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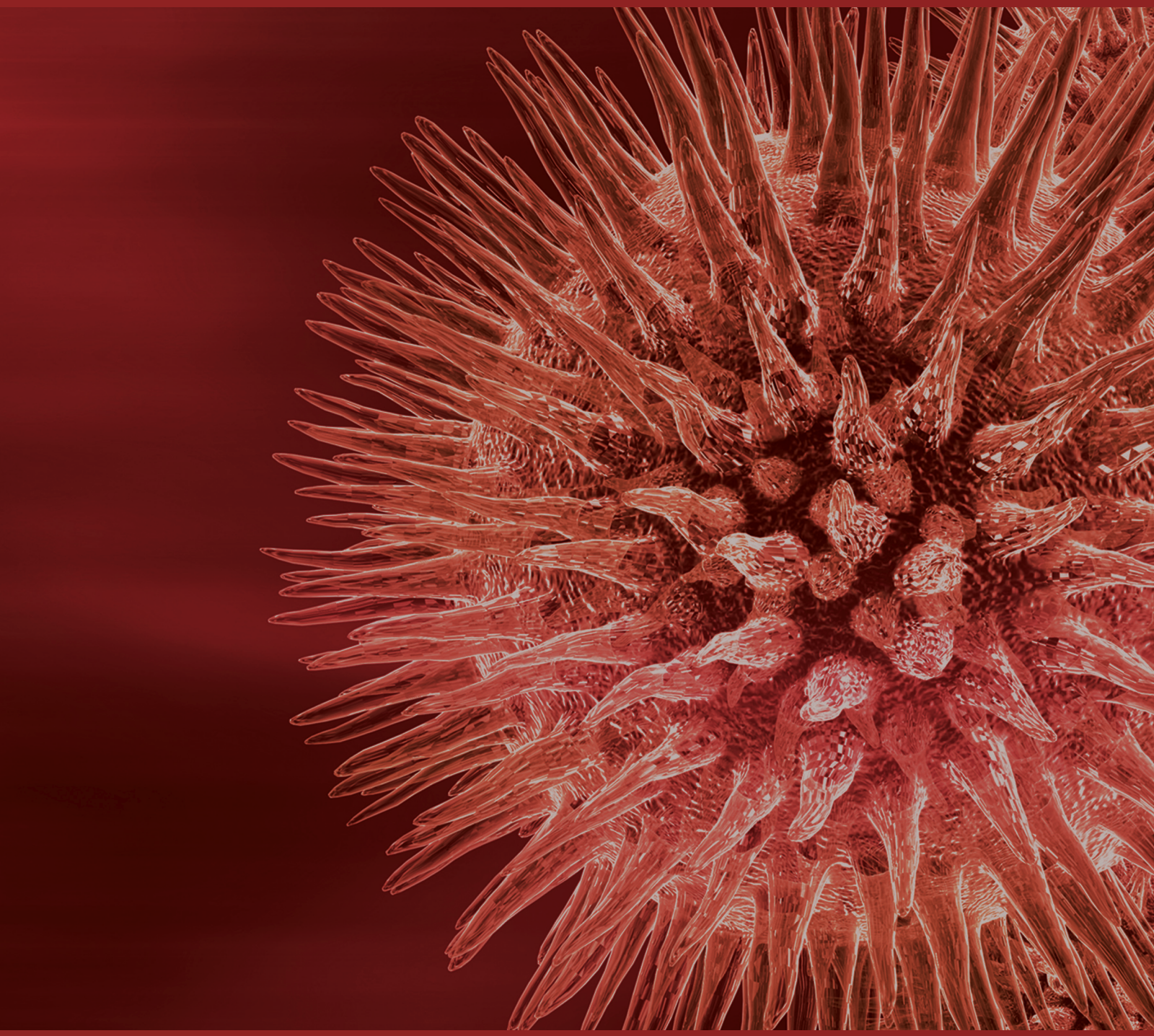
Bioactive Natural Matrices and Compounds

Guest Editors: Isabel C. F. R. Ferreira, Marina Soković, and Lillian Barros



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Contents

Bees' Honey Protects the Liver of Male Rats against Melamine Toxicity, Haddad A. El Rabey, Madeha N. Al-Seeni, and Suad M. Al-Solamy
Volume 2013, Article ID 786051, 8 pages

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 125964, 2 pages
http://dx.doi.org/10.1155/2014/125964

Editorial
Bioactive Natural Matrices and Compounds

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Received 9 December 2013; Accepted 9 December 2013; Published 3 February 2014

The demand for healthy ingredients and a natural way of preventing diseases is contributing to the increased use of natural matrices. There has been a lot of research indicating that general antioxidant properties are not the contributing factors to improving human health but rather the antioxidant properties of specific compounds involved in specific cell signaling mechanisms that need to be elucidated. In this way, beyond the composition of the usual macronutrients and micronutrients, it seems important to also provide information on the composition of natural bioactive compounds. Phytochemicals such as phenolic compounds, essential oils, steroids, vitamins, or pigments should assume high importance due to their documented antioxidant, antimicrobial, antitumour, and anti-inflammatory properties, among others, but the knowledge of the mechanisms of action involved is mandatory. The contributions for this special issue consist of five papers covering several aspects of nutraceuticals and phytochemicals in natural matrices with proven activities in various *in vitro* or *in vivo* models.

The paper by R. P. Constantin et al. evaluated the effects of three citrus flavanones (hesperidin, hesperetin, and naringenin) on several parameters linked to fatty acid oxidation in mitochondria, peroxisomes, and perfused livers of rats. These results confirm the hypothesis that citrus flavanones are able to induce a more oxidised state in liver cells, altering parameters related to hepatic fatty acid oxidation. The prooxidant effect is most likely a consequence of the ability of these substances to oxidise NADH upon production of phenoxyl radicals in the presence of peroxidases and hydrogen peroxide.

A. Chennuru and M. T. S. Saleem described the cardioprotective effects of sesamol against doxorubicin-induced cardiomyopathy in rats. The authors concluded that, sesamol has significant cardioprotection against doxorubicin that induced cardiomyopathy via amelioration of oxidative stress, lipid lowering, and membrane stabilization effect.

C. Chaotham et al. evaluated the oral toxicity of a partially purified plaunotol extract (PPE), which is traditionally consumed for its antigastric ulcer properties, using *in vivo* assays. The acute toxicity study demonstrated that PPE was practically nontoxic based on its high median lethal dose value. The chronic toxicity studies also showed the absence of mortality and clinical symptoms in all treated rats. Therefore, the authors concluded that these results suggest that PPE is potentially safe for further development as a therapeutic agent in humans.

In the research study presented by H. A. E. Rabey et al., the protective effect of natural bees' honey on the liver of male albino rats against melamine toxicity was reported. Treating the male albino rats (that were presupplemented regularly with 20000 ppm melamine) with natural bees' honey at a dose of 2.5 g/kg body weight for 28 days improved both liver functions and increased serum protein. In addition, a positive impact on the shape of the cells after treatment with honey, compared to the positive melamine supplemented group, was observed. The authors concluded that the use of natural bees' honey has the ability to protect the livers of rats against the toxic effects of melamine.

A. Rasul et al. reviewed recent data on primary flavonoids isolated from plants. Pinocembrin exhibits pharmacological effects on almost all systems, and the authors reviewed its pharmacological and therapeutic applications with specific emphasis on mechanisms of actions. The review suggested

that pinocembrin is a potentially promising pharmacological candidate, but additional studies and clinical trials are required to determine the specific intracellular sites of action and derivative targets in order to fully understand the mechanism of its anti-inflammatory, anticancer, and apoptotic effects to further validate pinocembrin medical applications.

Acknowledgments

We thank the authors of the submitted papers for their contribution. The preparation of this special issue would not have been possible without the generous support and dedication of experts that evaluated the submitted papers.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 391528, 5 pages
http://dx.doi.org/10.1155/2014/391528

Research Article

Induction of Epoxide Hydrolase, Glucuronosyl Transferase, and Sulfotransferase by Phenethyl Isothiocyanate in Male Wistar Albino Rats

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Received 12 September 2013; Revised 2 November 2013; Accepted 2 November 2013; Published 27 January 2014

Academic Editor: Lillian Barros

Phenethyl isothiocyanate (PEITC) is an isothiocyanate found in watercress as the glucosinolate (gluconasturtiin). The isothiocyanate is converted from the glucosinolate by intestinal microflora or when contacted with myrosinase during the chopping and mastication of the vegetable. PEITC manifested protection against chemically-induced cancers in various tissues. A potential mechanism of chemoprevention is by modulating the metabolism of carcinogens so as to promote deactivation. The principal objective of this study was to investigate in rats the effect of PEITC on carcinogen-metabolising enzyme systems such as sulfotransferase (SULT), N-acetyltransferase (NAT), glucuronosyl transferase (UDP), and epoxide hydrolase (EH) following exposure to low doses that simulate human dietary intake. Rats were fed for 2 weeks diets supplemented with PEITC at 0.06 μmol/g (low dose, i.e., dietary intake), 0.6 μmol/g (medium dose), and 6.0 μmol/g (high dose), and the enzymes were monitored in rat liver. At the Low dose, no induction of the SULT, NAT, and EH was noted, whereas UDP level was elevated. At the Medium dose, only SULT level was increased, whereas at the High dose marked increase in EH level was observed. It is concluded that PEITC modulates carcinogen-metabolising enzyme systems at doses reflecting human intake thus elucidating the mechanism of its chemoprevention.

1. Introduction

Phenethyl isothiocyanate (PEITC) is a phytochemical with an aromatic side chain, found in cruciferous vegetables such as watercress, where it is present as a glucosinolate, so called gluconasturtiin [1]. Whenever this vegetable is interrupted, for instance during mastication, the enzyme myrosinase (β-thioglucoside glucohydrolase) is released and induce the conversion of gluconasturtiin into PEITC as well as in the human intestine by microbial myrosinase [2]. Epidemiological studies reported an inverse association between cruciferous vegetable consumption and risk of cancers including lung [3], colorectal [4], and breast [5] cancers, all common cancers, mainly in developed and developing countries.

In animal-induced cancer model studies, PEITC has been reported to antagonise the carcinogenicity of chemicals in various tissues [6, 7]. It has exhibited a protection on chemically-induced carcinogenesis in the oesophagus, intestine, lung, and pancreas induced by azoxymethane, nitrosocompounds, and polycyclic aromatic hydrocarbons [6, 8–10], even though no beneficial effect was observed in colon cancer-induced by azoxymethane when the formation of aberrant crypt foci was used as biomarker [11]. Interestingly, the mercapturate of PEITC, an important metabolite, preserves its chemopreventive properties [12].

PEITC elicits its chemoprevention by blocking initiation [13] and post-initiation processes of tumour growth via modulating proliferation of cells cycle and induction of apoptosis [14–16]. Protecting against DNA damage and, thus,

suppressing tumour initiation step is a major importance anticarcinogenic mechanism of isothiocyanates [17]. This can be achieved by reducing the availability of the metabolite products of chemical carcinogens by averting their generation, thus inhibition of their cytochrome P450-mediated bio-activation [18–20], and/or by stimulating their detoxification, via induction of enzyme systems such as the quinone reductase and glutathione *S*-transferases [21]. Nevertheless, the chemopreventive properties of isothiocyanates at this stage are multiple depending on dose regimen, animal species [22], nature of isothiocyanate [23], target tissue [24], and treatment protocol [25].

In studies employing precision-cut liver slices, the ability of PEITC to modulate carcinogen-metabolising enzymes in rat and human liver has been established [26, 27] and as a result revealed its potency to function as an anticancer agent. It is important to assess whether carcinogen metabolising enzymes react similarly to PEITC in animal models *in vivo* following exposure to low doses that simulate human dietary intake.

Most studies have focussed on quinone reductase and the glutathione *S*-transferases, and the modulation of other major carcinogen-metabolising hepatic enzyme systems by PEITC still remains to be evaluated. The objective of the current study was to evaluate in rats the effect of PEITC intake, employing dietary levels of exposure, on carcinogen metabolising enzymes systems, for example, epoxide hydrolase, glucuronosyl transferase, sulfotransferase, and N-acetyltransferase. Modulation of carcinogen metabolising enzymes was investigated in a liver tissue, as the liver is the principal site of the bioactivation of carcinogens [28]. A marked induction of epoxide hydrolase was observed at the high dose, while at the low and medium doses, glucuronosyl transferase and sulfotransferase levels were elevated, respectively.

2. Materials and Methods

Phenethyl isothiocyanate (PEITC) (LKT Laboratories, MN, USA), benzo[a]pyrene 4,5-epoxide and benzo[a]pyrene 4,5-diol (Mid-West Research Institute, KS, USA), 1-naphthol, 2-naphthol, and 4-aminobenzoic acid (Sigma Co. Ltd., Poole, Dorset, UK) were all purchased.

Male Wistar albino rats (180 ± 20 g) were obtained from B&K Universal Ltd (Hull, East Yorkshire, UK). The animals were housed at 22 ± 2°C, 30–40% relative humidity, in an alternating 12 h light: dark cycle with light onset at 07.00 h. After a week's acclimatization, the rats were randomly assigned into 4 groups of 5 rats each. Animal doses were chosen so that the low dose responses the average human daily intake of glucosinolates (75 mg/person/day or 1.07 mg/kg bw) [29]. Three groups were administered diets supplemented with 0.06 (low dose), 0.6 (medium dose), and 6.0 (high dose) μmol PEITC/g diet, whereas one group served as control; animals were maintained on these diets for 14 days. At the end of the treatment period, rats were sacrificed and the livers were removed. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until required. Hepatic S9 of liver (25% w/v), in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, was prepared prior to microsomal and cytosolic separation by differential centrifugation. The following assays were carried out on isolated microsomes: glucuronosyl transferase (UDP) using 1-naphthol as substrate [30], epoxide hydrolase (EH) using benzo[a]pyrene 4,5-epoxide [31] as well as on the isolated cytosols, that is, sulfotransferase (SULT) using 2-naphthol as substrate [32], and N-acetyltransferase (NAT) using 4-aminobenzoic acid [33]; protein concentration was determined in both cellular subfractions using bovine serum albumin as standard [34].

Results are presented as mean ± standard deviation of groups of five rats each. Statistical evaluation was carried out by one-way ANOVA followed by the Dunnett's test.

3. Results and Discussion

The present study investigated the *in vivo* modulation of carcinogen-metabolising enzymes by PEITC, as this emerged as an impressing chemoprevention mechanism in the studies employing precision-cut rat [26] and human liver slices [27]. Rats were treated with diets supplemented with 3 different doses, 0.06 (low dose), 0.6 (medium dose), and 6.0 (high dose) μmol PEITC/g diet for 14 days. The low dose is proportionate with human dietary intake of total glucosinolates, 75.5 mg/person/day or 1.07 mg/kg body weight for a 70 kg individual [29], which is comparable to an intake of 300 g watercress, the primary source of PEITC, based on 100 g watercress releasing approximately 25 mg PEITC [35]. Earlier studies in animal models have utilised either a single high dose or chronic intake of higher doses than those employed in the current study [36, 37]. Studies were carried out in the liver as it the principal site of bioactivation of chemical carcinogens [28].

Even though the effects of isothiocyanates on quinone reductase and glutathione *S*-transferases have been well reported both *in vitro* and *in vivo*, their capability to modulate other phase II enzyme systems involved in carcinogen metabolism has received little attention. Glucuronosyl transferases are a very essential phase II detoxifying enzyme system involved in the metabolism of chemical carcinogens including aromatic amines and polycyclic aromatic hydrocarbons [38]. The current studies found that PEITC at the low dose has the potential to upregulate glucuronosyl transferase (Figure 1), which commensurate previous findings where cruciferous vegetable consumption led to increased glucuronidation of the heterocyclic amine PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) in human volunteers, even though other components in the vegetables are likely to have also contributed to the upregulation of this enzyme [39]. Similarly, the consumption of diets supplemented with cruciferous vegetables reduced serum bilirubin levels, the glucuronidation that was catalysed by glucuronosyl transferase 1A1 (UGT1A1) [40]. In addition, PEITC isolated from watercress enhanced the metabolism of nicotine due to increased glucuronidation among smokers [41], while in rat liver slices, PEITC led to increase in the glucuronidation of 1-naphthol [26].

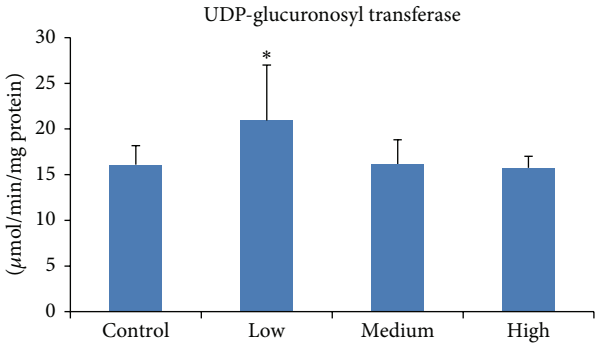

Figure 1: Effect of PEITC on glucuronosyl transferase activity in rat liver. Groups of five rats were exposed to diets supplemented with PEITC at 0.06 (low dose), 0.6 (medium dose), and 6.0 (high dose) μmol/g diet for 14 days, whereas another group served as control. At the end of the treatment period, hepatic S9 was prepared from which microsomes were isolated and used to determine glucuronosyl transferase activity. Results are presented as mean ± SD for five rats. $^{*}P < 0.05$.

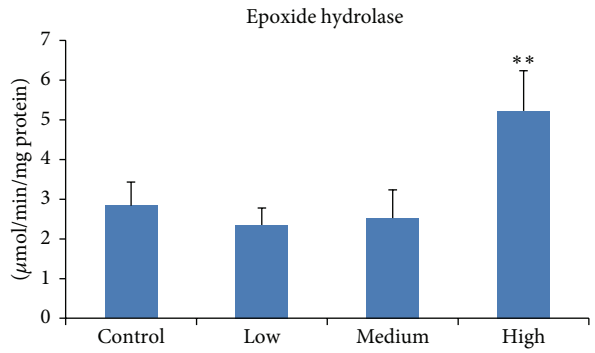

Figure 2: Effect of PEITC on epoxide hydrolase activity in rat liver. Groups of five rats were exposed to diets supplemented with PEITC at 0.06 (low dose), 0.6 (medium dose), and 6.0 (high dose) μmol/g diet for 14 days, whereas another group served as control. At the end of the treatment period, hepatic S9 was prepared from which microsomes were isolated and used to determine epoxide hydrolase activity. Results are presented as mean ± SD for five rats. $^{**}P < 0.01$.

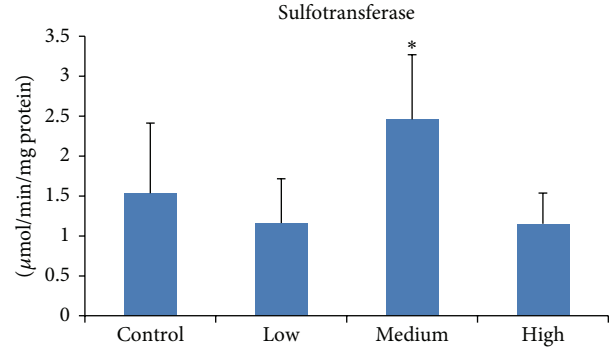

Figure 3: Effect of PEITC on sulfotransferase activity in rat liver. Groups of five rats were exposed to diets supplemented with PEITC at 0.06 (low dose), 0.6 (medium dose), and 6.0 (high dose) μmol/g diet for 14 days, whereas another group served as control. At the end of the treatment period, hepatic S9 was prepared, and then cytosol was isolated and used to determine sulfotransferase activity. Results are presented as mean ± SD for five rats. $^{*}P < 0.05$.

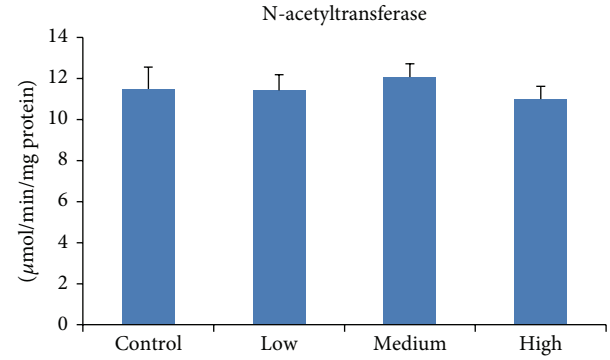

Figure 4: Effect of PEITC on N-acetyltransferase activity in rat liver. Groups of five rats were exposed to diets supplemented with PEITC at 0.06 (low dose), 0.6 (medium dose), and 6.0 (high dose) μmol/g diet for 14 days, whereas another group served as control. At the end of the treatment period, hepatic S9 was prepared and then cytosol was isolated and used to determine N-acetyltransferase activity. Results are presented as mean ± SD for five rats.

Epoxide hydrolase is the phase II enzyme involved in the detoxification of many epoxides, the reactive intermediates of chemical carcinogens including polycyclic aromatic hydrocarbons, aflatoxin B1, and halogenated aliphatic compounds [42]. The present study showed that at the high dose PEITC elevated epoxide hydrolase (Figure 2), in concordance with our previous findings [26]; an 8-fold rise in the activity was exhibited, rendering it one of the potent inducers of this enzyme.

Sulfotransferases are the enzymes that catalyse sulfonation, an important reaction involved in the metabolism of numerous xenobiotics, drugs, and endogenous compounds [43]. The process of sulfonation encompasses the transfer of a sulfonyl (SO_3^-) group, normally to a hydroxyl on an acceptor molecule, which is catalysed by sulfotransferases [44]. It was revealed that medium dose of PEITC increased the level of this enzyme indicating that PEITC accepting sulfonyl group to its hydroxyl (Figure 3). N-acetyltransferases are cytosolic conjugating enzymes which transfer an acetyl group from acetyl coenzyme A to a xenobiotic acceptor substrate [45]. Our finding showed that this enzyme was unaffected (Figure 4), elucidating that acetyl group was transferred to PEITC forming N-acetylcysteine (NAC) conjugate of phenethyl isothiocyanate (PEITC-NAC), the major metabolite of PEITC that is abundant in watercress [46].

4. Conclusions

The present studies allow us to infer that phenethyl isothiocyanate modulates carcinogen-metabolising enzyme systems at doses reflecting human intake as a marked induction of epoxide hydrolase which was seen at the high dose, while at the low and medium doses, glucuronosyl transferase

and sulfotransferase levels were upregulated, respectively. Increased levels of detoxification enzymes such as epoxide hydrolase, glucuronosyl transferase, and sulfotransferase are the likely one of the mechanisms for chemoprevention of PEITC.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

The authors would like to thank the Ministry of Education, Malaysia, for providing a research fund.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 943520, 6 pages
http://dx.doi.org/10.1155/2013/943520

Research Article

In Vitro Antiophidian Mechanisms of *Hypericum brasiliense* Choisy Standardized Extract: Quercetin-Dependent Neuroprotection

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Received 20 September 2013; Accepted 4 December 2013

Academic Editor: Marina Soković

The neuroprotection induced by *Hypericum brasiliense* Choisy extract (HBE) and its main active polyphenol compound quercetin, against *Crotalus durissus terrificus* (Cdt) venom and crotoxin and crotamine, was enquired at both central and peripheral mammal nervous system. Cdt venom (10 μg/mL) or crotoxin (1 μg/mL) incubated at mouse phrenic nerve-diaphragm preparation (PND) induced an irreversible and complete neuromuscular blockade, respectively. Crotamine (1 μg/mL) only induced an increase of muscle strength at PND preparations. At mouse brain slices, Cdt venom (1, 5, and 10 μg/mL) decreased cell viability. HBE (100 μg/mL) inhibited significantly the facilitatory action of crotamine (1 μg/mL) and was partially active against the neuromuscular blockade of crotoxin (1 μg/mL) (data not shown). Quercetin (10 μg/mL) mimicked the neuromuscular protection of HBE (100 μg/mL), by inhibiting almost completely the neurotoxic effect induced by crotoxin (1 μg/mL) and crotamine (1 μg/mL). HBE (100 μg/mL) and quercetin (10 μg/mL) also increased cell viability in mice brain slices. Quercetin (10 μg/mL) was more effective than HBE (100 μg/mL) in counteracting the cell lysis induced by Cdt venom (1 and 10 μg/mL, resp.). These results and a further phytochemical and toxicological investigations could open new perspectives towards therapeutic use of *Hypericum brasiliense* standardized extract and quercetin, especially to counteract the neurotoxic effect induced by snake neurotoxic venoms.

1. Introduction

An estimated 5.4-5.5 million people are bitten by snakes each year, resulting in about 400.000 amputations and about 125.000 deaths [1, 2]. The problem of human suffering by snake bite is actually so relevant that WHO has included it in the list of neglected tropical diseases in April, 2009 [3].

Snake venoms embody a complex mixture of toxic enzymes and proteins, such as myotoxins, neurotoxins, cytotoxins, hemorrhagic metalloproteases, clotting serineproteases, and others [4]. Among all snake venoms, the crotalic is one of the most neurotoxic, in which systemic effects reside primarily in the peripheral neurotoxicity. However, when injected directly on CNS of mammals it can induce convulsion and death [5]. Among other symptoms, the neurotoxicity induced by *Crotalus* poisoning in both central and peripheral nervous system is mainly related to the presence in the venom of the toxins crotoxin [6] and crotamine [7]. Thus, the search of novel venom inhibitors is therefore relevant, being natural or synthetic, in order to complement the current serum therapy and to neutralize the remaining damages of snake envenomation.

Hypericum brasiliense is an annual cycle plant, recurrent in the southern and southeastern Brazil, known by the common names of "milfurada", "milfacadas," and "alecrim bravo" [8, 9]. *H. brasiliense* extract has shown anti-inflammatory and analgesic [10] activities, with contradictory signs on the CNS [11] and protection of mice against lethality of *Bothrops jararaca* venom [12].

The present work demonstrates the ability of *Hypericum brasiliense* standardized extract and quercetin to counteract neurodegenerative insults induced by Cdt venom in brain and muscles preparations. In addition, it is shown that the major neurotoxic components of the *Crotalus durissus terrificus* venom, crotoxin and crotamine, also had their effects prevented in the neuromuscular paralysis at mouse nerve-muscle preparations.

2. Experimental

2.1. Reagents and Venom. All chemicals and reagents used were of the highest purity and were obtained from Sigma, Aldrich, Merck or BioRad. *Crotalus durissus terrificus* venom, crotamine and crotoxin were donated by Dr. S. Marangoni (UNICAMP) and quercetin by Dr. L. Rocha (UFF).

2.2. Animals. Adult Swiss white mice (28–35 g) from both sexes were supplied by the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and by the animal facility from Universidade Federal de Santa Maria (UFSM). The animals were housed at 25°C with access *ad libitum* to food and water. These studies have been done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.3. Plant Material. Hypericum brasiliense leaves were collected in the city of Nova Friburgo, RJ, Brazil, in 2001. A voucher specimen (n°19980) has been deposited at the herbarium of the Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil.

2.4. Chemical Analysis. The preparation of *H. brasiliense* EtOH extract (HBE) and detection of its chemical composition were carried out as described elsewhere [13]. Briefly, the chemical analysis was performed with a Liquid Chromatograph (GBC Scientific Equipment LLC, Hampshire, IL, USA), equipped with a Nucleosil MN 120-5 C_{18} silica column (Macherey-Nagel Inc., Bethelehem, PA, USA). The elution was made at room temperature using a linear gradient from 10–60% of acetonitrile in trifluoroacetic acid (0.05% v/v) at a flow rate of 1.0 mL/min in 30 minutes. Peaks were monitored at 254 nm in order to quantify the flavonoid quercetin.

2.5. Hippocampal Slices Preparation. Mice were decapitated, the brains removed immediately, and the hippocampus dissected on ice and humidified in cold HEPES-saline buffer gassed with O_2 (124 mm NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 12 mM glucose, 1 mM $CaCl_2$, and 25 mM HEPES pH 7.4). Hippocampal slices were obtained according to Vinadé & Rodnight [14], briefly: a Mcilwain tissue chopper was used to obtain the slices (0.4 mm) that were separated and preincubated at 37°C for 30 min in microwell plates filled with HEPES saline (200 μL/slice). Subsequently, fresh medium was replaced (200 μL/slice) for control condition and treatments with Cdt (1, 5 and 10 μg/mL), HBE (100 μg/mL), HBE + Cdt, quercetin (10 μg/mL), and quercetin + Cdt and incubated for 1 hour (37°C).

2.6. Hippocampal Slices Viability. Immediately after incubation with treatments, slices were assayed for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (0.05% in HEPES-saline) for 30 min (37°C) [15]. The MTT is converted into a purple formazan product after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by the addition of DMSO, resulting in a colored compound whose optical density (λ = 550 nm) was measured in an ELISA reader equipment [16].

2.7. Phrenic Nerve-Diaphragm Preparation. Whole diaphragms along with the phrenic nerves were removed from mice killed by carbon dioxide (CO_2) and exsanguinated. Both hemidiaphragms were mounted essentially as described for dal Belo et al. [17]. The preparations were suspended under a constant tension of 5 g in a 5 mL organ bath containing aerated (95%O_2–5%CO_2) Tyrode solution (pH 7.4, 37°C) of the following composition (mM): NaCl 137, KCl 2.70, $CaCl_2$ 1.80, $MgCl_2$ 0.490, NaH_2PO_4 0.420, $NaHCO_3$ 11.9, and glucose 11.1. Supramaximal stimuli (0.1 Hz, 0.2 ms) delivered by a Grass S4 electronic stimulator (Grass Instrument Co., Quincy, MA, USA) were applied through electrodes placed around the motor nerve, corresponding to an indirect stimulation.

2.8. Statistical Analysis. The results were expressed as the mean ± SEM and were compared statistically using ANOVA for repeated measures. A P value < 0.05 indicated significance.

3. Results

HBE was shown to be rich in flavonoids derivatives such as kaempferol, quercetin, and quercetin glycosides (quercitrin, isoquercitrin, guaijaverin, and hyperoside) [13]. The selective extraction of polyphenol compounds in HBE resulted, after hydrolysis, in not less than 6,7% of total flavonoids, expressed as quercetin. Incubation of mouse phrenic nerve-diaphragm preparation (PND) with Tyrode solution did not induce alterations in basal muscle twitch tension during 120 min recordings (n = 5, Figure 1). When *Crotalus durissus terrificus* venom (Cdt, 10 μg/mL) was added to (PND) preparation there was an increase of 160% in the muscle twitch tension followed by an irreversible and complete neuromuscular blockade after 70 min (n = 5, Figure 1). Incubation of PND preparation with HBE (10 and 100 μg/mL) produced no alteration in the amplitude of muscle twitch tension (n = 5), during 120 min observation. However, when preparations were assayed with a mixture of HBE (50 μg/mL and 100 μg/mL) and Cdt venom (10 μg/mL) previously incubated

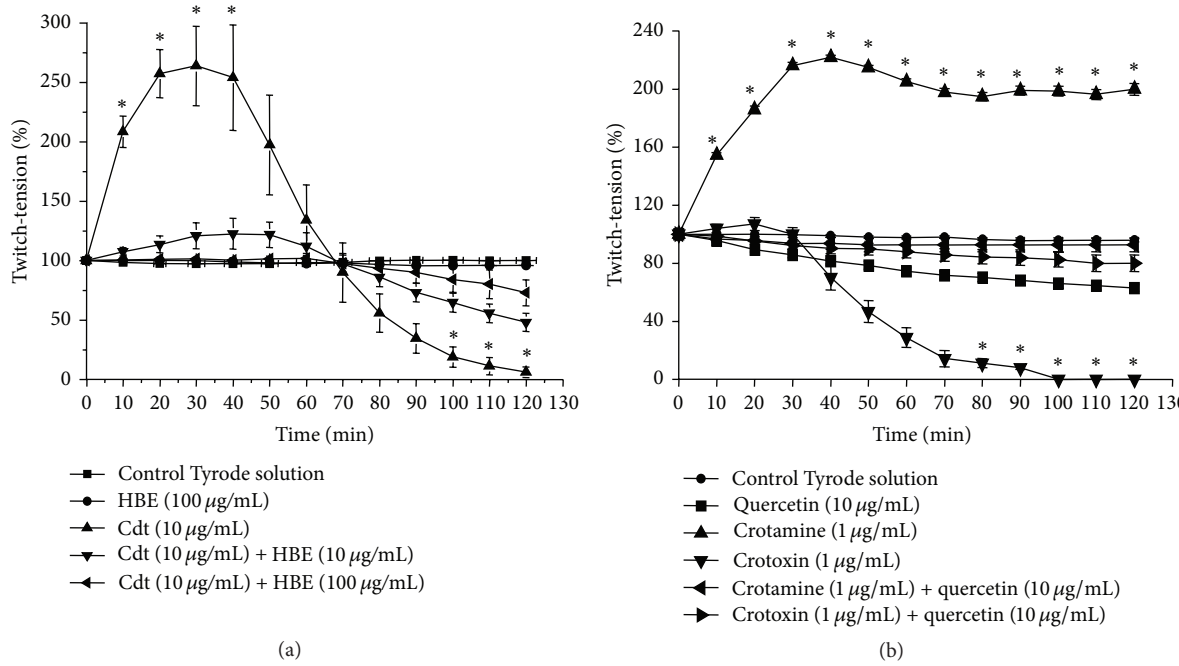

Figure 1: Neutralizing activity of *H. brasiliense* ethanolic plant extract (HBE) against Crotalic venom, crotoxin and crotamine at mouse phrenic nerve-diaphragm preparations. Panel (a) shows the inhibitory effect of HBE (10 and 100 μg/mL) against *C. d. terrificus* venom (10 μg/mL), crotoxin and crotamine. In Panel (b) effect of Quercetin (10 and 100 μg/mL) against Cdt (10 μg/mL), crotoxin (1 μg/mL), and crotamine (1 μg/mL). When HBE was applied alone in the organ-bath no alteration in the twitch-tension was observed. On both graphs control Tyrode solution lines show no alteration of normal nerve-muscle activity. The points on the graphs represent the mean ± S.E.M. of five experiments. On (b) note that quercetin mimicked the protective effect induced by HBE. HBE: *Hypericum brasiliense* standardized extract $^{*}P < 0.05$ compared to control Tyrode.

during 30 min at 37°C, the characteristic neuromuscular blockade was prevented in 75% with the highest concentration of the extract (Figure 1(a), $n = 5$, $P < 0.05$). The assay of the myotoxin crotamine (1 μg/mL) alone at PND preparations induced a significative increase of muscle twitch tension (~150%), that was maximum at 30 min ($P < 0.05$, $n = 6$, Figure 1(b)). On the contrary, the addition of the PLA_2 neurotoxin crotoxin isolated (1 μg/mL) at PND preparations caused a progressive and irreversible neuromuscular blockade during 120 min recordings ($P < 0.05$, $n = 6$). The assay of HBE (100 μg/mL) + crotamine (1 μg/mL) or crotoxin (1 μg/mL), previously incubated for 30 min at 37°C, inhibited 100% of the facilitatory actions induced by crotamine and 85% of the neuromuscular blockade caused by crotoxin (1 μg/mL), respectively, in 120 min recordings ($n = 5$, $P < 0.05$, data not shown). When quercetin (10 μg/mL) was incubated alone, there was a maximum decrease of muscle twitch tension of 20 ± 0.5% in 120 min recordings, although not significative (Figure 1(b), $P > 0.05$ compared to the control Tyrode). The addition of quercetin (10 μg/mL) with crotamine (1 μg/mL) or crotoxin (1 μg/mL) previously incubated for 30 min at 37°C showed a more potent antineurotoxic activity when compared to the HBE. This increased potency of quercetin compared to HBE must be due to a higher effective concentration of the flavonoid when compared to the whole extract (~7%). Quercetin was able to completely inhibit the facilitatory actions of crotamine (1 μg/mL) and decreased in 80 ± 5% the neuromuscular blockade induced by crotoxin (1 μg/mL) ($n = 5$, $P < 0.05$, Figure 1(b)).

The effect of HBE (100 μg/mL) or quercetin (10 μg/mL) alone was accessed at central nervous system (CNS) through hippocampal slices. In this set of experiments the cell viability was not modified after 1 h incubation with both vegetal extract and the pure flavonoid. On the other hand, the incubation of Cdt venom in doses of (1, 5, and 10 μg/mL) significantly decreased the cell viability (40 ± 3, 14 ± 1 and 28 ± 1%, $n = 3$, $P < 0.05$, resp.) (Figures 2(a) and 2(b)). The addition of HBE (100 μg/mL) with Cdt (10 μg/mL) to the slices produced a slight protection compared to the control Cdt ($n = 3$, $P < 0.05$) (Figure 2(a)). However, the blend of quercetin (10 μg/mL) and Cdt (1 μg/mL or 5 μg/mL), significantly inhibited the cell lysis showing a protection in the order of 46 ± 2% and 12 ± 1%, $n = 4$, $P < 0.05$, respectively (Figure 2(b)). The results in hippocampal slices confirm the HBE and quercetin potential role in the neuroprotection against Cdt poisoning. Therefore, the difference in potency between HBE and quercetin must also be related to the less amount of the flavonoid in the extract.

4. Discussion

In this work we described for the first time the effectiveness of the *H. brasiliense* extract (HBE) and its marked compound quercetin, against the neuromuscular paralysis induced by

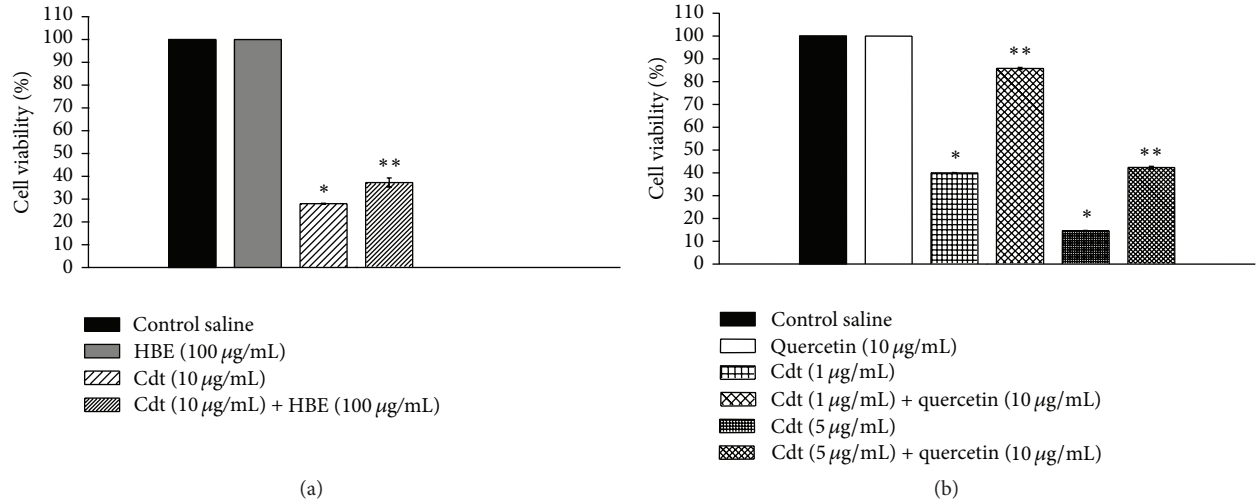

Figure 2: Effects of Cdt on the viability of hippocampal slices. (a) Hippocampal slices were incubated with HBE (100 μg/mL) in the presence or absence of Cdt (10 μg/mL) during 1 hour. (b) Hippocampal slices were incubated during 1 hour with Quercetin (10 μg/mL) in the presence or absence of Cdt (1 and 5 μg/mL). Cell viability was measured by MTT test. Values are expressed as % of control, which was defined as untreated slices (values are means ± S.E.M., $n = 4$). On (b) note that quercetin mimicked the HBE protective activity. HBE: *Hypericum brasiliense* standardized extract** $P < 0.05$ compared to control*.

Crotalus durissus terrificus snake venom (Cdt), crotoxin, and crotamine at mouse phrenic nerve-diaphragm preparations. Also, the effectiveness of HBE and quercetin was validated, to counteract the deleterious effects induced by *C. d. terrificus* venom, on cell viability of mouse brain slices. Crotalus venom induces neurotoxicity, coagulation disorders, systemic myotoxicity, and acute renal failure [18], with possible additional heart and liver damage [19]. This venom is a mixture of enzymes, toxins (crotoxin, crotamine, gyroxin, and convulxin), and several other peptides [19]. The characteristic pathophysiological pictures of neurotoxicity and systemic myotoxicity associated with *C. d. terrificus* envenomation are mainly related to the presence in the venom of crotoxin, a neurotoxic PLA_2 heterodimeric complex, which causes progressive paralysis, and in high concentrations myonecrosis [20, 21]. At nerve terminals, crotoxin induces triphasic alterations in the mean quantal content of transmitter release with a slow and progressive decrease of presynaptic release of the neurotransmitter acetylcholine that results in complete neuromuscular blockade [22, 23]. At mammal central nervous system, the injection of Cdt venom induces seizures [5], which is mainly associated with the presence of crotoxin [24]. At brain synaptosomes, crotoxin has also shown the ability of inhibiting L-glutamate and gamma aminobutyric acid (GABA) uptake [25]. Crotamine is the second major toxin in the Cdt venom; it is a basic, low molecular weight myotoxin devoid of PLA_2 activity [26], with a specific action on voltage-sensitive sodium channels of muscles [27] and brain cells [28].

Flavonoids are plant secondary metabolites that embrace a wealth of possibilities of hydrogen bonding arranged around a relatively small carbon skeleton, capable of interacting with molecular targets [29]. In the *H. brasiliense* extract, the flavonoid quercetin and its derivatives were shown to be the major secondary metabolites in the plant. Quercetin and several of its glycosides are the most often encountered flavonoids in anti-snake venom plants where *Albizia lebbeck*, *Achillea millefolium*, *Euphorbia hirta*, *Camellia sinensis*, and *Casearia sylvestris* are some examples. Flavonoids have been reported as snake venom phospholipase A_2 inhibitors [30].

Recent studies revealed that the treatment of the snake venom PLA_2 isoform from *Crotalus durissus cascavella* snake venom with the flavonoid quercetin produced a decrease in the pharmacological activity of the neurotoxin by inducing alterations in the secondary but not in tertiary structure composition of the molecule [31]. As discussed above, flavonoids have the ability of binding to biological polymers (e.g., enzyme inhibiting activities). Therefore, snake PLA_2 catalyzed the production of lysophospholipids and fatty acids that are involved in membrane damage [21]. We suggest that, in the case where biological activity is enzyme-dependent, the HBE antineurotoxic activity would involve the inactivation of PLA_2 activity by quercetin. However, the possibility that the HBE acts through a mechanistic intervention rather than an *in vitro* direct physical interaction with the venom is also a reasonable idea. This is likely to be the mode of action of many polyphenolic compounds found in plant extracts, which probably explains many of the "protective" effects of plant extracts when they are preincubated with venom before administration to the biological assay [32, 33].

Flavonoids derived from plants or tea extracts also affect acetylcholine release, muscle contraction, or neuromuscular junction activity [34]. In this regard, the muscle-type nicotinic acetylcholine receptor consists of $\alpha 1\beta 1\varepsilon$, in adult tissue [35]. It was found that quercetin inhibits the muscle type nicotinic acetylcholine receptor, by binding on the γ or ε subunities, which is a characteristic of a noncompetitive inhibitor [36]. Crotoxin also stabilizes the postsynaptic membrane of *Torpedo marmorata* by binding in non-ACh biding

sites [37]. Hence, these similarities in terms of binding sites would strengthen the hypothesis of a site-direct antagonism between quercetin and crotoxin at nerve terminals. In addition, quercetin actively participates in intracellular signaling, inhibiting phosphatidylinositol-3 kinase, protein kinase C, xanthine oxidase, and NADPH diaphorase [34]. In massive cellular insults like ischemia, involving metabolic failure, loss of Ca^{2+} homeostasis, and excitotoxicity, scavenger activity or one-target antioxidant mechanisms (NMDA receptor blockers, chain-breaking vitamin E, or pure scavenger molecules such as boldine) may fail to protect cells from free radical damage. Current explanation for the neuroprotective effect of quercetin is its antioxidant capacity and its ability to scavenge free radicals [34]. At moment there is no evidence that snake venoms induce cellular insults to increase free radicals in nerve terminals. However, the actions of Cdt venom on cell viability of brain slices is likely to be devoid to the presence of crotoxin and crotamine that ultimately account for the increase of excitatory neurotransmitters [22], resulting in excitotoxicity [38]. The decrease in neurotransmitter uptake by crotoxin is calcium independent [25], and quercetin potentiates neuronal excitability by increasing neuronal firing rates [39]. Ultimately, excitotoxicity is a result of synaptic dysfunction processes, which involves the excessive glutamate receptor activation and neuronal degeneration [38]. Based on the above considerations we suggest that the mechanism of the benefit of quercetin on snake venom-induced neuronal cellular death is complex and beyond the inhibition of presynaptic activity of snake PLA_2, and structural modifications, which may affect neurotransmitter uptake, involve the maintenance of neuronal mitochondrial transmembrane electric potential which would decrease the overstimulation of glutamate receptors [34]. However, in the case of crotamine, a direct inhibition of voltage-gated sodium channels by quercetin seems to be a coherent explanation [40].

Further investigation on *Hypericum brasiliense* isolated compounds will strengthen the understanding of its antiophidian activity. Preclinical assays, including safety assessment protocols, could also open the way towards therapeutic use of *Hypericum brasiliense* especially when neurotoxic venoms are involved.

Conflict of Interests

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

Acknowledgments

The authors thank Gildo Bernardo Leite for the excellence in technical assistance with mouse phrenic nerve-diaphragm preparations. Dr. Thais Posser for kind loan of MTT. This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundo de Apoio ao Ensino, à Pesquisa e Extensão (FAEPEX), and Coordenação de Aperfeiçoamento de Pessoal de nível Superior-CAPES Edital Toxinologia 063/2010.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 351602, 10 pages
http://dx.doi.org/10.1155/2013/351602

Research Article

Antioxidant and Toxicity Studies of 50% Methanolic Extract of *Orthosiphon stamineus* Benth

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Received 5 October 2013; Revised 23 November 2013; Accepted 24 November 2013

Academic Editor: Isabel C. F. R. Ferreira

The present study evaluated the antioxidant activity and potential toxicity of 50% methanolic extract of *Orthosiphon stamineus* (Lamiaceae) leaves (MEOS) after acute and subchronic administration in rats. Superoxide radical scavenging, hydroxyl radical scavenging, and ferrous ion chelating methods were used to evaluate the antioxidant properties of the extract. In acute toxicity study, single dose of MEOS, 5000 mg/kg, was administered to rats by oral gavage, and the treated rats were monitored for 14 days. While in the subchronic toxicity study, MEOS was administered orally, at doses of 1250, 2500, and 5000 mg/kg/day for 28 days. From the results, MEOS showed good superoxide radical scavenging, hydroxyl radical scavenging, ferrous ion chelating, and antilipid peroxidation activities. There was no mortality detected or any signs of toxicity in acute and subchronic toxicity studies. Furthermore, there was no significant difference in bodyweight, relative organ weight, and haematological and biochemical parameters between both male and female treated rats in any doses tested. No abnormality of internal organs was observed between treatment and control groups. The oral lethal dose determined was more than 5000 mg/kg and the no-observed-adverse-effect level (NOAEL) of MEOS for both male and female rats is considered to be 5000 mg/kg per day.

1. Introduction

Herbal medicines have received a great deal of attention as alternative medicines in recent years in Malaysia and are sold as dietary supplements. One of the Malaysian local herbs, scientifically known as *Orthosiphon stamineus* Benth (Lamiaceae) or locally called Misai Kucing, has attracted much attention for research purposes. *Orthosiphon stamineus* is also found in other countries such as Thailand, Indonesia, and Europe. It is widely used for the treatment of many diseases, especially those affecting the urinary tract, diabetes mellitus, hypertension, rheumatism, tonsillitis, and menstrual disorders [1]. The benefits of the traditional use of *Orthosiphon stamineus* have also been supported by the isolation and identification of several possible active chemical constituents from this plant, including flavonoids [2, 3], terpenoids [4, 5], saponins, hexoses, organic acids, caffeic acid derivatives, chromene, and myo-inositol [4–6]. Among the reported compounds, the most important components of *Orthosiphon stamineus* leaves are the polyphenols; polymethoxylated flavonoids such as sinensetin and eupatorine; caffeic acid derivatives, which include rosmarinic acid, cichoric acid, and caffeic acid [6].

Notwithstanding the widespread and long time usage of this plant, little toxicological information is available regarding the safety following chronic consumption of *O. stamineus* especially the bioactive 50% methanolic extract which has been reported to be effective in protection against alcohol-induced gastropathy, CCl_4-induced liver damage, antipyretic, anti-inflammatory, and analgesic effects [7–10]. Currently, Malaysian authorities are paying more and more attention on the safety and potential toxicity of botanicals, including medicinal plants and edible materials. Therefore, the aims of the present study were to evaluate the antioxidant

and provide scientific data on the safety of *O. stamineus*, focusing on the 14-day acute and 28-day subchronic toxicity of bioactive 50% methanolic extract of *O. stamineus,* using Sprague-Dawley (SD) rats, and hence, providing guidance for selecting a safe dose of *O. stamineus* in its use as a traditional medicine.

2. Materials and Methods

2.1. Chemical. Ferrous chloride ($FeCl_2$), hematoxylin, ferrozine, 2-deoxyribose, ethylenediaminetetraacetic acid (EDTA), ferrous sulfate ($FeSO_4$), trichloroacetic acid (TCA), nitroblue tetrazolium (NBT), xanthine, xanthine oxidase sodium phosphate (dibasic), sodium phosphate (monobasic), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), manganese chloride, xylenol orange, manganese chloride, and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Sigma (St. Louis, MO, USA). Absolute alcohol and eosin were purchased from Riedel-de Haën (Seelze, Germany). Paraplast was purchased from Oxford Labware (St. Louis, MO, USA). Xylene was purchased from Fisher Scientific (Leics, UK). Disposable microtome blades 818 were purchased from LEICA (Germany). Thiobarbituric acid (TBA) was purchased from AppliChem (Darmstadt, Germany). 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, sinensetin, and eupatorin were obtained from Indofine Chemical Company (NJ, USA). HPLC-grade acetonitrile and isopropyl alcohol were purchased from Merck (Darmstadt, Germany).

2.2. Plant Materials and Extract. Orthosiphon stamineus was grown from cuttings using standard agronomic practices at Kepala Batas, Pulau Pinang, Malaysia. The leaves of the plant were collected after flowering. The leaves were identified by Mr. Adenan Jaafar, School of Biological Sciences, Universiti Sains Malaysia. A voucher specimen (number 10810) was deposited at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia. The leaves were rinsed and dried in oven at 40°C for 2 days. The dried leaves were then ground by an electric grinder to a coarse powder and weighed. Subsequently, 300 g of powdered leaves of *O. stamineus* was extracted with 5 L methanol : water (50 : 50 v/v) at 60°C (for 8 hours) by maceration method. The resulting 50% methanolic extract of *O. stamineus* (MEOS) was concentrated using a Büchi-RE121 evaporator (Büchi Laboratorium-Technik, Switzerland) equipped with a Büchi-B169 vacuum system and then lyophilized in a Hetovac VR-1 (HETO Lab Equipment, Denmark) freeze dryer. The weight of the MEOS was recorded and the final plant-to-extract ratio was about 6%. The MEOS was then kept in desiccators in a refrigerator (0–4°C). The MEOS was freshly prepared daily by dissolving in distilled water.

2.3. HPLC Study

2.3.1. HPLC Instrumentation. HPLC analysis was performed using a Shimadzu-LC system (Shimadzu, Japan) equipped with a CBM-20A controller, LC-20AT pump, DGU-20A5 degasser, SIL-20A autosampler, SPD-20AV detector, and CTO-10ASvp column oven.

2.3.2. Chromatographic Conditions. Chromatographic separations were achieved using an Agilent Eclipse Plus C18 (250 × 4.6 mm i.d.; 5 μm). A Zorbax guard fittings kit packed with replaceable Eclipse Plus C18 Guard column (12.5 × 4.6 mm i.d.; 5 μm) was used to protect the analytical column. A reverse-phase HPLC assay was carried out using an isocratic system with a flow rate of 1 mL/min, a column temperature of 25°C, and a mobile phase of acetonitrile : isopropyl alcohol : 20 mM phosphate buffer (NaH_2PO_4) (30 : 15 : 55 v/v), with pH adjusted to 3.5 using 85% phosphoric acid. The UV detection was set at 340 nm. The injection volume was 20 μL of solution. Total run time was less than 20 min for each injection. Data was acquired and processed with LC-Solution software. The peaks were detected at 340 nm and identified using reference standards of 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, sinensetin, and eupatorin. Sinensetin and eupatorin were selected as standards because these two compounds were found to be the bioactive ingredients in the plant [11–13].

2.4. Antioxidant

2.4.1. Ferrous Ion Chelating Activity. Ferrous chelating activity was determined according to the method of Dinis et al. [14]. MEOS was added to a solution of 0.05 mL ferrous chloride ($FeCl_2$) (2 mM). The reaction was initiated by the addition of 0.2 mL ferrozine (5 mM) and the mixture was shaken vigorously and left standing at room temperature (24 ± 2°C) for 10 min. Absorbance of the solution was then measured spectrophotometrically (U-2000, Hitachi, Japan) at wavelength 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following formula:

$$\text{Ferrous ion chelating activity} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (1)$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of extracts. The experiment was run together with butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) as positive controls.

2.4.2. Hydroxyl Radical Scavenging Activity. Competition between deoxyribose and the extract against hydroxyl radical generated from the Fe^{3+}/ascorbate/ethylenediaminetetraacetic acid (EDTA)/hydrogen peroxide (H_2O_2) system was measured to determine the hydroxyl radical scavenging activity of MEOS [15]. The reaction mixture consisted of 0.30 mL of 0.02 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$, 0.15 mL of 10 mM EDTA, 0.15 mL of 10 mM H_2O_2, 0.525 mL of H_2O, and 0.075 mL of extract. The reaction mixture was incubated at 37°C for 2 h and the formed TBARS were measured. Seventy five milliliter of 2.8% trichloroacetic acid and 0.75 mL of 1.0% TBA in 50 mM NaOH were added to test tubes and boiled for 20 min. After cooling the mixture, absorbance was measured at 520 nm. The percentage of inhibition of inhibition rate of

2-deoxyribose oxidation by hydroxyl radical was given in the following formula:

$$\text{Hydroxyl radical scavenging activity} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (2)$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of extracts.

2.4.3. Superoxide Anion Radical Scavenging Activity. Superoxide anion radical generated by the xanthine/xanthine oxidase system was determined spectrophotometrically by monitoring the product of nitroblue tetrazolium (NBT) [16]. The reaction mixture consisted of 1.0 mL of 0.05 M phosphate buffer (pH 7.4), 0.04 mL of 3 mM xanthine, 0.04 mL of 3 mM EDTA, 0.04 mL of 0.15% bovine serum albumin, 0.04 mL of 15 mM NBT, and 0.04 mL of sample solution. After incubation at 25°C for 10 min, the reaction was initiated by adding 0.04 mL of 1.5 U/mL xanthine oxidase and carried out at 25°C for 20 min. After 20 min, the absorbance of the reaction mixture was measured at 560 nm. Decreased absorbance of the reaction mixture indicates increased superoxide anion radical scavenging activity.

2.4.4. Lipid Peroxidation Inhibition Study. Fresh livers were obtained from healthy male adult SD rats. They were then homogenized according to the method [17] for the preparation of 40% (w/v) liver homogenates in methanol. 80 μL of liver homogenate was mixed with 10 μL of Fenton's reagent (containing 5 μL of 5 mM manganese chloride and 5 μL of 50 mM hydrogen peroxide) and 10 μL of various concentrations of MEOS (for control, the extract was replaced with distilled water). The mixture was incubated for 30 min at 37°C to produce lipid peroxidation. The mixture was then added with 900 mL FOX reagent (containing 49 mg ammonium ferrous sulfate in 50 mL of 250 mM H_2SO_4, 0.397 g of BHT, and 0.038 g of xylenol orange in 950 mL methanol). Absorbance of the mixture was read at 560 nm after 30 min of incubation at room temperature. The percentage of inhibition was calculated with the following equation:

$$\%\ \text{of inhibition} = \left[\frac{(A_0 - A_1)}{A_1}\right] \times 100, \quad (3)$$

where A_0 was the absorbance in the presence of extracts and A_1 was the absorbance of the control.

2.5. Toxicology Studies

2.5.1. Experimental Animals. Eight-week-old male and female Sprague-Dawley rats (160–190 g) were purchased from the Animal House, School of Pharmaceutical Sciences, Universiti Sains Malaysia. The rats were acclimatised to laboratory conditions for 7 days prior to performing the experiments. All rats were kept at 26 ± 3°C, with a light/dark cycle of 12 hours. The rats were housed in a single polycarbonate cage (3 rats per cage) with free access to food (normal laboratory chow, Gold Coin) and tap water *ad libitum*. All the procedures were performed according to the Animal Ethics Guidelines of Universiti Sains Malaysia.

2.5.2. Acute Toxicity Study. The experiment was performed according to the Organisation for Economic Cooperation and Development (OECD) revised up and down procedure for acute toxicity testing [18]. All the rats were fasted overnight and weighed before the extract was administered. A maximum dose of 5000 mg/kg of MEOS was administered by oral gavage to 5 healthy female adult Sprague-Dawley rats. The administration volume was adjusted between 1 and 2 mL for each and every rat. After administration of MEOS to the first rat, the rat was observed for clinical signs of toxicity for the first hour, then hourly for three hours, and then periodically throughout 48 hours. Other rats were administrated sequentially at 48-hour intervals if the first rat survived after 48 hours of treatment. All the experimental rats were maintained under close observation throughout the 14 days and the number of mortality was recorded. The LD_{50} was predicted to be above 5000 mg/kg if three or more rats survived after the experimental period [19].

2.5.3. Subchronic Toxicity Study. Experimental Sprague-Dawley rats of either sex were randomly assigned to 4 groups ($n = 12$; six males and six females per group), and their weights were recorded. Different doses of MEOS (1250, 2500, or 5000 mg/kg) were prepared in distilled water and administered daily as single doses to different groups of rats: group 1 (5000 mg/kg), group 2 (2500 mg/kg), and group 3 (1250 mg/kg), while group 4 (control) received only distilled water. Toxic manifestations and mortality were monitored daily for 28 days. The bodyweights of all the rats were measured and recorded at the end of every week and the treated rats were anesthetised using CO_2 after 28 days of treatment. Blood samples were collected via cardiac puncture and transferred into nonheparinised and EDTA-containing tubes for both biochemical and haematological analyses, respectively [20]. Thereafter, the rats were killed by cervical dislocation. All organs, brain, heart, lungs, thymus, liver, kidneys, adrenal glands, sex organs (ovaries and uterus for female rats; testes for male rats), spleen, stomach, and gut (begining from small intestine until the end of large intestine), of all experimental rats were excised, weighed, and examined macroscopically. Vital organs such as lungs, kidneys, livers, and stomach were then preserved in 10% formalin for histopathological study [19].

2.5.4. Relative Organ Weight. The excised organs were weighed individually. The relative organ weight index of each organ to its bodyweight was calculated as (weight of organ/bodyweight of rats on the day of sacrifice) × 100% [20].

2.5.5. Blood Analyses. Haematological and biochemical analyses were performed at the Pathology Laboratory, Lam Wah Ee Hospital, Penang. Complete blood cell counts were determined using a fully automated haematological analyser Abbott Cell-Dyn 3500 (Abbott Laboratories, IL, USA), while

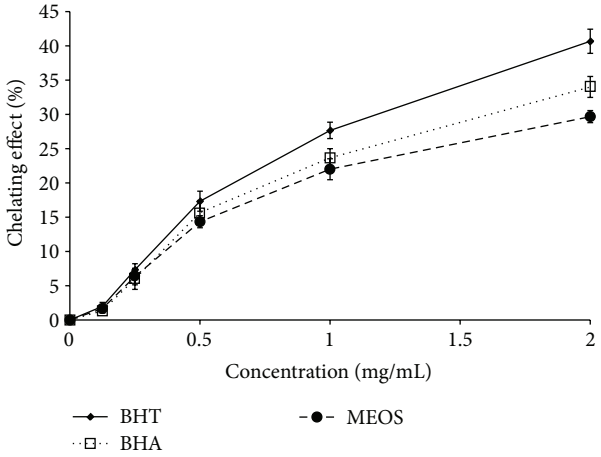

Figure 1: Ferrous ion chelating activity of MEOS ($n = 3$). Data are expressed as mean ± SD.

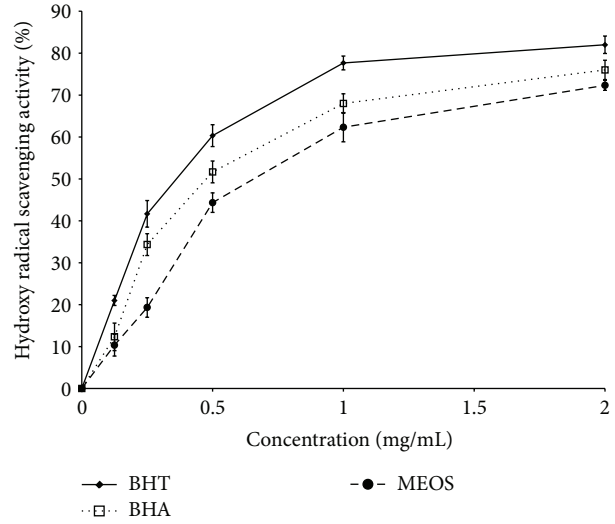

Figure 2: Hydroxyl radical scavenging activity of MEOS ($n = 3$). Data are expressed as mean ± SD.

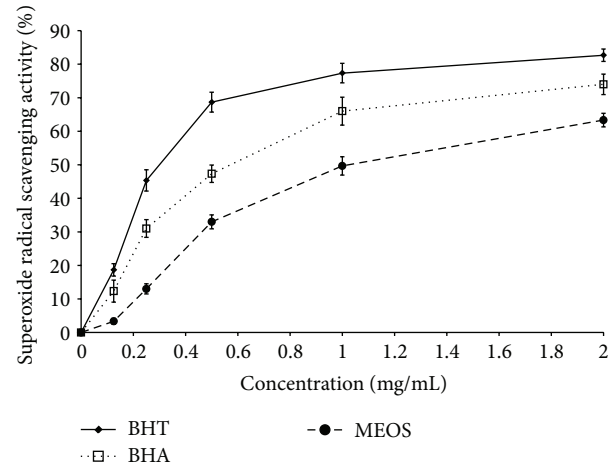

Figure 3: Superoxide radical scavenging activity of MEOS ($n = 3$). Data are expressed as mean ± SD.

serum biochemistry tests were performed using a COBAS Integra 800 (Roche, Germany) [20].

2.5.6. Histopathological Study. Vital organs (kidney and liver) were processed using a Citadel 1000 Histokinette (Shandon Scientific Ltd., Cheshire, UK). These tissues were embedded in paraffin using a Histo-Center II-N (Barnstead/Thermolyne Corp., Dubuque, IA, USA) and cut into 5 μm thick sections with a Reichert-Jung Histocut 820 II (Cambridge Instrument GmbH, Nussloch, Germany). All the sections were stained with haematoxylin and eosin and examined microscopically [20].

2.6. Statistical Analysis. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS). All the data are indicated as mean ± standard error of mean (SEM) and were analysed using one-way analysis of variance (ANOVA). Significant differences between the groups were determined using a Dunnett-comparison test with $P < 0.05$ taken as significant.

3. Results

3.1. HPLC Analysis. The objective of HPLC analysis in this experiment was to standardize the MEOS using sinensetin, eupatorin, and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone. From the results, the percentages of sinensetin, eupatorin, and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone determined in MEOS were 1.12 ± 0.026%, 0.94 ± 0.025%, and 0.46 ± 0.016% (w/w), respectively.

3.2. Antioxidant Activities

3.2.1. Ferrous Ion Chelating Activity. Ferrozine can quantitatively form the complexes with Fe^{2+}. Chelating activity of the MEOS with ferrous ion was 1.6% at 0.125 mg/mL. In addition, there was an increase of chelating activity to 30% at 2 mg/mL which was slightly lower than positive control (BHA and BHT) (Figure 1).

3.2.2. Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was investigated by using the Fenton reaction. As shown in Figure 2, addition of less than 2 mg/mL of MEOS effectively inhibited the formation of hydroxyl radicals linearly up to 72%.

3.2.3. Superoxide Anion Radical Scavenging Activity. Superoxide anion radical scavenging activity of MEOS was determined by the xanthine oxidase system. Figure 3 showed the percentage inhibition of superoxide radical generated by BHT, BHA, and MEOS. The inhibition activity of MEOS reached 63% when 2 mg/mL of extract was used.

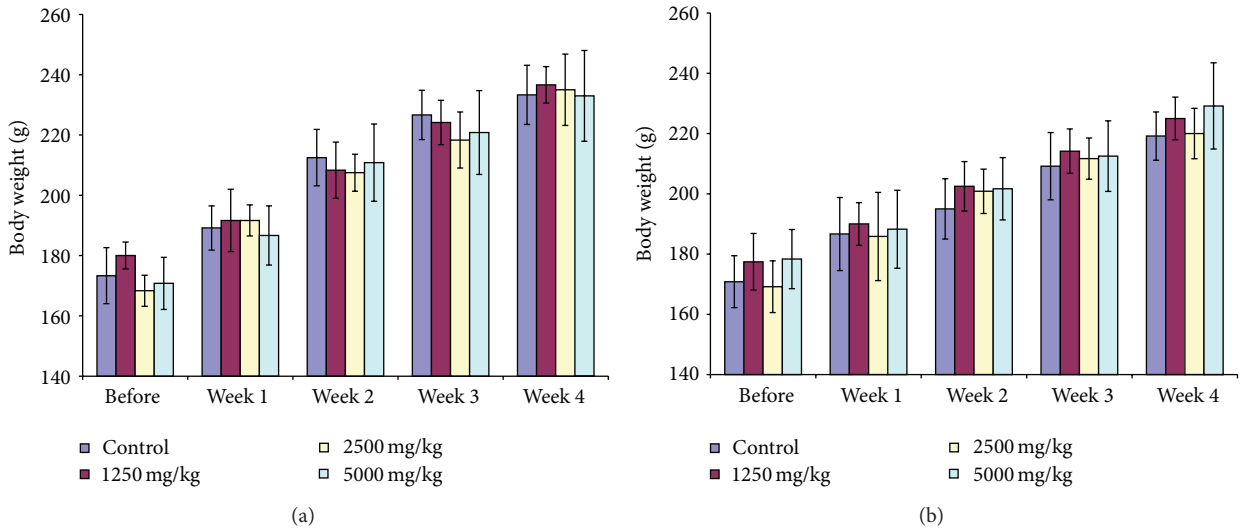

Figure 4: The effect of daily oral administration of MEOS on the bodyweight of (a) male and (b) female rats ($n = 6$). Data are expressed as mean ± SEM.

3.2.4. Lipid Peroxidation Inhibition Study. Antilipid peroxidation activity of MEOS was determined by the FOX method. IC_{50} of liver homogenate lipid peroxidation of MEOS was 0.34 ± 0.024 mg/mL.

3.3. Toxicology Studies

3.3.1. Acute Toxicity Study. In this experiment, oral administration of 5000 mg/kg of MEOS did not cause any visible signs of toxicity to the rats. No changes were observed in the behavior and mortality of the animals over the 14 days. All five female SD rats survived until the end of the experiment with no mortality were recorded. The LD_{50} determined was greater than 5000 mg/kg.

3.3.2. Subchronic Toxicity Study. In subchronic toxicity study, there were no observable changes in the general behaviour of all the treated rats (groups 1, 2, and 3) as compared to the control group. No significant changes were detected in either the bodyweights (Figure 4) or relative organ weights (Table 1) of all the treated rats. In addition, no death was recorded in both sexes of the treatment groups as well as their respective control group, during or after the course of the experiment. The controls and the treated rats appeared uniformly healthy at the end of the experiment and throughout the 28 days treatment period.

3.3.3. Blood Analyses. There were no significant differences observed in any of the haematological parameters tested (red blood cell count (RBC), haemoglobin concentration (Hgb), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total white blood cell count (WBC), or white blood cell differential count) in any of the treated rats as compared to control rats (Table 2). Meanwhile, analyses of biochemical parameters (alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), creatinine, bilirubin, urea, sodium, potassium, and chlorine) also showed no significant differences in any of the parameters tested between the control and the treated groups of both sexes (Table 3).

3.3.4. Histopathological Study. No lesions or pathological changes were observed in the organs of either sex of the MEOS treated rats as compared to their respective control groups.

4. Discussion

Orthosiphon stamineus Benth. (Lamiaceae) is an important medicinal plant in Malaysia. It has been widely used for treatment of various ailments. Many studies which involved both *in vivo* and *in vitro* experiments have reported that the *Orthosiphon stamineus* leaves possess a wide range of pharmacological properties such as, anti-inflammatory, analgesic, gastroprotective, antipyretic, and hepatoprotective effects. The present study was conducted to determine the antioxidant potential, which is responsible for the wide range of pharmacological properties of this plant and to investigate the possible harmful effects of MEOS in experimental animals at the specific doses chosen, since the safety use of the plant is of concern to the Malaysian authorities. The toxicity of MEOS in rats was evaluated by both acute 14-day and subchronic 28-day toxicological studies. Methanol was chosen as our solvent since many literature reviews showed that this extract was very effective in many pharmacological activities such as antipyretic, anti-inflammatory, and analgesic effects as well as a good protective agent against alcohol-induced gastropathy and CCl_4-induced liver damage [7–10].

Antioxidant capacity is one of the commonly used parameters to determine the bioactive components which exhibited pharmacological activities. *Orthosiphon stamineus* has been shown to be a potent scavenger of a variety of reactive radical species such as DPPH and ABTS [7]. Therefore, in the present study, the antioxidant potential of MEOS was tested by Ferrous ion chelating activity, hydroxyl radical scavenging

Table 1: (a) Relative organs weight of male rats orally treated daily with 50% methanolic extract of *Orthosiphon stamineus* for 28 days. (b) Relative organs weight of female rats orally treated daily with 50% methanolic extract of *Orthosiphon stamineus* for 28 days (cont').

(a)

% organ weight/bodyweight	Control	Treatment for 28 days 50% methanolic extract of *Orthosiphon stamineus*		
		1250 mg/kg	2500 mg/kg	5000 mg/kg
Male				
Brain	0.42 ± 0.02	0.39 ± 0.01	0.43 ± 0.02	0.42 ± 0.02
Heart	1.54 ± 0.03	1.44 ± 0.13	1.43 ± 0.08	1.37 ± 0.06
Liver	2.34 ± 0.10	2.42 ± 0.16	2.48 ± 0.15	2.46 ± 0.14
Thymus	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
Spleen	0.27 ± 0.02	0.27 ± 0.01	0.25 ± 0.01	0.27 ± 0.02
Kidney (right)	0.35 ± 0.01	0.37 ± 0.02	0.33 ± 0.01	0.35 ± 0.01
Kidney (left)	0.32 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
Adrenal gland	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Lungs	0.67 ± 0.05	0.71 ± 0.06	0.63 ± 0.02	0.63 ± 0.03
Testis (right)	0.56 ± 0.02	0.54 ± 0.01	0.55 ± 0.01	0.55 ± 0.02
Testis (left)	0.54 ± 0.03	0.55 ± 0.01	0.54 ± 0.02	0.56 ± 0.01
Stomach	2.74 ± 0.31	2.63 ± 0.06	2.93 ± 0.08	2.56 ± 0.11
Stomach (empty)	0.57 ± 0.01	0.59 ± 0.01	0.51 ± 0.02	0.54 ± 0.02
Gut	6.64 ± 0.21	6.38 ± 0.14	6.45 ± 0.14	6.32 ± 0.30
Gut (empty)	3.27 ± 0.03	3.44 ± 0.11	3.17 ± 0.04	3.24 ± 0.13

Data are expressed as mean ± SEM.

(b)

% organ weight/bodyweight	Control	Treatment for 28 days 50% methanolic extract of *Orthosiphon stamineus*		
		1250 mg/kg	2500 mg/kg	5000 mg/kg
Female				
Brain	0.40 ± 0.00	0.39 ± 0.00	0.40 ± 0.01	0.38 ± 0.01
Heart	1.70 ± 0.03	1.65 ± 0.02	1.68 ± 0.02	1.60 ± 0.04
Liver	2.61 ± 0.08	2.69 ± 0.09	2.81 ± 0.04	2.66 ± 0.06
Thymus	0.11 ± 0.01	0.09 ± 0.01	0.09 ± 0.00	0.10 ± 0.01
Spleen	0.22 ± 0.01	0.25 ± 0.02	0.23 ± 0.02	0.24 ± 0.01
Kidney (right)	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01
Kidney (left)	0.27 ± 0.00	0.27 ± 0.01	0.28 ± 0.00	0.26 ± 0.01
Adrenal gland	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Lungs	0.66 ± 0.01	0.63 ± 0.02	0.65 ± 0.02	0.67 ± 0.03
Ovaries	0.13 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
Uterus	0.21 ± 0.00	0.16 ± 0.01	0.19 ± 0.02	0.15 ± 0.01
Stomach	2.74 ± 0.18	2.71 ± 0.14	2.71 ± 0.06	2.69 ± 0.09
Stomach (empty)	0.58 ± 0.01	0.53 ± 0.01	0.51 ± 0.01	0.50 ± 0.01
Gut	7.29 ± 0.25	6.82 ± 0.21	6.80 ± 0.11	6.94 ± 0.30
Gut (empty)	4.06 ± 0.08	3.83 ± 0.11	3.63 ± 0.13	3.82 ± 0.15

Data are expressed as mean ± SEM.

activity, and superoxide anion radical scavenging activity. Our study showed that the antioxidant activity of MEOS was comparable to the positive controls: BHA and BHT. In the antioxidant activities, we reported that MEOS can strongly reduce the hydroxyl and supercritical onion radicals. This finding is comparable to the previous study which reported that MEOS inhibited lipid peroxidation in different animal models [7, 10]. In addition, it has been long reported that there is a strong association between the termination of free radical propagation in biological systems with the reduction

Table 2: (a) Haematological values of male rats treated with 50% methanolic extract of *Orthosiphon stamineus* for 28 days. (b) Haematological values of female rats treated with 50% methanolic extract of *Orthosiphon stamineus* for 28 days (cont').

(a)

	Unit	Control	Treatment for 28 days 50% methanolic extract of *Orthosiphon stamineus*		
			1250 mg/kg	2500 mg/kg	5000 mg/kg
Male					
White blood cell count	10^9/L	9.57 ± 0.32	11.05 ± 0.48	10.78 ± 0.59	10.73 ± 0.21
Neutrophils	10^9/L	2.46 ± 0.18	3.09 ± 0.22	3.15 ± 0.31	3.18 ± 0.43
Lymphocytes	10^9/L	6.67 ± 0.24	6.94 ± 0.46	6.72 ± 0.29	6.67 ± 0.41
Monocytes	10^9/L	0.24 ± 0.03	0.62 ± 0.09	0.53 ± 0.15	0.54 ± 0.05
Eosinophils	10^9/L	0.02 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Basophils	10^9/L	0.18 ± 0.03	0.35 ± 0.04	0.33 ± 0.08	0.30 ± 0.02
Red blood cell count	10^{12}/L	8.55 ± 0.12	8.36 ± 0.17	8.43 ± 0.20	8.24 ± 0.03
Hemoglobin	g/L	14.80 ± 0.21	14.76 ± 0.14	15.02 ± 0.32	14.49 ± 0.08
Hematocrit	%	0.75 ± 0.01	0.74 ± 0.02	0.75 ± 0.02	0.71 ± 0.00
Mean red blood cell volume	fL	88.03 ± 0.74	88.15 ± 1.27	88.51 ± 1.74	86.47 ± 0.81
Mean corpuscular Hb	pg	17.34 ± 0.19	17.70 ± 0.31	17.75 ± 0.22	17.58 ± 0.07
Mean corpuscular Hb concentration	g/L	19.71 ± 0.25	20.16 ± 0.40	20.12 ± 0.40	20.39 ± 0.16
Platelets	10^9/L	1205.61 ± 26.53	1149.19 ± 29.80	1145.28 ± 67.30	1125.36 ± 18.04
Mean platelet cell volume	fL	8.12 ± 0.12	8.46 ± 0.17	8.26 ± 0.19	8.11 ± 0.13

Data are expressed as mean ± SEM.

(b)

	Unit	Control	Treatment for 28 days 50% methanolic extract of *Orthosiphon stamineus*		
			1250 mg/kg	2500 mg/kg	5000 mg/kg
Female					
White blood cell count	10^9/L	7.66 ± 0.96	6.77 ± 0.43	6.31 ± 0.72	7.24 ± 0.35
Neutrophils	10^9/L	1.69 ± 0.32	1.63 ± 0.18	1.47 ± 0.16	1.78 ± 0.18
Lymphocytes	10^9/L	5.35 ± 0.68	4.62 ± 0.52	4.37 ± 0.55	4.26 ± 0.95
Monocytes	10^9/L	0.34 ± 0.08	0.30 ± 0.09	0.31 ± 0.05	0.30 ± 0.03
Eosinophils	10^9/L	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Basophils	10^9/L	0.27 ± 0.07	0.20 ± 0.04	0.22 ± 0.04	0.19 ± 0.02
Red blood cell count	10^{12}/L	7.76 ± 0.17	7.79 ± 0.06	7.25 ± 0.36	7.48 ± 0.19
Hemoglobin	g/L	14.16 ± 0.14	14.44 ± 0.12	13.56 ± 0.29	13.64 ± 0.50
Hematocrit	%	0.71 ± 0.01	0.71 ± 0.00	0.67 ± 0.02	0.68 ± 0.01
Mean red blood cell volume	fL	89.47 ± 1.64	90.93 ± 0.52	87.80 ± 1.95	91.09 ± 1.20
Mean corpuscular Hb	pg	18.30 ± 0.25	18.54 ± 0.14	18.01 ± 0.23	18.35 ± 0.56
Mean corpuscular Hb concentration	g/L	20.14 ± 0.18	20.41 ± 0.18	19.85 ± 0.23	20.16 ± 0.56
Platelets	10^9/L	1054.14 ± 57.95	1021.22 ± 14.30	987.46 ± 21.65	1035.83 ± 55.79
Mean platelet cell volume	fL	7.99 ± 0.10	7.78 ± 0.19	7.23 ± 036	7.55 ± 0.10

Data are expressed as mean ± SEM.

of chronic diseases, DNA damage, mutagenesis, carcinogenesis, and inhibition of pathogenic bacterial growth [21]. Hence, as a potent antioxidant, it is not surprising that *Orthosiphon stamineus* is effective in treating various oxidative stress-related chronic diseases such as diabetes, alcohol-induced stomach ulcer, and CCl_4-induced liver damage.

In acute 14-day toxicity study, a limited dose was selected and performed on the experimental rats. Its purpose was to determine a proper range of doses to be used in the subsequent subchronic 28-day toxicological study [19]. In this experiment, a single dose of 5000 mg/kg MEOS was given orally to five female Sprague-Dawley rats. No significant mortality or alteration in the behaviour patterns of the treated rats was observed compared to their respective controls.

During the subchronic 28-day toxicity study, the rats were treated orally with different doses of MEOS (1250, 2500, and 5000 mg/kg/day) for 28 days. The results obtained were comparable to those in the acute toxicity study. Both

Table 3: Biochemical values of male and female rats treated with 50% methanolic extract of *Orthosiphon stamineus* for 28 days.

	Unit	Control	Treatment for 28 days 50% methanolic extract of *Orthosiphon stamineus*		
			1250 mg/kg	2500 mg/kg	5000 mg/kg
Male					
Aspartate transaminase (AST)	U/L	122.74 ± 2.19	131.94 ± 3.53	126.79 ± 9.40	121.81 ± 2.45
Alanine aminotransferase (ALT)	U/L	64.51 ± 2.98	62.43 ± 2.59	64.50 ± 3.86	65.22 ± 2.73
Urea	U/L	6.31 ± 0.34	6.65 ± 0.34	6.50 ± 0.40	6.74 ± 0.14
Creatinine	μmol/L	40.56 ± 0.81	39.59 ± 0.30	41.08 ± 1.16	39.33 ± 0.45
Alkaline phosphatase (ALP)	μmol/L	300.56 ± 7.18	309.63 ± 10.08	309.50 ± 11.49	316.86 ± 13.42
Bilirubin	mmol/L	1.80 ± 0.00	1.80 ± 0.00	1.80 ± 0.00	1.80 ± 0.00
Sodium	g/L	142.53 ± 0.36	142.53 ± 0.22	141.96 ± 0.58	142.22 ± 0.31
Potassium	g/L	4.69 ± 0.12	4.64 ± 0.06	4.72 ± 0.07	4.65 ± 0.08
Chlorine	g/L	102.72 ± 0.60	102.71 ± 0.33	103.75 ± 0.28	103.53 ± 0.61
Female					
Aspartate transaminase (AST)	U/L	127.83 ± 2.51	127.72 ± 4.98	122.61 ± 10.41	117.28 ± 8.10
Alanine aminotransferase (ALT)	U/L	54.33 ± 2.32	52.17 ± 3.32	53.78 ± 3.41	52.94 ± 1.72
Urea	U/L	7.83 ± 0.46	7.39 ± 0.08	8.00 ± 0.39	7.19 ± 0.29
Creatinine	μmol/L	50.83 ± 2.41	47.72 ± 1.11	47.11 ± 1.27	44.50 ± 0.25
Alkaline phosphatase (ALP)	μmol/L	247.50 ± 8.33	249.72 ± 21.56	250.22 ± 21.23	267.33 ± 9.95
Bilirubin	mmol/L	1.80 ± 0.00	1.80 ± 0.00	1.80 ± 0.00	1.80 ± 0.00
Sodium	g/L	142.83 ± 0.60	142.94 ± 0.20	141.44 ± 0.20	141.11 ± 0.26
Potassium	g/L	4.23 ± 0.14	4.19 ± 0.15	4.04 ± 0.11	4.14 ± 0.15
Chlorine	g/L	102.50 ± 0.62	103.39 ± 0.54	103.17 ± 0.51	103.83 ± 0.46

Data are expressed as mean ± SEM.

control and treated rats of both sexes appeared generally healthy during and throughout the experimental period. No mortality was recorded and no toxicity signs were detected in any of the treated rats. Both evaluation of the acute and subchronic toxicity of MEOS had indicated that a single oral administration of MEOS up to 5000 mg/kg dose caused neither visible signs of toxicity nor mortality to experimental rats. Hence, the LD_{50} of MEOS determined is more than 5000 mg/kg.

Generally, an increase or decrease in bodyweight of an animal has been used as an indicator of adverse effect of drugs and chemicals [22]. In our study, all the treated animals survived beyond the observation period. The bodyweight of both control and treatment groups increased gradually throughout the experimental period with no significant difference. The increase in bodyweight was not significantly different as compared to the control group, in both acute and subchronic toxicity studies. All the rats at each dosage level continued to gain weight throughout the experimental period, suggesting that growth inhibition did not occur during this course of repeated dosage.

In addition to bodyweight, relative organ weight has also been used as another basic indicator to determine whether the rats have been exposed to harmful agents [19, 22]. Their organs will tend to swell or damage if they were subjected to toxic substances. This will subsequently alter their organ-to-bodyweight ratios as compared to the respective controls. In the present study, the relative organ weights of liver, lungs, spleen, sex organs, heart, and kidneys in all the treated male and female rats were not significantly different ($P < 0.05$) from those of the control groups.

Analysis of blood parameters in animal studies could be very useful when evaluating the risk of human toxicity as the changes in the haematological system provide a predictive value for toxicity [18]. These blood parameters are often called haematological parameters. Several important haematological parameters were selected in this study to evaluate the toxicity of MEOS. From the results, the haematological profile of all the treated rats showed no significant difference with the control group (Table 2). This indicated that the MEOS did not affect the haematopoiesis system of the treated rats.

Several important biochemical parameters were also included in this toxicity study. These biological parameters are indicators for organ toxicity. For example, kidney functions were evaluated by means of serum urea and creatinine. Increase of blood creatinine has been shown to be a good indicator of negative impact in kidney functions [23]. While the increase levels of AST and ALT in the blood are associated with damage of hepatic cells [24, 25]. The results of the experiment suggested that the kidney and liver functions were not altered for both the treated male and female rats. There were no statistically significant differences in creatinine, AST, and ALT levels between controls and treated animals at any dosage levels. Hence, these findings suggest that MEOS does not cause any hepatotoxicity and kidney damage to the rats. Both these organs were selected because kidneys and liver are the first organs to show toxicity when the rats are exposed to potential toxic substances. Based on

the statistical analyses of these biochemical parameters, no significant difference was observed in any of the parameters tested for either the treated or normal rats (Table 3).

Histopathological studies were conducted on vital organs of all the rats. According to Wang et al., impaired organs often have abnormal atrophy [26]. However, from our results, no related histopathological changes were observed. Gross examination in necropsy and microscopic examination revealed no changes that might be due to the administration of MEOS (data not shown). No significant changes or damages were observed in the morphology of all the isolated vital organs, either from the rats treated with MEOS or control rats (data not shown). The isolated organs showed normal architecture. These results again suggested that daily repeated oral administration of MEOS did not cause any detrimental changes or morphological disturbances to the rats or to their vital organs. The no-observed-adverse-effect level (NOAEL) of MEOS determined was 5000 mg/kg bodyweight/day (>3000 mg/kg bodyweight/day) and considered to be a non-hazard compound.

5. Conclusion

This finding is comparable with Mohamed et al.'s [19] and Abdullah et al.'s (2009) in which *O. stamineus* alcoholic extract did not show any toxic effect in rats during both acute and subchronic studies. Daily oral administration of 50% methanolic extract of *O. stamineus* (MEOS) at doses of 1250, 2500, and 5000 mg/kg to both male and female Sprague-Dawley rats for 28 days did not result in mortality and was not associated with adverse effects as reflected in the observation of general condition, growth, body and organ weights and hematological and biochemical values. Thus, its oral lethal dose for both male and female rats is in excess of 5000 mg/kg. There were no abnormalities in necropsy and histopathological findings. An NOAEL from the present study was determined to be 5000 mg/kg per day for rats under the condition tested. Further investigation on the preclinical and clinical studies of the extract will be necessary to determine a safe dose before it becomes a potential drug and to protect the population from possible toxic effects of MEOS. It would be interesting to assess the toxic effect of the compounds found in MEOS such as eupatorin and sinensetin.

Conflict of Interests

Mariam Ahmad declares that no competing interests existed for the authors or the institute before, during, and after preparing and summiting this paper for review.

Acknowledgments

This study was funded by Fundamental Research Grant Scheme (FRGS) 203/PFARMASI/6711306 and FRGS/FP31-2012A, UMRG Program (Sub-Program 3/RP003C-13ICT), and High Impact Research Grant (UM.C/625/1/HIR/MOHE/FCSIT/15) from Universiti Sains Malaysia, University of Malaya, and Ministry Education Malaysia.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 128594, 8 pages
http://dx.doi.org/10.1155/2013/128594

Research Article

Assessment of Cytotoxicity, Fetotoxicity, and Teratogenicity of *Plathymenia reticulata* Benth Barks Aqueous Extract

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Received 29 August 2013; Revised 12 November 2013; Accepted 13 November 2013

Academic Editor: Marina Soković

Scientific assessment of harmful interactions of chemicals over the entire reproductive cycle are divided into three segments based on the period: from premating and mating to implantation (I), from implantation to major organogenesis (II), and late pregnancy and postnatal development (III). We combined the segments I and II to assess *Plathymenia reticulata* aqueous extract safety. In order to investigate reproductive toxicity (segment I), pregnant rats received orally 0.5 or 1.0 g/kg of extract, daily, during 18 days. These concentrations were determined by a preliminary *in vitro* LD50 test in CHO-k1 cells. A control group received deionized water. The offspring was removed at the 19th day, by caesarean, and a teratology study (segment II) was carried out. The corpora lutea, implants, resorptions, live, and dead fetuses were then counted. Placenta and fetuses were weighted. External and visceral morphology were provided by the fixation of fetuses in Bouin, whereas skeletal analysis was carried out on the diaphanized ones. The increase in the weights of placenta and fetuses was the only abnormality observed. Since there was no sign of alteration on reproduction parameters at our experimental conditions, we conclude that *P. reticulata* aqueous extract is safe at 0.5 to 1.0 g/kg and is not considered teratogenic.

1. Introduction

Plathymenia reticulata Benth (Leguminosae) is a plant popularly known as "vinhático" (wine-like), found in "Cerrado" region in Brazil, and represents a good source of high quality wood. The plant coevolution with other species has led to the development of secondary metabolites for its self-defenses against pathogens (viruses, bacteria, and fungi) and predators like insects and mammals.

Among several secondary metabolites identified in *P. reticulata* trunk heartwood, plathyterpol [1], vinhaticyl acetate, and methyl vinhaticoate [2], 16,18-diacetoxycass-13(15)-ene and 16-hidroxy-18-acetoxycass-13(15)-ene [3] are the most common. The medicinal potential of *P. reticulata* as anti-inflammatory [4], antimicrobial [5, 6], and depurative of blood [7] is also highlighted by the inherent presence of tannins, flavonoids [5], and cassane diterpenes in its constitution [3].

However, in spite of their medicinal potential, secondary metabolites synthesized by plants can also be harmful for the aggressor. In mammals, they can induce toxicity to a number of different organs including skin, lung, liver, kidney and

bladder, blood, skeletal muscle, and central and peripheral nervous systems including the neuromuscular junctions [8].

In vitro studies showed that *P. reticulata* barks hydroalcoholic extract inhibits the irreversible neuromuscular blockade induced by *Bothrops jararacussu* (79.3 ± 7.5%) and *Crotalus durissus terrificus* (73.2±6.7%) venoms, on mouse neuromuscular junctions. This antivenom activity was mainly related to protein precipitation caused by the high content of tannins (4%) present in the extract [9].

Using mouse skeletal muscles, further investigations upon the anti-snake venom profile of *P. reticulata* barks secondary metabolites showed that the dichloromethane extract (0.4 mg/mL) inhibited the throphic muscle effects induced by *Bothrops jararacussu* venom [10].

In an attempt of investigating the risk assessment of *P. reticulata*, its mutagenic potential was evaluated by the *Salmonella* mutagenicity assay (Ames test) and the micronucleus test in CHO-K1 cells. Although the hydroalcoholic extract of *P. reticulata* barks showed mutagenicity, the Ames test also unveiled its anticarcinogenic potential [11].

In Brazil, local markets frequently sell herbal medicinal plants, in which tannin-rich trees, like *P. reticulata*, are commonly found. Plants rich in tannins are also described for treating diarrhea, hypertension, wounds, burns, kidney and stomach diseases, and inflammation [12]. However, in spite of their obvious clinical benefits, the oral administration of these remedies, associated with the lack of scientific proof of safety, put in risk the population health. Besides the potential adverse effects caused by the herb itself, teratogenicity is another important concern.

During pregnancy, one of the results of acute or chronic exposure to naturally occurring chemical agents can be an abnormal offspring development. Manifestations of the developmental toxicity include structural malformations, growth retardation, functional impairment, and/or death of the organism [13].

In this work we showed the safety evaluation of *P. reticulata* aqueous extract using developmental and reproductive toxicology protocols (segments I and II).

2. Materials and Methods

2.1. Vegetal Material and Extraction Procedure. Samples of *P. reticulata* Benth bark were collected from Miracema city herbarium (Miracema, Tocantins, Brazil) in December 2007. The specimens were deposited (protocols NRHTO 3327) at the herbarium of Federal University of Tocantins. The bark was dried at 40°C in an incubator with forced air circulation apparatus for 48 hours. The material was ground in a mill (MA 340, Marconi, Brazil), macerated for seven days (1276.32 g) in 70% ethanol (14.5 liters), and the suspension was protected from light and percolated at 20 drops/minute, resulting in a 20% (w/v) hydroalcoholic extract. This procedure was previously described by Farrapo et al. [10] and Della Torre et al. [11]. Briefly, the obtained extract contained 3.75% polyphenols and 0.16% flavonoids and showed positive reactions to tannins. The resulting material was concentrated in a rotary evaporator (TE-210, Tecnal, Brazil) and lyophilized (Multitasking Freeze Drying S, SNL216V-115, Thermo Fisher Scientific, USA).

2.2. Cell Line and Culture Conditions. As described by Della Torre et al. [11], Chinese hamster ovary cells (CHO-K1 lineage, American Type Cell Culture, ATCC number CCL-61) were maintained at 37°C in 5% CO_2 and 97% humidity in RPMI 1640 culture medium (Gibco, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) L-glutamine (L-Glu, Gibco), 1% (v/v) penicillin streptomycin (PS, Gibco) and 0.1% (v/v) amphotericin B (Gibco). For subculturing and experiments, the cells were harvested using 0.05% (w/v) trypsin and 0.02% (w/v) ethylene diamine tetracetic acid (EDTA) in a saline phosphate-buffered solution, pH 7.4. Each trypsinization was recorded as one passage. The test was performed at the third passage.

2.3. Cytotoxicity Evaluation (IC10 and IC50). The cytotoxicity evaluation was carried out by using the CellTiter 96 AQueous Non-radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), in which 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) is bioreduced to formazan by dehydrogenase enzymes in metabolically active cells. The amount of formazan produced by the cells was determined by measuring sample absorbance at 490 nm with a spectrophotometer SpectraMax 190 (Molecular Devices, São Paulo, SP, Brazil). Statistical analysis of data was performed by using one-way analysis of variance (ANOVA) between two different sample curves and solvent control curve. The binomial proportion confidence interval was adopted.

2.4. In Vitro LD50 of P. reticulata Barks Lyophilized Extract. The value of the LD50 (Lethal Dose to kill 50% of animals), an essential test for the controlled use of animals of assays *in vivo*, was determined in *P. reticulata* barks lyophilized extract. In this assay, the inhibitory concentration that kills 50% of the cells (IC 50), was (0.331 mg mL^{-1}), as describe by Della Torre et al. [15]. Applying the formula: log [LD50 (mg mL^{-1})] = 0.372 × log IC50 (μg mL^{-1}) +2.024 [16, 17], the value of the LD50 was calculated in order to orientate the *in vivo* experimental assays.

2.5. In Vivo Experiment

2.5.1. P. reticulata Aqueous Extract Preparation. The *P. reticulata* aqueous extract, to be administered via gavage in rats, was prepared daily using the previous lyophilized extract (see plant material and extraction) dissolved in deionized water.

2.5.2. Animals. Six males and fifteen female adult Wistar rats weighing 160 g to 200 g were supplied by Anilab, Animais de Laboratório (Paulínia, São Paulo, Brazil). All animals were maintained in groups (5 rats per cage), previously housed to laboratory conditions during one week before the experiments at 25 ± 3°C on a 12 h light/dark cycle and had access to food and water *ad libitum*. This project

(protocol number A011/CEP/2008) was approved by the institutional Committee for Ethics in Research of Vale do Paraiba University (UNIVAP), and the experiments were carried out according to the guidelines of the Brazilian College for Animal Experimentation.

2.5.3. The Reproduction and Fertility Study (Segment I). The method for reproductive evaluation was previously described elsewhere [18–20]. Briefly, 15 sexually naive rat females were mated with males (five females with two males per cage). Pregnancy was confirmed through the presence of spermatozoids in vaginal-washing rubbing observed by microscopy analysis [21]. The presence of spermatozoids was considered as the first day of pregnancy. Each pregnant female was kept in separate cage. Three experimental groups were analyzed, two treated and one control. The animals had free access to water and food during all the experiment and the consumption was monitored daily. For reproductive evaluation, each group of 5 females received 0.5 g/kg/day (group 1) or 1.0 g/kg/day (group 2) of *P. reticulata* extract or deionized water (group 3, control), from the first to the 18° day of pregnancy. The weight gain of pregnant females was monitored during the pregnancy.

2.5.4. The Teratology Study (Segment II). For the teratogenic study each group of 5 females received by gavage 0.5 g/kg/day (group 1), 1.0 g/kg/day (group 2) of *P. reticulata* extract, or deionized water (group 3) from days 1 to 18 of pregnancy. Pregnant rats were anesthetized with halothane (Halotano, Cristalia, Brazil), killed, and submitted to a rapidly excision of their uterus. The following macroscopic parameters were evaluated in order to observe the reproductive performance of rats [22]: (1) placenta weight (grams); (2) fetus weight (grams); (3) preimplantation loss (%) = corpora lutea number − implantation number/corpora lutea number; (4) postimplantation loss (%) = implantation number − alive fetus/implantation number; and (5) offspring vitality (%).

The offspring was anesthetized with halothane, killed, and fixed in Bouin's solution for 24–48 h, replaced by 70% hydroalcoholic solution. The following parameters were measured (cm): A: craniocaudal; B: tail; C: anteroposterior of cranio; D: laterolateral of cranio; E: anteroposterior of thorax; and F: laterolateral of thorax. The other offspring group was anesthetized with halothane, killed, eviscerated, and diaphanizated for posterior skeletal examination. The fetuses selected were fixed in ethanol, then "cleared" and stained by a KOH alizarin red-S technique [23]. Examination included enumeration of the vertebra, ribs, and other bone structures, degree of ossification, and any fusions or abnormalities in bone shape or position [24].

2.6. Statistical Analysis. Data from the different assays were first analyzed regarding distribution and variance homogeneity. Normally distributed data were submitted to comparison between both groups by using Student's t-test. Nonnormally distributed data were first transformed (log). One-way ANOVA or Fisher's exact tests were used for evaluation of physical development parameters. Significance level was set as 5%.

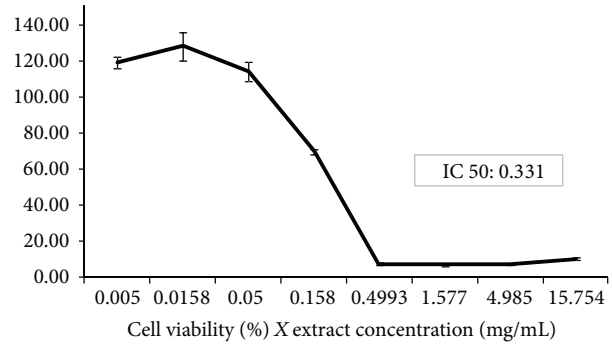

Figure 1: Effect of *P. reticulata* hydroalcoholic extract against Chinese hamster ovary cell viability (%). The graph shows the cell viability versus different concentrations of *P. reticulata* extract (mg/mL). The IC10 value was estimated in 0.113 mg/mL and the IC50 in 0.331 mg/mL, calculated via Phototox software program [14].

3. Results and Discussion

This study was designed to evaluate the safety of oral administration of *P. reticulata* aqueous extract in pregnant rats.

A prerequisite for understanding the abnormal development in mammals is the evaluation of the normal development that, in turn, is characterized by changes such as size, biochemistry and physiology, and in shape and functionality. In this view, gametogenesis is the process of forming the haploid germ cells, the egg, and sperm. These gametes fuse in the process of fertilization to form the diploid zygote,the embryo. It is well known that, in developmental toxicity studies, the major effects of prenatal exposure of a chemical compound are observed at the time of birth as embryolethality, malformations, and growth retardation. A disturbance on a single cell may induce an abnormal development at the zygote (one-cell) stage, the blastocyst stage (when only a few cells in the inner cell mass are embryo progenitors), or during organogenesis, when organ rudiments may consist of only few cells. Nevertheless, the relationship between these effects is too complex to evaluate and varies with the type of agent, the time of exposure, and the dose of the toxic compound [13].

Figure 1 shows the cell viability (%) compared to different plant extract concentrations. The value of IC10 was found to be 0.113 mg/mL, accounting for the concentration at which approximately 90% of cells survived (noncytotoxic concentration). The IC50 of 0.331 mg/mL corresponds to the concentration at which approximately 50% of the cells survived.

De Toledo et al. [6] evaluated *Plathymenia reticulata* cytotoxicity using VERO cells and found a cytotoxic concentration (CC50) of 156.67 μg/mL. A possible explanation for these different values may relay in the different cell line used and also the different colorimetric assays.

According to ICCVAM [16], the LD50 value can be determined based on the IC50, by applying the following formula: log LD50 (mg/kg) = 0.372 log IC50 (μg/mL) + 2.024. Thus, the advantages of using this analysis are the reduction of animal use during *in vivo* tests [14], and the knowledge

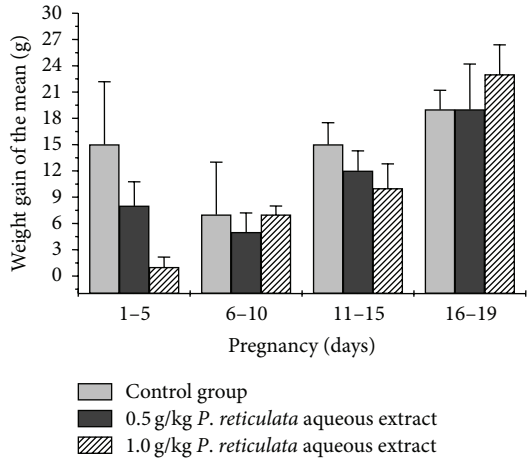

Figure 2: Effect of *P. reticulata* aqueous extract on weight gain of pregnant rats. On the graph each bar is the mean ± S.E.M. of five experiments. Note that there were no significant changes ($P > 0.05$, t-test, and one-way ANOVA test were applied in this assay) between the *P. reticulata*-treated and control groups.

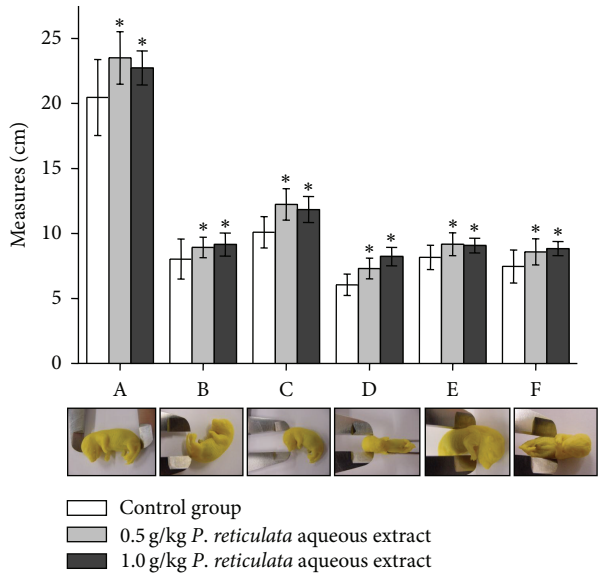

Figure 3: Effect of *P. reticulata* aqueous extract (0.5 g/kg and 1.0 g/kg) against the external morphological parameters of offspring. The graph is the mean ± S.E.M. of five experiments. (A: craniocaudal; B: tail; C: anteroposterior of cranio; D: laterolateral of cranio; E: anteroposterior of thorax; and F: laterolateral of thorax.) $^{*}P < 0.05$ compared to control group.

of the initial dose for *in vivo* studies, mainly when LD50 tests for acute oral toxicity, is required. Nowadays, most of the toxicological studies involving new drugs combine *in vivo* and *in vitro* assays in order to increase safety (i.e., in the case of a further approval for clinical use). For example, the evaluation of the development and safety of medicinal products require the estimation of IC50 values [17]. In our experimental conditions, together with the IC50 values in CHO-K1 cells, it was calculated the LD50 as 915 mg/kg.

Table 1: Reproductive performance of pregnant rats exposed to *Plathymenia reticulata* aqueous extract.

Teratogenicity parameters	Control	Experimental 0.5 g/kg	Experimental 1.0 g/kg
Preimplantation loss (%)	0	0	0
Postimplantation loss (%)	0	1.69	5.55
Placenta weight (grams)	0.494 ± 0.07 ($n = 59$)	0.542 ± 0.09* ($n = 58$)	0.530 ± 0.07* ($n = 51$)
Fetus weight (grams)	1.336 ± 0.25 ($n = 59$)	1.433 ± 0.20* ($n = 58$)	1.456 ± 0.15* ($n = 51$)

*$P < 0.05$.

The above calculations applied to the *in vivo* experimental assays permitted the determination of two concentrations (0.5 and 1 g/kg) of *P. reticulata* extract, that mimics the human consumption.

Figure 2 shows the graph of mean (±S.E.M.) weight gain during the gestation, considering water ingestion and food consumption *ad libitum*. At the 95% confidence level the two means (control compared to *P. reticulata extracts*) are not significantly different. The weight losses of control pregnant rats and treated groups and also of 6–10th day of pregnancy compared to 1–5th day can be explained by the habituation phenomenon [25], since rats are very sensitive to manipulation.

The relationship between maternal and developmental toxicity is not only a result of an insult to the conceptus at the cellular level. Insults may occur through different routes, including a direct effect on the embryo/fetus, indirect toxicity of the agent to the mother through the placenta, or a combination of direct and indirect effects. Maternal conditions could potentially harm the developing organism by altering the nutritional status, among several different factors [26, 27].

It is well known that intergenerational malnutrition is responsible for reducing the gain of weight during pregnancy in rats [28]. Therefore, the distinction between direct and indirect developmental toxicity is important to understand safety assessment tests in pregnant animals. In our experimental conditions, all animals had access to food and water *ad libitum*, in order to exclude this variable (Figure 3). Here, the concentrations assayed did not induce maternal toxicity. According to Rogers and Kavlock [13], a decrease in food or water intake would induce weight loss and other clinical signs. As an example, they have shown a significant maternal weight reduction at the end of pregnancy in the sibutramine nonoverweight drug-treated group, compared to the control (nonoverweight, no drug). This data can be linked to a significant increase in post-implantation loss and placental index, suggesting that sibutramine alone or the condition of excess weight in the absence of drugs has altered the reproductive performance [15].

The reproductive performance can be also evaluated by macroscopic parameters such as placenta weight (grams),

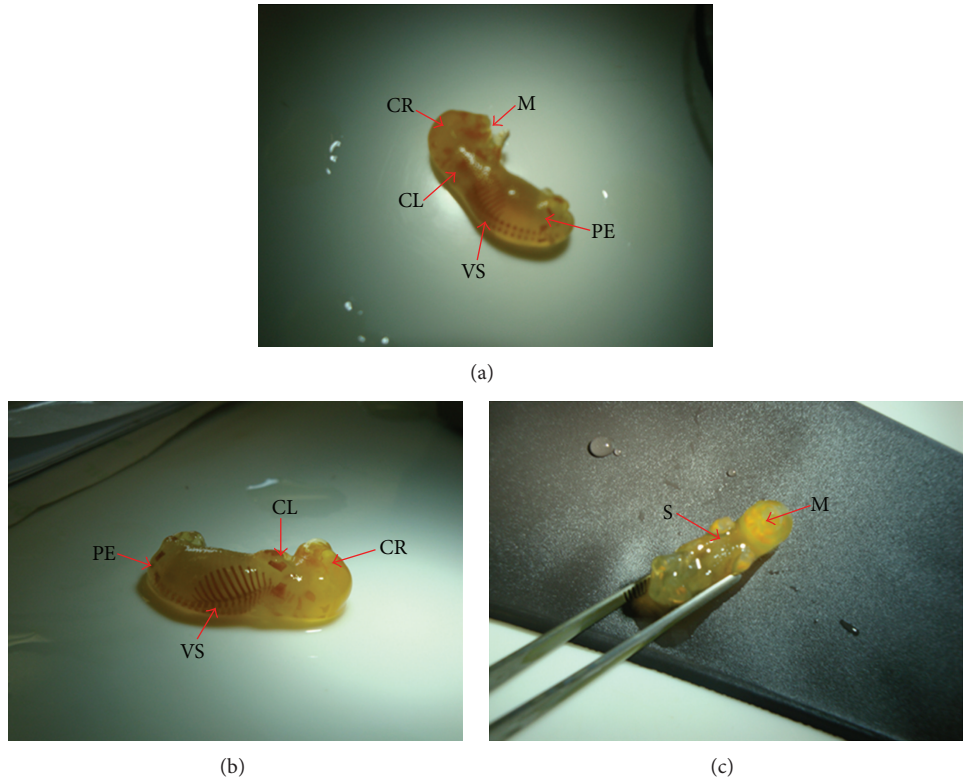

Figure 4: Representative pictures of 19th days gestation fetuses for teratogenicity test. Pregnant rats were treated daily with *P. reticulata* (1.0 g/kg) and the offspring removed surgically prior to birth. Diaphanized fetuses were analyzed by lateral (a), posterior (b), and frontal (c) views. The parameters of sternum ossification (S), clavicule (CL), cranio (CR), pelvis (PE), mandible (M), and vertebral spine (VS) were examined. Notice that no abnormality was observed.

fetus weight (grams), preimplantation loss (%), postimplantation loss, and offspring vitality (%). Regarding this later information, the data after administration of *P. reticulata* aqueous extract (0.5 g/kg or 1 g/kg) did not differ from control group (Table 1), except for placenta and fetuses weights. Under these parameters, *P. reticulata* treatment increased the gain (in grams) of placenta and fetuses. According to Langley and Jackson [29], low-protein intake induces intrauterine protein restriction during diet that could explain the gain (in grams) of placenta and fetuses in *P. reticulata* treatment. However, in our experimental conditions animals had access to food and water *ad libitum*. According to des Robert et al. [30], high protein intake via the enteral route could explain the enhanced weight gain in *P. reticulata* treatment.

The external morphological parameters of offspring were measured and compared to control group. All parameters evaluated were statistically different to the control, but not between the experimental treated-groups, via mother, that received 0.5 g/kg or 1.0 g/kg of *P. reticulata* aqueous extract (Figure 3).

No abnormality was seen in fetuses, except with the offspring sizes, demonstrating the safety of *P. reticulata* aqueous extract, in our experimental conditions. When cyclophosphamide (40 mg/kg), a well-known teratogenic agent, was used as a positive control, a strong teratogenic activity was observed (a dose 12.5 and 25 times lower than 0.5 and 1.0 g/kg *P. reticulata*, resp.). At this concentration cyclophosphamide was able to induce high resorption rate (approximately, 80%) and severe fetal malformations with retarded growth. These later phenomena were traduced by craniofacial alterations such as severe microcephaly, agnathia, open eyes, limb reduction, and trunk anomalies such as phocomelia or amelia, as well as, eventration of the abdominal wall and absence of the tail. Even at 15 mg/kg of cyclophosphamide, the teratogenic effect was observed in 70% of fetuses, which exhibited external tail and digit anomalies, such as short or crooked tail, syndactyly and ectrodactyly [31].

The analysis of external morphology carried out in conceptus exposed to *P. reticulata* aqueous extract (0.5 g/kg or 1/0 g/kg) just prior to birth showed no abnormalities upon skeletal examination of diaphanized fetuses (Figure 4). The parameters analyzed were soft tissues, cartilage calcification, vertebra and ribs quantification, bone shape or position, sternum ossification (S), and bones such as cranium (CR), pelvis (PE), vertebral spine (VS), clavicule (CL), and mandible (M).

Visceral malformations were not observed in organs such as liver, stomach, duodene, and kidneys. Morphological analysis in the head and neck regions showed that all structures were correctly implanted. The observation of structures such as palate, nostrils, ocular globe, inner ear, cortex, cerebral ventricle, marrow, trachea, and esophagus showed no sign of alterations; the oral cavity was delimited by palate and was

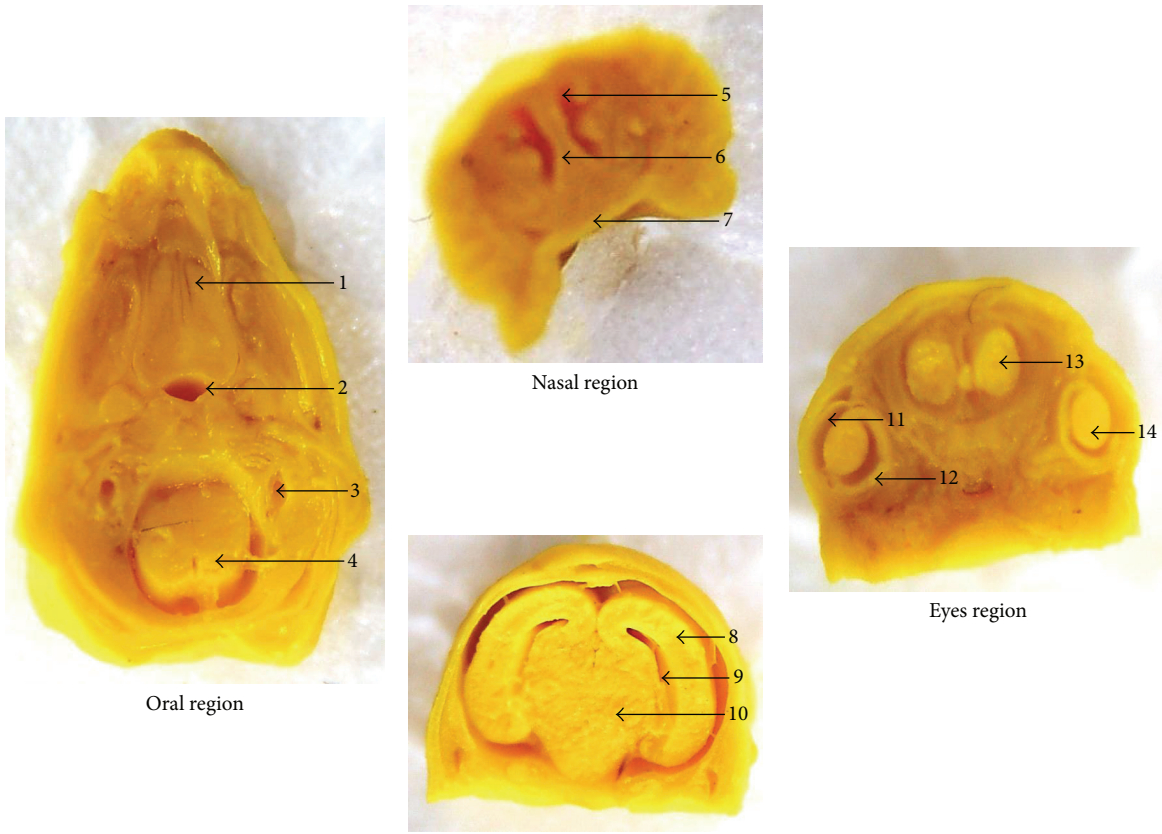

Figure 5: Representative sections from head and neck regions of fetuses exposed to *P. reticulata* aqueous extract (1.0 g/kg). Oral region transversally sectioned: 1: palate; 2: trachea; 3: inner ear; 4: marrow. Nasal region frontally sectioned: 5: nasal cavity; 6: nasal septum; 7: palate. Cerebral hemispheres region frontally sectioned: 8: cerebral hemisphere; 9: ventricles; 10: diencephalon. Eyes region frontally sectioned: 11: cornea; 12: retina; 13: olfactory bulb; 14: crystalline. After a careful analysis of the anatomical parameters, no abnormality was observed among the groups.

not obstructed in all fetuses examined, treated groups, and control group (Figure 5).

This study also included the daily observation of pregnant rats to a previous oral administration of *P. reticulata* aqueous extract (0.5 g/kg or 1.0 g/kg). In this protocol there were no signs of increased hair loss, excessive salivation, alteration in respiration, and abnormal gaits, tremors, or seizures. Although the gain of weight (grams) of pregnant rats was not statistically different (treated groups compared to the control group), there were significative changes in the weights of placenta and fetuses (n = S.E.M., $^{*}P < 0.05$). The fetal evaluation between treated and control groups showed no malformations, defined as "those structural anomalies that alter general body conformity, disrupt or interfere with body function, or are generally thought to be incompatible with life" [32].

4. Conclusions

Overall the results suggest that oral administration of *P. reticulata* aqueous extract at 0.5 and 1.0 g/kg is safe, related to reproduction and fertility parameters or even in terms of inducing teratogenicity. This paper also shows that the combination of *in vitro* assays to select the dosage, with *in vivo* experiments, which involved the segment I (the period of premating and mating to implantation) and segment II (the period from implantation to major organogenesis), can be useful for assessing safety parameters of new medicinal compounds.

Conflict of Interests

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

Acknowledgments

This work was supported by a Research Grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP): 04/09705-8; 07/53883-6; 08/52643-4; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES/PROSUP; Conselho Nacional de Desenvolvimento Científico e Tecnológico-(CNPQ): 35.0798/2004-4. Lia de Barros Leite Albuquerque is a student of post-Graduate

Course in Pharmaceutical Sciences (Master Level) from UNISO and has a scholarship from CAPES/PROSUP.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 158348, 10 pages
http://dx.doi.org/10.1155/2013/158348

Research Article

Safety Evaluation of Turmeric Polysaccharide Extract: Assessment of Mutagenicity and Acute Oral Toxicity

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Received 5 September 2013; Revised 26 November 2013; Accepted 26 November 2013

Academic Editor: Marina Soković

Curcuma longa Linn. (Zingiberaceae) commonly known as turmeric has long been used for centuries as a spice and household remedy. The present study was carried out to assess the possible mutagenic potential and acute oral toxicity of polysaccharide extract of turmeric rhizome (NR-INF-02) using standard tests. The standard battery of *in vitro* genotoxicity tests, bacterial reverse mutation test (BRMT), chromosome aberration (CA), and micronucleus (MN) tests were employed to assess the possible mutagenic activity of NR-INF-02 (Turmacin). The results showed no mutagenic effect with NR-INF-02 up to a dose of 5000 μg/mL in BRMT. The results on CA and MN tests revealed the non clastogenic activity of NR-INF-02 in a dose range of 250.36 to 2500 μg/mL with and without metabolic activation (S9). In acute oral toxicity study, NR-INF-02 was found to be safe up to 5 g/kg body weight in Wistar rats. Overall, results indicated that polysaccharide extract of *C. longa* was found to be genotoxically safe and also exhibited maximum tolerable dose of more than 5 g/kg rat body weight.

1. Introduction

Turmeric (*Curcuma longa*) has been used for centuries in Ayurvedic medicine, which amalgamate the medicinal goods of herbs with food. This astonishing herb has established its way into the attention in the west because of its wide range of medicinal benefits [1]. In *ayurveda*, rhizome of turmeric is used as medicines against skin, gastrointestinal, respiratory, hepatic, and biliary disorders [2]. The active constituents of *C. longa* are the flavonoid curcumin (diferuloylmethane) and various volatile oils, including tumerone, atlantone, and zingiberene. Other constituents include sugars, proteins, and resins. The best researched active constituent is curcumin, which comprises 0.3–5.4 percent of raw turmeric [1]. Components of turmeric especially curcumin has been shown to have anti-inflammatory, antiviral, and anticancer properties [3–5]. Among the phytoconstituents of *C. longa*, curcuminoids are considered as an important active molecule and also exhibited wide range of pharmacological activities. The literature review confirmed plethora of information available on safety aspects of curcumin and essential oil fractions of *C. longa* [3, 6–8].

While curcuminoids based extract were well studied for their pharmacological and safety aspects, polysaccharide extract of *C. longa* is gaining importance since it showed to have various pharmacological activities, which include antidiabetic, antitumour, antidepressant, antioxidant, antimicrobial, antifertility, hepatoprotective, and immunomodulatory properties [9–16]. NR-INF-02, a polysaccharide extract prepared from rhizome of *C. longa* had shown clinical efficacy in a randomized placebo controlled study on 120 human patients (37 males and 83 females) affected with primary osteoarthritis [17]. NR-INF-02 deserved as an effective option for the treatment of patients with primary painful knee and joint pains and also reduced the need of analgesics as a rescue medication [17]. Also, NR-INF-02 showed immunestimulatory and anti-inflammatory effects in *in vitro* models

by influencing various cytokines involved in immune regulation [18]. Similarly, the immunostimulatory effects of *C. longa* polysaccharides on peripheral blood mononuclear cells was investigated and the findings revealed the potential use of *C. longa* polysaccharide extract as an adjuvant supplement for cancer patients, whose immune activities were suppressed during chemotherapies [16].

Polysaccharide extract of *C. longa* needs evaluation for their safety due to its growing demand on reported preclinical therapeutic indications. Also, before setting of a clinical trial of an herbal product, its safety must be evaluated by toxicity test procedures. Also, this toxicity evaluation is helpful for the estimation of an initial safe starting dose and dose range for the human trials and the identification of parameters for clinical monitoring for potential adverse effects. Hence, the goal of the present investigation is to characterize the toxicological profile of polysaccharide extract from *C. longa* rhizome (NR-INF-02).

In order to evaluate toxicological profile, the ability of NR-INF-02 to induce mutations was assessed in indicator microorganisms using bacterial reverse mutation test. The effect of NR-INF-02 on the genetic system was weighed up by analyzing induced chromosomal aberrations and micronucleus in mammalian cells. An acute oral toxicity study was performed after single oral dose of NR-INF-02 to determine immediate toxic effect. All these tests were conducted based on recommendation of the OECD guidelines [19–22].

2. Materials and Methods

2.1. Collection and Identification of Curcuma longa. The rhizomes of *Curcuma longa* Linn. were collected from different parts of Tamil Nadu State, India and authenticated at NISCAIR (National Institute of Science Communication and Information Resources). A voucher specimen (no. 653) was deposited in our herbarium.

2.2. Preparation of Polysaccharide Extract of Curcuma longa Extract (NR-INF-02). NR-INF-02 is manufactured and registered as Turmacin by Natural Remedies Pvt. Ltd., Bangalore, India. Coarsely powdered rhizomes of *C. longa* were subjected to steam distillation and the turmeric oil was separated and collected. The rhizomes were further extracted by refluxing with water in a commercial extraction facility. The liquid water extract was concentrated by distillation under vacuum and the resultant concentrated liquid was spray dried to obtain a free flowing powder. NR-INF-02 was prepared by blending 99 parts of spray dried water extract of *C. longa* and 1 part of the turmeric oil followed by sieving. Yield of this extract is 10.5% w/w.

Details about the preparation and characterization of NR-INF-02 were described as earlier [18]. In brief, NR-INF-02 was standardized to contain polysaccharides (>10% w/w) by high performance liquid chromatography [23]. As reported previously, NR-INF-02 was found to contain negligible amount of curcumin [18]. NR-INF-02 was assessed for presence and absence of microbial (total aerobic microbial count, total yeast and mould count, bile tolerant gram negative bacteria, *E. coli*, Salmonella species, *S. aureus*), heavy metals (lead, arsenic, cadmium, and mercury), pesticides (75 different pesticides), and aflatoxin levels as per the method described by the United States Pharmacopeia [24]. Results indicated that NR-INF-02 is compliant to the limit set by United States Pharmacopeia (USP) and British Pharmacopeia [24, 25]. Stability of NR-INF-02 was assessed using accelerated and real time protocols of ICH guidelines [26]. NR-INF-02 did not undergo any major changes (physically, chemically, and microbiologically) when stored under accelerated and real time condition for 6 months and 12 months, respectively. Based on the stability study it is recommended to store NR-INF-02 below 30°C in an airtight container.

2.3. Source of Chemicals. 2-aminoanthracene (2-AA), benzo[a]pyrene, colchicine, cytochalasin B, D-glucose-6-phosphate, 3-(4,5Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, nicotinamide adenine dinucleotide phosphate, mitomycin C, 2-nitrofluorene (2-NF), 4-nitroquinolene-N-oxide (4-NQO), phytohemagglutinin, potassium chloride (KCl) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was procured from Hyclone. Aroclor 1254 induced S9 fraction was obtained from Moltox. The Ames II Automated System for High Throughput Screening kit containing Salmonella strains like TA98 strain and TAMix strain, ampicillin, exposure medium incubation bag, indicator medium and growth medium were purchased from Xenometrix. Ham's F12K medium, RPMI 1640 medium and Trypsin-EDTA were purchased from Gibco Life Technologies.

2.4. Bacterial Strains and Mammalian Cells. The bacterial reverse mutation test (Ames II) was performed using *Salmonella typhimurium* histidine auxotrophs, TA98 strain and TAMix strain. Genotyping on Salmonella strains like TA98 strain and TAMix strain was confirmed before conducting the mutagenicity study. Concentration of standard mutagen(s), S9 and incubation time were optimized and selected based on previous publications [27–31] which we have followed in the current genotoxicity study. 10 μL of freshly thawed frozen Salmonella strains like TA98 strain and TAMix strain were inoculated to 10 mL of growth medium and the cultures were grown overnight at 37°C in an environmental shaker set at 180 rpm in the presence of ampicillin (50 μg/mL). The human peripheral blood for chromosome aberration study was collected from healthy human volunteers. Lymphocytes of human peripheral blood cells were cultured in RPMI 1640 media supplemented with 20% heat-inactivated fetal bovine serum (FBS) and 2% phytohemagglutinin-M for 48 h in culture tubes fitted with loose caps at 37°C in a humidified atmosphere of 5% CO_2. Chinese Hamster Ovary cells (CHO-K1) for micronucleus test was procured from American Type Culture Collection. CHO-K1 cells were cultured in Ham's F12K media supplemented with 10% FBS, in 37°C incubator maintained at 5% CO_2.

2.5. Bacterial Reverse Mutation Test. The bacterial reverse mutation test (Ames II) was used to identify the ability of test substance to induce reverse mutation at histidine loci

in Salmonella strains like TA98 strain and TAMix strain (mixture of six base pair mutant strains TA7001-7006). This mutagenicity test was performed according to standard procedure [19, 27–32]. Briefly, *Salmonella typhimurium* tester strains like TA98 strain and TAMix strain were exposed to NR-INF-02 by fluctuation method with and without S9. Dulbecco's Phosphate Buffered Saline (DPBS) was used to solubilise NR-INF-02 and also designated as a vehicle control. NR-INF-02 at a maximum concentration of 5000 μg/mL and subsequent concentrations, 1582.28, 500.72, 158.46, 50.14, and 15.87 μg/mL were selected to assess the mutagenic effect. NR-INF-02 at concentration range of 15.87 to 5000 μg/mL, Salmonella tester strain like TA98/TAMix strain with and without S9 mix were added along with reversion indicator media into the wells of 384-well plates and incubated for 48 h at 37°C. All doses of the NR-INF-02, vehicle control (DPBS), and positive controls were plated in triplicates; 2-NF (2 μg/mL) + 4-NQO (0.5 μg/mL) and 2-AA (5 μg/mL) were used as positive controls in the absence and presence of S9 respectively. After incubation, the revertant colonies were counted and positive response was determined by significant increase in the mean revertant per plate of atleast one of the tester strains as compared to vehicle control.

2.6. Chromosome Aberration Assay Using Human Peripheral Blood Lymphocytes. Chromosome aberration test was performed as per OECD guideline no. 473 [20]. Human blood was collected aseptically from one healthy, non-smoking male donor. 0.5 mL of blood, 9.5 mL growth medium (RPMI 1640 + 20% heat inactivated fetal bovine serum) were added and cultured using phytohemagglutinin-M (2% v/v) for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The cultures were gently shaken daily. After 48 h, cells were treated with NR-INF-02 (250.35, 791.14, 2500 μg/mL) in presence and absence of S9. NR-INF-02 at a maximum concentration of 2500 μg/mL was used since it did not produce evident cytotoxicity to human lymphocytes. RPMI 1640 medium was used to dissolve NR-INF-02 and also designated as vehicle control. Mitomycin C (0.2 μg/mL) was used as positive control for both short term (4 h) and long term (36 h) exposure studies in the absence of S9, whereas, benzo[a]pyrene (20 μg/mL) was used as positive control for short term exposure (4 h) study only with S9. After short term exposure, the treatment medium was removed by centrifugation and cultures replenished with growth medium (RPMI 1640 + 10% FBS). The cells were again returned to the incubator to complete 1.5 cell cycle lengths. For long term, the cells are treated with test compound for the entire 1.5 cell cycle length. Cells were exposed to colchicine (0.3 μg/mL) for 3 h prior to harvest. After 3 h, cells were centrifuged, resuspended in hypotonic medium (75 mM KCl) and fixed (prechilled, 3 parts of ethanol and 1 part of glacial acetic acid). Approximately 3-4 drops of the fixed cell suspension from the height of 30 cm was dropped onto a clean microscope slide and stained with 10% Giemsa solution for 10 min. Two hundred metaphase chromosome spreads per treatment were examined under the microscope for chromosome break, chromatid break, deletion, ring, dicentric and rearrangements as indicated by Savage [33]. Only metaphases containing 46 ± 2 centromeres (chromosomes) were considered for analysis. The number of cells with aberrations and the number of aberrations were recorded. Experiments were performed in duplicates per treatment. Cytotoxicity was determined by calculating Mitotic index (MI) according to the formula

$$\text{percent MI} = \left(\frac{\text{no. of cells in metaphase}}{\text{total number of cells}}\right) \times 100. \quad (1)$$

2.7. In Vitro Micronucleus Test in Chinese Hamster Ovary (CHO-K1) Cells. Micronucleus test was carried out according to the OECD guideline no. 487 [21]. CHO-K1 cells were incubated with NR-INF-02 (250.36 μg/mL, 791.14 μg/mL and 2500 μg/mL) in both short (4 h) and long term (18 h) exposure studies with and without S9. Ham's F12K media was used to solubilise NR-INF-02 and also designated as a vehicle control. Mitomycin C (0.2 μg/mL) was used as positive control in the absence of S9 for both short and long term exposure studies. Benzo[a]pyrene (20 μg/mL), was used only with S9 in a short term exposure study. After short term exposure, treatment medium was removed by aspiration and the cells were rinsed with Hank's balanced salt solution (HBSS) and replenished with complete Ham's F12 medium containing cytochalasin B (3 μg/mL) to arrest cytoplasmic division of the cells. The cells were then returned to the incubator for an additional 18 h. For long term study, NR-INF-02 and cytochalasin B were added together and incubated for 18 h. After trypsinization, cells were suspended in 75 mM KCl and fixed using precooled fixative [ethanol and glacial acetic acid (3 : 1)]. Approximately 3-4 drops of the cell suspension were dropped from the height of 5 cm onto a clean microscope slide. The slides were air dried overnight and stained with 10% Giemsa for 10 min. Experiments were performed in duplicates. The criteria for selecting the binucleated cells were based on the report by Fenech [34]. Per treatment, 2000 cytoplasm division arrested cells were examined for the presence of micronucleus. Cytokinesis Block Proliferation Index (CBPI) was determined to calculate the cell toxic effect of treatment according to the formula

$$\begin{aligned}\text{CPBI} = {} & (\text{no. of mononucleated cells} \\ & + 2\,(\text{no. of binucleated cells}) \\ & + 3\,(\text{no. of multinucleate cells})) \\ & \times (\text{total number of cells})^{-1}.\end{aligned} \quad (2)$$

2.8. Acute Oral Toxicity. The animal experiment was conducted according to the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines and after approval by the Institutional Animal Ethics Committee (IAEC). Female albino Wistar rats (8–12 weeks) were accommodated in polypropylene cages and temperature was maintained between 25 ± 2°C with 12 h each of dark and light cycle. The rats were fed with standard laboratory pelleted feed (M/s Gold Mohur Foods & Feeds Ltd., Bangalore, India). The rats were fasted overnight before and 3 h after the administration of NR-INF-02. Acute oral toxicity

study was performed as per the OECD Guideline for the Testing of Chemicals (Test no. 420, Section 4: Health Effects) Acute Oral Toxicity—Fixed Dose Procedure [22]. NR-INF-02 solubilized in demineralized water was administered by oral route to rats at the limit dose of 5 g/kg body weight in a sequential manner. On the day of dosing, all the animals were observed for mortality and clinical signs for first 10 min, 30 min, 1 h, 2 h, 4 h, and 6 h after dosing and thereafter twice daily for mortality and once a day for clinical signs, for 14 days. Animals were sacrificed at the end of the study period of 14 days.

2.9. Statistical Analysis. Data are expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was performed on the results followed by a Dunnett's test for multiple comparisons using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) statistical software package. The significance level was chosen at $P < 0.05$ for all statistical analyses in comparison to the respective vehicle control.

3. Results

3.1. Bacterial Reverse Mutation Test. To detect possible point mutations by NR-INF-02, histidine requiring mutants of *S. typhimurium* strains likeTA98 strain and TAMix strain, with and without S9 were used. No significant bacterial cell toxicity was observed after treatment with NR-INF-02 up to a maximum concentration of 5000 μg/mL. The occurrence of spontaneous reversion for TA98 strain and TAMix strain is in agreement with previous reports [27–31]. ANOVA followed by Dunnett's multiple comparison tests indicated that treatment of NR-INF-02 at concentrations, namely, 15.87 μg/mL, 50.14 μg/mL, 158.46 μg/mL, 500.72 μg/mL, 1582.28 μg/mL, and 5000 μg/mL, did not show any significant increase in the number of revertant colonies in both Salmonella strains like TA98 strain and TAMix strain with and without S9 when compared to vehicle control. Positive controls, 2-NF + 4-NQO, 2-AA demonstrated a significant ($P < 0.05$) increase in the number of revertant colonies in the absence and presence of S9 respectively (Tables 1 and 2). These results confirmed the nonmutagenic property of NR-INF-02 in TA98 strain and TAMix strain of *S. typhimurium.*

3.2. In Vitro Chromosome Aberration Analysis in Human Peripheral Blood Lymphocytes. Mitotic index values indicated that NR-INF-02 at dose level, namely, 250.36 μg/mL, 791.14 μg/mL, and 2500 μg/mL, did not produce cell toxicity in both short term (4 h) and long term exposure (36 h) with and without S9 (Tables 3, 4, and 5). Treated cells arrested at metaphase stage were analyzed for possible structural chromosomal aberrations. Exposure of NR-INF-02 at the indicated concentrations (250.36 μg/mL, 791.14 μg/mL and 2500.0 μg/mL) to human lymphocytes did not induce a statistically significant increase in the number of cells with chromosome aberrations in the absence and presence of S9 in both short and long term exposure studies. As determined by ANOVA followed by Dunnett's multiple comparison tests, vehicle control cultures had an insignificant number of structural chromosomal aberrations which were within the limit of published data [28, 30, 31]. The positive controls, mitomycin C, and benzo[a]pyrene were found to induce statistically significant ($P < 0.05$) number of structural chromosomal aberrations, namely, chromosome break, chromatid break, deletion in absence and presence of S9 respectively in human peripheral blood lymphocytes (Tables 3, 4 and 5).

3.3. In Vitro Micronucleus Analysis in CHO-K1 Cells. Statistical analysis (ANOVA followed by Dunnett's tests) indicated that CBPI values did not differ significantly between the tested concentrations of NR-INF-02 and vehicle control. Mitomycin C treatment showed significant ($P < 0.05$) reduction on viability of CHO-K1 cells in both short term and long term exposure cultures as evident from decrease of CBPI values in comparison with vehicle control. In the micronucleus test, no statistically significant increase in the frequency of cells with micronucleus was observed upon treatment with NR-INF-02 at the indicated concentrations (250.36 μg/mL, 791.14 μg/mL and 2500 μg/mL), in the presence and absence of S9, in both short (4 h) and long term (18 h) exposure cultures. Vehicle control had an insignificant number of micronucleus which were within the limit of published data [28, 30, 31]. As evident, the positive controls mitomycin C and benzo[a]pyrene formed significant ($P < 0.05$) increase in the frequency of cells with micronucleus in absence and presence of S9 respectively. Percent micronucleated, binucleated cells, and CBPI values for all treatment cultures are presented in Table 6.

3.4. Acute Oral Toxicity. NR-INF-02 was evaluated for its acute oral toxicity by administering as a single oral dose to albino Wistar rats. NR-INF-02 was administered orally in a sequential manner to five rats at the limit dose level of 5000 mg/kg body weight. On the day of dosing, all the animals were observed for mortality and clinical signs for first 10 min, 30 min, 1 h, 2 h, 4 h, and 6 h after dosing and thereafter twice daily for mortality and once a day for clinical signs, for 14 days. The body weight of rats was recorded and weekly body weight gain was calculated. After the observation period of 14 days, all surviving rats were sacrificed and subjected to complete necropsy. The treated rats did not show any adverse clinical signs immediately following dosing and during the observation period of 14 days. In sighting and main studies, treatment with NR-INF-02 did not reveal any adverse effects on the body weight gain at first and second week of observation. Overall, the percent body weight gain during the complete 14 days observation period was found to be normal in all the treated animals. On necropsy, no major gross pathological changes were observed in NR-INF-02 treated rats (Tables 7 and 8). Based on the findings of the present study, NR-INF-02 was found to be safe after oral administration as a single dose of 5000 mg/kg to female albino Wistar rats.

4. Discussion

This study is focused on characterization of toxicological properties of *C. longa* polysaccharide extract: mutagenicity,

Table 1: Mutagenicity testing of NR-INF-02 in *Salmonella typhimurium* TA98 strain.

Treatment	Concentration (μg/mL)	S9	Number of revertant colonies				
			Individual colony counts			Mean ± S.D	Fold increase over baseline
			R1	R2	R3		
NR-INF-02	15.87	−	8	6	7	7.00 ± 1.00	0.83
		+	7	5	7	6.33 ± 1.15	0.90
	50.14	−	6	4	8	6.00 ± 2.00	0.71
		+	3	8	7	6.00 ± 2.65	0.86
	158.46	−	7	6	8	7.00 ± 1.00	0.83
		+	5	8	2	5.00 ± 3.00	0.71
	500.72	−	4	7	6	5.67 ± 1.53	0.67
		+	4	6	4	4.67 ± 1.15	0.67
	1582.28	−	8	8	8	8.00 ± 0.00	0.95
		+	3	7	4	4.67 ± 2.08	0.67
	5000.00	−	8	7	4	6.33 ± 2.08	0.75
		+	8	7	7	7.33 ± 0.58	1.05
Vehicle control	0	−	8	4	7	6.33 ± 2.08	—
		+	6	7	5	6.00 ± 1.00	—
Positive control 2-NF + 4-NQO	2.00 + 0.50	−	47	46	48	47.00 ± 1.00*	5.58
Positive control 2-AA	5.00	+	48	48	48	48.00 ± 0.00*	6.85

R: replicate; *$P < 0.05$; 2-NF: 2-nitrofluorene; 4-NQO: 4-nitroquinoline-N-oxide; 2-AA: 2-aminoanthracene.

Table 2: Mutagenicity testing of NR-INF-02 in *Salmonella typhimurium* TAMix strain.

Treatment	Concentration (μg/mL)	S9	Number of revertants colonies				
			Individual colony counts			Mean ± S.D	Fold increase over baseline
			R1	R2	R3		
NR-INF-02	15.87	−	0	2	0	0.67 ± 1.15	0.67
		+	0	0	0	0.00 ± 0.00	0.00
	50.14	−	0	0	1	0.33 ± 0.58	0.33
		+	0	2	0	0.67 ± 1.15	0.37
	158.46	−	0	0	2	0.67 ± 1.15	0.67
		+	2	1	2	1.67 ± 0.58	0.92
	500.72	−	0	1	0	0.33 ± 0.58	0.33
		+	0	0	1	0.33 ± 0.58	0.18
	1582.28	−	2	1	1	1.33 ± 0.58	1.33
		+	0	2	0	0.67 ± 1.15	0.37
	5000.00	−	0	3	2	1.67 ± 1.53	1.67
		+	0	2	0	0.67 ± 1.15	0.37
Vehicle control	0	−	1	1	1	1.00 ± 0.00	—
		+	0	0	2	0.67 ± 1.15	—
Positive control 2-NF + 4-NQO	2.00 + 0.50	−	48	48	48	48.00 ± 0.00*	48.00
Positive control 2-AA	5.00	+	45	41	47	44.33 ± 3.06*	24.35

R: replicate; *$P < 0.05$; 2-NF: 2-nitrofluorene; 4-NQO: 4-nitroquinoline-N-oxide; 2-AA: 2-aminoanthracene.

clastogenicity, and acute oral toxicity. These evaluations are a fundamental part of the clinical study aimed at determining the risk/benefit ratio of its use in the field of human health and subsequently introducing this extract into dietary supplement practice. Hence, this study was conducted to investigate the possible genotoxic potential of NR-INF-02 using bacterial reverse mutation, chromosomal aberration and micronucleus tests.

The significance of the bacterial reverse mutation test has been clearly confirmed as a suitable primary test for the detection of potential mutagens and carcinogens, and since midseventies this assay has been routinely used as

Table 3: Clastogenicity study upon short term exposure (4 h) of NR-INF-02 in the absence of metabolic activation in human blood lymphocytes.

Treatment	Vehicle control		Positive control MMC (0.20 μg/mL)		NR-INF-02 (2500.00 μg/mL)		NR-INF-02 (791.14 μg/mL)		NR-INF-02 (250.36 μg/mL)	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Total number of metaphase analyzed	100	100	100	100	100	100	100	100	100	100
Normal	99	100	92	92	98	97	98	96	98	100
Chromatid break	1	—	—	4	1	—	1	—	—	—
Chromosome break	—	—	—	1	—	1	—	—	—	—
Deletion	—	—	—	1	—	—	—	—	1	—
Ring	—	—	3	1	1	—	—	—	1	—
Dicentric	—	—	5	1	—	2	—	1	—	—
Fragment	—	—	—	—	—	—	—	1	—	—
Gap	—	—	—	—	—	—	1	1	—	—
Ploidy	—	—	—	—	—	—	—	1	—	—
Endoreduplication	—	—	—	—	—	—	—	—	—	—
Total aberrations	1	0	8	8	2	3	1	1	2	0
Total aberrations (Mean ± SD)	0.50 ± 0.70		8.00 ± 0.00*		2.50 ± 0.70		1.0 ± 0.00		1.00 ± 1.41	
Mitotic Index (Mean ± SD)	5.35 ± 0.49		2.63 ± 0.10*		3.75 ± 1.06		4.25 ± 0.35		4.0 ± 0.42	

R: replicate; *$P < 0.05$; MMC: mitomycin C; Ploidy, endoreduplication, gaps, and fragments are not considered as aberrations for calculations.

Table 4: Clastogenicity study upon short term exposure (4 h) of NR-INF-02 in the presence of metabolic activation in human blood lymphocytes.

Treatment	Vehicle control		Positive control B[a]P (20.00 μg/mL)		NR-INF-02 (2500.00 μg/mL)		NR-INF-02 (791.14 μg/mL)		NR-INF-02 (250.36 μg/mL)	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Total number of metaphase analyzed	100	100	100	100	100	100	100	100	100	100
Normal	100	97	93	89	100	96	97	97	98	98
Chromatid break	—	—	3	2	—	—	—	—	—	1
Chromosome break	—	—	1	1	—	—	1	—	—	—
Deletion	—	—	—	—	—	—	1	—	—	—
Ring	—	—	1	5	—	3	—	2	—	—
Dicentric	—	1	1	1	—	—	—	1	1	1
Fragment	—	—	—	—	—	—	—	—	—	—
Gap	—	—	—	—	—	—	1	—	—	—
Ploidy	—	2	1	2	—	—	—	—	1	—
Endoreduplication	—	—	—	—	—	1	—	—	—	—
Total aberrations	0	1	6	9	0	3	2	3	1	2
Total aberrations (Mean ± SD)	0.50 ± 0.70		7.50 ± 2.12*		1.50 ± 2.12		2.50 ± 0.70		1.50 ± 0.70	
Mitotic Index (Mean ± SD)	3.20 ± 0.14		2.60 ± 0.13		2.70 ± 0.28		3.0 ± 0.28		3.45 ± 0.35	

R: replicate; *$P < 0.05$; B[a]P: benzo[a]pyrene; Ploidy, endoreduplication, gaps, and fragments are not considered as aberrations for calculations.

a screening assay to predict carcinogens [32]. NR-INF-02 at dose range of 15.87–5000 μg/mL did not induce any significant increase in the revertant colonies both in the presence and absence of metabolic activation. These results confirmed the nonmutagenic activity in Salmonella strains like TA98 strain and TAMix strain. This is in agreement with the absence of genotoxicity of *C. longa* extracts tested *in vitro* by several researchers [6, 35–38]. Moreover, a substantial lack of genotoxic activity is reported in the literature for extracts obtained from polysaccharide fraction of other species of the genus Curcuma, such as *C. zedoaria* [39]. Apart from lack of mutagenicity, antimutagenic property of *C. longa* extracts against several classical mutagens were reported [35, 40].

The study of DNA damage at the chromosomal level and micronuclei formation is a vital part of genetic toxicity screening [20, 21]. Chromosome aberration and

Table 5: Clastogenicity study upon long term exposure (36 h) of NR-INF-02 in the absence of metabolic activation in human blood lymphocytes.

Treatment	Vehicle control		Positive control MMC (0.20 μg/mL)		NR-INF-02 (2500.00 μg/mL)		NR-INF-02 (791.14 μg/mL)		NR-INF-02 (250.36 μg/mL)	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Total number of metaphase analyzed	100	100	100	100	100	100	100	100	100	100
Normal	98	99	89	95	97	99	96	100	100	100
Chromatid break	—	—	4	2	—	—	1	—	—	—
Chromosome break	—	—	—	—	—	—	—	—	—	—
Deletion	—	—	4	3	1	—	—	—	—	—
Ring	1	1	1	—	1	1	1	—	—	—
Dicentric	—	—	1	—	—	—	2	—	—	—
Fragment	—	—	—	—	1	—	—	—	—	—
Gap	—	—	—	—	—	—	—	—	—	—
Ploidy	1	—	1	—	—	—	—	—	—	—
Endoreduplication	—	—	—	—	—	—	—	—	—	—
Total aberrations	1	1	10	5	2	1	4	0	0	0
Total aberrations (Mean ± SD)	1.00 ± 0.00		7.50 ± 3.53*		1.50 ± 0.70		2.00 ± 2.82		0.00 ± 0.00	
Mitotic Index (Mean ± SD)	3.41 ± 0.41		2.15 ± 0.07*		2.86 ± 0.23		2.85 ± 0.28		3.01 ± 0.01	

R: replicate; *$P < 0.05$; MMC: mitomycin C; Ploidy, endoreduplication, gaps, and fragments are not considered as aberrations for calculations.

Table 6: Effect of NR-INF-02 on micronucleus induction in CHO-K1 cells.

Treatment (μg/mL)	MN-BN cells (%) Mean ± SD	CBPI Mean ± SD
Without S9 short term exposure (4 h)		
Vehicle control	0.15 ± 0.07	1.80 ± 0.01
NR-INF-02 (250.36 μg/mL)	0.00 ± 0.00	1.78 ± 0.03
NR-INF-02 (791.14 μg/mL)	0.00 ± 0.00	1.78 ± 0.01
NR-INF-02 (2500.00 μg/mL)	0.00 ± 0.00	1.77 ± 0.01
Positive control MMC (0.2 μg/mL)	0.50 ± 0.14*	1.66 ± 0.03*
With S9 short term exposure (4 h)		
Vehicle control	0.00 ± 0.00	1.80 ± 0.01
NR-INF-02 (250.36 μg/mL)	0.05 ± 0.07	1.80 ± 0.04
NR-INF-02 (791.14 μg/mL)	0.00 ± 0.00	1.80 ± 0.01
NR-INF-02 (2500.0 μg/mL)	0.00 ± 0.00	1.80 ± 0.04
Positive control B[a]P (20.0 μg/mL)	1.6 ± 0.28*	1.70 ± 0.03
Without S9 long term exposure (18 h)		
Vehicle control	0.00 ± 0.00	1.86 ± 0.00
NR-INF-02 (250.36 μg/mL)	0.10 ± 0.00	1.87 ± 0.02
NR-INF-02 (791.14 μg/mL)	0.00 ± 0.00	1.86 ± 0.01
NR-INF-02 (2500.0 μg/mL)	0.15 ± 0.07	1.84 ± 0.01
Positive control MMC (0.2 μg/mL)	4.55 ± 0.78*	1.58 ± 0.04*

*$P < 0.05$; MMC: mitomycin C; B[a]P: benzo[a]pyrene.

micronucleus tests were carried out with NR-INF-02 using peripheral blood lymphocytes and CHO-K1 cells, respectively. The effect of NR-INF-02 on dividing cell population was examined by studying mitotic index and CBPI in peripheral blood lymphocytes and CHO-K1 cells, respectively. The mitotic index is used to quantify differences in cell division when an environmental parameter is changed [20]. CBPI indicates the number of cell cycles per cell during the period of exposure to cytochalasin B [21]. Treatment effect of NR-INF-02 on MI and CBPI indicated that cells did not differ in their capability to divide in comparison to vehicle control. Also, NR-INF-02 did not induce significant chromosome aberrations and micronuclei formation in mammalian cells. These results are in accordance with the absence of clastogenic effect of *C. longa* and *C. zedoaria* extracts tested and reported previously [39–42]. In addition, anticlatsogenic effect of *C. longa* was observed against benzo (a) pyrene induced micronucleus in mice and these results indicated that components of *C. longa* could help in cancer chemoprevention [35, 40].

Acute oral toxicity data on NR-INF-02 is used to satisfy hazard classification and labelling requirements for its risk assessment in human health and environment [43]. In the present study, acute oral toxicity assessment of NR-INF-02 did not cause mortality, abnormal clinical signs or any significant pathological changes at a dose level of 5000 mg/kg body weight. Also, the overall body weight gain was found to be normal in all the treated rats and hence resulted in labelling the NR-INF-02 as unclassified in the hazard category according to Globally Harmonised System [22]. Current acute oral toxicity study results are in agreement with previous study which demonstrated no toxic effects upon ingestion of turmeric extracts by rats [44].

In contrary, curcumin induced significant increases in sister chromatid exchanges and chromosomal aberrations

Table 7: Clinical signs and gross pathology findings in rats after treatment with NR-INF-02.

Study	Dose (g/kg)	Cage side observations		Total number of animals	Gross pathology findings
		Observed signs	Period of signs in days, from–to		
Sighting ($n = 1$)	5	Nil	0–14	1	No abnormality detected
Main ($n = 4$)	5	Nil	0–14	4	No abnormality detected

n: number of animals.

Table 8: Effect of NR-INF-02 on body weight and percent body weight gain in rats.

Study	Dose (g/kg)	Body weight			Percent body weight gain		
		Day 0	Day 7	Day 14	Days 0–7	Days 7–14	Days 0–14
Sighting ($n = 1$)	5	163	188	204	15.34	8.51	25.15
Main ($n = 4$)	5	163.75	195.75	216.25	19.54	10.47	32.06

n: number of animals.

in cultured Chinese hamster ovary cells [36]. In addition, hepatotoxicity was observed in rodents fed turmeric for chronic duration [45, 46]. National Toxicology Program conducted detailed two year safety study; results indicated an equivocal evidence of carcinogenic activity, increased incidences of ulcers, hyperplasia, and inflammation of the forestomach, cecum, and colon in rats of turmeric oleoresin exposed groups [36]. Differences on results observed between the current study and published results might be due to variation in phytochemical composition of test items.

NR-INF-02 is a polysaccharide containing extract and is a complex mixture of natural substances. As evident in our results, NR-INF-02 contains acceptable pharmacopeial limits for microbes, heavy metals, pesticides, and aflatoxin contents which further confirm the quality assurance of this extract.

The use of genotoxicity testing is to determine *C. longa* extract influences genetic material or may cause cancer. The results confirmed the genotoxic safety of *C. longa* extract in a battery of genotoxicity tests. Acute oral toxicity data on *C. longa* extract is used to satisfy hazard classification and labelling requirements for its risk assessment in human health and environment [43]. In the present study, acute oral toxicity assessment of *C. longa* extract did not cause mortality, abnormal clinical signs, or any significant pathological changes upto the dose level of 5000 mg/kg body weight. Also, the overall body weight gain was found to be normal in all the treated rats and hence resulted in labelling the *C. longa* extract as unclassified in the hazard category according to Globally Harmonised System [22]. Also, clinical effective dose of NR-INF-02 for pain management in human osteoarthritis patients was achieved at 1 g/day with no adverse effects after daily oral intake of 42 days [17].

5. Conclusion

In conclusion, polysaccharide extract of *C. longa* was found to be non-mutagenic to *S. typhimurium* strains like TA98 strain and TAMix strain. *C. longa* did not increase the occurrence of structural chromosomal aberrations in human peripheral blood lymphocytes and micronucleus formation in CHO-K1 cells. Also, it was found to be safe after oral administration as a single dose to female albino Wistar rats up to 5 g/kg body weight. Therefore, polysaccharide extract from rhizomes of *C. longa* is not mutagenic in the tested standard battery of genotoxicity tests and found to be safe in an acute oral toxicity study.

Conflict of Interests

Trade mark of the product under study is owned by the company for which all the authors work.

Disclosure

This research received no specific grant from any funding agency in the public and not-for-profit sectors.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 342973, 12 pages
http://dx.doi.org/10.1155/2013/342973

Research Article

Citrus Flavanones Affect Hepatic Fatty Acid Oxidation in Rats by Acting as Prooxidant Agents

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Received 22 August 2013; Revised 17 September 2013; Accepted 18 September 2013

Academic Editor: Isabel C. F. R. Ferreira

Citrus flavonoids have a wide range of biological activities and positive health effects on mammalian cells because of their antioxidant properties. However, they also act as prooxidants and thus may interfere with metabolic pathways. The purpose of this work was to evaluate the effects of three citrus flavanones, hesperidin, hesperetin, and naringenin, on several parameters linked to fatty acid oxidation in mitochondria, peroxisomes, and perfused livers of rats. When exogenous octanoate was used as substrate, hesperetin and naringenin reduced the mitochondrial NADH/NAD^+ ratio and stimulated the citric acid cycle without significant changes on oxygen uptake or ketogenesis. When fatty acid oxidation from endogenous sources was evaluated, hesperetin and naringenin strongly reduced the mitochondrial NADH/NAD^+ ratio. They also inhibited both oxygen uptake and ketogenesis and stimulated the citric acid cycle. Hesperidin, on the other hand, had little to no effect on these parameters. These results confirm the hypothesis that citrus flavanones are able to induce a more oxidised state in liver cells, altering parameters related to hepatic fatty acid oxidation. The prooxidant effect is most likely a consequence of the ability of these substances to oxidise NADH upon production of phenoxyl radicals in the presence of peroxidases and hydrogen peroxide.

1. Introduction

Citrus flavonoids are found at high concentrations in citrus fruits; the most common classes are flavanones, flavones, and flavonols [1]. These compounds exhibit a wide range of biological activities and positive health effects on mammalian cells, including antiinflammatory, antiatherogenic, and anticancer effects. The antioxidant properties of citrus flavonoids and their metabolites have been found to be at least partially responsible for these therapeutic actions [2–4].

The antioxidant activities of citrus flavonoids and their metabolites depend upon their molecular structures. The arrangements of hydroxyl, methoxy, and glycosidic side groups, as well as the conjugation between the aromatic rings are important features that govern antioxidant activity [5]. For example, the antioxidant capacities of quercetin and fisetin, two abundant dietary flavonols, are favoured by some structural peculiarities (Figure 1): (a) a high degree of hydroxylation; (b) presence of 2,3 unsaturation in conjugation with the 4-oxo group in the C-ring; (c) a dihydroxylated B-ring (catechol), which allows prompt hydrogen donation (electrons); and (d) the presence of both 3- and 5-hydroxyls in quercetin and 3-hydroxyls in fisetin [6–10]. However, not all studies confirm the antioxidant effects of quercetin, fisetin and other flavonoids, and several report that they have prooxidant capabilities [11–13]. Both quercetin and fisetin are able to promote NADH oxidation because of interactions with cellular enzymes and their abilities to shift cellular conditions to a more oxidised state [12, 13]. This state is reflected by changes in several parameters of

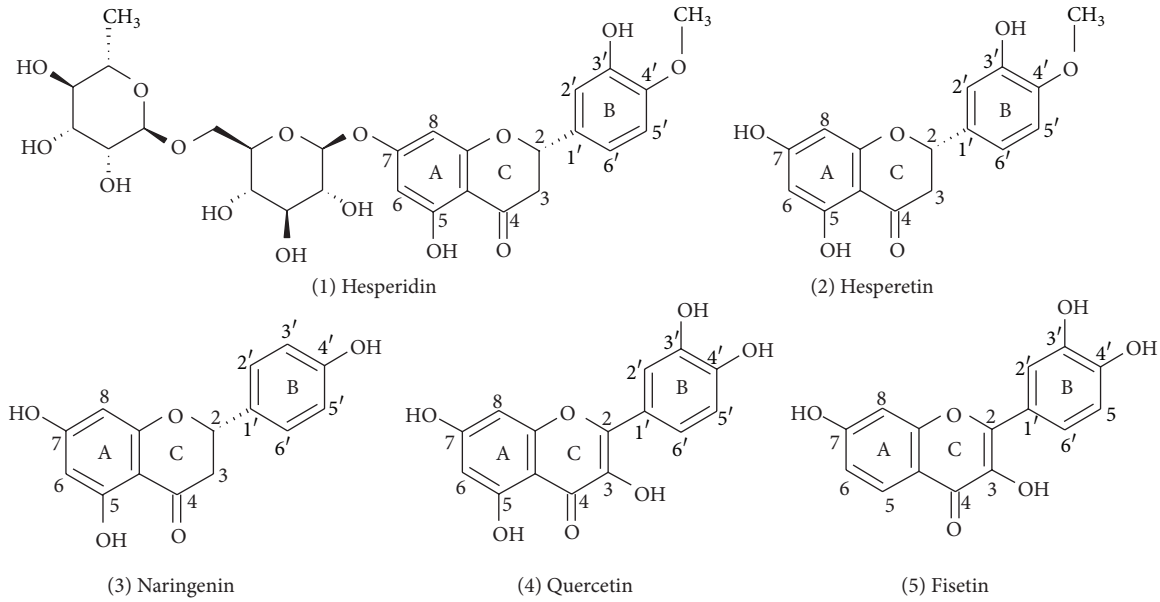

Figure 1: Chemical structures of the flavanones hesperidin (1), hesperetin (2), and naringenin (3) and of the flavonols quercetin (4) and fisetin (5). The rings of each compound are highlighted.

cellular metabolism, such as inhibition of gluconeogenesis and ketogenesis [12–16].

This prooxidant effect is apparently not restricted to quercetin and fisetin; indeed, previous studies have shown that citrus flavonoids belonging to the flavanones, for example, hesperidin, hesperetin, and naringenin (Figure 1), can form prooxidant metabolites that oxidise NADH upon oxidation by peroxidase/H_2O_2 [17, 18]. Despite obvious structural differences between flavonols and flavanones (e.g., flavanones are nonplanar, have a chiral centre at C2, and contain a saturated C2-C3 bond and a phenol B ring), they could have effects on liver metabolism similar to those caused by the flavonols quercetin and fisetin [12, 13].

The main purpose of this work was to assess whether three common flavanones, namely, hesperidin, hesperetin, and naringenin (Figure 1), have prooxidant activities in liver cells and determine in the effects of their differing structures on various metabolic parameters. For this study, we used isolated perfused rat liver and subcellular liver fractions.

A more detailed characterisation of the mode of action of citrus flavanones in mammalian cells (especially that of any potential prooxidant effects) is important because these substances have generally been considered to be effective antioxidants that are beneficial in the contexts of cancer, inflammation, and atherosclerosis [2–4].

2. Materials and Methods

2.1. Materials. Hesperidin, hesperetin, naringenin, NADH, NAD^+, succinate, L-malate, L-carnitine, palmitoyl-L-carnitine, octanoyl-L-carnitine, octanoyl-CoA, palmitoyl-CoA, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2′,7′-dichlorofluorescein (DCF), 2,4-dinitrophenol (DNP), 2,5-diphenyloxazole, 2,2-p-phenylenebis(5-phenyloxazole), fatty acid-free serum albumin, phenylmethylsulfonyl fluoride (PMSF), horseradish peroxidase (HRP) type VI-A, D-β-hydroxybutyrate, D-β-hydroxybutyrate dehydrogenase from *Pseudomonas lemoignei*, and labelled octanoate ([1-^{14}C]octanoate), (50 μCi/mmol) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). All other chemicals were of the best available grade.

2.2. Animals. Male Wistar rats weighing 180–220 g and fed *ad libitum* with a standard laboratory diet (Nuvilab CR-1) were housed in polycarbonate cages in a controlled environment, with a 12 : 12 h light-dark cycle starting at 06:00 AM, at 20–23°C. Overnight-fasted animals were used in all experimental protocols. All experiments were conducted in strict adherence with the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá (CEAE, Registered number 014/2009, Protocol 002/2009), which is in accordance with the internationally accepted recommendations for the care and use of animals.

2.3. Liver Perfusion. The liver perfusion apparatus was built in the workshops of the University of Maringá. For the surgical procedure, the animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The criterion for anaesthesia was lack of body or limb movement in response to a standardised tail clamp stimulus. The haemoglobin-free, nonrecirculating perfusion was performed according to the technique described by Scholz and Bücher [19]. After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. The hepatic artery was closed (monovascular perfusion) and the bile duct left open. Flow was maintained at a constant rate using a peristaltic pump (Miniplus 3, Gilson, France) and was adjusted to between 30 and 32 mL/min, depending on

the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% (w/v) bovine serum albumin. It was saturated with a mixture of oxygen and carbon dioxide (95 : 5) using a membrane oxygenator with a simultaneous temperature adjustment to 37°C. The composition of the Krebs/Henseleit-bicarbonate buffer was as follows: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 2.5 mM $CaCl_2$. After the stabilization of oxygen consumption the experiments were initiated and samples of the effluent fluid were collected at time intervals of 2 minutes. In some experiments, substrate-free perfusion fluid was used, and trace amounts of [1-^{14}C]octanoate (0.01 μCi/mL) were infused from the beginning of the perfusion experiments. In other experiments, a mixture of octanoate (0.2 mM) and [1-^{14}C]octanoate (0.01 μCi/mL) was infused starting at 10 minutes of perfusion. According to Soboll et al. [20], this procedure effectively measures the citric acid cycle via labelling of acetyl-CoA. Consequently, $^{14}CO_2$ production can be regarded as an indicator of citric acid cycle activity. These substrates were infused for a defined time period, according to experimental protocol, and this period corresponds to a control condition. Subsequently, the citrus flavanones were infused dissolved in the same preceding perfusion fluid. The solubilisation of these compounds was achieved by the simultaneous addition of an equivalent amount of 1.0 M NaOH. After this procedure, the pH of the perfusion fluid containing the flavanones was adjusted to 7.4 before use. Samples of the effluent perfusion fluid were collected at 2 min intervals and analysed for their metabolite content. Acetoacetate and β-hydroxybutyrate were assayed by means of standard enzymatic procedures using β-hydroxybutyrate dehydrogenase [21]. Interference by citrus flavanones (absorbance at 340 nm) was excluded by running blanks. The oxygen concentration in the outflowing perfusate was continuously monitored by employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber where the perfusate exited [22]. The carbon dioxide production from [1-^{14}C]octanoate was measured by trapping $^{14}CO_2$ in phenylethylamine [23]. Radioactivity was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/ethanol (2/1) containing 5 g/L 2,5-diphenyloxazole and 0.15 g/L 2,2-p-phenylenebis(5-phenyloxazole). Metabolic rates were calculated from the differences between the input and output and the total flow rates and were analysed in reference to the wet weights of the livers.

2.4. Isolation of Mitochondria and Peroxisomes. The livers were removed and cut into small pieces. These fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 1.0 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 7.4), 1.0 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 50 mg% (w/v) fatty acid-free bovine serum albumin. Homogenisation was carried out in the same medium by means of a Dounce homogeniser. After homogenisation, the mitochondria were isolated by differential centrifugation according to Voss et al. [24] and suspended in the same medium (protein concentration of 70–80 mg/mL), which was kept at 0–4°C.

Peroxisomes were isolated according to the method described by Natarajan et al. [25]. The livers were removed and cut into small pieces. These fragments were suspended in a medium containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.1 mM PMSF, and 3 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.4. Homogenisation was carried out in the same medium by means of a Dounce homogeniser. Peroxisomes were isolated by differential centrifugation. The homogenate was first centrifuged at 600 g for 10 minutes to remove cell debris, and mitochondria were pelleted by centrifugation at 15,000 g for 5 minutes. The postmitochondrial supernatant was then centrifuged at 39,000 g for 10 minutes to isolate the fraction containing peroxisomes, which was resuspended in 250 mM sucrose containing 1.0 mM EDTA, 0.1 mM PMSF, and 10 mM Tris-HCl (pH 7.3) and homogenised using a Dounce homogeniser. This suspension was centrifuged at 15,000 g for 10 minutes to remove mitochondrial contamination. Afterwards, the supernatant was again centrifuged at 39,000 g for 10 minutes to isolate the peroxisomes, which were resuspended, homogenised, and adjusted to a final protein concentration of approximately 1.0 mg/mL.

2.5. Determination of Oxygen Uptake by Isolated Mitochondria Oxidising Fatty Acids. Oxygen uptake by isolated mitochondria oxidising fatty acids was measured polarographically using a Teflon-shielded platinum electrode [22–24]. The incubation medium contained 2.0 mM KH_2PO_4, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH 7.2), 0.1 mM EGTA, 130 mM KCl, 5.0 mM $MgCl_2$, 0.1 mM 2,4-dinitrophenol (2,4-DNP), 2.5 mM L-malate, and 50 mg% (w/v) fatty acid-free bovine serum albumin [26]. Mitochondria (0.6–1.2 mg protein/mL) were incubated in final volumes of 2.0 mL. The reaction was initiated by the addition of (a) 20 μM octanoyl-CoA + 2.0 mM L-carnitine, (b) 20 μM octanoyl-L-carnitine, (c) 20 μM palmitoyl-CoA + 2.0 mM L-carnitine, or (d) 20 μM palmitoyl-L-carnitine. In the control series, oxygen uptake was followed by nearly five minutes and the rates of oxygen uptake were computed from the slopes of the recorder tracings. The citrus flavanones at final concentrations varying from 10 to 300 μM (dissolved in 0.1 M dimethylformamide) were added to the incubation medium, two minutes before the substrates. Control experiments were performed to exclude solvent effects. The data were expressed as nmol of O_2 consumed per min per mg of mitochondrial protein.

2.6. Mitochondrial Membrane-Bound Enzymatic Activities. Rat liver mitochondria, isolated as described above [24], were disrupted by successive freeze and thawing procedures using liquid nitrogen and used as an enzyme source for assaying membrane-bound enzymatic activities. NADH-oxidase and succinate-oxidase activities were assayed polarographically using a 20 mM Tris-HCl (pH 7.4) medium. The reactions were started by the addition of 1.0 mM NADH or 10 mM succinate

[27]. The same experimental procedure described above to measure the effects of citrus flavanones (100–300 μM) on fatty acid oxidation was conducted. The data were expressed as nmol of O_2 consumed per min per mg of mitochondrial protein.

2.7. Peroxisomal Fatty Acyl-CoA Oxidase Activity. The peroxisomal fatty acyl-CoA oxidase activity was measured fluorimetrically using a modification [28] of the method described by Small et al. [29]. The assay for acyl-CoA oxidase was based on the determination of H_2O_2 production, which was coupled to the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) into a highly fluorescent compound (2′,7′-dichlorofluorescein (DCF)) in a reaction catalysed by exogenous peroxidase. The enzyme activity was monitored in real time by recording the variation in fluorescence. After the addition of the peroxisome-enriched fraction (0.3-0.4 mg protein/mL), the reaction was started by addition of the substrate palmitoyl-CoA (to a final concentration of 30 μM). Citrus flavanones (25 or 200 μM) were added to the incubation medium as a solution in dimethylformamide (0.1 M). In the control experiments the solvent was included in the reaction medium instead of the citrus flavanones. The increase in fluorescence (excitation, 503; emission, 529 nm) was recorded over a period of 10 minutes, and the activity of fatty acid acyl-CoA oxidase was expressed as nmol of DCF produced per min per mg of peroxisomal protein. The rates of H_2O_2 production were calculated from the linear regression analysis of the curves after subtracting the values of the blank curves.

2.8. Protein Determination. The protein contents of the mitochondrial and peroxisomal suspensions were measured as described by Lowry et al. [30], using the Folin phenol reagent and bovine serum albumin as a standard.

2.9. D-β-Hydroxybutyrate Dehydrogenase Activity. The effect of citrus flavanones on the activity of D-β-hydroxybutyrate dehydrogenase from *Pseudomonas lemoignei* was assayed according to Bergmeyer [31]. The activity was measured by following the reduction of NAD^+ at 340 nm using a spectrophotometer. In a 1.0 mL reaction mix, the final concentrations were 147 mM tris(hydroxymethyl)aminomethane (Tris, pH 8.0), 29 mM D-β-hydroxybutyrate, 1.93 mM NAD^+, and 0.03 units β-hydroxybutyrate dehydrogenase. Citrus flavanones (50–200 μM) were added to the incubation medium dissolved in dimethylformamide (0.1 M). In all assays, the enzyme plus citrus flavanones were preincubated together at room temperature (25°C) for 2 minutes, and the reaction was started by the addition of D-β-hydroxybutyrate. The increase in absorbance at 340 nm resulting from NADH formation was measured and expressed as nmol/min. Interference by citrus flavanones (absorbance at 340 nm) was excluded by running blanks. Control experiments were performed to exclude solvent effects.

2.10. Measurement of NADH Oxidation in a Cell-Free System. The effect of citrus flavanones on NADH oxidation in a cell-free system was measured according to the method of Chan et al. [17]. The reaction mixtures contained 0.1 M tris-HCl/1.0 mM EDTA buffer (pH 7.4), H_2O_2 (25 μM), NADH (200 μM), and horseradish peroxidase (HRP) type VI-A (0.1 μM). Hesperidin and hesperetin (5–50 μM) and naringenin (0.5–1.2 μM) were added to the incubation medium dissolved in dimethylformamide (0.1 M). Reactions were started by the addition of H_2O_2 (25 μM), and the oxidation of NADH was followed at 340 nm using a spectrophotometer. Control experiments were performed to exclude solvent effects.

2.11. Treatment of Data. Data are shown as the means ± standard errors. The statistical significance of the differences between parameters obtained in the experiments was evaluated using Student's *t*-test or Newman-Keuls test according to the context. The results are discussed in the text using *P* values, where $P < 0.05$ was the criterion used for significance. The ID_{50} were computed by numerical interpolation using a cubic spline function. Statistical analyses were performed using Graphic Pad Prism software version 3.0.

3. Results

3.1. The Effects of Citrus Flavanones on Oxygen Uptake, $^{14}CO_2$ Production, and Ketogenesis from Exogenous Octanoate and Endogenous Sources. To investigate the effects of citrus flavanones on octanoate metabolism, 0.2 mM octanoate and tracer amounts of [1-^{14}C]octanoate (0.01 μCi/mL) were simultaneously infused into isolated perfused rat liver (10–42 minutes). The production of $^{14}CO_2$ was measured, along with oxygen consumption and production of ketone bodies (β-hydroxybutyrate and acetoacetate). All these parameters are related to fatty acid transformation. Figures 2(a), 2(b), and 2(c) illustrate the experimental protocol and show the time courses of the changes caused by 100 μM hesperidin, hesperetin, and naringenin. The livers responded rapidly to octanoate infusion, with clear signs that β-oxidation was enhanced. With the exception of acetoacetate production, which was slightly or not at all affected by octanoate, all of the parameters reached new steady states. Because the increase in β-hydroxybutyrate was higher than the increase in acetoacetate, there was a substantial increase in the β-hydroxybutyrate/acetoacetate ratio. Citrus flavanones (100 μM) were introduced 16 minutes after starting the infusion of octanoate (at 26 minutes in the time scale of Figures 2(a), 2(b), and 2(c)). Naringenin and hesperetin caused decreases in β-hydroxybutyrate production (by 32.1% and 49.3%, resp., $P < 0.05$) and increased acetoacetate production (by 17.9% and 35.9%, resp., $P < 0.05$). Oxygen consumption remained unaltered in the presence of the flavanones. The productions of $^{14}CO_2$ were increased by 39.0% ($P < 0.05$) and 49.3% ($P < 0.05$) by naringenin and hesperetin, respectively. Naringenin and hesperetin clearly decreased the β-hydroxybutyrate/acetoacetate ratio. Experiments such as those illustrated in Figures 2(a), 2(b), and 2(c), but without $^{14}CO_2$ measurements, were repeated with 50 and 200 μM hesperetin and naringenin and with 200 μM hesperidin to

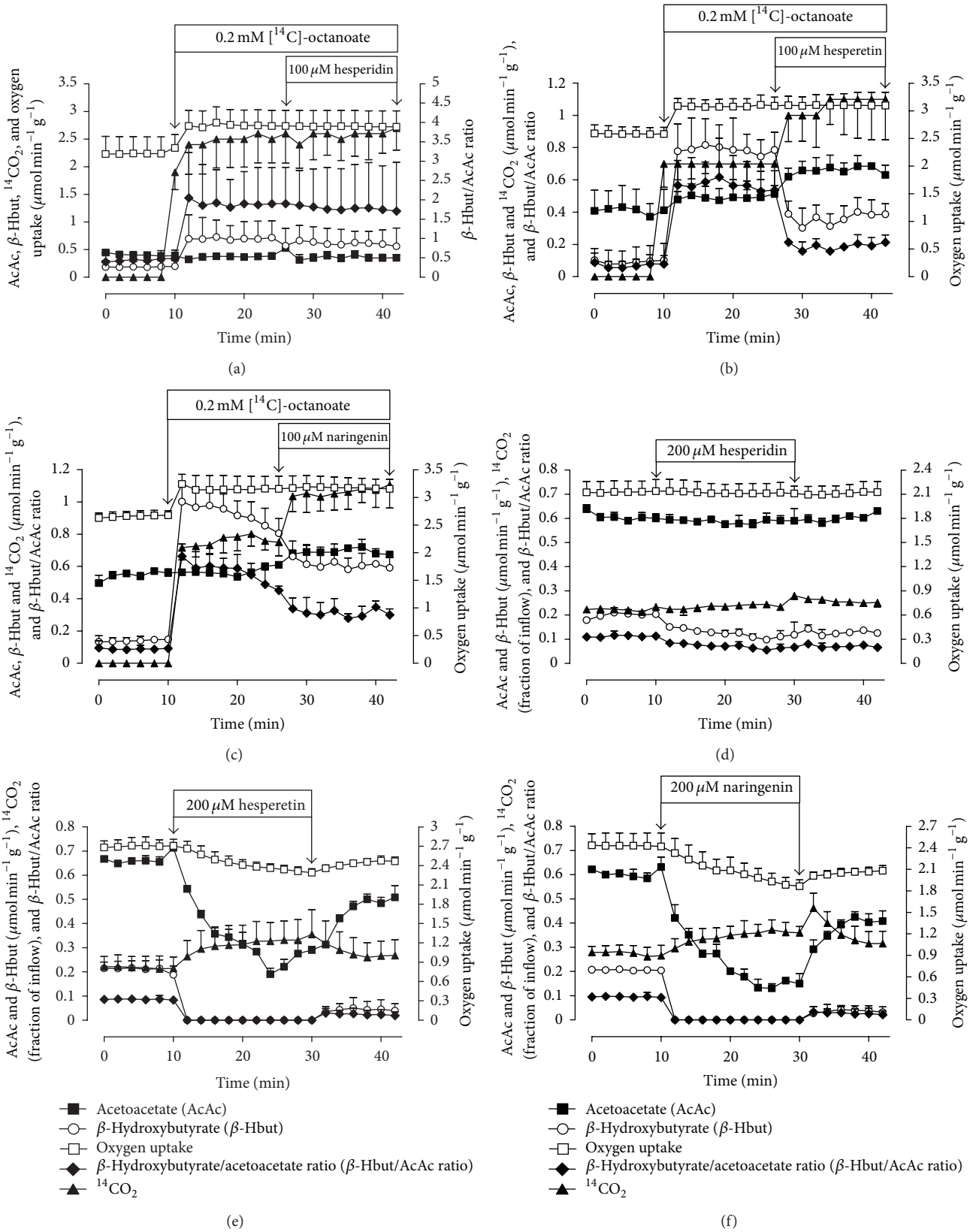

Figure 2: Comparison of the changes caused by hesperidin, hesperetin, and naringenin on the metabolic fluxes from exogenous octanoate ((a), (b), and (c)) and from endogenous fatty acids ((d), (e), and (f)) in perfused livers isolated from fasted rats. The effluent perfusate was analysed for acetoacetate (AcAc), β-hydroxybutyrate (β-Hbut), and $^{14}CO_2$. Oxygen uptake was followed polarographically. Values are expressed as the means ± standard errors of the mean of three animals (three liver perfusion experiments) for each experimental condition.

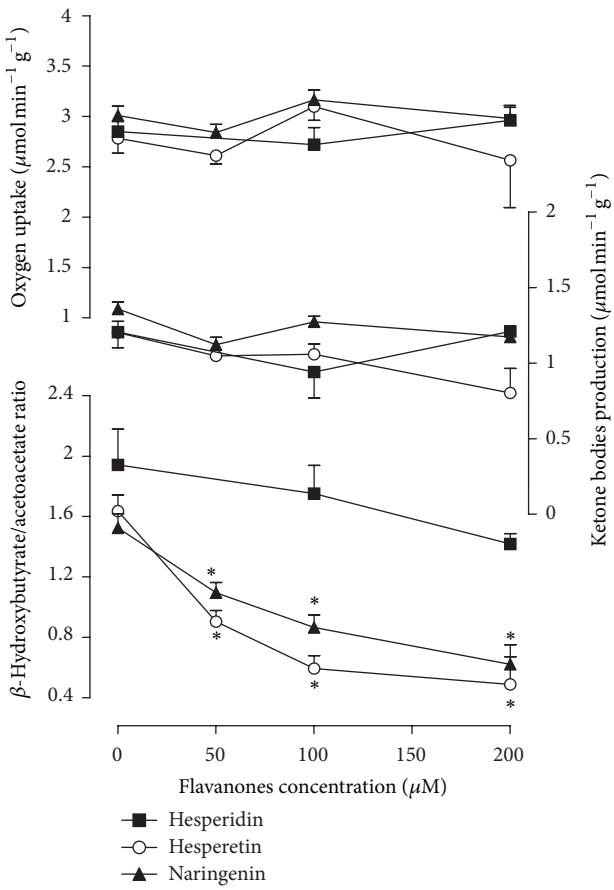

Figure 3: Concentration-dependent effects of hesperidin, hesperetin, and naringenin on octanoate metabolism. Data were obtained from experiments similar to those illustrated in Figure 2. Values in the absence of citrus flavanones (control values) are the mean values before the onset of citrus flavanones infusion. Values in the presence of citrus flavanones were computed at the end of the citrus flavanone infusion. Each experimental point is the mean ± standard error of three animals (three liver perfusion experiments) for each experimental condition. Asterisks indicate a statistically significant difference compared to the control condition, as revealed by variance analysis with post hoc Newman-Keuls testing ($^*P < 0.05$).

establish concentration dependences for the effects. The changes in the rates of ketogenesis (for β-hydroxybutyrate + acetoacetate) in the β-hydroxybutyrate/acetoacetate ratio and in the rates of oxygen uptake are summarised in Figure 3. The values at zero citrus flavanone concentration correspond to the mean values of the metabolic fluxes just before the onset of citrus flavanone infusion (i.e., the basal rates measured at 26 minutes of perfusion time); the rates in the presence of citrus flavanones were the values evaluated at 42 minutes of perfusion time. Hesperetin and naringenin had no significant effect on ketogenesis or oxygen uptake. The most pronounced effect was that on the β-hydroxybutyrate/acetoacetate ratio, which was reduced in a concentration dependent manner, decreasing from 1.63 ± 0.10 to 0.59 ± 0.08 ($P < 0.05$) at 100 μM hesperetin and from 1.52 ± 0.09 to 0.86 ± 0.08 ($P < 0.05$) at 100 μM naringenin. Half-maximal reductions are expected at concentrations of 58.99 ± 6.50 hesperetin and 136.70 ± 5.98 μM naringenin, as revealed by numerical interpolation. As shown in Figures 2(a), 2(b), 2(c), and 3, the oxidation of exogenous octanoate was not significantly modified by hesperidin.

Substrate-free perfused livers from fasted rats are entirely dependent on oxidation of endogenous fatty acids [32]. Due to high rates of fatty acid oxidation, the ketogenic activity of such livers is also pronounced. Because of the results obtained in the present work with an exogenous ketogenic substrate, the medium-chain fatty acid octanoate, the question arises whether the citrus flavanones hesperetin and naringenin also affect ketogenic activity that depends solely on endogenous substrates. The results of experiments performed under these conditions are shown in Figures 2(d), 2(e), and 2(f), which represents the rates of oxygen uptake, ketogenesis (β-hydroxybutyrate and acetoacetate production), $^{14}CO_2$ production, and the β-hydroxybutyrate/acetoacetate ratio in experiments in which 200 μM citrus flavanone (hesperidin, hesperetin, or naringenin) was infused for 20 minutes (10–30 minutes). Tracer amounts of [1-^{14}C]octanoate (0.01 μCi/mL) were infused from the beginning of the perfusion experiments (0–42 minutes). All metabolic fluxes responded to naringenin and hesperetin in a very similar manner but showed little or no change in response to hesperidin. While hesperetin and naringenin caused rapid and continuous decreases in the production of β-hydroxybutyrate (to nondetectable levels ($P < 0.05$)), the inhibition of β-hydroxybutyrate production in the presence of 200 μM hesperidin was 47.8% ($P < 0.05$). Acetoacetate production was also inhibited by hesperetin (61.1%, $P < 0.05$) and naringenin (76.4%, $P < 0.05$). In contrast, the acetoacetate production was not altered by hesperidin. We found that the total production of ketone bodies (β-hydroxybutyrate plus acetoacetate) and the β-hydroxybutyrate/acetoacetate ratio were reduced in the presence of any of the three citrus flavanones. The β-hydroxybutyrate/acetoacetate ratio was reduced 46.5% ($P < 0.05$), 100% ($P < 0.05$) and 100% ($P < 0.05$) by hesperidin, hesperetin, and naringenin, respectively. The oxygen uptake was progressively decreased by hesperetin (14.5%, $P < 0.05$) and naringenin (21.9%, $P < 0.05$), but no significant alteration was observed in the presence of hesperidin. It should be noted that these effects were accompanied by a significant increase in $^{14}CO_2$ production. The production of $^{14}CO_2$ was increased 9.9% ($P < 0.05$), 52.5% ($P < 0.05$), and 33.2% ($P < 0.05$) by hesperidin, hesperetin, and naringenin, respectively. In general, when the infusion of citrus flavanones was interrupted, the productions of acetoacetate, β-hydroxybutyrate and $^{14}CO_2$, and the oxygen uptake showed only partial recoveries during the subsequent 12 minutes.

If the effects of citrus flavanones on the β-hydroxybutyrate/acetoacetate ratio are due to interaction of these substances with the enzyme β-hydroxybutyrate dehydrogenase, which interconverts β-hydroxybutyrate and acetoacetate, it should be possible to reproduce these effects using the isolated enzyme. However, we found that the citrus

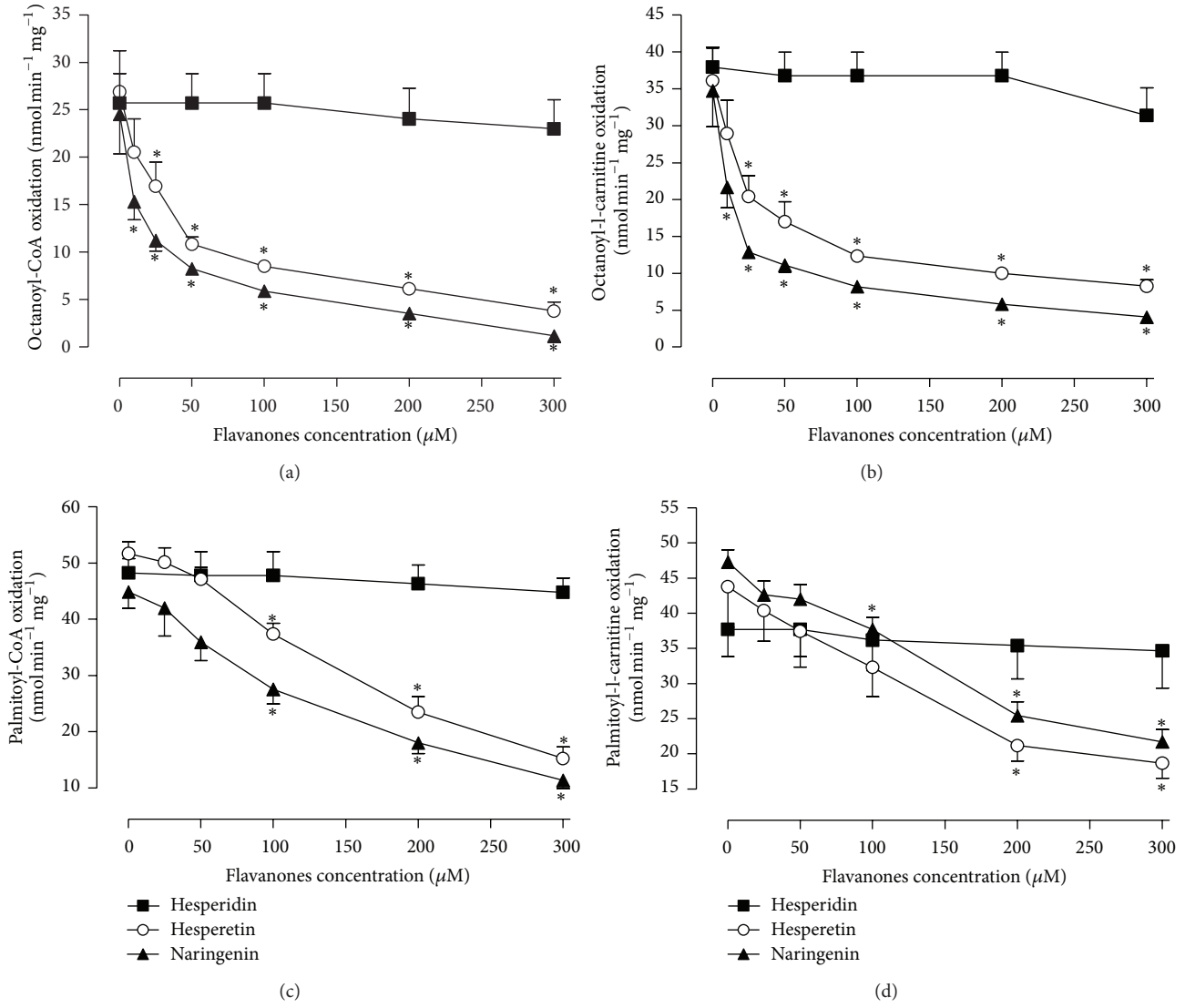

Figure 4: Determination of oxygen consumption by mitochondria oxidising fatty acids. The values are the means ± standard errors of the results obtained in three to six independent mitochondrial preparations, depending on the experimental series. Asterisks indicate a statistically significant difference compared to the control condition, as revealed by variance analysis with post hoc Newman-Keuls testing ($^*P < 0.05$).

flavanones, at concentrations up to 200 μM, had no direct effect on the enzyme β-hydroxybutyrate dehydrogenase (data not shown).

3.2. The Effects of Citrus Flavanones on Oxygen Uptake by Mitochondria Oxidising Fatty Acids, NADH, and Succinate. The medium-chain fatty acid octanoate was used as the oxidative substrate in some experiments with perfused liver. However, in those experiments in which the substrate-free perfused rat liver was used, respiration is mainly dependent on the oxidation of long-chain fatty acids [32]. For this reason, we also evaluated the effects of citrus flavanones (10–300 μM) on oxygen uptake by mitochondria oxidising the medium-chain fatty acid octanoate and the long-chain fatty acid palmitate. These fatty acids were used as acyl-CoA derivatives (octanoyl-CoA and palmitoyl-CoA) in the presence of carnitine. Octanoyl-L-carnitine and palmitoyl-L-carnitine were also tested. As shown in Figures 4(a)–4(d), hesperetin and naringenin inhibited oxygen uptake during the oxidation of all activated fatty acids, with well-defined concentration dependencies. The ID_{50} concentrations of hesperetin for the oxidation of the activated fatty acids were octanoyl-L-carnitine, 47.0 ± 16.3 μM; octanoyl-CoA, 47.9 ± 9.6 μM; palmitoyl-L-carnitine, 175.7 ± 23.4 μM; palmitoyl-CoA, 198.2 ± 20.7 μM. The ID_{50} concentrations of naringenin for the oxidation of the activated fatty acids were octanoyl-L-carnitine, 30.8 ± 13.0 μM; octanoyl-CoA, 21.3 ± 5.9; palmitoyl-L-carnitine, 225.6 ± 37.1 μM; palmitoyl-CoA, 146.1 ± 16.6 μM. In all cases, oxidation was not significantly altered by hesperidin.

The similar effects of hesperetin and naringenin on oxygen uptake observed in isolated rat liver mitochondria

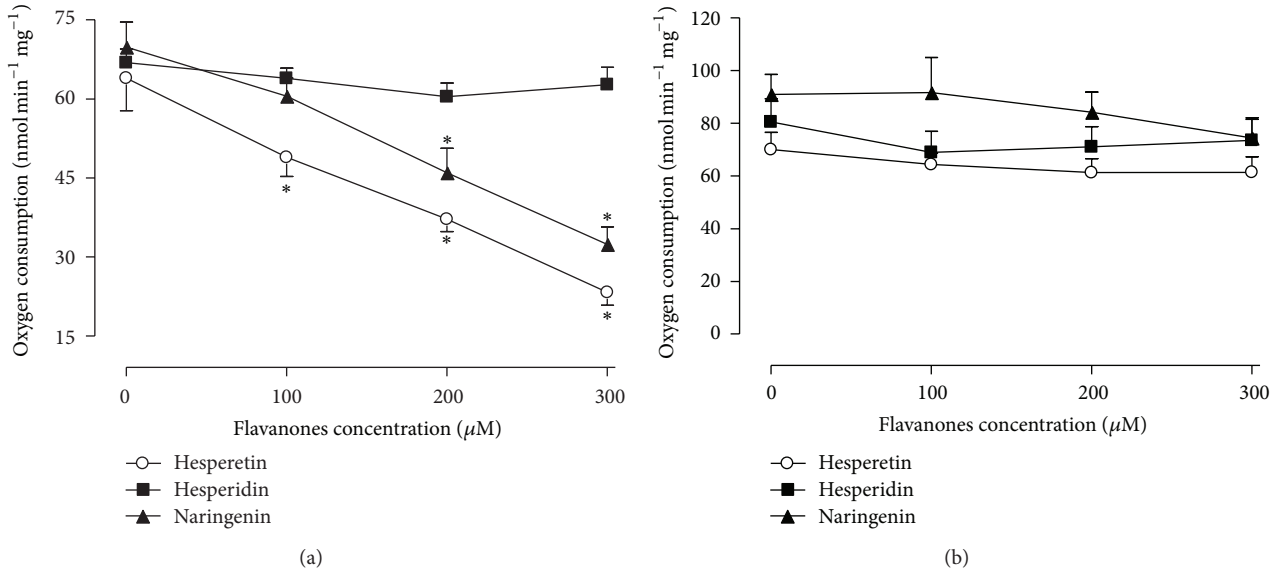

Figure 5: The effects of hesperidin, hesperetin and naringenin on NADH-oxidase (a) and succinate-oxidase (b) activities. The values are the means ± standard errors of the results obtained in six to seven independent mitochondrial preparations, depending on the experimental series. Asterisks indicate a statistically significant difference compared to the control condition, as revealed by variance analysis with post hoc Newman-Keuls testing ($^{*}P < 0.05$).

oxidising medium-chain and long-chain fatty acids raises the question of whether this effect is dependent on a direct action of citrus flavanones on mitochondrial electron flow. A way to evaluate this possibility is measurement of the respiratory activity in freeze/thaw-disrupted rat liver mitochondria using NADH (NADH-oxidase activity) or succinate (succinate-oxidase activity) as substrates for complex I and II, respectively. The illustration of Figure 5 shows that in the concentration range between 100 and 300 μM, hesperetin and naringenin progressively inhibited NADH-oxidase but not succinate-oxidase activity. The ID_{50} concentrations of hesperetin and naringenin were 238.4 ± 22.19 μM and 265.5 ± 23.47 μM, respectively. Hesperidin had no significant effect on mitochondrial membrane-bound enzymatic activities.

3.3. The Effects of Citrus Flavanones on Peroxisomal Fatty Acyl-CoA Oxidase Activity. Citrus flavanones may also interfere with peroxisomal β-oxidation, which supplies chain-shortened products for the complete mitochondrial fatty acid β-oxidation. To assess this possibility, the effects of two concentrations (25 and 200 μM) of citrus flavanones on the peroxisomal acyl-CoA oxidase activity were measured in preparations containing liver peroxisome-enriched fractions. The results demonstrated that the activity of oxidising palmitoyl-CoA, which is dependent on peroxisomal fatty acyl-CoA oxidase activity, was unaffected by citrus flavanones at concentrations of up to 200 μM (data not shown).

3.4. The Effects of Citrus Flavanones on the Catalytic Oxidation of NADH. Hesperidin, hesperetin, and naringenin promoted NADH oxidation in the presence of peroxidase and catalytic amounts of H_2O_2 (Figure 6). This action is consistent with previous reports indicating that these substances are able to form prooxidant metabolites that cooxidise NADH [17].

4. Discussion

Confirming the central hypothesis raised above, we found that the citrus flavanones hesperidin, hesperetin, and naringenin are able to induce a more oxidised state in liver cells by altering several parameters related to hepatic fatty acid oxidation, namely, oxygen uptake, citric acid cycle activity, and ketogenesis. This oxidative state was revealed by the decrease in the β-hydroxybutyrate/acetoacetate ratio in the presence of citrus flavanones, indicating a shift in the mitochondrial redox state to a more oxidised condition [33]. The β-hydroxybutyrate/acetoacetate ratio in the perfused liver is an indicator for the mitochondrial NADH/NAD^+ ratio because the enzyme β-hydroxybutyrate dehydrogenase is present solely in the mitochondria and also because it operates under near-equilibrium conditions [12]. Furthermore, the stimulation of $^{14}CO_2$ production indicated that the activity of the citric acid cycle was increased in the perfused livers. Under normal conditions, the rate of the citric acid cycle is strictly dependent on NADH reoxidation via the mitochondrial respiratory chain. However, a parallel increase in the oxygen consumption by the livers was not observed. Thus, a diversion of the NADH generated in the citric acid cycle from the respiratory chain to another oxidative reaction was raised as a possible explanation for such a phenomenon. The mechanisms by which citrus flavanones exert this effect are relatively complex and likely involve biotransformation reactions that occur in the liver, especially intracellular formation of phenoxyl radicals that can oxidise NADH in the presence of peroxidases and hydrogen peroxide [17, 18].

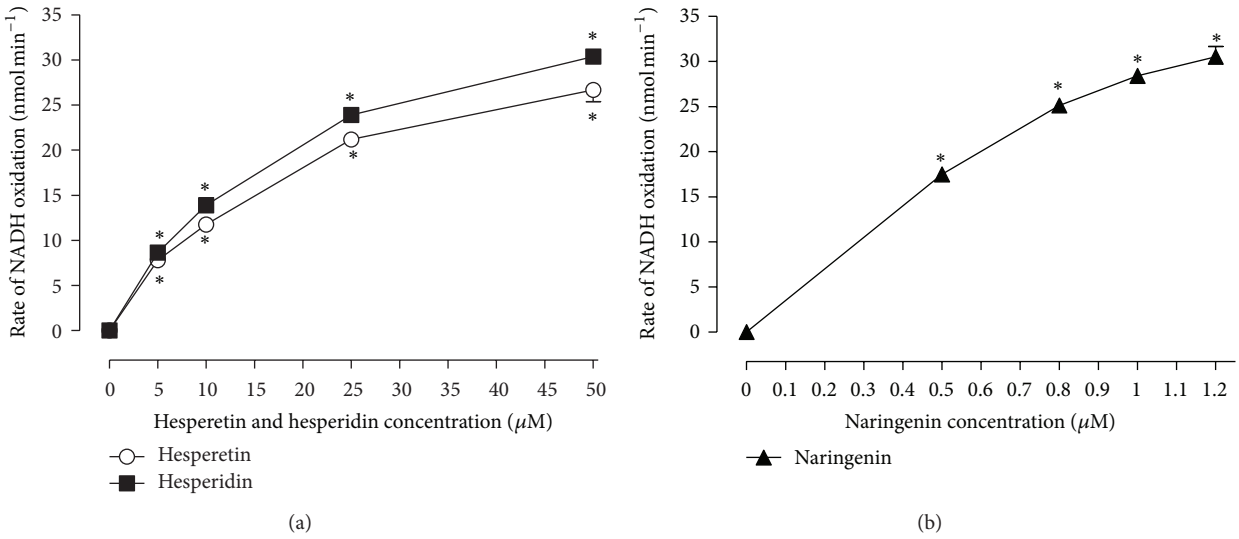

Figure 6: (a) and (b) show the dependence of the rate of peroxidase-catalysed NADH oxidation on the concentrations of the flavanones hesperidin, hesperetin, and naringenin. Values represent the means ± standard errors of three separate experiments. Asterisks indicate a statistically significant difference compared to the control condition, as revealed by variance analysis with post hoc Newman-Keuls testing ($^*P < 0.05$).

The fact that phenoxyl radicals have been implicated in the initiation of atherosclerosis and carcinogenesis by xenobiotic phenolic metabolites must be taken into consideration [17, 34–36].

As demonstrated in previous reports [17] and confirmed in the present work (Figure 6), the citrus flavanones have a strong ability to oxidise NADH in the presence of horseradish peroxidase (HRP) and hydrogen peroxide even when low flavanone concentrations are used. It is well known that hydrogen peroxide is always present in the liver, which possesses many enzymatic systems that are capable of producing free radicals from phenolic or polyphenolic compounds, so that it is quite possible that the same phenomena occur in the intact perfused liver [37–40]. It is unlikely that the citrus flavanones act directly on the mitochondrial enzyme β-hydroxybutyrate dehydrogenase because even at 200 μM they did not affect the activity of this enzyme in kinetic enzyme assays.

The effect of citrus flavonoids on the NADH/NAD$^+$ redox potential was accompanied by a number of other effects. In the isolated perfused rat liver, citrus flavonoids clearly inhibited ketogenesis from endogenous fatty acids, which is mainly dependent on oxidation of the long-chain fatty acids [32], but had little effect on ketogenesis from the exogenously supplied medium-chain fatty acid octanoate. Paradoxically, hesperetin and naringenin (but not hesperidin) were able to inhibit octanoate and palmitate oxidations in isolated mitochondria regardless of their chemical compositions (acyl-CoA or acylcarnitine derivatives). Thus, the inhibition of fatty acid oxidation in isolated mitochondria likely occurs at some common point in the oxidation process of these compounds. Impairments in the respiratory chain at complex I can lead to secondary inhibition of oxidation of the fatty acids derivatives whose oxidation depends on the direct action of NAD$^+$-dependent dehydrogenases [41–43]. Thereby, it is highly probable that naringenin and hesperetin inhibit the mitochondrial β-oxidation of fatty acids in isolated mitochondria by means of an inhibitory effect on the electron flow at complex I of the respiratory chain. It is unlikely, however, that the inhibition of ketogenesis observed in the substrate-free perfused liver is due to primary action of citrus flavanones on mitochondrial electron flow. If citrus flavanones were acting directly and solely on the respiratory chain, the β-hydroxybutyrate/acetoacetate ratio would be increased rather than decreased, as in similar perfusion experiments using isosteviol, an inhibitor of the respiratory chain [44].

The inhibitory actions of naringenin and hesperetin on ketone body production, oxygen uptake, and citric acid cycle stimulation in perfused livers are associated with the prooxidant effects of these flavanones. As previously demonstrated in experiments performed with isolated mitochondria [45], ketogenesis decreases with lower NADH/NAD$^+$ ratios because the near-equilibrium of 3-hydroxyacyl-CoA dehydrogenase is shifted toward acetoacetyl-CoA, which inhibits acetyl-CoA acetyltransferase. The same phenomenon, however, favours the citric acid cycle, mainly because a less reduced state of the NADH-NAD$^+$ pair shifts the near equilibrium catalysed by L-malate dehydrogenase in the direction of oxaloacetate, the acceptor of acetyl-CoA, thus favouring the action of citrate synthase. Oxygen uptake inhibition most likely occurs because the NADH concentration in the mitochondria is diminished. In addition, it is improbable that the effects of the citrus flavanones found in the present work reflect an action on peroxisomal β-oxidation because peroxisomal fatty acyl-CoA oxidase activity was unaffected by citrus flavanones at concentrations of up to 200 μM.

Another interesting point is the difference between the effects of citrus flavanones obtained in the presence of octanoate and in the substrate-free perfused liver. Though prooxidant effect of hesperetin and naringenin are present in both cases, as shown by reductions in the mitochondrial $NADH/NAD^+$ ratios, these effects were most prominent in the substrate-free perfused liver. This difference can be explained by the higher reducing power established by the introduction of 0.2 mM octanoate. If one looks at the absolute values of the β-hydroxybutyrate/acetoacetate ratios in the absence of citrus flavanones, it is clear that the maximal value in the presence of octanoate is higher than the value in its absence (i.e., in the substrate-free perfused liver). This is due to the enormous increases in the mitochondrial $NADH/NAD^+$ ratio that are produced by the flow of reducing equivalents from exogenous octanoate. In this case, the competition for NADH between the respiratory chain and the flavanone metabolites (phenoxyl radicals) is minimised, favouring the respiratory chain. This assumption is consistent with the observation that neither oxygen uptake nor ketone body production was significantly affected by treatment with the citrus flavanones in the presence of exogenous octanoate.

Though hesperidin strongly oxidises NADH in the presence of horseradish peroxidase and hydrogen peroxide, as revealed in an *in vitro* incubation system, this flavanone did not directly affect the mitochondrial or peroxisomal activities. It exerted only little to no metabolic effects in the liver, even in the substrate-free perfused liver, most likely because of its low lipophilicity and interactions with membranes. As previously demonstrated in a biophysical study, hesperetin (i.e., the aglycone form) interacts with membranes more strongly than hesperidin (the glycoside form) does. Hesperidin, due to its rutinoside moiety, is located at the level of the polar headgroup, whereas hesperetin interacts better with acyl chains and adopts a more planar conformation [46]. Indeed, this difference could impair hesperidin transport across cellular and organelle membranes, thus decreasing its prooxidant action within hepatocytes. Our results also indicated that although hesperetin and naringenin differ in the substituents of the B-ring (hesperetin has an additional methoxy functional group ($-OCH_3$) at the paraposition and its hydroxyl group is substituted at the metaposition), these differences did not have a great influence on their metabolic effects in the liver. Moreover, in general terms, comparison of the effects of the citrus flavanones (with the exception of hesperidin) revealed in the present work and those reported for the flavonols quercetin and fisetin [12, 13] show that the structural differences between these specific citrus flavonoids does not significantly alter their metabolic effects in the liver.

It is noteworthy that the concentration range of citrus flavanones used in the present study is in line with the concentrations used in cell culture systems [47] and in animals fed diets supplemented with citrus flavanones [48]. In addition, it is also known that the portal concentration of xenobiotics can achieve levels much higher than in the systemic circulation and also that the bile concentration can reach values 100 times higher than the plasma [49, 50]. Thus, it is highly probable that the portal concentrations used in the aforementioned studies reach values that are similar to those used in this work.

5. Conclusion

In conclusion, at least in isolated perfused rat liver, the prooxidant effects of the citrus flavanones hesperetin, naringenin and, to a lesser extent, hesperidin seem to predominate over their antioxidant effects [1], affecting the liver metabolism in several ways. To exert these prooxidant effects, the citrus flavanones most likely require cellular peroxidases, which in the presence of hydrogen peroxide, oxidise phenols to phenoxyl radicals [17, 18]. The information provided in this study counteracts the widely disseminated idea that citrus flavonoids, acting as antioxidants, exhibit beneficial effects in inflammation, atherosclerosis, and cancer [2–4]. While the effects of hesperidin, hesperetin, and naringenin on liver metabolism are certainly very complex, and while it is difficult to predict the net effects that the substances will exert *in vivo*, these results aim to improve our understanding of the mode of action of citrus flavanones on mammalian cells, particularly liver cells. However, since citrus flavanones are intensively metabolized when orally administered [51, 52], the possibility that some of their metabolites could be responsible for part of their effects (*in vivo*) cannot be excluded. Thus, despite the findings presented, further experimental investigations are necessary to provide detailed information about the effects of citrus flavanones metabolites and other citrus flavanones concentrations on hepatic metabolism and the physiological significance of such effects on the whole organism.

Conflict of Interests

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

Acknowledgments

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Araucária (FA), and Coordenação de Aperfeiçoamento do Pessoal de Nível Superior (CAPES). The sponsors had no involvement in the study design, collection, analysis and interpretation of data, writing or revision of the report or in the decision of publishing. The authors would like to express their gratitude for the technical assistance of Aparecida Pinto Munhos Hermoso, Célia Akemi Gasparetto, Irene Aparecida Bernardino, and Luiz Saraiva Arraes.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 934239, 5 pages
http://dx.doi.org/10.1155/2013/934239

Research Article

Antioxidant, Lipid Lowering, and Membrane Stabilization Effect of Sesamol against Doxorubicin-Induced Cardiomyopathy in Experimental Rats

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Received 23 August 2013; Revised 12 September 2013; Accepted 13 September 2013

Academic Editor: Lillian Barros

The present study was designed to evaluate the cardioprotective effect of sesamol against doxorubicin-induced cardiomyopathy in rats. In this study, the cardioprotective effect of sesamol against doxorubicin induced cardiomyopathy in experimental rats was evaluated at the dosage of 50 mg/kg bw. Doxorubicin was administered to rats at a total cumulative dose of 15 mg/kg through intraperitoneal route for 2 weeks in six-divided dose on 8th, 10th, 14th, 16th, 18th, and 21st day. After the last dose administration, the endogenous antioxidants and lipid peroxidation were estimated in heart tissue homogenate. Cardiac biomarkers such as troponin T, LDH, CK, and AST and lipid profiles such as cholesterol, triglycerides, HDL, LDL, and VLDL were estimated in serum. Sesamol has cardioprotective activity through normalization of doxorubicin-induced-altered biochemical parameters. Biochemical study was further supported by histopathological study, which shows that sesamol offered myocardial protection from necrotic damage. From these findings, it has been concluded that the sesamol has significant cardioprotection against doxorubicin induced cardiomyopathy via amelioration of oxidative stress, lipid lowering, and membrane stabilization effect.

1. Introduction

Doxorubicin (DOX) is one of the most efficient anticancer antibiotics. The clinical use of DOX is limited due to the extensive adverse effects. Chronic administration of DOX to cancer patients causes dose-dependent cardiotoxicity which leads to heart failure and cardiomyopathy [1, 2]. It is reported that 41% of cancer patients who received DOX are affected with various cardiac problem. DOX treatment increases the morbidity and mortality of cancer patients due to the heart failure [3].

The heart is distinctively susceptibility to oxidative damage. DOX-induced cardiomyopathy is strongly linked to an increase in cardiac oxidative stress, as indicated by the depletion of endogenous antioxidant enzymes, and accumulation of free radicals in the myocardium which increases the chance of DOX-induced cardiomyopathy [4]. Several therapeutic interventions implemented to protect the heart from DOX-induced cardiomyopathy. However, higher mortality through abnormal cardiac activity limited the ability of protective role of these therapies [2]. So the search of novel molecule to ameliorate the DOX-induced cardiotoxicity is exceedingly urgent. Administration of antioxidant drugs to protect the heart from free radical damage getting more attention in cardiovascular disease research [1].

Sesamol is a potent phenolic antioxidant which is a component of sesame oil. It is white crystalline powder, sparingly soluble in water, and miscible with most of oils. Antioxidant property of sesamol has been shown earlier to exhibits radioprotective [5], antimutagenic [6], gastroprotective [7], neuroprotective [8], and antiplatelet activity [9]. It is reported that administration of sesamol protect the myocardium from isoproterenol-induced myocardial injury via antioxidative mechanism [10]. In the light of the above

literature, the present study was undertaken to evaluate the effect of sesamol on DOX-induced cardiomyopathy.

2. Materials and Methods

2.1. Chemicals. Sesamol and doxorubicin was purchased from Sigma-Aldrich, India. All chemicals were of analytical grade purchased from Sigma-Aldrich, India.

2.2. Animals. Healthy albino Wistar rats of either sex weighing between 180–200 g of 3 months of age were used. Animals were housed individually in polypropylene cages, maintained under standard conditions (12:12 L:D cycle; 25 ± 3°C; and 35–60% humidity), and fed with standard rat pellet diet (SaiDurga Feeds and Foods, Bangalore) and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee (File number ANCP/IAEC/01-2013).

2.3. Experimental Protocol. After 1 week of acclimatization, the animals were randomly divided into four groups with six animals in each group. Group 1 (G1) served as normal control and received normal saline (10 mL/kg body weight p.o.). Group 2 (G2) received sesamol 50 mg/kg body weight i.p. (dose selected based on previous report by Vennila and Pugalendi [10]) for 7 days and then alternatively with vehicle for the next 2 weeks. Group 3 (G3) was treated with DOX (total cumulative dose of 15 mg/kg i.p. for 2 weeks in six divided dosage on 8th, 10th, 14th, 16th, 18th, and 21st day). Group 4 (G4) was pretreated with sesamol 50 mg/kg body weight i.p. for 7 days followed by DOX administration as in G3.

All animals from respective groups were observed for heart weight, body weight changes, and mortality. After 24 h of last treatment, rats were anaesthetized with pentobarbitone sodium (60 mg/kg^{-1}), and serum was separated from the blood for the estimation of cardiac biomarkers like troponin-T, lactate dehydrogenase (LDH), creatinine kinase (CK), and aspartate transaminase (AST) and lipid parameters like total cholesterol (TCH), triglyceride (TGL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), and high density lipoprotein (HDL) by using enzyme kits (Transasia Bio-Medicals Limited, Solan). Animals from respective groups were sacrificed, and the heart tissue was quickly dissected, washed in ice cold saline, dried on filter paper, and weighed immediately. A portion of each heart was taken from all the groups, and homogenate was prepared in 0.3 M phosphate buffer (pH 7.4) for the estimation of thiobarbituric acid reactive substance (TBARS) [11], reduced glutathione (GSH) [12], superoxide dismutase (SOD) [13], catalase [14], and protein [15].

2.4. Histopathological Examination. The heart was isolated and washed immediately with ice cold saline then fixed in 10% formalin. After fixation tissues were embedded in paraffin-wax, and thick sections were cut into thin sections and stained with hematoxylin and eosin. These slides were then observed under light microscope for histopathological changes.

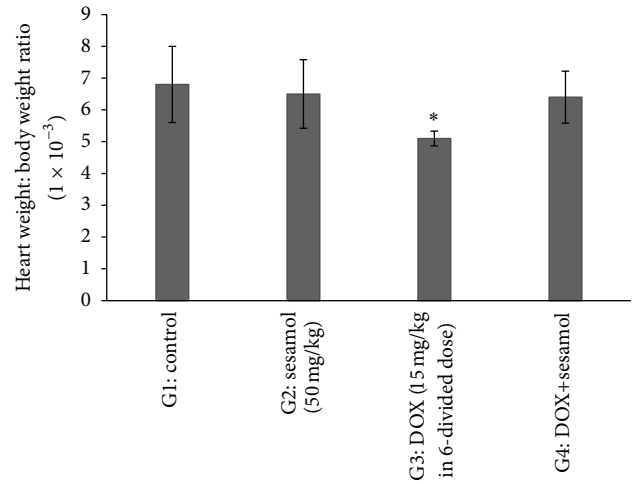


Figure 1: Effect of sesamol on heart weight : body weight ratio. $^{*}P < 0.05$ versus G1.

2.5. Statistical Analysis. Values are expressed as mean ± SD and analyzed using Graph Pad prism version 5.1 using ANOVA fallowed by Tukey's multiple comparison test. $P < 0.05$ was considered significant.

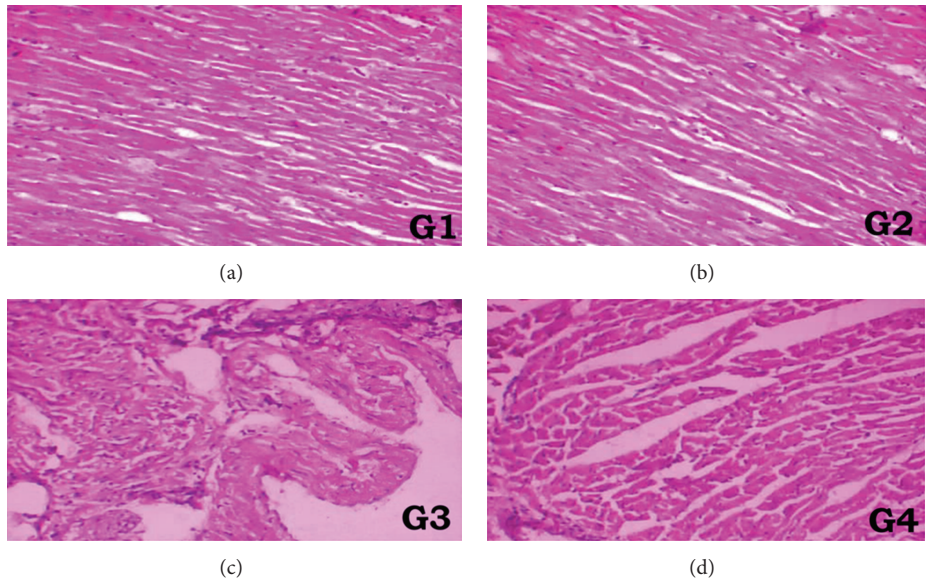
3. Results

There was no mortality observed in any of the treatment group.

3.1. Effect of Sesamol on Heart Weight : Body Weight Ratio (1×10^{-3}). There was a significant ($P < 0.05$) decrease in the heart weight : body weight ratio in DOX treated group (G3) compared to control group (G1). There was no significant fall in the heart weight : body weight ratio in sesamol alone (G2) and sesamol+DOX (G4) treated groups compared to G3 (Figure 1).

3.2. Effect of Sesamol on Serum Lipid Profile. There was no significant difference in the level of TCH, TGL, LDL, VLDL and HDL in sesamol treated group (G2) when compared to control group (G1). There was significant ($P < 0.05$) increase in the level of TCH, TGL, LDL, and VLDL and significant ($P < 0.05$) decrease in the level of HDL in DOX (G3) treated group when compared to the control group. There was significant ($P < 0.05$) decrease in the level of TCH, TGL, LDL, VLDL and significant ($P < 0.05$) increase in the level of HDL in sesamol+DOX (G4) treated group when compared to the DOX treated group (Table 1).

3.3. Effect of Sesamol on Serum Troponin-T, LDH, CK, and AST. There was no significant difference in the level of troponin-T, LDH, CK, and AST between control (G1) and sesamol (G2) alone treated rats. Rats that were treated with DOX (G3) significantly ($P < 0.0001$) increased the level of troponin-T, LDH, CK, and AST when compared with control (G1). Rats that were pretreated with sesamol followed by DOX (G4) ($P < 0.0001$) significantly decreased the level of



(a) (b) (c) (d)

Figure 2: Light microscopical analysis of heart tissue: (G1) heart of rat received saline, (G2) heart of rat received sesamol, (G3) heart of rat received DOX, and (G4) heart of rat received sesamol+DOX.

Table 1: Effect of sesamol on serum TCH, TGL, LDL, VLDL, and HDL.

Group	TGL (mg/dL)	TCH (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)
G1: control	103.0 ± 5.8	103.0 ± 1.7	51.5 ± 2.2	20.5 ± 0.76	26.3 ± 0.42
G2: sesamol (50 mg/kg)	105.0 ± 2.2	110.8 ± 2.9	56.0 ± 3.2	19.5 ± 0.76	27.5 ± 0.76
G3: DOX (15 mg/kg in 6-divided dose)	169.0 ± 4.4[a]	131.3 ± 3.4[a]	73.5 ± 2.2[a]	28.0 ± 3.2[a]	19.0 ± 3.0[a]
G4: DOX+sesamol	79.00 ± 2.2[b]	110.0 ± 2.0[b]	62.5 ± 0.76[b]	20.5 ± 0.76[b]	27.6 ± 0.42[b]

All values expressed as mean ± SEM. [a]$P < 0.05$ versus G1. [b]$P < 0.05$ versus G3.

troponin-T, LDH, CK, and AST when compared with DOX (G3) treated rats (Table 2).

3.4. Effect of Sesamol on TBARS. There was no significant difference in the level of TBARS between control (G1) and sesamol (G2) alone treated rats. Rats that were treated with DOX (G3) significantly ($P < 0.0001$) increased the level of TBARS when compared with control (G1). Rats that were pretreated with sesamol followed by DOX (G4) ($P < 0.001$) significantly decreased the level of TBARS when compared with DOX (G3) treated rats (Table 3).

3.5. Effect of Sesamol on GSH, SOD, and Catalase. There was no significant difference in the level of GSH and SOD between control (G1) and sesamol (G2) alone treated rats, and there was significant ($P < 0.0001$) difference in the level of catalase in sesamol (G2) alone treated rats when compared to control (G1) group. Rats that were treated with DOX (G3) significantly ($P < 0.0001$) decreased the level of GSH, SOD, and catalase when compared to control (G1). Rats were pretreated with sesamol followed by DOX (G4) significantly ($P < 0.0001$) decreased the level of GSH, SOD, and catalase when compared to DOX (G3) treated rats (Table 3).

3.6. Histopathology. Light microscopical section of heart tissue (Figure 2) of saline treated rat (G1) and sesamol treated rat (G2) show normal architecture of myocardium. Rats treated with DOX (G3) shows severe myocardial necrosis with subendocardial loss of muscles. Rats treated with sesamol+DOX show well-preserved myocardium when compared to DOX (G3) treated group.

4. Discussion

The present study endows with the significant indication of the beneficial effects of sesamol on DOX-induced cardiomyopathy. The outstanding remarkable findings were that the sesamol prevented the DOX-induced-altered lipid parameters, myocardial marker enzymes, and antioxidant parameters. Our findings suggest that sesamol protects the myocardium through numerous aspects against DOX-induced cardiomyopathy.

Generation of reactive oxygen species (ROS) is one of the mechanism for DOX-induced myocardial toxicity. DOX metabolically converted into its semiquinone form by CYP450 and flavin monooxygenase enzymes. This metabolite interacts with mitochondrial oxygen to generate ROS. In another way DOX reacts with iron (Fe^{2+}) to make free radical complex, which involves in the generation of hydroxyl radical ($OH^{\bullet}$) [16, 17].

Oxidative stress plays major role in DOX-induced cardiotoxicity by generation of lipid peroxidation. Myocardial

Table 2: Effect of sesamol on serum LDH, CK, AST, and troponin-T.

Group	LDH IU/L	CK IU/L	AST IU/L	Troponin-T μg/mL
G1: control	109.2 ± 6.493	114.3 ± 6.766	121.5 ± 2.9	0.50 ± 0.21
G2: sesamol (50 mg/kg)	119.0 ± 2.309	128.3 ± 1.7	138.0 ± 0.89	0.59 ± 0.34
G3: DOX (15 mg/kg in 6-divided dose)	299.0 ± 20.67[a]	249.3 ± 4.2[a]	345.0 ± 17.8[a]	1.7 ± 0.63[a]
G4: DOX+sesamol	107.5 ± 5.696[b]	185.7 ± 0.88[b]	119.0 ± 16.9[b]	0.81 ± 0.14[b]

All values expressed as mean ± SD. [a]$P < 0.0001$ versus G1. [b]$P < 0.0001$ versus G3.

Table 3: Effect of sesamol on TBARS, GSH, SOD, and catalase.

Groups	TBARS (nmol/g wet wt)	GSH (μg/g wet wt)	SOD (IU/mg protein)	CAT (IU/mg protein)
G1: control	6.9 ± 1.7	227.7 ± 6.2	102.2 ± 3.7	33.3 ± 2.2
G2: sesamol (50 mg/kg)	6.3 ± 1.2	237.9 ± 10.3	103.8 ± 6.04	38.8 ± 1.3***
G3: DOX (15 mg/kg in 6-divided dose)	15.8 ± 2.8[a]	130.2 ± 2.9[a]	47.7 ± 8.27[a]	22.09 ± 1.9[a]
G4: DOX+sesamol	11.07 ± 1.8[b]	150.5 ± 5.8[b]	94.02 ± 5.5[b]	29.3 ± 1.9[b]

All values expressed as mean ± SD. ***$P < 0.0001$ versus G1, [a]$P < 0.0001$ versus G1, and [b]$P < 0.001$ versus G3.

tissue susceptible to free radical damage due to less amount of antioxidants like SOD and catalase present in the heart [18, 19]. Administration of DOX at cumulative dose (15 mg/kg) increases the lipid peroxidation and depleted the endogenous antioxidants in the myocardium. Similar biochemical changes have been reported by several other studies [20, 21]. In the present study, generation of lipid peroxidation is conformed by elevated level of TBARS in DOX administered rat. Elevated level of TBARS significantly decreased by sesamol indicated the protective role of sesamol via reduction of oxidative stress. Moreover, sesamol that enhanced the GSH, SOD, and catalase in the myocardium supported the antioxidative effect of sesamol against DOX-induced oxidative damage. In the present study we have observed the putative antioxidant property of sesamol against DOX-induced cardio toxicity. Previous studies have demonstrated that sesamol exhibits antioxidant property in various oxidative conditions that cause tissue injury [10, 22–24].

Generation of free radical extensively damage the myocardium result in increased membrane permeability leads to leakage of LDH, CK and AST [25]. In the present study, sesamol significantly decreases the elevated level of serum LDH, CK, and AST. These results were consistent with the previous studies reported by other investigators [25, 26]. Another important findings observed in the present study are that sesamol significantly decreases the elevated level of serum troponin-T. Troponins are myocardial regulatory proteins, which regulate the calcium mediated actin and myosin interaction. Troponin-T is widely used as specific marker to diagnose myocardial infarction. Sesamol pretreatment significantly decreased the elevated level of serum troponin-T near to normal level which conformed the membrane stabilizing effect of sesamol against DOX-induced myocardial damage.

Lipids plays important role in cardiovascular disease complications. Drugs with lipid lowering agent protect the myocardium from DOX-induced cardiotoxicity [27]. DOX interferes with metabolism and biosynthesis of lipids there by TGL, TCH, LDL, VLDL levels were increased and HDL level were decreased in serum. In the present study, we have observed that the DOX significantly increased the level of TGL, TCH, LDL, and VLDL and decreased the level of HDL. Sesamol pretreatment that significantly reverted these lipid parameters near to normal indicated that sesamol may be lowering the lipids due to the inhibition cholesterol biosynthesis and increase in the uptake of LDL from blood by liver [3]. Similar effect was observed by Vennila and Pugalendi [28] in isoproterenol-induced myocardial infarction.

The biochemical data was supported by histopathological report, which showed severe myocardial necrosis with subendocardial loss of muscles in DOX administered rat. Sesamol shows that well-preserved myocardium was the indication of cardio protection.

Nayak et al. [29] reported the cardioprotective effect of sesamol against DOX-induced cardiotoxicity by *in vitro* experimental model. In this study they have reported that sesamol pretreatment increased the antioxidant status by preventing free radical generation. Similar effect has been observed in the present research conformed the significant effect of sesamol in ameliorating the toxic effects of DOX associated with cancer chemotherapy.

5. Conclusion

From these findings it has been concluded that our study, for the first time, demonstrates that sesamol protects the myocardium from DOX-induced cardiomyopathy in rat. The protective role may be due to its antioxidative, lipid lowering, and membrane stabilizing effect.

Disclosure

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. Research was conducted in Annamacharya College of Pharmacy, Department of Pharmacology.

Conflict of Interests

The authors declared no potential conflict of interests with respect to the research, authorship, and/or publication of this paper.

Acknowledgment

The authors are thankful to Management and Principal of Annamacharya College of Pharmacy, Rajampet, for the providing facilities for present work.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 303162, 12 pages
http://dx.doi.org/10.1155/2013/303162

Research Article

Acute and Chronic Oral Toxicity of a Partially Purified Plaunotol Extract from *Croton stellatopilosus* Ohba

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Received 6 August 2013; Accepted 22 August 2013

Academic Editor: Lillian Barros

Plaunotol, an acyclic diterpenoid with highly effective antigastric ulcer properties, has been commercially isolated from leaves of *Croton stellatopilosus* Ohba. This Thai medicinal plant was traditionally used in the form of crude extracts, suggesting that it is possible to administer these plaunotol-containing extracts without toxicity. To confirm its safety, the oral toxicity of a partially purified plaunotol extract (PPE) was evaluated *in vivo*. The PPE was simply prepared by 95% ethanol reflux extraction followed by hexane partition. The obtained extract was analyzed and found to contain 43% w/w of plaunotol and another compound, likely a fatty acid-plaunotol conjugate that is considered a major impurity. Oral administration of PPE to ICR mice and Wistar rats was conducted to evaluate acute and chronic toxicity of the plaunotol extract, respectively. The acute toxicity study demonstrated that PPE was practically nontoxic based on its high median lethal dose value (LD_{50} = 10.25 g/kg). The chronic toxicity studies also showed the absence of mortality and clinical symptoms in all rats treated with 11–1,100 mg/kg/day of PPE during a 6-month period. Histopathological and hematological analyses revealed that altered liver and kidney function and increased blood platelet number, but only at the high doses (550–1,100 mg/kg/day). These results suggest that PPE is potentially safe for further development as a therapeutic agent in humans.

1. Introduction

Croton stellatopilosus Ohba, a Thai medicinal plant (Plau-noi), has been acknowledged for its traditional remedy in the treatment of helminthes and topical infection [1]. A major constituent isolated from leaves of Plau-Noi is plaunotol [2]. This natural acyclic diterpene has been used as an antigastric ulcer medication based on its pharmacotherapeutic effects in inducing prostaglandin E_2 (PGE_2) and eradicating *Helicobacter pylori* bacteria [3–6]. However, in addition to its application as a single compound that requires a complicated process of extraction and purification [7], plaunotol used in form of a partially purified plaunotol extract (PPE) is also of interest. This is due to the wide distribution of Plau-Noi plant in Thailand [8] and the reliability of pharmacological benefits of natural terpenoid provides in human healthcare [9, 10]. Furthermore, the use of PPE can minimize the cost of production and increase the access to an effective drug at a low price.

At a dose of 240 mg/day or 4.8 mg/kg/day, plaunotol has been recommended to achieve antigastric ulcer effects in humans [11]. Because equivalent pharmacological activities could potentially come from higher amounts of PPE than those of pure plaunotol, it is necessary to assess toxicity profiles of such a high doses of PPE. Therefore, this study aims to

evaluate oral toxicity of PPE in order to generate a safety data in animal models for extrapolation to human toxicity profiles.

2. Materials and Methods

2.1. Preparation of PPE. PPE was prepared from dried leaf powder of *Croton stellatopilosus* Ohba by modifying a previously described method [7]. The leaf powder was refluxed with 95% ethanol (1 : 1 ratio by weight) at 70°C for 1 h. After filtration, the extract was concentrated by using a rotary evaporator, followed by partitioning with hexane (ratio of 1 : 1) to remove highly nonpolar constituents. With plaunotol remaining in ethanol layer, the ethanol was evaporated in a vacuum to obtain PPE, which was then collected and stored at 4°C. For use in this toxicity study, PPE was suspended in a 0.5% tragacanth solution and adjusted to various concentrations as indicated.

2.2. Plaunotol Content Analysis. Quantitative analysis of plaunotol in PPE was conducted using the method of thin-layer chromatography (TLC) reported previously [12, 13]. Briefly, a silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany) was spotted with PPE and standard plaunotol (obtained from Thai Sankyo Co., Ltd., which is currently under Tipco Biotech Co. Ltd., Prachuab Khiri Khan, Thailand). The plate was then developed using a solvent system of chloroform:n-propanol (96 : 4). The developed TLC plate was scanned by a densitometer at 220 nm using a Camag TLC Scanner 3 with Wincats software V 1.3.4 (Camag, Muttenz, Switzerland). Structural analysis of each constituent was carried out using gas chromatography-mass spectrometry (GC-MS: Agilent Model 6890N-5973N, Agilent Technologies, CA, USA). A DB-5 capillary column (30 × 0.25 mm) with helium as a carrier gas at a flow rate of 13.8 mL/min was heated from 150°C up to 300°C with an increasing temperature rate of 15°C/min. Temperatures of both detector and the injector were set at 250°C [12]. Compound identification was performed by comparing the resulting mass spectra with those available in the Wiley Registry of Mass Spectral Data 7th edition (McLafferty, 2000), Agilent Part no. G1035B.

2.3. Animals and Housing. All ICR (Imprinting Control Region) mice and Wistar rats were obtained from the National Laboratory Animal Center (Mahidol University, Nakorn Pathom, Thailand). They were housed in the conventional hygienic animal rooms of the Laboratory Animal Center, Department of Medical Sciences, Nonthaburi, Thailand. Room conditions were maintained at 25°C, 60% humidity and 12 hour-light-dark cycle. The animals were given a commercial pellet diet 082 CP feed (Perfect Companion Group, Thailand) and clean water ad libitum. Prior to the experiment, the animals were allowed two weeks to acclimate to the environment.

2.4. Acute Toxicity Study. Five male and five female ICR mice were treated with either water for the control group or PPE at 2.5, 5, 10, and 20 g/kg for each treatment group. All animals were food restricted for 2 h prior to oral administration of single doses of the substances. Observations of clinical presentation and mortality were conducted at 15 min, 30 min, 3 h, 6 h, and 24 h following treatment and daily for 14 days thereafter. Finally, the mean lethal dose value (LD_{50}) was calculated. This study was authorized by the Institutional Animal Care and Use Committee, Department of Medical Sciences as Permission no. 52-002.4.

2.5. Chronic Toxicity Study. The protocol used for the chronic toxicity study was approved by the Institution of Animal Care and Use Committee, Department of Medical Sciences as Permission no. 53-014. Wistar rats were randomized into six groups, each with 15 male and 15 female rats. Four experimental groups were administered the PPE suspension orally at doses of 11, 110, 550, and 1100 mg/kg/day for six months. Two control groups received distilled water and 0.5% tragacanth solution orally at the volume of 10 mL/kg. During the experimental period, animals were observed daily for general appearance and signs of toxicity. Body weight and food consumption were measured weekly. At the end of the study, animals were fasted overnight and were then killed using diethyl ether inhalation. Blood samples were collected from the posterior vena cava for hematological and biochemical value measurements.

Hematological analysis was performed using the hematological analyzer Cell Dyn 3500 (Abbot Laboratories Ltd., IL, USA). The parameters examined included red blood cells (RBC), hematocrit (Hct), hemoglobin, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cells (WBC), lymphocytes, neutrophils, eosinophils, basophils, monocytes, and platelets. Biochemical values were measured using Cobas integra 400 (Hoffmann-La Roche Ltd., Basel, Switzerland), which assessed levels of alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), total protein, albumin, bilirubin, blood urea nitrogen (BUN), creatinine, glucose, uric acid, triglyceride, cholesterol and sodium, potassium, and chloride ions.

A complete necropsy was performed to assess gross lesions of visceral organs. Brain, lung, heart, liver, kidney, stomach, spleen, testis, uterus, urinary bladder, and adrenal glands were isolated and weighed using Mettler (Mettler Toledo PB 153 balance, Mettler Toledo Intl. Inc., Zurich, Switzerland). Relative organ weight was calculated. The above mentioned organs, including trachea, were fixed in 10% phosphate-buffered formalin and subjected to conventional histological processing and stained with hematoxylin for further histopathological examination [14].

2.6. Statistical Analysis. A one-way ANOVA was used to evaluate significant differences between groups via the Bonferroni test. Fisher's exact was applied to compare histopathological differences between groups. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Plaunotol and a Plaunotol-Like Compound Are Major Constituents of PPE. By modifying the conventional method

Table 1: Observed acute toxicity symptoms and mortality in mice receiving different single doses of PPE.

Dose (g/kg)	Symptoms/Time (Onset to recovery)				Mortality	
	Dyspnea	Dullness	Abdominal cramp	Diarrhea	Death/Total	Latency period
Control	None	None	None	None	0/10	—
2.5	None	None	None	6–72 h	0/10	—
5	None	None	None	6–72 h	0/10	—
10	15 min–24 h	15 min–24 h	15 min–24 h	6–96 h	4/10	24 h
20	15 min–24 h	15 min–24 h	15 min–24 h	6–72 h	10/10	24–72 h

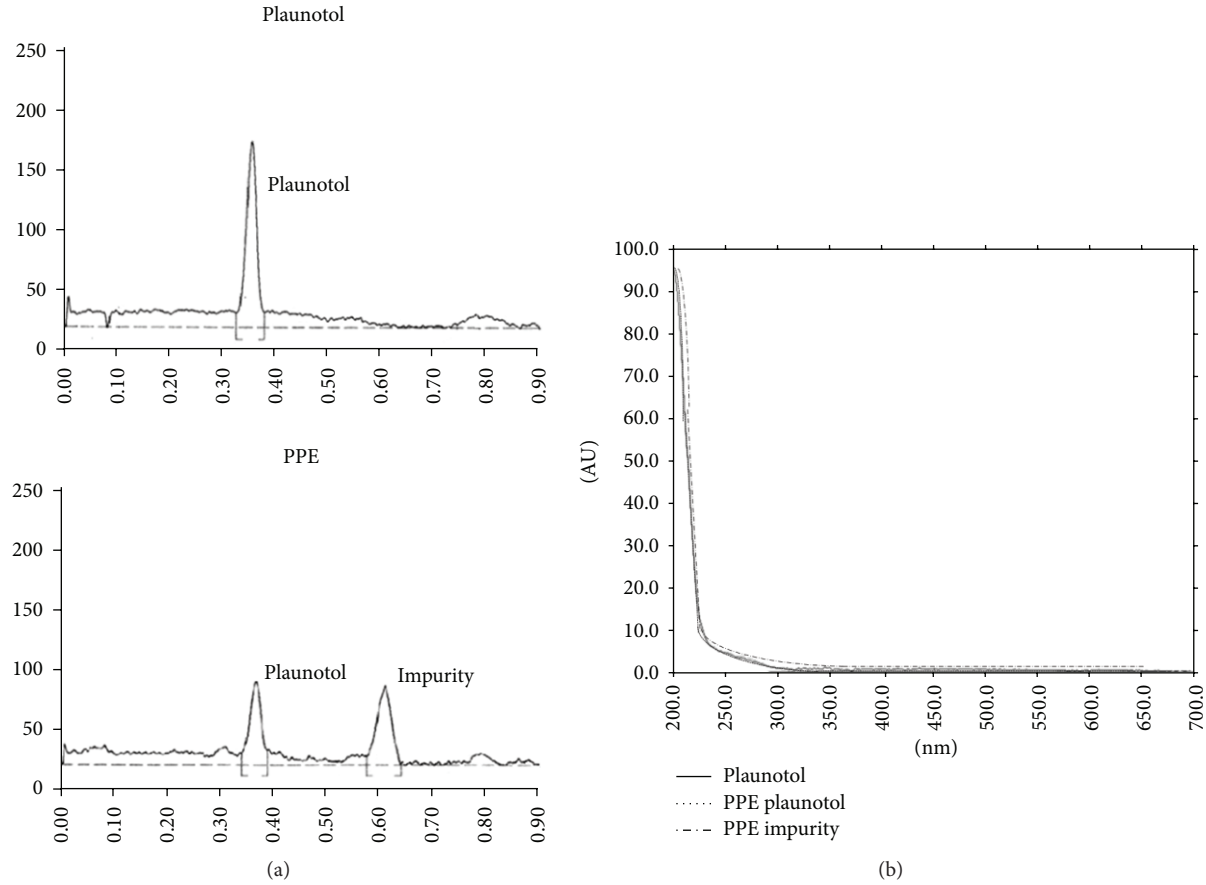

Figure 1: Densitometric TLC for separation of PPE constituents in PPE and quantitative analysis of plaunotol. (a) TLC chromatogram of PPE showing two major peaks of plaunotol and a main impurity. (b) Comparison of the UV absorption spectra obtained from the wavelength scanning at the spots of standard plaunotol, PPE plaunotol, and the PPE impurity shown in (a).

of plaunotol extraction and limited purification, PPE was obtained in 2% yield (w/w) as a brownish-yellow translucent oil. The extract appeared to contain plaunotol and an unknown compound as the two major constituents and a few minor impurities, as shown by the chemical profiles in the TLC (Figure 1(a)) and GC (Figure 2(a)) chromatograms. The UV absorption spectrum (Figure 1(b)) and mass spectrum (Figure 2(b)) of the unknown compound were similar to those of plaunotol, suggesting that the major impurity in PPE was a plaunotol-like compound. Because fatty acid conjugates of plaunotol are known to be present in the leaves of *C. stellatopilosus* [15], it is likely that the major impurity was one of the fatty acid-plaunotol conjugates that were extracted with plaunotol. Quantitative analysis indicated that plaunotol was present at approximately 43.0% w/w in the PPE preparation.

3.2. Acute Oral Toxicity and LD_{50}. All of the observed PPE-induced acute toxicity symptoms are summarized in Table 1. The results were based on oral doses of PPE at 2.5, 5, 10, and 20 g/kg, calculated to be 200, 400, 800, and 1,600 times the recommended dose of plaunotol for human therapeutic use, respectively [11]. Acute diarrhea was detected early, 6 h after extract administration, at all doses and remained until 72–96 h. With the high doses of 10 and 20 g/kg, other clinical symptoms were observed, including dyspnea (shortness of breath), dullness, and stomach cramp.

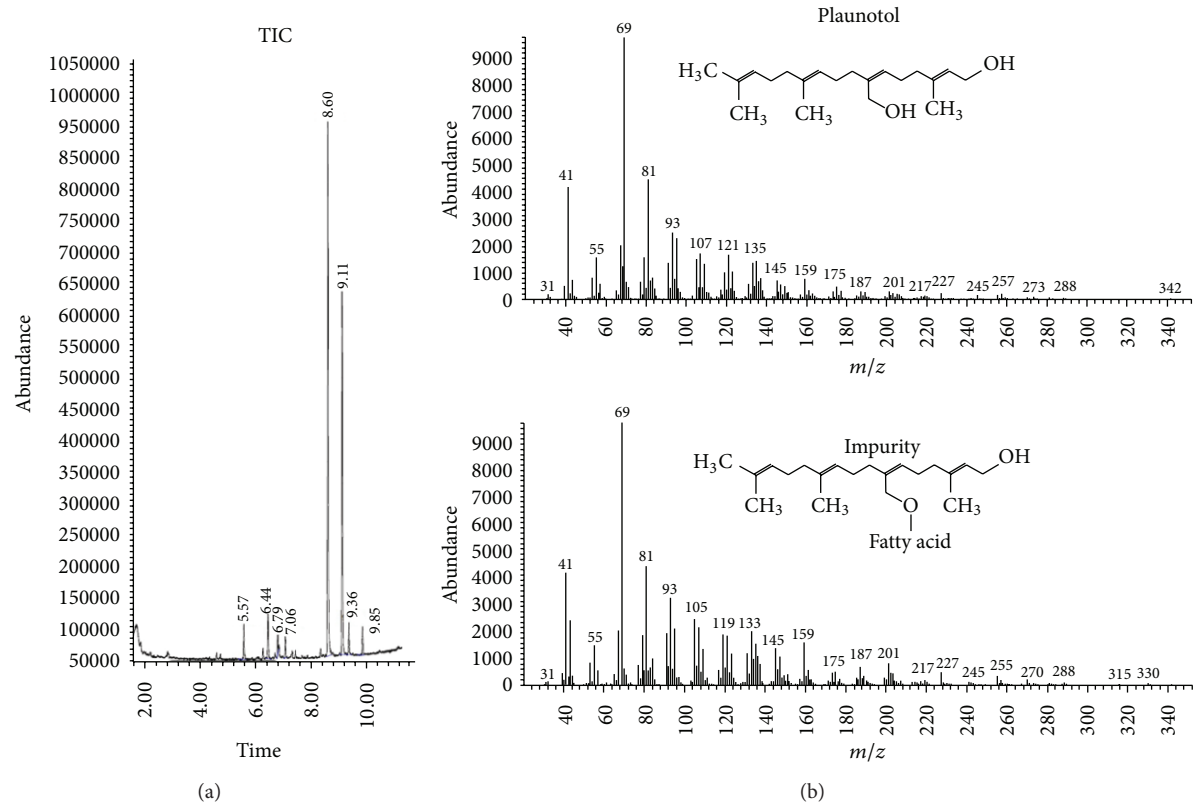

Figure 2: GC-MS chromatogram of PPE (a) and the mass spectra (b) of the two major peaks of plaunotol and the impurity identified based on the total ion chromatograms (TIC) of plaunotol and the major impurity in PPE.

Mortality was not observed in mice treated with water (control) or PPE at 2.5 and 5 g/kg; however, it was observed in four (one male and three females) and 10 (five males and five females) mice between 24 and 72 h at the 10 and 20 g/kg doses, respectively. Probit analysis revealed that the LD_{50} of PPE was 10.25 g/kg with 95% confidence limit between 7.37 and 14.63 g/kg.

3.3. Chronic Oral Toxicity

3.3.1. Effect of PPE on Body Weight, Food Consumption, and Health Status. In this chronic toxicity study, rats were fed equal volumes of PPE to minimize the effect of administration differences. PPE was diluted in 0.5% w/w of tragacanth to achieve the desirable concentrations used for gavage feeding the rats. The selected concentrations of PPE were 11, 110, 550, and 1,100 mg/kg/day, which are equivalent to 1, 10, 50, and 100 times the therapeutic dose of plaunotol, respectively. Mortality and significant clinical symptoms were not observed in any of the rats fed with PPE, water, and 0.5% w/w tragacanth (Figure 3). It is important to note that, for male rats given the high dose of PPE at 1,100 mg/kg/day, a trend in altered food consumption was not apparent even though there was a significant decrease in body weight (Figure 4). Similarly, the body weight and food consumption among female rats showed no significant difference.

3.3.2. Effect of PPE on Relative Organ Weight Values. The values of relative organ weight, expressed as g/1000 g body weight, are summarized in Tables 2 and 3. For male rats, relative organ weight values of the heart, liver, stomach, spleen, and kidney were significantly increased at doses of 110, 550, and 1,100 mg/kg/day, compared with the water and tragacanth control groups (Table 2). Furthermore, relative weights of the brain, lung, bladder, adrenal gland, and testis were also higher in the male rats given 550 and 1,100 mg/kg/day of PPE. Table 3 summarizes the values of relative organ weight for female rats. It can be seen that PPE treatment at high doses (550 and 1,100 mg/kg/day) caused an increase in the relative weight of the liver and kidney compared to both control groups. An additional organ that appeared to be affected in the female group was the stomach, which showed significantly higher relative weights in the 550 and 1,100 mg/kg/day PPE treatment groups.

3.3.3. Effect of PPE on Altered Hematological Values. After feeding PPE at 11 and 110 mg/kg/day for 6 months to both male and female rats, no significant alterations in any of the hematological values were observed (Tables 4 and 5). At the high doses of 550 and 1,100 mg/kg/day, male rats had significantly higher platelet numbers but a lower percentage of eosinophils compared to both controls. The hematocrit, RBC, and hemoglobin values were significantly higher only in

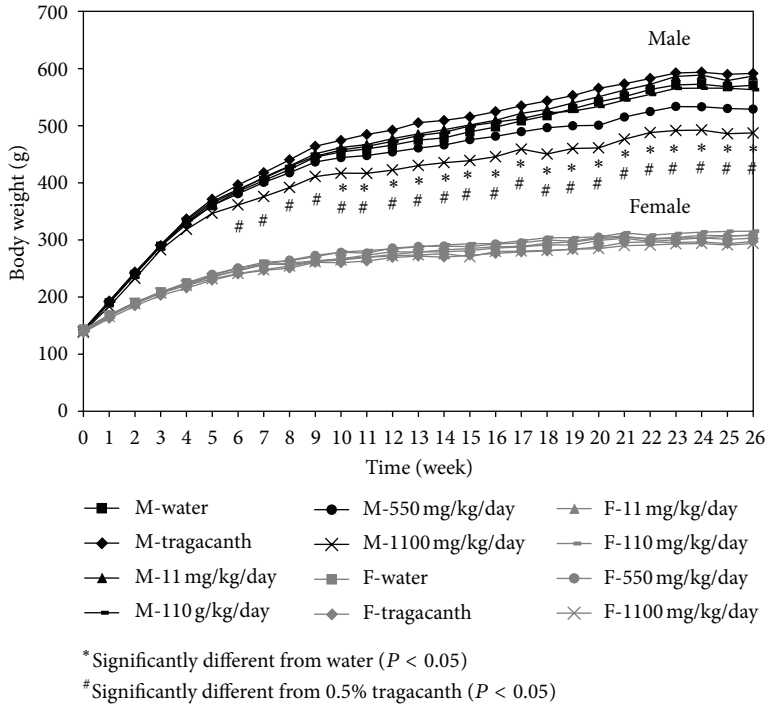

Figure 3: Growth curves of the male and female rats receiving PPE for 6 months.

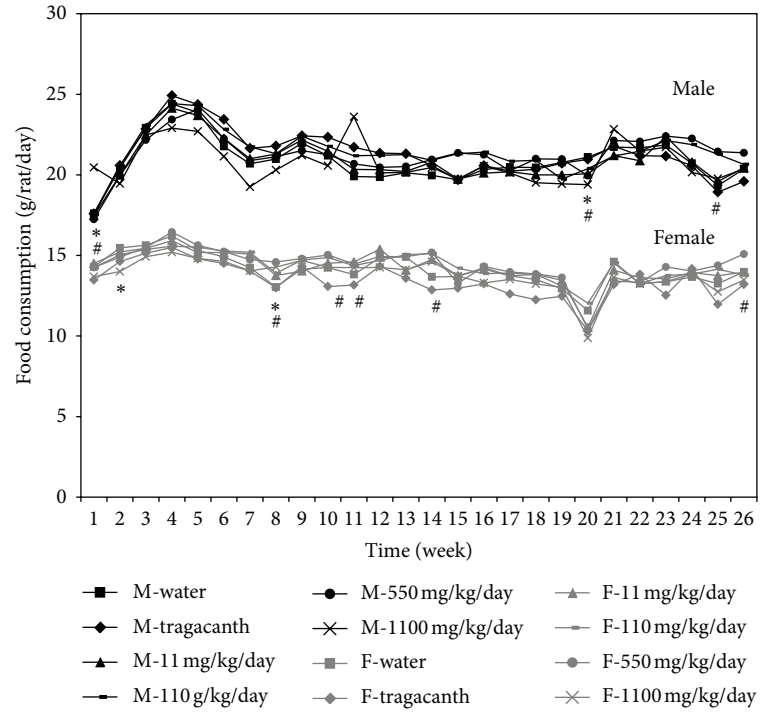

Figure 4: Food consumptions for male and female rats receiving PPE for 6 months.

Table 2: Relative organ weight (g/1000 g of body weight) and body weight (g) of male rats receiving PPE for 6 months.

Organs	Dose of PPE administered (mg/kg/day)					
	Control	Tragacanth	11	110	550	1100
	$n = 15$	$n = 15$	$n = 15$	$n = 14$[a]	$n = 15$	$n = 15$
Brain	3.88 ± 0.39	3.72 ± 0.33	3.78 ± 0.22	3.91 ± 0.31	4.21 ± 0.26[#]	4.55 ± 0.28[*,#]
Heart	2.63 ± 0.28	2.74 ± 0.12	2.68 ± 0.28	2.89 ± 0.13[#]	3.40 ± 0.25[*,#]	3.31 ± 0.27[*,#]
Lung	3.07 ± 0.31	3.18 ± 0.19	3.45 ± 1.04	3.31 ± 0.19	3.70 ± 0.29[*,#]	4.02 ± 0.43[*,#]
Liver	23.86 ± 2.02	24.38 ± 1.82	25.57 ± 1.68	33.65 ± 3.62[*,#]	49.65 ± 2.37[*,#]	59.81 ± 3.17[*,#]
Stomach	4.01 ± 0.40	3.81 ± 0.50	4.06 ± 0.39	4.53 ± 0.47[#]	5.20 ± 0.43[*,#]	5.64 ± 0.39[*,#]
Spleen	1.68 ± 0.20	1.55 ± 0.16	1.60 ± 0.15	1.77 ± 0.18[#]	2.14 ± 0.29[*,#]	2.18 ± 0.14[*,#]
Right kidney	2.33 ± 0.17	2.27 ± 0.21	2.38 ± 0.21	2.71 ± 0.18[*,#]	3.12 ± 0.25[*,#]	3.43 ± 0.31[*,#]
Left kidney	2.21 ± 0.19	2.20 ± 0.22	2.22 ± 0.22	2.63 ± 0.20[*,#]	3.04 ± 0.22[*,#]	3.35 ± 0.24[*,#]
Right testis	5.52 ± 0.53	5.16 ± 0.70	5.40 ± 0.43	5.48 ± 0.52	6.25 ± 0.55[*,#]	6.61 ± 0.49[*,#]
Left testis	5.60 ± 0.64	5.24 ± 0.82	5.41 ± 0.44	5.50 ± 0.56	6.27 ± 0.58[#]	6.62 ± 0.46[*,#]
Right adrenal	0.071 ± 0.01	0.071 ± 0.02	0.067 ± 0.01	0.080 ± 0.01	0.088 ± 0.01[*,#]	0.080 ± 0.01
Left adrenal	0.075 ± 0.01	0.082 ± 0.01	0.077 ± 0.02	0.087 ± 0.02	0.095 ± 0.01[*]	0.092 ± 0.02[*]
Bladder	0.328 ± 0.06	0.327 ± 0.06	0.313 ± 0.06	0.365 ± 0.09	0.418 ± 0.04[*,#]	0.368 ± 0.12
Initial body weight	113.89 ± 4.57	113.25 ± 4.70	114.19 ± 5.91	114.46 ± 8.98	113.15 ± 4.70	113.50 ± 6.34
Final body weight	533.07 ± 55.27	573.47 ± 52.91	569.32 ± 42.54	545.16 ± 49.74	506.27 ± 30.97[#]	461.69 ± 29.35[*,#]

The values are expressed as the mean ± SD.
[*] Significantly different from water ($P < 0.05$).
[#] Significantly different from 0.5% tragacanth ($P < 0.05$).
[a] Died from feeding extract into respiratory tract.

Table 3: Relative organ weight (g/1000 g body weight) and body weight (g) of female rats receiving PPE for 6 months.

Organs	Dose of PPE administered (mg/kg /day)					
	Control	Tragacanth	11	110	550	1100
	$n = 15$	$n = 15$	$n = 15$	$n = 14$[a]	$n = 15$	$n = 15$
Brain	6.88 ± 0.65	7.00 ± 0.62	6.82 ± 0.67	6.61 ± 0.54	6.72 ± 0.47	7.20 ± 0.39
Heart	3.30 ± 0.26	3.30 ± 0.33	3.27 ± 0.34	3.32 ± 0.20	3.55 ± 0.35	3.58 ± 0.23
Lung	4.58 ± 0.49	4.54 ± 0.50	4.49 ± 0.56	4.31 ± 0.31	4.62 ± 0.51	4.70 ± 0.30
Liver	27.08 ± 3.46	26.24 ± 2.93	25.60 ± 1.90	28.96 ± 3.38	44.80 ± 8.19[*,#]	54.68 ± 2.79[*,#]
Stomach	5.64 ± 0.80	5.58 ± 0.58	5.37 ± 0.65	5.61 ± 0.53	6.66 ± 1.00[*]	6.88 ± 0.60[*,#]
Spleen	2.22 ± 0.24	2.28 ± 0.26	2.19 ± 0.23	2.22 ± 0.27	2.29 ± 0.16	2.26 ± 0.29
Right kidney	3.03 ± 0.20	3.00 ± 0.28	2.99 ± 0.33	3.01 ± 0.26	3.51 ± 0.60[*]	3.59 ± 0.28[*,#]
Left kidney	2.86 ± 0.21	2.85 ± 0.21	2.85 ± 0.37	2.90 ± 0.19	3.43 ± 0.54[*,#]	3.44 ± 0.29[*,#]
Right adrenal	0.153 ± 0.02	0.145 ± 0.03	0.147 ± 0.03	0.130 ± 0.02	0.163 ± 0.06	0.136 ± 0.02
Left adrenal	0.155 ± 0.02	0.153 ± 0.03	0.156 ± 0.03	0.154 ± 0.03	0.170 ± 0.09	0.145 ± 0.02
Bladder	0.318 ± 0.04	0.321 ± 0.06	0.312 ± 0.06	0.339 ± 0.09	0.374 ± 0.08	0.389 ± 0.06
Uterus	2.31 ± 0.67	2.69 ± 1.05	2.53 ± 0.64	2.17 ± 0.64	2.55 ± 0.76	2.40 ± 0.58
Right ovary	0.279 ± 0.05	0.284 ± 0.07	0.277 ± 0.06	0.267 ± 0.07	0.309 ± 0.07	0.288 ± 0.06
Left ovary	0.291 ± 0.05	0.305 ± 0.09	0.303 ± 0.07	0.257 ± 0.07	0.303 ± 0.06	0.315 ± 0.09
Initial body weight	125.05 ± 14.22	122.79 ± 12.06	123.22 ± 11.99	124.06 ± 10.67	123.27 ± 14.54	121.77 ± 11.17
Final body weight	290.75 ± 29.24	283.54 ± 27.76	294.23 ± 30.84	301.79 ± 24.01	294.16 ± 22.49	275.07 ± 16.64

The values are expressed as the mean ± SD.
[*] Significantly different from water ($P < 0.05$).
[#] Significantly different from 0.5% Tragacanth ($P < 0.05$).
[a] Died from feeding extract into respiratory tract.

the 1,100 mg/kg/day treatment group, although their MCHC was significantly lower.

The reduced value of %eosinophil was observed in female rats administered 550 and 1,100 mg/kg/day of PPE. These two groups also appeared to have significantly higher values of platelet numbers and percentage of basophil compared to both controls. Moreover, the female rats treated with 1,100 mg/kg/day had significantly lower MCV, MCH, and MCHC compared to the controls, while the RBC and WBC values were significantly higher.

Table 4: Hematological values for male rats receiving PPE for 6 months.

Parameters	Dose of PPE administered (mg/kg/day)					
	Water	0.5% tragacanth	11	110	550	1100
	$n = 15$	$n = 15$	$n = 15$	$n = 14$[a]	$n = 15$	$n = 15$
Hematocrit (%)	31.23 ± 1.84	31.63 ± 1.82	31.47 ± 0.93	32.11 ± 1.80	32.82 ± 1.88	33.61 ± 1.83[*,#]
RBC ($\times 10^6$ cells/mm^3)	9.66 ± 0.71	9.90 ± 0.51	9.70 ± 0.39	9.97 ± 0.67	10.33 ± 0.76	10.59 ± 0.75[*]
Hemoglobin (g/dL)	16.82 ± 0.96	16.90 ± 0.98	16.97 ± 0.53	17.12 ± 0.88	17.49 ± 0.98	17.82 ± 0.92[*]
MCV (μm^3/red cell)	53.85 ± 0.73	53.47 ± 0.55	53.94 ± 0.79	53.32 ± 0.89	53.31 ± 0.57	53.06 ± 0.70
MCH (pg/red cell)	17.44 ± 0.71	17.07 ± 0.49	17.51 ± 0.69	17.20 ± 0.56	16.96 ± 0.48	16.86 ± 0.55
MCHC (g/dL RBC)	32.39 ± 1.26	31.92 ± 0.99	32.46 ± 1.08	32.25 ± 1.10	31.83 ± 0.91	31.79 ± 1.05[*]
WBC ($\times 10^3$ cells/mm^3)	3.79 ± 1.29	3.97 ± 1.13	4.40 ± 1.65	4.87 ± 1.45	5.21 ± 1.62	4.45 ± 0.77
Neutrophil (%)	26.17 ± 5.81	29.36 ± 6.99	30.67 ± 13.68	27.59 ± 10.65	30.11 ± 11.32	25.31 ± 6.58
Eosinophil (%)	1.52 ± 0.66	1.33 ± 0.47	1.16 ± 0.42	1.00 ± 0.40	0.77 ± 0.25[*,#]	0.74 ± 0.21[*,#]
Lymphocyte (%)	67.95 ± 9.14	66.73 ± 6.23	65.06 ± 14.21	69.44 ± 9.91	67.66 ± 11.01	72.01 ± 6.47
Monocyte (%)	2.62 ± 3.39	1.62 ± 2.82	1.84 ± 2.76	0.98 ± 1.64	0.61 ± 0.29	0.58 ± 0.17
Basophil (%)	1.75 ± 1.95	0.95 ± 0.99	1.28 ± 1.25	0.98 ± 0.91	0.86 ± 0.39	1.35 ± 0.45
Platelet ($\times 10^3$ cells/mm^3)	1,020.57 ± 102.85	1,122.97 ± 118.94	1,052.83 ± 111.57	1,133.82 ± 140.14	1,297.57 ± 128.87[*,#]	1,455.37 ± 205.83[*,#]

The values are expressed as the mean ± SD.
[*]Significantly different from water ($P < 0.05$).
[#]Significantly different from 0.5% tragacanth ($P < 0.05$).
[a]Died from feeding extract into respiratory tract.

3.3.4. Effect of PPE on Clinical Chemistry Values. An increase in blood glucose levels was observed in male rats treated with PPE at 11 to 550 mg/kg/day compared to the water control group. Surprisingly, only the male rats fed 1,100 mg/kg/day showed lower blood glucose levels (Table 6), whereas the female rats had higher blood glucose levels following treatment with 110 to 1,100 mg/kg/day of PPE, compared to the tragacanth control group (Table 7).

Increased ALP values were observed in male rats that received 110 to 1,100 mg/kg/day and in female rats that received the highest dose of 1,100 mg/kg/day. Decreased AST levels were observed only at the 550 mg/kg/day dose in both genders of rat. Total protein level was significantly increased in the male rats receiving 11 and 550 mg/kg/day, as well as in female rats receiving 550 and 1,100 mg/kg/day. Increased albumin and BUN levels were observed in both male and female rats given 550 and 1,100 mg/kg/day of PPE compared to both control groups.

The cholesterol values were significantly higher in both male and female rats that received PPE at 550 and 1,100 mg/kg/day. In contrast, male rats treated with 1,100 mg/kg/day of the extracts had lower triglyceride levels compared to both water and tragacanth control groups. Lower chloride levels were observed in male rats given 110 and 550 mg/kg/day and female rats given 550 and 1,110 mg/kg/day. High levels of uric acid were measured in male rats, but only at the doses of 11 and 550 mg/kg/day. Female rats receiving PPE at 550 mg/kg/day showed a lower level of total bilirubin compared to both control groups.

3.3.5. Histopathological Evaluation of PPE Treatment. For all rats, remarkable macroscopic lesions were not observed during the autopsy process. Histopathological evaluation also indicated that remarkable lesions were not found in most organs of the rats treated with PPE. Minor lesions were found on specific organs, as summarized in Tables 8 and 9.

For male rats, the groups treated with 11, 110, and 550 mg/kg/day of PPE showed significantly higher incidence of cortical fat infiltration in the adrenal glands when compared to the water control group. Only the group receiving the highest dose of PPE showed a significant increase in bile duct hyperplasia compared to both control groups. In the female rats, hyperplasia of the mammary gland was found in both control groups but not in the 11 and 110 mg/kg/day PPE-treated groups. The significant lesion in the kidney was congested tubules in female rats given 1,100 mg/kg/day. Furthermore, dilated tubules were also observed in some of the female rats receiving 550 and 1,100 mg/kg/day of PPE.

4. Discussion

The partially purified plaunotol extract (PPE) used in this acute and chronic toxicity study was prepared from the leaves

Table 5: Hematological values for female rats receiving PPE for 6 months.

Parameters	Dose of PPE administered (mg/kg/day)					
	Water	0.5% tragacanth	11	110	550	1100
	$n = 15$	$n = 15$	$n = 15$	$n = 14$[a]	$n = 15$	$n = 15$
Hematocrit (%)	30.86 ± 0.82	30.85 ± 1.40	30.88 ± 0.92	30.82 ± 1.28	31.86 ± 1.51	31.62 ± 1.44
RBC ($\times 10^6$ cells/mm^3)	8.93 ± 0.37	8.97 ± 0.52	8.98 ± 0.34	8.80 ± 0.38	9.29 ± 0.60	9.65 ± 0.37$^{*,\#}$
Hemoglobin (g/dL)	16.60 ± 0.42	16.72 ± 0.60	16.79 ± 0.53	16.73 ± 0.64	17.00 ± 0.72	16.73 ± 0.74
MCV (μm^3/red cell)	53.77 ± 0.50	54.23 ± 0.72	54.40 ± 0.59	54.27 ± 0.52	53.37 ± 0.96	52.90 ± 0.55$^{*,\#}$
MCH (pg/red cell)	18.61 ± 0.47	18.69 ± 0.58	18.71 ± 0.49	19.00 ± 0.47	18.34 ± 0.89	17.34 ± 0.53$^{*,\#}$
MCHC (g/dL RBC)	34.60 ± 0.85	34.45 ± 0.83	34.38 ± 0.74	35.05 ± 0.74	34.33 ± 1.32	32.78 ± 0.93$^{*,\#}$
WBC ($\times 10^3$ cells/mm^3)	1.95 ± 0.50	2.05 ± 0.61	2.40 ± 0.55	2.49 ± 0.78	2.56 ± 0.69	2.82 ± 1.04*
Neutrophil (%)	22.36 ± 4.49	25.03 ± 9.50	24.65 ± 8.51	19.86 ± 4.82	21.35 ± 11.41	20.94 ± 3.38
Eosinophil (%)	1.64 ± 0.56	1.50 ± 0.79	1.27 ± 0.70	1.20 ± 0.47	0.73 ± 0.34$^{*,\#}$	0.74 ± 0.55*
Lymphocyte (%)	72.25 ± 5.08	71.95 ± 9.23	72.53 ± 8.55	76.97 ± 4.91	75.19 ± 12.68	75.80 ± 3.49
Monocyte (%)	1.94 ± 2.89	0.89 ± 0.30	0.93 ± 0.36	1.05 ± 0.65	1.43 ± 1.63	0.93 ± 0.67
Basophil (%)	0.83 ± 1.02	0.62 ± 0.41	0.63 ± 0.40	0.92 ± 0.46	1.29 ± 0.62$^{\#}$	1.59 ± 0.61$^{\#}$
Platelet ($\times 10^3$ cells/mm^3)	1,068.73 ± 121.33	1,039.23 ± 102.92	1,012.80 ± 71.82	1,015.57 ± 97.02	1,240.60 ± 106.72$^{*,\#}$	1,334.80 ± 101.78$^{*,\#}$

The values are expressed as the mean ± SD.
*Significantly different from water ($P < 0.05$).
$^{\#}$Significantly different from 0.5% tragacanth ($P < 0.05$).
[a]Died from feeding extract into respiratory tract.

Table 6: Biochemical values for male rats receiving PPE for 6 months.

Parameters	Dose of PPE administered (mg/kg/day)					
	Water	0.5% tragacanth	11	110	550	1100
	$n = 15$	$n = 15$	$n = 15$	$n = 14$[a]	$n = 15$	$n = 15$
ALP (U/L)	51.67 ± 9.03	48.13 ± 7.28	55.53 ± 22.60	55.29 ± 4.29$^{\#}$	70.53 ± 9.51$^{*,\#}$	84.53 ± 14.39$^{*,\#}$
ALT (U/L)	43.67 ± 15.99	38.47 ± 11.91	43.87 ± 23.19	33.71 ± 4.75	41.20 ± 7.63	43.93 ± 5.60
AST (U/L)	99.60 ± 33.66	86.33 ± 13.70	92.27 ± 39.18	74.29 ± 10.03	69.20 ± 6.94*	74.53 ± 15.57
Total protein (g/dL)	6.41 ± 0.18	6.67 ± 0.24	6.70 ± 0.18*	6.67 ± 0.22	6.84 ± 0.33*	6.66 ± 0.29
Albumin (g/dL)	4.51 ± 0.19	4.54 ± 0.09	4.61 ± 0.14	4.67 ± 0.23	5.03 ± 0.27$^{*,\#}$	5.27 ± 0.24$^{*,\#}$
Total bilirubin (mg/dL)	0.074 ± 0.03	0.070 ± 0.02	0.072 ± 0.02	0.104 ± 0.17	0.051 ± 0.01	0.071 ± 0.01
BUN (mg/dL)	18.75 ± 4.60	18.77 ± 3.11	16.71 ± 2.28	20.46 ± 3.13	22.59 ± 3.51$^{*,\#}$	26.53 ± 4.98$^{*,\#}$
Creatinine (mg/dL)	0.50 ± 0.08	0.52 ± 0.07	0.47 ± 0.06	0.47 ± 0.07	0.48 ± 0.07	0.49 ± 0.07
Glucose (mg/dL)	208.59 ± 36.28	265.05 ± 59.11	285.36 ± 36.34*	286.70 ± 63.09*	271.40 ± 51.29*	177.17 ± 34.34$^{*,\#}$
Uric acid (mg/dL)	4.07 ± 1.18	5.43 ± 1.65	5.90 ± 1.33*	5.26 ± 1.33	6.23 ± 1.49*	4.73 ± 0.90
Triglyceride (mg/dL)	74.91 ± 21.16	81.59 ± 27.43	82.31 ± 25.37	99.32 ± 45.27	77.24 ± 26.90	37.45 ± 16.70$^{*,\#}$
Cholesterol (mg/dL)	62.95 ± 10.31	75.38 ± 13.26	73.28 ± 14.85	84.36 ± 28.95	91.62 ± 19.62*	86.79 ± 16.99*
Sodium	143.20 ± 1.15	143.20 ± 1.26	143.27 ± 1.33	143.00 ± 0.88	143.40 ± 1.12	142.67 ± 1.05
Potassium	7.41 ± 1.02	7.40 ± 0.65	7.18 ± 0.66	6.93 ± 0.69	7.32 ± 0.56	7.67 ± 0.74
Chloride	105.00 ± 1.41	103.93 ± 1.49	103.20 ± 1.86	102.50 ± 0.85*	102.40 ± 1.68*	103.27 ± 1.67

The values are expressed as the mean ± SD.
*Significantly different from water ($P < 0.05$).
$^{\#}$Significantly different from 0.5% tragacanth ($P < 0.05$).
[a]Died from feeding extract into respiratory tract.

Table 7: Biochemical values for female rats receiving PPE for 6 months.

Parameters	Dose of PPE administered (mg/kg/day)					
	Water	0.5% tragacanth	11	110	550	1100
	$n = 15$	$n = 15$	$n = 15$	$n = 14$[a]	$n = 15$	$n = 15$
ALP (U/L)	22.93 ± 8.00	25.07 ± 7.29	23.80 ± 5.72	21.62 ± 7.68	22.38 ± 6.75	47.33 ± 12.97[*,#]
ALT (U/L)	27.33 ± 4.45	28.60 ± 9.05	26.20 ± 4.99	23.54 ± 4.96	25.07 ± 9.76	29.00 ± 3.68
AST (U/L)	82.67 ± 9.77	87.40 ± 17.22	86.73 ± 11.74	75.15 ± 13.72	63.93 ± 6.57[*,#]	73.60 ± 10.54
Total protein (g/dL)	6.63 ± 0.26	6.49 ± 0.28	6.51 ± 0.35	6.72 ± 0.33	6.99 ± 0.29[*,#]	7.14 ± 0.27[*,#]
Albumin (g/dL)	4.91 ± 0.20	4.82 ± 0.19	4.92 ± 0.26	5.15 ± 0.29[#]	5.49 ± 0.70[*,#]	5.81 ± 0.14[*,#]
Total bilirubin (mg/dL)	0.090 ± 0.02	0.098 ± 0.02	0.093 ± 0.02	0.089 ± 0.02	0.077 ± 0.01[#]	0.087 ± 0.02
BUN (mg/dL)	20.62 ± 5.44	21.91 ± 5.98	22.73 ± 4.99	25.23 ± 5.97	27.46 ± 5.99[*]	33.39 ± 7.34[*,#]
Creatinine (mg/dL)	0.49 ± 0.08	0.50 ± 0.10	0.51 ± 0.09	0.53 ± 0.13	0.55 ± 0.09	0.68 ± 0.13[*,#]
Glucose (mg/dL)	120.53 ± 16.41	110.66 ± 20.68	113.85 ± 12.41	138.09 ± 24.65[#]	156.02 ± 33.38[*,#]	136.70 ± 21.60[#]
Uric acid (mg/dL)	3.15 ± 0.84	3.22 ± 0.55	2.83 ± 0.55	3.18 ± 0.65	4.16 ± 1.48	3.83 ± 1.16
Triglyceride (mg/dL)	42.47 ± 9.81	40.74 ± 9.16	40.71 ± 6.06	40.20 ± 10.36	41.81 ± 7.07	33.94 ± 6.50
Cholesterol (mg/dL)	65.30 ± 25.78	59.60 ± 15.74	54.69 ± 10.64	74.42 ± 20.54	89.14 ± 17.89[*,#]	115.28 ± 26.26[*,#]
Sodium	142.40 ± 1.18	141.53 ± 1.55	142.20 ± 1.01	142.31 ± 1.11	141.93 ± 0.88	142.20 ± 1.26
Potassium	7.38 ± 0.83	7.66 ± 1.06	7.13 ± 0.56	6.74 ± 0.88	6.81 ± 0.65	6.53 ± 0.68[#]
Chloride	106.60 ± 1.45	105.80 ± 1.47	106.07 ± 0.96	105.15 ± 1.41	103.27 ± 2.71[*,#]	103.93 ± 1.16[*,#]

The values are expressed as the mean ± SD.
[*] Significantly different from water ($P < 0.05$).
[#] Significantly different from 0.5% tragacanth ($P < 0.05$).
[a] Died from feeding extract into respiratory tract.

Table 8: Histopathological results of male rats receiving PPE for 6 months.

Organs	Microscopic findings	Dose of PPE administered (mg/kg/day)					
		Water	0.5% tragacanth	11	110	550	1100
Lung	BALT proliferation	5/15	5/15	2/15	5/14	4/15	5/15
Liver	Centrilobular fatty degeneration	0/15	2/15	2/15	1/14	2/15	0/15
	Centrilobular hydropic degeneration	0/15	0/15	2/15	1/14	0/15	0/15
	Bile ductule hyperplasia	0/15	0/15	0/15	0/14	1/15	10/15[*,#]
Small intestine	GALT proliferation in submucosa	1/15	1/15	6/15	2/14	1/15	3/15
Large intestine	GALT proliferation in submucosa	1/15	1/15	0/15	1/14	2/15	3/15
Adrenal gland	Cortical fatty infiltration	0/15	2/15	6/15[*]	6/14[*]	4/15[*]	0/15

The results were expressed as the number of rats with pathological findings per total number of rats treated.
[*] Significantly different from water ($P < 0.05$).
[#] Significantly different from 0.5% tragacanth ($P < 0.05$).
BALT: bronchiole-associated lymphoid tissue; GALT: gut-associated lymphoid tissue.

Table 9: Histopathological results of female rats receiving PPE for 6 months.

Organs	Microscopic findings	Dose of PPE administered (mg/kg/day)					
		Water	0.5% tragacanth	11	110	550	1100
Lung	BALT proliferation	4/15	2/15	3/15	5/15	1/15	2/15
Liver	Bile ductule hyperplasia	0/15	0/15	0/15	0/15	1/15	2/15
	Dilated sinusoid and congestion	0/15	0/15	0/15	0/15	2/15	0/15
Kidney	Congestion	0/15	0/15	1/15	0/15	2/15	4/15[*,#]
	Dilated tubules	0/15	0/15	0/15	0/15	1/15	3/15
Mammary gland	Glandular hyperplasia	4/15	4/15	0/15[*,#]	0/15[*,#]	1/15	6/15
Adrenal gland	Medullary congestion	2/15	3/15	3/15	3/15	1/15	2/15

The results were expressed as the number of rats with pathological findings per total number of rats treated, 1100: the satellite group.
[*] Significantly different from water ($P < 0.05$).
[#] Significantly different from 0.5% tragacanth ($P < 0.05$).
BALT: bronchiole-associated lymphoid tissue; GALT: gut-associated lymphoid tissue.

of *C. stellatopilosus*. It was extracted by 95% ethanol under reflux, followed by liquid-liquid partition with hexane to remove nonpolar impurities. The major constituents of the resulting ethanol extract of PPE appeared to be 43% w/w of plaunotol with a similar amount of a plaunotol-like compound, presumably a fatty acid-plaunotol conjugate. Kitazawa et al. in 1982 reported on the presence of various geranylgeraniol derivatives in the extracts of *C. stellatopilosus*, including plaunotol (18-hydroxyl geranylgeraniol) and a number of fatty acid esters of geranylgeraniol [15].

The PPE obtained from processing was expected to be usable as a natural health product with its therapeutic benefits similar to that of plaunotol. This acyclic diterpenoid, originally found to possess antigastric ulcer activity, has continuously been reported to have bactericidal activity against *Helicobacter pylori* and potential as an anticancer agent [5, 16–18]. In this study, PPE was calculated to contain an equivalent amount of plaunotol for antigastric ulcer treatment in humans (4.8 mg/kg/day), which is equivalent to a dose of 11 mg/kg/day of PPE [11]. Our results obtained from the acute toxicity studies revealed that the tolerated dose of PPE was approximately 2.5 g/kg, or 200-fold higher than the recommended therapeutic dose. With this high dose, death was not observed in ICR mice, but mild diarrhea was the only observed side effect. In referring to toxicity classification, PPE can be categorized as a practical, non-toxic agent [19], with an LD_{50} of 10.25 g/kg. This LD_{50} value and the observed clinical signs following PPE treatment were not different from the data on pure plaunotol, which has previously been reported to have LD_{50} values of 8.8 and 8.1 g/kg for male and female mice, respectively [16].

Based on the LD_{50} of 10.25 g/kg, PPE was administered by oral gavage at doses of 11, 110, 550, and 1,100 mg/kg/day to a total of 180 Wistar rats, male and female, for chronic oral toxicity evaluation. Clinical and health status of the rats were observed for 6 months during treatment. The results showed that PPE did not suppress food consumption in all groups of rats, although significant body weight loss was observed in male rats treated with 1,100 mg/kg/day. These results suggest that the high concentration of acyclic diterpenoids in PPE may increase the metabolic rate in male rats, as reported previously [20]. However, this weight loss was only observed in male rats, and not in females. The differential responses may be gender dependent. In terms of organ weights, the relative weights of most organs were increased due to PPE treatment mainly in the male rats, whereas relative weights of the liver, kidney, and stomach were increased in both male and female rat groups. The latter may be due to responses at the subcellular level, such as stimulation of proteins or enzymes production [21]. Plaunotol exposure has been shown to induce COX2 and PGE_2 expression in gastric epithelial cells of rats [6]. Furthermore, the observed congestion of blood in renal venules, as well as the dilated renal tubules, may have affected the relative weight of kidneys in both male and female rats.

The altered hematological values were not significantly different from reference values for normal rats [21, 22], except the increased platelet number in the highest dose-treated group. The underlying mechanism for this observed increase in platelet levels by plaunotol and the fatty acid-plaunotol conjugate in PPE remains to be understood.

The significant increase in ALP levels caused by disorders of the hepatobiliary duct and osteoblast activity has been reported previously [23]. This response was observed in male rats treated with PPE at 1,100 mg/kg/day and in female rats treated with PPE at 550 and 1,100 mg/kg/day. Although the geranylgeraniol compound has been reported to have influence on osteoblast formation [24], abnormalities in bone formation were not observed in any of the rats. Additionally, the albumin values were increased male rats treated with 110 to 1,100 mg/kg/day and female rats given 550 and 1,100 mg/kg/day. This may be due to the stimulation of insulin, growth hormone, and corticosteroids [25]. The relationship between the plaunotol extract and hormonal expression is still unclear and requires further studies [26].

The values of AST, uric acid, and bilirubin were not related to the administered concentrations of PPE, so these alterations may not be due to the plaunotol extract. In contrast, the increase in BUN values in female rats treated with 550 and 1,100 mg/kg/day may be the result of the high reabsorption of enlarged renal tubules.

Recently, Gutierrez et al. in 2011 demonstrated an interesting effect of geranylgeraniol derivatives on blood glucose levels in diabetic rats. Specifically, hyperglycemia was apparent in rats treated with geranylgeranyl octadecanoate [20]. While the hydrophilic derivatives, such as geranylgeranyl acetate, lower blood glucose levels [20], our present study showed the hyperglycemic activity of PPE in male rats given doses of 11 to 550 mg/kg/day and in female rats treated with 110 to 1,100 mg/kg/day. The lowered blood glucose was only observed following treatment with 1,100 mg/kg/day in male rats. The varied effect on blood glucose may be gender dependent following treatment with the geranylgeraniol derivatives including plaunotol and its fatty acid conjugate in PPE.

Although there were altered levels of chloride, potassium, protein, and creatinine in the blood, these biochemical values are still within the normal clinical ranges [22]. The increased cholesterol levels in female rats treated with 550 and 1,100 mg/kg/day may be caused by the high dose of PPE that was administered. The essential role geranylgeraniol derivatives play in cholesterol synthesis has been reported previously [27, 28].

It is known that, following oral administration, plaunotol is metabolized via the liver and excreted through bile and urine [11]. Thus, large doses of PPE may result in pathological changes in liver and kidney cells. Histopathological evaluation revealed apparent bile duct hyperplasia in both male and female rats treated with 1,100 mg/kg/day. Congestion and dilation of renal tubules were observed at doses of 1,100 mg/kg/day of PPE in female rats. Incidence of fat accumulation in the adrenal gland in male rats and mammary gland hyperplasia in female rats were not related to the concentration of PPE and therefore may not be due to treatment with the extract.

5. Conclusion

This research demonstrated the safety of using PPE, supporting the traditional use of plaunotol in the form of

crude extracts. It was apparent that PPE treatment at the therapeutic dose did not induce any lethal pathological or clinical signs following chronic exposure. Therefore, it may be possible to develop this extract as a human healthcare product. Nevertheless, administration of high doses of PPE over a long period of time may cause deterioration of the liver and renal tissue, including increased blood platelet levels. Therefore, hematological and biochemical values should be monitored during use of high doses of the extract over a long period of time.

Acknowledgments

Chatchai Chaotham would like to thank The Thailand Research Fund and The Royal Golden Jubilee Ph.D. program (Grant no. 5.Q.CU/50/C.1). They would also like to thank Nalinpat Saktiyasunthorn for providing experimental facilities at the Medical Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand. This study was financially supported by the Thailand's National Innovation Agency.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 379850, 9 pages
http://dx.doi.org/10.1155/2013/379850

Review Article

Pinocembrin: A Novel Natural Compound with Versatile Pharmacological and Biological Activities

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Received 29 April 2013; Revised 1 July 2013; Accepted 9 July 2013

Academic Editor: Isabel C. F. R. Ferreira

Pinocembrin (5,7-dihydroxyflavanone) is one of the primary flavonoids isolated from the variety of plants, mainly from *Pinus* heartwood, *Eucalyptus, Populus, Euphorbia*, and *Sparattosperma leucanthum*, in the diverse flora and purified by various chromatographic techniques. Pinocembrin is a major flavonoid molecule incorporated as multifunctional in the pharmaceutical industry. Its vast range of pharmacological activities has been well researched including antimicrobial, anti-inflammatory, antioxidant, and anticancer activities. In addition, pinocembrin can be used as neuroprotective against cerebral ischemic injury with a wide therapeutic time window, which may be attributed to its antiexcitotoxic effects. Pinocembrin exhibits pharmacological effects on almost all systems, and our aim is to review the pharmacological and therapeutic applications of pinocembrin with specific emphasis on mechanisms of actions. The design of new drugs based on the pharmacological effects of pinocembrin could be beneficial. This review suggests that pinocembrin is a potentially promising pharmacological candidate, but additional studies and clinical trials are required to determine its specific intracellular sites of action and derivative targets in order to fully understand the mechanism of its anti-inflammatory, anticancer, and apoptotic effects to further validate its medical applications.

1. Introduction

Throughout the history of civilization, natural products have served human beings as a primary source of medicine [1]. The term "natural products" comprises chemical compounds that are derived from living organisms such as plants, fungi, bread molds, microorganisms, marine organisms, and terrestrial vertebrates and invertebrates [2]. In 2008, of the 225 drugs being developed, 164 were of natural origin, with 108 being derived from plants, 25 from bacterial sources, 7 from fungal, and 24 from animal sources, and, to throw some more numbers around, of the 108 plant-based drugs, 46 were in preclinical development, 14 in phase I, 41 in phase II, 5 in phase III, and two had already reached preregistration stage [3]. An analysis of medical indications by resource of compounds has established that natural products and related drugs, including anticancer, antibacterial, antiparasitic, anticoagulant, and immunosuppressant agents, are being used to treat 87% of all categorized human diseases [4]. Plants provide an extensive reservoir of natural products, demonstrating important structural diversity, and offer a wide variety of novel and exciting chemical entities in modern medicine [2, 4–9]. Historical experiences with plants as therapeutic tools have led to discoveries of many important, effective, and novel drugs including older drugs such as quinine and morphine and newer drugs such as paclitaxel (taxol), camptothecin, topotecan, and artemisinin [10].

The significance of natural products in health care is supported by a report that 80% of the global population still relies on plant derived medicines to address their health care needs [11]. It is also reported that 50% of all drugs in clinical use are natural products, or their derivatives, or their

analogs [12], and 74% of the most important drugs consist of plant-derived active ingredients [13]. Until the 1970s, drug discovery was based on screening of a large number of natural and synthetic compounds, with the advent of computer and other molecular biology techniques, resulting in the modern and rational drug discovery [14]. Plant-based drugs have provided the basis of traditional medicine systems that have been employed in various countries such as Egypt, India, and China since prehistoric times [12]. The medicinal properties of plants have been documented already on Assyrian clay tablets dated about 2000 B.C. and reported in the Egyptian culture, the Indian Ayurveda [15], and traditional Chinese medicines (TCMs) [16].

All this said is implying that natural products including plants are important and valuable resources for drug development of natural origin [17]. Furthermore, a large number of natural compounds have been reported, which have been isolated from plants possessing wide variety of biological functions such as total glucosides of astragalus showing anti-inflammatory activity, tripterygium wilfordii multiglycoside, sinomenine [18], and camptothecin, taxol, vinblastine, vincristine, podophyllotoxin, and colchicine that demonstrate antineoplastic activity [19]. Indeed, molecules derived from natural sources including plants, marine organisms, and microorganisms have played and continue to play a dominant role in the discovery of leads for the development of conventional drugs for the treatment of the majority of human diseases. Chemoprevention was defined as the administration of agents to prevent induction, to inhibit, or to delay the progression of disease [20]. Mainly several scientific studies have been carried out on *Euphorbia hirta* Linn., widely spread in south China, which is extensively used in folk Chinese medicines for several ailments such as dysentery, eczema, hematuria, hypersensitivity, and gastroenteritis [21]. In addition, many studies have also reported that natural products have antimicrobial [22, 23], anticancer [24, 25], antioxidant [26, 27], anti-inflammatory [28, 29], and antifungal properties [30, 31]. The yield extract of leaves of *Sparattosperma leucanthum* (Vell.) K. Schum, that is, a native tree of Brazil, is popularly known as "caroba branca" or "ipê branco." Previous phytochemical studies on the genus *Sparattosperma* described the isolation of the flavanone pinocembrin-7-O-(-d-neohesperidoside). Pinocembrin, one of the most important phytochemicals among flavonoids, acts as anti-inflammatory, antimicrobial, and antioxidant agent [24, 26, 32]. The extensive research indicated that pinocembrin has potential biological activities, which have made further interest among the chemists and biologists.

This review summarizes the recent researches on pinocembrin focusing on its biological and pharmacological activities. The literature was screened through various e-sites including PubMed, Scopus, and Elsevier Science Direct. Access to the Elsevier Science Direct Journals was made possible through the library of Northeast Normal University, Changchun, China. The searched literature mainly focused on recent advances, and additional manual searches were carried out on relevant medical journals and the google search Engine. Key words used for search were "pinocembrin," "pinocembrin and biological activity," "anticancer activity," "inflammatory activity," "cytotoxicity," and "medicinal plants." The data collected from primary sources and/or from data that superseded earlier work were included.

2. Natural Sources of Pinocembrin

Pinocembrin (Figure 1) has been identified in several plants such as the numerous genera of the *Piperaceae* family, which comprises fourteen genera and 1950 species that are reported as the rich source of pinocembrin. Of which, two genera, *Peperomia* and *Piper*, have been proved to be the most widespread and most diverse with 600 and 700 species, respectively [30, 33]. In addition to this family, pinocembrin has been also isolated from plants of Lauraceae and Asteraceae families, which comprise a large number of species. Of which, about 250 species of genus *Cryptocarya* are mainly distributed in tropical and subtropical regions, and 600 species of *Helichrysum* are located in Africa, of which some 244 species are found in South Africa [32]. Pinocembrin was also isolated from aerial parts of *Flourensia oolepis* S.F. Blake (Asteraceae) [34] and honey [35]. Further, pinocembrin, being a flavonoid natural compound, is located in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, tea, and red wine [36, 37]. It has also shown a variety of pharmacological properties of interest in the therapy of several diseases including inflammation by inhibiting bacterial colonization, cancer, or vascular ailments [38, 39]. The summary of plants containing pinocembrin, parts used, and biological/pharmacological activities is shown in Table 1. As shown in Figure 1, accumulated data indicate that pinocembrin was isolated from many plant species such as *Alpinia mutica* [40, 41], *Litchi chinensis* [42], *Lippia graveolens* [43], *Lippia origanoides* [44, 45], *Dalea elegans* [46], *Oxytropis falcate* [47, 48], *Glycyrrhiza glabra L.* [49, 50], *Sparattosperma leucanthum* [51], *Cleome droserifolia* [52], *Lychnophora markgravii* [53], *Helichrysum gymnocomum* [54], *Syzygium samarangense* [55], *Centaurea eryngioides* [56], *Cistus incanus* [27], *Turnera diffusa* [57], and *Eriodictyon californicum* [58].

Apart from natural sources, it has been noted that pinocembrin can be biosynthesized. The strategy to produce pinocembrin, a flavanone, by microorganisms was to design and express an artificial phenylpropanoid pathway. This was accomplished by assembling of phenylalanine ammonia-lyase (PAL) from the yeast *Rhodotorula rubra*; 4-coumarate: CoA ligase (4CL) from the actinomycete *S. coelicolor*; chalcone synthase (CHS) from the licorice plant *Glycyrrhiza echinata*; and chalcone isomerase (CHI) from the plant *Pueraria lobata* on a single pET plasmid in *E. coli* [37–39, 59–61].

3. Biological Activity of Pinocembrin and Mechanisms of Action

The biological activity of natural compounds is generally investigated with emphasis on the mechanisms of actions. Several studies have been conducted *in vitro* and *in vivo* to determine the biological properties ascribed to pinocembrin and to elucidate its mechanisms of actions. In this case, some researchers pointed out the effect of functional groups on the

Table 1: Plants containing pinocembrin with their mode of actions.

Plants name		Part used/extract	Functions	References
Botanical name	Common name			
Alpinia mutica	Orchid ginger	Air-dried Rhizome	Antiplatelet, antioxidant	[40, 41]
Alpinia katsumadai	Katsumadai	Seeds	Antibacterial, antiinflammatory	[96–99]
Alpinia pricei	Prospero Alpini	Roots	Antiinflammatory	[100, 101]
Alpinia galangal	Siamese ginger	Roots	Anticancer	[24]
Alpinia rafflesiana	Raffles' alpinia	Ripe fruits	DPPH free radical scavenger	[102]
Boesenbergia pandurata	Ginger	Fingerroot Rhizome	Antiinflammatory, antioxidant	[25, 85, 103, 104]
Centaurea eryngioides	Centory	—	Antitumor	[56]
Cleome droserifolia	Black thorn/egy	Aerial parts	Antirheumatic Antifever and antiinflammation	[52]
Combretum collinum	Combretum	Pulverized leaves	Antimicrobial, antimalarial	[105]
Cryptocarya chinensis	—	Air-dried Leaves	Antituberculosis	[106]
Cryptocarya konishii	Brown Laurel	Woods	Antibacterial, anticancer	[107]
Cystus incanus	—	—	Antioxidant/antiestrogenic	[27]
Dalea elegans	Prairie clover/indigo bush	Roots	Antibacterial	[46]
Dysphania graveolens	Fetid goosefoot	—	Antimicrobial, larvicidal, hepatoprotective, antihyperlipidaemic	[108]
Eriodictyon californicum	Yerba santa	Leaves	Chemopreventive agents	[58]
Euphorbia hirta Linn	Asthma herb	Aerial part	Antitumour, antifilarial	[109]
Glycyrrhiza glabra L.	Liquorice	Aerial parts	Cognitive functions, cholinesterase activity	[49, 50]
Helichrysum gymnocomum	—	Flowers	Antimicrobial	[54]
Lippia graveolens	Oregano		Antigiardial	[43]
Lippia origanoides	Wild marjoram	Flowers, leaves, stems	Antimicrobial	[44, 45]
Litchi chinensis	Lychee	Seeds		[42]
Lychnophora markgravii	—	Aerial parts	Antileishmania	[53]
Oxytropis falcate	—	Whole plants	Antipain, antiarthritis	[47, 48]
Piper chimonantifolium	—	Leaves	Antifungal	[62, 110]
Piper lanceaefolium	—	Leaves	Antibacterial	[30, 62]
Piper sarmentosum	—	Aerial parts	Antifeedant, anticarcinogenic	[111]
Sparattosperma leucanthum	—	Leaves	—	[51]
Syzygium samarangense	Champoo	Pulp, seeds of the fruits	Antioxidants	[55]
Turnera diffusa	Damiana	Leaves	Antiaromatase	[57]

biological activity of certain molecules to evaluate the effect of hydroxyl group on biological activity of pinocembrin and its analogues.

3.1. Antibacterial Activity. For centuries, natural products, including pinocembrin, have been used to treat microbial infections. Drewes and van Vuuren [54] investigated the antibacterial effect of pinocembrin with three kinds of Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *and K. pneumoniae*) and three kinds of Gram-positive bacteria (*B. subtilis*, *S. aureus*, and *S. lentus*) by measuring the minimal inhibitory concentrations in microgram of DMSO extract (mg of extract/mL) determined by an adjustment of the agar streak dilution method based on radial diffusion. Another investigation was conducted to evaluate the effect of pinocembrin by the metabolic engineering technique for the production in bacteria under cultural conditions which were *E. coli* at a cell density of 50 g/L, incubated in the presence of 3 μM tyrosine or phenylalanine; the yields of pinocembrin reached about 60 mg/L. Phenylalanine ammonia lyase (PAL) from the yeast *Rhodotorula rubra*, 4-coumarate: CoA ligase (4CL) from an actinomycete *Streptomyces coelicolor*, and chalcone synthase (CHS) from a licorice plant *Glycyrrhiza echinata*, taken individually are each an active ingredient for fermentation production of flavanones; such as pinocembrin in *Escherichia coli* via different pathway including phenylpropanoid pathway. In the construction of set, they are placed in order under the control of pT7 promoter and the ribosome binding sequence (RBS) in the pET vector. These pathways bypassed cinnamate-4-hydroxylase (C4H), a cytochrome P-450 hydroxylase, because the bacterial 4CL enzyme legated coenzyme A to both cinnamic acid and

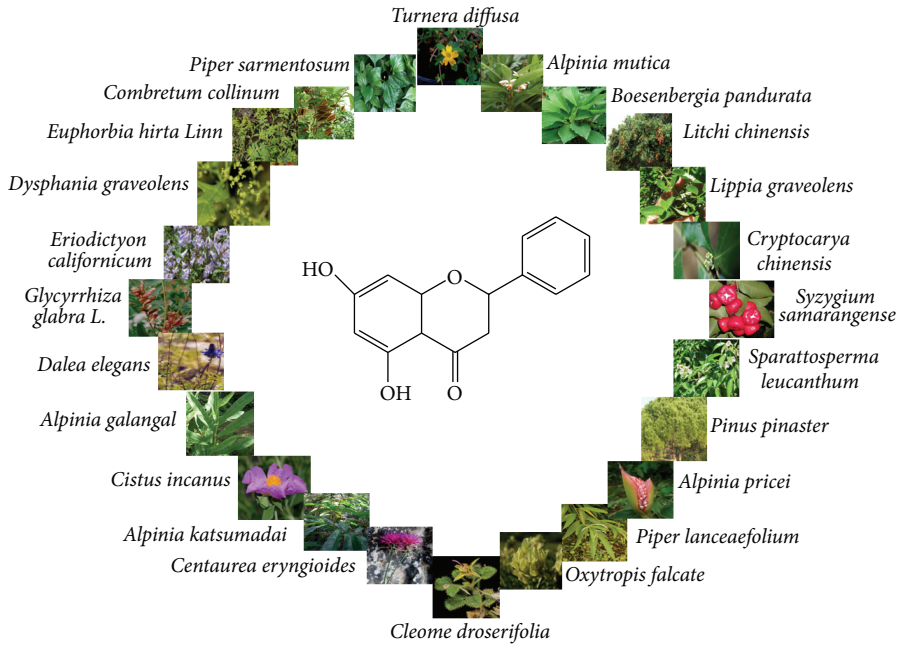

Figure 1: Chemical structure and natural sources of pinocembrin.

4-coumaric acid. *E. coli* cells containing the gene clusters produced two flavanones. The fermentative production of flavanones in *E. coli* is the sine qua non provided in the construction of a library of unnatural flavonoids in bacteria [37, 60, 61].

The mechanisms of actions of pinocembrin were studied to evaluate its effect on the bacterial membranes of *Neisseria gonorrhoeae, E. coli, P. aeruginosa, B. subtilis, S. aureus, S. lentus,* and *K. pneumoniae* by observing changes in membrane composition and monitoring the metabolic engineering technique, which revealed that pinocembrin induced cell lysis through a metabolic engineering technique [37, 60–62].

3.2. Anti-Inflammatory Activity. Although the type of inflammatory responses may differ among diseases, inflammation and disease conditions are linked through the production of inflammatory mediators by macrophages and neutrophils. Overexpression activity of the enzyme cyclooxygenases-(COX-) 1 and COX-2 produces inflammatory mediators such as prostaglandin E 2 (PGE 2). Anti-inflammatory drugs together with nonsteroidal anti-inflammatory drugs (NSAIDs) suppress the inflammatory response by inhibiting infiltration and activation of inflammatory cells as well as their synthesis or, secondly, release of mediators or effects of inflammatory mediators themselves [63].

The anti-inflammatory activity of pinocembrin against sheep red blood cell-induced mouse paw oedema as a model of delayed-type hypersensitivity reaction *in vitro* and in the mouse model of LPS-induced acute lung injury inhibited significantly enzymatic and nonenzymatic lipid peroxidation (IC_{50} = 12.6 and 28 μM, resp.) [28]. Pulmonary edema, histological severities, and neutrophil, lymphocyte, and macrophage infiltration increased by LPS administration; this would mean that pinocembrin exhibited anti-inflammatory activity in the sheep red blood cell-induced delayed-type hypersensitivity reaction. Although it downregulated TNF-α, IL-1β, and IL-6 and significantly suppressed IκBα, JNK, and p38MAPK with (20 or 50 mg/kg, i.p.) in LPS-induced lung injury, having regard to the foregoing, pinocembrin is a natural compound recommended for the modulation of inflammatory responses [28, 29, 64].

3.3. Antimicrobial Activity. Flavonoid compounds in general and in particular pinocembrin are well-known plant compounds that have antimicrobial and anti-inflammatory properties [65]. Scientists and clinicians have demonstrated *in vitro* and *in vivo* the biological or pharmacological properties of pinocembrin and have elucidated mechanisms of action [23]. In this momentum, production of glucosyltransferase from microorganisms according to the results obtained on *Staphylococcus aureus; Escherichia coli, Candida albicans, Bacillus subtilis, Candida albicans, Trichophyton mentagrophytes, Streptococcus mutans, Neisseria gonorrhoeae*, treatment with pinocembrin at daily doses of 100 mg/kg body weight the animals as well as the controls died between the 6th and 24th day after beginning. The possible mechanisms of the antimicrobial action of pinocembrin demonstrated the highest inhibition of the enzyme activity, and growth of the bacteria indicates that pinocembrin inhibited 100% of the *Neisseria gonorrhoeae* panel at 64 g/mL and 128 g/mL, respectively, whereas cyclolanceaefolic acid methyl ester inhibited 44% of the strains at 128 g/mL [22, 66–68].

3.4. Anticancer Activity. Due to the toxic effects of synthetic drugs, accumulated data indicate that prevailing treatment

Table 2: Molecular targets of pinocembrin in different cancer types.

Cancer types	Cell lines	IC_{50}/concentration	Major targets	References
Colon	HCT-116, HT-29	26.33 to 143.09 $\mu g\,mL^{-1}$ 1.6 to 13.6 μM	Superoxide anion radical↓, Bax↑, NO_2↓, ΔΨm↓	[24, 111, 112]
Leukaemia	HL-60	IC_{50} < 100 ng/mL	Fas↑, FasL↑, caspase-3/8/9↑, tBid↑	[100, 113]

↓: downregulation; ↑: upregulation.

options have limited therapeutic success in human cancers; therefore, there is considerable emphasis on identifying novel natural products that selectively induce apoptosis and growth arrest in cancer cells without cytotoxic effects in normal cells [69]. Apoptosis is defined as an extremely synchronized mode of cell death and is characterized by distinct morphological features, including cell membrane blabbing, chromatin condensation, and nuclear fragmentation [70, 71]. The normal cell regulation and during disease conditions the importance of signaling has been recognized, [72, 73] and many well-known targets at the signaling levels have been identified that are critical rapid proliferate of cancer cells. It is believed that in normal cells, certain cellular signals control and regulate their growth and all growth mechanisms, and when these signals are altered due to various mutations that prevent cells to undergo apoptosis, normal cells are transformed into cancerous cells and undergo hyperproliferation. Therefore, to arrest cancerous cell proliferation, regulation of apoptosis plays a critical role [74–76]. Accumulated data suggest that various anticancer chemopreventive agents can induce apoptosis which causes death in cancerous cells [77–84]. Although several studies revealed that pinocembrin can inhibit, delay, block, or reverse the initiation; promotional events associated with carcinogenesis are needed for the prevention and/or treatment of cancer. Here, we reviewed studies related to anticancer activity of pinocembrin to allow scientists and researchers to have a clearer view of this natural compound.

Based on the research anterior made, pinocembrin has shown cytotoxicity against certain cancer cell lines such as colon cancer cell line (HCT116), with relatively less toxicity toward human umbilical cord endothelial cells [24]. In colon cancer cell line (HCT116), pinocembrin increased increased the activity of heme oxygenase, caspase-3 and -9, and mitochondrial membrane potential (MMP) but did not affect the activities of cytochrome P450 reductase, quinone reductase, UDP glucuronosyltransferase, and glutathione-S-transferase [24, 25]. Although some *in vivo* and *in vitro* studies reveal that pinocembrin can promote the differentiation of EPCs and improve the biological functions in rat liver micronucleus and medium-term carcinogenicity; interestingly, pinocembrin slightly increased the number of GST-P positive foci, PI3 K-eNOS-NO signaling pathway when given prior to diethylnitrosamine injection, and adhesion of EPCs. The effect of pinocembrin may help to protect against chemical-induced hepatocarcinogenesis and suggest that the promoting effect of this compound might be due to lipid peroxidation [85]. The details of all the information regarding the molecular targets of pinocembrin in different cancer types are recorded in Table 2.

3.5. Antifungal Activity. Microbial infections especially fungal are a common public health problem ranging from superficial to deep infections. The superficial mycoses sometimes reach high endemic levels, especially in tropical areas. The treatment of fungal infections or mycoses is becoming more and more problematic due to the development of antimicrobial resistance to some kind of drugs. It is for that reason the natural products have been used to treat these infections and to demonstrate the ability to inhibit the growth of various pathogens agents. The antimicrobial activity against *P. italicum* and *Candida albicans*, with a minimal inhibitory concentration value of 100 microg/mL, shows that pinocembrin exhibited antifungal activity and inhibited the mycelial growth of *P. italicum* by interfering energy homeostasis and cell membrane damage of the pathogen [30, 31].

3.6. Neuroprotective Activity. The diverse array of bioactive nutrients present in the natural products plays a pivotal role in prevention and cure of various neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and other neuronal dysfunctions [86]. Cerebral ischemic injury is a debilitating disease that can occur with great morbidity, during asphyxiation, shock, brain injury, extracorporeal circulation, and cardiac arrest [87, 88]. The neuroprotective effects of naturally occurring compound, pinocembrin, are being evaluated in this review. Previous studies demonstrated that pinocembrin can be used as neuroprotective against cerebral ischemic injury with a wide therapeutic time window, which may be attributed to its antiexcitotoxic effects [89] and decreased glutamate-induced SH-SY5Y cell injury and primary cultured cortical neuron damage in oxygen-glucose deprivation/reoxygenation (OGD/R). Pinocembrin alleviates cerebral ischemic injury in the middle cerebral artery occlusion rats [90–92] and also enhanced cognition by protecting cerebral mitochondria structure and function against chronic cerebral hypoperfusion in rats [93]. In another attempt to understand the mechanism of action of pinocembrin, it increased ADP/O, glutathione, state 3 respiration state, neuronal survival rates, and oxidative phosphorylation rate in NADH/FADH2 and decreased LDH release, reactive oxygen species, nitric oxide, neuronal nitric oxide synthase (nNOS), inducible NOS (iNOS), and 4 respiration state (V4) in NADH. Moreover, pinocembrin enhanced ATP content in brain mitochondria in SH-SY5Y cells; DNA laddering and caspase-3 are downregulated and increased PARP degradation [89, 94] and resulted in the alleviation of brain injury in the global cerebral ischemia/reperfusion (GCI/R) rats [94]. Furthermore, pinocembrin decreased neurological score and reduced brain edema induced by

GCI/R. Pinocembrin also lessened the concentrations of Evan's blue (EB) and fluorescein sodium (NaF) in brain tissue of the GCI/R rats and alleviated the ultrastructural changes of cerebral microvessels, astrocyte end-feet, and neurons and improved cerebral blood flow (CBF) in the GCI/R rats. In addition, pinocembrin increased the viability and mitochondrial membrane potential of cultured rat cerebral microvascular endothelial cells (RCMECs) induced by oxygen-glucose deprivation/reoxygenation (OGD/R) [95]. Therefore, pinocembrin may be a novel therapeutic strategy to reduce cerebral ischemia [89, 94].

4. Conclusions and Future Perspectives

This review suggests that pinocembrin is a good pharmacological drug with potential antioxidative, anti-inflammatory, antitumor, and antimicrobial properties. Several research results demonstrated the potential applications of pinocembrin both *in vitro* and *in vivo*. Pinocembrin is a natural product with a small molecular weight and is a biologically active constituent of honey, an edible nutrient, which ensures safety of pinocembrin during long-term administration, combined with its cost and future therapeutic potential, making it an ideal therapeutic agent. Pinocembrin analogues with improved pharmacokinetic and pharmacodynamics may also encourage further advances. Many studies have shown that pinocembrin induces apoptosis of many types of cancer cells, but mechanisms of actions have not been fully elucidated. This review suggests that pinocembrin may establish direct medicinal application as a pharmaceutical agent or may serve as chemical templates for the design, synthesis, and semisynthesis of new substances for the treatment of human diseases. Additional studies and clinical trials are required to determine its specific intracellular sites of action and derivative targets in order to fully understand the mechanisms of its anti-inflammatory, anticancer, and apoptotic effects to further validate this compound in medical applications and to make clear the potential role of pinocembrin as a medicinal agent in the prevention and treatment of various diseases.

Acknowledgments

This work was supported by Ministry of Science and Technology (no. 2010DFA31430), Ministry of Education of China (NCET-10-0316; 10SSXT147), Jilin Provincial Science & Technology Department (20130521010JH, YYZX201241, 20070719, and 200905116), Changchun Science & Technology Department (no. 2011114-11GH29) and National Natural Science Foundation of China (no. 30871301).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 786051, 8 pages
http://dx.doi.org/10.1155/2013/786051

Research Article

Bees' Honey Protects the Liver of Male Rats against Melamine Toxicity

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Received 17 April 2013; Revised 19 June 2013; Accepted 22 June 2013

Academic Editor: Isabel C. F. R. Ferreira

The protective effect of natural bees' honey to the liver of male albino rats against melamine toxicity was studied. Melamine supplementation at a dose of 20000 ppm in the diet for 28 days induced adverse effects on the liver, decreased serum total protein and increased liver enzyme: alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. Histological changes of the melamine supplemented group showed necrosis in the hepatic tissues around the central veins of the liver and precipitation of melamine crystals. Treating the male albino rats (that were presupplemented regularly with 20000 ppm melamine) with natural bees' honey at a dose of 2.5 g/kg body weight for 28 days improved both liver functions and increased serum protein. In addition, a positive impact on the shape of the cells after treatment with honey compared to the positive melamine supplemented group was observed. In conclusion, the results of this study revealed that the use of natural bees' honey has the ability to protect the liver of rats against the toxic effects of melamine.

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine, 1,3,5-triazine-2,4,6-triamine, 2,4,6-triamino-s-triazine, melamine amide or cyanuric triamide) is a nitrogen heterocyclic triazine compound, referred to as triamines or protein essence [1]. Melamine contains 66% nitrogen by mass, so it is sometimes illegally added to food products in order to increase the apparent protein content that has recently become a serious concern [1, 2]. Standard tests such as the Kjeldahl and Dumas tests estimate protein levels by measuring the nitrogen content, so they can be misled by adding nitrogen-rich compounds such as melamine [3]. The illegal use of melamine as a food ingredient has led to many poisoning incidents of cats and dogs in the United States, as well as renal function failure of Chinese infants [4, 5].

The toxicity of melamine alone is very low, and greater than 90% of the ingested melamine is eliminated within 24 h in an animal experiment [6]. In spite of this lower toxicity of melamine, most animal studies showed effects on health following subacute or chronic melamine exposure [2, 6, 7]. Data of serum chemistry after one month of melamine-contaminated artificial food indicated severe renal and mild hepatic failure [8, 9]. Hau et al. [10] reported that more than 90% of ingested melamine is excreted within 24 h, the half-life of melamine excretion in animal studies ranged from 2.7 to 4.04 h, and the levels of melamine in blood, liver, or plasma are similar.

The Council of Agriculture in Taiwan ordered an emergency recall of the suspected pet food, and a surveillance committee was constituted to investigate [9]. Melamine contamination of baby milk-based products was detected in China [11]. In general, approximately 300,000 people were affected, more than 52,000 were hospitalized, and six died due to global marketing of melamine-contaminated products in almost 70 countries, including the United States [10, 12, 13].

Gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) combined with ultraviolet (UV) or mass spectrometry (MS) detector are the most common methods used to detect melamine in foods and feedstuff [1, 14–16].

Honey is a sweet food made by bees using nectar from flowers. It is composed of a complex mixture of carbohydrates, proteins, enzymes (invertase, glucose oxidase, catalase, and phosphatases), amino and organic acids (gluconic acid, acetic acid, etc.), lipids, vitamins (ascorbic acid, niacin, pyridoxine, etc.), volatile chemicals, phenolic acids, flavonoids, and carotenoid-like substances and minerals which may function as antioxidants [17]. The composition of honey depends on the plant species visited by the honeybees and the environmental, processing, and storage conditions [18, 19]. The maillard reactions between amino groups of amino acids/proteins and reducing sugars lead to formation of a-dicarbonyl compounds such as glyoxal and methylglyoxal [20]. Honey is a natural antioxidant which may contain flavonoids, ascorbic acid, tocopherols, catalase, and phenolic compounds all of which work together to provide a synergistic antioxidant effect, scavenging and eliminating free radicals [21]. Its protective role against the kidney dysfunctions induced by sodium nitrite, a known food additives, hepatoprotective, hypoglycemic, reproductive, antihypertensive, and of course antioxidant effects has also been reported [22, 23].

The current study aimed to test the protecting effect of the natural bees' honey to the liver of male albino rat against melamine toxicity.

2. Materials and Methods

All animal experiments were carried out under protocols approved by the Institutional Animal House of the University of King Abdulaziz, Jeddah, Saudi Arabia.

2.1. Animals. Male albino rats (*Rattus rattus*) weighing about 180–200 g were obtained from King Fahd Center for Medical Research.

2.2. Diets. Conventional animal basal diet was obtained from a Grain Silos & Flour Mills in Jeddah. This diet contains the following: crude protein (20.00%), crude fat (4.00%), crude fiber (3.50%), ash (6.00%), salt (0.50%), calcium (1.00%), phosphorus (0.60%), vitamin A (20,0 IU/g), vitamin D (2.20 IU/g), vitamin E (70.00 IU/g), and energy ME K Cal/kg (2850.00). Trace elements (cobalt, copper, iodine, iron, manganeses, selenium, and zinc) were added. Basal diet food was stored in a dry place out of direct sunlight.

Melamine (99%) was purchased from Sigma-Aldrich cat. no. M2659-5G.

Sidr bees' honey (from flowers of *Ziziphus spina-christi* trees, from Hadramout, Yemen) was purchased from a specialized shop in Jeddah, Saudi Arabia.

2.3. Experimental Design. Rats were held for approximately two weeks before the study began for acclimatization and then housed five per polycarbonate cages. Cages, bedding, and glass water bottles (equipped with stainless steel sipper tubes) were replaced twice per week. Test diets, control diets, and tap water are available *ad libitum*. Stainless steel feed containers were changed once per week.

Forty rats were distributed into four groups each consists of 10 rats as follows.

(1) Rats of the first group (G1) were fed diets containing normal basic diet served as untreated control group.

(2) Rats of the second group (G2) were fed diets containing 20,000 ppm melamine to induce toxicity [24].

(3) Rats of the third group (G3) received daily 2.5 g/Kg body weight of a 25% natural bees' honey in an aqueous solution by gastric tube [25] for 28 days.

(4) Rats of the fourth group (G4) received the same dose of melamine as in the second group together with natural honey dose as in the third group for 28 days.

2.4. Biochemical Blood Tests. At the end of each specified period (28 days), rats were anesthetized using diethyl ether, and then, blood samples were collected from the orbital sinus [26]. Blood was collected in EDETA tube for CBC analysis and in plain tubes for chemistry analyses. Serum was obtained by centrifuging the blood samples at 1000 rpm for 10 min at room temperature and then stored at −20°C until analysis was performed.

Complete blood count (CBC) tests; hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelet count (PLT), red blood cell (RBC), and white blood cell (WBC) were measured using human reagent, Cell Dyne 17000, blood analysis system, Diagnostics Abbott, China, according to the instruction of the supplier.

Serum protein and albumin were measured using human reagent, Cobas—C111, fully automated clinical analyzers, Diagnostics Roche, India, according to the instruction of the supplier.

Serum liver enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), were measured using human reagent, Cobas—C111, fully automated clinical analyzers, Diagnostics Roche, India, according to the instruction of the supplier.

2.5. Weight Gain, Body Weight Gain Ratio (BWG%), and Food Efficiency Ratio (FER). Food intake and body weight per cage were recorded once per week. The mean body weight of each group was calculated by dividing the total weight of all surviving animals by the number of surviving animals in the group. Weight gain (g), body weight gain ratio (BWG%), and food efficiency ratio (FER) were calculated as follows:

(i) weight gain = final weight (g) − initial weight (g),

(ii) BWG% = final weight − initial weight/initial weight × 100, and

(iii) FER = weight (g)/food intake (g).

2.6. Histopathology. Animals were then scarified by cervical dislocation. The abdomen was opened, and the liver was rapidly dissected out, washed in sterile saline, and kept in 10% formalin. For microscopic preparations, the liver

Table 1: Effect of honey treatment on CBC in rats supplemented with melamine for 28 days.

Test	Statistics	G1	G2	G3	G4
Hb (g/dL)	Mean ± SE	17.40 ± 0.39	15.54 ± 0.27	17.77 ± 0.29	15.67 ± 0.14
	t-test		3.04**	4.80***	−0.70 N.S
HCT%	Mean ± SE	52.70 ± 0.23	46.26 ± 1.54	53.00 ± 0.47	46.62 ± 0.66
	t-test		3.87**	5.29***	−0.20 N.S
RBC mil/cmm	Mean ± SE	9.66 ± 0.08	8.79 ± 0.31	10.17 ± 0.13	9.31 ± 0.06
	t-test		2.44*	4.73***	−1.85 N.S
MCV (fL)	Mean ± SE	54.40 ± 0.71	50.30 ± 1.39	54.50 ± 0.91	50.46 ± 0.12
	t-test		2.43*	2.05 N.S	−0.11 N.S
MCH (pg)	Mean ± SE	17.36 ± 0.22	16.16 ± 0.31	18.22 ± 0.33	16.47 ± 0.17
	t-test		2.87**	4.31**	−1.50 N.S
MCHC (g/dL)	Mean ± SE	31.88 ± 0.15	31.36 ± 0.22	32.87 ± 0.37	32.58 ± 0.04
	t-test		1.98 N.S	3.06**	−6.31***
WBC/cmm	Mean ± SE	7.800 ± 0.68	13.80 ± 0.81	8.47 ± 0.60	10.72 ± 1.00
	t-test		−11.01***	−4.12**	3.15**
PLT/cmm	Mean ± SE	466.43 ± 208.02	575.34 ± 157.51	482.00 ± 215.18	308.80 ± 168.47
	t-test		−0.52 N.S	0.31 N.S	1.25 N.S

*Significant at 5% ($P < 0.05$), **highly significant at 1% ($P < 0.01$), ***very highly significant at 0.1% ($P < 0.001$), and N.S: nonsignificant.

was dehydrated in gradual ethanol (50–99%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin dye for microscopic investigation [27]. The stained sections were examined and photographed using a light microscope.

2.7. Statistical Analysis. Data were analyzed using the Statistical Program for Sociology Scientists (SPSS) Statistics Version 17.0 for computing the mean values, the standard errors (SE), and the test of significance (t-test). Histograms were plotted using Excel program.

3. Results

3.1. CBC. The effect of honey treatment on the CBC in rats supplemented with melamine for 28 days is illustrated in Table 1. The mean values of hemoglobin in the positive control (G2, rats supplemented with 20000 ppm melamine) were significantly lower ($P < 0.01$) than those of the negative control. In G3 (rats supplemented with honey), the mean values of hemoglobin were significantly higher ($P < 0.001$) than those of the negative control, while the mean values of hemoglobin in G4 (rats supplemented with melamine and treated with honey) were nonsignificantly lower than those of the negative control and higher than those of the positive control.

The mean values of hematocrit in the positive control were significantly lower ($P < 0.01$) than those of the negative control. In G3, the mean values of HCT were significantly higher ($P < 0.001$) than those of the negative control, whereas the mean values of HCT in G4 were nonsignificantly lower than those of the negative control and higher than those of the positive control.

The mean values of RBC in the positive control were significantly lower ($P < 0.05$) than those of the negative control. In G3, the mean values of RBC were significantly higher ($P < 0.001$) than those of the negative control, while the mean values of RBC in G4 were nonsignificantly lower than those of the negative control and higher than those of the positive control.

The mean values of MCV in the positive control were significantly lower ($P < 0.05$) than those of the negative control. In G3, the mean values of MCV were non significantly higher than those of the negative control. While the mean values of MCV in G4 were non significantly lower than those of the negative control and higher than those of the positive control.

The mean values of MCH in the positive control were significantly lower ($P < 0.01$) than those of the negative control. In G3, the mean values of MCH were significantly higher ($P < 0.01$) than those of the negative control, while the mean values of MCH in G4 were non significantly lower than those of the negative control and higher than those of the positive control.

The mean values of MCHC in the positive control were nonsignificantly lower than those of the negative control. In G3, the mean values of MCHC were significantly higher ($P < 0.01$) than those of the negative control. While the mean values of MCHC in G4 were significantly higher ($P < 0.001$) than those of the negative and positive controls.

The mean values of WBC in the positive control were significantly higher ($P < 0.001$) than those of the negative control. In G3, the mean values of WBC were significantly higher ($P < 0.01$) than those of the negative control, while the mean values of WBC in G4 were significantly higher ($P < 0.01$) than those of the negative control and lower than those of the positive control.

The mean values of platelet count in G2 and G3 were nonsignificantly higher than those of the negative control, while the mean values of PLT in G4 were non significantly lower than those of the negative control and positive control.

Table 2: Effect of honey treatment on serum protein in rats supplemented with melamine for 28 days.

Test	Statistics	G1	G2	G3	G4
Total protein g/dL	Mean ± SE	6.04 ± 0.38	5.79 ± 0.17	6.34 ± 0.10	6.28 ± 0.16
	t-test		0.19 N.S	1.56 N.S	1.00 N.S
Albumin g/dL	Mean ± SE	4.25 ± 0.18	4.10 ± 0.130	4.14 ± 0.11	4.20 ± 0.06
	t-test		1.53 N.S	0.24 N.S	0.72 N.S

N.S: nonsignificant.

3.2. Serum Proteins. Table 2 shows the effect of honey treatment for 28 days on serum proteins in rats supplemented with melamine. The mean values of total protein in the positive control were nonsignificantly lower than those of the negative control, whereas in G3, the mean values of total protein were nonsignificantly higher than those of the negative control. The mean values of total protein in G4 were nonsignificantly higher than those of the negative control and the positive control.

The mean values of albumin in G2 and G3 were nonsignificantly lower than those of the negative control, while the mean values of albumin in G4 were lower than those of the negative control and higher than those of the positive control.

3.3. AST, ALT, and ALP. Figure 1 shows the effect of honey treatment for 28 days on liver enzymes in rats presupplemented with melamine. The mean values of AST in the positive control were nonsignificantly higher than those of the negative control (118.20 ± 9.78 and 107.80 ± 2.65 U/L, resp.). In G3, the mean values of AST were nonsignificantly lower than that of the negative control (103.66 ± 4.31 and 107.80 ± 2.65 U/L, resp.), while the mean values of AST in G4 were nonsignificantly higher than those of the negative control and lower than those of the positive control (115.46 ± 2.78, 107.80 ± 2.65, and 118.20 ± 9.78 U/L, resp.).

The mean values of ALT in the positive control were significantly higher ($P < 0.01$) than those of the negative control (43.40 ± 3.14 and 29.75 ± 1.98 U/L, resp.). In G3, the mean values of ALT were significantly lower ($P < 0.01$) than those of the negative control (28.32 ± 2.15 and 29.75 ± 1.98 U/L, resp.), while the mean values of ALT in G4 were significantly higher ($P < 0.01$) than those of the negative control and lower than those of the positive control (35.00 ± 0.83, 29.75 ± 1.98, and 43.40 ± 3.14 U/L, resp.).

The mean values of ALP in the positive control were significantly higher ($P < 0.05$) than those of the negative control (149.75 ± 10.01 and 137.60 ± 9.42 U/L, resp.). In G3, the mean values of ALP were significantly lower ($P < 0.05$) than those of the negative control (128.75 ± 4.61 and 137.60 ± 9.42 U/L, resp.), while the mean values of ALP in G4 were nonsignificantly higher than those of the negative control and lower than that of the positive control (143.50 ± 10.51, 137.60 ± 9.42, and 149.75 ± 10.01 U/L, resp.).

3.4. Food Intake, Weight Gain, BWG%, and FER. The effect of melamine toxicity and honey treatment for 28 days on food intake, weight gain, BWG%, and FER in rats under study is shown in Table 3. The mean values of food intake (FI) were increased in the positive control (G2) compared to those of the negative control group. Meanwhile, G4 showed a decreased in food intake compared to the positive control group (G2).

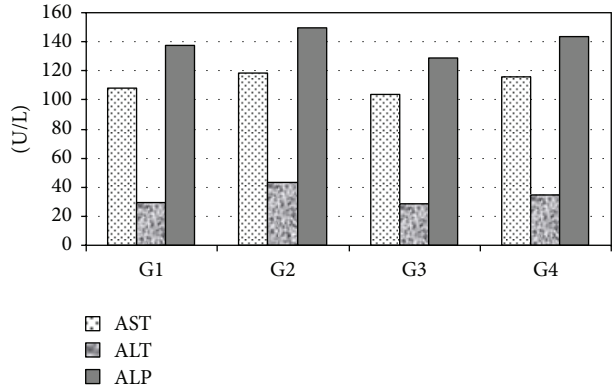

Figure 1: Effect of honey treatment on serum liver enzyme in rats supplemented with melamine for 28 days.

As shown in Table 3, weight gain, body weight gain ratio, and food efficiency ratio were decreased in positive control compared to the negative control group, while G4 showed a decrease in weight gain and increase in body weight gain ratio and food efficiency ratio compared with the positive control group.

Figure 2(a) shows the normal hepatic tissues of the control group with hepatic strands of cells around the central vein leaving blood sinusoids, whereas the liver tissue of (G2) which was regularly supplemented with 20000 ppm melamine (Figure 2(b)) shows massive fatty changes, necrosis, and broad infiltration of the lymphocytes comparable to those of the control group. The histological architecture of liver sections also showed more or less abnormal patterns, with a mild degree of necrosis and slightly lymphocyte infiltration, almost comparable to those of the control group with well-preserved cytoplasm and prominent nucleus that contains clusters of round blue melamine crystals. Also the hepatic tissue treated with honey showed normal hepatic tissues with normal hepatic strands (Figure 2(c)). The histological examination of liver sections supplemented with 20000 ppm melamine and honey-treated animals (Figure 2(d)) showed slightly degree of necrosis and clear accumulation of hepatic strands.

Table 3: Effect of honey treatment on food intake, weight gain, BWG%, and FER in rats supplemented with melamine for 28 days.

Test	Statistics	G1	G2	G3	G4
Food intake g/week	Mean ± SE	1137.20 ± 51.34	1406.30 ± 192.99	1057.30 ± 9.27	1055.90 ± 62.69
	t-test		−1.12 N.S	−1.73 N.S	−1.53 N.S
Weight gain (g)	Mean ± SE	110.20 ± 16.55	92.48 ± 9.74	90.16 ± 14.07	108.72 ± 26.24
	t-test		1.05 N.S	−0.15 N.S	0.54 N.S
BWG%	Mean ± SE	54.66 ± 9.13	52.72 ± 6.85	56.09 ± 4.44	64.93 ± 20.50
	t-test		0.19 N.S	0.91 N.S	0.55 N.S
FER	Mean ± SE	0.119 ± 0.017	0.081 ± 0.008	0.105 ± 0.017	0.128 ± 0.031
	t-test		2.14*	1.40 N.S	1.36 N.S

*Significant at 5% ($P < 0.05$) and N.S: nonsignificant.

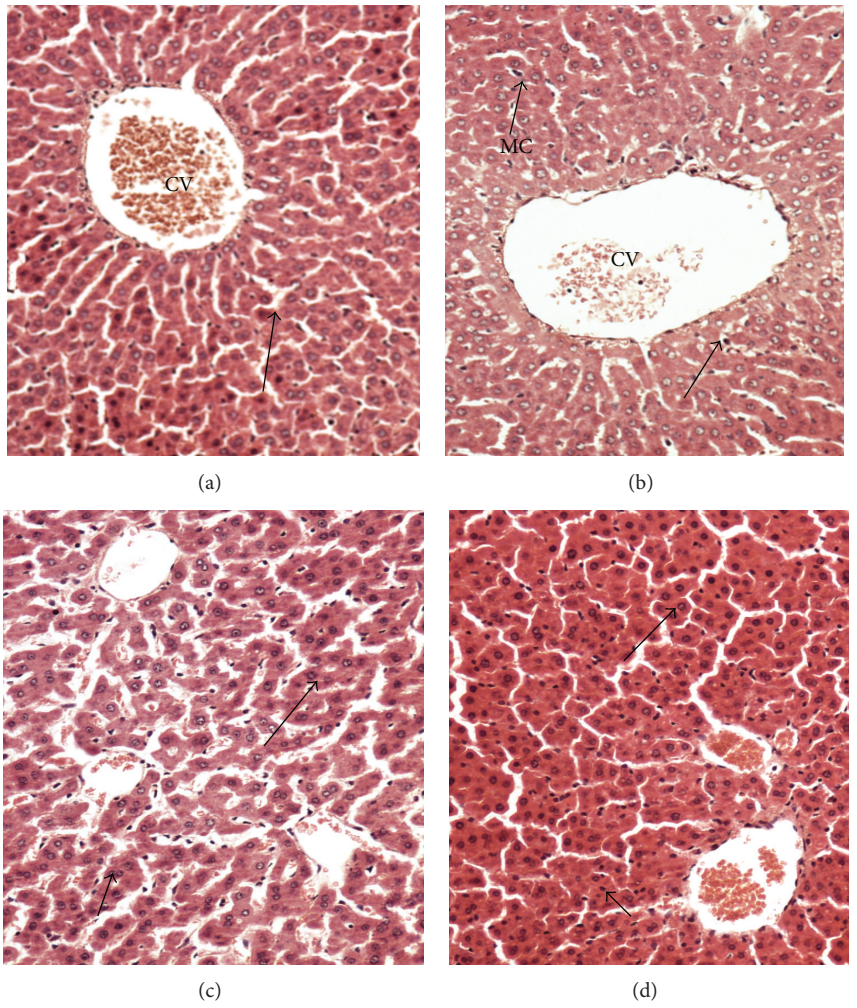

Figure 2: (a) Normal hepatic tissues showing hepatic strands of cells around the central vein (CV) leaving blood sinusoids (arrow). (b) Hepatic tissues pretreated with melamine show highly degree of necrosis hepatic tissues around the central veins (short arrow), highly degenerated hepatic tissues, and accumulation of granules in hepatic tissues (long arrow). Note dramatic pathological changes and accumulation of hepatic granules in most tissue and melamine crystals (MC). (c) Hepatic tissue of rats treated with honey showing normal hepatic tissues with normal hepatic strands (arrows). (d) Hepatic tissues supplemented with melamine and treated with honey show slightly necrotic hepatic tissues (short arrow) with normal hepatic strands (long arrow). ×200 (H&E stains).

4. Discussion

The present investigation has been focused on testing the protective effects of oral administration of honey on melamine-induced liver dysfunction. In the present study, rats treated with melamine showed a significant decrease in hemoglobin, hematocrit, mean corpuscular hemoglobin concentration, and red blood cells after 28 days compared to the control. In addition, the mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) values were decreased after the consumption of melamine in diet of rats for 28 days. These findings agree with other investigations that showed significant decline in the mean cell volume and mean cell hemoglobin values in rats exposed to pet food contaminated with melamine and cyanuric acid [9]. The observed elevation in white blood cell after melamine supplementation for 28 days is also in agreement with the above-mentioned study [9].

Treating rats with honey during melamine supplementation for 28 days increased the mean values of serum hemoglobin and red blood cell, compared to that of the negative control. This result disagree with another study that showed that honey administration tends to have stabilizing effects as there were no effects on hemoglobin, red blood cell counts at 20% (v/v)-treated group corroborate the honey as an antianemic and immunostimulant agent [28]. Also, the data obtained from the present study showed an increase in white blood cell and mean corpuscular hemoglobin values after oral administration of bees' honey for the experiment time span. This observation is concordant with [28], who recorded an increase in mean corpuscular hemoglobin and white blood cell counts at 20% (v/v) treated group in male rats. This might be due to the protective effect of honey which contains moisture, sugars such as glucose and fructose, enzymes such as catalase and glutathione reductase, trace essential elements such as iron, copper, zinc, and calcium, vitamins such as vitamin A, C, and E, and some flavonoids and phenolic acids [29–31]. In addition, administration of honey has significantly attenuated the detrimental effect of poisonous materials on different organs of the rat [28].

The increase in the mean values of platelet count after the oral supplementation of bees' honey in the current study is concordant with other investigations [32], which showed an increase in platelet count and disagree with the decrease in MCV and MCHC in rats that were not treated with bees' honey [32].

The decrease in serum total protein and albumin mean values of male rats under study after melamine supplementation in diet for 28 days is concordant with other results [9], which revealed a significant decline in albumin levels to pet food contaminated with melamine and cyanuric acid.

The noticeable increase in serum contents of total protein and albumin with decrease in the activities of serum liver enzymes detected after oral administration of bees' honey to rats that received melamine is consistent with other investigations [33], which indicated a marked hepatoprotection induced by bees' honey.

On the other hand, melamine toxicity increased the levels of AST, ALT, and ALP in the melamine-treated group, compared to the negative control group. This finding agrees with other investigations [8], which showed an outbreak of pet food-associated severe renal failure and mild hepatic damage. The result of ALP disagrees with another study that showed that decrease of alkaline phosphatase in cats consumed melamine and cyanuric acid contaminated food [6].

Body weight gain was decreased in rats treated with melamine for 28 days compared to that of the negative control. This result is in agreement with other investigations [34, 35]. The decrease in body weight gain after oral administration of bees' honey is also consistent with other investigations [36, 37], suggesting that some other bioactivity of the honey plays a part such as insulinmimetic effects of the hydrogen peroxide produced by the honey [36], but it is unclear if hydrogen peroxide reaches sufficient levels in vivo to elicit such response. Alternatively, differences in weight gain may be a result of the high antioxidant content of honey such as the effect of green tea on rats [38]. The decrease of BWG% and FER as a result of melamine supplementation is also consistent with previous investigations [35]. In contrast, BWG% and FER were elevated after treatment with honey.

Liver sections of rats supplemented with 20000 ppm melamine showed degenerated hepatic tissues, necrosis, and changes in massive fatty and broad infiltration of the lymphocytes. These results may indicate an increase in the release of the liver enzyme in the blood stream [39]. The concurrent administration of honey and melamine showed improvement in renal and hepatic tissues. These results are consistent with those reported in the previous literature [40].

In conclusion, this work revealed the protective effect of honey to the liver of rats against melamine toxicity. This was concluded from the improvement in all biochemical tests and histopathological signs compared with the melamine-supplemented rats. This protective effect of honey may be attributed to the biologically active compounds such as vitamins, flavonoids, and antioxidants that work together to scavenge free radicals. Therefore, bees' honey can be used to protect animals and humans against the adverse effects of melamine toxicity.

Conflict of Interests

The authors of this paper have no conflict of interests.

Acknowledgment

Thanks and appreciation are due to King Abdulaziz City for Science and technology for the financial support of this research (0763-11-ط-ت).

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