

Prophylactic Effect of Aqueous Propolis Extract against Acute Experimental Hepatotoxicity *in vivo*

Aiman S. El-Khatib[§], Azza M. Agha, Laila G. Mahran and Mohamed T. Khayyal*

Department of Pharmacology, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo 11562, Egypt. Fax: (00202) 7743491. E-mail: mtkhayyal@hotmail.com

[§] Present address: Department of Pharmacology, College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia

* Author for correspondence and reprint requests

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Propolis has been extensively used in folk medicine for the management of a wide spectrum of disorders. In a previous study, we demonstrated the protective effect of the aqueous propolis extract (APE) against the injurious effects of carbon tetrachloride (CCl₄) on hepatocytes *in vitro*. The present investigation was carried out to show whether the hepatoprotective effect of the extract could also be manifested *in vivo*. Rats were given APE orally for 14 consecutive days, before being subjected to a single intraperitoneal injection of CCl₄. One day after the CCl₄ injection, the animals were sacrificed, hepatocytes were isolated and liver homogenates were prepared for the assessment of liver injury. In isolated hepatocytes, APE afforded protection against CCl₄-induced injury as manifested by a decrease in the leakage of the cytosolic enzyme lactate dehydrogenase (LDH), decreased generation of lipid peroxide and maintenance of cellular reduced glutathione (GSH) content. In principle, similar findings were observed in liver homogenates. The present findings show that APE has *in vivo* hepatoprotective potential which could be attributed at least in part to the maintenance of cellular GSH content. The latter effect seems to play an important role in conserving the integrity of biomembranes as it was associated with a decrease in lipid peroxidation and reduced leakage of cytosolic LDH.

Introduction

Propolis or “bee-glue” contains a number of natural active constituents that have been shown to exert a variety of medical properties, such as anti-microbial activity (Koo *et al.*, 2000), protective effect against radiation-induced damage (El-Ghazaly and Khayyal, 1995), anti-mutagenic effect (Varanda *et al.*, 1999), anti-hyperalgesic action (de Campos *et al.*, 1998) and anti-inflammatory activity (Khayyal *et al.*, 1993; Ozturk *et al.*, 2000). Most of these effects have been related to the anti-oxidant and free radical scavenging properties of propolis (Basnet *et al.*, 1997).

Hepatic disease is often associated with oxidative stress. In a previous study conducted by the authors, the aqueous propolis extract (APE) was shown to have a protective effect on hepatocytes against carbon tetrachloride (CCl₄)-induced injury *in vitro* (Mahran *et al.*, 1996). The present investigation was carried out to see whether the hepatoprotective effect of the extract might also be exerted *in vivo*. Accordingly, the extract was

intended to be given to rats orally for 2 weeks before subjecting them to the injurious effect of CCl₄. Hepatocytes were then isolated and examined for leakage of the cytosolic lactate dehydrogenase enzyme (LDH), depletion of cellular reduced glutathione (GSH) and lipid peroxidation. The last two parameters were also measured in liver homogenates of the treated animals.

Materials and Methods

Animals

Adult male albino rats, each weighing 200–250 g were used in the present investigation. They were purchased from the National Research Center (Giza, Egypt) and housed in groups of 6–8 rats per cage. Animals were housed at the animal facility of the Faculty of Pharmacy, Cairo University at a temperature of 25 ± 1 °C and humidity of 60 ± 5% and left for two weeks for acclimatization before use. They were fed on a standard rat pellet diet (El-Nasr Chemical Company, Cairo, Egypt)

and allowed free access to water. The study was carried out according to international guidelines and approved by the Ethical Committee for Animal Experimentation at the Faculty of Pharmacy, Cairo University.

Chemicals

The following chemicals were used: collagenase type IV for hepatocyte isolation, bovine serum albumin (BSA) fraction V, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), ethylene glycol-bis-(β -aminoethylether)-N,N'-tetraacetic acid (EGTA), 2-thiobarbituric acid (TBA), metaphosphoric acid, and 5-5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) were purchased from Sigma Chemical Company (St. Louis, MO., USA). CCl_4 was obtained from Prolabo (Paris, France), while urethane was supplied by E. Merck (Darmstadt, Germany). All other chemicals used in the present study were of analytical grade. The aqueous extract of propolis was prepared as a 13% solution by Propharma (Havdrup, Denmark) and supplied by kind courtesy of Friesland-Coberco (Leeuwarden, Holland). According to the manufacturers, the product was prepared by aqueous decoction of crude propolis, collected from Denmark, China, Uruguay and Brazil, and standardised to contain not less than 0.05% of organic aromatic acids, mainly caffeic, ferulic, iso-ferulic, cinnamic and 3,4-dimethoxy-cinnamic acids in addition to trace amounts of various flavonoids.

Experimental design

Rats were randomly allocated to five groups, 14 animals each. Groups 1, 2 and 3 received APE in daily oral doses of 1, 5 and 10 ml/kg, respectively, for 14 consecutive days. Groups 4 and 5 were kept without treatment during this time period. On day 14, all animals from APE-treated groups and those from group 4 received a single intraperitoneal injection of CCl_4 in a dose of 1 ml/kg of a 50% (v/v) solution in liquid paraffin. Group 4 thus served as the control CCl_4 -treated group. Group 5 received only the vehicle viz., liquid paraffin and served as a normal untreated group. Twenty-four hours later, each animal group was subdivided into two equal subgroups: one was used for the isolation of hepatocytes and the other for the preparation of liver homogenates.

Assessment of hepatotoxicity

The parameters chosen to assess injury of the isolated hepatocytes were the leakage of the cytosolic LDH enzyme, the depletion of intracellular GSH and the overproduction of lipid peroxide. The last two parameters were selected to assess injury in liver homogenates as well.

Isolation of hepatocytes

Hepatocytes were isolated by the collagenase perfusion method, as described by Moldéus *et al.* (1978). Rats were anaesthetized with urethane, injected intraperitoneally in a dose of 1.3 g/kg. The peritoneal cavity was opened and heparin (500 units in 0.1 ml) was injected into the caval vein. The portal vein was cannulated and connected to a perfusion pump. The liver was first slowly perfused (5 ml/min) in situ with Hank's I buffer (composition g%: NaCl 0.8, KCl 0.04, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.006, KH_2PO_4 0.006, NaHCO_3 0.21, HEPES 0.3, EGTA 0.023, BSA 0.667) and was then carefully dissected out. The rate of perfusion was increased after isolation to 80 ml/min and the buffer was allowed to circulate for 5 min through the liver to wash out blood until the liver became pale. The liver was then perfused with Hank's II buffer (composition g%: NaCl 0.8, KCl 0.04, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.006, KH_2PO_4 0.006, NaHCO_3 0.21, HEPES 0.3, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.029, collagenase 0.075) for about 10 min. The perfusion was performed at 37 °C and the perfusion buffers were continuously bubbled with carbogen gas (95% O_2 , 5% CO_2). At the end of the perfusion time, the liver was detached from the cannula and placed in Krebs-Henseleit solution (composition g%: NaCl 0.69, KCl 0.035, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.028, KH_2PO_4 0.016, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.029, NaHCO_3 0.21), pH 7.4, supplemented with HEPES and BSA to give final concentrations of 0.3% and 1%, respectively. The hepatocytes were dispersed by mechanical teasing and gentle shaking. The cells were filtered through two layers of gauze and the filtrate was left for 2 min to allow the cells to settle forming a loose pellet. The cells were counted in a Neubauer chamber in the presence of 0.16% trypan blue. Viability of the cells was assessed by recording the percentage of cells, which excluded the dye. A yield of 100–300 mil-

lion cells per liver with viability greater than 85% was routinely obtained.

Suspensions of hepatocytes were prepared in Krebs-Henseleit solution, pH 7.4, containing HEPES and BSA as described above. The number of cells in suspensions was adjusted to give 5–7 million cells per ml. They were incubated for 30 min in rotary round-bottomed flasks kept in a water bath at 37 °C under an atmosphere of carbon dioxide. Two aliquots (0.5 ml each) were then withdrawn from each suspension of hepatocytes. One aliquot (aliquot A) was used for the determination of the level of lipid peroxide, while the other was centrifuged at $1000 \times g$ for 5 min before being subdivided into 2 portions. The residual cells after centrifugation were utilized for the estimation of cellular GSH content (aliquot B) and the clear supernatant was used for the assessment of the leaked LDH (aliquot C).

Preparation of liver homogenates

The animals were sacrificed by cervical dislocation and their livers were exposed, dissected free from extraneous tissues and rinsed with chilled 1.15% KCl solution (pH 7.4). The latter solution was employed to prepare 10% tissue homogenates, which were then used for the estimation of GSH content and lipid peroxide level.

Assessment of reduced glutathione content

Colorimetric estimation of GSH was performed as described by Beutler *et al.* (1963). To the pellet in aliquot B (see above) and to a sample of the liver homogenates, metaphosphoric acid was added to precipitate the protein content. The sample was then centrifuged at $1000 \times g$ and to the supernatant Ellman's reagent was added to form a stable colour which was then measured at 412 nm. Results were expressed as nmol of GSH per one million cells in case of isolated hepatocytes and as $\mu\text{mol/g}$ tissue in case of liver homogenates.

Assessment of lipid peroxide formation

Lipid peroxide was determined colorimetrically in aliquot A (see above) and in a sample of the liver homogenates as thiobarbituric acid-reactive substances (TBA-RS) according to the method of Uchiyama and Mihara (1978). The absorbances of

the formed coloured product at 520 and 535 nm were measured and the results were expressed as the difference in absorbance at the two wavelengths ($\Delta A_{535-520}$).

Assessment of lactate dehydrogenase activity

A kinetic assay following the recommendations of the German Society for Clinical Chemistry (1970, 1972), using the test reagent kit purchased from Randox Laboratories Ltd. (Antrim, UK) was performed. The assay was carried out on aliquot C (see above) and is based on the conversion of L-lactate to pyruvate by LDH with the subsequent reduction of NAD^+ to NADH. The absorbance of the latter was measured at zero, one and two min at 340 nm. The LDH activity expressed as units/l was determined by measuring the mean increase in absorbance per min.

Statistical analysis

Comparisons between different groups were carried out by one way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons test. The level of significance was set at $p < 0.05$.

Results

Acute exposure to a single intraperitoneal dose of CCl_4 resulted in a severe depletion of GSH content in isolated hepatocytes by 75%. Prior administration of APE for 14 consecutive days afforded different degrees of protection against such depletion, approaching nearly complete protection with the smallest dose of the extract (Fig. 1A). Essentially similar results were obtained in liver homogenates where CCl_4 produced 66% depletion of GSH content, which again was prevented to varying degrees by the different doses of APE, such that the depletion reached 42% with the 1 ml/kg dose (i.e. a protection of 58%) and only 20% with the higher doses, indicating 80% protection (Fig. 1B).

The depletion of GSH by CCl_4 was associated with a nearly two- to three-fold increase in lipid peroxidation as measured by the level of TBA-RS in both liver homogenates (Fig. 2A) and isolated hepatocytes (Fig. 2B), respectively. Pretreatment with APE completely normalized lipid peroxida-

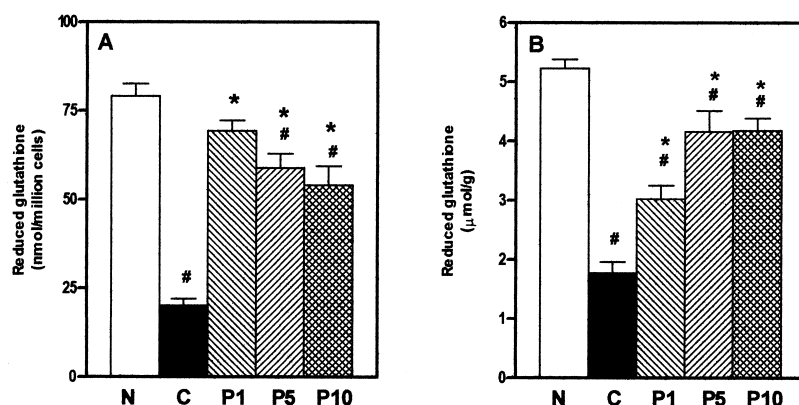


Fig. 1. Effect of different doses of aqueous propolis extract, given orally for 14 successive days in daily doses of 1 ml/kg (P1), 5 ml/kg (P5) and 10 ml/kg (P10), on the content of reduced glutathione in (A) isolated suspended hepatocytes and in (B) liver homogenates prepared from CCl_4 -treated rats. N = normal untreated animals, C = control animals given only CCl_4 .

Each column represents the mean of 6–7 experiments \pm s.e.mean, # $p < 0.05$ vs N, * $p < 0.05$ vs C.

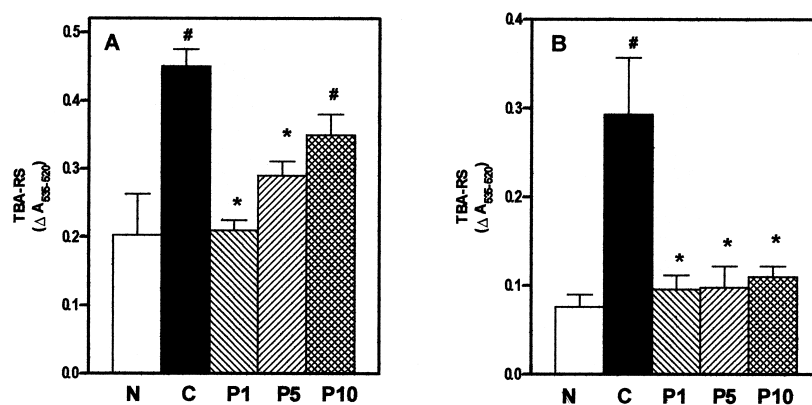


Fig. 2. Effect of different doses of aqueous propolis extract, given orally for 14 successive days in daily doses of 1 ml/kg (P1), 5 ml/kg (P5) and 10 ml/kg (P10), on the level of lipid peroxide determined as thiobarbituric acid reactive substances (TBA-RS) in (A) liver homogenates or in (B) isolated suspended hepatocytes prepared from CCl_4 -treated rats. N = normal untreated animals, C = control animals given only CCl_4 .

Each column represents the mean of 6–7 experiments \pm s.e.mean, # $p < 0.05$ vs N, * $p < 0.05$ vs C.

tion in the latter, even with the smallest dose (Fig. 2B). The lowest dose was also most effective in normalizing lipid peroxidation in liver homogenates, while higher doses afforded less protection (Fig. 2A).

Liver injury induced by CCl_4 was also evidenced by a 90% increase in leakage of the cytosolic LDH enzyme from isolated hepatocytes. A dose-related protective effect against LDH leakage was shown with increasing doses of APE, approaching nearly full protection with the highest dose (Fig. 3).

Discussion

In a previous study, it was shown that APE inhibited oxidative stress induced by CCl_4 when the extract was added to the isolated hepatocytes *in vitro* (Mahran *et al.*, 1996). Since in many cases, however, propolis extract is intended for oral use, it was important to show whether it would still exert its hepatoprotective effect against CCl_4 -induced liver injury after being administered orally and being subjected to possible biotransformation or degradation, or whether the previously ob-

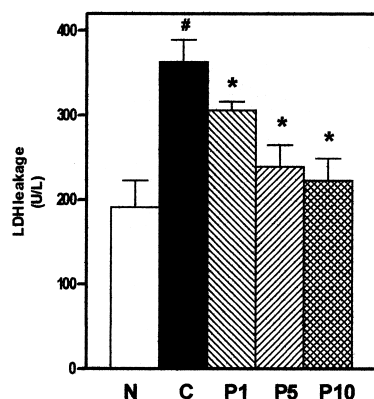


Fig. 3. Effect of different doses of aqueous propolis extract, given orally for 14 successive days in daily doses of 1 ml/kg (P1), 5 ml/kg (P5) and 10 ml/kg (P10), on the leakage of lactate dehydrogenase (LDH) from suspended hepatocytes isolated from CCl_4 -treated rats. N = normal untreated animals, C = control animals given only CCl_4 .

Each column represents the mean of 6–7 experiments \pm s.e.mean, # $p < 0.05$ vs N, * $p < 0.05$ vs C. One unit (U) is equivalent to 1 μmol NADH formed /min.

served effects *in vitro* were only evident in the presence of the aqueous extract in its entirety.

Carbon tetrachloride is a useful agent for inducing hepatic injury (Stacey and Priestly, 1978; Chenery *et al.*, 1981; Long and Moore, 1986; Moody *et al.*, 1990). It needs to be first bioactivated in the liver to form trichloromethyl radical, which in the presence of oxygen gives rise to the highly reactive trichloromethylperoxy radical. These free radicals can react with cell membrane phospholipids initiating a chain of extensive intrahepatic destructive peroxidation reactions (Recknagel, 1967; Plaa and Witschi, 1976; Kalf *et al.*, 1987; Yamamoto, 1990).

In our experiments, acute exposure to CCl_4 exhibited overt hepatotoxicity that was manifested as a dramatic depletion of the GSH content and a marked increase in the generation of lipid peroxide in both the isolated hepatocytes and the liver homogenates. The increased leakage of LDH that has been shown in the isolated hepatocytes reflects the decreased biomembrane integrity after being exposed to the hepatotoxin.

Prophylactic treatment with APE succeeded to protect against the acute hepatotoxicity induced by CCl_4 , as evidenced by the reduction in the level of lipid peroxide, the maintenance of intracellular

level of GSH and the decreased leakage of LDH. These effects could be, at least partly, explained by the anti-oxidant capability of the extract (Volpert and Elstner, 1993; Merino *et al.*, 1996; Basnet *et al.*, 1997).

Propolis has previously been reported to ameliorate hepatic dysfunction (Sugimoto *et al.*, 1999) and to decrease chronic alcohol-induced hepatocellular fatty degeneration (Lin *et al.*, 1999). Its anti-oxidant properties were considered responsible for preventing the cellular alterations seen in rough endoplasmic reticulum, golgi complex, nucleus and plasma membrane of the hepatocytes that have been subjected to galactosamine (Rodriguez *et al.*, 1997).

Crude propolis contains a mixture of a large number of biologically active substances (Walker and Crane, 1987) that belong chemically to the terpenes, caffeic acid and its esters, flavonoids, free amino acids, aldehydes and ketones. The aqueous extract of propolis that has been used in the current study exhibits higher anti-oxidant effects than the alcoholic extract (Volpert and Elstner, 1993) due to its much higher content of caffeic acid and its esters, which are known for their anti-oxidant properties (Krol *et al.*, 1990; Chopra *et al.*, 1995). Caffeic acid phenethyl ester was shown to possess strong anti-oxidant activity and to protect against spinal cord ischaemia/reperfusion injury, which is known to be mediated via oxygen-derived free radicals (Ilhan *et al.*, 1999). Propol, another component of APE, was also shown to possess potent anti-oxidant property (Basnet *et al.*, 1997).

It could be concluded, therefore, from the present findings that the previously reported protective effects of APE against CCl_4 toxicity to hepatocytes *in vitro* could also be exhibited when the extract is given orally prior to exposure of the liver to CCl_4 . Given systemically, APE does not seem to undergo metabolic changes that would hinder its hepatoprotective properties. The lower doses of APE appear more effective in conserving the GSH content of isolated hepatocytes and in reducing lipid peroxidation induced by CCl_4 . The effect of APE in liver homogenates was, in principle, similar to that in isolated hepatocytes. The hepatoprotective effect of the extract *in vivo* is probably related to its anti-oxidant properties, resulting in the maintenance of the GSH content that ultimately leads to inhibition of lipid peroxidation. This effect

appears to play an important role in reinforcing the integrity of biomembranes as evidenced by the decreased leakage of the cytosolic LDH enzyme.

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