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BEES WAX POLYPHENOLS AS SUPPRESSOR OF CCl_4-INDUCED OXIDATIVE STRESS IN RATS

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Abstract : Bee's wax produced by honeybees is rich in polyphenols. As the polyphenols are thought to protect cell constituents against oxidative damage through scavenging of free radicals, the present work was undertaken to evaluate the effects of polyphenols extracted from bees wax on the oxidative stress induced by carbon tetrachloride (CCl_4) in rats. The polyphenols extracted by 80% methanol from bee wax (PBW) were fed to Wistar rats at 100 mg/kg body weight and 200 mg/kg body weight for 14 days in order to study its antioxidative and antihepatotoxic effects against CCl_4 (1.5 ml/kg body weight) -induced stress. On 15th day all the rats were sacrificed, blood was collected for serum and organs/tissues were excised for biochemical analysis. The results showed a significant decrease in hepatic antioxidant enzyme activities viz. catalase, glucose-6-phosphate dehydrogenase (G-6-PDH), glutathione peroxidase (GSH-Px), glutathione reductase, superoxide dismutase (SOD) and a significant increase in glutathione S-transferase (GST) and γ-glutamyl transpeptidase (GGT) by CCl_4, probably due to the peroxidative effects. The prophylactic use of PBW at 200 mg/kg level resulted in a significant increase in CCl_4-induced reduction in catalase, G-6-PDH, GSSGR and SOD. The hepatic levels of lipid peroxides viz. malondialdehyde, conjugated dienes and lipid hydroperoxides, enhanced by the administration of CCl_4 were brought down by the ingestion of PBW at a level of 200 mg/kg. The hepatotoxicity caused by the administration of CCl_4 was reduced significantly. Hence, it is concluded that the polyphenols from bees wax exhibit hepatoprotective and antioxidative properties in rats.

Key words : bee wax, polyphenols, oxidative stress, antioxidants

INTRODUCTION

Honey has been used since ancient times and has gained appreciation as the only concentrated form of sugar available worldwide. The wax secreted by the wax glands of bee is a result of the digestion of carbohydrates. Bees wax is a valuable byproduct of bee keeping industry and is used in the cosmetic industry for the

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manufacture of cold creams, lipsticks, lip pomades and lotions. It is also used in folk medicine (1) and its use in pharmaceutical industry is increasing probably due to its rich content of polyphenols and other phytoconstituents. Polyphenols are thought to protect cell constituents against oxidative damage through scavenging of free radicals (2). Polyphenolic compounds have a wide range of beneficial effects such as anti-inflammatory responses, prevention of low-density lipoprotein oxidation, antihypertensive, antioxidant, antithromobtic neuroprotective and anticarcinogenic actions (3, 4). Propolis, a honeybee product produced from the waxes of *Apis mellifera,* a nonexisting bee's species in India, is rich in more than 150 polyphenolic compounds (5). The water extract of propolis shows hepatoprotective activity and a wide spectrum of activities such as anticancer, antioxidant, antimicrobial, anti-inflammatory and antibiotic activity (6–9). The information on the polyphenols of wax produced by *Apis dorsetta* is sparse. Hence we wanted to extract polyphenols from the bees, *Apis dorsetta* and study its effect against oxidative stress induced by carbon tetrachloride (CCl_4). Carbon tetrachloride is selected in this work as it is generally used as model toxicant for screening of antioxidative and hepatoprotective drugs.

MATERIAL AND METHODS

Extraction of polyphenols from bees wax

Honey bee wax obtained from colonies of *Apis dorsetta* available at Bilirangan Hills, Karnataka, India and was processed using following procedure:

After removing honey by draining, the comb was melted in hot water. The wax was allowed to accumulate on the water surface, filtered to remove gross impurities viz. dead bees, cocoons etc, and allowed to solidify in moulds.

Polyphenols were extracted from bees wax by the method of Xia et al (10). About 100 g of the bees wax was mixed with 2 volumes of 80% methanol and kept for 5 days at room temperature. After 5 days, it was filtered and evaporated under the hood. The residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with 1% glacial acetic acid prepared in ethyl acetate. Extraction of polyphenols was carried out for 36 hours at room temperature and the combined ethyl acetate layer was concentrated. The yield was found to be 0.2%. The residue dissolved in water was used for *in vivo* studies.

Experimental schedule

Male Wistar rats (110 g–130 g) were allocated randomly into six groups, consisting of six animals in each. The grouping of rats is as given below:

Group I : Treated as control

Group II : Treated with CCl_4 (1.5 ml/kg body weight)

Group III : Treated with 100 mg PBW/kg body weight

Group IV : Treated with 100 mg PBW/kg body weight + CCl_4 (1.5 ml/kg body weight)

Group V : Treated with 200 mg PBW/kg body weight

Group VI : Treated with 200 mg PBW/kg body weight + CCl_4 (1.5 ml/kg body weight)

All the rats were housed in individual stainless steel, wire-bottomed cages at a temperature of 27±2°C, and fed *ad libitum* with free access to the laboratory stock diet and water. The polyphenols extracted from bees wax (1.0 ml) was administered to groups III, IV, V and VI by gavage for 14 days. Weekly food intake and weight gain were monitored. After 24h of the last treatment, CCl_4 (1.5 ml/kg body weight) was administered i.p. to groups II, IV and VI while groups I, III and V were injected with saline. All the rats were sacrificed after 24h of CCl_4/saline injection, under mild anesthesia (sodium pentobarbitone, 50 mg/kg body weight i.p.). Blood was collected by cardiac puncture. The organs were quickly excised and stored in liquid nitrogen until analysis. Clearance of experimental design by Institutional Ethical Committee for rats was taken.

Chemical analysis

The flavonoids (11) in the extract of polyphenols from bees wax were estimated according to prescribed methods. For the assay of malondialdehyde (MDA), liver homogenate (0.5 g) was precipitated with trichloroacetic acid (10%) and assayed as reported earlier (12). The lipid isolated from liver was used to assay the amount of conjugated dienes (12) and the upper layer of the same assay was utilized for the estimation of hydroperoxides (13). The content of ascorbic acid on liver was determined by the method of Roe and Keuther (14) using 2, 4-dinitrophenyl hydrazine. Lipids isolated from liver were used for estimation of tocopherol (15). Activities of glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transminase (SGPT) and alkaline phosphate (ALP) in serum were assayed as described earlier (16). Hepatic glutathione (GSH) content was determined by the method of Ellman (17) using 5, 5'–dithiobis-2-nitrobenzoic acid reagent. For the assay of catalase, liver was homogenized in phosphate buffer and assayed (18). Hepatic glutathione reductase (GSSGR) and GSH-Px activities were determined by the method of Weiss et al (19). Hepatic glutathione S-transferase (GST) activity was determined by the procedure of Habig et al (20). Superoxide dismutase was measured by the inhibition of cytochrome C reduction mediated via superoxide anions generated by xanthine-xanthine oxidase and monitored at 550 nm. One unit of SOD was defined, as the amount required to inhibit the reduction of cytochrome C by 50% (21). The assay mixture for the estimation of hepatic glucose-6-phosphate dehydrogenase (G-6-PDH) consisted of the enzyme source prepared in 0.1 mM of Tris buffer, 2 mM of glucose-6-phosphate, and 0.3 mM of NADP and ÄA was monitored at 340 nm (22). Gamma glutamyl transpeptidase (GGT) was estimated by the method of Meister et al (23). Protein in tissues was determined according to Lowry et al (24). Statistical analysis was carried out by Duncan's multiple comparison tests using one-way analysis of variance (ANOVA). Significance of differences between mean values was determined at 5% level.

RESULTS

The absolute weights of liver, kidney,

heart and brain were not affected by the oral feeding of polyphenols or by the i.p. injection of CCl_4 (Table I).

Table II presents the hepatic levels of GSH, ascorbic acid, tocopherol, conjugated dienes, hydroperoxides and MDA in the rats. The CCl_4 administration resulted in a decrease in GSH; an increase in MDA, conjugated dienes, and hydroperoxides. There was no change in ascorbic acid and total tocopherol content. The oral feeding of PBW did not produce any change in hepatic peroxides and antioxidant contents viz. GSH, ascorbic acid and tocopherol. The PBW at both the levels of administration resulted in decreased MDA, conjugated dienes and hydroperoxides with enhanced ascorbic acid content in the co-administered group (PBW + CCl_4). The CCl_4-induced reduction in hepatic GSH was attenuated significantly by co-administration of PBW (200 mg/kg body weight).

The data for the effect of PBW and CCl_4 on hepatic transminase activities viz. SGOT, SGPT and alkaline phosphatase are given Table III. There was significant increase in these enzyme activities while no change was noted with the oral ingestion of PBW. It was interesting to note that the both the levels of PBW intake brought down the

TABLE I: Effect of PBW and CCl_4 treatments on rat organ weights (g/100 g body weight).

Rat Groups	*Liver*	*Kidney*	*Heart*	*Brain*
Control	3.50±0.341	0.879±0.081	0.354±0.041	0.932±0.097
CCl_4-alone	3.88±0.371	0.842±0.091	0.335±0.032	0.879±0.091
PBW (100 mg/kg)	333±0.320	0.962±0.093	0.380±0.036	1.008±0.123
PBW (100 mg/kg) + CCl_4	3.88±0.401	0.965±0.082	0.360±0.035	0.892±0.090
PBW (200 mg/kg)	4.06±0.540	0.905±0.091	0.370±0.032	0.863±0.085
PBW (200 mg/kg) + CCl_4	3.31±0.353	0.810±0.079	0.334±0.041	0.803±0.070

Dose of CCl_4: 1.5 ml/kg body weight. Values are Mean±SD of 6 rats in each group.

TABLE II: Effect of PBW and CCl_4 treatments on hepatic antioxidants and lipid peroxides.

Rat Groups	*GSH mmol/g*	*MDA n mol/g*	*Conjugated dienes µ mol/g*	*Lipid hydroperoxides µ mol/g*	*Ascorbic acid mg/g*	*Tocopherol mg/g*
Control	9.44±0.810	8.31±0.090	0.261±0.052	0.194±0.032	0.425±0.041	20.29±3.29
CCl_4 -alone	5.88±0.591*	42.67±3.921*	0.719±0.063*	0.673±0.033*	0.442±0.050	19.56±2.79
PBW (100 mg/kg)	9.05±0.802	8.04±0.901	0.202±0.072	0.194±0.041	0.300±0.014*	18.88±1.75
PBW (100 mg/kg) + CCl_4	5.80±0.562*	28.91±2.631#*	0.493±0.074#*	0.496±0.060# *	0.530±0.032#*	19.63±3.30
PBW (200 mg/kg)	9.35±0.940	7.82±0.830	0.196±0.017	0.194±0.030	0.420±0.400	17.14±2.59
PBW (200 mg) + CCl_4	7.45±0.761*#	12.01±1.191#*	0.534±0.072#*	0.467±0.056#*	0.516±0.031#*	19.84±1.94

Dose of CCl_4: 1.5 ml/kg body weight. Values are Mean±SD of 6 rats in each group. *indicates P<0.05 as compared to Control; # indicates P<0.05 as compared to CCl_4 alone. (Statistical test: Duncan's multiple comparisons Test using ANOVA).

TABLE III: Effect of PBW and CCl_4 treatments on hepatic SGOT, SGPT and alkaline phosphatase activities.

Rat Groups	*SGOT ΔA/min/l*	*SGPT ΔA/min/1*	*Alkaline phosphatase (KA units)*
Control	11.42±1.39	0.70±0.11	100.5±06.2
CCl_4-alone	26.13±2.01*	6.54±0.83*	258.0±19.5*
PBW (100 mg/kg)	12.00±1.93	0.83±0.10	103.5±09.8
PBW (100 mg/kg) + CCl_4	12.02±2.55*	4.73±0.32*	178.7±19.1*
PBW (200 mg/kg)	11.18±2.11	0.86±0.06	108.5±10.3
PBW (200 mg/kg) + CCl_4	14.15±2.09*	1.94±0.13*	139.2±15.69*

Dose of CCl_4: 1.5 ml/kg body weight. Values are Mean±SD of 6 rats in each group. *indicates $P<0.05$ as compared to Control; (Statistical test: Duncan's multiple comparisons Test using ANOVA).

TABLE IV: Effect of PBW and CCl_4 treatments on hepatic antioxidant enzymes.

Rat Groups	*Catalase × 10^4 ΔA of 0.1/ min/mg protein*	*GSH-Px mmol NADP/ min/mg protein*	*GSSGR mmol NADP/ min/mg protein*	*SOD × 10^2 Unit/ min/mg protein*	*G-6-PDH μmol NADP reduced/ min/mg protein*	*GST mmol conjugate min/mg/ protein*	*GGT nmol p-NA/ min/mg protein*
Control	0.91±0.08	5.63±0.42	3.96±0.28	1.92±0.17	9.53±1.64	1.15±0.16	0.88±0.081
CCl_4-alone	0.50±0.03*	3.28±0.43*	2.24±0.21*	0.88±0.09*	4.26±0.68*	2.83±0.12*	1.49±0.065*
PBW (100 mg/kg)	1.32±0.15*	5.58±0.47	4.70±0.33*	2.02±0.03	19.04±1.00*	1.52±0.24	0.83±0.095
PBW (100 mg/ kg) + CCl_4	0.48±0.06*	3.18±0.34*	4.28±0.32#	0.84±0.07*	9.39±1.08#*	2.17±0.20*	1.48±0.050*
PBW (200 mg/kg)	1.29±0.10	6.87±0.52	5.08±0.46	1.95±0.02	28.60±3.55	1.45±0.14	0.78±0.085
PBW (200 mg/ kg) + CCl_4	1.21±0.12*#	3.44±0.45*#	4.80±0.38*#	1.31±0.11*#	10.08±1.61*#	1.89±0.25*#	1.41±0.040*#

Dose of CCl_4: 1.5 ml/kg body weight. Values are Mean±SD of 6 rats in each group. *indicates $P<0.05$ as compared to Control; #indicates $P<0.05$ as compared to CCl_4-alone. (Statistical test: Duncan's multiple comparisons Test using ANOVA). p-NA=p-nitroanilide.

CCl_4-induced increase in transaminases significantly.

The effect of pretreatment of PBW on CCl_4-induced changes on hepatic antioxidant enzymes has been given in Table IV. There was a significant decrease in hepatic G-6-PDH, catalase, GSH-Px, GSSGR and SOD activities by CCl_4 as compared to control group of rats. PBW at both the levels of administration significantly increased the catalase and G-6-PDH activites. Co-administration of both the levels of PBW and CCl_4 resulted in a significant increase in activities of G-6-PDH and GSSGR as compared to CCl_4 exposed rats. At the same time the catalase and SOD activities were increased significantly only in the co-administered group of rats exposed to the higher levels of PBW and CCl_4. There was a significant increase in hepatic GST and GGT activities by the administration of CCl_4. PBW *per se* did not influence the hepatic GST and GGT activities. There was no change in GGT activity in the liver of rats administered with PBW; the increase observed was due to the injection of CCl_4 only. The GST activity was reduced significantly due to the co-

administration of PBW (200 mg/kg body weight) and CCl_4 as compared to CCl_4 injected rats.

DISCUSSION

Carbon tetrachloride is generally used as a hepatotoxic model to screen hepato protective drugs. Enhanced activities of serum transaminases in CCl_4-intoxicated rats as observed in the present study can be attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage (25). The increase in these enzyme leakages as demonstrated by increased levels of serum SGOT, SGPT and alkaline phosphatase has been noted, indicating liver cell damage by CCl_4 (26). The extract of polyphenols prevents leakage of these enzymes and restoring the activity of enzymatic variables. These findings are also substantiated by studies with *Ginko biloba* (27) and propolis (28).

The administration of CCl_4 reduced the hepatic GSH content with a concomitant increase in MDA, conjugated dienes and hydroperoxides. The CCl_4 administration resulted in increased MDA, conjugated dienes, hydroperoxides with decrease in GSH. GSH can be depleted either by a conjugation reaction with electrophiles or by inhibition of GSH generated from the oxidized GSSG and or its biosynthesis. PBW was found to reduce these adverse effects implying that polyphenols from the wax may be capable of reducing the CCl_4-induced lipid peroxidation. There is an increased hepatic G-6-PDH activity by the PBW intake. This might help in achieving a proper GSH status by coupling with $NADP^+$/NADPH redox pair in the HMP shunt pathway.

Carbon tetrachloride produced significant reduction in catalase, GSH-Px, GSSGR, SOD and G-6-PDH with a significant elevation in GST and GGT activities. The endogenous antioxidant defence includes the enzymatic (SOD, catalase, and peroxidase) and non-enzymatic (ascorbic acid, tocopherol, GSH, etc.) (29). Hence the enhanced catalase, GSSGR and SOD activities in the liver of co-administered group of rats vis-à-vis CCl_4 administered rats might provide nucleophilic environment to the cell for providing protection.

GSTs are a group of enzymes that catalyze the binding of various electrophiles to GSH to form conjugates and it has an integral role in the metabolism of xenobiotics (29). The PBW (200 mg/kg body weight) intake reduced the CCl_4-induced raise in hepatic GST activity. Abnormally high levels of GGT have been observed in cells of a variety of hepatocellular toxicity and carcinomas. However, the PBW did not modulate the CCl_4-induced rise in hepatic GGT activity.

The extract used in this study was found to contain 8% of flavonoids. It has been demonstrated that flavonoids can scavenge singlet oxygen and terminate peroxidation and they are therefore considered as free radical scavengers. Lipid peroxidation together with the covalent binding of CCl_4 reactive metabolites to lipids and proteins is a critical process underlying CCl_4-hepatotoxicity.

The partial restoration of lipid peroxidation seen when pretreated with PBW

might be due to its capacity to scavenge free radicals and improve cellular thiols, thereby elevating the redox status of the cells. Hence, it is suggested that the polyphenols extracted from bees wax possesses antioxidative and hepatoprotective properties. Further studies are warranted to characterize the type of polyphenols present in the extract.

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