

Original article

Fractionation using supercritical CO₂ influences the antioxidant and hepatoprotective activity of propolis against liver damage induced by *tert*-butyl hydroperoxideBe-Jen Wang,^{1*} Yen-Hui Lien,¹ Chun-Li Su,² Chien-Ping Wu³ & Zer-Ran Yu⁴

1 Department of Food Science, National Chiayi University, Chiayi, Taiwan

2 Department of Nursing, Chang Jung Christian University, Tainan, Taiwan

3 Department of Animal Science, National Chiayi University, Chiayi, Taiwan

4 Graduate Institute of Natural Healing Science, Nanhua University, Chiayi, Taiwan

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Summary The ethanolic extract (E) of propolis was further fractionated with supercritical CO₂ into four fractions (R, F1, F2 and F3). The extracts and the four fractions were characterised in terms of antioxidant and hepatoprotective activity against *tert*-butyl hydroperoxide (t-BHP)-induced damage *in vitro* and in a rat model. The *in vitro* study revealed that pre-treatment with propolis extract or its fractions significantly protected the primary hepatocytes against damage by t-BHP ($P < 0.05$). The hepatoprotective capacity increased with the dose of propolis. The R and F1 fractions had the highest flavinoid contents and most effectively protected the liver from damage by t-BHP. This study also demonstrated that the oral pre-treatment with propolis (50 and 100 mg kg⁻¹) 5 days before a single dose of t-BHP (1.5 mM kg⁻¹, s.c. injection) was administered significantly kept the serum levels of hepatic enzyme markers (aspartate aminotransferase and alanine aminotransferase) low, even after treatment with t-BHP ($P < 0.05$). A pathological examination showed that lesions of liver were partially protected by treatment with propolis extract and fractions. Oxidative stress induced by t-BHP led to lipid peroxidation (malondialdehyde) and changes in the levels of the antioxidant enzymes. However, all the fractions, except F3 at low concentration (50 mg kg⁻¹), markedly suppressed lipid peroxidation and any increase in the activity of antioxidant enzymes.

Keywords Antioxidant enzymes, hepatoprotective, lipid peroxidation, primary hepatocytes, propolis, supercritical fluid extractive fractionation, *tert*-butyl hydroperoxide.

Introduction

Propolis is a resinous wax-like material collected by honeybees from various plant sources. The composition of propolis varies with the plant sources. In general, propolis contains 50% resin and vegetable balsam, 30% wax, 10% essential oils, 5% pollen and 5% various other substances (Burdock, 1998). Propolis exhibits a wide spectrum of biological activities including the antimicrobial and anti-tumour properties (Crocnan *et al.*, 2000; Chen *et al.*, 2001; Keskin *et al.*, 2001); thus, it is extensively used in food and beverages to improve the health and prevent diseases. Propolis also exhibits hepatoprotective effects against chemically

induced liver injury, including chronic alcohol-induced liver injury in rats (Lin *et al.*, 1997), carbon tetrachloride-induced liver damage (Merino *et al.*, 1996), galactosamine-induced hepatitis (Banskota *et al.*, 2001), and econazole-induced liver injury (Liu *et al.*, 2004). Those protective effects may be, at least in part, related to the antioxidant activity of propolis (Liu *et al.*, 2004). Some investigations have demonstrated that the antioxidant activity is attributed to the presence of flavinoids (Russo *et al.*, 2002). Flavinoids exhibit strong scavenging capacity for free radicals and also suppress the formation of free radicals by chelating with heavy metal ions, which have been demonstrated to catalyse many processes leading to the appearance of free radicals (Torel *et al.*, 1986).

The constituents of extracts differ with the extraction methods. Matsuno *et al.* (1997) found that the

*Correspondent: Fax: 886 5 2777922;
e-mail: bejen@mail.nycu.edu.tw

supercritical extract of propolis contains more diterpens and fewer flavinoids than ethanol extracts. A low dose of supercritical extract of propolis was enough to effectively prevent mammary carcinogenesis induced by chemicals (Kimoto *et al.*, 1999). Fractionation is a separation method used to minimise the co-extraction of unwanted compounds and yield fractions with different compositions (Yang & Wang, 1999). In our previous study (Wang *et al.*, 2004), supercritical carbon dioxide (SC-CO₂) extractive fractionation successfully fractionated the ethanolic extract of propolis into four fractions (R, F1, F2, and F3), containing various total flavinoid contents and thus involved various antioxidant capabilities.

The intention of this experiment was designed to further confirm the protective effects of propolis fractions rich in flavinoid contents on the liver and their antioxidant effects. *tert*-Butylhydroperoxide (t-BHP), a well-known inducer of oxidant stress, can be metabolised into free radical intermediates, which initiate lipid peroxidation and subsequently cause cell injury (Rush *et al.*, 1985). Antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), represent one means of protection against oxidative damage, which convert active oxygen molecules including the superoxide and hydroxyl radicals into non-toxic compounds (Halliwell & Gutteridge, 1990). Hence, hepatotoxicity induced by t-BHP in cultured rat hepatocytes *in vitro* and rat models *in vivo* was evaluated to elucidate the hepatoprotective and antioxidant activities of propolis extract and fractions. Simultaneously, the protective effects of propolis were compared with those treated with silymarin, which is an active constituent of the fruit of the milk thistle (*Silybum marianum*, Compositae) and has been used to treat toxic liver disease in clinical practice for over two decades.

Materials and methods

Fractionation using supercritical carbon dioxide (SC-CO₂)

Propolis obtained from Naturais Ltd (Parana, Brazil) was mixed with ethanol in a ratio of 1:10 (v/w) and left to stand without agitation for 24 h to yield ethanol extract (E). A continuous pilot-scale supercritical fluid system for fractionation of extract as described by Rizvi *et al.* (1993) was used. Both extracts and SC-CO₂ were continuously fed countercurrently into a continuous pilot-scale supercritical fluid system equipped with a 500-mL cylinder (316L-50DF4-500; Swagelok Co., Solon, OH, USA) packed with glass balls (3 mm, Kimax, Kimble, Vineland, NY, USA) and three separation vessels in series at 20, 15, 10, and 5 MPa and 60 °C to yield residual (R) (material insoluble in SC-CO₂) and three fractions (fraction 1, fraction 2 and fraction 3,

respectively) (material soluble in SC-CO₂). A cold-trap, 316-stainless steel sampling vessel submerged in an ice bath, collected the remaining solutes prior to release of CO₂ to the atmosphere. The collected samples were then freeze-dried and stored at -70 °C until use.

Preparation of rat hepatocytes

Eight-week-old male Sprague-Dawley (SD) rats (250–300 g) were used to isolate the hepatocytes, by two-stage collagenase perfusion, as described by Berry & Friend (1973) and Bonney *et al.* (1974). Rats were anaesthetised by an i.p. injection of pentobarbital (100 mg kg⁻¹ body weight). The liver was perfused with 150-mL pH 7.6 perfusion buffers, which contained 25-mM sodium phosphate buffer, 3.1-mM KCl, 119-mM NaCl, 3.5-mM glucose, 0.1% BSA and 0.005% phenol red, at a flow rate of 25 mL min⁻¹. After the blood was removed, the buffer was replaced with 200 mL of the same buffer but supplemented with 0.05% collagenase (Sigma Chemical Co., St Louis, MO, USA), 40-mM CaCl₂, and 5-mg trypsin inhibitor (Gibco BRL, Grand Island, NY, USA) for 10 min at a flow rate of 18 mL min⁻¹. To obtain a single-cell suspension of hepatic parenchymal cells, the liver was removed into a 10 cm dish, sieved, washed with L-15 washing medium (pH 7.6) (Gibco BRL), which contained 18 mM *N*-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES; Gibco BRL), 0.2% bovine serum albumin (BSA), 0.05% glucose and 5 mg mL⁻¹ insulin, and then suspended in buffered Percoll (Sigma Chemical Co.) and centrifuged at 130 × *g* for 15 min at 4 °C (Kreamer *et al.*, 1986). With the final wash, the hepatocytes were resuspended in L-15 culture medium (pH 7.6), which consisted of L-15 medium, 18 mM HEPES, 0.005 mg mL⁻¹ insulin-transferrin (Gibco BRL), 1-mg mL⁻¹ galactose, 1-mM dexamethasone, 100-U mL⁻¹ penicillin, 100-mg mL⁻¹ streptomycin, 5-ng mL⁻¹ Na₂SeO₃ and 2.5% foetus bovine serum at a density of 5 × 10⁴ cells mL⁻¹.

Hepatocytotoxic assay on primary hepatocytes

The viability of hepatocytes assayed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-based colorimetric assay (Matsuda *et al.*, 1997; Wang *et al.*, 2000) was measured to quantify the effects of propolis extract and fractions fractionated with SC-CO₂ against *tert*-butyl hydroperoxide (t-BHP) (Sigma Chemical Co.) hepatotoxin. Hepatocytes in 200 µL of medium at a concentration of 1 × 10⁴ cells per well were placed in a 96-well microplate. After the plates were incubated at 37 °C in an atmosphere of 5% CO₂ in air for 20 h (Matsuda *et al.*, 1997; Wang *et al.*, 2000), an aliquot of 10 µL of various concentrations (25, 50, 100 or 300 µg mL⁻¹) of propolis extract (E) and fractions (R, F1, F2 or F3) was added to the plates.

After the propolis extract and fractions were treated for 4 h, the toxicity of the primary hepatocytes was induced by t-BHP (1.5 mM). After 4 h in the presence of t-BHP, the cells were washed with phosphate buffered saline (PBS) twice and then 200 μL of medium that contained 0.5 mg mL^{-1} MTT was added to each well. Another 4 h of incubation allowed MTT formazan to be produced, and the absorbance was measured using an ELISA reader (Stat-Fax 2100; Awareness Technology Inc., FL, USA) at a test wavelength of 540 nm and a reference wavelength of 690 nm.

The protective effect of propolis extract (E) and fractions was evaluated by comparing the viability of the cell lines with that of the induced hepatoxin or the control. The viability (%) was calculated according to the following formula. Viability (%) = $[\text{OD (sample)} - \text{OD (induced)}] [\text{OD (control)} - \text{OD (induced)}]^{-1} \times 100\%$. OD (sample), OD (induced) and OD (control) represent the absorbances of the test samples, the t-BHP induced, and the (vehicle) control (dimethyl sulfoxide, DMSO), respectively (Wang *et al.*, 2000).

Animal treatment

Male SD rats, weighing 200–250 g, were purchased from the Animal Center, National Science Council (Taipei, Taiwan). The animals were housed individually and maintained under standard laboratory conditions of a 12 h light/dark cycle and fixed temperature (25 ± 2 °C). Rats were randomly distributed into thirteen groups of six animals. Extract (E) and fractions of propolis (50 and 100 mg kg^{-1}) were given daily by gavage to the animals on five consecutive days to elucidate the protective effect against t-BHP-induced hepatotoxicity (Wang *et al.*, 2000). On day six, t-BHP (1.5 mm kg^{-1}) in 0.9% saline was s.c. injected into the animals, which were sacrificed 24 h later. Animals in the normal control and t-BHP groups were s.c. injected with 0.9% saline (3 mL kg^{-1}) and t-BHP (1.5 mm kg^{-1}), respectively, without oral dose. Rats in the silymarin treatment group were administered silymarin (25 mg kg^{-1}) by a gavage 24 h before t-BHP s.c. injection.

Analysing hepatotoxicity

Animals were anaesthetised with ethyl ether 24 h after t-BHP was administered and blood was collected by cardiac puncture. Serum samples were obtained by centrifuging all of the blood at $3000 \times g$ and 4 °C for 10 min. Hepatic enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were used as the biochemical markers of the hepatic damage. The serum activities of AST and ALT were assayed using the commercial kits (Raichem Co., San Diego, CA, USA) and a photometer (Chiron/Diagnostics Express Plus, East Walpole, MA, USA).

Histopathological examination

Hepatic tissues of rats pre-treated with 100- mg kg^{-1} propolis, saline (control) or silymarin were collected from the same lobe of the livers and fixed in 10% neutral formalin solution. Hepatic tissue was dehydrated and embedded in paraffin. Cross-sections were stained using the haematoxylin and eosin for photomicroscopic observation.

Lipid peroxidation assay

The lipid peroxidation product malondialdehyde (MDA) of the liver was assayed by the method improved by Fraga *et al.* (1988) and Sano *et al.* (1986). The liver was excised immediately and rinsed in ice-cold 0.1 M Tris-HCl buffer. Liver homogenate was prepared by homogenising 1 g of hepatic tissue sample with 10 mL of ice-cold homogenising buffer (8- $\text{mM Na}_2\text{HPO}_4$, 12- $\text{mM NaH}_2\text{PO}_4$, 1.5% KCl, pH 7.4) in a Potter-Elvehjem homogeniser and centrifuging the product at $105\,000 \times g$ and 4 °C for 20 min. The thiobarbituric acid fluorometric method was performed at 555 nm with excitation at 515 nm using 1,1,3,3-tetramethoxypropane as a standard. The protein concentration was determined using a commercial kit (Raichem Co.). The results were expressed as the amount of MDA formed per milligram protein.

Activity of antioxidant enzymes

Liver homogenate was used for the following assay of antioxidant enzyme activity. The activities of SOD and GPx in the liver were evaluated using the commercial kits (Randox Lab Ltd, Crumlin Co., Antrim, UK) and a photometer (Chiron/Diagnostics Express Plus). The specific enzyme activities of SOD and GPx were expressed as U mg^{-1} protein. Catalase activity in the liver was measured following the method of Aebi (1984). Diluted liver homogenate (2 mL), which consisted of 0.01 mL of homogenate, 0.09 mL of 1% Triton X-100 and 1.9 mL of 20 mM of phosphate buffer, was added to a crystal cuvette containing 1 mL of 0.03 M H_2O_2 . Changes in absorbance were read at 240 nm for 1 min. Using the reaction time interval (Δt) of absorbance (A_1 and A_2), the rate constant (K) was calculated following the equation $K = (2.3\Delta t^{-1})\log(A_1A_2^{-1})$. The specific activity of the enzyme was expressed as mK mg^{-1} protein.

Statistical analysis

Data were presented as mean \pm SEM. The Student's *t*-test was used to compare the difference between the induced and the treatment groups. The one-way ANOVA test was used to compare the mean differences among extract and fractions. The differences between

groups of $P < 0.05$ were considered to be statistically significant.

Results

Effect of propolis on t-BHP-induced hepatocytotoxicity

Adding propolis to the primary cultured hepatocytes protected the cells from the cytotoxicity induced by t-BHP as measured by their viability (Fig. 1). The viability was set to zero and 100% for the groups with t-BHP-induced damage and the control group, respectively. The results indicate a significant hepatoprotective effect of propolis extract and the fractions ($P < 0.05$). The hepatoprotective effects increased with the dose of propolis. Of these fractions, R and F1 were more effective in protecting against the damage by t-BHP.

Effect of propolis on t-BHP-induced hepatotoxicity of rats

Figure 2 presents the effects of the extract and all fractions on t-BHP-induced hepatotoxicity of rats. A single dose of t-BHP caused liver damage, as revealed by an increase in the serum AST (Fig. 2a) ($211.2 \pm 25.0 \text{ U L}^{-1}$) and ALT (Fig. 2b) ($90.8 \pm 5.5 \text{ U L}^{-1}$) values over those of the control group (72.9 ± 2.2 and $28.7 \pm 4.5 \text{ U L}^{-1}$, respectively) ($P < 0.05$). Significant declines in AST and ALT levels pre-treated with 100-mg kg^{-1} propolis extract and fractions, except those of propolis pre-treated with F3

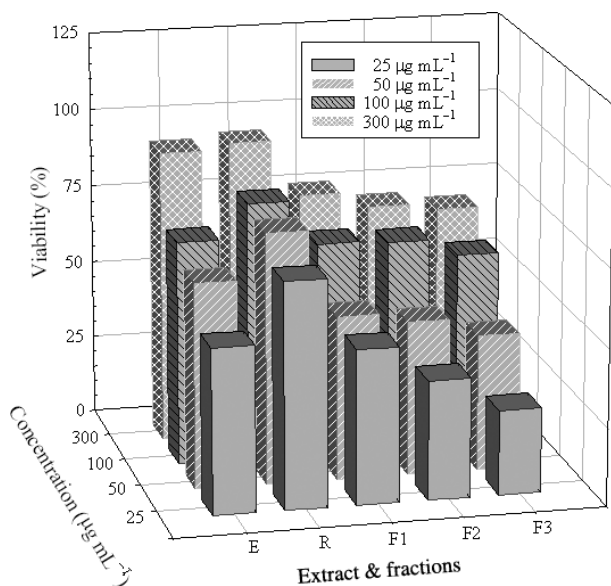


Figure 1 Effect of various concentrations of propolis extract and its fractions on *tert*-butyl hydroperoxide (t-BHP)-induced viability (%) in the primary hepatocytes.

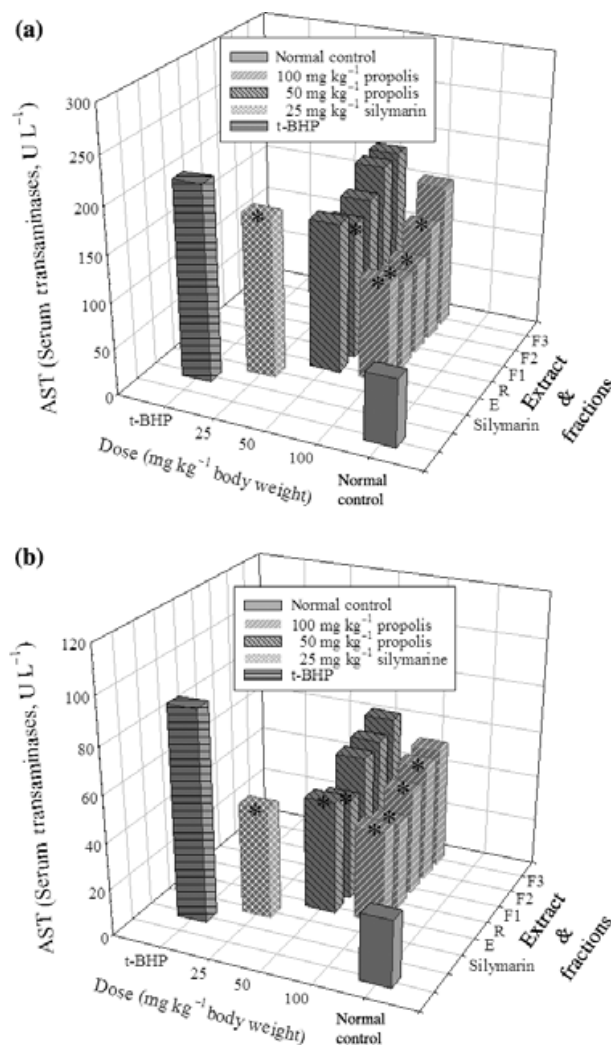


Figure 2 Effect of propolis on serum aspartate aminotransferase (a) and alanine aminotransferase (b) activities in t-BHP-treated rats. *Significant differences ($P < 0.05$) between the t-BHP treated and various concentration of propolis group by Student's *t*-test.

($P < 0.05$), indicated that propolis can protect the liver from t-BHP damage. The R fraction exhibited the most effective decline in the levels of AST and ALT. A low concentration, such as 50 mg kg^{-1} , of R significantly decreased AST and ALT levels when compared with the t-BHP-induced group. Silymarin (25 mg kg^{-1}) also significantly decreased AST ($173.2 \pm 33.0 \text{ U L}^{-1}$) and ALT ($47.6 \pm 14.9 \text{ U L}^{-1}$) levels when compared with the t-BHP group.

Histopathology of the liver

Histological changes, including increased degeneration, necrosis, hepatitis and portal triaditis, were investigated.

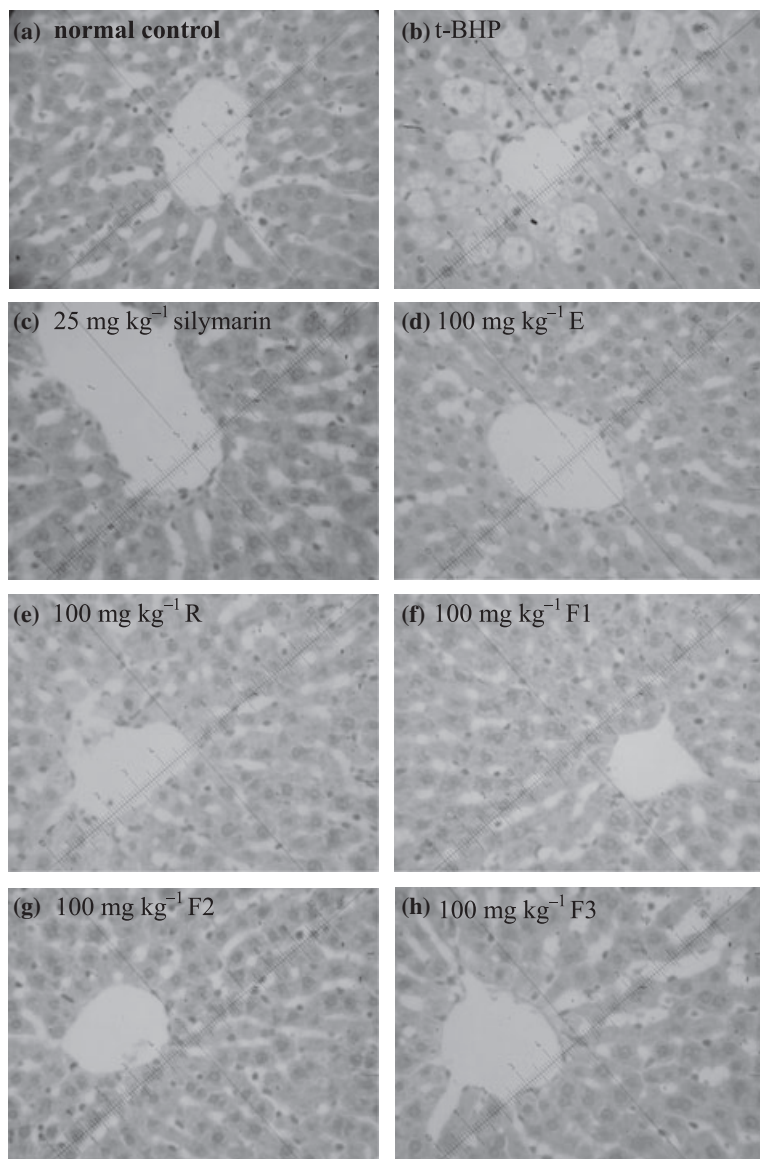


Figure 3 Effects of propolis on *t*-BHP-induced liver damage. (a) Normal control group; (b) animals treated with *t*-BHP; (c) animals treated with *t*-BHP and then with 25 mg kg⁻¹ silymarin; (d–h) animals pre-treated with 100-mg kg⁻¹ propolis extract (E) and its fractions (R, F1, F2 and F3), respectively, and then with *t*-BHP.

All rats except those in the control group exhibited the ballooning degeneration in the centrilobular zone and the necrosis of hepatocytes. However, the group with *t*-BHP-induced damage and the group pre-treated with the F3 fraction suffered more severely than the groups pre-treated with the other fractions (Fig. 3). This finding was consistent with the levels of the enzymes markers.

Effect of propolis on lipid peroxidation

Treatment with *t*-BHP for 24 h promoted the lipid peroxidation, the extent of which was specified by the level of MDA (4.1 ± 0.2 and 18.5 ± 4.1 nmole mg⁻¹ vs. the normal control and *t*-BHP groups, respectively).

Pre-treatment with 50- or 100-mg kg⁻¹ body weight of propolis extract and fractions markedly reduced the formation of MDA (Fig. 4), indicating that the administration of propolis effectively inhibited the lipid peroxidation as induced by *t*-BHP. Silymarin (25 mg kg⁻¹) also significantly decreased MDA (10.3 ± 4.9 nmole mg⁻¹ protein) levels when compared with the *t*-BHP group.

Effect of propolis on the activity of antioxidant enzymes

The activities of the antioxidant enzymes, GPx (Fig. 5a), SOD (Fig. 5b) and catalase (Fig. 5c) in the liver of the animals in the group with *t*-BHP-induced damage were

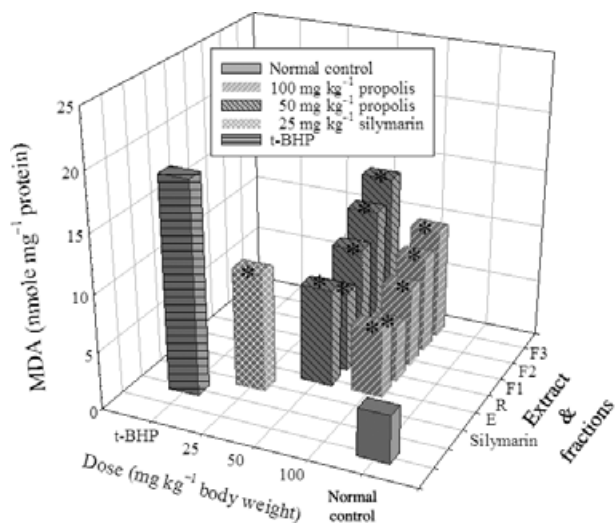


Figure 4 Effect of propolis on MDA levels in t-BHP-treated rats. *Significant differences ($P < 0.05$) between the t-BHP treated and various concentration of propolis group by Student's *t*-test.

substantially higher than those in the normal control group (Fig. 5). Propolis extract and fractions dose dependently remained GPx, SOD and catalase levels low even after t-BHP treatment. Similarly, pre-treatment with silymarin (25 mg kg^{-1}) significantly maintained the levels of antioxidant enzymes at the normal control levels.

Discussion

In an SC-CO₂ extractive fractionation system, the solubility (extractive efficiency) of active compounds depends on the vapour pressure and polarity of these compounds. However, the solubility increases with the operational pressure for a particular compound at a particular temperature (Sato *et al.*, 1998). In an earlier study (Wang *et al.*, 2004), the ethanolic extract (E) of propolis was subjected to successive partitioning using the SC-CO₂ on the scale of a pilot-plant at 60 °C and pressures of 20, 15, 10 and 5 MPa to yield R, F1, F2 and F3 fractions, respectively. The total flavinoid contents of E, R, F1, F2 and F3 were 98.6 ± 3.0 , 136.5 ± 10.0 , 90.1 ± 2.3 , 82.9 ± 1.5 and $76.2 \pm 0.5 \text{ mg quercetin g}^{-1}$ dry weight, respectively (Wang *et al.*, 2004). Flavonoids have been reported to display an antioxidant effect; thus, high total flavinoid contents in R and F1 fractions imply strong antioxidant activity.

This *in vitro* work clearly demonstrates that the extract and the fractions of propolis significantly prevented the hepatotoxicity in primary hepatocytes caused by t-BHP. t-BHP (t-BuOOH), an organic hydroperoxide, is widely used as a model compound to induce the oxidative stress. In this work, viability was

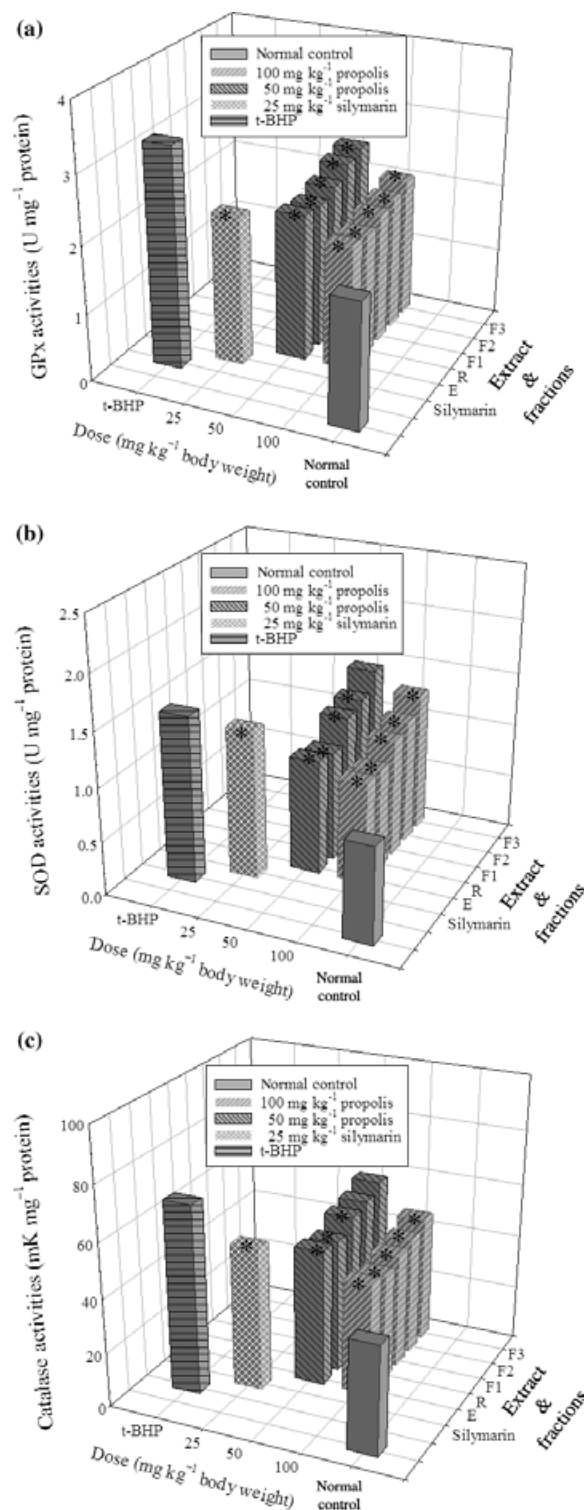


Figure 5 Effect of propolis on (a) glutathione peroxidase, (b) superoxide dismutase and (c) catalase activity in rat liver. *Significant differences ($P < 0.05$) between the t-BHP treated and various concentration of propolis group by Student's *t*-test.

set to zero and 100% for the t-BHP-induced and control groups, respectively. Accordingly, the survival of the cells showed that pre-administration of propolis (extract or fractions) significantly protected the hepatocytes against the damage caused by t-BHP ($P < 0.05$). The hepatoprotective effects increased with the dose of propolis. Of these fractions, R, E and F1 were the most effective in protecting liver cells from chemical damage.

Administering t-BHP to rats causes a marked elevation of serum transaminase (AST and ALT) levels. This increase often reflects the degree of liver injury. The leakage of large quantities of transaminase into the blood stream was associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. In this work, substantial increases in AST and ALT were observed after s.c. administration of 1.5 mm kg^{-1} t-BHP. However, the elevated levels of AST and ALT were markedly reduced by pre-treatment with 100 mg kg^{-1} of propolis extract and $50\text{--}100 \text{ mg kg}^{-1}$ of the fractions (except F3), indicating that propolis tended to prevent damage and suppressed the leakage of enzymes although cellular membranes. Pre-treatment of t-BHP-intoxicated rats with propolis reduced the severity of the aforementioned histopathological injuries and the increase in the serum transaminase levels.

Most of the hepatoprotective drugs belong to the group of free radical scavengers, and their mechanism of action involves membrane stabilisation, neutralisation of free radicals and immuno-modulation (Cai *et al.*, 2000). t-BHP has been demonstrated to permeate the cell membrane readily and induce oxidative stress both by 2-electron oxidation and by metal ion and metallo-protein-catalysed free radical processes (Cai *et al.*, 2000). The cleavage of t-BuOOH leads to the formation of unstable free radicals (alkoxyl and peroxy radicals) to initiate peroxidation (Latour *et al.*, 1995). Flavinoids and their esters in propolis are pharmacologically active molecules and have been hypothesised to influence the antioxidant activity of propolis (Bankova *et al.*, 1995; Lahouel *et al.*, 2004). Flavinoids may reduce free radical formation and consequently might have a protective effect against the oxidation in model and biological systems (Ferrali *et al.*, 1997). Lipid peroxidation in cell membranes occur in the presence of trace quantities of transition metal ions owing to oxygen-free radicals attacking the polyunsaturated fatty acids present in and/or released from the cell membrane phospholipids (Halliwell & Gutteridge, 1990). Metal ions also aided the decomposition of t-BOOH to free radicals (Guidarelli *et al.*, 1997). Hence, flavinoids in propolis extract and fractions may chelate trace metals to protect the damage caused by t-BHP.

A range of studies has demonstrated that flavinoids act as scavengers of reactive oxygen species (ROS), including superoxide, hydroxyl radicals and hydrogen

peroxide (Vuillaume, 1987). ROS are generated and react with biological molecules to initiate lipid peroxidation, eventually damaging membranes and other tissues (Afanas'ev *et al.*, 1989). Antioxidant enzymes (SOD, GPx, GRd, GST and catalase) represent one protection against the oxidative tissue damage. SOD converts $\text{O}_2^{\bullet-}$ into H_2O_2 . GPx and catalase metabolise H_2O_2 to non-toxic products (Halliwell & Gutteridge, 1990). Our results indicate the increased MDA levels in the liver in response to t-BHP treatment, implying the increased oxidative damage to the liver. t-BHP also caused an increase in GPx, SOD and catalase activities in the liver compared with the control group. Under the oxidative stress, some endogenous protective factors such as GPx and catalase are activated in the defence against oxidative injury (Kyle *et al.*, 1987). Pre-treatment with vitamin E scavenges ROS and thus the lower oxidative stress consequently reversed the increased SOD and catalase activities caused by pesticides in rats erythrocytes (John *et al.*, 2001). In this work, t-BHP causes oxidative stress and the consequent up-regulation of antioxidant enzymes, to render cells more resistant to subsequent oxidative damage. Propolis treatments returned the increased MDA and antioxidant enzymes levels back to their control levels, implying that propolis may prevent the peroxidation of lipids by t-BHP.

This *in vitro* and *in vivo* antioxidant and hepatoprotective study demonstrated that R and F1 were the most effective fractions for protecting against t-BHP-induced hepatotoxicity. A low dose of R or F1 fraction was enough to effectively prevent the liver damage and oxidant stress induced by t-BHP. Flavinoids appeared primarily in the fraction R and F1. Rich flavinoids contents in fractions R and F1 had the strongest antioxidant capacities, including the metal chelating and radicals scavenging effects (Wang *et al.*, 2004). Thus, flavinoids in propolis may relate to their ability to protect the damage of t-BHP and this large-scale supercritical fluid extractive fractionation method can be applied to yield the products with most of the active ingredients.

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