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Effect of propolis extract on acute carbon tetrachloride induced hepatotoxicity

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Ethanolic extract of propolis was administered to rats intoxicated by carbon tetrachloride. Administration of bolus dose of CCl_4 (1.5 ml/kg, ip) resulted in elevation of serum transaminases and serum alkaline phosphatase activities. Levels of hepatic lipid peroxidation were significantly increased. On the contrary, there was significant decrease in hepatic reduced glutathione level. The propolis extract (100 and 200 mg/kg, po) exhibited recoupment in both pre- and post-treatment (prophylactic and curative studies) of biochemical changes induced by CCl_4. The post treatment of 200 mg/kg, po extract showed most significant hepatoprotective effect. Histopathological studies showed damage in hepatocytes and disturbed chord arrangement after toxicant administration. Propolis extract (200 mg/kg, po) was found to be more effective in restoring CCl_4 induced histopathological alterations.

Keywords: Propolis, Carbon tetrachloride (CCl_4), Hepatoprotection.
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Propolis has been used for thousands of years in folk medicine[1]. It is a mixture of sticky plant substances which is collected by honey bees (*Apis mellifera*) from vegetal exudates and from pellets with their mandibles and mix it with bees wax along with their salivary secretions[2]. Bees use this material to seal hive to strengthen the border of the combs, and embalm dead invaders[3]. It is a resinous or wax like beehive product that has been used by man since ancient time for its pharmaceutical properties[4]. It contains esters of phenolic acids and is used as a popular herbal medicine[5]. It possesses several biological activities such as anti-inflammatory[6], antifungal[4], anticancer[7], antiviral[8] and tissue regenerative[9] etc. It has been suggested that the biological activities of propolis may be due to the presence of a large number of flavonoids. Propolis showed free radical scavenging activity and is used as a source of natural antioxidant[3]. The ethanolic extract of propolis has a profound anti-inflammatory effect on both chronic and acute inflammation[10]. The present study has been undertaken with the objective to investigate *in vivo* effects of ethanolic extract of propolis against carbon tetrachloride induced liver injury in rats.

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Materials and Methods

Propolis from the hive of *Apis mellifera* was collected from BSF Academy, Tekanpur (Gwalior) and identification was confirmed by a senior entomologist of the school. Propolis extract thus obtained had a concentration of 68.4 % (w/v) in 95% ethanol[11] and stored at 4°C. Aqueous suspension of propolis was prepared in gum acacia and was administered (100 and 200 mg/kg) to the animals orally. Control animals received equal amount of vehicle only. Female albino rats (35) of Sprague Dawley strain weighing 130±10 g body weight were used. Animals were housed under standard husbandry conditions (25°±2°C, 60-70% RH and 12:12 hr L:D photoperiod) and allowed standard rat feed (Pranav Agro Industries, New Delhi) and water *ad libitum*.

The rats were divided into following 7 groups of 5 animals each: Gr. 1 served as normal control, Gr. 2 was administered propolis extract *per se* (200 mg/kg, po). Animals of Gr. 3 received CCl_4 (1.5 ml/kg, ip) once only[12]. Groups 4 and 5 received propolis extract (100 and 200 mg/kg, po) 24 hr prior to CCl_4 administration (as in group 3). Groups 6 and 7 received propolis extract (100 and 200 mg/kg, po) after 24 hr of CCl_4 administration (as in group 3). The rats were sacrificed 24 hr after the last treatment.

Blood samples were withdrawn by puncturing the retro-orbital venous sinus. Serum was separated and used for quantitative estimations of aspartate

aminotransferase (AST), alanine aminotransferase (ALT)[13] and serum alkaline phosphatase (SALP)[14]. The livers from all the rats were removed to determine the level of hepatic lipid peroxidation[15] and reduced glutathione[16]. The lipid peroxidation was measured by the concentration of TBARS in liver. The malondialdehyde (MDA) formed was quantitated by reaction with thiobarbituric acid (TBA) and used as an index of lipid peroxidation. Reduced glutathione was estimated in the liver homogenate using dithio nitro benzoic acid (DTNB).

For histopathological study, the liver was fixed in Bouin's solution for 24 hr. The tissue blocks were prepared and cut into 5μm thick sections and stained with haematoxylin and eosin dye (Merck) and mounted with dibutyl polystyrine xylene (DPX). Photomicrographs were shot for histopathological observation. The results were calculated using Student's '*t*' test and one way analysis of variance (ANOVA)[17]. *P* values ≤ 0.05 were considered as statistically significant.

Results

Biochemical changes—Carbon tetrachloride caused significant increase in the activity of serum transaminases and alkaline phosphatase (Table 1). There was significant increase in MDA concentration of liver tissue after CCl_4 administration. However, a marked depletion was observed in hepatic reduced glutathione level (Table 1). There was no significant variation in the activities of serum transaminases, serum alkaline phosphatase, hepatic lipid peroxidation and hepatic reduced glutathione level in propolis *per se* administered group.

Pre-treatment study (prophylactic) and post treatment study (curative) of propolis extract prevented progression of CCl_4 induced acute liver injury. Significant recoveries were noted in the activity of serum transaminases in curative study and in serum alkaline phosphatase both in prophylactic and curative studies. Hepatic lipid peroxidation was suppressed and hepatic reduced glutathione was recovered. Analysis of variance ($P \leq 0.05$) showed maximum recoupment in LPO and GSH level with extract at a higher dose of 200 mg/kg in curative study.

Histopathological changes—Normal histoarchitecture of liver is shown in Fig. 1. Liver of rats treated with CCl_4 at a dose of 1.5 ml/kg, ip exhibited an extensive degenerative lesion (Fig. 2). Vacuolization of hepatocytes was very common; periportal fibrosis, bile duct, hyperplasia and fatty degeneration were also observed. Leucocytic infiltration was common. The central sinus showed congestion. Administration of propolis extract (200 mg/kg, curative study) showed well-maintained histoarchitecture (Figs 3 and 4). Sinusoids were normal. Nuclei of hepatocytes were well maintained. There was no granulation and perinuclear vacuolation in the hepatocytes, however, at some places kupffer cells were observed. The biliary ductules were normal.

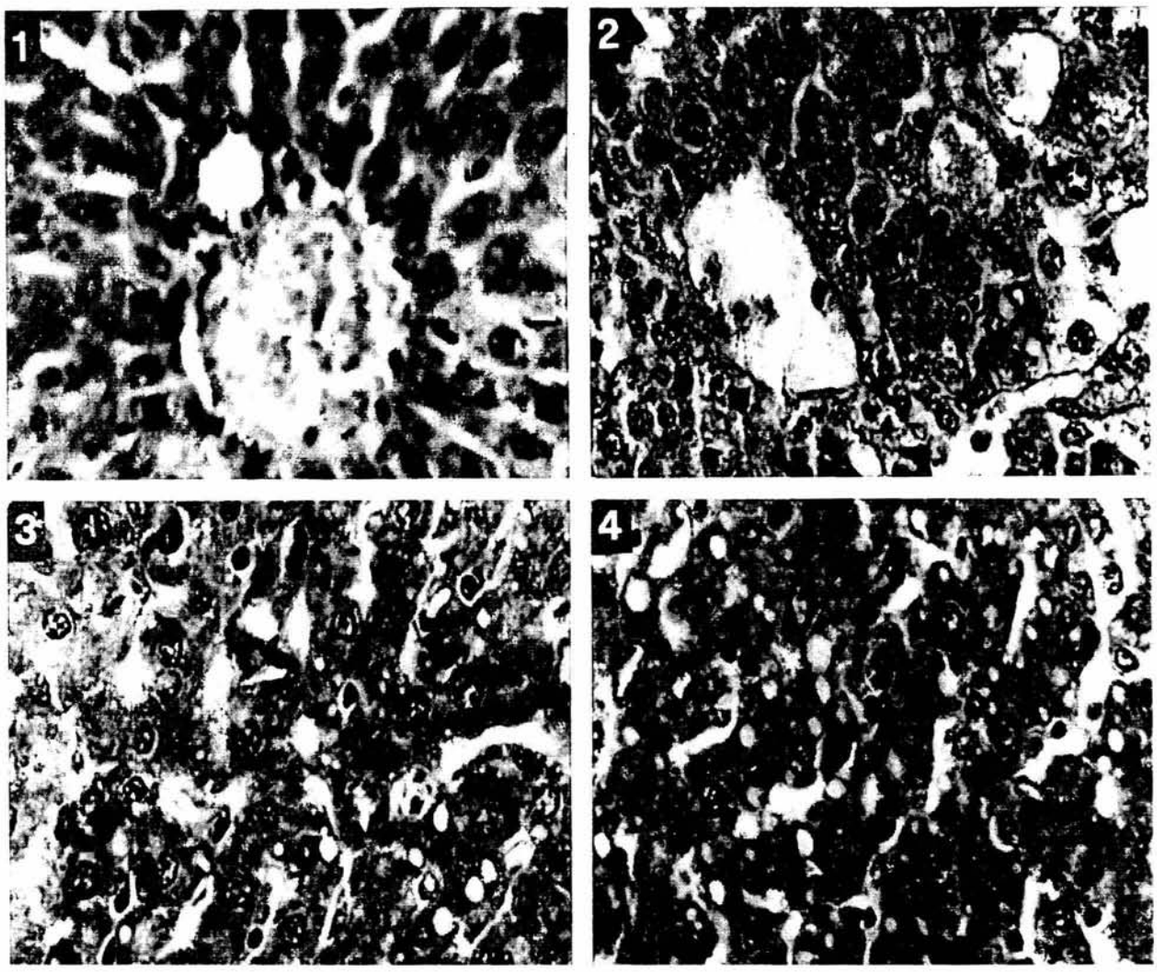
Discussion

CCl_4 is commonly used as model toxicant for the screening of hepatoprotective drugs. Raised activity of serum transaminases in intoxicated rats as found in present study can be attributed to the damaged structural integrity of the liver, because these are

Table 1—Effectiveness of propolis against carbon tetrachloride treated rats
[Values are mean ± SE, from 5 rats in each group]

Treatments	AST (IU/L)	ALT (IU/L)	Serum alkaline phosphatase (mg Pi /100ml /hr)	Hepatic lipid peroxidation (n moles MDA /mg protein)	Hepatic reduced glutathione (μ moles /g)
Control	65.5± 3.56	47.4± 3.85	206.0± 10.31	0.25± 0.01	8.01+ 0.43
Propolis *per se*	64.6± 3.56	54.0± 3.16	214.0± 11.27	0.30± 0.02	8.20+ 0.48
CCl_4	180.0± 12.54[b]	240.0± 16.42[b]	1077.0± 105.6[b]	1.50± 0.11[b]	4.32+ 0.42[b]
(Prophylactic study)					
Propolis (100 mg/kg) + CCl_4	163.0± 8.86	218.0± 12.30	842.2± 66.57	0.95± 0.06[d]	5.60+ 0.54
Propolis (200 mg/kg) + CCl_4	128.0± 11.42[c]	204.0± 11.07	613.0± 55.32[d]	0.82± 0.05[d]	6.10+ 0.33[c]
(Curative Study)					
CCl_4+ propolis (100 mg/kg)	107.0± 5.93[d]	170.0± 13.31[c]	406.0± 21.46[d]	0.64± 0.05[d]	6.96+ 0.48[d]
CCl_4+ propolis (200 mg/kg)	68.8± 4.49[d]	69.8± 5.35[d]	288.0± 15.16[d]	0.29± 0.01[d]	7.20+ 0.37[d]
F Variance at 5 % level	38.545*	63.933*	44.114*	67.367*	11.530*

* Significant *P* value CCl_4 Vs control at [b]<0.01; *P* value Drugs Vs CCl_4 at [c]< 0.05, [d]<0.01



Figs. 1-4—Effect of propolis on CCl_4 treated rats—(1) Photomicrograph of control rat liver. Showing normal chord arrangement well developed hepatocytes with Kupffer cells (H&E, 200×); (2) CCl_4 induced hypertrophy in hepatocytes after 24 hr (H&E, 200×); (3) Effect of propolis therapy after 24 hr on CCl_4 administered rats, showing recoupment in the structure but hypertrophy of nucleus was observed at some places (H&E, 200×); (4) Propolis treatment after 24 hr shows normal hexagonal hepatocytes (H&E, 200×).

cytoplasmic in location and are released into circulation after cellular damage[18]. Bolus doses of CCl_4 result in cell lysis and cytoplasmic hepatic enzymes are released into blood circulation[19]. Many fold increase of enzyme leakage as demonstrated by an increased level of serum enzymes ALT, AST and ALP has been noted, indicating liver cell damage by CCl_4[20]. Propolis prevents leakage of these enzymes and restoring the activity of enzymatic variables. These findings are also substantiated by studies with the treatment of *Ventilago leiocarpa*[21] and *Ginkgo biloba* [22].

Significant increase in hepatic LPO and decreased level of reduced GSH were observed after CCl_4 exposure. Carbon tetrachloride toxicity requires cleavage of the carbon-chlorine bond and cleavage takes place after binding of carbon tetrachloride to cytochrome P-450 apoprotein in the mixed function system located in the hepatocellular endoplasmic reticulum[23]. CCl_4 is metabolized into trichloromethyl radical ($CCl_3°$), which further reacts with molecular oxygen, resulting in the formation of trichloromethyl peroxy radicals ($CCl_3O_2°$)[24,25]. Trichloromethyl radical and trichloromethyl peroxy radical combine with cellular lipid and proteins to induce lipid peroxidation by hydrogen abstraction[26,27].

Lipid peroxidation (LPO) is oxidative deterioration of polyunsaturated lipids and it involves reactive oxygen species and transition metal ions. It is a molecular mechanism of cell injury leading to

generation of peroxides and lipidperoxides, which can be decomposed to yield a wide range of cytotoxin products, most of which are aldehydes, as exemplified by malondialdehyde (MDA), 4-hydroxynonenal etc.

Propolis extract inhibited lipid peroxidation significantly. The extract could reduce the extent of toxin induced lipid peroxidation. This is due to scavenging property of various free radicals that may have been generated within the system due to the presence of the toxin. It may be presumed that free radical scavengers present in the propolis may have a role in reducing the toxicities of CCl_4, caused by enhanced lipid peroxidation. In animal with impaired endogenous antioxidant defense, exogenous administration of the antioxidants may be helpful to counteract the toxic reaction of the CCl_4. In states of oxidative stress, reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) and depletion in it leads to lipid peroxidation. Therefore, the role of GSH as a reasonable marker for evaluation of oxidative stress is important as it acts as an antioxidant, both extracellularly and intracellularly, and is produced in the liver[28]. Propolis extract inhibited lipid peroxidation significantly and recovered the decreased hepatic GSH level induced by CCl_4 towards normal. Generation of malondialdehyde like substances are inhibited and delayed by the presence of the propolis extract during initiation and propagation phases of lipid peroxidation in serum[29].

Lipid peroxidation generated after CCl_4 intoxication is eliminated by the conversion of reduced GSH to oxidized GSH in presence of enzyme glutathione peroxidase, thus curbing the propagation of lipid peroxidation[30]. Administration of Turkish folk remedies[31] and *Salvia miltorrhize*[30] reduced CCl_4 induced hepatotoxicity that leads to depletion of hepatic glutathione.

Histopathological studies demonstrated that CCl_4 induces degenerative lesion, vacuolation, periportal fibrosis and fatty degeneration in hepatocytes. These findings are further supported by earlier reports[31, 32] showing degeneration in hepatocytes and hepatic cords. Focal necrosis, congestion in central vein and sinusoids, infiltration of lymphocytes and Kupffer cell proliferation were seen. Significant recoupment in histoarchitecture was seen with propolis administration at a dose of 200 mg/kg in post-treatment.

Propolis contains a wide variety of phenolic compounds[33], mainly flavonoids[34]. Propolis extract may hinder the formation of CCl_3 and CCl_3O_2 free radical and shows its hepatoprotective effectiveness. Propolis possesses antioxidative properties[35], which may inhibit deleterious effects of free radicals generated by CCl_4, influencing the membrane rigidity by inhibition of membrane peroxidation. Thus, it can be concluded that propolis extract possesses potent antioxidant and significant hepatoprotective activity with marked dose dependent effects, which may be due to presence of a variety of flavonoids.

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