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Antioxidative natural product protect against econazole-induced liver injuries

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Abstract

The study objective of this research is in order to investigate the hepatoprotective and therapeutic effects of propolis ethanol extract (PEE) on acute econazole-induced liver injury. Positive control of various concentrations of PEE on liver function and the dose-response relationship of liver injury induced by various doses of econazole were firstly observed from biochemical assay of serum level of aspartate transaminase (SGOT) and serum alanine transaminase (SGPT) and histopathological microscopic examination. The hepatoprotective effects of various concentration of PEE on liver damage induced by hepatotoxic dose (300 mg/kg) of econazole were observed by the obvious decrement of SGOT and SGPT level and further confirmed by hepatohistological microscopic examination. The inhibitory effects of PEE on $FeCl_2$-induced (in vitro) or econazole-induced (in vivo) lipid peroxidation were investigated from the measurement of the formed malonic dialdehyde (MDA) level in the rat liver homogenate. The IC_{50} (μM) of various concentrations of PEE in the superoxide scavenging activity in econazole (300 mg/kg)-damaged rat liver homogenate were assessed by cytochrome *c* reduction method and compared with that of (+)-alpha-tocopherol. It could be postulated that the hepatoprotective effect of PEE may be, at least in part, due to their inhibitory ability on membrane lipid peroxidation and free radical formation or due to their free radical scavenging ability.
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Keywords: Propolis ethanol extract; Malonic dialdehyde; Cytochrome *c*; Econazole

1. Introduction

Propolis is made up of a mixture of gums, resins and balms, of viscous consistency, which is found in certain parts (buds and bark, mainly) of vegetables (especially coniferous trees) and gathered by

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honeybees. They bring it back to the hive, where it is modified and mixed with other substances (essentially their own wax and salivary secretions). Propolis were found to have the medical properties of anti-inflammatory, anti-viral, immunostimulatory, anti-carcinogenic activity (Hladon et al., 1980) and hepatoprotective activity (Lin et al., 1997, 1999).

Antifungal drugs abuse is a serious clinical problem in Taiwan, especially econazole. Acute econazole abuse in curing tinea pedis athlete's foot or gray fingers often lead to tissue damage, especially to liver (Raab and Hogl, 1982). For this reason, trying to find some effective methods for the treatment of econazole-induced liver injuries are considered very important in health care priorities. In this study, the influences of PEE on SGOT, SGPT and superoxide level after acute econazole administration was investigated.

2. Materials and methods

2.1. Animals

Male Wistar strain rats (110–130 gm) were purchased from the animal center, College of Medicine, National Yang-Ming university. They were kept at least 1 week on commercial diets (Fu-So Co., Taipei) under environmentally control conditions ($25 \pm 1\,^{\circ}C$, $55 \pm 5\%$ humidity) with free access to food and water. A 12 h light/dark cycle was maintained and hard wood chips was used as bedding. In this study, animal were randomly divided into four groups, including control (vehicle treated), econazole-treated, propolis ethanol extract (PEE)-treated groups.

2.2. Drugs and chemicals

Econazole, glutamic-pyruvic transaminase (GPT) kit, glutamic oxaloacetic transaminase (GOT) kit, thiobarbituric acid (TBA), xanthine oxidase, cytochrome *c*, sodium dodecyl sulfate, ferric chloride, *n*-butanol were all purchased from Sigma (St. Louis, MO 63178, USA). Acetic acid was obtained from Yon-Chi chemical company in Taipei, Taiwan. Propolis ethanol extract was a gift of Meei-Jaw Life Enterprises Ltd., Taipei, Taiwan.

2.3. Preparation of propolis ethanol extract

Take one or two parts propolis which has been cleaned and removed of impurities. It was preferably crushed, for 10 parts 35 proof ethanol. It was then placed in an opaque and closed container. It was macerated at room temperature for several days (usually 8–10 days), with regular shaking the mixture at 2–3 times a day. On the last day, the solution was filtered through with great care. The filtrate was collected for study while the residue was discarded.

2.4. Positive control

In order to investigate whether the propolis ethanol extract itself can damage the liver. The rats were p.o. administrated with PEE (10, 30, 100%) in the absence of econazole. Serum biochemical assay and liver histopathological microscopic examination were conducted.

2.5. Assessment of liver function

All blood sample were collected by carotid artery cut, and then allowed to coagulate in room temperature for 1 h. Serum was separated by centrifugation at $4\,^{\circ}C$, 15,000 rpm for 10 min. Determination of SGOT and SGPT levels were assayed according to the method described by Bergmeyer et al. (1978) using a CH100 photometer (Texas International Laboratory).

2.6. Econazole-induced liver injury

Econazole was dissolved in normal saline, and then administrated p.o., at doses of 100, 200 and 300 mg per 10 ml/kg body weight to rats. The hepatoprotective effect was investigated by administering the PEE (10 and 30%, diluted from the original 35% ethanol solution which will be considered as 100%) three times, that were at 2, 4 and 8 h after p.o. administration of econazole. Before the rats were killed, they were fasted for 24 h after the administration of econazole.

2.7. $FeCl_2$-Stimulated lipid peroxidation in rat liver homogenate (in vitro)

The effect of PEE on rat liver homogenate with $FeCl_2$ and lipid peroxidation was determined by the

MDA-TBA adduct according to the modified method described by Yuda et al. (1991). A mixture containing 0.5 ml of liver homogenate, 0.1 ml of Tris–HCl buffer (pH 7.2), 0.05 ml of 4 mM $FeCl_2$ and 0.05 ml of 4 mM of various concentrations of PEE (1, 5 and 10%) were incubated for 1 h at 37 °C. After incubation, 9 ml of distilled water and 2 ml of 0.6% TBA were added to 0.5 ml of the incubated mixture and were shaken vigorously. The mixture was heated for 30 min in a boiling water bath. After cooling, 5 ml of *n*-BuOH was added and the mixture was again shaken vigorously. The *n*-BuOH layer was separated by centrifugation at $1000 \times g$. for 10 min. The malonic dialdehyde (MDA) production was measured at 532 nm (Wong et al., 1987).

2.8. Assessment of lipid peroxidation activity (in vivo)

In order to evaluate the inhibitory activity of PEE on lipid peroxidation generated assay system, a portion of rat liver tissue was sliced and homogenated (13,000 rpm, 3 min) with 25 mM Tris–HCl buffer (pH 7.2) (10%, w/v). In a glass test tube, 0.1 ml of the liver homogenates was incubated with shaking for 1 h at 37 °C, in Tris–HCl buffer (pH 7.2), in the presence of various concentrations of PEE (1, 5 and 10%). After 1.5 ml of 1.0% TBA and 1.5 ml of 20% acetic acid were added, the mixture was further incubated for 1 h at 95 °C. The upper layer of the mixture was collected and measured at 532 nm.

2.9. SOD-like (sscavenging of free radical) test

To evaluate free radical scavenging activity of PEE in econazole generated superoxide anion assay system, a cytochrome *c* reduction method described by McCord and Fridovich (1968) was used. Xanthine oxidase converts xanthine to uric acid to yield the byproduct: superoxide anions, followed by directly reducing ferri-cytochrome *c* to ferro-cytochrome *c*, which has a UV-absorbance at 550 nm. When a substance shows superoxide scavenging activity, there is a decrease in the ferri-cytochrome *c*, and hence the production of ferro-cytochrome *c* and a lower UV-absorbance peak.

2.10. Histopathological examination

After blood sample was collected, part of the hepatic tissue was excised immediately from the same lobe of the liver and fixed in 10% neutral formalin solution for at least 1 week. Subsequently, the hepatic tissue was dehydrated with a series of ethanol solution from 75 to 100% before embedding in paraffin. Cross-sections (5 μm thick) were stained with hematoxylin and eosin (H.E.) for photomicroscopic assessment.

2.11. Statistical analysis

Statistical significance was calculated by one-way analysis of variance coupled with Dunnett's test. *P* value less than 0.05 was taken as significance.

3. Results

3.1. Positive control of propolis ethanol extract

Propolis ethanol extract (10, 30 and 100%) could has significant improvement on the normal liver function. From Table 1, both SGOT and SGPT

Table 1
Positive control of the p.o. administration of propolis ethanol extract (PEE) (10, 30 and 100%), dose-response relationship of econazole-induced liver injury, and hepatoprotective effect of various concentrations of PEE (10, 30 and 100%) on the elevation of serum aspartate transaminase (SGOT) and alanine transaminase (SGPT) levels induced by hepatotoxic dose of econazole (300 mg/kg)

Groups	Transaminases	
	SGOT	SGPT
(A) Normal saline p.o.	404.0 ± 12.6	115.4 ± 4.3
(B) PEE (10%)	276.4 ± 14.9**	88.0 ± 7.1**
(C) PEE (30%)	315.3 ± 3.9**	69.0 ± 4.6**
(D) PEE (100%)	320.2 ± 3.7**	42.0 ± 2.7**
(E) Econazole (100 mg/kg)	893.4 ± 28.6**	201.8 ± 5.7**
(F) Econazole (200 mg/kg)	1445.3 ± 80.5**	283.0 ± 3.9**
(G) Econazole (300 mg/kg)	1955.6 ± 63.4**	348.6 ± 17.8**
(H) Econazole (300 mg/kg) + PEE (10%)	601.8 ± 28.6##	163.6 ± 5.5##
(I) Econazole (300 mg/kg) + PEE (30%)	520.5 ± 26.1##	120.0 ± 10.1##
(J) Econazole (300 mg/kg) + PEE (100%)	451.2 ± 23.1##	119.0 ± 6.1##

Serum was collected at 24 h after the last p.o. administration of various doses of TMP or econazole. Values are presented as mean ± S.E. ($n = 10$). One-way analysis of variance coupled with Dunnett's test.

** $P < 0.01$, significantly different from the A group.

$P < 0.01$ significantly different from the G group.

concentrations of PEE treated groups were less than those of normal control group; the hepato-histological microscopic examination did also show more clear and more health histological architecture in PEE liver than in normal control liver (data not shown).

3.2. Hepatoprotective effect of propolis ethanol extract on econazole-induced acute hepatotoxicity

P.O. administration of econazole significantly and dose-dependently increase the concentration of serum transaminases (SGOT, SGPT) (Table 1). Nevertheless, both 10, 30 and 100% of PEE significantly decrease the level of econazole-induced serum transaminases (SGOT, SGPT) (Table 1).

3.3. Histopathological assessment

Fig. 1A shows the histopathological observation of normal control rat liver, of which no necrosis and no inflammation could be observed. In econazole (100, 200 and 300 mg/kg) intoxicated rat liver, a different degree of hepatic tissue changes, namely coagulative necrosis, lytic change, cloudy swelling or hydropic degeneration, microvesicular (fatty) degeneration, focal necrosis, inflammatory cell infiltration, and mitotic figures increase were noted. Econazole at 300 mg/kg (Fig. 1B1), coagulative necrosis, lytic change could be obviously observed, but did not appear in any special area, whereas cloudy swelling and hydropic degeneration appeared most easily in the periportal area, and more obviously appeared in 300 mg/kg than in 200 and 100 mg/kg; foamy appearance was often observed, microvesicular change and focal necrosis could also be observed at all liver parenchyma.

In econazole 200 mg/kg (Fig. 1B2), coagulative necrosis and lytic change were few observed, but cloudy swelling was often observed, and mainly appeared in the periportal area, hydropic degeneration and foamy appearance could be observed occasionally, other changes was not obviously observed.

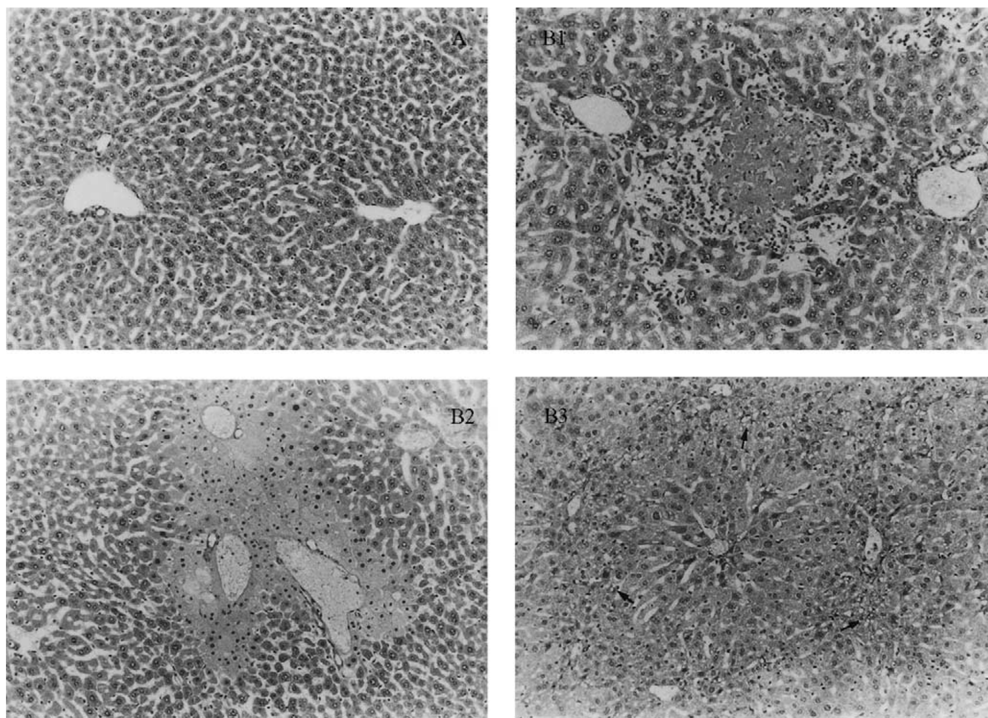

Fig. 1. (A) Normal control. (B1) Econazole 300 mg/kg, (→) coagulative necrosis with inflammatory cell infiltration. (B2) Econazole 200 mg/kg, (→) swelling change with pynotic nuclei of the hepatocyte in the central part. (B3) Econazole 100 mg/kg, (→) microvesicular change of the hepatocyte (H.E. stain, 100×).

In econazole 100 mg/kg (Fig. 1B3), microvesicular degeneration could be observed mainly, and often appeared in the periportal area, coagulative necrosis, lytic change, cloudy swelling and hydropic change were all not appeared.

Econazole-induced liver injuries could be significantly ameliorated with 10, 30 and 100% of PEE solution (original 35% PEE solution was considered as 100%). After the treatment of PEE, the degree of microvesicular degeneration decreased in the order of propolis 30% > propolis 100% > propolis 10%, whereas the mitotic figure numbers increased as propolis 10% > propolis 100% > propolis 30% (Fig. 2C–E).

3.4. Inhibitory effect of PEE on tissue lipid peroxidation in econazole-induced acute hepatotoxicity (in vitro)

PEE at 1, 5, and 10% inhibit dose-dependently the $FeCl_2$-induced lipid peroxidation in rat liver homogenate (Table 2). From Table 2, it is interesting to find that the PEE could inhibit dose-dependently the lipid peroxidation-induced MDA formation in vitro.

3.5. Inhibitory effect of PEE on tissue lipid peroxidation in econazole-induced acute hepatotoxicity (in vivo)

Various concentrations of PEE (1, 5 and 10%) at 1 ml/mg inhibit dose-dependently the econazole-induced lipid peroxidation and hence inhibit the MDA formation in rat liver homogenate in vivo (Table 3).

3.6. SOD-like (superoxide scavenging assay) test

In cytochrome *c* test, as shown in Table 4, (+)-alpha-tocopherol (Vitamin E) was used as positive control. It has been reported to have the ability in scavenging the econazole-induced superoxide free radical production (Suzuki et al., 1998). Compared with that of Vitamin E, the IC_{50} (μM) of various concentrations of PEE in scavenging the econazole (300 mg/kg)-induced superoxide free radical production in rat liver homogenate were found all less then that of Vitamin E. Among the different PEE concentrations tested, ranging from 0.223 to 0.188 μM, PEE at 10% exhibited the strongest scavenging activity.

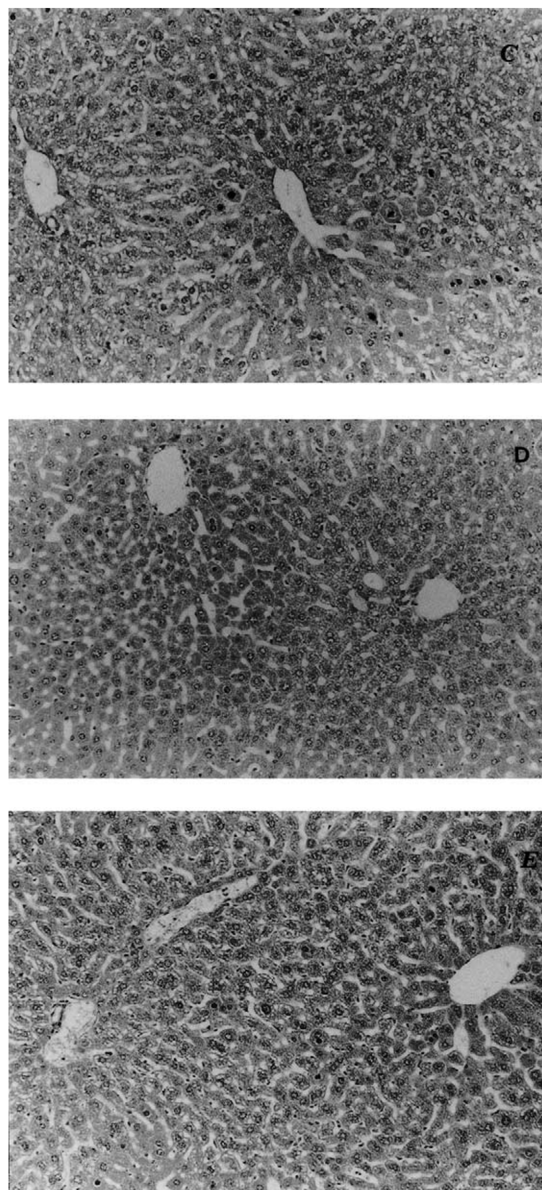

Fig. 2. (C) Econazole (300 mg/kg) + propolis (10%) group (EP10). (→) Microvesicular change in the periportal and middle zones. Mitotic figure: >20/10 HPF. (D) Econazole (300 mg/kg) + propolis (30%) group (EP30), (→) nearly normal liver parenchyma. Mitotic figure: <3/10 HPF. (E) Econazole (300 mg/kg) + propolis (100%) group (EP100), (→) focal microvesicular change was noted. Mitotic figure: 10/10 HPF. No atypical mitotic figure is found in all the live tissues (H.E. stain, 100×).

Table 2
Inhibitory effect of propolis ethanol extract (PEE) on $FeCl_2$-induced lipid peroxidation in the rat homogenate in vitro

Groups	Concentration of PEE (%)	MDA (nmole/mg protein)	Inhibition rate (%)
Normal control	–	1.73 ± 0.11	–
$FeCl_2$-treated control	–	2.63 ± 0.08**	–
$FeCl_2$ + propolis	1	0.87 ± 0.04##	67.06
$FeCl_2$ + propolis	5	0.61 ± 0.04##	76.93
$FeCl_2$ + propolis	10	0.38 ± 0.03##	85.72

Each value represents mean ± S.E. ($n = 6$). One-way analysis of variance coupled with Dunnett's test.

** $P < 0.01$, significantly different from the normal control group.

$P < 0.01$, significantly different from the $FeCl_2$-treated control group.

Table 3
Inhibitory effects of propolis ethanol extract (PEE) on econazole-induced lipid peroxidation in the rat liver homogenate in vivo

Groups	Concentration of PEE (%)	MDA (nmole/mg protein)	Inhibition rate (%)
Normal control	–	1.73 ± 0.11	–
Econazole-treated control	–	3.63 ± 0.08**	–
Econazole + propolis	1	0.97 ± 0.04##	73.3
Econazole + propolis	5	0.71 ± 0.04##	80.4
Econazole + propolis	10	0.48 ± 0.03##	86.8

Each value represents mean ± S.E. ($n = 6$). One-way analysis of variance coupled with Dunnett's test.

** $P < 0.01$, significantly different from the normal control group.

$P < 0.01$, significantly different from the $FeCl_2$-treated control group.

Table 4
The superoxide scavenging activity of various concentrations of propolis ethanol extract (PEE) in econazole (300 mg/kg)-damaged rat liver homogenate

Groups	IC_{50} (μM)
(A) Vitamin E (8 mg/kg)	0.244 ± 0.0002
(B) PEE (1%)	0.223 ± 0.0001
(C) PEE (5%)	0.202 ± 0.0002
(D) PEE (10%)	0.188 ± 0.0001

(+)-Alpha-tocopherol (Vitamin E) was used as positive control. Each data represents mean ± S.E. ($n = 3$).

4. Discussion

In recent years, the biological and pharmacological properties of propolis have reeived great attention in the scientific community. This is mainly stemmed from the focus that propolis could exhibit a broad spectrum of biological activities, for instance, antibacterial, antifungal, anti-viral, anti-inflammatory, immunostimulatory, anti-carcinogenic activity (Hladon et al., 1980), hepatoprotective activity (Lin et al., 1997, 1999) and anti-oxygen stress activities (Raab and Hogl, 1982). Propolis have been reported to contain cinnamic acid, benzoic acid and their esters, substituted phenolic acids and esters, flavonoid glycones, bee wax, and caffeic acid phenethyl ester (CAPE) (Su et al., 1994). In 1987, Giurgea et al. reported that standardized propolis extract (SPE) possesses a remarkable anti-oxidative and hepatoprotective activity as good as silymarin on the ethanol-induced rat liver damage (Giurgea et al., 1987).

From the positive control of Table 1, it was noticed that various concentrations of PEE (10, 30 and 100%) was shown to improve the liver function, even in the normal health control rat liver. Both SGOT and SGPT concentrations of PEE treated groups was found to be less than those of normal control group.

From the hepatohistological microscopic examination, PEE also found to improve the liver function. Econazole is one of the commonly used imidazole antifungal drugs. Structurally, it very closely related to another imidazole derivative called miconazole (Heel et al., 1978). Econazole was previously shown to increase the mitotic cross-overs (Raab and Hogl, 1982), affects chromosome segregation by an indirect mechanism, which is possibly related to induced structural chromosome damage, and often lead to tissue damage, especially to liver (Raab and Hogl, 1982). In the present study, the hepatoprotective and therapeutic effects of propolis ethanol extract on econazole-induced liver injury were investigated in Wistar rat. Serum levels of transaminases (SGOT, SGPT), histopathological changes and superoxide scavenging activity were used as indicators of hepatoprotective and therapeutic efficacy.

The acute administration of econazole (100, 200 and 300 mg/kg) to rats leads to a different degree of hepatic tissue changes, namely coagulative necrosis, lytic change, cloudy swelling or hydropic degeneration,

microvesicular (fatty) degeneration, focal necrosis, inflammatory cell infiltration, and mitotic figure numbers increase within the liver, and dose-dependently caused a marked increase in the serum transaminase (SGOT, SGPT) levels (Table 1). This elevation of the transaminases levels reflects the degree of liver injury. Post-treatment of econazole-intoxicated rats with PEE significantly decreased the severity of the above histopathological injuries (Fig. 1) and the increase in the serum transaminases (SGOT, SGPT) levels (Table 1). These results demonstrated that the PEE has a remarkable hepatoprotective effect on econazole-induced liver injuries. From Table 2, it was found that the PEE exhibited a remarkable and dose-dependent inhibition on $FeCl_2$-induced lipid peroxidation, which was indicated by a significant decrease in the malonic dialdehyde formation. What is more, in the cytochrome *c* test, PEE was further confirmed to have the dose-dependent antioxidant activity (Table 4). In conclusion, it could be postulated that the hepatoprotective effect of PEE may be, at least in part, due to its inhibitory ability on membrane lipid peroxidation and free radical formation or due to its free radical scavenging activity. The propolis ethanol extract could be benefical to apply them clinically in the treatment of econazole-induced hepatitis in the nearest future, although further detail study should be continuously processed.

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