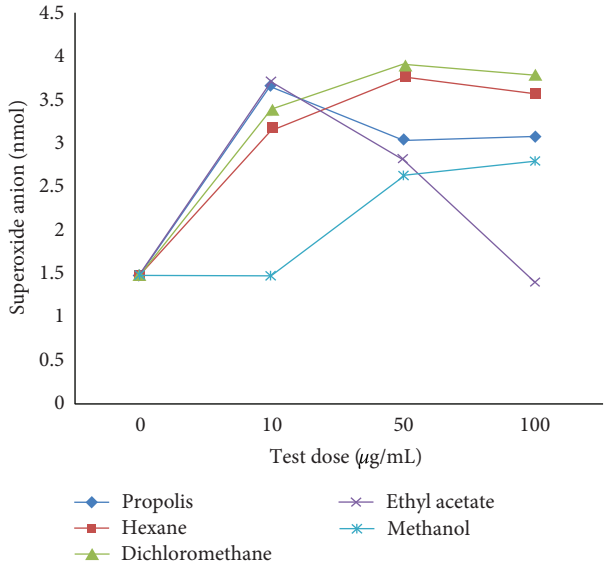


Figure 2: Dose-response curve determined by the superoxide anion release assay for the following dosages of propolis and its fractions: 0 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL.

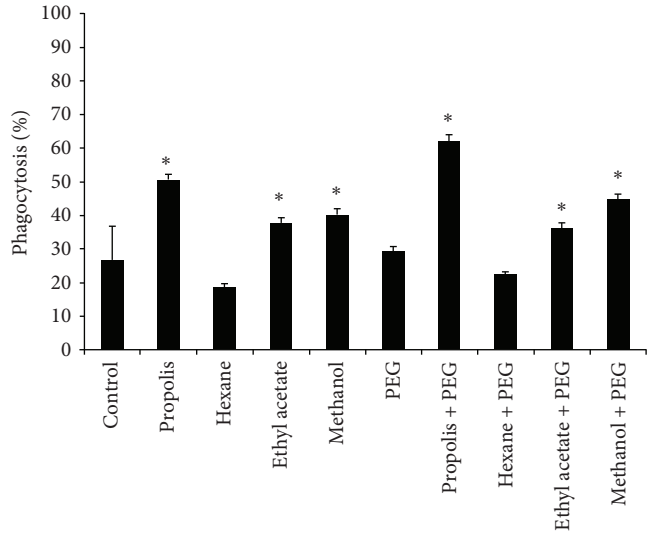


Figure 3: Phagocytic index of phagocytes stimulated with propolis and its fractions, alone or adsorbed to PEG microspheres. ($^*P < 0.05$).

fungicidal activity was observed when the phagocytes were incubated with PEG microspheres alone ($P < 0.05$, Figure 4).

4. Discussion

In the present study, we determined the effect of propolis and the propolis fractions obtained by a polarity gradient and adsorbed to PEG microspheres on the functional activity of phagocytes from human blood.

PEG microspheres are a type of copolymer used for the clinical administration of therapeutics because of their capacity to incorporate drugs [42], their ability to increase the duration of drug exposure or the production of other substances such as enzymes [43], and their role as an important signaling vehicle in immunity [44].

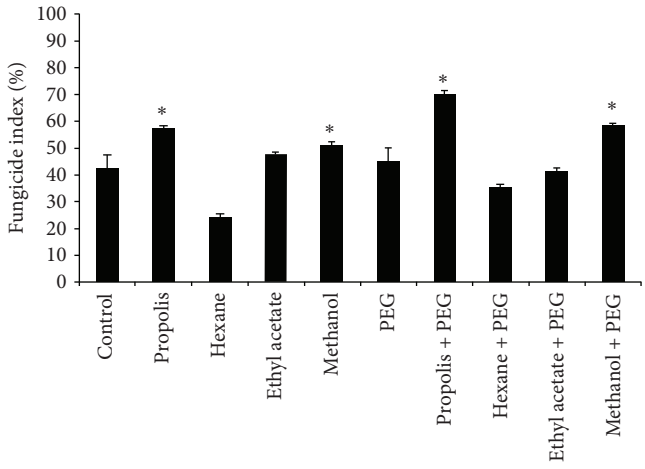


Figure 4: Fungicidal index of phagocytes stimulated with propolis and its fractions, alone or adsorbed to PEG microspheres. ($^*P < 0.05$).

In this study, analysis by fluorescence microscopy showed the PEG microspheres to be of regular size, be easily separated from a suspension, and to readily adsorb propolis. The characterization of PEG microspheres using flow cytometry has been previously reported and has shown that this polymer is approximately 5.8 μm of diameter [24, 33].

The use of a polymeric microsphere delivery system for natural products has been described in the literature, and this system is a method that may be useful to deliver a variety of medicinal natural products that could provide additional protection against infection [24, 33].

The literature describes the importance of natural products in discovering new drugs [45]. One of the numerous medicinal properties of natural products is the ability to modulate the immune system, by either stimulating or suppressing certain immune response events [14, 46–51].

Most studies have been based on the action of propolis without determining the scientific basis of their cytotoxic properties. Propolis is a natural product produced by bees from resinous material collected from a variety of plant species, and it is a complex mixture [52]. In this study the main chemical constituents of this propolis showed were tannins, phenols, flavones, flavonoids and xanthones, flavanones, resins, and to a lesser degree, flavanones and resins. Because it is a mixture, the isolation of the active components that cause this effect is a very long and complex process. Alternatively, fractionation performed with solvents of different polarities allows partial purification of the substances in the mixture, and the examination of the fractions obtained via biological assays enables the determination of some of the metabolites that show therapeutic activity [53].

Here, we demonstrated that the dichloromethane fraction is toxic to phagocytes. This may be caused by the presence of toxic metabolite(s) at higher concentrations in this fraction because, according to Cechinel-Filho and Yunes [53], some compounds exhibit cytotoxic effects in high doses. On the other hand, the fractions obtained from elution with hexane, ethyl acetate, and methanol contain important substances that are able to modulate the activity of phagocytes.

The various types of metabolites obtained by the fractionation of propolis by hexane, dichloromethane, ethyl acetate, and methanol are well known [54–56]. The hexane fraction concentrates steroids, terpenes, and acetophenones. The dichloromethane extract contains lignans, flavonoids, desmethoxy sesquiterpenes, triterpenes, and coumarins. Ethyl acetate isolates flavonoids, tannins, xanthones, triterpene acids, saponins, and phenolic compounds and the methanol extract concentrates glycosylated flavonoids, tannins, saponins, and carbohydrates [53].

Mononuclear phagocytes play an important role in host defense. They produce phagocytic NADPH oxidase, which forms superoxide, and this process is necessary to microbicidal activity and for the success of immune and inflammatory reactions [57].

During oxidative stress, cells generate high levels of superoxide radicals. Free radical generation has been reported as an important mechanism for body protection from infections, mainly intestinal infections [58].

Phagocytosis is an important defense mechanism, especially for bacterial and fungal infections. The microbicidal activity of phagocytes is mediated by the production of reactive oxygen species (ROS) and the release of lysosomal enzymes [59]. ROSs are highly reactive molecules as a result of their unpaired electrons. These molecules rapidly react with various biomolecules, leading to DNA damage and the deterioration of membranes through lipid peroxidation and eventually causing cell death [60]. The ROS superoxide anion is a major component of this process, as it is a precursor to other oxygen radicals and essential for effective phagocytosis [61].

In this study, it was found that the phagocytes exposed to the fungus *Candida albicans* release a superoxide. This release was not modified in the presence of both the fungus and propolis or propolis fractions. In contrast, propolis or the ethyl acetate fraction adsorbed onto PEG microspheres increased a superoxide release by the phagocytes. This increased effect of propolis when adsorbed to PEG microspheres may be associated with possible protective properties of the PEG microsphere against chemical, physical, or biological degradation (enzymatic action, hydrolysis, oxidation, and changes in pH, among others). Several drugs that are associated with PEG and currently on the market, such as interferon alpha (Pegasys, PEG-Intron), growth hormone (Somavert), asparaginase (Oncaspar), and insulin, have prolonged residence time in the plasma relative to the corresponding free drugs, and the association with PEG has been shown to potentiate the pharmacological effects of the drug [62].

The results of this study confirm the importance of the superoxide anion in fungicidal death. The increase in superoxide release in the presence of PEG microspheres with adsorbed propolis affected the phagocytic and microbicidal activity.

We found that mononuclear phagocytes exposed to propolis adsorbed onto PEG microspheres present the highest levels of superoxide release, phagocytosis, and microbicidal activity. These findings indicate that PEG microspheres with adsorbed propolis stimulate the microbicidal activity of phagocytes in the blood. Similar results were shown using other medicinal plants or hormones [24, 33, 63].

Notably, the phagocytes exposed to PEG microspheres adsorbed the ethyl acetate fraction exhibited an increased superoxide release but presented a low microbicidal activity. This result may suggest that the immunosuppressive action of propolis is related to the presence of a molecule in a group of compounds that are normally isolated in the ethyl acetate fraction, such as flavonoids, tannins, xanthones, triterpene acids, saponins, and phenolic compounds.

Evaluating the results of the tests performed with propolis, the unfractionated extract demonstrates a better efficacy than the fractions, suggesting a possible synergistic effect between multiple chemical components, which is not observed to the same extent when the components are separated by fractionation. According to Bussmann et al. [64], the therapeutic success of the mixtures of natural origin may be associated with an intrinsic relationship between the compounds they contain, given that the studies of the activity of individual substances that comprise these mixtures have been shown to be inactive or toxic.

5. Conclusions

In conclusion, the results presented here suggest that propolis adsorbed to PEG microspheres has immunostimulatory effects on phagocytes in human blood, and this system might be used for a variety of therapies based on natural products and could reveal an additional mechanism for treating infections.

Acknowledgments

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Research Article

Hormonal Receptors in Skeletal Muscles of Dystrophic Mdx Mice

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Introduction. Several evidences show that muscles have an endocrine function. Glucocorticoid, estrogen, progesterone, and testosterone receptors have already been found in normal skeletal muscles, but not in dystrophic muscles. *Methods*. The gene expression of hormone receptors was compared between dystrophic and healthy muscles in mdx and C57BL6 mice strains. *Results*. The mdx mice showed a significant increase in the steroid receptors mRNA when compared to the C57BL6 mice: levels of androgen(s) receptors in the heart, estrogen receptors alpha in the EDL, and estrogen receptors beta in the quadriceps were increased. In addition, significant lowered levels of some other hormone receptors were found: corticosteroid receptors in the EDL and estrogen receptors alpha in the quadriceps. *Conclusion*. Dystrophic muscles bear significant differences in the expression of hormone receptors when compared to the C57BL6 mice strain. The importance of such differences is yet to be better understood.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder resulting in a defect in the muscle membrane protein called dystrophin. In the literature, there are more than thirty catalogued types of dystrophies. However, DMD is the most common kind, which affects 1 in every 3,500 male births. The disease initially manifests itself by muscular strength alterations, making the individual progressively lose the ability to walk and finally causing a reduction of the cardiac function and the respiratory muscles strength [1].

The most widely used experimental model for studying the disease is the mdx mouse. This kind of mouse bears a point mutation in the dystrophin gene. The mdx mutation occurred spontaneously due to a premature stop codon resulting in a termination in exon 23 of the dystrophin gene. The absence of dystrophin in mice produces a variety of phenotypes, higher than that observed in humans. Under normal conditions, the mdx mice show very few symptoms of the disease, and when subjected to intensive exercises, they present aggravation of the pathological alterations [2].

Corticosteroids are the only recommended treatment for DMD. The drug increases muscle strength and slows the onset of complications [1]. As muscles have an endocrine function and due to the fact that this disease causes muscle degeneration, the study of hormone receptors in these organs

are of paramount importance. The mechanism of action of corticosteroids in DMD has not been fully elucidated [3].

Several evidences demonstrate that muscles have an endocrine function. Therefore, the study of hormone receptors in muscles has shown to be important. These hormones have an important role in the protein synthesis and degradation, in the increase of muscle strength, and in the muscle protection against the damage caused by exercises, facts that make relevant the quantification of hormone receptors in dystrophic muscles.

The aim of this study was to evaluate the expression of hormone receptors in dystrophic muscles in mdx mice, comparing it with the expression of receptors in healthy muscles in C57BL6 mice.

2. Material and Methods

All procedures used in this study were approved and performed in accordance with the guidelines of the Animal Ethic Committee of FMABC.

Ten four-month-old mice, being five mdx and five C57BL6 strains, were studied. The latter was classified as control group. The animals were kept in spontaneous activity, with food and water ad libitum, and later sacrificed in a CO_2 chamber. Some muscles, like the cardiac, the gastrocnemius, the diaphragm, the quadriceps, the soleus, and the extensorum digitalis longus (EDL), from both mice strains were dissected, frozen in liquid nitrogen, and stored at −80°C.

Total tissue RNA was extracted using Trizol Reagent (Invitrogen Co., USA), according to the manufacturer's instructions, quantified by absorbance at 260 nm, and stored in diethylpyrocarbonate-treated water at −80°C. The integrity of RNA was verified by agarose gel electrophoresis. Total RNA (2 ug) was used for first-strand cDNA synthesis by reverse transcriptase. MMLV and RNaseOUT were also added to protect the RNA during the process.

An aliquot of the reaction was then submitted to PCR amplification with the appropriate primers. Alpha-actin was used as an internal control for the coamplification. The conditions for PCR were as follows: initial denaturation at 94°C for 5 minutes, followed by cycles of 94°C for 45 seconds, annealing of the temperature for 45 seconds, and 72°C for 1 minute. The final extension step occurs at 72°C for 10 minutes. The primers sequence(s), size of the PCR products, the annealing temperature, the number of cycles, the accession numbers of the target sequences, and the reactions conditions are presented in Table 1.

Three pooled RNA aliquots were omitted from the reverse transcriptase reaction to ensure the absence of products other than those originated from the reverse-transcribed mRNAs. PCR products were loaded in 1% or 2% agarose gel electrophoresis, stained with 0.2 ug/mL ethidium bromide. The gel was subjected to ultraviolet light and photographed. The bands intensity was quantified by video densitometry and the signals were expressed in relation to the intensity of the alpha-actin amplicon in each coamplified sample. All primers and enzymes used in this protocol were purchased from Invitrogen Co. The results were subjected to analysis of variance, using the program GBStat and considering the level of significance $P < 0.05$.

3. Results

The results of the mRNA of the mice were placed in Table 2.

Mdx mice showed significant raise in the expression of steroidal receptors mRNA when compared to the C57BL6 mice: androgen receptors in the heart, estrogen receptors alpha in the EDL, and estrogen receptors beta in the quadriceps were increased. In addition, significant lowered levels of some other hormone receptors were found: corticosteroid receptors in the EDL and estrogen receptors alpha in the quadriceps.

4. Discussion

Muscles express steroid receptors that belong to the superfamily of intracellular receptors which control gene transcription. The effect is to induce or repress particular genes [4–6].

The biological effects of corticosteroids are mediated by glucocorticoid receptors [6]. These effects include control of salt/water homeostasis, blood pressure regulation, metabolic alterations, and cellular immunity [5]. Glucocorticoids have catabolic effect on skeletal muscles and, in high doses, they can cause a steroid myopathy [6]. The muscles most affected by corticosteroids are the quadriceps and the other muscles of the pelvic girdle, being type IIb fibers the most susceptible to this effect. Exercises are effective in preventing steroid myopathy [7].

The catabolic effects of glucocorticoids are mediated by numerous mechanisms. Glucocorticoids inhibit glucose uptake in skeletal muscles and may contribute to the degradation of muscle proteins. They may also act directly to inhibit the protein synthesis or increase its degradation. One of the genes responsible for this effect encodes the glutamine synthetase, an enzyme responsible for catalyzing the formation of glutamine that is exported from the muscle in catabolic conditions. In muscular atrophy induced by glucocorticoids, the efflux of glutamine is 25–30% of the total exported protein of the muscle. In rats, an increase of glutamine synthetase and the level of mRNA were observed after the administration of glucocorticoids. Another effect of glucocorticoids is the activation of genes responsible for the ubiquitin-proteasome system, increasing the proteolytic activity in the muscle. It is clear that the transactivation of many genes must be involved in muscle atrophy by corticosteroids. The increase of myostatin, a well-known protein inhibitor of muscle mass, has also been observed with the use of glucocorticoids [6, 8].

Androgens, unlike glucocorticoids, exert an anabolic effect on muscles. The anabolic effects of androgens in skeletal muscles have been a source of controversy for more than six decades. Testosterone supplementation increases muscle mass in men; this effect is associated with hypertrophy of fibers type I and II with an increase of satellite cells and myonuclei. The mechanism by which testosterone increases muscle mass is poorly understood. Although the expression of androgen receptors have been described in muscle cells,

Table 1: RT-PCR analysis of various mRNAs: primes, size of the PCR products and conditions of the PCR reactions.

Gene	Primers sequence	Size of PCR product	Temperature (°C)	Number of cycles	Accession number at http://ncbi.nlm.nih.gov/
Androgen receptor	Sense: TACAACTTTCCGCTGGCTCT Antisense: CCGGAGTAGTTCTCCATCCA	464	58	30	NM013476.2
Glucocorticoid receptor	Sense: CGAGAGTCCTTGGAGGTCAG Antisense: GATCCTGCTGCTGAGAAAGG	410	55	24	X13358
Estrogen receptor Alpha	Sense: AATTCTGACAATCGACGCCAG Antisense: GTGCTTCAACATTCTCCCTCCTC	344	55	30 or 40*	NM007956
Estrogen receptor Beta	Sense: ACAGTCCTGCTGTGATGAAC Antisense: ACTAGTAACAGGGCTGGCAC	271	55	30 or 40*	U81451
Progesterone receptor	Sense: CCAGCATGTCGTCTGAGAAA Antisense: AAACACCATCAGGCTCATCC	426	60	40	NM008829
α-Actin	Sense: TGTGATGGTGGGAATGGGTCAG Antisense: TTGATGTCACGCACGATTTCC	513	55	28	AK151136

* Number of cycles varies with the saturation curve by the cycle of each tissue.

it is not clear which cells express this receptor and what the target of its action is [9]. Recent studies have shown that androgens promote the differentiation of lineages of mesenchymal pluripotent cells into myogenic cells and inhibit the differentiation into cell lineages of adipose tissue. This effect is blocked by androgen receptor antagonists, indicating that this effect may be mediated through these receptors [9]. Testosterone receptor expression is observed in almost all satellite cells and in 50% of myonuclei [9]. Physical activity also plays a role in the expression of the androgen receptor. In rats subjected to prolonged and intense physical activity, there was an increase of the expression of androgen receptors in the soleus, but not in the EDL [10].

Estrogens also play an important role in skeletal muscles, regulating lipid and carbohydrate metabolism and acting on the muscle growth and strength [11–14].

Alpha and beta receptors are expressed in skeletal muscles in both men and women [15]. Many studies suggest that estrogens increase muscle strength, and variations in the voluntary muscle strength were observed in humans during the menstrual cycle [13, 14]. In other studies, there was no change in the muscle function with increased estrogen levels or fluctuations during the menstrual cycle [16, 17]. In female rats, the eccentric muscle contractions cause less histological changes than in male rats [18]. In ovariectomized rats, there is a greater expression of proteins related to stress and a greater degree of injury compared to normal female rats. This difference disappears upon cessation of estrogen treatment [19, 20]. Intense and prolonged physical activity increases the expression of alpha receptor mRNA in the gastrocnemius muscle in female rats, but not in male rats [21]. The expression of estrogen receptors alpha and beta is increased in men undergoing physical exercises. The mRNA of estrogen receptors alpha and beta is increased in highly trained men than in moderately trained ones, and that expression is correlated with muscle oxidative capacity. Estrogen receptors can be regulated by physical activities and may be involved in the process of adaptation to physical exercises [22]. Estrogen receptor alpha mRNA expression is higher in the soleus than in the gastrocnemius or the EDL [23].

There are few studies of hormone receptors and hormone effects in dystrophic muscles.

Dubois and Almon [24] described a 100% increase in corticosteroid receptors in the leg muscles of mice with muscular dystrophy in relation to the control group; this increase was also found in chickens with muscular dystrophy [25].

The use of anabolic androgens was also tested in muscular dystrophy. DMD patients treated with oxandrolone were compared with a placebo group [26]; although patients treated with oxandrolone did not experience the loss of strength of the control group, the difference between both groups was not significant. However, the average QMT (quantitative muscle testing of four muscles) showed a significant increase in patients treated with oxandrolone compared with those treated with placebo. Besides the great anabolic power of oxandrolone in DMD, the drug promotes increased expression of genes that might partly explain its effect [27]. Skeletal muscles of mice with muscular dystrophy have testosterone receptors with the same characteristics of activity and affinity as those of normal mice.

Table 2: Comparison of hormone receptor mRNA in different muscle groups studied in mdx mice and C57BL6 mice.

Receptor	Glucocorticoid		Androgen		Estrogen alpha		Estrogen beta		Progesterone	
	C57BL/6	mdx	C57BL/6	mdx	C57BL/6	mdx	C57BL/6	mdx	C57BL/6	mdx
Solium	1.49 ± 0.16	1.60 ± 0.89	0.99 ± 0.28	1.79 ± 0.83	1.86 ± 0.29	1.72 ± 0.64	1.15 ± 0.52	1.18 ± 0.46	0.79 ± 0.12	0.71 ± 0.43
EDL	1.90 ± 0.29	1.20 ± 0.16**	1.27 ± 0.79	1.34 ± 0.19	0.99 ± 0.18	1.40 ± 0.10**	0.78 ± 0.22	1.16 ± 0.26	#	#
Gastrocnemius	2.42 ± 0.68	2.87 ± 0.50	2.62 ± 0.34	2.21 ± 0.23*	4.34 ± 0.82	3.94 ± 0.54	1.00 ± 0.21	1.00 ± 0.17	#	#
Quadriceps	0.42 ± 0.10	0.35 ± 0.03	1.30 ± 0.28	0.89 ± 0.07*	1.52 ± 0.29	1.04 ± 0.12*	5.14 ± 0.92	9.17 ± 1.95*	#	#
Diaphragm	1.47 ± 0.16	1.25 ± 0.24	0.80 ± 0.33	0.91 ± 0.33	1.82 ± 0.49	1.25 ± 0.18	5.72 ± 1.60	4.11 ± 0.58	0.58 ± 0.16	0.34 ± 0.07*
Heart	1.37 ± 0.19	1.64 ± 0.35	0.39 ± 0.16	1.61 ± 0.52*	0.91 ± 0.34	1.57 ± 0.70	#	#	#	#

nonexpression; ** significance statistical.

In DMD patients, estrogen levels were higher than a control group matched by age. LH levels were normal and FSH levels were low [28]. The use of estrogens has already been evaluated in DMD. In eleven DMD patients treated with diethylstilbestrol, a reduction in the levels of CK and LDH was observed with no analysis of the muscular strength parameters [29]. Another study showed anabolic effect of diethylstilbestrol in 7 DMD patients [30]. Young female mdx mice show less myonecrosis and more fiber regeneration; mdx ovariectomized females show a reduction of fiber regeneration. Older mdx females present extensive fibrosis, increase of the permeability of the sarcolemma, and marked deposition of extracellular matrix components compared to mdx males [31]. This increased regeneration of mdx females at younger ages may be explained by a mitogenic effect of estrogen that binds to nuclear receptors. This effect has been confirmed in vivo with estradiol activating the proliferation of muscle satellite cells [32]. In spite of the fact that myoblasts from mice express the estrogen receptor in vitro, estradiol does not influence the proliferation of myoblasts [32]. It is suggested that estrogen would act indirectly, by inducing growth factors like IGF-1 and IL-6 [31, 32]. In our study, we observed that the expression of glucocorticoid receptors is reduced in the EDL muscle of dystrophic mice. As the treatment with glucocorticoids increases the expression of these receptors, we could speculate that this would be one of the probable mechanisms of action of corticosteroids. We also note that the expression of androgen receptors is reduced in the quadriceps and the gastrocnemius of mice with a mutation in the dystrophin gene. Since the expression of these receptors increases with testosterone administration, this would also be a likely benefic mechanism of treatment with androgens in muscular dystrophy.

Another observation from our study was that the expression of estrogen receptors was increased in the EDL (estrogen alpha) and the quadriceps (estrogen beta) of dystrophic mice. This effect may explain the worst outcome of female mdx mice, which present extensive fibrosis, increased permeability of the sarcolemma, and marked deposition of extracellular matrix components, once they had been suffering a prolonged action of estrogens [33].

Another fact found, which requires more studies, was the reduction in progesterone receptor expression in the diaphragm of dystrophic mice compared with healthy mice.

5. Conclusion

This is a preliminary study and it represents the first study of hormone receptors in differentmuscles of mdx mice. The analysis of different muscle groups showed that dystrophic muscles have some significant differences in hormone receptor expression when compared to normal mice. Changes in expression of hormone receptors in dystrophic muscles reported here could explain some of the events related to the pathophysiology of DMD and the response to the therapy already described in the literature. Hence, carrying out more studies on the hormone receptor expression in DMD patients would open new path for the discovery of new drugs that might, more effectively, slow the disease progression and improve the quality of life of these patients.

Abbreviations

cDNA:	Complementary deoxyribonucleic acid
CK:	Creatine kinase
DMD:	Duchenne muscular dystrophy
EDL:	Extensor digitorum longus
FSH:	Follicle-stimulating hormone
IGF1:	Insulin growth factor 1
IL-6:	Interleukin-6
LDH:	Lactase dehydrogenase
LH:	Luteinizing hormone
MMLV:	Moloney murine leukemia virus reverse transcriptase
mRNA:	Messenger ribonucleic acid
PCR:	Polymerase chain reaction
QTM:	Quantitative muscle testing of four muscles
RNA:	Ribonucleic acid
RNaseOUT:	Ribonuclease inhibitor
RT:	Reverse transcriptase.

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Research Article

Modulation of the Antibiotic Activity by Extracts from *Amburana cearensis* A. C. Smith and *Anadenanthera macrocarpa* (Benth.) Brenan

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The aim of this study was to verify the possible interactions between ethanol extracts of *Amburana cearensis* A. C. Smith and *Anadenanthera macrocarpa* (Benth.) Brenan, combined with six antimicrobial drugs against multiresistant strains of *Staphylococcus aureus* and *Escherichia coli* isolated from humans. The antibacterial activity of the extracts was determined using the minimum inhibitory concentration (MIC). The microdilution assay was performed to verify the interactions between the natural products and the antibiotics using a subinhibitory concentration. The activity of amikacin associated with the extract of *Anadenanthera macrocarpa* against EC 27 was enhanced, demonstrating an MIC reduction from 128 to 4 μg/mL. Among the β-lactams, no potentiation on its activity was observed, with exception to the antagonism of the natural products with ampicillin against *S. aureus* 358.

1. Introduction

The research for new antibacterial substances becomes necessary due to increase of the antibiotic resistance of clinically important pathogens [1]. Due to this fact, substances derived from plants could be attractive alternatives [2, 3]. Natural products from plant may change or modulate the action of the antibiotic, enhancing or reducing the activity of this drug [4]. In recent years, many plants have been evaluated not only for the direct antibacterial action, but also as a modulator of the antibiotic activity [5, 6].

The use of natural products, mainly the chemical components of plants with antimicrobial properties have contributed to significant results in therapeutic treatments [7–9].

The *Amburana cearensis* (German) A. C. Smith, Fabaceae, is a tree which reaches 10–12 m of height [10]. Also known as "Cumaru", it has been explored for use in fine furniture making, sculpture and carpentry, being listed as a threatened species [11]. Moreover, due to their medicinal properties, the bark and seeds are used to produce popular drugs for the treatment of cough, asthma, bronchitis, and pertussis. The species is still used in the perfume

Table 1: Origin of the bacterial strains and profile of resistance to antibiotics.

Bacteria	Origin	Profile of resistance
Escherichia coli 27	Surgical wound	Ast, Ax, Amp, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Chlo, Im, Kan, Szt, Tet, Tob
Escherichia coli ATCC10536	ATCC	—
Staphylococcus aureus 358	Surgical wound	Oxa, Gen, Tob, Ami, Kan, Neo, Para, But, Sis, Net
Staphylococcus aureus ATCC25923	ATCC	—

Ast: Aztreonam; Ax: Amoxicillin; Amp: Ampicillin; Ami: Amikacin; Amox: Amoxicillin; Ca: Cefadroxil; Cfc: Cefaclor; Cf: Cefalotin; Caz: Ceftazidime; Cip: Ciprofloxacin; Chlo: Chloramphenicol; Im: Imipenem; Kan: Kanamycin; Szt: Sulfametim; Tet: Tetracycline; Tob: Tobramycin; Oxa: Oxacillin; Gen: Gentamicin; Neo: Neomycin; Para: Paramomycin; But: Butirosin; Sis: Sisomicin; Net: Netilmicin; (—): sensitivity. ATCC: american type culture collection.

Table 2: Botanical families, species, and number of the title of the plants used in this study.

Family	Species	Number HCDAL	Herbarium
Leguminosae	*Amburana cearensis*	5545	Vale do São Francisco-UNIVASF
Fabaceae	*Anadenanthera macrocarpa*	6490	Dárdano Andrade Lima-URCA

industry [12]. Medical trials have demonstrated preclinical anti-inflammatory, bronchodilator and analgesic activity for the hydroalcoholic extract, being possible to associate these effects of coumarin and flavonoidic fraction [13, 14].

The *Anadenanthera macrocarpa* is a species belonging to Mimosoideae [15]. This is a species of "angico" with larger geographic areas, occurring from southern Bolivia to northern Argentina, in Brazil, and is not only found in southern region [16]. Popular medicine has been used against several diseases through the preparation of syrups and lickers, it is used for the treatment of coughs, bronchitis, fads, external wounds and inflammation [17].

The objective of this study was to realize the phytochemical prospecting and assay of the *in vitro* ethanolic extracts of leaves of *A. Cearensis* and *A. macrocarpa* to determine the antibacterial activity and the modifying antibiotic activity of aminoglycosides and beta-lactams against the *Escherichia coli* and *Staphylococcus aureus*.

2. Material and Methods

2.1. Bacterial Material. The bacterial strains used were *E. coli* (EC-ATCC10536 and EC27) and *S. aureus* (SA-ATCC25923 e SA358) with a resistance profile identified in Table 1. All strains were maintained on heart infusion agar (HIA, Difco Laboratories Ltd.). Before the tests, the strains were grown for 18 h at 37°C in broth brain heart infusion (BHI, Difco Laboratories Ltd.).

2.2. Plant Material. Leaves of *Amburana cearensis* and *Anadenanthera macrocarpa* were collected at Penaforte, Ceara, Brazil. The plant material was identified and a voucher specimen was placed in the respective herbal collections (Table 2).

2.3. Preparation of Ethanol Extracts of Amburana Cearensis and Anadenanthera Macrocarpa. For the preparation of extracts, leaves were collected and weighed (Table 3). The material was powdered and wrapped in a container with an amount of solvent to submerge the plant material by 72 hours. After this time, the eluent was filtered and concentrated in a rotary vacuum condenser (model Q-344B-Quimis, Brazil) and in an ultrathermal bath (model Q-214 M2-Quimis, Brazil) [18]. For the tests, the solutions used were prepared from extracts in a concentration of 10 mg/mL dissolved in DMSO (dimethyl-sulfoxide), then diluted with distilled water to a concentration of 1024 μg/mL.

2.4. Phytochemical Prospecting. The phytochemicals tests to detect the presence of heterosides, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids, and alkaloids were performed according to the method described by Matos [19]. The tests were based on visual observation of the change in color or formation of precipitate after the addition of specific reagents.

2.5. Antibacterial Activity Test. The MIC (minimal inhibitory concentration) was determined in a microdilution assay utilizing an inoculum of 100 μL of each strain, suspended in brain heart infusion (BHI) broth up to a final concentration of 10^5 CFU/mL in 96-well microtiter plates, using twofold serial dilutions. Each well received 100 μL of each extract solution. The final concentrations of the extracts varied from 512 to 8 μg/mL. MICs were recorded as the lowest concentrations required to inhibit growth. The minimal inhibitory concentration for the antibiotics was determined in BHI by the microdilution assay utilizing suspensions of 10^5 CFU/mL and a drug concentration range from 2.5 to 0.0012 mg/mL (twofold serial dilutions). MIC was defined as the lowest concentration at which no growth was observed. For the evaluation of the extracts as modulators of the resistance to the antibiotics, MIC of the antibiotics was determined in the presence or absence of EEAC and EEAM at subinhibitory concentrations (8 μg/mL) and the plates were incubated for 24 h at 37°C. Each antibacterial assay for MIC determination was carried out in triplicate.

Table 3: Dry weight and yield of ethanolic extracts (g).

Biological species	Solvent	Leaves (mass)	Extract gross (yield)
Anadenanthera macrocarpa	Ethanol (EEAM)	50 g	9,24%
Amburana cearensis	Ethanol (EEAC)	50 g	8,15%

EEAM: ethanolic extract of *Anadenanthera macrocarpa*; EEAC: ethanolic extract of *Amburana cearensis*.

Table 4: Phytochemical prospecting of the ethanolic extracts of *Anadenanthera macrocarpa* and *Amburana cearensis.*

	Metabolites														
Extracts	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EEAC	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+
EEAM	−	+	−	+	+	+	+	+	+	+	+	+	+	+	−

1: phenols; 2: tannin pyrogallates; 3: tannin phlobaphenes; 4: anthocyanins; 5: anthocyanidins; 6: flavones; 7: flavonols; 8: xanthones; 9: chalcones; 10: aurones; 11: flavonols; 12: leucoanthocyanidins; 13: catechins; 14: flavonones; 15: alkaloids; (+): presence; (−): absence. EEAC: etanolic extract of *Amburana cearensis;* EEAM: Ethanolic Extract of *Anadenanthera macrocarpa.*

Table 5: Minimal inhibitory concentration (MIC) of the ethanolic extracts of *Anadenanthera macrocarpa* and of *Amburana cearensis* (μg/mL).

Extracts and antimicrobials	EC 27	EC-ATCC 10536	AS 358	SA-ATCC 25923
EEAM	≥1024	≥1024	≥1024	≥1024
EEAC	≥1024	≥1024	≥1024	512

EEAM: ethanolic extract of *Anadenanthera macrocarpa*; EEAC: ethanolic extract of *Amburana cearensis*. EC: *Escherichia coli*, SA: *Staphylococcus aureus*.

2.6. Evaluation of the Modulation of Extracts on the Resistance to Aminoglycosides and β-Lactams Antibiotics. To evaluate the extracts as modulators of antibiotic action, the MICs of antibiotics of the class aminoglycoside and beta-lactams, were evaluated in the presence and absence of the extracts in sterile microplates. The antibiotics were evaluated at concentrations ranging from 512 to 0.5 mg/mL. All antibiotics tested were obtained from Sigma. The extracts were mixed in BHI broth at 10% sub—inhibitory concentrations obtained and determined after the test evaluation of MIC, and for the modulation test concentration was used a concentration of extract referring the MIC diluted 8 times (MIC/8). The preparation of the antibiotic solutions was performed by adding sterile distilled water in a double concentration (1024 μg/mL) in relation to the initial concentration set volume of 100 μL and serially diluted 1 : 1 in 10% BHI broth. In each well with 100 μL of culture medium containing the bacterial suspension diluted (1 : 10). The same controls used in the evaluation of MIC for the extracts were used for the modulation [4]. The plates were filled and incubated at 35°C for 24 hours, and after that the reading was evidenced by the use of resazurin as previously mentioned in the test of determination of the MIC.

3. Results and Discussion

The extracts evaluated in this work showed the yields demonstrated in Table 3, where we observed a higher yield of extract *A. macrocarpa* compared to *A. cearensis*. To perform the microdilution assay, the extracts were diluted in DMSO obtaining a solution of concentration of 10 mg/mL. A pilot study was conducted using only DMSO, but no antibacterial or modulatory activity was observed, indicating nontoxic effect.

Table 4 shows the presence of various potentially bioactive compounds in the extracts evaluated, like phenols, tannin pyrogallatos, tannin phlobaphenes, anthocyanins, anthocyanidins, flavones, flavonols, xanthones, chalcones, aurones, flavononols, leucoanthocyanidins, catechins, flavonones, and alkaloids. Through phytochemical prospecting of extracts, it was possible to identify the presence of several classes of secondary metabolites that exhibit a wide variety of biological activities such as antimicrobial [20–22], antioxidant [23], antitumor and antiophidic [24].

Table 5 shows the determination of minimum inhibitory concentration (MIC) of ethanol extracts tested against *E. coli* and *S. aureus* of reference and multiresistant. Comparatively, the extracts EEAM and EEAC showed the same MIC with the exception of EEAC against SA-ATCC 25923 which showed a better MIC of 512 μg/mL.

Several medicinal plants were used as a source of many antimicrobial drugs used in the treatment of infectious diseases, including against bacteria multiresistant to antibiotics [25]. It is known that the synergistic action of the natural products with antimicrobial agents is commonly used in the therapeutic treatment [26, 27].

Table 6 shows the interference of the extracts on the activity of aminoglycosides, demonstrating a modulation in the activity of antibiotics, reducing the MICs. The more representative effect was observed with the association of EEAM and amikacin, an increase being observed in the antibiotic activity against EC27, reducing the MIC of the antibiotic from 128 to 4 μg/mL.

Due to absorption into the intracellular space, the cell toxicity is common to all aminoglycosides (except to streptomycin). Nephrotoxicity, ototoxicity, and neuromuscular blockade are the most important toxic effects of aminoglycosides [7, 28]. The reported frequency of these side effects

Table 6: MIC of the aminoglycosides in the presence and absence of ethanolic extracts of *A.cearensis* and *A. macrocarpa* at a concentration 128 μg/mL.

Antibiotics	EC 27			SA 358		
	MIC	MIC combined		MIC	MIC combined	
		EEAC	EEAM		EEAC	EEAM
Gentamicin	64	4	4	16	16	4
Amikacin	128	8	4	64	64	16

EEAC: ethanolic extract of *Amburana cearensis*; EEAM: ethanolic extract of *Anadenanthera macrocarpa*. EC: *Escherichia coli*, SA: *Staphylococcus aureus*.

Table 7: Minimal inhibitory concentration (MIC) of beta-lactam in the presence and absence of ethanolic extracts of *Amburana cearensis* and *Anadenanthera macrocarpa* and a concentration of MIC/8 (128 μg/mL).

Antibiotics	EC 27			SA 358		
	MIC	MIC combined		MIC	MIC combined	
		EEAC	EEAM		EEAC	EEAM
Benzetacil	≥1024	≥1024	≥1024	512	512	512
Cephalothin	16	16	16	≤0.5	≤0.5	≤0.5
Ampicillin	≥1024	≥1024	≥1024	128	512	512
Oxacillin	≥1024	≥1024	≥1024	≤0.5	≤0.5	≤0.5

EEAC: ethanolic extract of *Amburana cearensis*; EEAM: ethanolic extract of *Anadenanthera macrocarpa*. EC: *Escherichia coli*, SA: *Staphylococcus aureus*.

is highly variable due to different criteria used for diagnosis [29]. The combination of the aminoglycosides with natural products can be an alternative to minimize the side effects of this class of antibiotics, since the association leads to a synergistic effect significantly reducing the MIC of these drugs, decreasing the dose needed for therapeutic usage.

Many β-lactam antibiotics can penetrate Gram-negative bacteria via protein channels present in the outer membrane. Through these channels, the drug can reach its receptor on the cell wall and exert its bactericidal action [30]. Although extracts have in its constitution secondary metabolites such as tannins and flavonoids, which are synthesized by plants in response to microbial infections [31, 32], modifying the cell wall or disrupting the bacterial cell membrane [33, 34]. However, Gram-negative strains were not susceptible to association between the extracts and the antibiotics (Table 7). This fact can be explained by other resistance mechanisms present in these bacteria as efflux pump, production of enzymes that cleave the beta-lactam ring (β-lactamases), changes in PBP, among other [35, 36].

A similar fact occurs with the combination of natural products with beta-lactams against the Gram-positive strains. No modulatory effect was observed against these strains, with exception to the combination with ampicillin, which resulted in an antagonism with the MIC enhancing from 128 to 512 μg/mL (Table 7).

4. Conclusion

The present results indicate that ethanolic extracts of *A. cearensis* and *A. macrocarpa* are an alternative source of natural products with antibacterial action, due to the presence of several antibacterial, which can be responsible for the observed modulatory effects, indicating the possibility of using natural products combined with aminoglycosides to increase the antimicrobial potential of these drugs against multiresistant microorganisms.

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Research Article

Essential Oils from Fruits with Different Colors and Leaves of *Neomitranthes obscura* (DC.) N. Silveira: An Endemic Species from Brazilian Atlantic Forest

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Neomitranthes obscura (DC.) N. Silveira is an endemic plant of Brazilian Atlantic Forest and widely spread in the sandbanks of "Restinga de Jurubatiba" National Park. It is popularly known by local population as "camboim-de-cachorro" or "cambuí-preto" and recognized by its black ripe fruits. However, specimens with yellow ripe fruits were localized in the "Restinga de Jurubatiba" National Park. The aim of the present study was to evaluate chemical composition of essential oils obtained from leaves and fruits of *N. obscura* specimens with different fruit color (black and yellow) by GC and GC-MS. Essential oils from leaves of specimens with black and yellow fruits indicated a predominance of sesquiterpenes (81.1% and 84.8%, resp.). Meanwhile, essential oil from black fruits presented a predominance of monoterpenes (50.5%), while essential oil from yellow fruits had sesquiterpenes (39.9%) as major substances. Despite previous studies about this species, including essential oil extraction, to our knowledge this is the first report on *N. obscura* fruits with different colors. Our results suggest the occurrence of unless two different varieties for this species.

1. Introduction

"Restinga" is a type of habitat originated from Quaternary marine deposits and is represented by herbaceous and arbustive-arboreal vegetation covering typically sandy soils [1, 2]. It is characterized by large sandy coastal plains of sedimentary origin that are rippled by rows of dunes isolating lagoons, lakes, ponds, bogs, and marshes. Such a several physical conditions give rise to a diversity of habitats that are colonized by a great variety of vegetal communities [3]. "Restinga" contains many species in common with the Atlantic forest but presents diverse physiological responses to a drier habitat [4].

On this context, "Restinga de Jurubatiba" National Park is an area for permanent preservation of "restinga" habitats (Brazilian sandy coastal plain vegetation) on Rio de Janeiro State, Brazil. This area (22° to 22° 23′ S and 41° 15′ to 41° 45′ W) comprises the municipalities of Macaé, Carapebus and Quissamã [2].

The Myrtaceae family has a great diversity, mainly found across tropical and temperate areas of the globe. It comprises about 4630 species, distributed among about 144 genera [5]. In Brazil, this family is mainly constituted by wood species, being one of the dominant families in Atlantic Forest [6], and it is represented by about 23 genera and 976 species, of which 749 are endemic [7]. Despite the high number of

Myrtaceae species found at "restinga" habitats and numerous voucher specimens deposited at herbaria, intricacy related to identification of many species from this family remains [8].

Chemical composition of essential oils from some Myrtaceae species from the "Restinga de Jurubatiba" National Park was evaluated. Meanwhile, essential oil from *Eugenia sulcata* Spring ex Mart. and *Myrciaria floribunda* (H.West ex Willd.) O.Berg exhibited anticholinesterase activity [9, 10].

Many species from this family are cultivated due to their edible fruits, source of scents, essences, and as ornamentals, such as Eucalyptus spp. *Eugenia uniflora* L., *Psidium guajava* L. and *Syzygium jambos* (L.) Alston [11]. In addition, numerous species from this family are used in folk medicine, such as *Psidium gajava*, which is used as antiparasitic, anti-inflammatory, antimicrobial, and treatment of intestinal diseases [12]. Ethnobotanical studies conducted with another species from this family in "restinga" areas indicate several popular uses for them, such as the treatment of diarrhea, sore throat, gout, rheumatism, influenza, urinary tract diseases, diarrhea and diabetes [13]. Species from this family also have great ecological value, since they provide important reward and attract pollinators [14, 15].

The genus *Neomitranthes* is restricted to Brazil, with sixteen identified species [7]. *Neomitranthes obscura* (DC.) N. Silveira is an endemic species of the Brazilian Atlantic Forest and widely spread in the sandbanks of "Restinga de Jurubatiba" National Park [16]. It is popularly known by local population as "camboim-de-cachorro" or "cambuí-preto" and commonly used for intestinal disorders as well as food [17, 18].

In the literature, ripe fruits of *Neomitranthes obscura* are described with black pericarp [8, 19]. However, specimens with yellow ripe fruits were localized during this study in the "Restinga de Jurubatiba" National Park. Both populations occur together, and there are not vegetative distinctions. This species is easily identified by the green cylindrical galls on the apical/axial bud leaf [20] and the globoses fruits crowned by the calyx tube [8]. Fruit color varieties can be found in some Myrtaceae species, but according to Moreno [21], varieties are not easily definable entities in this family and are not widely accepted.

The aim of the present study was to evaluate chemical composition of essential oils obtained from specimens of *Neomitranthes obscura* (DC.) N. Silveira with different fruit color.

2. Materials and Methods

2.1. Plant Material. Leaves and ripe fruits of *Neomitranthes obscura* (DC.) N. Silveira were collected from three individuals of each fruit color specimens in "Restinga de Jurubatiba" National Park (Rio de Janeiro, Brazil), in open *Clusia* scrub vegetation (Black fruits specimens: S 22° 13′ 4.32″-W 41° 35′ 14.18″; S 22° 13′ 4.67″-W 41° 35′ 13.74″; S 22° 13′ 3.92″-W 41° 35′ 13.28″/Yellow fruits specimen: S 22° 13′ 4.43″-W 41° 35′ 14.83″; S 22° 13′ 4.00″-W 41° 35′ 13.96″; S 22° 13′ 4.04″-W 41° 35′ 14.17″). The ripening of the fruits was characterized by the softening, sweetening, and coloring of the tissue (black or yellow pericarp in the different specimens). This species was identified by the botanist Dr. Marcelo Guerra, and voucher of the yellow fruit specimens (L. Rocha 03, 04, 06) and black fruit specimens (L. Rocha 02, 05, 07) were deposited at the herbarium of the Faculdade de Formação de Professores (Universidade do Estado do Rio de Janeiro, Brazil).

2.2. Extraction of the Essential Oils. Fresh leaves from the black fruit specimen (LBF) (2.720 kg), fresh leaves from the yellow fruit specimens (LYF) (2.790 kg), fresh black fruits (BF) (0.896 kg), and fresh yellow fruits (YF) (0.914 kg) were individually ground with distilled water using an automatic blender (Ética Equipamentos Científicos S.A., Brazil). Hydrodistillation method was employed using Clevenger type apparatus, and each plant material was placed in a 5 L flask [9]. The extraction was performed for 4 hours, and after this period, essential oils were collected, dried over anhydrous sodium sulphate, and stored at 4°C for further analyses.

2.3. Gas Chromatography/Mass Spectrometry Analysis. Essential oils were analyzed by a QP2010 (SHIMADZU) gas chromatograph equipped with a mass spectrometer using electron ionization. The gas chromatographic (GC) conditions were as follows: injector temperature, 260°C; detector temperature, 290°C; carrier gas (Helium), flow rate 1 mL/min and split injection with split ratio 1 : 40. Oven temperature was initially 60°C and then raised to 290°C at a rate of 3°C/min. One microliter of each sample, dissolved in CH_2Cl_2 (1 : 100 mg/μL), was injected at RTX-5 column (i.d. = 0.25 mm, length 30 m, film thickness = 0.25 μm). Mass spectra were recorded at 70 eV with a mass range from m/z 35 to 450 and scan rate of 1 scan/s. The retention indices (AI) were calculated by interpolation of retention times of the substances to the retention times of a mixture of aliphatic hydrocarbons (C7-C40) (Sigma) analyzed in the same conditions [25]. The identification of substances was performed by comparison of their retention indices and mass spectra with those reported in the literature [26]. The MS fragmentation pattern of compounds was also checked with NIST mass spectra libraries. Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (GC/FID) with a QP2010 (SHIMADZU) gas chromatograph, under the same conditions of GC/MS analysis and percentages obtained by FID peak-area normalization method.

3. Results and Discussions

According to Souza and Morim [8], the species *N. obscura* has globoses fruits crowned by the calyx tube with black pericarp when ripe. These characteristics are helpful in the identification of this species [8]. During our study, it was observed not only specimens with the characteristic black ripe fruits, but also populations with ripe yellow fruits (Figure 1). The use of essential oils in studies of intra- and interspecific genetic diversity and geographic patterns

Table 1: Chemical constituents of essential oil from leaves and fruits of *Neomitranthes obscura* (DC). N. Silveira.

Compound	AI	Plant part			
		Leaves		Fruits	
		Black	Yellow	Black	Yellow
Hexanal	803	—	—	—	0.6
Unidentified[1]	812	0.3	1.7	—	—
Unidentified[2]	854	—	—	1.8	0.8
α-Pinene	936	0.9	1.7	11.0	1.8
Unidentified[3]	954	—	—	0.9	—
(3Z)-Octen-2-ol	968	—	—	0.9	—
β-Pinene	980	1.2	2.0	13.5	3.2
Myrcene	993	0.5	—	1.6	—
α-Phellandrene	1008	0.4	—	—	—
δ-3-Carene	1013	1.0	2.1	—	—
o-Cymene	1027	—	—	0.7	0.6
Limonene	1031	1.3	2.7	2.4	0.7
Unidentified[4]	1106	—	—	—	0.6
α-Camphollenal	1129	—	—	1.3	0.6
Trans-pinocarveol	1142	—	—	3.4	1.5
Trans-verbenol	1148	—	—	1.0	0.7
Pinocarvone	1166	—	—	1.0	—
p-Cymen-8-ol	1188	—	—	2.6	2.0
α-Terpineol	1194	—	—	4.1	1.7
Myrtenol	1200	—	—	3.0	1.5
Verbenone	1213	—	—	1.2	—
1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-ol [22]	1226	—	—	1.3	—
Unidentified[5]	1278	—	—	1.2	—
Unidentified[6]	1317	—	—	2.6	—
Trans-p-menth-6-en-2,8-diol	1381	—	—	1.2	—
Sativene	1397	3.1	2.4	—	1.2
Unidentified[7]	1403	—	—	2.4	—
(E)-Caryophyllene	1424	7.0	6.2	—	—
Carvone hydrate	1428	—	—	1.2	—
γ-Elemene	1437	2.3	—	—	—
α-Guaiene	1443	1.5	—	—	—
α-Humulene	1458	0.6	—	—	—
γ-Gurjunene	1481	1.0	—	—	—
Unidentified[8]	1485	—	—	—	0.9
β-Selinene	1491	6.3	6.0	1.6	2.1
Unidentified[9]	1496	1.9	—	—	—
Unidentified[10]	1498	—	—	1.9	—
α-Selinene	1500	6.4	5.7	—	—
α-Bulnesene	1510	1.1	—	—	—
β-Bisabolene	1512	—	3.4	—	—
2,4-di-tert-butylphenol [23]	1516	1.9	13.4	—	—
7-epi-α-Selinene	1523	2.5	2.3	—	—
(E)-γ-Bisabolene	1536	—	4.4	—	—
Selina-3,7,(11)diene	1540	14.1	18.7	—	—

Table 1: Continued.

Compound	AI	Plant part			
		Leaves		Fruits	
		Black	Yellow	Black	Yellow
Unidentified[11]	1543	4.7	5.0	—	—
Trans-dauca-4 (11)7-diene	1547	11.4	13.9	—	—
Unidentified[12]	1558	—	—	—	0.8
Germacrene B	1563	21.8	8.4	—	—
Unidentified[13]	1568	0.8	—	—	—
Caryophyllene oxide	1589	—	—	1.7	12.6
Unidentified[14]	1594	—	—	—	2.5
Humulene epoxide II	1615	—	—	—	2.2
Unidentified[15]	1619	—	—	—	1.5
Unidentified[l6]	1624	0.9	—	—	—
Unidentified[17]	1633	—	—	1.9	2.6
Unidentified[18]	1638	—	—	—	1.3
Desmetoxy encecalin	1650	—	—	2.3	3.6
β-Eudesmol	1656	—	—	—	1.0
Unidentified[19]	1658	0.6	—	—	0.6
Unidentified[20]	1659	0.2	—	—	—
Selin-11-en-4α-ol	1661	1.2	—	3.5	6.0
Allohimachalol	1663	—	—	1.4	—
Unidentified[21]	1671	0.7	—	—	—
α-(z)-Santalol	1677	—	—	—	2.0
Cadalene	1681	—	—	—	1.2
Unidentified[22]	1687	—	—	—	0.9
Unidentified[23]	1689	—	—	—	1.1
Unidentified[24]	1693	—	—	1.3	1.2
Juniper camphor [24]	1702	—	—	—	1.1
Unidentified[25]	1723	—	—	—	1.3
Unidentified[26]	1733	—	—	—	1.5
Cyclocolorenone	1757	—	—	—	1.9
Unidentified[27]	1764	—	—	3.5	1.1
Aristolone	1768	—	—	—	2.1
Unidentified[28]	1778	—	—	—	1.7
Unidentified[29]	1791	—	—	4.1	4.5
Unidentified[30]	1796	—	—	—	0.7
Unidentified[31]	1801	—	—	—	0.9
Unidentified[32]	1804	—	—	—	1.2
Unidentified[33]	1808	—	—	—	1.2
Unidentified[34]	1814	—	—	1.98	3.1
Unidentified[35]	1826	—	—	—	2.4
Unidentified[36]	1872	0.8	—	—	—
Unidentified[37]	1875	—	—	—	1.2
Unidentified[38]	1879	—	—	2.0	—
Unidentified[39]	1906	—	—	1.2	2.0
Unidentified[40]	1910	—	—	—	1.1
Unidentified[41]	1924	0.8	—	2.4	—
Unidentified[42]	1952	—	—	4.1	—
Unidentified[43]	1955	—	—	—	5.1

Table 1: Continued.

Compound	AI	Plant part			
		Leaves		Fruits	
		Black	Yellow	Black	Yellow
Unidentified[44]	1963	—	—	2.1	1.6
Unidentified[45]	1967	—	—	2.9	1.9
Unidentified[46]	2034	—	—	—	1.0
Unidentified[47]	2347	0.7	—	—	—
Total identified		86.1	93.3	70.0	70.2

[1]MS *m/z* (Relat. int.): 40; 41; 45 (100); 69; 71; 87.
[2]MS *m/z* (Relat. int.): 41; 43 (100); 69; 83; 97; 98.
[3]MS *m/z* (Relat. int.): 55 (100); 69; 70; 97; 98; 112.
[4]MS *m/z* (Relat. int.): 41; 43; 57 (100); 70; 96; 98; 114; 207.
[5]MS *m/z* (Relat. int.): 41; 43; 69; 83 (100); 97; 125; 135; 207.
[6]MS *m/z* (Relat. int.): 41; 43 (100); 59; 71; 84; 100; 123.
[7]MS *m/z* (Relat. int.): 41; 43 (100); 59; 71; 95; 109; 123; 133; 151; 166.
[8]MS *m/z* (Relat. int.): 41; 55; 67; 79; 93; 107; 121; 135; 145; 163; 178 (100); 2004.
[9]MS *m/z* (Relat. int.): 41; 57; 71; 91; 105; 119; 133; 147; 161 (100); 175; 189; 204.
[10]MS *m/z* (Relat. int.): 41; 43; 59; 71; 97; 108; 112; 126; 155; 189; 204.
[11]MS *m/z* (Relat. int.): 41; 55; 67; 81; 91; 105; 119; 133; 147; 161; 175; 189 (100); 204.
[12]MS *m/z* (Relat. int.): 41; 69; 79 (100); 91; 106; 121; 135; 149; 159; 177; 187; 205; 220.
[13]MS *m/z* (Relat. int.): 41; 55; 69; 79; 91; 75; 119; 133; 148; 161; 189 (100); 204.
[14]MS *m/z* (Relat. int.): 41; 55; 67; 79; 91; 108; 119; 135; 147; 163; 178 (100); 192; 202; 220.
[15]MS *m/z* (Relat. int.): 41; 43 (100); 67; 81; 95; 109; 123; 135; 147; 161; 178; 189; 204; 222.
[16]MS *m/z* (Relat. int.): 41; 43; 67; 81; 93; 105; 123 (100); 135; 147; 161; 189; 204.
[17]MS *m/z* (Relat. int.): 40; 44; 69; 81; 91; 105; 120; 131; 145; 159; 173; 187; 202 (100).
[18]MS *m/z* (Relat. int.): 41; 43; 59; 79; 91; 105; 119; 135; 145 (100); 161; 177; 185; 204; 218.
[19]MS *m/z* (Relat. int.): 41; 69; 81; 95; 100; 125 (100); 135; 147; 164; 177; 187; 205; 220.
[20]MS *m/z* (Relat. int.): 44; 55; 81; 93; 107; 133 (100); 149; 161; 173; 187; 202.
[21]MS *m/z* (Relat. int.): 41; 43; 67; 81; 93; 105; 122; 133; 148; 161; 189 (100); 104.
[22]MS *m/z* (Relat. int.): 41; 43; 69 (100); 79; 93; 109; 123; 133; 151; 160; 175; 187; 207; 220; 236.
[23]MS *m/z* (Relat. int.): 41; 55; 67; 81; 96 (100); 107; 123; 126; 149; 165; 179; 194; 205; 222; 252.
[24]MS *m/z* (Relat. int.): 41; 43 (100); 69; 71; 93; 109; 121; 153; 167; 178; 196.
[25]MS *m/z* (Relat. int.): 41; 43; 67; 79; 91; 105; 123; 138; 151 (100); 161; 179; 194; 205; 218.
[26]MS *m/z* (Relat. int.): 41; 43 (100); 67; 83; 93; 111; 123; 135; 149; 161; 175; 193; 208; 221; 236.
[27]MS *m/z* (Relat. int.): 41; 43; 60 (100); 73; 85; 99; 115; 129; 143; 157; 171; 185; 199; 228.
[28]MS *m/z* (Relat. int.): 41; 43 (100); 67; 79; 93; 108; 119; 138; 145; 161; 176; 194; 208; 218; 231.
[29]MS *m/z* (Relat. int.): 41; 59; 81; 95; 105; 119; 133; 147; 161; 179 (100); 187; 205; 220.
[30]MS *m/z* (Relat. int.): 41; 55; 67; 79; 96 (100); 107; 121; 135; 149; 162; 176; 189; 208; 218; 234.
[31]MS *m/z* (Relat. int.): 41; 43 (100); 59; 79; 91; 107; 123; 135; 147; 160; 177; 187; 205; 223; 232; 238.
[32]MS *m/z* (Relat. int.): 41; 67; 79; 93 (100); 108; 119; 133; 151; 161; 179; 187; 203; 221; 238.
[33]MS *m/z* (Relat. int.): 41; 43; 69; 81; 91; 107; 123 (100); 135; 145; 159; 177; 187; 205; 220; 236.
[34]MS *m/z* (Relat. int.): 41; 43; 69; 81; 95; 109; 123 (100); 135; 141; 162; 177; 191; 205; 220; 238.
[35]MS *m/z* (Relat. int.): 41; 43; 67; 83; 97; 105; 121 (100); 133; 145; 161; 176; 194; 196; 218; 236.
[36]MS *m/z* (Relat. int.): 40; 57; 76; 93; 104; 149 (100); 167; 207; 223.
[37]MS *m/z* (Relat. int.): 43 (100); 59; 71; 95; 99; 119; 137; 151; 159; 177; 193; 199; 219; 234; 252.
[38]MS *m/z* (Relat. int.): 41; 69; 81; 95; 105; 123; 135; 150; 164 (100); 179; 205; 220; 238.
[39]MS *m/z* (Relat. int.): 43; 69; 81; 97; 109; 121; 135; 150; 161; 179 (100); 197.
[40]MS *m/z* (Relat. int.): 41; 69; 79; 95; 107; 123; 138 (100); 149; 159; 173; 191; 201; 216; 234.
[41]MS *m/z* (Relat. int.): 41; 55; 57 (100); 71; 85; 99; 113; 127; 141; 155; 175; 189; 205; 217; 232; 261.
[42]MS *m/z* (Relat. int.): 41; 43; 59; 81; 97; 109 (100); 121; 135; 147; 161; 179; 191; 197; 219; 234.
[43]MS *m/z* (Relat. int.): 41; 43; 59; 81; 97; 109 (100); 121; 135; 147; 161; 179; 191; 197; 219; 233; 238.
[44]MS *m/z* (Relat. int.): 41; 43 (100); 60; 73; 87; 101; 115; 129; 143; 157; 174; 185; 199; 213; 256.
[45]MS *m/z* (Relat. int.): 41; 43; 69; 81; 97; 109; 121; 135; 149; 161; 179; 187; 197 (100); 220; 238.
[46]MS *m/z* (Relat. int.): 43; 67; 81; 93; 105; 119; 133; 149; 159; 177; 195 (100); 217.
[47]MS *m/z* (Relat. int.): 41; 55; 69; 85; 99; 113; 127; 165; 179 (100); 207; 261; 343.

of variation in several plant species is well recognized [27]. Thus, an investigation was performed to analyze the chemical pattern of essential oils from these different colorful fruit specimens.

After extraction, the essential oils obtained from LBF and LYF yielded 0.50% (w/w) and 0.37% (w/w), respectively. Essential oils from BF and YF yielded 0.02% (w/w) and 0.07% (w/w), respectively.

The chemical analysis performed by GC-MS/GC-FID indicated a predominance of sesquiterpenes on both leaves essential oils, corresponding to 81.1% and 84.8% of relative composition of LBF and LYF, respectively. These contents

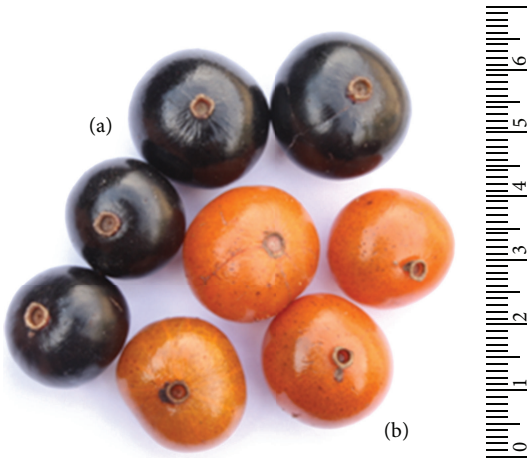

Figure 1: Ripe fruits of specimens from *Neomitranthes obscura* (DC.) N. Silveira in "Restinga de Jurubatiba" National Park. (a) Black fruits specimen. (b) Yellow fruits specimen.

were mainly constituted by sesquiterpenes hydrocarbons. Oxygenated sesquiterpenes and monoterpene hydrocarbons also appeared on leaves oils, while aliphatic compounds appeared just on LBF specimens.

This correlation of major constituents was not observed for each fruits analyzed. BF essential oil showed a predominance of monoterpenes (50.5%) and was constituted by 10.5% of sesquiterpenes. Meanwhile, YF essential oil presented sesquiterpenes (39.9%) as major substances and was constituted by 16.1% of monoterpenes. In all, 27 substances were identified on this essential oil. Both essential oils from fruits presented aliphatic compounds, corresponding to 0.9% and 1.4% of relative composition of BF and YF, respectively.

Germacrene B was the major substance found in LBF essential oil, corresponding to 21.8% of relative composition. The major substances found in the essential oil from LYF were Selina-3,7(11)diene (18.7%), Trans-dauca-4,(11),7-diene (13.9%), and 2,4-di-tert-butylphenol (13.4%), while germacrene B corresponded to 8.4% of relative composition of this essential oil. On another study carried out with leaves from *N. obscura*, De Ramos et al. [28] showed that sesquiterpenes corresponded to 87% of relative composition of this essential oil, which is in accordance with our results.

β-pinene (13.5%) and α-pinene (11.0%) were the major substances found in the essential oil from BF, however, appeared in lower amounts in the essential oil from YF (β-pinene, 3.2%/α-pinene, 1.8%). Caryophyllene oxide, which appeared as the major substance in the essential oil from YF (12.6%), corresponded to 1.7% of relative composition of essential oil from BF. The substances found in the essential oils from leaves and fruits of *N. obscura* are presented in Table 1.

In the tropical and subtropical zones, fruits mainly consumed by mammals are often yellow or orange, while fruits consumed by birds are often red or black [29, 30]. The occurrence of two different colors for a fruit species may increase its dispersal. On another study, Gomes et al. [4] concluded that availability peak of caloric fruits coincides with energy-demanding seasons for resident and nonbreeding birds in "Restinga de Jurubatiba" National Park, despite the fact that there was no mention of different fruit colors. In addition, regarding the literature data, we can observe that essential oil from *N. obscura* was investigated but without any mention of differences in fruit color [28].

4. Conclusions

Varieties are not easily definable entities in the Myrtaceae family; however, a study carried out for different coloring fruits of *Eugenia brasiliensis* Lam. indicated that analysis of its volatiles corroborated the concept of different varieties for this species [21]. To our knowledge, this is the first report about different fruit colors of *N. obscura*. It is interesting from a chemical overview that the content of predominant volatiles from the two analyzed fruits had an inverse relation between sesquiterpenes and monoterpenes. These results suggest that preferable metabolic production of monoterpenes or sesquiterpenes is followed for each specimen. Thus, the color and chemical constituents of different fruits of *N. obscura* suggest that this Myrtaceae species contains at least two different varieties.

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Research Article

Preparation of ACE Inhibitory Peptides from *Mytilus coruscus* Hydrolysate Using Uniform Design

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The angiotensin-I-converting enzyme (ACE) inhibitory peptides from mussel, *Mytilus coruscus*, were investigated and the variable factors, protease concentration, hydrolysis time, pH, and temperature, were optimized using Uniform Design, a new statistical experimental method. The results proved that the hydrolysate of alkali proteases had high ACE-inhibitory activity, especially the alkali protease E1. Optimization by Uniform Design showed that the best hydrolysis conditions for preparation of ACE-inhibitory peptides from *Mytilus coruscus* were protease concentration of 36.0 U/mL, hydrolysis time of 2.7 hours, pH 8.2, and Temperature at 59.5°C, respectively. The verification experiments under optimum conditions showed that the ACE-inhibitory activity (91.3%) were agreed closely with the predicted activity of 90.7%. The amino acid composition analysis of *Mytilus coruscus* ACE-inhibitory peptides proved that it had high percent of lysine, leucine, glycine, aspartic acid, and glutamic acid.

1. Introduction

About 30% of Americans are suffering hypertension and risk of cardiovascular disease development as an independent factor [1]. Hypertension is one of the most frequent chronic diseases and the incidence of this disease was increased in recent years. This disease affected about 65% of 65–75-year-old people in Western developed countries and its incidence was increased with age [2]. Angiotensin-I-converting enzyme (EC 3.4.15.1; ACE) is one of the metalloproteases and zinc is neccessary for its activity [3]. ACE cleaves dipeptides from oligopeptide's carboxylic terminus, which plays important physiological role in blood pressure regulation [4]. Functional foods, containing ACE inhibitory peptides, may control blood pressure moderately. Many ACE inhibitory peptides *in vitro* have been isolated from various food derived proteins hydrolysate, such as milk [5], seed protein [6], blue mussel protein [7], bovine blood plasma [8], casein [9–11], zein [12], sardine [13], and tuna muscle [14]. ACE inhibitors have also been isolated from fermented foods, such as yoghourt [15], soy sauce [16], and soybean [17].

Mytilus coruscus is one of the most important bivalves in both Chinese aquaculture and Chinese market [18]. Like other marine animals, some biactive peptides have been reported from *Mytilus* mussel protein, such as *Mytilus* inhibitory peptides [19], antimicrobial peptides [20], and anticoagulant peptide [21]. In addition, an ACE inhibitory peptide has been purified by chromatography method and identified from blue mussel sauce [7]. However, there was no report to obtain ACE inhibitory peptides from *Mytilus coruscus* mussel protein hydrolysate.

Uniform Design method, a new experimental technique, is established together by Fang [22]. One of the most important advantages of the Uniform Design is that many factors and levels can be desined simulataneously. Uniform Design offers many convenient experimental tables [23]. But, unlike orthogonal design, the largest possible amount of levels for each factor can be allowed in Uniform Design, and so much so that the number of levels sometimes can be equal to the number of experiment runs [24]. As a statistical and experiment design technique, Uniform Design method has been successfully used for many experiments, especially in optimizing processes [23, 25, 26].

In the present study, we want to optimize the hydrolysis conditions for achieving ACE inhibitory peptides from *Mytilus coruscus* muscle protein. Uniform Design method

was applied to investigate the effects of protease concentration, hydrolysis time, hydrolysis temperature, and hydrolysis pH for the ACE inhibitory activity of hydrolysates from *Mytilus coruscus*.

2. Materials and Methods: ACE from Rabbit Lung

2.1. Materials. Mussel, *Mytilus coruscus*, was obtained from local aquatic product market (Hangzhou, China). Hippuryl-histidyl-leucine (HHL) was used as substrate of ACE. The HHL and ACE were purchased from local chemical company (Hangzhou, China). Five kinds of proteases (E1 to E5) were purchased from local food additives market (Hangzhou, China). The labeled optimum hydrolysis temperature and pH were shown in Table 1. All other reagents were analytical grade chemicals.

2.2. Preparation of Hydrolysates. Mussels, *Mytilus coruscus,* were washed with water to remove salt and other materials. The mussels were filleted and defatted with petroleum ether at 50°C by reflux extraction. Then the mussels were minced and mixed with distilled water (ratio of 1 : 10). The mixture was homogenate and then was boiled for 10 minutes to inactive the inner protease. Then the mixture was digested by five proteases at designed conditions, respectively. The pH of the reaction mixture was maintained stably by addition of either 1 N NaOH or HCl. Then, the mixture was incubated at 90°C for 10 min to terminate the reaction. After centrifugation (12,000 ×g, 4°C) for 10 min, the supernatant of the hydrolysate was collected for test the ACE inhibitory activity.

2.3. Determination of ACE Inhibitory Activity. The ACE inhibitory activity was determined by Wang et al. method [27] with slight modifications. All samples were diluted to the same protein content (1.0 mg/mL), which was determined by Biuret assay method [28]. Sample solution (10 μL) and ACE solution (50 units/mL, 30 μL) were mixed together. After the mixture being preincubated at 37°C for 5 min, 50 μL 7.6 mmol/L HHL substrate solution, which was solved in 50 mM sodium borate buffer and 6.8 mM NaCl at pH 8.3, was added. The mixture was incubated at 37°C for 25 min. The reaction was terminated after addition of 10 μL of 20% trifluoroacetic acid (TFA). The solution was filtrated through 0.22 μm membrane. The hippuric acid liberated by ACE was analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) on an Inertsil ODS C_{18} (4.6 mm × 300 mm, 5 μm) column. The mobile phase was 30% methanol, which contained 0.1% TFA and 0.05% acetic acid. The flow rate was 1.0 mL/min. The UV detection wavelength was 228 nm. The ACE inhibitory activity was obtained from peak area and expressed as percent.

2.4. Choice of Protease. Five kinds of proteases were used for hydrolysis at their labeled optimum temperature and pH (shown in Table 1). And the protease was added at 50 U per mL mixture and the hydrolysis time was fixed at 4.0 h. Then ACE inhibitory activity of hydrolysate was determined.

Table 1: Hydrolysis conditions of the five proteases for producing ACE inhibitory peptides from *Mytilus coruscus*.

Protease	Temperature (°C)	pH	Time (h)
Protease E1	55	8.5	4.0
Protease E2	55	8.5	4.0
Protease E3	55	7.0	4.0
Protease E4	55	3.0	4.0
Protease E5	55	9.0	4.0

2.5. Uniform Design. A Uniform Design table of $U_7(7^4)$ was applied to determine the optimum hydrolysis conditions for obtaining ACE inhibitory activity peptides from *Mytilus coruscus*. The combination effects of independent variables X_1 (protease concentration, U/mL), X_2 (hydrolysis time, h), X_3 (hydrolysis pH), and X_4 (hydrolysis temperature, °C) at 7 variation levels in the hydrolysis process were shown in Table 2. A total of 21 combinations (three replicates) for four factors were chosen according to Uniform Design table. The actual values were also shown in Table 2. The responses functions (Y) were ACE inhibitory activity. These values were related to the variables by a second-order polynomial (1) below:

$$Y = \beta_0 + \sum_{i-1}^{m} \beta_i X_i + \sum_{i-1}^{m} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j, \quad (1)$$

where Y is the predicted response. X_i and X_j are the independent variables. β_0, β_i, β_{ii}, and β_{ij} were the regression coefficients.

The significance was evaluated by Student's t-test. The actual values were compared with model predictions. The optimum hydrolysis conditions were verified by additional triplicate experiments under these conditions.

2.6. Amino Acid Composition Analysis. The amino acid analyses were conducted by the method of Noreen and Salim [29]. Briefly, the 10 mL of the sample was hydrolyzed under vacuum by addition of 10 mL concentrated HCl at 110°C for 24 h. When the free amino acids were analyzed, the sample did not hydrolyzed by HCl. Amino acids were analyzed in a Shimadzu HPLC system by separation in an ion-exchange column and post-column reaction with ninhydrin.

2.7. Statistical Analysis. Data were expressed as means ± standard deviation of triplicate. A probability value of $P < 0.05$ was considered significantly.

3. Results and Discussion

3.1. Choice of Protease. ACE inhibitory peptides generally were short peptides and enzymatic hydrolysis of food derived protein was one of important measures to obtain ACE inhibitory peptides. A lot of ACE inhibitory peptides had been reported from food derived proteins hydrolysates. In this investigation, five kinds of commercial proteases, including three alkali proteases, one neutral protease, and one acid protease, were chosen to obtain ACE inhibitory

Table 2: Uniform Design with the observed responses and predicted values.

Treat	Variable levels				Experimental Y_e	Predicted Y	LRE (%)
	X_1	X_2	X_3	X_4			
1	25	2	7.0	60	87.98 ± 3.14	88.08	4.8
2	50	4	8.5	55	77.21 ± 2.18	77.49	3.7
3	75	6	6.5	50	36.92 ± 1.91	36.83	5.0
4	100	1	8.0	45	26.54 ± 1.47	26.28	2.1
5	125	3	6.0	40	39.46 ± 2.31	39.02	3.6
6	150	5	7.5	35	58.13 ± 2.35	58.47	4.4
7	175	7	9.0	65	20.17 ± 1.17	20.46	3.2

X_1: protease concentration, U/mL; X_2: hydrolysis time, h; X_3: hydrolysis pH; X_4: hydrolysis temperature, °C; experimental Y_e was expressed as mean ± standard deviation of three determinations; LRE: largest relative error = 100 × |the largest or the lowest $Y_e - Y$|/average Y_e .

peptides from *Mytilus coruscus*. The ACE inhibitory activity of various enzymatic hydrolysates was shown in Figure 1. From the results, it was shown that the hydrolysate produced by alkali protease E1 had the highest ACE inhibitory activity. In addition, alkali proteases (E1, E2 and E3) were more effective for hydrolysis *Mytilus coruscus* mussel protein to obtain ACE inhibitory peptides than other two proteases (E4 and E5). Therefore, alkali protease E1 was chosen to next experiments to optimize hydrolysis conditions for producing ACE inhibitory peptides from *Mytilus coruscus*.

3.2. Data Analysis of Uniform Design. A regression analysis was conducted to fit a mathematical model to the experimental data. The results of regression analysis were summarized (Table 2), and a regression equation was given in

$$\begin{aligned} Y = {} & 0.1023 + 0.2626X_1 + 0.1083X_2 + 0.1100X_3 + 0.1592X_4 \\ & + 0.0114X_1^2 + 0.0937X_2^2 + 0.1751X_3^2 + 0.0020X_4^2 \\ & - 0.1404X_1X_2 - 0.0829X_1X_3 - 0.0314X_1X_4 \\ & + 0.1561X_1X_3 + 0.2367X_2X_4 + 0.1960X_3X_4. \end{aligned} \quad (2)$$

The statistical analysis indicated the predicted model was adequate, possessing significant P value ($P = 0.047 < 0.05$) and satisfactory values of the regression coefficient R^2 ($R^2 = 0.9712$) for the response. The high regression coefficient make clear that the experimental values of the ACE inhibitory activity agreed with predicted values, which meant that the predicted model seemed to reasonably represent the observed values. The largest relative error of predicted value was less than 5% shown in Table 2. The significance was tested by Student's t-test and P value in Table 3. It was shown that temperature, pH, and protease added quantity affected significantly the ACE inhibitory activity of hydrolysates.

3.3. Verification Experiments. Then the optimum hydrolysis conditions of protease E1 and the prediction ACE inhibitory activity were obtained by (2). The optimum hydrolysis conditions of protease E1 were protease concentration (X_1): 36.0 U/mL; hydrolysis time (X_2): 2.7 h; hydrolysis pH (X_3): 8.2; hydrolysis temperature (X_4): 59.5°C. The predicted ACE inhibitory activity was 90.7% at optimum hydrolysis conditions. Under this optimum hydrolysis conditions, other three verification experiments were conducted and the average actual ACE inhibitory activity was 91.3%, which was in agreement with the predicted values of 90.7%.

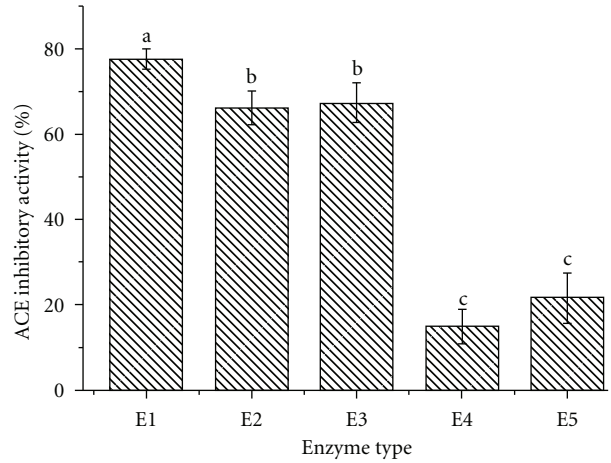

Figure 1: The ACE inhibitory activity of hydrolysates by five commercial proteases. The data was expressed as mean ± standard deviation of three determinations. Means sharing the same lowercase letter was not significantly different at $P < 0.05$.

3.4. Amino Acid Composition. The compositions of free amino acid and amino acid in peptides of the ACE inhibitory peptides from *Mytilus coruscus* were determined and the results were shown in Table 4. From the results, it was seen that the ACE inhibitory peptides solution had only a few free amino acid content, not equal with amino acid in peptides. The peptides, not amino acid, might contributed to high activity. The ACE inhibitory peptides had high percent of glutamic acid, taking 0.578 mmol/g, which could improve the breath ability of brain cell and be favorable to the expulsion of ammonia in brain and regulation of body metabolism, and these phases could impact the blood pressure directly. Also the ACE inhibitory peptides had high percent of lysine, leucine, glycine, and aspartic acid. These amino acids might play crucial role in the inhibitory

Table 3: Significance of regression coefficient for the ACE inhibitory activity.

Variables	Standard error	Computed t value	Significance level P value
X_1	0.6374	5.2987	0.0501
X_2	1.0897	0.9872	0.4619
X_3	1.2345	5.6426	0.0478
X_4	0.8766	7.2358	0.0342
X_1X_2	−0.9234	4.4760	0.0323
X_1X_3	−0.8768	3.9765	0.05926
X_1X_4	−1.0236	7.0626	0.0355
X_2X_3	0.7931	3.2617	0.0635
X_2X_4	0.8942	6.2932	0.0433
X_3X_4	0.6745	6.7869	0.0408
X_1^2	0.5679	5.4876	0.0496
X_2^2	0.9236	1.2381	0.3763
X_3^2	1.0111	4.2635	0.0543
X_4^2	0.8765	6.9367	0.0374

Table 4: Amino acid compositions of ACE inhibitory peptides from *Mytilus coruscus*.

Amino acid	Free amino acid content (mmol/g)	Amino acid in peptides (mmol/g)
Aspartic acid	0.009	0.449
Threonine	0.016	0.209
Serine	0.012	0.246
Glutamatic acid	0.013	0.578
Glycine	0.052	0.565
Alanine	0.027	0.558
Valine	0.009	0.155
Methionine	0.013	0.359
Isoleucine	0.018	0.157
Leucine	0.069	0.336
Tyrosine	0.000	0.089
Phenylalanine	0.020	0.146
Histidine	0.104	0.106
Lysine	0.068	0.419
Arginine	0.029	0.230
Cysteine	Not detected	Not detected
Proline	Not detected	Not detected
Tryphtophan	Not detected	Not detected

activity. Cheung et al. [30] reported that dipeptides having hydrophobic amino acids such as valine (Val) and isoleucine (Ile) at the amino temerninus have higher ACE inhibitory activities. The amino acids, such as lysine, leucine, glycine, aspartic acid, and glutamic acid, were key constitutes with tall frequency appeared among many reported ACE inhibitory peptides [31–35].

4. Conclusions

Alkali protease was a good choice for hydrolyzing *Mytilus coruscus* protein for producing ACE inhibitory peptides. The factors, including protease concentration, hydrolysis time, hydrolysis pH, and temperature, affected the ACE inhibitory peptides of hydrolysates. Uniform Design was chosen to investigate the effects of preceding variables on ACE inhibitory activity. And the best hydrolysis conditions of alkali protease E1 optimized by Uniform Design were protease concentration of 36.0 U/mL, hydrolysis time of 2.7 hours, pH 8.2, temperature at 59.5°C. The optimal predicted ACE inhibitory activity of 90.7% was obtained at the optimum conditions. The experimental activity (91.3%) under optimized conditions was agreed closely with the predicted activity. The amino acid composition analysis of the ACE inhibitory peptides proved that it had high percent of lysine, leucine, glycine, aspartic acid, and glutamic acid. It was suggested that the ACE inhibitory peptides derived from *Mytilus coruscus* could be utilized to develop nutraceuticals and pharmaceuticals.

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Research Article

Chemical Composition and Antibacterial and Cytotoxic Activities of *Allium hirtifolium* Boiss

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Allium hirtifolium Boiss. known as Persian shallot, is a spice used as a traditional medicine in Iran and, Mediterranean region. In this study, the chemical composition of the hydromethanolic extract of this plant was analyzed using GC/MS. The result showed that 9-hexadecenoic acid, 11,14-eicosadienoic acid, and n-hexadecanoic acid are the main constituents. The antibacterial activity of the shallot extract was also examined by disk diffusion and microdilution broth assays. It was demonstrated that Persian shallot hydromethanolic extract was effective against 10 different species of pathogenic bacteria including methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Escherichia coli, Escherichia coli* O157:H7, *Salmonella typhimurium, Proteus mirabilis*, and *Klebsiella pneumoniae*. Specifically, the minimum concentration of the extract which inhibited bacterial growth (MIC values) was 1.88 mg/mL for most of the gram-positive bacteria. This concentration was not much different from the concentration that was safe for mammalian cells (1.50 mg/mL) suggesting that the hydromethanolic extract of Persian shallot may be a safe and strong antibacterial agent.

1. Introduction

The problems of multidrug resistances exhibited by human pathogenic microorganisms and the side effects of antibiotics have led scientists to search for alternatives such as medicinal plants. The phenolic compounds present in most plants have a broad spectrum of biological activities whereby their antimicrobial actions stand out [1, 2].

The use of *Allium* genus members such as garlic and onion in the treatment of various ailments has been reported worldwide. Many members in this genus have been proved to possess antibacterial, antifungal, antiprotozoal, and anthelmintic activities [3–6]. In addition, *Allium* plants are believed to heal diabetes, arthritis, colds and flu, stress, fever, coughs, headache, hemorrhoids, asthma, arteriosclerosis, cancer, rheumatic, and inflammatory disorders [7–12].

Allium hirtifolium Boiss. (Persian shallot) is one of the Iranian native spices [6, 13] which belong to the same biological genus of *Allium sativum* (garlic) and other onions [14]. It is one of the valuable members of *Allium* with its bulbs commonly used as a traditional remedy [14, 15]. Besides, the dried bulb slices are used as additives to yogurt as well as pickling mixtures. The Persian shallot (also known as "mooseer") is different from the common shallot (*Allium ascalonicum*)

in many characteristics. For instance, the bulbs of common shallot are pear shaped with the skin reddish brown in color and its cluster may contain as many as 15 bulbs [16], while for the Persian shallot its bulbs are oval shaped and have white color of skin and normally consists of single or sometimes two bulbs [17]. In this work, we report the chemical composition and antibacterial as well as cytotoxic effects of this spice.

2. Materials and Methods

2.1. Plant Material. The bulbs of *Allium hirtifolium* Boiss were collected from Ilam Province of Iran during September to October 2009. The plant specimen (number CMN10) was deposited in the Herbarium of Materia Medica, Research Center of Agriculture and Natural Sources (RCANS) of Ilam Province.

2.2. Extract Preparation. The bulbs of Persian shallot were washed with tap water and cut into small slices. The air-dried bulbs were grinded into powder by using a blender. The extraction was carried out using percolation procedure with 60% (v/v) hydromethanol for 48 hours. Then it was filtered and evaporated to dryness under reduced pressure in a rotary vacuum evaporator at 40°C. The extract was stored at 4°C until being used.

2.3. GC-MS Analysis. The GC-MS analysis of the *Allium hirtifolium* extract was carried out by Gas Chromatography Mass Spectrometer Shimadzu QP 5050A. The GC conditions were splitless modes of injection, injector/interface temperature 250/260°C. Helium at a flow rate of 1.0 mL/min was employed as a carrier gas. The oven temperature was programmed as follows: 100°C for 3 minutes and gradually increased to 250°C for 15 minutes. The identification of the constituents was performed according to the Wiley and Nist mass spectral library.

2.4. Bacterial Strains. The following strains were purchased from ATCC and tested in the screening.
Gram-Positive Bacteria: methicillin resistant Staphylococcus aureus (MRSA) ATCC 700698, methicillin sensitive Staphylococcus aureus (MSSA) ATCC 29247, Staphylococcus aureus ATCC 25923, Staphylococcus epidermidis ATCC 12228, Streptococcus pneumonia ATCC 10015.
Gram-Negative Bacteria: *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7 ATCC 35159, *Salmonella typhimurium* ATCC 13311, *Proteus mirabilis* ATCC 29906, and *Klebsiella pneumoniae* ATCC 13883.

All microorganisms were stored at −80°C as a stock and maintained at 4°C on Mueller-Hinton Agar (Merck, Germany).

2.5. Antibacterial Susceptibility Test

2.5.1. Disk Diffusion Assay. The *in vitro* antimicrobial activity was screened by the disc diffusion method according to the protocol by Zaidan et al. [18] with minor modifications. Three hundred μL of bacterial culture (suspended in tryptic soy broth) (Merck, Germany) adjusted to 0.5 McFarland standard was spread on Muller-Hinton agar plates evenly using a sterile swab and allowed to dry for 15 minutes. The different concentrations of extract (120, 60 mg/mL) filtered by 0.45 μm millipore filters (Orange Scientific, Belgium) were impregnated on 6 mm sterile discs (Whatman paper number 1) with 20 μL per disc. Then, the loaded discs were placed on the surface of inoculated medium. The plates were incubated at 37°C for 24 hours. At the end of incubation, the plates were examined and recorded for inhibition zone. Gentamycin (Sigma) was used as the positive control. The average of each zone of inhibition was calculated and recorded.

2.5.2. Microdilution Broth Assay. The procedure was implemented as described by Khan et al. [19] with minor modifications. Different concentrations of extract in two-fold serial dilutions were prepared in tryptic soy broth. One hundred μL of inoculum suspension with the optical density in the range of 0.08–0.10 (adjusted using spectrophotometer (Beckman Coulter Inc., Fullerton) at 600 nm or 0.5 Mcfarland standard (1.5×10^8 CFU/mL)) was added into each well of a 96-well microtitre plate. Afterwards, 100 μL of extract was added to the previous wells giving a final volume of 200 μL. The 96-well microtiter plate was shaken for 20 seconds at 300 rpm and incubated at 37°C for 24 hours. The inoculum suspension was used as the negative control. Gentamycin (10 μg/mL) was used as the positive control, while tryptic soy broth alone was used as blank.

MIC is the lowest concentration which inhibits bacterial growth or it is the lowest concentration of the extract at which the microorganism does not demonstrate visible growth.

Confirmatory test, MBC, (minimum bactericidal concentration) was performed by loading 5 μL of each well onto nutrient agar (Merck, Germany). MBC is the concentration at which there was no bacterial growth and the result was recorded.

2.6. Cells Viability Assay

2.6.1. Cell Line. The Vero (African green monkey kidney cells) was purchased from ATCC and maintained in RPMI media (Sigma, USA) that incorporated with 1% Penicillin-Streptomycin, and Amphotericin B. The growth media was supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, USA) and incubated inside 37°C, 5% CO_2, and 95% of humidity.

2.6.2. MTT Test. Monolayer cells were trypsinized, followed by washing with PBS (Sigma, USA), and seeded in each well of the 96-well flat-bottomed plates with density of 6.0×10^4 cells/mL. After 24 hours of incubation time, the serial two-fold dilutions of extract were added to the confluent monolayer cells (except for the control) and the plate was incubated for further 24, 48, and 72 hours in a humidified incubator at 37°C and 5% CO_2. The cells viability was determined by the MTT colorimetric technique [45] by adding 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) solution (5 mg/mL in PBS) into each

Table 1: Composition of the hydromethanolic extract of *Allium hirtifolium* Boiss bulbs.

Peak number	Compound	SI	Retention indices (type of column) (references)	MW	Percentage
1	2-pentanone,4-hydroxy-4-methyl	92	818 (methyl silicone) [20]	116	0.72
2	Acetic acid anhydride	95	699 (methyl silicone) [21]	88	4.06
3	2,3-Butanediol	97	793 (DB-5), 1492 (Carbowax 20 M) [22, 23]	90	3.16
4	1,3-Butanediol	98	784 (DB-Wax) [24]	90	2.39
5	2,4,5-Trithiahexane	85	1613 (Carbowax 20 M), 1098 (HP-1), 1106 (OV-1), 1211 (HP-5), 1666 (HP-INNOWax) [25–29]	140	2.03
6	Hexanoic acid	93	981 (HP-5MS) [30]	116	0.38
7	S-Methyl methanethiosulphonate	90	934 (HP-1) [29]	126	0.43
8	2,4-Dithiapentane	80	900 (HP-5MS), 871 (OV-1) [27, 31]	108	1.15
9	Benzene,1,2-Dimethoxy-4-(2-propenyl)-	90	1401 (DB-5) [32]	178	6.67
10	(Diisopropylamino)ethanol	74		145	0.38
11	Methyl P-vinylbenzoate	81		162	0.26
12	2-Pyridinethione	85		111	1.16
13	n-Decanoic acid	80	1347 (DB-5) [33]	172	0.26
14	Benzene,1,2-dimethoxy-4-(2-propenyl)-	80	1401 (DB-5) [32]	178	0.23
15	Pentadecanoic acid, 14-methyl-, methyl ester	96	1884 (DB-5) [34]	270	1.71
16	1,3-Dioxolane,2,4,5-trimethyl	84	745 (DB-5) [35]	116	5.22
17	2,4,5-Trithiahexane	85	1613 (Carbowax 20 M), 1098 (HP-1), 1106 (OV-1), 1211 (HP-5), 1666 (HP-INNOWax) [25–29]	140	1.16
18	Phenol-3,5-bis(1,1-dimethylethyl)-	93		206	1.42
19	s-Triazolo[4,3-a] Pyridine,7-methyl-	65		133	0.36
20	D-Glucitol, 1,4:3,6-dianhydro-	75		146	0.63
21	m-Dioxane, 2,4-dimethyl-	76	786 (OV-101) [36]	116	0.51
22	Methyl elaidate	95	2084 (DB-5) [33]	296	2.94
23	9-Octadecenoic acid, ethyl ester	90	2110 (DB-5) [37]	310	0.93
24	9,12-Octadecadienoic acid, methyl ester	94	2064 (HP-5MS) [38]	294	2.44
25	Hydroxymethylfurfurole	84	1812 (DB-Wax) [39]	126	1.90
26	9,12-Octadecadienoic acid, methyl ester	90	2064 (HP-5MS) [38]	294	1.17
27	Linolenic acid, methyl ester	85		292	0.38
28	Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate	79		292	0.69
29	3,4,5-Trimethoxybenzoic acid, methyl ester	84		226	0.69
30	L-Glutamic acid	90		147	1.05
31	Methane,(chloromethylthio)-(methylthio)-	80		142	0.49
32	Tetradecanoic acid	84	2585 (DB-Wax) [39]	228	0.44
33	Phenol, nonyl	66	1720 (HP-5) [40]	220	0.12
34	Ethyl N-(O-anisyl)formimidate	67		179	0.34
35	Cinnamic acid	75	1386 (HP-1) [41]	148	0.26
36	n-Hexadecanoic acid	95	1962 (DB-5) [42]	256	15.26
37	1-Tetradecanol	80	1673(DB-5) [43]	214	0.35
38	Octadecanoic acid	87	2182 (DB-5) [33]	284	0.52
39	9-Hexadecenoic acid	93	1949 (HP-5) [44]	254	18.09
40	11,14-Eicosadienoic acid, methyl ester	92		322	16.24
41	Linolenic acid, methyl ester	91		292	1.38

Table 2: *In vitro* antibacterial activity of hydromethanolic extract of *Allium hirtifolium* Boiss against particular pathogens.

Pathogens	Diameter of inhibition zone (mm)		
	Concentration of extract (mg/mL)		Gentamycin[a]
	120[b]	60[c]	
Gram-positive bacteria			
MRSA ATCC 700698	18.17 ± 0.75	15.70 ± 0.52	23.50 ± 0.55
MSSA ATCC 29247	17.50 ± 0.55	15.30 ± 0.52	22.70 ± 0.52
Staphylococcus aureus ATCC 25923	15.00 ± 0.60	11.50 ± 0.55	23.70 ± 0.52
Staphylococcus epidermidis ATCC 12228	19.20 ± 0.75	15.80 ± 0.41	23.50 ± 0.55
Streptococcus pneumonia ATCC 10015	21.50 ± 0.84	18.30 ± 0.52	19.50 ± 0.84
Gram-negative bacteria			
Escherichia coli ATCC 25922	15.70 ± 0.52	14.00 ± 0.63	14.80 ± 0.41
Escherichia coli O157:H7 ATCC 35159	14.70 ± 0.52	11.50 ± 0.55	12.80 ± 0.41
Salmonella typhimurium ATCC 13311	14.70 ± 0.52	12.50 ± 0.84	22.50 ± 0.84
Proteus mirabilis ATCC 29906	11.30 ± 0.82	10.00 ± 0.63	14.50 ± 0.84
Klebsiella pneumoniae ATCC 13883	16.50 ± 0.84	14.20 ± 0.75	19.00 ± 0.63

Note: [a]commercial antibiotic disk (Sigma) at 30 μg/disk against gram-positive and gram-negative bacteria; [b]potential of disk 2.4 mg/mL; [c]potential of disk 1.2 mg/mL; $n = 6$; values are means ± SD of two replicates from three experiments.

well. After 3 hours incubated in 37°C, 150 μL of DMSO (dimethyl sulfoxide) (Merck, Germany) was added to each well in order to dissolve the MTT crystals. The plate was placed on the shaker for 15 minutes and the optical density was recorded at the wavelength of 590 nm (620 nm reference wavelength) using a microtitre plate reader (Tecan, Austria). The samples were performed in triplicate. The percentage of viable cells is calculated as (B/A) × 100, whereby A and B are the OD_{590} of untreated and treated cells, respectively [45]. The 50% cytotoxicity (CC_{50}) of the test extract was determined from a curve of the cells viability (in percentage) versus the concentration of extract. CC_{50} is defined as the concentration that of reduces the OD_{590} of treated cells to 50% of that of untreated cells [46].

3. Results and Discussion

3.1. Chemical Composition of the Extract. The chemical constituents of the extract are presented in Table 1. In general, most chemical compositions found in the extract are fatty acids where the highest is 9-hexadecenoic acid, 18.09%, followed by 11, 14-eicosadienoic acid, 16.24%, and n-hexadecanoic acid for 15.26% while the other fatty acids are not more than 3%. Other organic compounds' percentages are less than 7%. The detection of fatty acids has also been reported by Ebrahimi et al. [17] who found that linolenic acid and linoleic acid with the percentages 14.66% and 78.88%, respectively are present in the *Allium hirtifolium* Boiss plant.

The individual chemical compounds were identified by matching their mass spectra of peaks with those obtained from the Wiley and Nist library mass spectra library. In a study performed by Ghodrati Azadi et al. [13], the presence of allicin (an organosulfur compound) compound was shown in *A. hirtifolium* Boiss by the TLC method. In contrast only a low percentage of sulfur compounds including s-methyl methanethiosulphonate, 2,4,5-trithiahexane, 2,4-dithiapentane, 2-pyridinethione and methane (chloromethylthio) (methylthio)- were detected in the current study. As reported by Iranshahi [47], these volatile sulfur compounds were formed due to the cleavage of odorless S-alk(en)yl cysteine sulfoxide flavor precursors by the enzymes alliinase and lachrymatory-factor synthase.

3.2. Antibacterial Effect of the Extract. The initial screening of antibacterial activity of the *Allium hirtifolium* Boiss hydromethanolic extract was conducted *in vitro* using the disk diffusion method. In general, the inhibition zones were observed for both gram-positive and gram-negative bacteria indicating their sensitivity to the extract as shown in Table 2. The inhibition zones ranged 11.5–21.5 mm and 10.0–16.5 mm for gram-positive and gram-negative bacteria, respectively. The extract also showed inhibitory activity on bacterial growth in a dose dependent manner. The hydromethanolic extract presented an antibacterial activity stronger than that of Gentamycin (positive control) against *Streptococcus pneumonia* ATCC 10015, *Escherichia coli* ATCC 25922, and *Escherichia coli* O157:H7 ATCC 35159. As shown in Table 3, the MIC and MBC values varied among the tested bacteria, and in most cases, the MIC values were lower than MBC values, indicating a bacteriostatic activity of the extract.

In terms of the range of the inhibition zone as well as the MIC and MBC values, it was generally observed that the extract was more effective against gram-positive bacteria compared to the gram-negative bacteria which might be due to the differences in their cell wall structures. Specifically, gram-positive bacteria lack outer membrane while the outer membrane possessed by gram-negative bacteria might act as a barrier to many types of environmental substances which also include antibiotics [48]. The findings of the present study are in agreement with the previous report that showed that the gram-positive bacteria were more sensitive to the different types of *Allium roseum* extracts with the mean of growth inhibition zone ranged between 8 and 15 mm [49].

Table 3: MIC and MBC (mg/mL) for antibacterial activity of *Allium hirtifolium* Boiss against particular pathogens.

Pathogens	Extract	
	MIC	MBC
Gram-positive bacteria		
MRSA ATCC 700698	1.88	7.50
MSSA ATCC 29247	1.88	7.50
Staphylococcus aureus ATCC 25923	1.88	3.75
Staphylococcus epidermidis ATCC 12228	1.88	15.00
Streptococcus pneumonia ATCC 10015	3.75	15.00
Gram-negative bacteria		
Escherichia coli ATCC 25922	7.50	30.00
Escherichia coli O157:H7 ATCC 35159	3.75	15.00
Salmonella typhimurium ATCC 13311	3.75	7.50
Proteus mirabilis ATCC 29906	7.50	30.00
Klebsiella pneumonia 13883	3.75	15.00

Note: MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration.

Table 4: Minimum amounts of *Allium hirtifolium* hydromethanolic extract giving 50% of cell inhibition for normal monkey kidney cells (Vero).

Incubation time (hours)	IC_{50} value (mg/mL) (Vero cells)
24	6.90
48	2.90
72	2.60

Chikwem et al. [50] also reported that the *Allium sativum* extract could inhibit the growth of gram-positive bacteria with a larger size of inhibition zone which was more than 20 mm in diameter by disk diffusion assay compared to gram-negative bacteria. This result was in contrast to other *Allium sativum* extracts, which mainly exhibited their antibacterial activity on gram-negative bacteria [51, 52]. Studies have shown that fatty acids, particularly methyl ester (FAME) found in plants such as *S. brachiate,* possess antibacterial and antifungal activities [53]. Therefore, we speculate that the antibacterial effect of *A. hirtifolium* Boiss extract might also be due to the presence of fatty acids such as 9-hexadecenoic acid,11,14-eicosadienoic acid, and n-hexadecanoic acid.

3.3. Effect of the Extract on Normal Cells Viability. As shown in Figure 1, the hydromethanolic extract of Persian shallot was not toxic to the Vero cells at concentrations of 1.50 mg/mL and below (1 mg/mL) as more than 60% of cells remained viable at different incubation times 24, 48, and 72 hours (Figure 1). The extract also showed concentration and time dependent inhibition for the growth of the cells. As for the CC_{50}, the values were 6.90, 2.90, and 2.60 mg/mL for 24, 48, and 72 hours, respectively, as indicated in Table 4.

With regard to antibacterial effect through microdilution broth assay, the minimum inhibitory concentration (MIC) of the extract against all gram-positive bacteria except for *Streptococcus pneumonia* was 1.88 mg/mL which is almost similar to nontoxic concentration to normal mammalian cells. As for other pathogens used in this study, the MIC and MBC values

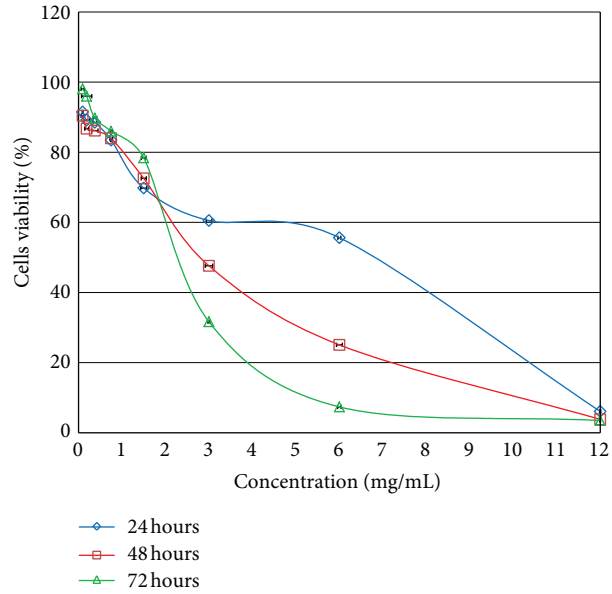

Figure 1: Effect of *Allium hirtifolium* Boiss. hydromethanolic extract on the viability of normal monkey kidney cell line (Vero) after 24, 48, and 72 hours of incubation time. Data show means ± SD of three replicates.

were higher than the nontoxic concentration to normal cells. However, this controversy might be solved if further study on the mode of actions of individual compounds of the extract is carried out [54] or some appropriate chemical and structure modifications are applied on the extract [55, 56]. Moreover, in a study by Levison [57], it was shown that the effective MIC and MBC values used *in vivo* were lesser than obtained values *in vitro*. Therefore, further investigation is required for the Persian shallot extract to be used as a safe antibacterial agent.

Azadi et al. [58] reported that the nontoxic concentration of chloroformic extract of *Allium hirtifolium* to normal mouse fibroblast cell line L929 was 1.00 mg/mL. Besides that,

a previous study showed that the garlic and onion, which both belong to the genus *Allium hirtifolium,* exhibited a significant activity as cytoprotective agents on normal cells [59]. Cao et al. [60] also reported that DADS compound from *Allium sativum* has shown to be less harmful against normal cell line (type of normal cell used was not stated).

4. Conclusion

The present study revealed that the hydromethanolic extract of *Allium hirtifolium* Boiss exhibited antibacterial activity. In general, the results indicated that gram-positive bacteria appeared to be more sensitive to the extract compared with gram-negative bacteria. It was also shown to be noncytotoxic to normal cells at concentration below 1.50 mg/mL. More detailed studies such as separation of the hydromethanolic extract, isolation of single compound from the extract, and fractionation might be useful in the development of a Persian shallot-based medicine. Besides, further *in vivo* studies are also required to validate these *in vitro* observations.

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Research Article

Quantitative Determination of Fatty Acids in Marine Fish and Shellfish from Warm Water of Straits of Malacca for Nutraceutical Purposes

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This study was conducted to quantitatively determine the fatty acid contents of 20 species of marine fish and four species of shellfish from Straits of Malacca. Most samples contained fairly high amounts of polyunsaturated fatty acids (PUFAs), especially alpha-linolenic acid (ALA, C18:3 n3), eicosapentaenoic acid (EPA, C20:5 n3), and docosahexaenoic acid (DHA, C22:6 n3). Longtail shad, yellowstripe scad, and moonfish contained significantly higher ($P < 0.05$) amounts of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA), respectively. Meanwhile, fringescale sardinella, malabar red snapper, black pomfret, Japanese threadfin bream, giant seaperch, and sixbar grouper showed considerably high content (537.2–944.1 mg/100g wet sample) of desirable omega-3 fatty acids. The polyunsaturated-fatty-acids/saturated-fatty-acids (P/S) ratios for most samples were higher than that of Menhaden oil (P/S = 0.58), a recommended PUFA supplement which may help to lower blood pressure. Yellowstripe scad (highest DHA, $\omega - 3/\omega - 6 = 6.4$, P/S = 1.7), moonfish (highest ALA, $\omega - 3/\omega - 6 = 1.9$, P/S = 1.0), and longtail shad (highest EPA, $\omega - 3/\omega - 6 = 0.8$, P/S = 0.4) were the samples with an outstandingly desirable overall composition of fatty acids. Overall, the marine fish and shellfish from the area contained good composition of fatty acids which offer health benefits and may be used for nutraceutical purposes in the future.

1. Introduction

Fish and shellfish are widely accepted as highly nutritious and healthy foods. However, people usually think that different types of fish are of similar nutritional value, and fish selections are made only based on availability, freshness, flavor, and other physical factors [1]. Based on the Malaysian Adult Nutrition Survey (2002-2003), the prevalence of daily consumption of marine fish among rural and urban adults were quite high at 51% and 34%, respectively [2]. Therefore, it is crucial to increase the awareness of different nutrient contents of fish and shellfish species by providing complete nutritional value information, especially for fatty acid content, which is associated with various health-related effects.

Nutritional analysis of fatty acids can be classified as qualitative and quantitative. Qualitative analysis of fatty acid produces data regarding the fatty acid composition in the form of percentages of total fatty acids (% of total fatty acids). Meanwhile, quantitative analysis is able to quantify the actual amount (weight) of each fatty acid that is present in the food. Quantitative data are often presented in the form of weight of the fatty acid per weight of food or fat (e.g., mg/g oil). Currently, there are limited qualitative fatty acid data on marine fish and shellfish from warm water area; meanwhile,

Figure 1: Location of sample collection sites.

no quantitative data are available especially as a mean to utilize source of natural product for nutraceutical purposes.

Reliable analytical data are prerequisite for correct interpretation of findings in nutritional content analysis, since unreliable results may lead to over- or underestimations, false interpretations, and unwarranted conclusions [3]. Thus, validation procedures of analytical methods were also performed to provide reliable quantitative data on the fatty acid contents of various species of local marine fish and shellfish.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents used for analysis were of analytical and gas chromatography (GC) grade. Menhaden oil, 37 components FAME mix 47885-U (Supelco, Germany), and tridecanoic acid (C13, internal standard, Sigma, USA) were used as standards in the fatty acid content analysis.

2.2. Preparation of Sample. A stratified random sampling procedure was used as it was the most suitable method in database work [4]. To ensure representativeness, ten fish landing areas along the Straits of Malacca were identified with the help of Malaysian Fisheries Development Authority (LKIM). The locations are marked as L1 through L10, respectively (Figure 1).

At each of the collection sites, available samples were collected randomly according to species. All samples were from free-roaming fish and shellfish; and they were collected fresh (caught within a period of 0 to 36 hours). All samples were immediately placed on ice, kept cold, and transported in polystyrene boxes to maintain freshness. Upon arrival at Universiti Putra Malaysia, the temperature of ice boxes was checked to ensure samples were still within the range of −4°C to 0°C. Then, samples for nutrient determination were individually measured for total body weight and length. Only samples of a weight within the narrow range for each species were included as primary samples (Table 1). Then, the samples were beheaded, gutted, washed, and filleted. These primary samples were placed in sealed plastic bags and frozen at −20°C. The actual degree of freshness of samples during transportation and time of analysis may not be assured as there was no analysis of freshness or quality index of samples was done. However, appropriate precautions were performed to sustain freshness of samples and minimize oxidation throughout the study by performing procedures in chillers' room (4°C) and under minimal light exposures.

A small-scale experiment performed independently showed insignificant differences in nutrient contents of samples from different locations. This observation justified that units of primary samples can be combined or composited by geographical locations to minimize the number of analytical measurements and yet represent the contribution of the unit to the estimate of central tendency [4]. Thus, before analysis, three composite samples were prepared by mixing individual samples (n = 8–12, whole fillets) of the same weight for each species. Individual samples from L1, L2, L3, and L4 constituted as Composite 1; individual samples from L5, L6, and L7 constituted as Composite 2; while individual samples from L8, L9, and L10 constituted as Composite 3 (Figure 1). All composite samples were analyzed separately and data presented are the mean values of each of the species.

2.3. Extraction of Fat. Extraction of fat was done following Bligh and Dyer [6], with slight modifications by Kinsella et al. [7]. Representative samples of fish filets (30 g) were homogenized in a Waring blender for 2 min with a mixture of methanol (60 mL) and chloroform (30 mL). One volume of chloroform (30 mL) was added to the mixture and after blending for additional 30 seconds, distilled water (30 mL) was added. The homogenate was stirred with a glass rod and filtered through Whatman No. 1 filter paper on a Buchner funnel with slight suction. The filtrate was transferred to a separatory funnel. The lower clear phase was drained into a 250 mL round-bottom flask and concentrated with a rotary evaporator at 40°C. To minimize oxidation, the extracted lipids were kept in solvents containing 0.05% butylated hydroxytoluene (B.H.T.), in glass bottles, flushed with nitrogen, and wrapped with aluminium foils to avoid any light exposure. Then, the bottles were stored immediately at −20°C and only being taken out from freezer just before analysis.

2.4. Analysis of Fatty Acid Content. Lipid samples were converted to their constituent FAMEs following the method used in previous study [8]. Approximately 25 mg (±0.1 mg) of oil was weighed and added with 1.5 mL of NaOH 0.50 M in methanol in a 15 mL capped centrifuge tube. The mixture was heated in a water bath at 100°C for 5 min and then cooled at room temperature. The mixture was added with 2.0 mL of boron trifluoride (BF_3, 12%) in methanol and heated again in a water bath at 100°C for 30 minutes. Next, the tube was cooled in running water at room temperature before adding 1 mL of isooctane. It was vigorously stirred for 30 seconds

Table 1: List of samples with narrow range of weight, length, and fat content.

Category	Common name	Local name	Scientific name	Habitat	Range of weight (g)	Range of length (cm)	Fat content (%)
Lean fish (<2% fat)	Hardtail scad	Cencaru	*Megalapsis cordyla*	Pelagic	100–250	21–28	1.53 ± 0.15^{a}
	Golden snapper	Jenahak	*Lutjanu sjohnii*	Demersal	490–510	30–35	1.29 ± 0.41^{a}
	Indian mackerel	Kembung	*Rastrelliger kanagurta*	Pelagic	50–100	14–20	1.80 ± 0.62^{a}
	Indian threadfin	Kurau	*Polynemus indicus*	Demersal	350–1450	36–59	0.85 ± 0.21^{a}
	Malabar red snapper	Merah	*Lutjanus argentimeculatus*	Demersal	580–760	28–37	1.37 ± 1.10^{a}
	Dorab wolfherring	Parang	*Chirocentrus dorab*	Pelagic	200–900	40–71	1.22 ± 0.22^{a}
	Long-tailed butterfly ray	Pari	*Gymnura spp.*	Demersal	1300–1700	32–36	0.93 ± 0.12^{a}
	Large-scale tongue sole	Sebelah/Lidah	*Cynoglossusarel*	Demersal	50–100	24–32	0.70 ± 0.10^{a}
	Spanish mackerel	Tenggiri papan	*Scromberomorus guttatus*	Pelagic	200–450	30–42	1.05 ± 0.06^{a}
Low fat fish (2–4% fat)	Black pomfret	Bawal hitam	*Parastromateus niger*	Pelagic	780–1040	33–42	2.33 ± 0.11^{a}
	Silver pomfret	Bawal putih	*Pampus argentus*	Pelagic	100–200	15–25	2.09 ± 0.93^{a}
	Sixbar grouper	Kerapu	*Epinephelus fasciatus*	Demersal	480–750	33–36	3.46 ± 3.46^{ab}
	Japanese threadfin bream	Kerisi	*Nemipterus japonicus*	Demersal	100–230	18–25	2.70 ± 0.37^{ab}
	Yellowstripe scad	Selar kuning	*Selaroides leptolepis*	Pelagic	50–100	16–20	2.12 ± 0.50^{a}
	Gray eel-catfish	Sembilang	*Plotosus spp.*	Demersal	350–600	40–50	3.04 ± 0.59^{ab}
	Fourfinger threadfin	Senangin	*Eleutheronema tetradactylum*	Pelagic	150–300	27–32	2.10 ± 0.25^{a}
	Giant seaperch	Siakap	*Lates calcarifer*	Demersal	700–1000	38–42	2.68 ± 0.79^{ab}
	Fringescale sardinella	Tamban	*Clupea fimbriata*	Pelagic	20–40	13–17	3.00 ± 2.40^{ab}
Medium fat fish (4–8% fat)	Moonfish	Nyior-nyior	*Trachinotus blochii*	Demersal	400–1400	31–47	6.89 ± 2.76^{b}
High fat fish (>8% fat)	Longtail shad	Terubuk	*Hilsa macrura*	Pelagic	928	40–45	23.15 ± 0.00^{c}
Shellfish	Cuttlefish	Sotong	*Sepia officinalis*	—	20–45	12–18	1.35 ± 0.28^{a}
	Prawn	Udang putih	*Metapenaeus affinis*	—	10–20	12–17	1.06 ± 0.10^{a}
	Cockles	Kerang	*Anadara granosa*	—	10–20	2–5	1.93 ± 1.28^{a}
	Oyster	Tiram	*Ostrea spp.*	—	100–300	14–48	1.24 ± 0.00^{a}

[1] Categories of fish samples were based on the fat content [5].

[2] Different superscript lowercase letters ($^{a-c}$) in the same column show significant differences at $P < 0.05$ (Tukey post-hoc test) [5].

before adding 5.0 mL of a saturated sodium chloride solution to facilitate the phase separation. The esterified sample was placed in a refrigerator and left to rest for better phase separation. After collecting the supernatant, another 1.0 mL of isooctane (containing 0.05% B.H.T. as antioxidant) was added into the tube and stirred. The supernatant was collected and added to the previous fraction. The sample was concentrated to a final volume of 1.0 mL for later injection into the gas chromatograph. As precautions, amber vials were used in order to minimize oxidation during analysis.

Analysis of Gas Chromatography (GC). Analysis of methyl esters was performed by a capillary gas chromatograph model Agilent 6890 (USA Agilent Technology) equipped with a split-splitless injector, flame ionization detection (FID) system used to separate and quantify each FAME component. FAMEs were separated using a highly polar HP88 column (Agilent, USA) column (100 m length × 0.25 mm I.D. × 0.2 μm D.F.). Carrier gas was helium at a linear velocity of 30.0 mL/min. Split injection with a split ratio (volume of gas passing to waste: volume of gas passing down the capillary column) of 10 : 1 and 99.9 mL/min split flow was used. The operating conditions were 250°C injection port, 250°C flame ionization detector, and 200°C column temperature. Compounds were identified by comparison of the retention times of 37 components FAME mix 47885-U (Supelco, Germany) and Menhaden oil standards (Supelco, Germany).

Quantification of Fatty Acids. The concentration of fatty acids in mg/g of total lipids was measured against tridecanoic acid methyl ester (C13:0, Sigma, USA) as an internal standard. This followed procedures described in a previous study [6] with slight modifications; in which the Empirical Response Factor (R_i) of FID (flame ionization detector) was calculated and used instead of theoretical response factor (C_{FX}). Calculation of the empirical response factor (R_i) was done as described in the literature [9]. The following formulae were used in the quantification of fatty acids:

(i)

$$\text{empirical response factor } \left(R_i\right) = \frac{(\text{Psi})\,(\text{WISm})}{(\text{PsIS})\,(\text{Wi})}, \quad (1)$$

where Psi: peak area of individual fatty acids in mixed FAME standard solution, PsIS: peak area of fatty acid internal standard in mixed FAME standard solution, WISm: weight of fatty acid internal standard in mixed FAME standard solution, and Wi: weight of individual FAME in mixed FAME standard solution;

(ii)

$$\text{fatty acid } \left(\text{mg/g}\right) \text{ total lipid} = \frac{(\text{AX})\,(\text{WIS})\,\left(R_i\right)\,(1000)}{(\text{AIS})\,(\text{WX})\,(1.04)}, \quad (2)$$

where AX: peak area of fatty acid, AIS: peak area of internal standard (IS) WIS: weight (mg) of IS added to the sample (mg), WX: sample weight (mg), Ri: empirical response factor, and 1.04: conversion factor necessary to express results in mg fatty acid/g oil (rather than as methyl ester).

2.5. Analysis of Method Validation

Linearity Test. Three fatty acid standards, myristic acid, heptadecanoic acid, and linoleic acid were prepared at different concentrations: myristic acid (0.4, 2.0, and 4.0 mg/mL), heptadecanoicacid (0.4, 2.0, and 4.0 mg/mL), linoleic acid (0.5, 1.0, and 5.0 mg/mL). These fatty acid standards were used as they represent both saturated and unsaturated fatty acids present in samples. Calibration curves were formed for each of the compounds. The linearity parameters, which included linear regression ($y = mx + c$) and the correlation coefficient (R^2) were obtained from the linear relationship between the peak area and the concentrations of the fatty acid standards.

Precision Test. Two precision tests of repeatability (within-day) and reproducibility (between-day) were performed. Two fatty acid standards were quantified in three randomly selected samples in both tests. For repeatability (within-day precision), four replicates of each sample were analyzed in a single day using the same procedures as in the fatty acid analysis. Data are reported as relative standard deviations (RSD) of four replicates, with minimum and maximum values of both palmitic and linolenic acids quantified in each sample. For reproducibility (between-day precision), samples were analyzed using the same procedures as in the fatty acid analysis on three different days, representing three replicates of each sample. Palmitic acid and linolenic acids were quantified and presented as relative standard deviations (RSD) of three replicates, with minimum and maximum values of each fatty acid.

Recovery Test. The recovery of the method was determined using three different concentrations (0.4, 2.0, 4.0 mg/mL) of myristic acid, heptadecanoic acid, and stearic acid standards. Two samples were randomly selected and used for the recovery analysis of each of the fatty acid standards. During the sample preparation, each standard was added together with lipid extract and internal standards and prepared following the same procedures used in normal sample preparation. Recoveries of different standards were performed separately to avoid overlapping of peaks in chromatograms which could lead to biased results. Recoveries of fatty acid standards at different concentrations were determined by comparing the content of the fatty acids in samples with and without the addition of the standard. Data are presented as the percentage of recovery.

Statistical Analysis. Data were analyzed using SPSS (Scientific Package of Social Science) version 17.0. The mean, standard deviation (SD), and one-way ANOVA test followed by Tukey post-hoc analysis were performed to compare differences in the mean fatty acid contents of different species of fish and shellfish. Bivariate correlation (Pearson's r) was performed to

explore the relationships between different fatty acid classes in samples.

3. Results and Discussion

3.1. Fatty Acid Content in Samples. Data from this study are reported in the form of milligrams per 100 grams of wet muscle. Tables 2, 3, and 4 show the sample content of saturated, monounsaturated, and polyunsaturated fatty acids, respectively.

Based on the previous findings, the studied fish samples can be categorized into lean-, low-, medium-, and high-fat fish based on the fat content [5]. Among the lean-fish, hardtail scad was high in saturated fatty acids (SFAs), with palmitic acid as the highest (531.7 mg/100 g wet sample) (Table 2). The monounsaturated fatty acid (MUFA) of the fish was 121.2 mg/100 g wet sample; with fairly high oleic acid (C18:1n9c) and heptadecenoic acid (C17:1), at 63.9 and 45.7 mg/100 g wet sample, respectively (Table 3). For PUFA, hardtail scad contained quite high amounts of DHA (C22:6n3), at 196.0 mg/100 g wet sample, followed by ALA (C18:3n3) at 117.3 mg/100 g wet sample (Table 4). Meanwhile, malabar red snapper was higher in PUFA, characterized by high DHA (C22:6n3) at 209.9 mg/100 g wet sample, followed by ALA (C18:3n3) at 335.0 mg/100 g wet sample (Table 4). For SFA, palmitic acid was the dominant fatty acid (373.7 mg/100 g wet sample), followed by caprylic acid at 112.8 mg/100 g wet sample (Table 2). The MUFA content was 141.8 mg/100 g wet sample, with fairly high oleic acid (C18:1 n9c) at 119.5 mg/100 g wet sample (Table 3).

Among the low-fat fish, yellowstripe scad contained a very high PUFA content, with significantly higher ($P < 0.05$) levels of DHA (782.1 mg/100 g wet sample) compared to other samples (Table 4). The DHA amount was about 2.7 times higher than Japanese threadfin bream (2nd highest DHA, 293.0 mg/100 g wet sample); and about 86.6 times higher than long-tailed butterfly ray (lowest DHA, 9.0 mg/100 g wet sample). Additionally, yellowstripe scad also had a high ALA (C18:3n3) level at 338.1 mg/100 g wet sample. For SFA and MUFA, the most dominant fatty acids were palmitic acid (560.8 mg/100 g wet sample) and oleic acid (188.8 mg/100 g wet sample), respectively (Tables 2 and 3).

The medium-fat fish, moonfish, showed palmitic acid as the most prominent fatty acid (1308.0 mg/100 g wet sample) (Table 2). This was followed by α-linolenic acid (C18:3n3) at 1046.8 mg/100 g wet sample, which was significantly higher ($P < 0.05$) compared to the others (Table 4). Other $\omega - 3$ fatty acids, such as eicosatrienoic acid (C20:3n3), EPA (C20:5n3), and DHA (C22:6n3) were at 257.6, 176.7, and 122.6 mg/100 g wet sample, respectively. Its $\omega - 6$ fatty acid content, linolelaidic acid (C18:2n6t, 637.7 mg/100 g wet sample) and linoleic acid (C18:2n6c, 215.3 mg/100 g wet sample), were the second highest after level in longtail shad (linolelaidic acid at 2254.1 mg/100 g and linoleic acid at 1327.4 mg/100 g wet sample), respectively. Meanwhile, for MUFA, the most dominant fatty acid was oleic acid (C18:1n9c), at 398.5 mg/100 g wet sample. Moonfish also contained significantly higher (Tukey post-hoc test, $P < 0.05$) of heptadecenoic acid (C17:1, 81.5 mg/100 g wet sample) compared to others (Table 3).

The high-fat fish, longtail shad also showed palmitic acid (C16:0) as the dominant fatty acid (12542.6 mg/100 g wet muscle, Table 2). For MUFA, longtail shad contained significantly higher (Tukey post-hoc test, $P < 0.05$) of palmitoleic acid (C16:1, 458.1 mg/100 g wet sample) and oleic acid (C18:1n9c, 2554.4 mg/100 g wet sample) compared to others (Table 3). While for PUFA, the fish contained significantly higher (Tukey post-hoc test, $P < 0.05$) of EPA (C20:5 n3, 2041.8 mg/100 g wet sample) and eicosatrienoic acid (C20:3n3, 437.5 mg/100 g wet sample) compared to others (Table 4). Besides, longtail shad was also high in $\omega - 6$ fatty acids; with linolelaidic acid (C18:2n6t) at 2254.1 mg/100 g wet muscle, and linoleic acid (C18:2n6c) at 1327.4 mg/100 g wet muscle.

As in most of fish samples, shellfish including cuttlefish, cockles, and oyster also showed palmitic acid (C16:0) as the most prominent fatty acid (Table 2). Meanwhile, prawn showed caprylic acid (C8:0) as the highest fatty acid (139.9 mg/100 g wet sample), followed by palmitic acid (C16:0) at 125.3 mg/100 g wet sample. For MUFA, cockles contained higher oleic acid (C18:1n9c, 303.8 mg/100 g wet sample) compared to other shellfish (Table 3). While for PUFA, cockles were found to be higher in both EPA (C20:5n3, 343.0 mg/100 g wet sample) and DHA (C22:6n3, 123.4 mg/100 g wet sample), compared to other shellfish (Table 4). However, the content of alpha-linolenic acid (C18:3n3) in cockles (18.6 mg/100 g wet sample) was the lowest among all samples; with cuttlefish (251.1 mg/100 g wet sample), prawn (320.4 mg/100 g wet sample), and oysterr (100.7 mg/100 g wet sample).

3.2. Fatty Acids of Fish and Shellfish from Local and Other Countries. Most of previous local studies have focused on qualitative aspects of fat content in various samples of marine and freshwater origin samples [10–15]. A previous study found very high percentage of PUFA; with omega-3 PUFA (29.7–48.4%), other PUFA (27.7–40.0%), and omega-6 PUFA (11.0–20.0%) in ten common fish species with the current study [9]. Meanwhile, percentages of SFA and MUFA were quite low; at 3.63–11.4% and 1.37–9.12%, respectively. This study found hexadecadienoic acid as the most prominent fatty acid (18.1–24.9%) in six samples, hexadecatrienoic acid (C16:3n4) in two samples, and hexadecadienoic acid (C16:2) in other two samples [10]. Another local study reported PUFA as the dominant group of fatty acid in seven species, and SFA as the dominant fatty acid group in another six species of their fish samples [11]. Palmitic acid (C16:0) was the most prominent fatty acid (17.6–32.1%) in eight samples; meanwhile, DHA (C22:6n3) was the highest fatty acid (19.3–24.0%) in another five samples [11]. Same findings were reported by other study of 16 species of local pelagic fish; with palmitic acid (C16:0) as the most prominent fatty acid (20.9–34.5%) in nine species, meanwhile, DHA (C22:6n3) was the highest fatty acid (26.0–29.8%) in another seven species [12]. The current study, however, focuses on the quantitative aspect of samples fat thus allow limited comparison be made with these previous local data. However,

Table 2: Saturated fatty acids (SFAs) content of samples.

Category	Samples	Saturated fatty acids (SFA) content (mg/100 g wet sample)								
		8:0	12:0	14:0	15:0	16:0	17:0	18:0	24:0	∑SFA
Lean fish (<2% fat)	Hardtail scad	42.2^{ab}	1.4^{abcd}	69.8^{a}	18.6^{abc}	531.7^{a}	45.2^{a}	nd	nd	708.8
	Golden snapper	152.9^{bcd}	0.7^{abc}	9.6^{a}	8.5^{ab}	203.7^{a}	21.5^{a}	1.8^{a}	20.1^{abcd}	418.7
	Indian mackarel	139.0^{abcd}	0.7^{abc}	64.1^{a}	9.5^{ab}	313.7^{a}	22.8^{a}	35.7^{bcd}	2.3^{ab}	587.8
	Indian threadfin	98.9^{abcd}	0.9^{abc}	10.3^{a}	6.8^{a}	134.7^{a}	17.7^{a}	4.8^{ab}	33.4^{bcde}	307.4
	Malabar red snapper	112.8^{abcd}	0.9^{abc}	28.8^{a}	5.7^{a}	373.7^{a}	31.8^{a}	nd	4.3^{ab}	557.9
	Dorab wolfherring	72.2^{abc}	1.4^{abcd}	39.3^{a}	7.6^{a}	395.9^{a}	21.1^{a}	nd	nd	537.4
	Long-tailed butterfly ray	140.1^{abcd}	nd	4.6^{a}	2.9^{a}	129.8^{a}	11.3^{a}	nd	nd	288.8
	Large-scale tongue sole	80.7^{abc}	0.3^{ab}	7.5^{a}	5.4^{a}	95.1^{a}	15.2^{a}	1.5^{a}	36.8^{cde}	242.4
	Spanish mackarel	86.3^{abcd}	0.4^{ab}	16.1^{a}	5.9^{a}	183.7^{a}	9.5^{a}	nd	21.0^{abcde}	322.8
Low fat fish (2–4% fat)	Black pomfret	214.8^{d}	nd	46.9^{a}	18.0^{abc}	549.5^{a}	48.5^{a}	6.9^{ab}	50.4^{def}	935.0
	Silver pomfret	40.0^{ab}	1.1^{abc}	102.4^{a}	15.5^{ab}	590.2^{a}	38.6^{a}	11.2^{abc}	79.7^{f}	878.7
	Sixbar grouper	186.1^{cd}	1.6^{abcd}	65.6^{a}	19.8^{abc}	775.6^{a}	62.1^{a}	6.4^{ab}	191.6^{g}	1308.7
	Japanese threadfin bream	34.0^{ab}	5.2^{de}	102.6^{a}	28.2^{abcd}	996.5^{a}	36.8^{a}	8.0^{ab}	nd	1211.2
	Yellowstripe scad	101.1^{abcd}	1.7^{abcd}	69.5^{a}	19.9^{abc}	560.8^{a}	65.7^{a}	nd	10.9^{ab}	829.6
	Gray eel-catfish	35.3^{ab}	3.9^{bcde}	159.7^{a}	24.3^{abc}	717.8^{a}	359.0^{a}	40.9^{cd}	nd	1340.8
	Fourfinger threadfin	52.9^{ab}	2.5^{abcd}	127.2^{a}	33.7^{bcd}	677.0^{a}	31.9^{a}	7.0^{ab}	21.1^{abcde}	953.4
	Giant seaperch	79.5^{abc}	4.4^{cde}	102.5^{a}	15.5^{ab}	928.2^{a}	76.7^{a}	8.0^{ab}	52.4^{ef}	1267.2
	Fringescale sardinella	21.5^{a}	2.2^{abcd}	242.0^{a}	43.1^{cd}	822.2^{a}	108.1^{a}	33.5^{bcd}	nd	1272.8
Medium fat fish (4–8% fat)	Moonfish	383.9^{e}	7.2^{e}	335.3^{a}	53.4^{d}	1308.0^{a}	431.6^{a}	31.1^{abcd}	21.9^{abcde}	2572.4
High fat fish (>8% fat)	Longtail shad	20.3^{a}	21.1^{f}	2805.4^{b}	113.8^{e}	12542.6^{b}	2034.5^{b}	82.5^{e}	nd	17620.2
Shellfish	Cuttlefish	93.7^{abcd}	0.7^{abc}	30.9^{a}	9.3^{ab}	394.5^{a}	7.0^{a}	nd	33.0^{bcde}	569.1
	Prawn	139.9^{abcd}	0.3^{ab}	7.0^{a}	11.4^{ab}	125.3^{a}	24.5^{a}	nd	nd	308.4
	Cockles	37.0^{ab}	2.8^{abcd}	69.9^{a}	12.4^{ab}	359.2^{a}	130.3^{a}	9.5^{abc}	22.2^{abcde}	643.3
	Oyster	30.2^{ab}	0.5^{abc}	41.4^{a}	10.0^{ab}	325.8^{a}	80.3^{a}	62.6^{de}	13.0^{abc}	563.7

[1] Categories of fish samples were based on the fat content [5].

[2] Different superscript lowercase letters ($^{a–g}$) in same column show significant differences at $P < 0.05$ (Tukey post-hoc test).

[3] nd: not detected.

Table 3: Monounsaturated fatty acids (MUFAs) content of samples.

Category	Samples	Monounsaturated fatty acids (MUFAs) content (mg/100 g wet sample)					
		14:1	15:1	16:1	17:1	18:1 n9c	∑MUFA
Lean fish (<2% fat)	Hardtail scad	2.5^{abc}	1.5^{ab}	7.5^{abc}	45.7^{ef}	63.9^{ab}	121.2
	Golden snapper	2.7^{abc}	4.6^{abc}	40.7^{de}	6.6^{ab}	39.5^{a}	94.0
	Indian mackarel	1.8^{abc}	7.5^{c}	116.4^{f}	10.4^{ab}	158.7^{ab}	294.8
	Indian threadfin	1.0^{ab}	1.3^{ab}	3.3^{ab}	14.1^{abc}	18.5^{a}	38.3
	Malabar red snapper	1.0^{ab}	1.0^{ab}	4.4^{ab}	16.0^{abc}	119.5^{ab}	141.8
	Dorab wolfherring	1.0^{ab}	0.6^{a}	1.1^{a}	20.1^{abcd}	123.1^{ab}	145.9
	Long-tailed butterfly ray	1.6^{abc}	0.8^{a}	1.9^{ab}	7.7^{ab}	72.2^{ab}	84.6
	Large-scale tongue sole	0.6^{a}	1.4^{ab}	nd	10.6^{ab}	20.8^{a}	33.4
	Spanish mackarel	0.4^{a}	0.3^{a}	2.8^{ab}	13.8^{abc}	53.2^{ab}	70.6
Low fat fish (2–4% fat)	Black pomfret	1.8^{abc}	4.1^{abc}	13.6^{abcd}	20.6^{abcd}	98.8^{ab}	138.8
	Silver pomfret	1.6^{abc}	1.2^{ab}	30.9^{bcde}	36.0^{cdef}	81.5^{ab}	151.1
	Sixbar grouper	4.5^{bcd}	15.7^{d}	116.0^{f}	nd	151.0^{ab}	287.2
	Japanese threadfin bream	6.9^{de}	1.5^{ab}	105.3^{f}	28.8^{bcdef}	144.7^{ab}	287.2
	Yellowstripe scad	2.8^{abc}	1.9^{ab}	43.4^{e}	49.9^{f}	188.8^{ab}	286.8
	Gray eel-catfish	11.3^{f}	5.7^{bc}	nd	47.4^{ef}	103.7^{ab}	168.1
	Fourfinger threadfin	5.1^{cd}	2.4^{ab}	35.3^{cde}	43.2^{def}	241.5^{ab}	327.4
	Giant seaperch	3.6^{abcd}	2.6^{ab}	43.4^{e}	25.2^{bcde}	155.3^{ab}	230.0
	Fringescale sardinella	3.5^{abcd}	3.5^{abc}	53.0^{e}	51.3^{f}	192.0^{ab}	303.3
Medium fat fish (4–8% fat)	Moonfish	9.5^{ef}	14.8^{d}	nd	81.5^{g}	398.5^{b}	504.2
High fat fish (>8% fat)	Longtail shad	10.7^{ef}	nd	458.1^{g}	36.1^{cdef}	2554.4^{c}	3059.3
Shellfish	Cuttlefish	0.8^{ab}	2.62^{ab}	4.0^{ab}	17.2^{abc}	89.5^{ab}	114.2
	Prawn	0.7^{a}	1.67^{ab}	7.4^{abc}	16.9^{abc}	87.8^{ab}	114.5
	Cockles	2.9^{abc}	13.52^{d}	nd	75.9^{g}	303.8^{ab}	396.1
	Oyster	0.7^{ab}	2.65^{ab}	nd	25.1^{bcde}	53.9^{ab}	82.4

[1] Categories of fish samples were based on the fat content [5].
[2] Different superscript lowercase letters ($^{a-g}$) in same column show significant differences at $P < 0.05$ (Tukey post-hoc test).
[3] nd: not detected.

there were a few studies focused on the quantitative aspects of fatty acids in other cold water species of marine and freshwater fish and shellfish performed in other countries. The comparisons made with the previous findings would be useful in giving better overview of the content of fatty acids in local marine fish and shellfish.

One of the previous quantitative studies of fatty acids had reported the $\omega - 3$ and $\omega - 6$ fatty acids content in different types (wild, farmed, supermarket, and feed) of salmon from North America and Europe [16]. Linoleic acid (C18:2n6) were at 67, 647, 604, and 1719 mg/100 g muscles for wild salmon, farmed salmon, supermarket salmon, and salmon feed [16]; which were higher than most of the samples (4.3–215.3 mg/100 g wet sample) in current study (Table 4). However, longtail shad is comparable in linoleic acid content with salmon feed, at 1327.4 mg/100 g wet sample [16]. This previous study also reported low γ-linolenic acid (C18:3n6) at 3 mg/100 g (wild salmon), 14 mg/100 g (farmed salmon), 13 mg/100 g (supermarket salmon), and 40 mg/100 g (salmon feed) [16]. Only three samples in current study were found to contain this fatty acid; hard tail scad (1.6 mg/100 g), silver pomfret (10.9 mg/100 g), and black pomfret (29.1 mg/100 g), which were comparable with their findings [16]. For α-linolenic acid (C18:3n3), most samples in current study contained about 100–300 mg/100 g of this fatty acid; which were higher than wild salmon (50 mg/100 g), comparable with farmed salmon (181 mg/100 g) and supermarket salmon (168 mg/100 g), meanwhile were lower when compared to salmon feed (505 mg/100 g) [16].

Most samples in current study contained lower EPA (C20:5n3, 2.7–343.0 mg/100 g wet sample) compared to wild salmon (414 mg/100 g), farmed salmon (1079 mg/100 g), supermarket salmon (969 mg/100 g), and salmon feed (2638 mg/100 g) [16]. Only longtail shad was found to contain comparable amount of EPA with salmon feed; at 2638 mg/100 g wet sample [16]. It is really interesting to find such a high level of EPA in longtail shad, as high intake of this fatty acid had been related with protective effects to the occurrence of asthma, coronary heart problems and many other diseases [17–19]. Meanwhile, compared to DHA values (629–2633 mg/100 g) reported by the previous study [16], the content of this fatty acid in most samples of the

Table 4: Polyunsaturated fatty acids (PUFA) content of samples.

Category	Samples	Polyunsaturated fatty acids (PUFA) content (mg/100 g wet sample)							
		18:2 n6t	18:2 n6c	18:3 n6	18:3 n3	20:3 n3	20:5 n3	22:6 n3	∑PUFA
Lean fish (<2% fat)	Hardtail scad	22.5^{a}	30.8^{a}	1.6^{a}	117.3^{a}	nd	18.9^{a}	196.0^{cdefg}	387.0
	Golden snapper	105.2^{ab}	9.6^{a}	nd	365.6^{ab}	nd	7.3^{a}	18.6^{ab}	506.3
	Indian mackarel	23.1^{a}	29.3^{a}	nd	53.9^{a}	7.3^{ab}	53.6^{a}	23.3^{ab}	190.5
	Indian threadfin	33.2^{a}	5.2^{a}	nd	271.7^{a}	nd	23.7^{a}	82.2^{abcde}	415.9
	Malabar red snapper	86.1^{ab}	60.4^{a}	nd	335.0^{ab}	9.2^{ab}	24.1^{a}	209.9^{defg}	724.7
	Dorab wolfherring	36.3^{a}	6.4^{a}	nd	204.0^{a}	11.6^{ab}	23.9^{a}	54.3^{abcd}	336.5
	Long-tailed butterfly ray	11.2^{a}	15.1^{a}	nd	337.5^{ab}	nd	2.7^{a}	9.0^{a}	375.5
	Large-scale tongue sole	25.9^{a}	4.3^{a}	nd	111.6^{a}	2.5^{ab}	8.3^{a}	113.4^{abcde}	266.2
	Spanish mackarel	18.2^{a}	19.7^{a}	nd	178.9^{a}	nd	27.7^{a}	69.8^{abcde}	314.2
Low fat fish (2–4% fat)	Black pomfret	16.8^{a}	45.1^{a}	29.06^{c}	272.8^{a}	nd	73.5^{a}	277.1^{fg}	714.3
	Silver pomfret	104.7^{ab}	49.5^{a}	10.91^{b}	136.2^{a}	6.1^{ab}	116.3^{a}	148.0^{abcdefg}	571.6
	Sixbar grouper	139.2^{ab}	119.3^{a}	nd	645.5^{b}	nd	100.9^{a}	197.7^{cdefg}	1202.5
	Japanese threadfin bream	81.6^{ab}	51.0^{a}	nd	112.2^{a}	nd	258.7^{a}	293.0^{g}	796.5
	Yellowstripe scad	78.6^{ab}	113.6^{a}	nd	338.1^{ab}	7.5^{ab}	97.1^{a}	782.05^{h}	1417.0
	Gray eel-catfish	257.4^{ab}	133.6^{a}	nd	185.0^{a}	nd	145.8^{a}	88.8^{abcde}	810.7
	Fourfinger threadfin	148.3^{ab}	48.7^{a}	nd	114.3^{a}	nd	96.2^{a}	53.1^{abc}	460.6
	Giant seaperch	142.5^{ab}	94.4^{a}	nd	245.6^{a}	215.7^{d}	139.5^{a}	95.4^{abcde}	933.0
	Fringescale sardinella	33.2^{a}	164.2^{a}	nd	69.3^{a}	31.0^{c}	211.5^{a}	225.4^{efg}	734.6
Medium fat fish (4–8% fat)	Moonfish	637.7^{ab}	215.3^{a}	nd	1046.8^{c}	257.6^{e}	176.7^{a}	122.6^{abcdef}	2456.7
High fat fish (>8% fat)	Longtail shad	2254.1^{b}	1327.4^{b}	nd	68.9^{a}	437.5^{f}	2041.8^{b}	168.7^{bcdefg}	6298.4
Shellfish	Cuttlefish	13.4^{a}	17.5^{a}	nd	251.1^{a}	14.9^{b}	121.9^{a}	85.0^{abcde}	503.8
	Prawn	16.3^{a}	31.3^{a}	nd	320.4^{ab}	nd	48.7^{a}	45.3^{abc}	461.9
	Cockles	71.5^{ab}	55.0^{a}	nd	18.6^{a}	nd	343.0^{a}	123.4^{abcdef}	611.4
	Oyster	62.0^{a}	35.9^{a}	nd	100.7^{a}	nd	54.0^{a}	82.8^{abcde}	335.4

[1] Categories of fish samples were based on the fat content [5].

[2] Different superscript lowercase letters ($^{a–h}$) in same column show significant differences at $P < 0.05$ (Tukey post-hoc test).

[3] nd: not detected.

Table 5: Polyunsaturated/saturated fatty acids (P/S) and $\omega - 3/\omega - 6$ ratio of samples.

Classification	Samples	SFA	MUFA	PUFA	PUFA/SFA	$\omega - 3$	$\omega - 6$	$\omega - 3/\omega - 6$
Lean fish (<2% fat)	Hardtail scad	708.8	121.2	387.0	0.5	332.1	54.8	6.1
	Golden snapper	418.7	94.0	506.3	1.2	391.5	114.8	3.4
	Indian mackarel	587.8	294.8	190.5	0.3	138.1	52.4	2.6
	Indian threadfin	307.4	38.3	415.9	1.4	377.5	38.4	9.8
	Malabar red snapper	557.9	141.8	724.7	1.3	578.3	146.5	3.9
	Dorab wolfherring	537.4	145.9	336.5	0.6	293.7	42.7	6.9
	Long-tailed butterfly ray	288.8	84.6	375.5	1.3	349.3	26.3	13.3
	Large-scale tongue sole	242.4	33.4	266.2	1.1	235.9	30.2	7.8
	Spanish mackarel	322.8	70.6	314.2	1.0	276.3	37.9	7.3
Low fat fish (2–4% fat)	Black pomfret	935.0	138.8	714.3	0.8	623.3	91.0	6.8
	Silver pomfret	878.7	151.1	571.6	0.7	406.6	165.1	2.5
	Sixbar grouper	1308.7	287.2	1202.5	0.9	944.1	258.5	3.7
	Japanese threadfin bream	1211.2	287.2	796.5	0.7	663.9	132.6	5.0
	Yellowstripe scad	829.6	286.8	1417.0	1.7	1224.8	192.2	6.4
	Gray eel-catfish	1340.8	168.1	810.7	0.6	419.6	391.1	1.1
	Fourfinger threadfin	953.4	327.4	460.6	0.5	263.6	197.0	1.3
	Giant seaperch	1267.2	230.0	933.0	0.7	696.2	236.9	2.9
	Fringescale sardinella	1272.8	303.3	734.6	0.6	537.2	197.4	2.7
Medium fat fish (4–8% fat)	Moonfish	2572.4	504.2	2456.7	1.0	1603.7	853.0	1.9
High fat fish (>8% fat)	Longtail shad	17620.2	3059.3	6298.4	0.4	2716.9	3581.5	0.8
Shellfish	Cuttlefish	569.1	114.2	503.8	0.9	472.9	30.9	15.3
	Prawn	308.4	114.5	461.9	1.5	414.3	47.6	8.7
	Cockles	643.3	396.1	611.4	1.0	484.9	126.5	3.8
	Oyster	563.7	82.4	335.4	0.6	237.5	97.9	2.4

[1] Categories of fish samples were based on the fat content [5].

current study were lower (9.0–277.1 mg/100 g wet sample). However, yellowstripe scad (782.1 mg/100 g wet sample) was found to contain slightly higher DHA content compared to wild salmon (629 mg/100 g) [16].

Data from National Nutrient Data by United States Department of Agriculture (USDA) showed Greenland halibut, farmed catfish, wild catfish, farmed salmon, and wild salmon contained EPA + DHA at 1177.6, 177.6, 236.5, 2147.1, and 1840 mg/100 g muscles [20]. Generally, most samples in the current study contained EPA + DHA amounts (11.8–551.7 mg/100 g wet sample); which were lower compared to Greenland halibut, farmed salmon, and wild salmon; but comparable with farmed and wild catfish. However, longtail shad (2210 mg/100 g wet sample) was found to contain EPA + DHA at comparable amount with farmed and wild salmon [20]. This could be due to the high fat content of the fish (23.2% fat) [5].

The current study is novel as it provides new quantitative findings for warm water species of fish and shellfish. Moreover, the findings are of necessary representativeness, which resulted from systematic sampling procedures performed. Data from the current study is also highly important as it represents samples from the Straits of Malacca, which aligns the west coast of Peninsular Malaysia, the main contributor of marine landings production in the country which produced 50.16% of the total marine landings production of Malaysia and 67.34% of the marine landings production of peninsular areas (Department of Fisheries Malaysia, 2007).

3.3. Ratios of Polyunsaturated/Saturated (P/F) and ω-3/ω-6 Fatty Acids. Overall, seventeen species of samples contained SFA as the dominant group of fatty acid (Table 5), with palmitic acid (C16:0) as the highest fatty acid quantified in most of samples (Table 2). The high amount of palmitic acid is due to its function as a key metabolite in fish, and the level is not influenced by the diet [21]. Meanwhile, seven of the samples (golden snapper, Indian threadfin, malabar red snapper, long-tailed butterfly ray, large-scale tongue sole, yellowstripe scad, and prawn) contained PUFA as the dominant group of fatty acid. Generally, the PUFA to SFA (P/S) ratios of most of the samples of this study (Table 5) were above the value for Menhaden oil (0.58); as suggested by Food and Drug Association (FDA) as PUFA supplement [22]. Besides, ratios exceeding 0.50 have also been shown to lower blood cholesterol level [23].

Among the lean fish, hardtail scad and Indian mackerel contained fairly high SFA but fairly low PUFA; which resulted in low P/S ratio of 0.6 and 0.3, respectively. Meanwhile, other lean fish showed higher P/S ratios, between 0.6 and 1.4. For low-fat fish, four-finger threadfin showed low P/S ratio of 0.5. Other low-fat fish showed P/S ratio between 0.6 and 1.7. While for the medium fat fish, moonfish contained quite high

Table 6: Repeatability (within-day precision) data of fatty acids ($n = 4$).

Sample	n	Palmitic acid (C16:0)					Linolenic acid (C18:3 n3)				
		Mean of fatty acid content (mg/g oil)	SD	RSD (%)	Minimum	Maximum	Mean of fatty acid content (mg/g oil)	SD	RSD (%)	Minimum	Maximum
Gray eel-catfish	4	321.06	4.60	1.43	315.91	326.43	55.40	0.19	0.34	55.23	55.66
Giant seaperch	4	334.48	12.04	3.59	319.45	345.48	92.50	1.73	1.87	91.00	94.02
Cockles	4	146.36	3.00	2.05	142.12	149.19	109.22	1.86	1.70	107.52	111.33

Table 7: Reproducibility (between-day precision) data of fatty acids ($n = 3$).

Sample	n	Palmitic acid (C16:0)					Linolenic acid (C18:3 n3)				
		Mean of fatty acid content (mg/g oil)	SD	RSD (%)	Minimum	Maximum	Mean of fatty acid content (mg/g oil)	SD	RSD (%)	Minimum	Maximum
Gray eel-catfish	3	319.27	3.48	1.09	315.91	322.85	55.31	0.09	0.16	55.23	55.40
Giant seaperch	3	339.49	8.19	2.41	330.15	345.48	91.99	1.71	1.86	91.00	93.96
Cockles	3	147.77	1.25	0.85	146.84	149.19	108.51	1.48	1.36	107.52	110.22

P/S ratio of 1.0. The high-fat fish, longtail shad contained higher SFA content compared to PUFA that resulted in low P/S ratio of 0.4. Overall, most of samples contained favorable ratio of fatty acids, which was beneficial for PUFA intake and lowering blood cholesterol [22, 23].

There is no specific trend in levels of saturation or unsaturation relative to the fat content of samples. On the other hand, a very high positive correlation was found between fat content of samples with all three classes of fatty acids; SFA (Pearson's $r = 0.989$, $P = 0.001$), MUFA (Pearson's $r = 0.984$, $P = 0.001$), and PUFA (Pearson's $r = 0.983$, $P = 0.001$). This suggested that increment in fat content is followed by increment in the content of all forms of fatty acids: SFA, MUFA, and PUFA in the samples.

The $-3 : \omega-6$ ratio has been suggested as a useful indicator for comparing relative nutritional values of fish oils. The ratio is also expressed in the form of $\omega - 3/\omega - 6$. It has been suggested that an $\omega-3 : \omega-6$ ratio of 1 : 1 to 1 : 5 (or $\omega-3/\omega-6$ between 0.2 and 1.0) would constitute a healthy human diet [22]. High $\omega - 3 : \omega - 6$ ratio is preferred, as excess $\omega - 6$ fatty acids can counteract the health benefits of $\omega - 3$ fatty acid intake [24].

Overall, long-tailed butterfly ray and cuttlefish had very high ratios of $\omega - 3/\omega - 6$, at 13.3 and 15.3, respectively (Table 5). However, the high ratio was not contributed to by high $\omega - 3$ fatty acids, but by the low content of $\omega - 6$ fatty acids. These fish contained 349.3 and 472.9 mg/100 g wet samples of $\omega - 3$ fatty acids, respectively, which were within the common range of other samples. Meanwhile, their $\omega - 6$ fatty acids contents were only 26.3 mg/100 g wet sample for long-tailed butterfly ray, and 30.9 mg/100 g wet sample for cuttlefish. The same reason applied to the fairly high ratio of $\omega - 3/\omega - 6$ at 9.8, 8.7, 7.8, and 7.3 for another four samples; namely, Indian threadfin, prawn, large-scale tongue sole, and Spanish mackerel. These samples contained $\omega - 3$ fatty acids between 235.9 and 414.3 mg/100 g wet samples; meanwhile their $\omega-6$ fatty acids were below than 40 mg/100 g wet sample. While for yellowstripe scad, the high ratio of $\omega - 3/\omega - 6$ was contributed by its high content of $\omega - 3$ fatty acids (1224.8 mg/100 g wet sample) compared to $\omega - 6$ fatty acids (192.2 mg/100 g wet sample). In contrast, longtail shad contained high content of $\omega - 6$ fatty, resulted in fairly low ratio of $-3 : \omega - 6$ (0.8), which was the lowest among all samples.

3.4. Validation Procedures. A few validation analyses were performed to ensure the reliability of the method used for fatty acid analysis in this study; these measures included linearity, precision (repeatability and reproducibility), and recovery tests. Linear relationships were observed for myristic acid (C14:0) standard (linearity parameters: $y = 221.25x - 23.04$; $R^2 = 0.999$), heptadecanoic acid (C17:0) standard (linearity parameters: $y = 7552x - 1060$; $R^2 = 0.9976$), and linoleic acid (C18:2n6) standard (linearity parameters: $y = 325.23x + 18.996$; $R^2 = 0.9985$).

The precision test assessed both repeatability (within-day) and reproducibility (between-day) tests. Table 6 shows the RSD values for the repeatability test of fatty acids; these values ranged between 1.4–3.6% for palmitic acid and 0.2–1.9% for linolenic acid. Meanwhile, Table 7 shows the RSD values for the reproducibility test, which ranged between 0.9–2.4% for palmitic acid, and 0.16–1.86% for linolenic acid. The RSD values in this study were lower compared to most RSD values (3.1–13.3%) as reported previously [25], but were higher when compared with excellent RSD values (<2.0%) reported by another study [26]. However, from overall, the RSD values in all repeatability and reproducibility tests were satisfactory and showed that the methods used were reliable to produce precise data for multiple determinations of fatty

Table 8: Recovery rates of fatty acids in samples.

Fatty acids standards	Concentrations (mg/mL)	Samples	Recovery (%)
Myristic (C14:0)	0.4	a	105.61
		b	106.84
	2.0	a	105.40
		b	119.35
	4.0	a	90.34
		b	114.80
Heptadecanoic (C17:0)	0.4	a	94.63
		b	114.13
	2.0	a	111.92
		b	112.84
	4.0	a	87.09
		b	115.92
Stearic (C18:0)	0.4	a	116.15
		b	106.75
	2.0	a	105.40
		b	108.81
	4.0	a	110.39
		b	93.39

acids (saturated and unsaturated) in fish and shellfish samples, both in a single-day and multiple-day determinations.

Meanwhile, the percentages of recoveries for different concentrations of myristic acid were between 90.3–105.6% in golden snapper and 106.8–119.4% in cockles (Table 8). Meanwhile for heptadecanoic acid, the percentages of recoveries were between 87.1–111.9% in golden snapper and 112.8–115.9% in cockles. For stearic acid, the range of 93.4–108.8% in cockles. Overall, the recovery percentages were satisfactory, at about 90–120%, which is comparable to the values (most showed 80–120%) reported by previous studies [25, 27]. This shows that the methods used in the analysis are highly accurate for determinations of fatty acid contents at various concentrations in fish and shellfish samples.

4. Conclusion

Overall, most of the marine fish and shellfish samples contained desirable compositions of fatty acids with a fairly high amount of $\omega-3$ fatty acids, a suitable ratio of $\omega-3/\omega-6$ fatty acids, and preferable P/S ratios which were higher than the level in the recommended PUFA supplement, Menhaden oil. Three samples were identified as being outstanding in their desirable overall composition of fatty acids: yellowstripe scad (highest DHA, $\omega-3/\omega-6=6.4$, P/S = 1.7), moonfish (highest ALA, $\omega-3/\omega-6=1.9$, P/S = 1.0), and longtail shad (highest EPA, $\omega-3/\omega-6=0.8$, P/S = 0.4). These findings showed that marine fish and shellfish from warm water area contain a good composition of fatty acids and could provide many health benefits if consumed regularly. These reliable and representative data are very useful to develop a nutritional database of marine fish and shellfish from warm water area and as reference to people for intake locally and globally. Additionally, the findings also showed that some identified species of fish and shellfish from this area may have possible value in terms of future manipulation for various nutraceutical purposes. However, further studies should be developed on this matter.

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Research Article

Harmine and Harmaline Downregulate TCDD-Induced Cyp1a1 in the Livers and Lungs of C57BL/6 Mice

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We previously demonstrated that *Peganum harmala* L. extract and its main active constituents, harmine and harmaline inhibit the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-mediated induction of the carcinogen-activating enzyme, Cyp1a1, *in vitro*. However, the effect of both alkaloids on Cyp1a1 *in vivo* has not been investigated. Therefore, the aim of this study is to examine the effect of harmine and harmaline on TCDD-mediated induction of Cyp1a1 in mice livers and lungs. C57BL/6 male mice were distributed into four groups ($n = 6$). First group received vehicle, while the second group received TCDD (i.p.). The third and fourth groups received either harmine or harmaline (i.p.) × 3 times along with TCDD one time with the mid dose of harmine and harmaline. All mice were sacrificed after 14 h from TCDD injection, and livers and lungs were isolated. The effect of harmine and harmaline on TCDD-mediated induction of Cyp1a1 mRNA, protein, and activity levels was determined using real-time PCR, Western blot analysis, and 7-ethoxyresurofin as a substrate, respectively. Our results showed that harmine and harmaline significantly decreased the TCDD-mediated induction of Cyp1a1 in both the livers and lungs. We concluded that harmine and harmaline are promising candidate to inhibit TCDD-mediated induction of Cyp1a1 in mice hepatic and extrahepatic tissues.

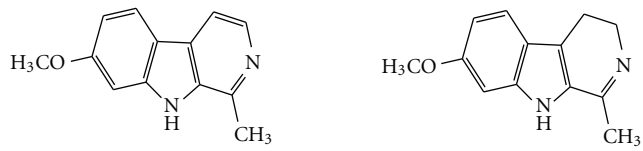
1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a widely distributed environmental contaminant that possesses multiple species- and tissue-specific adverse effects such as tumor promotion, teratogenicity, and immune-, hepato-, cardio- and skin toxicity. TCDD is a known carcinogen to animals and humans, and it was classified as a human carcinogen (Group 1) by International Agency of Research on Cancer (IARC) since 1997. In 2009, IARC confirmed that TCDD is a human carcinogen that is correlated to the increased mortality from all types of human cancers combined [1].

The adverse effects of TCDD are mainly mediated through binding and activation of ubiquitous transcription factor called aryl hydrocarbon receptor (AhR). AhR is found inactive in the cytoplasm until it binds to its ligand such as TCDD. Upon ligand binding, AhR gets activated and translocated to the nucleus where it heterodimerizes with another protein called AhR nuclear translocator (ARNT). The formed complex binds to its cognate DNA sequence that is found upstream of several AhR-regulated genes including CYP1A1 [2].

CYP1A1 is a carcinogen-activating enzyme that catalyzes the metabolic activation of several procarcinogens to their ultimate carcinogenic forms. The activity of CYP1A1 has been correlated not only to the exposure to several environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) but also to the activation of several pro-carcinogenic agents [3]. Moreover, several studies have demonstrated the positive relationship between the induction of CYP1A1 and the incidence of several human cancers such as lung, colon, and rectal cancers [4, 5]. Additionally, it has been demonstrated that the inhibition of AhR activity and its regulated gene, CYP1A1, could result in the prevention of toxic effects caused by the AhR ligands, including carcinogenicity [6].

Harmine, 7-methoxy-1-methyl-9H-pyrido (3,4-b)indole and harmaline, 4,9-dihydro-7-methoxy-1-methyl-3H-pyrido (3,4-b)indole (Figure 1) are common β-carboline alkaloids that are present in several plants such as *Peganum*



Harmine Harmaline

Figure 1: Chemical structure of harmine (7-methoxy-1-methyl-9H-pyrido [3,4-b]indole) and harmaline (4,9-dihydro-7-methoxy-1-methyl-3H-pyrido [3,4-b]indole).

harmala L. (Nitrariaceae). Harmine and harmaline possess several pharmacological effects such as hypothermic, antimicrobial, antioxidant, hallucinogenic, cytotoxic, and antitumor properties [7]. We previously demonstrated that *Peganum harmala* extract and its main active constituents, harmine and harmaline decreased the TCDD-mediated induction of Cyp1a1 activity in mouse hepatoma Hepa-1c1c7 cells [8]. Moreover, we reported that both harmine and harmaline inhibited the TCDD-mediated induction of the carcinogen-activating enzyme, CYP1A1 in human hepatoma HepG2 cells through transcriptional and posttranslational mechanisms [9, 10]. However, the effect of both alkaloids on Cyp1a1 *in vivo* remains to be examined. Therefore, the aim of this study is to examine the effect of harmine and harmaline on TCDD-mediated induction of Cyp1a1 in mice livers and lungs.

2. Material and Methods

2.1. Chemicals and Reagents. Harmine hydrochloride (>98% pure) and 7-ethoxyresorufin (7ER) were purchased from Sigma-Aldrich (St. Louis, MO). TRIzol was obtained from Invitrogen (Carlsbad, CA). Primary antimouse Cyp1a antibody, primary rabbit antimouse actin and goat anti-rabbit IgG peroxidase secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). TCDD, >99% pure, was obtained from Cambridge Isotope Laboratories (Woburn, MA). Harmaline hydrochloride dihydrate (>90% pure) was supplied by ACROS Organics (Morris Plains, NJ). Goat antimouse IgG peroxidase secondary antibody was purchased from R&D Systems (Minneapolis, MN). High-Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were obtained from Applied Biosystems (Foster City, CA). Nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA). Chemiluminescence Western blotting detection reagents were obtained from GE Healthcare Life Sciences (Piscataway, NJ). Primers were purchased from Integrated DNA technologies (IDT, Coralville, IA). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

2.2. Animals. All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Twenty-four male C57BL/6 mice weighing 20–25 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were exposed to 12 h light/dark cycles and were allowed free access to food and water.

2.3. Mice Treatment and Tissues Isolation. Twenty-four male C57BL/6 mice were divided randomly into four groups (n = 6). The first group served as weight-matched controls and received the same volume of vehicle for the indicated time points. The second group were treated intraperitoneally (i.p.) with TCDD (15 μg/kg) dissolved in corn oil at 0 h. The third and fourth groups were treated either with harmine hydrochloride or harmaline hydrochloride (10 mg/kg, i.p.) dissolved in normal saline with sonication and heating for 30 minutes at 40°C and TCDD (15 μg/kg, i.p.). Harmine hydrochloride and harmaline hydrochloride were administered to mice at −4, 0, and +4 h, while TCDD was treated once at 0 h (Table 1). All animals were sacrificed by cervical dislocation after +14 h from TCDD treatment. Liver and lung tissues were excised and divided in two separate parts; one smaller part was kept for total RNA isolation and the larger part was used for microsomal fraction isolation, immediately frozen in liquid nitrogen and stored at −80°C until analysis.

2.4. RNA Isolation and cDNA Synthesis. The total RNA was isolated from frozen tissues using TRIzol reagent, according to the manufacturer's instructions (Invitrogen) and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio, and the first strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μL of 10X reverse transcriptase buffer, 0.8 μL of 25X dNTP mix (100 mM), 2.0 μL of 10X reverse transcriptase random primers, 1.0 μL of MultiScribe reverse transcriptase, and 4.2 μL of nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 s, and finally cooled to 4°C [11].

2.5. Quantification of mRNA Expression by Real-Time Polymerase Chain Reaction (Real-Time PCR). Real-time PCR reactions were performed using an ABI 7500 system (Applied Biosystems, Inc., Foster City, CA) as described previously [12]. The primers used in the current study were chosen from previously published studies [8, 13] and were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Mouse Cyp1a1: forward primer 5′-GGT TAA CCA TGA CCG GGA ACT-3′, reverse primer 5′-TGC CCA AAC CAA AGA GAG TGA-3′, and mouse 18S: forward primer 5′-GTA ACC CGT TGA ACC CCA TT-3′, reverse primer 5′-CCA TCC AAT

Table 1: Representation of different groups of C57BL/6 mice and the dose schedule for each group.

Treatments groups	Time (h)			
	−4	0	+4	+14
Group 1	Saline	Saline + corn oil	Saline	sacrifice
Group 2	Saline	Saline + TCDD (15 μg/kg)	Saline	sacrifice
Group 3	Harmine (10 mg/kg)	Harmine (10 mg/kg) + TCDD (15 μg/kg)	Harmine (10 mg/kg)	sacrifice
Group 4	Harmaline (10 mg/kg)	Harmaline (10 mg/kg) + TCDD (15 μg/kg)	Harmaline (10 mg/kg)	sacrifice

CGG TAG TAG CG-3′. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The real-time PCR data were analyzed using the relative gene expression (ΔΔCt) method, as described in Applied Biosystems User Bulletin No. 2 [14].

2.6. Microsomal Fraction Isolation and Western Blot Analysis. Liver and lung microsomal fractions were isolated as described previously [15]. Briefly, frozen tissues were cut into small pieces and homogenized separately in cold sucrose solution (1 g of tissue in 5 mL of 0.25 M sucrose). Thereafter, the microsomal fractions were separated using differential ultracentrifugation process. The resulted microsomal pellets were reconstituted in ice-cold sucrose solution and stored at −80°C till used. Protein content of each microsomal fraction was determined by Lowry method using bovine serum albumin as a standard [16]. Western blot analysis was performed as described previously [8]. Briefly, microsomal proteins (2 μg) were loaded onto a 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The protein blots were blocked for 24 h at 4°C in blocking buffer (5% skim milk powder, 2% bovine serum albumin, and 0.05% (v/v) Tween 20 in Tris-buffered saline solution (0.15 M sodium chloride, 3 mM potassium chloride, and 25 mM Tris base)). Thereafter, the protein blots were incubated with primary antimouse Cyp1a antibody for 2 h at room temperature or primary rabbit antimouse actin for 24 h at 4°C. Finally, the membranes were incubated with peroxidase-conjugated secondary antibodies for another 1 h, namely, goat antimouse IgG for Cyp1a, or goat anti-rabbit IgG for actin detection. The formed bands were visualized with the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The intensity of protein bands was quantified relative to the signals obtained for actin protein using Java-based image-processing software, ImageJ (W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

2.7. Microsomal Incubation and Determination of Cyp1a1 Enzymatic Activity. Microsomes from Liver (0.25 mg/mL) or lung (0.15 mg/mL) were suspended in incubation buffer containing 3 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH 7.4 at 37°C in a shaking water bath (100 rpm). 7-Ethoxyresorufin was used as a substrate and its final concentration was 2 μM. A pre-equilibration period of 5 min was performed before initiating the reaction with 1 mM NADPH. After an incubation period of 3 min for liver microsomes and 30 min for lung microsomes, the reaction was stopped by adding 1 mL of ice-cold methanol. The reaction was carried out in duplicate using a reaction mixture without NADPH as a blank for each microsome sample. The amount of resorufin in the supernatant of each reaction mixture was determined using the Baxter 96-well fluorescence plate reader using excitation and emission wavelengths of 545 and 575 nm, respectively. Formation of resorufin was linear with incubation time and protein amount. The amount of resorufin was calculated using a standard curve of known resorufin concentrations, and the final amount was calculated by subtracting the amount of resorufin formed in each blank from its corresponding reaction mixture. The final enzymatic activities were expressed as picomole of resorufin formed per minute and per milligram of microsomal proteins.

2.8. Statistical Analysis. All results are presented as mean ± S.E.M., and statistical differences between treatment groups were determined using one way ANOVA followed by the Student-Newman-Keuls post hoc test using SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA).

3. Results

3.1. Effect of Harmine and Harmaline on Cyp1a1 mRNA, Protein, and Enzymatic Activity in C57BL/6 Mice Livers. Our results showed that treatment of mice with TCDD significantly increased the level of Cyp1a1 mRNA by approximately 200,000% compared to the control group. Moreover, treatment with harmine significantly decreased TCDD-mediated induction of Cyp1a1 mRNA expression by 15% (Figure 2(a)).

To examine whether the effect of harmine on hepatic Cyp1a1 mRNA is translated to a relevant effect at protein and enzymatic activity levels, microsomal fractions were isolated from livers and the effect of harmine on Cyp1a protein and Cyp1a1 enzymatic activity was determined using Western blot analysis and 7ER as a substrate, respectively. Our results showed that TCDD induced Cyp1a protein by 250% relative to the control group. On the other hand, treatment of harmine significantly decreased TCDD-mediated induction of Cyp1a protein by 17% (Figure 2(b)). Moreover, TCDD induced Cyp1a1 enzymatic activity by 2000% relative to the control group, whereas harmine treatment significantly decreased TCDD-mediated Cyp1a1-dependent enzymatic activity by 60% (Figure 2(c)).

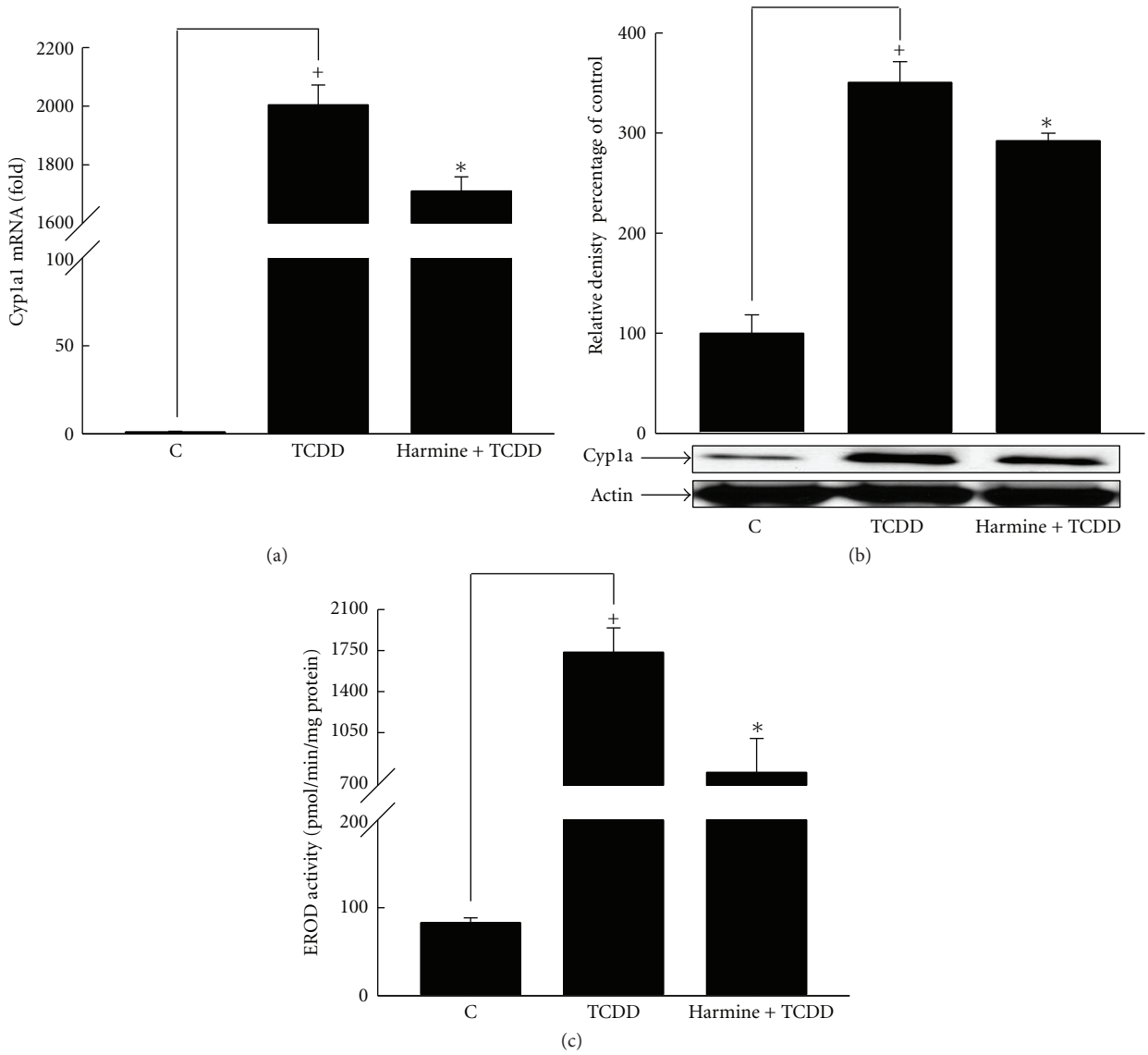


Figure 2: Effect of harmine on TCDD-mediated induction of hepatic Cyp1a1 at mRNA (a), protein (b), and catalytic activity (c) in C57BL/6 mice. Mice were distributed into several groups, receiving the used vehicle (weight-matched control), TCDD, or TCDD and harmine. After 14 h from TCDD treatment, mice were sacrificed, and the livers were isolated. Total RNA was isolated using TRIzol reagent, and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR (a). Furthermore, Cyp1a protein and Cyp1a1 catalytic activity were determined in microsomal fractions using Western blot analysis (b) and 7ER as a substrate (c), respectively. Values represent the mean ± S.E.M. ($n = 6$). (+) $P < 0.05$ compared to control (c), (∗) $P < 0.05$ compared to T.

On the other hand, our results showed that harmaline decreased the level of TCDD-mediated induction of Cyp1a1 mRNA expression by 9%; however, the effect was not significant (Figure 3(a)). Furthermore, harmaline significantly decreased Cyp1a protein by 20% and Cyp1a1 enzymatic activity by 32% using Western blotting and 7ER as a substrate, respectively (Figures 3(b) and 3(c)). Collectively, both alkaloids decreased TCDD-mediated induction of Cyp1a1 in liver tissues; however, harmine showed a more pronounced effect especially at Cyp1a1 enzymatic activity level (Table 2).

3.2. Effect of Harmine and Harmaline on Cyp1a1 mRNA, Protein, and Enzymatic Activity Levels in C57BL/6 Mice Lungs. In an effort to examine whether the effect of harmine and harmaline is not specific to liver tissues, lung tissues were isolated, and the effect of both alkaloids on TCDD-mediated induction of Cyp1a1 was determined at mRNA, protein, and enzymatic activity levels. Our results showed that TCDD significantly induced the lung Cyp1a1 mRNA expression by 43,000% compared to control group, whereas harmine significantly decreased TCDD-mediated induction of lung Cyp1a1 mRNA expression by 44% (Figure 4(a)). Moreover, TCDD caused induction of lung Cyp1a protein and Cyp1a1 enzymatic activity by 440% and 792%, respectively (Figures 4(b) and 4(c)). On the other hand, harmine significantly decreased TCDD-mediated induction of lung Cyp1a protein and enzymatic activity by 43% and 60%, respectively (Figures 4(b) and 4(c)). Taken together, these data demonstrate that

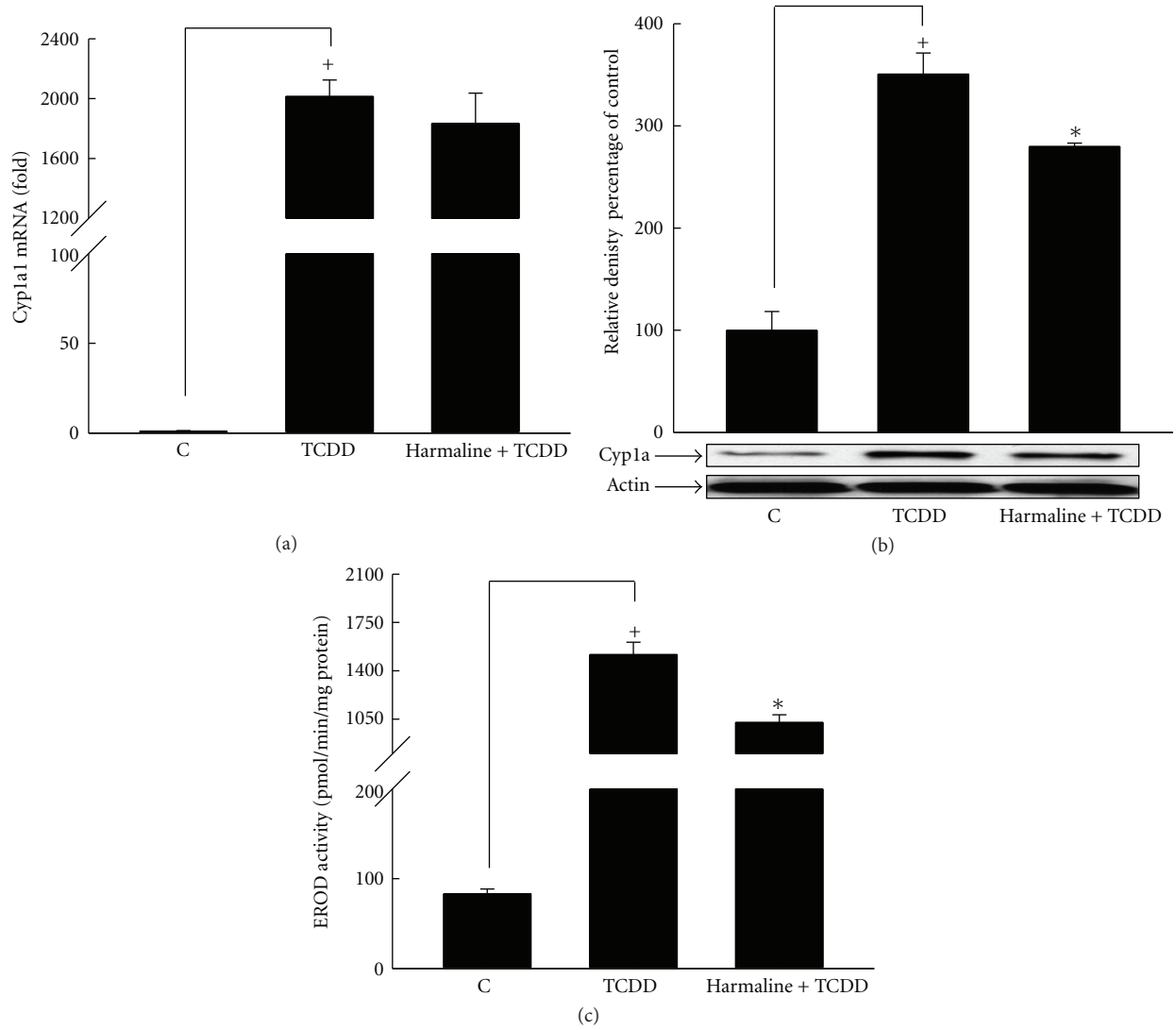


Figure 3: Effect of harmaline on TCDD-mediated induction of hepatic Cyp1a1 at mRNA (a), protein (b), and catalytic activity (c) in C57BL/6 mice. Mice were distributed into several groups, receiving the used vehicle (weight-matched control), TCDD, or TCDD and harmaline. After 14 h from TCDD treatment, mice were sacrificed, and the livers were isolated. Total RNA was isolated using TRIzol reagent, and microsomal proteins were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR (a). Furthermore, Cyp1a protein and Cyp1a1 catalytic activity were determined in microsomal fractions using Western blot analysis (b) and 7ER as a substrate (c), respectively. Values represent the mean ± S.E.M. ($n = 6$). (+) $P < 0.05$ compared to control (c), (∗) $P < 0.05$ compared to T.

the effect of harmine on TCDD-mediated Cyp1a1 is similar in liver and lung tissues especially at Cyp1a1 enzymatic activity level.

Similar to harmine, we tested the effect of harmaline on TCDD-mediated induction of lung Cyp1a1 at mRNA, protein and enzymatic activity levels. Our results demonstrated that harmaline decreased TCDD-mediated induction of lung Cyp1a1 mRNA expression by 34% (Figure 5(a)), whereas, it significantly decreased TCDD-mediated Cyp1a protein and Cyp1a1 enzymatic activity by 44% and 40%, respectively (Figures 5(b) and 5(c)). Taken together, these data demonstrate that the effect of harmaline on TCDD-mediated Cyp1a1 enzymatic activity is almost the same in lung and liver tissues. Similar to liver tissue, harmine showed a more pronounced effect in decreasing TCDD-mediated induction of lung Cyp1a1 enzymatic activity than that observed with harmaline (Table 2).

4. Discussion

The present study demonstrates for the first time that harmine and harmaline significantly decreased the TCDD-mediated induction of the carcinogen-activating enzyme Cyp1a1 in livers and lungs of C57BL/6 mice.

Cancer development is a multistage process that involves several factors. Inherited genetic factors can explain the incidence of 5–15% of most cancers, but environment and lifestyle are the major factors contributing to cancer

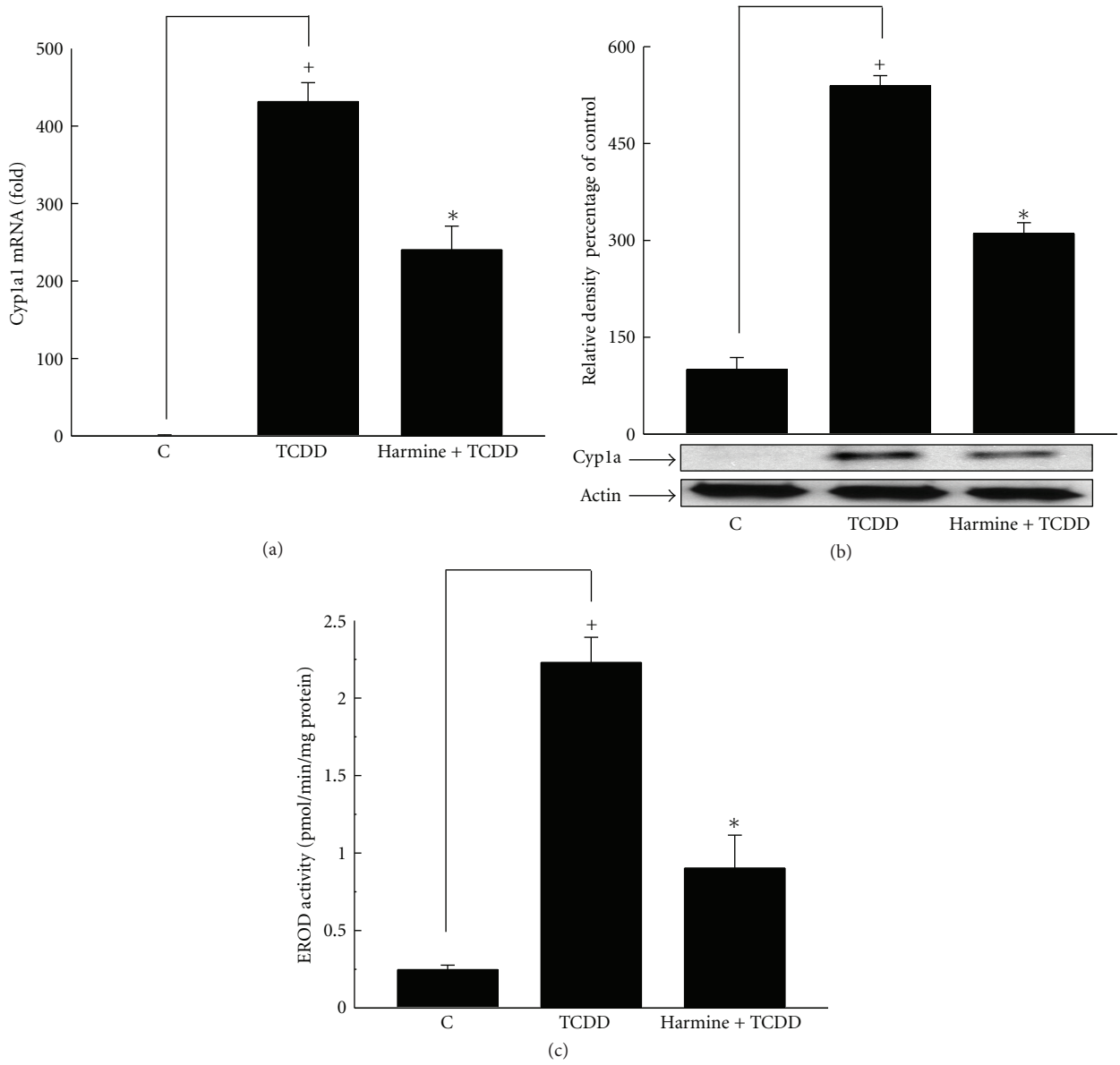


Figure 4: Effect of harmine on TCDD-mediated induction of lung Cyp1a1 at mRNA (a), protein (b), and catalytic activity (c) in C57BL/6 mice. Mice were distributed into several groups, receiving the used vehicle (weight-matched control), TCDD, or TCDD and harmine. After 14 h from TCDD treatment, mice were sacrificed, and the lungs were isolated. Total RNA was isolated using TRIzol reagent, and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR (a). Furthermore, Cyp1a protein and Cyp1a1 catalytic activity were determined in microsomal fractions using Western blot analysis (b) and 7ER as a substrate (c), respectively. Values represent the mean ± S.E.M. ($n = 6$). (+) $P < 0.05$ compared to control (c), (∗) $P < 0.05$ compared to T.

development [17]. TCDD is a widely distributed environmental pollutant that is usually released in the environment from several sources such as waste incinerators, ferrous and non-ferrous metal production, herbicides manufacturing, and power generation [18]. Several accidents and occupational exposures to TCDD demonstrated the role of TCDD in the increased risk of cancer incidence and mortality [1]. TCDD is a metabolically stable AhR ligand, and several adverse effects of TCDD exposure are related to the persistent activation of the AhR signaling pathway. In agreement with this hypothesis are the results of experiments using transgenic mice in which AhR function has been compromised. It has been demonstrated that the TCDD-mediated adverse effects are attenuated in mice possessing disrupted AhR function [2, 19].

Most of the chemical carcinogens in the environment are chemically inert by themselves and require metabolic activation by cytochrome P450 (CYP) enzymes to more reactive metabolites in order to exhibit carcinogenicity in experimental animals and humans [20]. It is well known that AhR

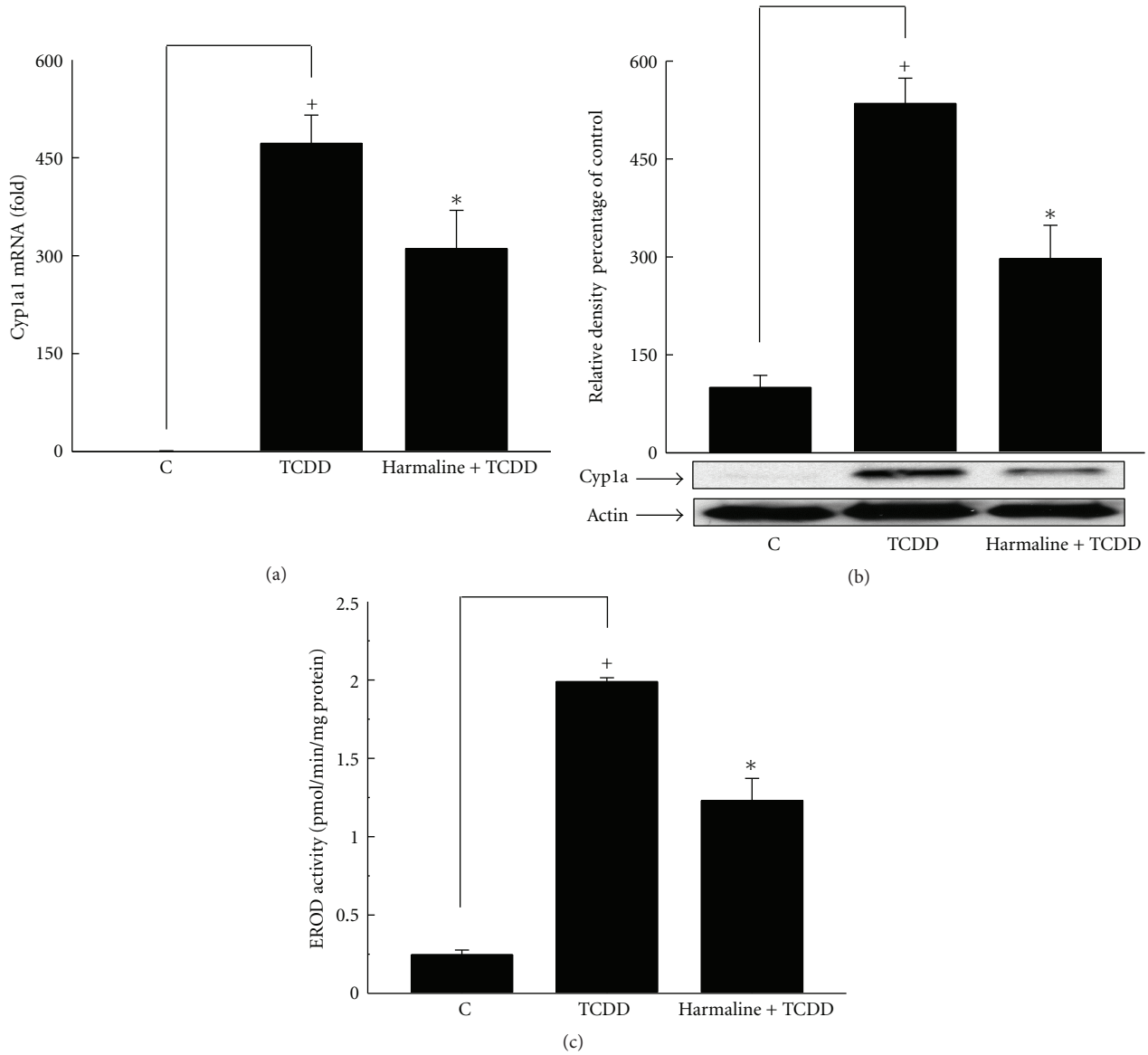


Figure 5: Effect of harmaline on TCDD-mediated induction of lung Cyp1a1 at mRNA (a), protein (b), and catalytic activity (c) in C57BL/6 mice. Mice were distributed into several groups, receiving the used vehicle (weight-matched control), TCDD, or TCDD and harmaline. After 14 h from TCDD treatment, mice were sacrificed, and the lungs were isolated. Total RNA was isolated using TRIzol reagent, and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR (a). Furthermore, Cyp1a protein and Cyp1a1 catalytic activity were determined in microsomal fractions using Western blot analysis (b) and 7ER as a substrate (c), respectively. Values represent the mean ± S.E.M. ($n = 6$). (+) $P < 0.05$ compared to control (c), (∗) $P < 0.05$ compared to T.

regulates numerous CYP, such as CYP1A1, that participate in the metabolic activation of several procarcinogens and their conversion to ultimate carcinogenic forms [2]. Consistent with this hypothesis, it has been previously reported that TCDD induces expression of persistent high level of CYP1A1 that leads to increased metabolism of exogenous and endogenous chemicals, generation of reactive oxygen species and induce oxidative stress that results in increased DNA damage [2, 21]. Moreover, recent reports have highlighted the use of AhR as a target for new chemopreventative agents. In this context, several AhR antagonists have shown promising results against numerous carcinogenic agents. It has been previously reported that the genotoxicity associated with benzo(a)pyrene in mice was inhibited by AhR antagonists such as 3′-methoxy-4′-nitroflavone and resveratrol [22, 23].

Harmine is an aromatic β-carboline compound that is structurally similar to its dihydro-β-carboline analogue, harmaline (Figure 1). Both compounds are found naturally in several plants such as *Peganum harmala*, and they possess several pharmacological effects including antitumor properties. Harmine and harmaline are metabolized in liver and extrahepatic tissues to their main metabolites, harmol, and harmalol, respectively, mainly by CYP2D6 and CYP1A2 (Figure 1) [24]. We have previously demonstrated that both

Table 2: Summary of the effects of harmine and harmaline on TCDD-mediated induction of Cyp1a1 in livers and lungs of C57BL/6 mice.

		Harmine	Harmaline
Liver	TCDD-induced		
	(i) mRNA Cyp1a1	↓ 15%	↓ 9%
	(ii) Protein Cyp1a	↓ 17%	↓ 20%
	(iii) Activity Cyp1a1	↓ 60%	↓ 32%
Lung	TCDD-induced		
	(i) mRNA Cyp1a1	↓ 44%	↓ 34%
	(ii) Protein Cyp1a	↓ 43%	↓ 44%
	(iii) Activity Cyp1a1	↓ 60%	↓ 40%

harmine and harmaline are capable of inhibiting TCDD-mediated induction of Cyp1a1 in murine hepatoma Hepa 1c1c7 cells [8]. Moreover, we demonstrated that both compounds act as AhR antagonists. Finally, we confirmed that harmine and harmaline possess posttranslational modification by which they reduce the CYP1A1 protein stability in human hepatoma HepG2 cells [9, 10]. Therefore, we hypothesized that the effect of harmine and harmaline can be translated *in vivo* using a responsive C57BL/6 mouse strain. Moreover, we tested whether or not the effect of harmine and harmaline can be demonstrated in other extrahepatic tissues, using lung as a representative tissue.

In the current study, we have chosen the C57BL/6 mouse strain as it contains a responsive AhR allele (AhR^b) [2]. Regarding the selection of TCDD dose, it is known that TCDD is a metabolically stable compound, and its half-life has been previously determined in mice to be around 20 days [25]. Several concentrations of TCDD have been examined previously for Cyp1a1 induction and AhR activation in the same mouse strain, and it was demonstrated that 15 μg/kg (i.p.) provides a submaximal saturation/activation of the AhR [26]. Additionally, harmine and harmaline doses have been selected according to their half-lives. It has been demonstrated previously that harmine possesses a short half-life in rodents estimated to be around 20 min, whereas harmaline possesses a relatively longer half-life, around 60 min [27]. Therefore, we thought that multiple doses of both alkaloids would be advantageous. Most importantly, 10 mg/kg body weight for three doses has been selected based on preliminary experiments, in which we could not detect any effect with lower doses. On the other hand, it is well established that the major drawbacks of using these β-carboline alkaloids are their tremorgenic side effects [7]. In our study, slight to moderate tremors have been detected for harmine and harmaline in the first dose with higher effect with harmaline. However, these tremors decreased dramatically in the subsequent doses.

There are several reasons behind the choice of liver tissue in our study. First, we have previously studied the effect of harmine and harmaline using different human and murine hepatoma cells. Second, it is well established that TCDD is concentrated in the body mainly in adipose tissue and liver [28]. TCDD is sequestered in the liver by liver-specific microsomal binding proteins [29]. Third, active AhR is an important factor for developing TCDD-mediated hepatocellular toxicity [30]. Finally, the liver is the place of maximum metabolism and highest amounts of CYP enzymes, with a maximum level of CYP1A1 induction. On the other hand, lung has been selected in our study because it is one of the highly exposed organs to environmental pollutants through smoking and air pollutants. Furthermore, several studies have correlated the induction of CYP1A1 enzymatic activity with the development of lung cancer [4].

In the current study, harmine and harmaline significantly decreased TCDD-mediated Cyp1a1 induction in mice livers and lungs. Harmine showed a greater effect than harmaline in both liver and lung tissues. The differences between the effect of harmine and harmaline can be attributed to two main reasons. First, there is a structural difference between both alkaloids. Harmine has an aromatic planar structure that can enhance its binding ability to AhR, whereas harmaline possesses a coplanar structure. In this context, we have previously demonstrated that harmine efficiently displaced radiolabeled-TCDD in a ligand competition binding assay, whereas harmaline showed a modest effect [9, 10]. Second, harmine and harmaline possess different pharmacokinetic parameters. It was estimated that the ability of harmine to concentrate in lung is more than that observed for harmaline in rodents [27].

5. Conclusion

Harmine and harmaline decrease the TCDD-mediated induction of the carcinogen-activating enzyme Cyp1a1 in C57BL/6 mice livers and lungs. These data provide the first evidence that harmine and harmaline can prevent the adverse effect of dioxins and other AhR ligands *in vivo*.

Abbreviations

AhR: Aryl hydrocarbon receptor
CYP1A1: Cytochrome P450 1A1
7ER: 7-Ethoxyresorufin
TCDD: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.

Conflict of Interests

The authors have declared no conflict of interests.

Acknowledgments

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Research Article

Triterpene Esters and Biological Activities from Edible Fruits of *Manilkara subsericea* (Mart.) Dubard, Sapotaceae

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Manilkara subsericea (Mart.) Dubard (Sapotaceae) is popularly known in Brazil as "guracica." Studies with *Manilkara* spp. indicated the presence of triterpenes, saponins, and flavonoids. Several activities have been attributed to *Manilkara* spp. such as antimicrobial, antiparasitic and antitumoral, which indicates the great biological potential of this genus. In all, 87.19% of the hexanic extract from fruits relative composition were evaluated, in which 72.81% were beta- and alpha-amyrin esters, suggesting that they may be chemical markers for *M. subsericea*. Hexadecanoic acid, hexadecanoic acid ethyl ester, (E)-9-octadecenoic acid ethyl ester, and octadecanoic acid ethyl ester were also identified. Ethanolic crude extracts from leaves, stems, and hexanic extract from fruits exhibited antimicrobial activity against *Staphylococcus aureus* ATCC25923. These extracts had high IC_{50} values against Vero cells, demonstrating weak cytotoxicity. This is the first time, to our knowledge, that beta- and alpha-amyrin caproates and caprylates are described for *Manilkara subsericea*.

1. Introduction

Manilkara subsericea (Mart.) Dubard (Sapotaceae) is popularly known in Brazil as "guracica," "maçaranduba-pequena," "maçaranduba-vermelha," "maçarandubinha," or "paraju" (Figure 1). This species is widely spread on the sandbanks of eastern Brazil, from the states of Espírito Santo to Santa Catarina. *M. subsericea* has edible fruits, being consumed *in natura*, and local population also use its wood for construction [1, 2]. Studies with species from the genus *Manilkara* indicated the presence of triterpenes [3], saponins [4], and flavonoids [5]. Several activities have been attributed to *Manilkara* spp., such as antimicrobial [6, 7], antiparasitic [8, 9], and antitumoral [10], which indicates the great biological potential of the genus.

Figure 1: *Manilkara subsericea* (Mart.) Dubard, Sapotaceae at Restinga de Jurubatiba National Park (RJ, Brazil).

On the present study, we evaluated the antibacterial and cytotoxicity activity of extracts from *Manilkara subsericea*. We also made the phytochemical characterization of the hexanic extract from edible fruits of *M. subsericea*.

2. Materials and Methods

2.1. Plant Material. Aerial parts with fruits of *Manilkara subsericea* (Mart.) Dubard (Sapotaceae) were collected at Restinga de Jurubatiba National Park (RJ, Brazil) in January 2009 and were identified by the botanist Dr. Marcelo Guerra Santos. A voucher specimen of *M. subsericea* was deposited at the herbarium of the Faculdade de Formação de Professores (Universidade do Estado do Rio de Janeiro, Brazil) under the register number RFFP 13.416.

2.2. Preparation of Extracts. Extracts were obtained from fruits, leaves, and stems. The *M. subsericea* freshly fruits (1.14 kg) were crushed and macerated with ethanol (EtOH) 96% (v/v) at room temperature until exhaustion. This ethanolic extract was concentrated in vacuum using a rotary evaporator to obtain ethanolic crude extract from fruits (170 g). This extract was dissolved into 500 mL EtOH/H_2O 90% (v/v) mixture and partitioned with hexane (2 × 600 mL) to obtain, after evaporation of the hexanic portion, 14.0 g of hexanic extract from fruits (FH). The hydroalcoholic portion from this partition was evaporated in vacuum and resuspended in 500 mL distilled water. This aqueous suspension was successively partitioned with ethyl acetate (2 × 600 mL) and butanol (2 × 600 mL), furnishing, after evaporation, 4.5 g of ethyl acetate extract (FEA), and 6.8 g of butanol extract (FB) from fruits. Leaves (1.93 kg) and stems (0.96 kg) were individually dried at 40°C for two days, triturated and macerated with ethanol (EtOH) 96% (v/v) at room temperature until exhaustion. Each ethanolic extract was concentrated in vacuum using a rotary evaporator to obtain 530 g of ethanolic crude extract from leaves (LET) and 169.3 g of ethanolic crude extract from stems (SET).

2.3. Analysis of FH by Gas Chromatography-Mass Spectrometry. The hexanic extractfrom fruits (FH) was analyzed by a GCMS-QP5000 (SHIMADZU) gas chromatograph equipped with a mass spectrometer using electron ionization, according to these experimental conditions: injector temperature, 270°C; detector temperature, 290°C; carrier gas, Helium; flow rate 1 mL/min; split injection with split ratio 1 : 50. The oven temperature was programmed from 60°C (isothermal for 3 min), with an increase of 10°C/min to 290°C, ending with a 59 min isothermal at 290°C. One microliter of the sample, dissolved in $CHCl_3$ (1 : 100 mg/μL), was injected into a ZB-5MS column (i.d. = 0.25 mm, length 30 m, film thickness = 0.25 mm). Mass spectrometry (MS) conditions were ionization voltage, 70 eV and scan rate, 1 scan/s. The identification was performed by comparison of the MS fragmentation pattern of the substances of FH with NIST mass spectra libraries. Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (CG/FID), under same conditions of GC/MS analysis and percentages obtained by FID peak area normalization method.

2.4. Antimicrobial Activity

2.4.1. Microbial Strain. *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC36298, obtained from the culture collections of the Laboratório de Controle Microbiológico, Faculdade de Farmácia, Universidade Federal Fluminense, were used for the antibacterial activity experiments. Overnight cultures were prepared by inoculating approximately 2 mL Tryptic soy broth (TSB; Difco) with 2-3 colonies of each organism. Bacterial strains were cultured overnight at 37°C. Inocula were prepared by diluting overnight cultures in saline to approximately 10^8 CFU/mL.

2.4.2. Diffusion Disk Assay. Qualitative antimicrobial tests were carried out by disk diffusion method [11]. Briefly, a suspension of microorganism (10^8 UFC/mL) was spread on the solid media plates of Muller-Hinton agar (Difco). Disks (6 mm in diameter) were impregnated, until saturation, with the ethanolic crude extracts from leaves and stems, hexanic, ethyl acetate, and butanol extracts from fruits. Then, disks were placed on the inoculated agar. Vancomycin (30 μg) and ampicillin (30 μg) were used as positive reference standards of the test. Disks impregnated with solvents used for solubilization of extracts were used as negative control. The inoculated plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each experiment made in triplicate.

2.4.3. Minimum Inhibitory Concentration (MIC). A microdilution technique using 96 well micro-plates, as described by Eloff [12] was used to obtain MIC values of extracts against *S. aureus*. The method comprised of filling all the wells of a 96 well microplate with 100 μL of Muller-Hinton broth (Vetec). Triplicates (100 μL) of the samples (ethanolic crude extracts from leaves and stems, hexanic, ethyl acetate, and butanol extracts from fruits) at starting concentrations of 2 mg/mL in DMSO were introduced into the first well.

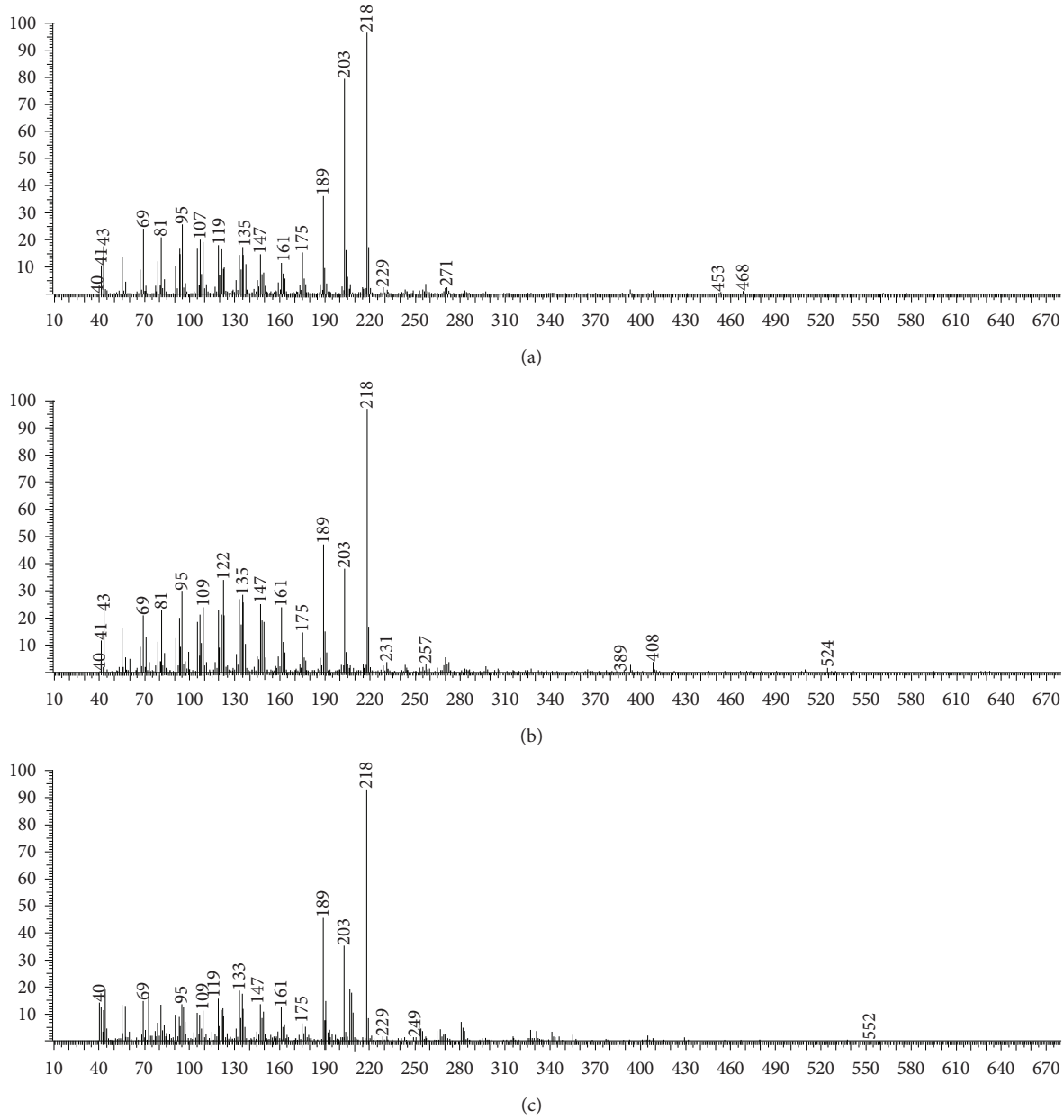


Figure 2: Fragmentation pattern for Δ12-oleane/Δ12-ursane series. (a) Beta-amyrin acetate. (b) Alpha-amyrin caproate. (c) Alpha-amyrin caprylate.

Serial doubling dilutions were then performed, rejecting 100 μL from each well and adding a mixture of test microorganism (100 μL) having an inoculum size of approximately 1×10^6 CFU/mL. The final concentrations per well were 500, 250, 125, 64, and 32 μg/mL. The microplates were sealed and incubated at 37°C for 24 h. After incubation, 50 μL of a 2.5% solution of the biological indicator TTC (Triphenyl Tetrazolium Chloride) solution was added, and the plates were incubated again for 2 h to visualize growth inhibition. The lowest concentration of the sample that inhibited the bacterial growth (colourless) after incubation was taken as the MIC. Vancomycin and DMSO were used as positive and negative controls, respectively.

2.5. Cytotoxic Assay

2.5.1. Mammalian Vero Cell Line Culture Condition. Vero cell line (ATCC CCL-81) was cultured at 37°C, 5% CO_2 in DMEM medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 0.1 mg/mL streptomycin (GIBCO), and 100 U/mL penicillin (GIBCO).

2.5.2. Cell Viability by LDH Assay. To evaluate the toxicity of extracts, Vero cell line was incubated with samples (ethanolic crude extracts from leaves and stems, hexanic, ethyl acetate, and butanol extracts from fruits) for 24 hours and cell viability measured using LDH assay (Doles). In brief,

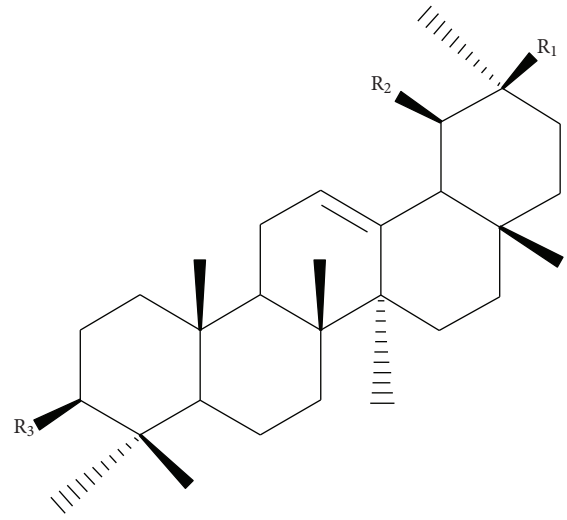
5×10^4 cells/well were seeded in a 96-well microplate and incubated for 24 hours to attach. In the following day, cells were washed with PBS, and fresh media DMEM without serum were replaced containing the plant extract at different concentrations (500–31.25 μg/mL). Plates were incubated for further 24 hours and LDH activity measured by colorimetric assay using spectrophotometer (micronal-B582) at 510 nm. As control for maximum LDH release, cells were treated with 0.1% triton-X100 in DMEM medium for 10 min before running the assay. To determine the normal LDH release, cells were cultured in serum-free medium in presence of DMSO. Cell viability was determined using absorbance of treated cells at DMSO as a reference for 100% viability (absorbance of extract-treated cells × 100/absorbance of DMSO-treated cells).

2.6. Statistical Analysis. For antibacterial assay, statistical analysis was performed by ANOVA (one-way Anova) with 95% confidence interval, using the GraphPad Prism 5.0 software package. Differences were considered significant when P values were ≤0.05. Vero cell viability (%) was determined by averaging three repeated experiments and IC_{50}, representing the concentration at which cell viability was reduced by 50%, was calculated by linear regression using the GraphPad Prism 5.0 software package.

3. Results and Discussion

Analysis of the chromatogram obtained from the hexanic extract from fruits of *M. subsericea* indicated the elution of 20 compounds. Substances with retention time (min) of 16.84, 16.99, 18.71, and 18.94 corresponded, respectively, to hexadecanoic acid (A) (5.41%), hexadecanoic acid ethyl ester (B) (3.57%), (E)-9-octadecenoic acid ethyl ester (C) (3.95%), and octadecanoic acid ethyl ester (D) (1.45%). These compounds were identified by comparison of their MS fragmentation pattern with NIST mass spectra.

On another study, we described the obtainment and identification of a mixture containing beta-amyrin acetate and alpha-amyrin acetate from edible fruits of this species [13]. Thus, comparison of the previously fragmentation pattern obtained for these substances confirmed the major substances, with retention time (min) of 34.63 and 36.15, as beta-amyrin acetate (E) (10.27%) and alpha-amyrin acetate (F) (42.34%), respectively. The substances with retention time (min) of 53.31/56.55 and 71.70/76.99 also showed a typical fragmentation pattern for pairs of triterpenes from the Δ12-oleane/Δ12-ursane series. The characteristic peaks at m/z 218 (base peak), 203 and 189 due to Retro-Dials-Alder fragmentation [14] were observed for these substances. Beta-amyrin type triterpenes presented peak at m/z 203 higher than peak at m/z 189, while alpha-amyrin type triterpenes showed an equal abundance (Figure 2). According to Oyo-Ita et al. [15], the amyrin caproates have molecular ion peak (M^+) at m/z 524, followed by loss of CH_3 or the acid moiety to m/z 509 and 408, respectively. Thus, the substances with retention time (min) of 53.31 and 56.55 could be identified as beta-amyrin caproate (G) (5.46%) and alpha-amyrin caproate (H) (7.26%). The mass fragment at m/z 408, due to the loss of 144 (caprylic acid) mass unit from the molecular ion peak (M^+) at m/z 552 was in accordance with literature data [16, 17] and suggested substances with retention time (min) of 71.70 and 76.99 as beta-amyrin caprylate (I) (2.44%) and alpha-amyrin caprylate (J) (5.04%), respectively.



(E) R_1=CH_3, R_2=H, R_3=AcO
(F) R_1=H, R_2=CH_3, R_3=AcO
(G) R_1=CH_3, R_2=H, R_3=$CH_3(CH_2)_4COO$
(H) R_1=H, R_2=CH_3, R_3=$CH_3(CH_2)_4COO$
(I) R_1=CH_3, R_2=H, R_3=$CH_3(CH_2)_6COO$
(J) R_1=H, R_2=CH_3, R_3=$CH_3(CH_2)_6COO$

Figure 3: Chemical structures of the amyrin esters: beta-amyrin acetate (E), alpha-amyrin acetate (F), beta-amyrin caproate (G), alpha-amyrin caproate (H), beta-amyrin caprylate (I), and alpha-amyrin caprylate (J) from the hexanic extract from fruits of *Manilkara subsericea*.

It is interesting for the chemotaxonomic consideration that several studies carried out for *Manilkara* species, such as *Mimusops littoralis* Kurz (*Manilkara littoralis* (Kurz) Dubard) [18], *Mimusops manilkara* G.Don (*Manilkara kauki* (L.) Dubard) (Misra and Mitra, 1969), and *Mimusops hexandra* Roxb *Manilkara hexandra* (Roxb.) Dubard) [19] indicated the presence of triterpene esters. Thus, the chemical substances identified on the hexanic extract from fruits of *M. subsericea* (Figure 3) are in accordance with the chemical markers of the genus *Manilkara*.

The identified substances corresponded to 87.19% of the total relative composition of the hexanic extract from fruits of *M. subsericea*. The individual amounts of each substance are illustrated in Figure 4. Furthermore, to our knowledge, this is the first time that the beta- and alpha-amyrin caproates and caprylates are described for the *Manilkara subsericea* species.

Antibacterial assay was performed against *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC36298. There were significant differences ($P < 0.05$) in the antibacterial activity of ethanolic crude extract from leaves (7 ± 0.28 mm), ethanolic crude extract from stems (8 ± 0 mm), and hexanic

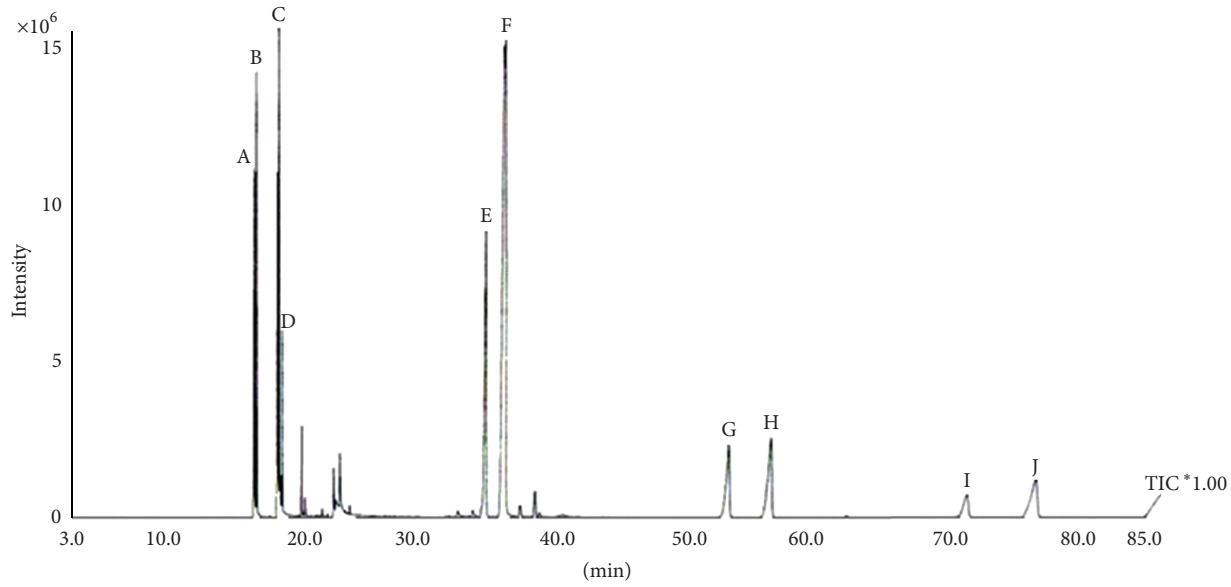


Figure 4: CG-FID chromatogram of the hexanic extract from fruits of *Manilkara subsericea*, Sapotaceae. A: hexadecanoic acid (5.41%), B: hexadecanoic acid ethyl ester (3.57%), C: (E)-9-octadecenoic acid ethyl ester (3.95%), D: octadecanoic acid ethyl ester (1.45%), E: beta-amyrin acetate (10.27%), F: alpha-amyrin acetate (42.34%), G: beta-amyrin caproate (5.46%), H: alpha-amyrin caproate (7.26%), I: beta-amyrin caprylate (2.44%), J: alpha-amyrin caprylate (5.04%). For analysis conditions see Materials and Methods, Section 2.3.

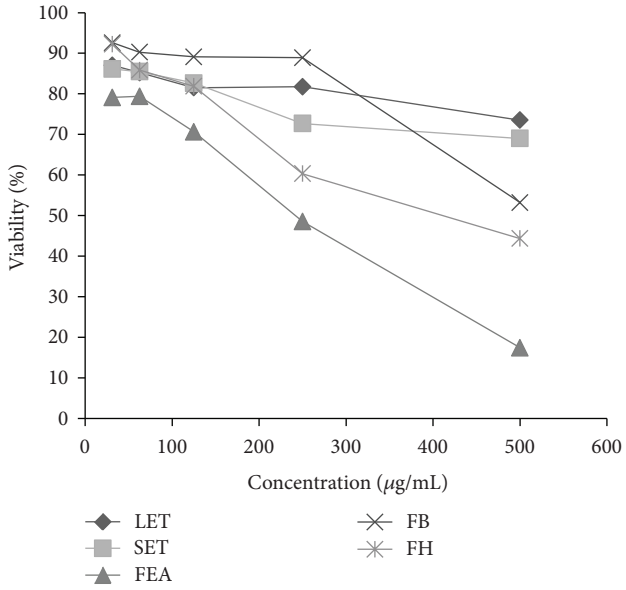


Figure 5: Vero cell viability in the presence of extracts for 24 h measured by LDH assay. LET: ethanolic crude extract from leaves, SET: ethanolic crude extract from stems, FEA: ethyl acetate extract from fruits, FB: butanol extract from fruits, FH: hexanic extract from fruits.

Table 1: Means of the inhibition halos (mm ± SD) for *Staphylococcus aureus* and *Escherichia coli* tested with extracts (100 mg/mL) from *Manilkara subsericea*. LET: ethanolic crude extract from leaves, SET: ethanolic crude extract from stems, FH: hexanic extract from fruits, FEA: ethyl acetate extract from fruits, FB: butanol extract from fruits. Vanc: vancomycin (30 μg), ampicillin: (30 μg).

	Inhibition halo (mm) ± SD	
	Staphylococcus aureus	*Escherichia coli*
LET	7 ± 0.28^{b}	0^{b}
SET	8 ± 0^{c}	0^{b}
FH	6 ± 0^{d}	0^{b}
FEA	0^{e}	0^{b}
FB	0^{e}	0^{b}
Vanc	18 ± 0.28^{a}	Not tested
Amp	Not tested	32 ± 7^{a}

Means in the same column with different superscripts are significantly different ($P < 0.05$).

extract from fruits (6 ± 0 mm), which were considered active against *S. aureus*. (Table 1). Ethyl acetate and butanol extracts from fruits did not inhibit the *S. aureus* growth. All extracts were considered inactive against *E. coli*. (Table 1).

The extracts that exhibited antimicrobial activity during the disk diffusion method were evaluated for their *Minimum inhibitory concentration* (MIC). All extracts tested inhibited the bacterial growth of the *S. aureus* strain with MIC of 250 μg/mL. Terpenoids are active against bacteria but the mechanism of action of terpenes is not fully understood, although it is speculated to involve membrane disruption by lipophilic compounds [20]. The isomeric mixture of beta-amyrin and alpha-amyrin is known by its antimicrobial activity [21], and 72.81% of the relative amount of the hexanic extract from fruits is constituted by esters of these substances. Beta- and alpha- amyrin acetates are also known by their anti-inflammatory activity and also inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) in Raji cells [22]. Furthermore, according to Hichri et al. [23], the triterpene

beta-amyrin acetate was able to inhibit the bacterial growth of the *Staphylococcus aureus* ATCC25923 reference strain at 90 μg/mL. Thus, our results suggest that the antibacterial activity found in the hexanic extract from fruits may be modulated by the beta- and alpha- amyrin esters identified.

All tested extracts demonstrated weak cytotoxic effects on the mammalian Vero cells. The Cell viability on treatment with hexanic and ethyl acetate extracts from fruits was 69.66% and 56.07% in concentration of 250 μg/mL, respectively (Figure 5). Ethanolic crude extract from leaves (1658 μg/mL; 1164–2525) had highest IC_{50} value, followed by ethanolic crude extract from stems (1112 μg/mL; 757–2525), butanol extract from fruits (683.4 μg/mL; 451–2200), hexanic extract from fruits (482.6 μg/mL; 385–677), and ethyl acetate extract from fruits (307.6 μg/mL; 276–346).

The triterpene beta-amyrin acetate was reported to have cytotoxicity against A2780 ovarian cancer cell line with IC_{50} of 12.1 μg/mL [24]. This compound was not considered active against A549, SK-OV-3, SK-MEL-2, XF498, and HCT15 cancer cell lines [25]. Moreover, some beta- and alpha-amyrin esters were able to induce cell apoptosis in HL-60 leukemia cells [26].

4. Conclusions

Although *Manilkara subsericea* fruits are used as food, to our knowledge, only one article regarding its phytochemicals and biological activities was published [13]. The present study describes the identification of a high percentage of substances from the hexanic extract from edible fruits of *Manilkara subsericea*, in which beta- and alpha- amyrin caproates and caprylates are reported for the first time for this species. Our results suggest that this hexane extract from fruits and ethanolic crude extract from leaves and stems presented antimicrobial activity against *S. aureus* ATCC25923. In addition, these extracts had low cytotoxicity on Vero cells, in the same concentration which inhibited *S. aureus* growth. Several biological studies are carried out for mixtures of beta- and alpha- amyrin type triterpenes [21, 27, 28], since their separation by conventional chromatographic methods is quite difficult [29].

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Research Article

Evaluation of the Toxicity of *Pradosia huberi* Extract during the Preimplantation in Wistar Rats

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The treatment during the embryonic preimplantation phase of Wistar rats with the *Pradosia huberi* extract did not interfere with the water and feed consumption, as well as upon the body-weight gain. However, it has expressed a decrease of the uterine implant number, followed by the preimplantation losses at all applied doses (1.22, 6.1, and 30.5 mg/kg), and the number of embryonic resorptions in the two highest doses (6.1 and 30.5 mg/kg). After the organ weighing (hypophysis, ovaries, and uterus), only the relative weight of the hypophysis was raised at the different doses (1.22, 6.1, and 30.5 mg/kg). It was concluded that the hydroalcoholic extract of *Pradosia huberi* compromises the reproductive ability during the embryonic preimplantation phase, suggesting a possible toxic effect upon the reproductive system of Wistar rats.

1. Introduction

The occurrence of biologically adverse effects upon the female reproductive system which affect the fertility and the reproductive ability can be expressed as changing the production and transport of gametes, in the reproductive cycle, endocrine system, sexual behavior, gestation, parturition and lactation, or alterations in other functions which depend of the integrity of the reproductive system [1, 2].

Numerous plants are known by presenting potential toxic effects upon the mammals' reproduction such as previous placenta, postterm pregnancy, premature uterine contractions, and even abortion [3, 4]. *Pradosia huberi* Ducke (Sapotaceae) is popularly known as "casca doce" or "pau doce" in the folk medicine [5] and it is widely used in the treatment of ulcers and gastritis due its anti-inflammatory properties upon the gastrointestinal system [6]. Phytochemical studies of the *P. huberi* bark revealed the presence of the following flavonoids: 2.3-dihydromyricetin 3-α-L-rhamnoside, astilbin, engelitin and dihydromyricetin-2.3 [7], and 2.3-dihydromyricetin-3-O-rhamnoside acetate and 2.3-dihydromyricetin-7-O-rhaminoside acetate [8]. Despite flavonoids having a wide representation in the Plantae kingdom associated to their broad therapeutic potential [9, 10], numerous surveys have revealed and alerted the population about their possible systemic toxicological effects, including the reproduction [11]. The glabridin—isolated flavonoid of *G. glabra*—showed antiestrogen activity, compromising the reproductive hormone physiology [12]. Besides that, the *Ginkgo biloba* extract which also has flavonoids in its composition induced apoptosis in the embryonic stem cells of rats [13].

Considering the vast use of the studied plant associated to the presence of substances with possible deleterious activity upon the reproductive process, this study aimed to evaluate the effects of hydroalcoholic extract of *P. huberi* during the uterine preimplantation time of Wistar rats.

2. Material and Methods

2.1. Animals and Experimental Groups. Adult Wistar rats (*Rattus norvegicus* Berkenhout, 1769) were used, aged 90 days and weighing approximately 200–250 g, and were provided by the vivarium of the Biotechnology Center of UFPB. The rats were housed in polyethylene cages and kept under controlled temperature conditions (21 ± 2°C), obeying to a light-dark cycle of 12 hours each, without medication and with free access to pelleted feed of Purina and drinking water during the whole experimental time.

This study was approved by the Ethics Committees on Animal Research of the CB/UFPB, number 0205/10.

The animals were randomly distributed into four experimental groups, three being treated and one a control, containing 16 females each.

2.2. Botanical Material and Extract Preparation. The bark of *Pradosia huberi* was collected in the city of Porto Grande, AP, Braul, where a voucher specimen number 012519 was deposited in the Herbarium of Amapá (HAMAB) of the Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá-IEPA (Institute of Scientific and Technological Research of Amapá). The plant material was prepared in the Chemistry Lab of IEPA. In order to obtain the hydroalcoholic extract (HAE) the plant material was held under controlled-temperature kiln drying (38°C), with EtOH mixture (70%): H_2O at room temperature (25–30°C) for 72 hours. Later, the obtained mixture was filtered and concentrated under vacuum in a rotary evaporator in a temperature of 50°C until obtaining the hydroalcoholic extract [14]. Then the HAE was diluted in distilled water to obtain solutions in appropriate concentrations for correct administration of doses used in this study (1.22, 6.1, and 30.5 mg/kg) [15], which were based on prior studies that have evaluated the antiulcerogenic activity of *P. huberi* in rats and mice [6].

2.3. Evaluation of the Reproductive Toxicity

2.3.1. Mating. The mating system was polygamous, in which three maiden females were placed in the box of each male, where they were kept during the dark cycle. Next morning, the animals were separated and the verification of pregnancy was performed by the presence of sperm in a vaginal smear, carried out daily between 7 and 8:0 AM and analyzed in a 10x optical microscope. Twenty-four hours after the observation of the presence of sperm in the vaginal smear was set as the first day of pregnancy. The mating was repeated until a sufficient number of progenitor cells for performing the experiments was obtained [16, 17].

2.3.2. Exposure during the Preimplantation and Tests of Reproductive Toxicity. The pregnant females ($n = 64$) were treated with HAE of *P. huberi* from the first to the seventh day of pregnancy, comprising the phase of embryonic preimplantation in rats which occurs from five to six days after fertilization [18]. During this period, the animals were monitored for the analysis of the maternal systemic toxicity, such as irritability, seizures, ataxia, sedation, diarrhea, cyanosis, hair loss, and deaths, besides the water and ration consumption and weight gain [19].

On the eighth day of pregnancy, females were sacrificed by an excessive dose of ketamine (König) and the reproductive variables were investigated through the analysis of the following parameters: number of corpora lutea on ovaries; number of uterine implants; number of resorptions; gestation index ((number of pregnant females/number of inseminated females) × 100) and the index of preimplantation losses ((number of corpora lutea − number of implants/ number of corpora lutea) × 100) [1, 20–22].

In addition, the absolute and relative weight ((organ weight/body weight) × 100) of the hypophysis, left and right ovaries, and the pregnant uterus were recorded [14, 23], and the dosage of serum levels of progesterone was performed by the enzyme reaction method by microparticles (automatic biochemical analyzer-Axsym).

2.3.3. Statistical Analysis. The data were analyzed by the variance analysis (ANOVA) and the differences among the groups were determined by Tukey test. The variables listed as index were analyzed by chi-square test, excepting the relative weight of the organs which was also analyzed using ANOVA followed by Tukey test.

All data obtained were statistically analyzed by the program GraphPad Prism, version 4.0 (GraphPad Software Inc., San Diego, CA, USA). The difference among the groups was considered as significant for $P < 0.05$.

3. Results

3.1. Exposure during the Preimplantation and Tests of Reproductive Toxicity

3.1.1. Systemic Signs of Maternal Toxicity. During the seven days of pregnancy, a period which corresponds to the preimplantation, the rats daily treated with the hydroalcoholic extract of *P. huberi* showed no symptomatic signs of systemic toxicity, such as irritability, seizures, ataxia, sedation, diarrhea, cyanosis, and hair loss; also no deaths were reported, as well as no change of water consumption (Figure 1) and food (Figure 2) swallowed by females treated when compared to the control groups. Similarly, the weight development of animals exposed to the extract from the first to the seventh day of pregnancy showed a normal growth pattern among the groups treated with doses of 1.22, 6.1, and 30.5 mg/kg when compared to the control group (Figure 3).

3.1.2. Reproductive Variables. As shown in Table 1, the daily exposure to the extract significantly decreased the number

Table 1: Reproductive variables of rats treated with the hydroalcoholic extract of *P. huberi* during the preimplantation.

Variables	Hydroalcoholic extract of *Pradosia huberi*			
	Control	1.22 mg/kg	6.1 mg/kg	30.5 mg/kg
Number of inseminated rats	16	16	15	16
Number of pregnant rats	15	11	12	13
Number of corpora lutea	6.4 ± 0.2	5.5 ± 0.4	5.8 ± 0.3	6.9 ± 0.4
Number of uterine implants	11.0 ± 0.1	6.6 ± 1.3*	8.2 ± 1.4*	8.7 ± 1.4*
Number of resorptions	0.1 ± 0.1	0.3 ± 0.2	1.0 ± 0.5*	1.5 ± 0.6*
Gestation index (%)	93.7	68.7	80.0	81.2
Preimplantation loss (%)	14.1	39.2*	28.7*	33.3*

Values are mean ± S.E.M. *$P < 0.05$ versus control.
Gestation index = (number of pregnant females/number of inseminated females) × 100.
Index of preimplantation loss = (number of corpora lutea − number of implants/number of corpora lutea) × 100.

Table 2: Body weight and absolute and relative weights of rats treated with the hydroalcoholic extract of *P. huberi* during the preimplantation.

Variables	Hydroalcoholic extract of *Pradosia huberi*			
	Control ($n = 16$)	1.22 mg/kg ($n = 16$)	6.1 mg/kg ($n = 15$)	30.5 mg/kg ($n = 16$)
Body weight (g)	252.3 ± 7.2	242.1 ± 9.7	236.8 ± 7.8	248.3 ± 5.5
Absolute weight (mg)				
Hypophysis	10.1 ± 0.1	11.3 ± 0.2	11.6 ± 0.3	11.9 ± 0.3
Right ovary	34.6 ± 0.6	31.7 ± 0.8	32.2 ± 1.1	31.1 ± 0.8
Left ovary	32.7 ± 0.8	31.4 ± 0.8	30.8 ± 0.9	32.1 ± 0.8
Uterus	783.6 ± 35.7	693.2 ± 39.0	690.0 ± 42.1	724.9 ± 58.9
Relative weight (%)				
Hypophysis	0.003 ± 0.0001	0.004 ± 0.0001*	0.005 ± 0.0002*	0.005 ± 0.0002*
Right ovary	0.01 ± 0.0004	0.01 ± 0.0004	0.01 ± 0.0007	0.01 ± 0.0004
Left ovary	0.01 ± 0.0006	0.01 ± 0.0006	0.01 ± 0.0006	0.01 ± 0.0004
Uterus	0.30 ± 0.002	0.24 ± 0.002	0.25 ± 0.003	0.26 ± 0.002

Values are mean ± S.E.M. *$P < 0.05$ versus control.

of uterine implantations, being followed by the increase of the preimplantation rates in the three applied doses (1.22, 6.1, and 30.5 mg/kg) and the index number of resorptions for the animals which received the two highest doses (6.1 and 30.5 mg/kg). The other variables were not changed.

3.1.3. Body Weight and Relative and Absolute Weights of Organs. According to the data presented in Table 2, the rats treated during preimplantation showed no significant difference in their body weight during sacrifice time, nor in the absolute weight of organs (hypophysis, ovaries, and uterus); however, the relative weight of the hypophysis gland expressed statistically significant increase in the three applied doses (1.22, 6.1, and 30.5 mg/kg) when compared to the control group. The other organs (ovaries and uterus) did not express changes in their relative weights.

3.1.4. Hormone Dosage. The concentration of serum progesterone did not suffer significant change in female rats that received the extract in different doses: 1.22 mg/kg (34.4 ± 2.5), 6.1 mg/kg (33.4 ± 2.5), and 30.5 mg/kg (34.7 ± 2.5) when compared to the control group (39.0 ± 0.9) during the preimplantation (Figure 4).

4. Discussion

This study investigated the possible toxicological effects of hydroalcoholic extract of *P. huberi* upon the reproductive system of Wistar rats during the embryonic preimplantation time, since this is a very delicate stage which requires perfect coordination of physiological events for the maintenance and the success of pregnancy [24, 25]. In addition, the medicinal plants are a source of active compounds able to exert therapeutic and toxic activities [26]. For example, *P. huberi* also has gastroprotective activity [6] but revealed high toxicity, causing behavioral changes, decreased weight of organs, and death in rodents [27].

Signs of systemic toxicity are defined from the reduction of body weight of animals, food and water consumption, and the outbreak of physical and behavioral changes, since the modification of such parameters reflects the toxic potential of a substance upon the organ systems, including the reproductive system [28]. Because this under hormonal influence

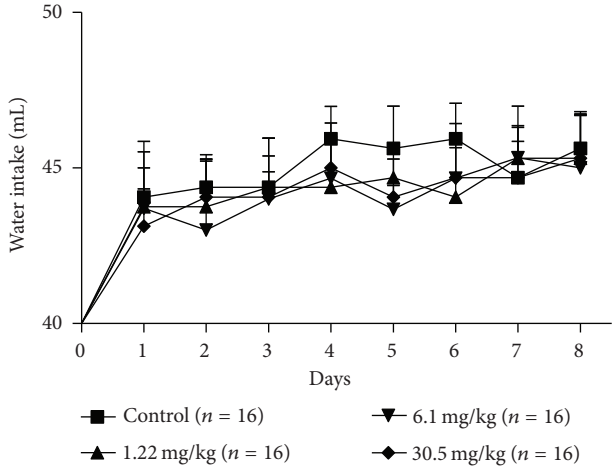

Figure 1: Water intake of female rats treated with the hydroalcoholic extract of *P. huberi*, during the preimplantation. The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ versus control group. ANOVA followed by Tukey test. The *n* represents the number of progenitors.

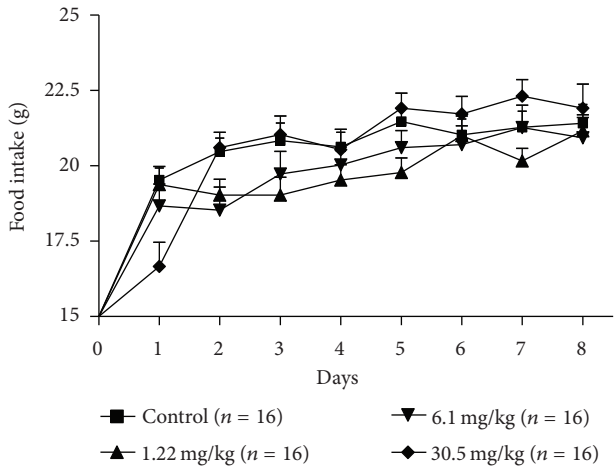

Figure 2: Food intake of female rats treated with the hydroalcoholic extract of *P. huberi*, during the preimplantation. The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ versus control group. ANOVA followed by Tukey test. The *n* represents the number of progenitors.

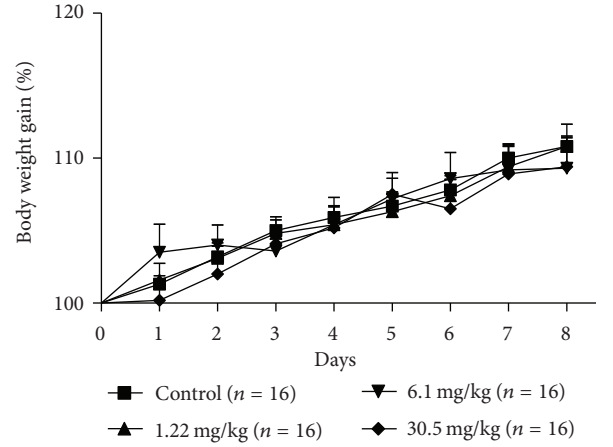

Figure 3: Body weight gain of female rats treated with the hydroalcoholic extract of *P. huberi* during the preimplantation. The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ versus control group. ANOVA followed by Tukey test. The *n* represents the number of progenitors.

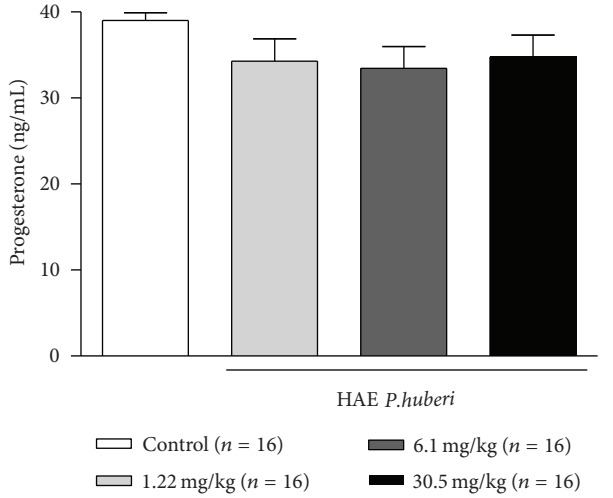

Figure 4: Progesterone dosage of female rats treated with the hydroalcoholic extract of *P. huberi*, during the preimplantation. The values are expressed as mean ± S.E.M. $^{*}P < 0.05$ versus control group. ANOVA followed by Tukey test. The *n* represents the number of progenitors.

of estrogen and progesterone interferes with the water and food ingestion, energy balance, fluid retention, and fat deposition by the female organism [29].

In this study the animals treated with the hydroalcoholic extract of *P. huberi* in different doses (1.22, 6.1, and 30.5 mg/kg) during the preimplantation did not show symptomatic systemic signs of maternal toxicity, suggesting the nonphysiological commitment of the central and autonomous nervous system [30].

The analysis of the reproductive variables goes through the ovaries' evaluation, which allows the investigation, besides its hormonal function, important reproductive indices by counting the number of corpora lutea which has direct relation to the amount of oocytes released during ovulation, allowing this way an analysis of the actual number of fertilized oocytes, besides thoughtful observation of the uterus in order to count the implantation sites and resorptions [1, 31].

The daily treatment with *P. huberi* extract during the preimplantation presented evidence of reproductive toxicity, since the preimplantation losses were significantly increased, as indicated by data which were monitored by the decreasing of uterine implants in rats treated with different doses of extract (1.22, 6.1, and 30.5 mg/kg) and an increase in the number of resorptions for those animals which received the two highest doses (6.1 and 30.5 mg/kg). Such results

suggest embryotoxicity, thus jeopardizing the reproductive capacity of females treated with the extract, to an order that caused loss of embryos at the development stage in the fallopian tubes [32].

In a similar study, rats treated with the hydroalcoholic extract of *Baccharis trimera* and flavonoids isolated from this plant have expressed significant reduction in the implantation and an increase in the preimplantation loss rate, suggesting a relaxing effect on the smooth muscles of the fallopian tube, with interference in the transport of the blastocyst up to the uterus [33].

In addition, the investigation of the toxic potential of a substance upon the reproductive system must include the dosage of serum levels of hormones involved in the homeostasis of the hypothalamic axis, hypophysis, and gonads [1]. First, the hypothalamus produces and secretes the GnRH which stimulates the hypophysis to release the FSH and LH; these, in turn, have a direct action upon the ovaries promoting follicular development and release of the oocyte [34].

The animals treated with the hydroalcoholic extract of *P. huberi* had the relative weight of their hypophysis increased, when compared to the control group, suggesting a possible effect of the extract on the hypophysis function. Several studies have demonstrated the adverse effect of substances in the hypothalamic, pituitary, and gonadal axis. For example, the treatment of Wistar rats with bisphenol A (insecticide) in high doses increased the weight of the hypophysis and elevated the prolactin levels compared to castrated rats [35–37]. However, the increased weight of the hypophysis caused by using the extract of *P. huberi* did not present direct correlation with hormone production, because the serum levels of progesterone and the number of corpora lutea were not changed, demonstrating a non-antiestrogenic effect, at least regarding the ovarian production of progesterone. Through ovarian weight and number of corpora lutea it is possible to deduct indirectly the hormonal conditions related to maternal progesterone, because the ovary weight is directly proportional to the number of corpora lutea which is the largest structure found in this organ [38].

Therefore, the analysis of the data obtained in this study indicates that the maternal hormone homeostasis, essential for embryonic development, of rats treated with the extract of *P. huberi* was not changed by inadequate levels of progesterone, whose reduction would undermine the viability of the embryo, by preventing the endometrium from being prepared to ensure the maintenance of pregnancy. However, the possibility of the extract of *P. huberi* being an endocrine disrupter cannot be ruled out, due to its interference of the embryonic preimplantation in Wistar rats, as previously evidenced by a reduction in the number of uterine implantations and increased resorptions. However, more studies should be carried out in order to monitor hormone production during the pregnancy period, because the success of a pregnancy requires a perfect physiological harmony among the hypothalamic, pituitary, and gonadal functions to ensure the transportation and integrity of the gamete and the zygote, and to enable the success of fertilization and embryo survival [39].

5. Conclusions

Based upon the obtained results and under adopted experimental conditions, the treatment of Wistar rats with the hydroalcoholic extract of *P. huberi* during the preimplantation induced suggestive reproductive changes of toxicity on the reproductive system of Wistar rats. However, further studies are necessary to elucidate the mechanism of action behind the observed effects.

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Research Article

Antihyperglycemic Effect of *Ginkgo biloba* Extract in Streptozotocin-Induced Diabetes in Rats

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The *Ginkgo biloba* extract (GBE) has been reported to have a wide range of health benefits in traditional Chinese medicine. The aim of this study was to evaluate the antihyperglycemic effects of GBE on streptozotocin- (STZ-) induced diabetes in rats. Diabetes was induced in male *Wistar* rats by the administration of STZ (60 mg/kg b.w.) intraperitoneally. GBE (100, 200, and 300 mg/kg b.w.) was administered orally once a day for a period of 30 days. Body weight and blood glucose levels were determined in different experimental days. Serum lipid profile and antioxidant enzymes in hepatic and pancreatic tissue were measured at the end of the experimental period. Significant decreases in body weight and antioxidant ability and increases in blood glucose, lipid profile, and lipid peroxidation were observed in STZ-induced diabetic rats. The administration of GBE and glibenclamide daily for 30 days in STZ-induced diabetic rats reversed the above parameters significantly. GBE possesses antihyperglycemic, antioxidant, and antihyperlipidemia activities in STZ-induced chronic diabetic rats, which promisingly support the use of GBE as a food supplement or an adjunct treatment for diabetics.

1. Introduction

Diabetes is a global epidemic with an estimated worldwide prevalence of 246 million people in 2007 and forecasts to rise to 300 million by 2025 [1]; consequently, diabetes presents a major challenge to healthcare systems around the world. Diabetes is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [2]. Many oral antihyperglycemic agents, such as sulfonylurea and biguanides, are available along with insulin for the treatment of diabetes, but these agents have significant side effects, and some are ineffective in chronic diabetes patients [3]. Thus, there is an increasing need of new natural antihyperglycemic products especially nutraceuticals with less side effects, safe, and high antihyperglycemic potential.

Previous studies have demonstrated that diabetes exhibits enhanced oxidative stress and high reactive oxygen species (ROS) in pancreatic islets due to persistent and chronic hyperglycemia, thereby depletes the activity of antioxidative defense system, and thus promotes free radical generation [4]. A number of mechanisms or pathways by which hyperglycemia, the major contributing factor of increased ROS production, causes tissue damage or diabetic complications have been identified [5]. Also, reduced antioxidant levels as a result of increased free radical production in experimental diabetes have been reported [6]. The rise in free radical activity is suggested to play an important role in lipid peroxidation and protein oxidation of cellular structures resulting in cell injury and implicated in the pathogenesis of vascular disease which are the mainly cause of morbidity and mortality in diabetes [7]. Streptozotocin (STZ) is frequently used to induce diabetes in experimental animals through its toxic effects on pancreatic β-cells [8, 9] and as a potential inducer of oxidative stress. It has been reported that diabetes induced by STZ is the best characterized system of xenobiotic-induced

diabetes and the commonly used model for the screening of antihyperglycemic activities.

Traditional medicines derived mainly from plants played an important role in the management of diabetes. *Ginkgo biloba* is a dioecious tree with a history of use in traditional Chinese medicine and has many pharmacologic effects. The mechanism of action of *Ginkgo* is believed to be linked with its functions as a neuroprotective agent, an antioxidant, a free-radical scavenger, a membrane stabilizer, and an inhibitor of the platelet-activating factor, and so on [10–13]. *Ginkgo biloba* extract (GBE) from *Ginkgo biloba* leaves is commonly used in dietary supplements for ailments and has showed excellent clinical effects in many cases. The goal of this study was to evaluate the effects of GBE on the antihyperglycemic ability in STZ-induced diabetes rats. Furthermore, the positive roles of natural products in the correction of oxidative stress and hyperlipidaemia, which are diabetes-related complication, were also assessed.

2. Materials and Methods

2.1. Materials. The powder form of GBE was purchased from Hangzhou Greensky Biological Tech (Hangzhou, China). All reagents used in this research were of analytical grade and obtained from Shenyang Biotechnology Co. Ltd.

2.2. Induction of Diabetes to Experimental Rats. Diabetes was induced by a single intraperitoneal injection of a freshly buffered (0.1 M citrate, pH 4.5) solution of STZ at a dosage of 60 mg/kg body weight (b.w.). After 72 h of STZ administration, the tail vein blood was collected to determine fasting blood glucose level. Only rats with fasting blood glucose over 250 mg/dL were considered diabetic and included in the experiments. Treatment with GBE started after the last STZ injection. Blood samples were drawn at 48 hours, 15 days, and 30 days till the end of the study (30 days).

2.3. Experimental Design. A total of 70 matured normoglycemic male *Wistar* rats (12–14 weeks of age, weighing about 180 ± 10 g) were collected for this experiment. Animals were acclimated for a period of 7 days in our laboratory condition prior to the experiment. The rats were fed with standard laboratory diet and allowed to drink water *ad libitum*. Animal experiments were carried out in accordance with institutional ethical guidelines for the care of laboratory animals of the China Medical University.

Rats randomly selected were divided into seven groups, comprising ten rats each. The treatment schedule was as follows: (1) normal control group (N group), (2) STZ control group (D group), (3) GBE- (200 mg/kg b.w.) treated control group (N + G group), (4) GBE- (100 mg/kg b.w.) treated STZ group (D + LG group), (5) GBE- (200 mg/kg b.w.) treated STZ group (D+MG group), (6) GBE- (300 mg/kg b.w.) treated STZ group (D + HG group), and (7) glibenclamide- (5 mg/kg b.w.) treated STZ group (D + GLI group). The treatment with GBE and glibenclamide started after the last STZ injection, where the vehicle, GBE, and glibenclamide were administrated orally to the respective group rats. After 48 hours, 15 days, and 30 days of treatment, the rats were fasted overnight, and blood glucose and body weight were measured in the morning. The serum lipid profile was determined after 30 days of treatment. At the end of 30-day experiment, all rats were anesthetized with pentobarbital sodium (35 mg/kg) and euthanized by cervical decapitation. The liver and pancreas were excised immediately from the animals, washed with ice-chilled physiological saline, and stored at −80°C.

2.4. Determination of Serum Glucose and Lipid Profile. The serum concentrations of glucose, triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined using commercially available kits (BIOSINO Biotechnology and Science INC, China).

2.5. Determination of Oxidative Stress Markers. The superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) activities, and glutathione (GSH) level in hepatic and pancreatic tissue were measured using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). Lipid peroxidation was measured as malondialdehyde (MDA) level in hepatic and pancreatic tissue according to Jain's method [14].

2.6. Statistical Analysis. Results are expressed as the means ± SD. Statistical analysis was performed by ANOVA for multiple comparisons (SPSS, Version 15.0). A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of GBE on Body Weight in Experimental Groups. Body weights of rats in the seven groups were monitored during the experimental period. As shown in Table 1, there is no difference in the different groups before treatment and after 48 h ($P > 0.05$). Body weights of rats in D group were lower than those in other groups after 15 days and 30 days ($P < 0.01$). STZ caused a significant weight loss of rats in D group while treatment with GBE at different concentrations or glibenclamide suppressed the decrease in the body weight.

3.2. Effect of GBE on Blood Glucose in Experimental Groups. The STZ-induced diabetic rats exhibited a significant increase in fasting blood glucose (299 ± 10 mg/dL) as compared to non-STZ-treated rats (49 ± 6 mg/dL) before the initial treatment of GBE or glibenclamide ($P < 0.01$). After GBE and glibenclamide treatment, the changes of blood glucose levels in different experimental groups were shown in Figure 1. At 48 hours, the levels of blood glucose in D group (298.3 ± 11.2 mg/dL), D+LG group (291.6±8.8 mg/dL), D + MG group (286.4 ± 9.2 mg/dL), D + HG group (283.5 ± 5.6 mg/dL), and D + GLI group (252.9 ± 9.1 mg/dL) were higher than N group (46.8 ± 4.6 mg/dL) and N + G group (45.3 ± 3.3 mg/dL) ($P < 0.01$). The administration of GBE or glibenclamide for 30 days in D + LG group (155.5 ± 11.6 mg/dL), D + MG group (143.1 ± 9.6 mg/dL), D + HG group (85.0 ± 6.7 mg/dL), and D + GLI group (40.9 ± 5.4 mg/dL) caused a significant

Table 1: Changes of the body weight (g) of rats in the seven groups during the experimental period of 30 days.

	Before treatment	48 h	15 days	30 days
N group	178.3 ± 5.6	183.4 ± 6.5	214.3 ± 7.7	220.3 ± 6.3
D group	180.1 ± 6.9	177.3 ± 5.9	174.1 ± 10.2[a]	169.3 ± 11.6[a]
N + G group	176.9 ± 5.8	186.1 ± 8.8	209.6 ± 9.6[b]	220.8 ± 10.0[b]
N + LG group	179.3 ± 7.6	183.0 ± 7.2	187.6 ± 5.5[a,b]	206.3 ± 5.9[a,b]
N + MG group	177.4 ± 5.1	180.6 ± 4.6	190.6 ± 4.9[a,b]	213.6 ± 9.9[b]
N + HG group	178.6 ± 7.7	186.9 ± 5.8	199.5 ± 7.1[a,b]	216.2 ± 7.8[b]
N + GLI group	180.2 ± 6.3	187.9 ± 5.0	196.3 ± 6.1[a,b]	212.1 ± 11.8[b]

Values are means ± SD for 10 rats in each group. N group: normal control; D group: diabetes group; N + G group: normal control plus GBE; D + LG group: diabetes plus low GBE treatment; D + MG group: diabetes plus middle GBE treatment; D + HG group: diabetes plus high GBE treatment; D + GLI group: diabetes plus glibenclamide treatment.

[a]Indicates statistical significance of $P < 0.01$ compared to N group; [b]$P < 0.01$ compared to D group.

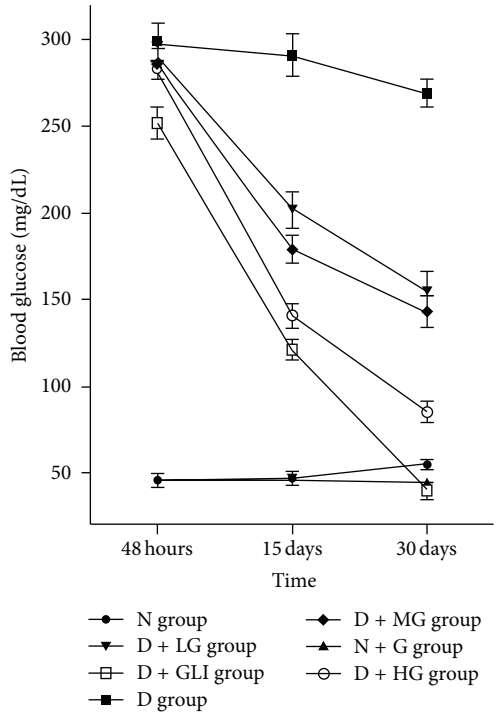

Figure 1: Changes of blood glucose of rats in the seven groups during the experimental period of 30 days. Values are mean ± SD ($n = 10$ animals).

decrease in blood glucose levels when compared with D group (269.7 ± 8.4 mg/dL) ($P < 0.05$), but all GBE-treated group retained high blood glucose (>100 mg/dL) after 15 days. GBE caused a significant dose- ($P < 0.01$) and time-dependent reduction ($P < 0.01$) in blood glucose levels of diabetic rats. The blood glucose values of diabetic rates showed a tendency to normal levels after administration of GBE at 300 mg/kg b.w. and glibenclamide, 5 mg/kg b.w. in 30 days.

3.3. Effect of GBE on Serum Lipid Profiles in Experimental Groups. Our observation provides further support to the growing body of evidence showing that STZ-induced diabetes can also induce anomaly of serum TC, TG, HDL-C, and LDL-C. Table 2 showed the levels of serum TG, TC, LDL-C, and HDL-C of rats in different experimental groups. Rats in D group displayed a significant increase in the levels of TG, TC, and LDL-C in comparison with N group ($P < 0.01$). However, serum HDL-C level of rats in D group was significantly lower than that of rats in N group ($P < 0.01$). Similar with the glibenclamide-treated STZ group, GBE administration showed a significant decrease in the levels of serum TG, TC, and LDL-C or a significant increase in the level of HDL-C after 30-day treatment when compared with D group.

3.4. Determination of Oxidative Stress Marker in Hepatic and Pancreatic Tissue. As shown in Table 3, a marked increase of MDA production and decrease of antioxidant level (GSH) and antioxidant enzyme activity (SOD, CAT, GSH-Px) were observed in the hepatic and pancreatic tissues of rats in D group when compared with N group ($P < 0.01$). GBE and glibenclamide treatment significantly inhibited the formation of MDA and raised antioxidant level (GSH) and antioxidant enzyme activity (SOD, CAT, GSH-Px) in a dose-dependent manner. Likewise, GBE exhibited the same antioxidation effects as glibenclamide at the dose of 5 mg/kg b.w.

4. Discussion

Diabetes is increasing at an alarming rate worldwide, which can mainly be attributed to the sedentary life style and calorie-rich diet. Diabetes is often linked with abnormal lipid metabolism and is considered as a major factor for the development of atherosclerosis and cardiovascular complication [15]. Recently, the WHO Expert Committee recommended the importance to investigate and explore hypoglycemic agents from plant origin because plants used in the traditional medicine have fewer side effects than synthetic drugs [16]. Currently, GBE is widely used for medicine in China and showed excellent clinical effects in many aspects, and the pharmacological mechanisms including modification of Ca^{2+} signaling [17], clearing oxygen free radical [18], decreasing lipid peroxidation, and promoting the synthesis and release

Table 2: Effect of GBE treatment on serum lipid profile of rats in experimental groups.

	N group	D group	N + G group	D + LG group	D + MG group	D + HG group	D + GLI group
TG (mmol/L)	1.48 ± 0.35	2.32 ± 0.51[a]	1.46 ± 0.38[b]	1.75 ± 0.40[c]	1.65 ± 0.41[b]	1.52 ± 0.28[b]	1.36 ± 0.40[b]
TC (mmol/L)	1.69 ± 0.26	2.71 ± 0.48[a]	1.61 ± 0.30[b]	2.02 ± 0.51[b]	1.83 ± 0.44[b]	1.77 ± 0.33[b]	1.65 ± 0.31[b]
HDL-C (mmol/L)	1.35 ± 0.23	0.92 ± 0.30[a]	1.21 ± 0.28[b]	0.98 ± 0.25	1.19 ± 0.23[c]	1.28 ± 0.30[c]	1.20 ± 0.22[b]
LDL-C (mmol/L)	0.56 ± 0.09	0.73 ± 0.11[a]	0.51 ± 0.08[b]	0.68 ± 0.09	0.67 ± 0.03	0.62 ± 0.06[c]	0.57 ± 0.10[b]

Values are means ± SD for 10 rats in each group. N group: normal control; D group: diabetes group; N + G group: normal control plus GBE; D + LG group: diabetes plus low GBE treatment; D + MG group: diabetes plus middle GBE treatment; D + HG group: diabetes plus high GBE treatment; D + GLI group: diabetes plus glibenclamide treatment.

[a]Indicates statistical significance of $P < 0.01$ compared to N group; [b]$P < 0.01$ compared to D group.
[c]$P < 0.05$ compared to D group.

of epoprostenol [6]. Increasing evidence has demonstrated that GBE has a potential efficacy in glucose metabolism and lipid metabolism. Therefore, the present study was aimed to assess the effect of GBE on hyperglycemia, lipid profile, and enzymatic and nonenzymatic antioxidants in STZ-induced diabetic rats.

In our study, Figure 1 shows the changes in body weight of normal and streptozotocin-induced diabetic rats. STZ-injection-induced diabetes is associated with the characteristic loss of body weight which is due to increased muscle wasting and due to loss of tissue proteins [19]. As expected in D group, the body weight of rats was progressively reduced, and the treatment of diabetic rats with glibenclamide and GBE improved body weight significantly, which indicates the prevention of muscle tissue damage due to hyperglycemic condition. In addition, STZ injection caused diabetes, which may be due to destruction of β-cells of the islets of Langerhans [20]. G. B. Kudolo has already reported that ingestion of 120 mg of GBE as a single dose for 3 months for individuals leads to an increase in pancreatic β-cell function [21, 22]. Zhou et al. proposed that GBE improved insulin sensitivity mainly by enhancing insulin receptor substrate 2 transcription and preventing insulin resistance. Similarly, our data showed that the daily administration of GBE for 30 consecutive days abolished the blood glucose increase in the STZ-induced diabetic rats. This effect was dose dependent and time dependent. The reduction of blood glucose may be either due to the increased level of plasma insulin in diabetic rats, which may influence the stimulation of pancreatic insulin secretion from β-cells in islets of Langerhans, or due to the enhanced transport of blood glucose to peripheral tissue. Our results supported the reported evidence that GBE has the potential to prevent insulin resistance and is a promising antidiabetic drug [23].

Previous studies have demonstrated that GBE exerts multidirectional lipid-lowering effects on the rat metabonome, including limitation of the absorption of cholesterol, inactivation of HMG-CoA, and favorable regulation of profiles of essential polyunsaturated fatty acid [24]. In diabetes, hyperglycemia is accompanied with dyslipidemia [25] characterized by increase in TC, LDL, VLDL, and TG and fall in HDL. This altered serum lipid profile was reversed towards normal after treatment with GBE. The possible mechanism through which GBE exerts its anti-hyperlipidemic effect might include the changed activity of cholesterol biosynthesis enzymes and/or the changed level of lipolysis which are under the control of insulin [26]. It is reported that GBE treatment could decrease the capacity of LDL to carry free cholesterol to various tissues without affecting the capacity of HDL to carry cholesterol back to the liver in rats [27]. In addition, GBE treatment can partially reverse ethanol-induced dyslipidemia at dose levels of 48 and 96 mg/kg b.w. in rats by reducing the lipid peroxidation induced by ethanol [28]. Our results indicated that the lipid-lowering effect of GBE could be an indirect consequence of amelioration of insulin resistance or direct hypolipidemic effect mediated through other mechanisms [29].

Hyperglycemia is a main cause for elevated free radical levels, followed by production of ROS, which can lead to increased lipid peroxidation and altered antioxidant defense and further impair glucose metabolism in biological system [30]. An imbalance between oxidation and antioxidant status has been shown to play an important role in mediating insulin resistance [29]. Overwhelming free radicals generated due to oxidative stress may develop several adverse effects commonly seen in diabetes such as neuropathy, nephropathy, retinopathy, and vascular disorders [31]. The major antioxidant enzymes, including SOD, CAT, and GSH-Px, are regarded as the first line of the antioxidant defense system against ROS generated in vivo during oxidative stress and act cooperatively at different sites in the metabolic pathway of free radicals [32]. In our study, reduced activities of SOD, CAT, and GSH-Px in the liver and pancreas have been observed in diabetic rats. The administration of GBE for 30 days increased the SOD, CAT, and GSH-Px activity and GSH level in the liver and pancreas of diabetic rats. Robertson et al. demonstrated that antioxidants have been shown to brake the worsening of diabetes by improving β-cells function in animal models and suggested that enhancing antioxidant defense mechanisms in pancreatic islets may be a valuable pharmacologic approach to managing diabetes [33]. Modak MA et al. reported that the control of hyperglycemia leads to improvement in oxidative stress profile, and enhancing antioxidant defense mechanisms in pancreatic islets helps them to cope better with oxidative stress. Since GBE is a complex mixture of ingredients with a unique broad spectrum of pharmacological activities, it probably acts through several different mechanisms covering ROS scavenger and/or enhancing antioxidant ability. Moreover, Naik et al. have demonstrated that GBE is scavengers of

Table 3: Effect of GBE treatment on hepatic and pancreatic oxidative stress markers of rats in experimental groups.

	N group	D group	N + G group	D + LG group	D + MG group	D + HG group	D + GLI group
Liver							
GSH (μg/mg protein)	512.36 ± 91.25	365.50 ± 84.77[a]	529.36 ± 101.23[b]	400.28 ± 79.46	451.33 ± 89.44[c]	486.36 ± 98.31[b]	482.09 ± 106.30[c]
CAT (U/mg protein)	362.63 ± 18.52	302.05 ± 22.82[a]	374.47 ± 26.36[b]	313.25 ± 24.47	322.52 ± 17.52[c]	349.96 ± 25.01[b]	344.80 ± 20.91[b]
SOD (U/mg protein)	542.35 ± 24.21	235.27 ± 34.22[a]	602.0 ± 40.50[b]	322.21 ± 32.65[b]	414.11 ± 35.33[b]	505.20 ± 41.52[b]	523.36 ± 36.66[b]
GSH-Px (U/mg protein)	3023 ± 217	2236 ± 259[a]	3111 ± 285[b]	2567 ± 198[b]	2758 ± 252[b]	2904 ± 220[b]	2808 ± 244[b]
MDA (nmol/mg protein)	9.03 ± 1.25	16.91 ± 1.81[a]	8.85 ± 1.33[b]	14.28 ± 1.57[b]	10.05 ± 1.01[b]	9.66 ± 1.41[b]	10.36 ± 1.55[b]
Pancreas							
GSH (μg/mg protein)	444.08 ± 71.44	294.14 ± 69.27[a]	408.50 ± 66.85[b]	312.58 ± 75.55	360.00 ± 57.19[c]	405.81 ± 63.36[b]	406.16 ± 56.39[b]
CAT (U/mg protein)	298.02 ± 21.30	174.22 ± 14.52[a]	287.34 ± 22.71[b]	196.35 ± 18.90[b]	209.42 ± 23.00[b]	212.56 ± 20.66[b]	274.30 ± 21.94[b]
SOD (U/mg protein)	415.00 ± 33.20	197.38 ± 36.55[a]	385.52 ± 32.11[b]	222.44 ± 36.40	356.78 ± 37.80[b]	379.91 ± 38.92[b]	364.82 ± 40.24[b]
GSH-Px (U/mg protein)	658 ± 89	462 ± 92[a]	711 ± 101[b]	555 ± 74[c]	596 ± 94[b]	623 ± 78[b]	539 ± 79[b]
MDA (nmol/mg protein)	8.77 ± 1.03	14.91 ± 1.44[a]	8.05 ± 1.11[b]	12.50 ± 1.48[b]	10.87 ± 1.61[b]	9.22 ± 1.31[b]	8.99 ± 1.40[b]

Values are means ± SD for 10 rats in each group. N group: normal control; D group: diabetes group; N + G group: normal control plus GBE; D + LG group: diabetes plus low GBE treatment; D + MG group: diabetes plus middle GBE treatment; D + HG group: diabetes plus high GBE treatment; D + GLI group: diabetes plus glibenclamide treatment.

[a]Indicates statistical significance of $P < 0.01$ compared to N group; [b]$P < 0.01$ compared to D group.

[c]$P < 0.05$ compared to D group.

free radicals by increasing levels of free radical scavenging enzymes [34]. It is also reported that GBE may reduce the oxidative stress in the reperfused myocardium and increase the antioxidant activity in ischemia reperfusion rats. Further, lipid peroxidation measurement is a more practical and safer method to evaluate the factors causing cellular injury and the activation of the common pathway. Tissue MDA content, the final product of lipid breakdown caused by oxidative stress, is an important indicator of free radical-induced lipid peroxidation [35–37]. GBE-treated rats showed decreased level of MDA, suggesting that GBE has antioxidant capacity.

5. Conclusion

In summary, GBE possesses antihyperglycemic, antioxidant, and antihyperlipidemic activities in STZ-induced chronic diabetic rats, which promisingly support the use of GBE as a food supplement or an adjunct treatment for diabetics. Moreover, further work is necessary to elucidate in detail the mechanism of action of the GBE at the cellular and molecular levels.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Antioxidant and Cytotoxic Effects of Crude Extract, Fractions and 4-Nerolidylcathecol from Aerial Parts of *Pothomorphe umbellata* L. (*Piperaceae*)

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The crude ethanolic extract from aerial parts of *Pothomorphe umbellata* L. (*Piperaceae*) and fractions obtained by partitions sequentially among water-methanol, methylene chloride, and ethyl acetate, as well as the major constituent, 4-nerolidylcatechol, were, respectively, evaluated and evidenced for antioxidant and cytotoxic effects through fluorometric microplate and microculture tetrazolium assays in HL-60 cells. The crude ethanolic extract demonstrated the preeminent antioxidant activity (IC_{50} = 1.2 μg/mL) against exogenous cytoplasmic reactive oxygen species, followed by the water-methanolic (IC_{50} = 4.5 μg/mL), methylene chloride (IC_{50} = 5.9 μg/mL), ethyl acetate (IC_{50} = 8.0 μg/mL), 4-nerolidylcatechol (IC_{50} = 8.6 μg/mL), and the sterol fractions (IC_{50} > 12.5 μg/mL). Vitamin C, the positive control used in this assay, presented IC_{50} value equivalent to 1.7 μg/mL. 4-Nerolidylcatechol (IC_{50} = 0.4 μg/mL) and methylene chloride fraction (IC_{50} = 2.3 μg/mL) presented considerable cytotoxicity probably because of the presence of an *o*-quinone, an auto-oxidation by product of the catechol. Polar compounds, present in the ethanol extract, appear to increase the solubility and stability of the major active constituent, acting synergistically with 4-nerolidylcatechol, improving its pharmacokinetic parameters and increasing significantly its antioxidant activity which, in turn, suggests that the aqueous-ethanolic extract, used in folklore medicine, is safe and effective.

1. Introduction

Plants belonging to *Piperaceae* family are reputed in the Indian Ayurvedic system of medicine and in folklore medicine of Latin America and West Indies for their medicinal properties [1]. *Piper umbellata*, former *Pothomorphe umbellata* L. (*Piperaceae*), known in Brazil as "caapeba" or "pariparoba," is a shrubby plant which thrives spontaneously in moist and shady places from Amazon to the south of Brazil [2]. Its species, described in the first Brazilian Pharmacopoeia [3], has been assigned to different pharmacological activities currently verified, such as an anti-inflammatory and analgesic [4], and antiulcer and gastroprotective [5], antimalarial [6] and antioxidant [7].

Free radicals and reactive oxygen species have been proven to mediate processes involved in the pathogenesis of

a variety of ailments including inflammation, cancer, diabetes, liver cirrhosis, cardiovascular diseases, and premature aging [8, 9]. Free radicals, with their unpaired electrons, can attack and damage almost any molecule found in the body. They are so active that, once formed, bind to different compounds in seconds. Therefore, they can deliver their nonpaired electron or capture another molecule electron to form a pair. Then, radicals become stable and, consequently, the molecule previously attacked becomes a radical, which promotes a chain reaction that act destructively in human tissues [10].

Reactive oxygen species, in turn, are significantly present in biological processes of energy production and phagocytosis [11]. The main superoxide anions are (O_2^-), hydroxyl radical (OH^-), nitric oxide (NO), hydrogen peroxide (H_2O_2), and lipid radical (L^-). Among these, hydroxyl radical is more reactive in the induction of lesions in cellular molecules whilst hydrogen peroxide is sufficiently able to cross the nuclear membrane and cause damage to the DNA molecule [12]. Thus, effective and safe antioxidants acquired sustainably from the biodiversity can diminish the threat of free radicals and reactive oxygen species damage over lifetime [13].

Nowadays, attention is outlined to antioxidants originating from natural products which offer new treatment possibilities for diseases mediated by oxidative stress. Many enzymes and secondary metabolites of higher plants have been demonstrated in *in vitro* and *in vivo* experiments to be capable of protecting tissues against oxidative stress through the free radicals and oxygen reactive species inhibition or capture [14], besides its consumption has been associated with the decreased risk of degenerative diseases occurrence [15]. Brazil has a flora that is extremely rich in medicinal plants with immense potential for supplying these antioxidant agents. Among these plants, members of *Piperaceae* family are rich in phenolic compounds [16], in particular, *Pothomorphe umbellata* L.

In relation to the antioxidant activity of *Pothomorphe umbellata* L., its crude extract has been evaluated by *in vitro* assays, using as model the self-oxidation of mouse brains, and such activity was partly attributed to the presence of 4-nerolidylcathecol [17], a phenolic compound, isolated from the vegetable roots and leaves [18]. Commonly, the defensive effects of natural antioxidants are related to the presence of phenolic compounds [19]. However, in another *in vitro* assay, the *Pothomorphe umbellata* L. crude extract showed an antioxidant potential significantly larger than the one of this isolated compound, suggesting the presence of additional compounds with antioxidant activity [20].

Based on these data, this study was undertaken to evaluate and evidence the antioxidant activity of crude ethanolic extract, sterol fraction, and 4-nerolidylcathecol from aerial parts of *Pothomorphe umbellata* L. as well as its cytotoxic effect to investigate and ascertain toxic or antiproliferative actions of these test samples in HL-60 cells and, then, to verify if the crude aqueous-ethanol extract prepared from the aerial parts of *Pothomorphe umbellata* L. and widely used in folklore medicine is, in fact, secure and efficient.

2. Materials and Methods

2.1. Plant Material. Leaves from *Pothomorphe umbellata* L. were collected in March 2011 on the campus of the University of São Paulo in Ribeirão Preto, São Paulo, Brazil. The botanical identification of the leaves was made by Prof. Pedro Melillo de Magalhães and a voucher sample with registration number UEC 127123 was deposited in the herbarium of the Botany Department of State University of Campinas, Campinas, Brazil.

2.2. Preparation of Extract and Fractions. The crude ethanolic extract and fractions from *Pothomorphe umbellata* L. were prepared as described preliminarily [4]. The ethanolic extract was suspended in methanol-water (9 : 1) and extracted with methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc), in sequence, to furnish methanol (MeOH), CH_2Cl_2, and EtOAc fractions. The CH_2Cl_2 was separated into hexane soluble and insoluble parts. The hexane insoluble part, analyzed by GC-MS, was found to be composed of β-sitosterol, campesterol and stigmasterol.

2.3. Isolation and Identification of 4-Nerolidylcatechol. The 4-nerolidylcatechol was isolated as reported previously [18] and properly identified by nuclear magnetic resonance. NMR spectra were obtained on Bruker DPX 400 MHz apparatus, operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The samples were dissolved in deuterated chloroform (Aldrich).

2.4. Microplate Assay for Oxidative Products Detection Using DCFH-DA in HL-60 Cells. A fluorometric microplate assay [21] was established for the detection of oxidative products using 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) in human promyelocytic leukemia cells (1×10^6 HL-60 cells/mL, ATCC) which were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37°C in 5% CO_2: 95% air. Then, 125 μL of the cell suspension were added into each well on 96-well microtiter plates. After treatment with different concentrations of the test material for 30 minutes, the cells were stimulated with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 minutes. Then, the cells were incubated for 15 minutes after the addition of 5 μg/mL of DCFH-DA (Molecular probes). The ability of the test materials to inhibit exogenous cytoplasmatic reactive oxygen species-catalyzed oxidation of DCFH-DA in HL-60 cells was measured by PMA treated control incubations with and without the test materials. The levels of DCFH-DA were measured using a CytoFluor 2350 fluorescence measurement system (Millipore) with an excitation wavelength at 485 nm (bandwidth 20 nm) and an emission at 530 nm (bandwidth 25 nm). Vitamin C and trolox were used as the positive controls in this assay.

2.5. XTT Assay for Cytotoxicity in HL-60 Cells. Cellular growth in the presence or absence of experimental agents was determined using the previously described microculture tetrazolium assay [22]. The tetrazolium reagent (XTT)

was designed to yield a suitably colored, aqueous-soluble, nontoxic formazan upon metabolic reduction by viable cells. After sample (25 μL) exposure on cells for 48 hours, the XTT assay was performed. Briefly, rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations (100-μL volume) into 96-well microtiter plates using a multichannel pipet. Accordingly, 25 μL of XTT-PMS solution (1 mg/mL XTT solution supplemented with 25 μM of PMS) was added to HL-60 cells (2×10^4 cells in 225 μL of medium) into each well on the microtiter plates. After incubation for 4 hours at 37°C, absorbance at 450 nm was measured by a microplate reader (reference absorbance at 630 nm). Vitamin C and trolox were used as the positive controls in this assay.

2.6. Statistical Analysis. The statistical analyses were established using Analysis of Variance (ANOVA) followed by the Tukey-Kramer multiple comparison test [23]. Results with $P < 0.05$ were considered to be significant. Data are expressed as mean ± S.D.

3. Results

3.1. Isolation and Identification of 4-Nerolidylcathecol. Analysis of the ^{1}H NMR spectrum evidences the presence of aromatic group by chemical shift and coupling constants relative for the three hydrogens, H-3 at δ 6.87 ppm (d, J = 2 Hz), H-5 at δ 6.75 ppm (dd, J = 8.4 Hz and J = 2 Hz), and H-6 at δ 6.80 ppm (d, J = 8.4 Hz). The data from ^{13}C NMR spectrum permitted the verification of aliphatic chain of catechol and nerolidyl groups by the presence of two methyl groups attached to carbon sp^2 not containing hydrogen (δ 124.4 ppm and δ 124.6 ppm). The methyl hydrogens linked to sp^2 carbon (Me11′) presented chemical shift between δ 1.5 ppm and δ 1.7 ppm (*singlet*), as well as protons of the methyl group linked to sp^3 carbon with δ 1.3 ppm. The data obtained are similar to data presented by Kijjoa et al. [18] suggesting that 4-nerolidylcathecol has been appropriately isolated and identified.

3.2. Microplate Assay for Oxidative Products Detection Using DCFH-DA in HL-60 Cells. Test samples and the positive controls, vitamin C and trolox, were evaluated for the inhibition of exogenous cytoplasmic reactive oxygen species-catalyzed oxidation using 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) in human promyelocytic leukemia cells (HL-60 cells). IC_{50} concentrations were established for the purpose of verifying *Pothomorphe umbellata* L. antioxidant effect which is displayed in Table 1.

The crude ethanolic extract from aerial parts of *Pothomorphe umbellata* L. demonstrated the best antioxidant activity (IC_{50} = 1.2 μg/mL). This activity was higher than that observed for vitamin C (IC_{50} = 1.7 μg/mL). The MeOH and EtOAc fractions presented, respectively, the lowest (IC_{50} = 4.5 μg/mL) and the highest antioxidant activity (IC_{50} = 8.0 μg/mL) among the assayed fractions, while the CH_2Cl_2 fraction demonstrated an intermediate effect (IC_{50} = 5.9 μg/mL). All these fractions exhibited lower activity than the crude extract. 4-Nerolidylcatechol, the major constituent present in this species, which is recognized to have a noteworthy antioxidant potential, displayed an activity even lower (IC_{50} = 8.6 μg/mL) in this assay. The sterol fraction did not present a significant antioxidant effect (IC_{50} > 12.5 μg/mL), inhibiting the oxidative products formation by 28% at concentration equivalent to 62.5 μg/mL.

Table 1: Antioxidant effect evaluation of *Pothomorphe umbellata* L.

Experimental samples	Antioxidant effect IC_{50} (μg/mL)
P. umbellata L. crude extract	1.2 ± 0.2
P. umbellata L. CH_2Cl_2 fraction	5.9 ± 0.4
P. umbellata L. EtOAc fraction	8.0 ± 0.6
P. umbellata L. MeOH fraction	4.5 ± 0.3
P. umbellata L. sterol fraction	>12.5
4-Nerolidylcatechol	8.6 ± 0.3
Vitamin C	1.7 ± 0.1
Trolox	0.9 ± 0.2

Table 2: Cytotoxic effect evaluation of *Pothomorphe umbellata* L.

Experimental samples	Cytotoxic effect IC_{50} (μg/mL)
P. umbellata L. crude extract	5.3 ± 0.4
P. umbellata L. CH_2Cl_2 fraction	2.3 ± 0.1
P. umbellata L. EtOAc fraction	>10.0
P. umbellata L. MeOH fraction	>10.0
P. umbellata L. sterol fraction	8.9 ± 0.7
4-Nerolidylcatechol	0.4 ± 0.05
Vitamin C	>10.0
Trolox	>10.0

3.3. XTT Assay for Cytotoxicity in HL-60 Cells. Test samples and the positive controls, vitamin C and trolox, were evaluated for cytotoxicity through cellular growth in the presence or absence of these experimental agents using the XXT-microculture tetrazolium assay in human promyelocytic leukemia cells (HL-60 cells). IC_{50} concentrations were established for the purpose of verifying *Pothomorphe umbellata* L. cytotoxic effect which is displayed in Table 2.

4-Nerolidylcathecol demonstrated the preeminent cytotoxicity (IC_{50} = 0.4 μg/mL) in HL-60 cells, followed by CH_2Cl_2 fraction (IC_{50} = 2.3 μg/mL), crude ethanolic extract (IC_{50} = 5.3 μg/mL), and sterol fraction (IC_{50} = 8.9 μg/mL). The patterns of vitamin C and trolox had not evidenced cytotoxicity at the highest tested dose. However, 4-nerolidylcatechol and CH_2Cl_2 fraction demonstrated a significant cytotoxic effect.

4. Discussion

Analysis of the crude ethanolic extract and fractions from aerial parts of *Pothomorphe umbellata* L. indicated that most of the 4-nerolidylcatechol molecules and sterols are concentrated in the CH_2Cl_2 fraction. The major reason for the observed lesser activity of 4-nerolidylcatechol and the CH_2Cl_2 fraction in comparison with the crude ethanolic extract must be correlated to solubility and stability. These pharmacokinetic properties are closely related to the pharmacological effectiveness once the antioxidant efficacy depends on the ability of compounds to penetrate the cell membrane [13]. Then, the probable reason for the lower activity of 4-nerolidylcathecol and sterol fraction, compared with the crude extract, should be correlated to solubility and stability. Therefore, other compounds, present in the crude extract, must act synergistically with 4-nerolidylcathecol, improving its pharmacokinetic parameters and increasing significantly its antioxidant activity.

Pure 4-nerolidylcatechol is labile in ambient light, air, and room temperatures [24] and, thus, tends to undergo rapidly an autooxidation to an *o*-quinone when exposed. This compound appears to be more stable as a constituent in the crude ethanolic extract. Additionally, high polar compounds which are present in the MeOH fraction could act synergistically with 4-nerolidylcatechol to enhance its significant antioxidant potential. The antioxidant activity of phenols can be attributed to the presence of phenolic groups [19], which are extremely susceptible to oxidation in function of their structures. Besides the presence of highly oxidizable catecholic group, the presence of an unsaturated aliphatic chain can also contribute to the high antioxidant potential of 4-nerolidylcatechol. This compound alone and fractions, rich in phenolic compounds, are able to neutralize free hydroxyl radicals and reactive oxygen species by reducing the oxidative stress that induces DNA damages [20].

Mongelli et al. [25] reported the cytotoxicity of 4-nerolidylcathecol demonstrating that, by a mechanism of inhibition of the activity of topoisomerase I, this substance induces growth inhibition of KB cells. Afterward, the same group has investigated its larvicidal activity, showing that the 4-nerolidylcathecol has a considerable activity [26]. A detailed analysis of the molecular structure of 4-nerolidylcathecol suggests that the catechol moiety would appear to be relevant to the observed cytotoxicity in 4-nerolidylcatechol, while the nerolidyl side chain would appear not to be a necessary structural element for the observed cytotoxicity [24] once 4-nerolidylcatechol can undergo an autooxidation by product of the catechol to yield an *o*-quinone which is probably responsible for the cytotoxic effect observed of both 4-nerolidylcatechol and CH_2Cl_2 fraction [27, 28]. Therefore, these mechanisms should contribute to its considerable cytotoxic effect.

5. Conclusion

The data presented in this work corroborate the significant antioxidant potential of the crude ethanol extract obtained from the aerial parts of *Pothomorphe umbellata* L. and of its major compound, 4-nerolidylcathecol. Considering its noteworthy antioxidant effect and low cytotoxicity, it must be stated that the crude aqueous-ethanol extract prepared from the aerial parts of *Pothomorphe umbellata* L. and widely used in folklore medicine appears to be a safe and effective natural remedy.

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