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RESEARCH ARTICLE

Hepatoprotective Efficacy of Propolis Extract: A Biochemical and Histopathological Approach

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ABSTRACT

Hepatoprotective efficacy of propolis extract (honeybee hive product, 200 mg/kg, p.o.) was studied against biochemical and histopathological changes induced by carbontetrachloride (CCl_4, 0.15 ml/kg, i.p.). Silymarin, a known hepatoprotective drug was used as positive control. Subchronic exposure to CCl_4 for 3 weeks (5 days a week) caused sharp elevation in the activity of liver marker enzymes *i.e.*, serum transaminases, alkaline phosphatase and lactate dehydrogenase. CCl_4 administration significantly decreased blood glucose level and increased serum proteins. Tissue biochemistry revealed significant reduction in total protein and glycogen contents, activity of alkaline phosphatase and adenosine triphosphatase; and significant increase in acid phosphatase activity in liver and kidney. CCl_4-induced oxidative stress was measured by estimating reduced glutathione level and amount of thiobarbituric acid reactive substances (TBARS) formed as an index of lipid peroxidation. Hepatorenal glutathione level showed marked depletion, whereas lipid peroxidation was enhanced significantly. A 5-day treatment of propolis extract after toxicant administration reversed alterations in blood and tissue biochemical variables including liver function test and markers of oxidative stress almost as same as in silymarin-treated positive control. Histopathological studies of liver and kidney showed improved cellular architecture after propolis therapy and confirmed its hepatoprotective efficacy as a natural miracle.

Keywords: *Propolis, Carbon tetrachloride, Hepatoprotective efficacy, Lipid peroxidation, Liver function tes*t

In recent years, studies are increasing in the field of free-radical-induced oxidative damage in human diseases [1]. Free radicals have been shown to modify biological molecules, which may result in various pathological conditions [2]. Various antioxidants, vitamins and other natural products are being investigated to encounter oxidative events [3]. Thus, identification of naturally-occurring inhibitors of peroxidation to be taken in diet can lead to important strategies for disease prevention. Plant-derived natural products have received considerable attention in recent years due to their diverse pharmacological actions including antioxidant and hepatoprotective activity [4-5]. Antioxidants play an important role in inhibiting and scavenging oxidative and peroxidative radicals; providing protection against free radicals adverse effects. Realizing this fact, propolis was taken into account as a natural product for its emerging hepatoprotective efficacy.

Propolis is prepared by honeybees from plant materials. It mainly contains sticky plant substances mixed with bee wax and other bee secretions [6]. It has gained popularity as a healthy food and is used extensively in food and beverages in various part of world including Japan, United States of America and Europe and it is believed that it can cure heart disease [7] and even cancer [8]. Propolis balsam (70% alcoholic extract of propolis) is used as a popular herbal medicine [9] and still used as a remedy in folk medicine as a constituent of 'bio-cosmetic' and 'health food' [10]. It is an extremely complicated mixture of substances from the plant kingdom and the only food on the earth containing 22 nutrients needed by body for perfect health [11]. Esters of phenolic acids and flavonoids have been identified as its main constituents having antibacterial, antiviral, antifungal, antioxidant and free radical scavenging activities [12-15]. Caffeic acid phenethyl ester (CAPE) is one of the main components of ethanolic extract of propolis, which is reported to prevent cisplatin-induced nephrotoxicity [16] and inhibits the growth of different types of transformed cells [17]. We have already reported the dose-dependant hepatoprotective activity of propolis extract against CCl_4-induced acute liver damage [18, 19]. In the present study, an attempt has been made to confirm hepatoprotective efficacy of propolis against

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Table 1. Efficacy of propolis extract on hepatorenal total protein and glycogen contents against carbon-tetrachloride (CCl4)-exposed rats.[Values are mean ± SE from six rats in each group]

Groups	Total protein contents (mg /100 mg)		Glycogen contents (mg /100 g)	
	Hepatic	Renal	Hepatic	Renal
Control	15.6 ± 0.86	14.1 ± 0.77	3190 ± 176	87.3 ± 4.82
CCl_4 (without rest)	11.1 ± 0.61[b]	12.2 ± 0.67	1076 ± 59.4[b]	59.7 ± 3.30[b]
CCl_4 (with rest)	11.6 ± 0.64[b]	12.8 ± 0.70	1156 ± 63.9[b]	63.5 ± 3.51[b]
CCl_4 + P200	14.2 ± 0.78[c]	13.8 ± 0.76	2999 ± 165[d]	78.4 ±4.33[c]
% Protection	*65.00%*	*76.92%*	*90.60%*	*62.60%*
CCl_4 + S50	14.1 ± 0.77[c]	13.6 ± 0.75	3004 ± 166[d]	81.3 ± 4.49[c]
% Protection	*62.50%*	*61.53%*	*90.85%*	*74.78%*
df value	4, 25	4, 25	4, 25	4, 25
F Variance	7.876*	1.350ns	73.287*	9.900*

*Significant; [ns]Not significant; Analysis of variance*F=$p \leq 0.05$
[b]p value ≤0.01 for CCl4-treated vs control; [c]p value ≤0.05 and [d]p value ≤0.01 for Drugs vs CCl4
Abbreviations: CCl_4= Carbon tetrachloride; P200= Propolis (200 mg/kg); S50= Silymarin (50 mg/kg)

biochemical and histopathological alterations induced by subchronic exposure to CCl_4.

MATERIALS AND METHODS

Animals and chemicals

Female albino rats of Sprague-Dawley strain (9-12 weeks old having 140±10 g body wt) were used in the study. They were housed under standard husbandry conditions (25±2°C temp, 60-70% relative humidity and 12 h photoperiod) and had access to standard rat feed and drinking water *ad libitum.* Experiments were conducted in accordance with the guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India. Experimental protocols were approved by the Institutional Ethical Committee (CPCSEA/501/01/A) of Jiwaji University, Gwalior, India. CCl_4, silymarin and other chemicals were procured from Sigma-Aldridge Company, Ranbaxy, New Delhi and Himedia Laboratories Ltd. Mumbai, India.

Induction of hepatorenal injury

Carbon tetrachloride (0.15 ml/kg, i.p.) was mixed with liquid paraffin [20] and animals were exposed to CCl_4 for a period of 3 weeks (5 days/week) to induce subchronic injury. Control animals received equal amount of liquid paraffin as vehicle.

Preparation of extract

Crude propolis from the hive of *Apis mellifera* was obtained by Prof. O.P. Agrawal, Senior Entomologist, School of Studies in Zoology, Jiwaji University, Gwalior (India). About 90% ethanolic extract was obtained as described previously [18]. Yield of dried residue was 61.4% (w/w) and kept at 4°C for further use. Aqueous suspension of propolis was prepared in 1% gum acacia solution (GAS) and selected optimum dose was administered (200 mg/kg, p.o.) to the animals on

Table 2. Hepatoprotective potential of propolis extract on the activities of hepatorenal adenosine triphosphatase, acid phosphatase and alkaline phosphatase against carbon tetrachloride administered rats. [Values are mean + SE from six rats in each group]

Groups	Adenosine triphosphatase (mg Pi /100mg /min)		Acid phosphatase (mg Pi /100mg /h)		Alkaline phosphatase (mg Pi /100mg /h)	
	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal
Control	2000 ± 110	2400 ± 132	240 ± 13.2	272 ± 15.0	75.9 ± 4.19	2600 ± 143
CCl_4 (without rest)	1288 ± 71.2[b]	1035 ± 57.2[b]	438 ± 24.2[b]	492 ± 27.1[b]	45.6 ± 2.52[b]	1382 ± 76.3
CCl_4 (with rest)	1456 ± 80.4[b]	1155 ± 63.8[b]	417 ± 23.1[b]	480 ± 26.5[b]	48.4 ± 2.67[b]	1564 ± 86.4
CCl_4 + P200	1780 ± 98.4[c]	1814 ± 100[d]	305 ± 16.8[d]	330 ± 18.2[d]	72.5 ± 4.00[d]	2237 ± 123
% Protection	*59.55%*	*52.93%*	*63.27%*	*72.11%*	*87.63%*	*64.96%*
CCl_4 + S50	1805 ± 99.7[c]	1926 ± 106[d]	298 ± 16.4[d]	318 ± 17.5[d]	73.1 ± 4.04[d]	2383 ± 131
% Protection	*64.15%*	*61.92%*	*67.23%*	*77.88%*	*89.81%*	*79.05%*
df value	4, 25	4, 25	4, 25	4, 25	4, 25	4, 25
F Variance	11.432*	41.672*	23.133*	26.302*	20.654*	25.429*

*Significant; Analysis of variance*F=$p \leq 0.05$,
[b]p value ≤0.01 for CCl_4-treated vs control; [c]p value ≤0.05 and [d]p value ≤0.01 for Drugs vs CCl_4
Abbreviations: CCl_4= Carbon tetrachloride; P200= Propolis (200 mg/kg); S50= Silymarin (50 mg/kg)

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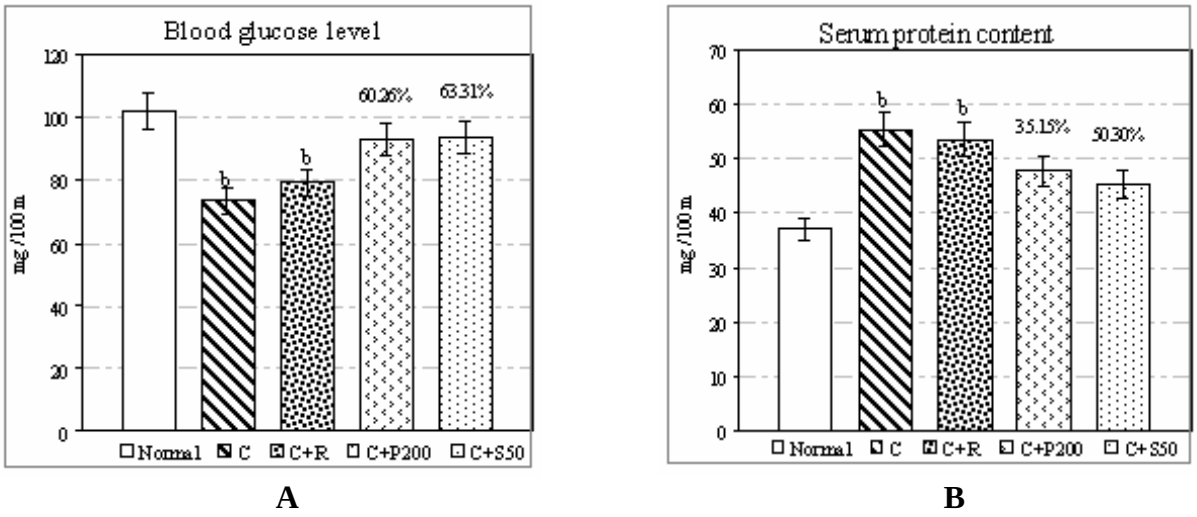


Fig 1. Blood sugar (A) and serum protein (B) of groups: normal, CCl_4 without rest (C), CCl_4 with rest (C+Rest), CCl_4+Propolis (C+P200) and CCl_4+Silymarin (C+S50). For each group n=6. ^{b}p values ≤0.01 for CCl_4-treated vs control group were considered significant based on ANOVA at 5% *using df* value 4, 25; blood sugar=6.725*, serum protein=8.933*. Percent protection by propolis and silymarin treatment is represented on their related bars.

the basis of our previous study [19]. Silymarin (50 mg/kg), a known hepatoprotective agent was administered as positive control. Experimental and control animals received equal amount of GAS (5 ml/kg) as vehicle.

Experimental procedure

Female rats were divided into five groups of six animals each. Group 1 received liquid paraffin and 1% GAS as vehicles and served as normal control. Groups 2-5 were administered CCl_4 (0.15 ml/kg, i.p.) for 3 weeks (5 days/week). Animals of group 2 were sacrificed after toxicant administration and treated as experimental control-I (without rest). Animals of group 3 received 1% GAS for 1 week after toxicant administration and were designated as experimental control-II (with rest). Groups 4 and 5 were orally administered propolis (200 mg/kg) and silymarin (50 mg/kg) respectively for 1 week (5 days/week) after CCl_4 administration. Animals of all the groups were sacrificed after 48 h of the last treatment.

Blood biochemistry

Blood samples were collected from retro-orbital venous sinus [21] and used for estimation of blood glucose level [22]. Briefly, 0.1 ml of blood was taken in 3.8 ml of isotonic solution and 0.1 ml sodium tungstate (10%) was added immediately followed by centrifugation at 2000 rpm for 10 min. Then, 1 ml of supernatant and 1 ml of alkaline tartarate solution were added and incubated in boiling water bath for 10 min. After cooling, 3 ml of phosphomolybdic acid and 3 ml of distilled water were added and optical density was noticed at λ680 nm against blank. Blood samples were allowed to stand at room temperature for 30 min and serum was isolated by centrifugation at 2000 rpm for 15 min and used for estimation of protein [23], transaminases (AST and ALT) [24], alkaline phosphatase (SALP) [25] and lactate dehydrogenase (LDH) [26].

For serum transaminases, 0.5 ml of AST and ALT substrates were incubated for 5 min, followed by adding 0.1 ml of serum (for experimental tubes) and further incubated for 60 and 30 min for AST and ALT respectively at 37°C. Equal amount of serum, working standard and distilled water were added respectively in control, standard and blank tubes. Dinitrophenylhydrazine (DNPH) was allowed to react at room temperature in all the tubes for 20 min. 5 ml of NaOH (0.4N) was added followed by incubation for 10 min at room temperature and absorbance was recorded at λ510 nm against blank. SALP activity was measured using sodium potassium phosphate ($NaK\text{-}PO_4$) as standard. Freshly-prepared amino naphthol sulfonic acid (ANSA) reagent was used to produce blue colored complex with phosphate group of enzymes and absorbance was recorded at λ620 nm.

For LDH measurement, 1.0 ml buffered substrate was mixed with 0.1 ml serum and incubated at 25°C in water bath. After 2 minutes, 0.1 ml NADH solution was added and further incubated for 15 min followed by addition of 1.0 ml DNPH solution. Buffered substrate (1.0 ml), phosphate buffer (0.2 ml) and DNPH (1.0 ml) were added to control tubes. Phosphate buffer (1.2 ml) was added with DNPH solution (1.0 ml) in blank tubes. All tubes were allowed to stand at room temperature for 20 minutes, and then 10.0 ml NaOH (0.4N) was added. Produced color was compared after 10 min at λ510 nm against blank.

Tissue biochemistry

Immediately after necropsy, liver and kidney were excised, rinsed in ice cold normal saline and blotted to dry for tissue biochemical estimations. Fresh tissues were processed for estimation of glycogen contents using anthrone reagent [27]. Pre-weighed small pieces of liver and kidney were dropped in 1 ml of 30% KOH followed by digestion in water bath for 10 min. Extraction process involved repetitive addition of 1.25 ml of 95% ethanol. Contents were mixed thoroughly and

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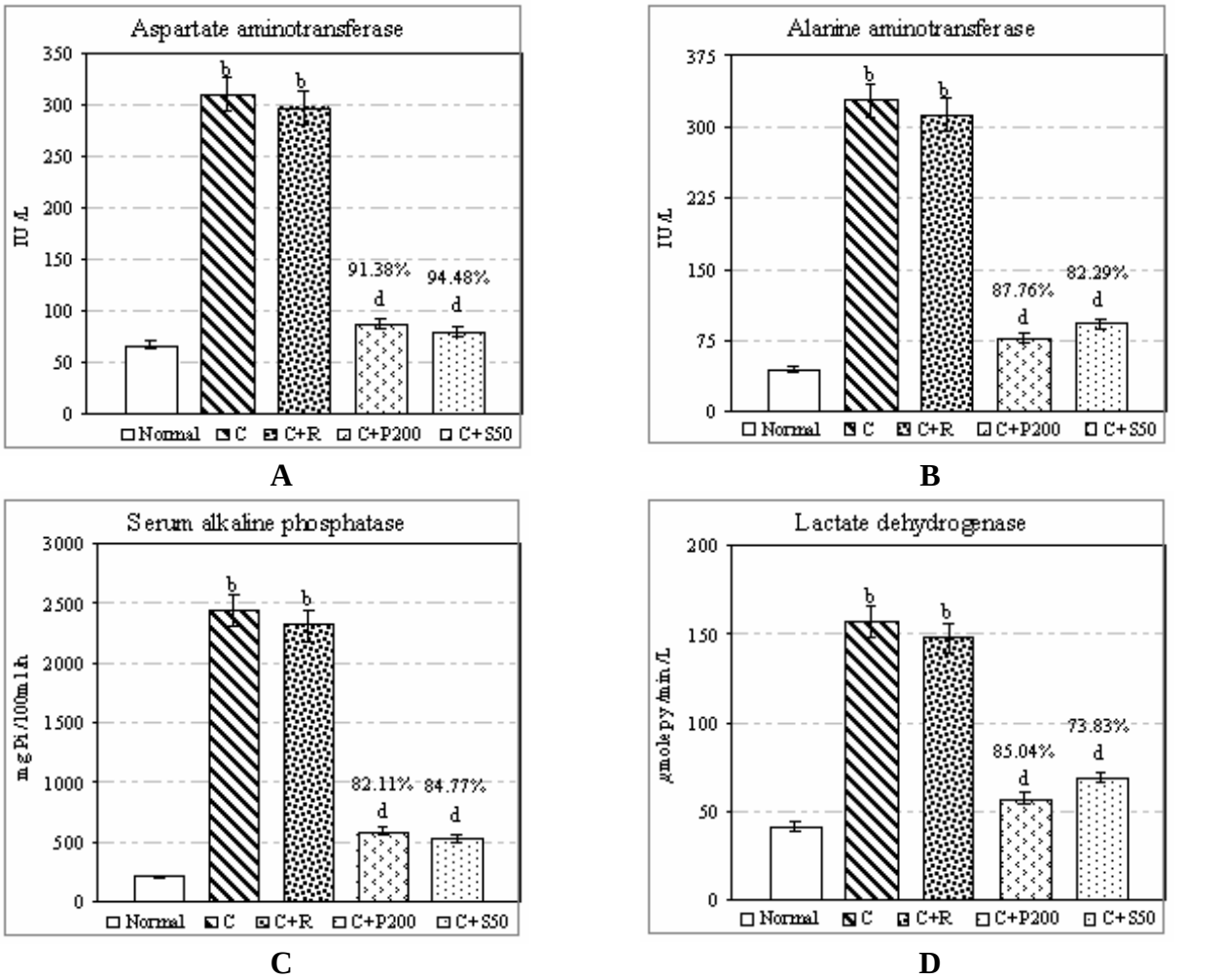


Fig 2. Aspartate aminotranfearse (AST), alanine transferase (ALT), serum alkaline phosphatase (SALP) and lactate dehydrogenase (LDH) of groups: normal, CCl_4 without rest (C), CCl_4 with rest (C+Rest), CCl_4+Propolis (C+P200) and CCl_4+Silymarin (C+S50). For each group n=6 ^{b}p value $\leq$0.01 for CCl_4-treated vs control, ^{d}p value $\leq$0.01 for Drugs- vs CCl_4 treated were considered significant. *Significant for ANOVA at 5% using *df* value 4, 25; AST=149.01*, ALT=168.08*, SALP=187.32*, LDH=101.99*. Percent protection by propolis and silymarin treatment is represented on their related bars.

brought to boil in a water bath and finally centrifuged at 3,000 rpm for 20 minutes. Precipitation process was repeated and the sedimented glycogen was dissolved in 5 ml of distilled water. Then, 10 ml of freshly prepared anthrone reagent (2%) was added and tubes were incubated for 10 min at 100°C to produce a green-colored complex and absorbance was recorded at λ620 nm.

Tissue homogenates (10%) of liver and kidney were prepared in chilled hypotonic solution for estimation of total protein contents and other enzymatic variables. Tissue and serum proteins were precipitated by adding 10% trichlroacetic acid (TCA) and finally colour was developed in dark by Folin's reagent. Bovine serum albumin (BSA) was used as standard and absorbance was read at λ625 nm [23].

Activities of adenosine triphosphatase (ATPase) [28], acid phosphatase and alkaline phosphatase (ACPase/ ALPase) [29] were measured using $NaKPO_4$ as standard. For assessment of ACPase and ALPase, 0.1 ml of triton-X and 0.5 ml of homogenate were added in 4.0 ml of acid/ alkaline buffer and incubated at 37°C for 1 h. About 0.5 ml TCA (30%) was added before and after incubation in control and experimental tubes respectively. Centrifugation was carried out at 2000 rpm for 15 min and supernatants were collected. About 2 ml of distilled water and $NaKPO_4$ were used for blank and standard respectively. About 2 ml supernatant, 6.6 ml distilled water and 2 ml ammonium molybdate were added with 0.4 ml ANSA solution and absorbance was noted at λ620 nm against blank after 10 minutes of incubation. For ATPase, 0.2 ml of tris buffer (*p*H 7.4) and 0.2 ml of ATP (0.1M) were added into 0.05 ml homogenate and volume was made up to 2 ml with distilled water followed by incubation at 37°C for 15 minutes. The reaction was stopped by adding 1 ml of TCA (10%). After centrifugation at 2000 rpm for 15 min, supernatant was processed similarly as in the case of ACPase/ ALPase.

Study of oxidative stress

Lipid peroxidation (LPO) [30] and reduced glutathione (GSH) [31] contents were measured to assess oxidative stress in tissue samples. For estimation of hepatorenal LPO, 10% homogenates were prepared in

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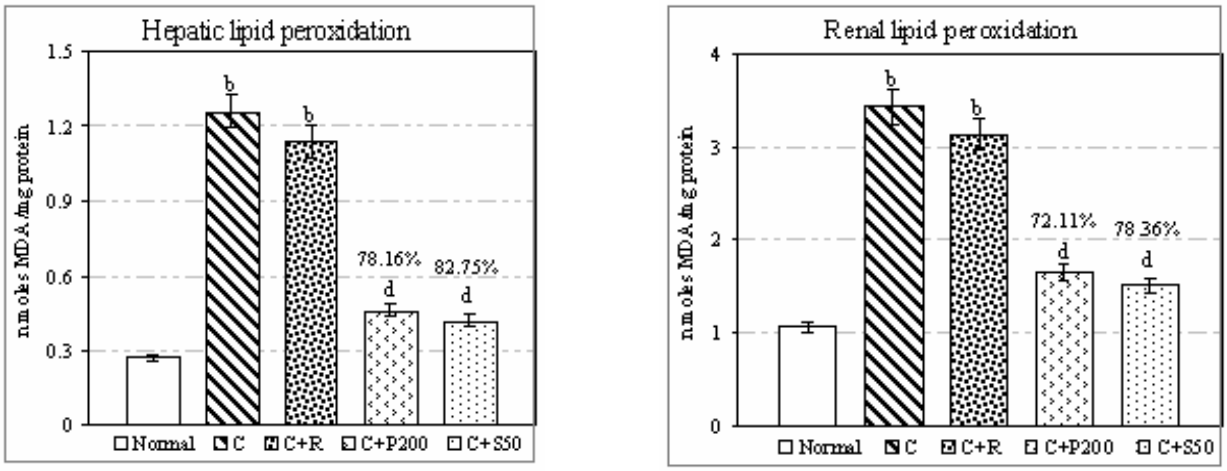


Fig 3. Hepatic and renal lipid peroxidation (LPO) of groups: normal, CCl_4 without rest (C), CCl_4 with rest (C+Rest), CCl_4+Propolis (C+P200) and CCl_4+Silymarin (C+S50). For each group n=6. ^{b}p values ≤0.01 for CCl4-treated vs control; ^{d}p values ≤0.01 for Drugs- vs CCl_4-treated. *Significant for ANOVA at 5% using df value 4, 25; hepatic LPO=121.32*, renal LPO=79.264*. Percent protection by propolis and silymarin treatment is represented on their related bars.

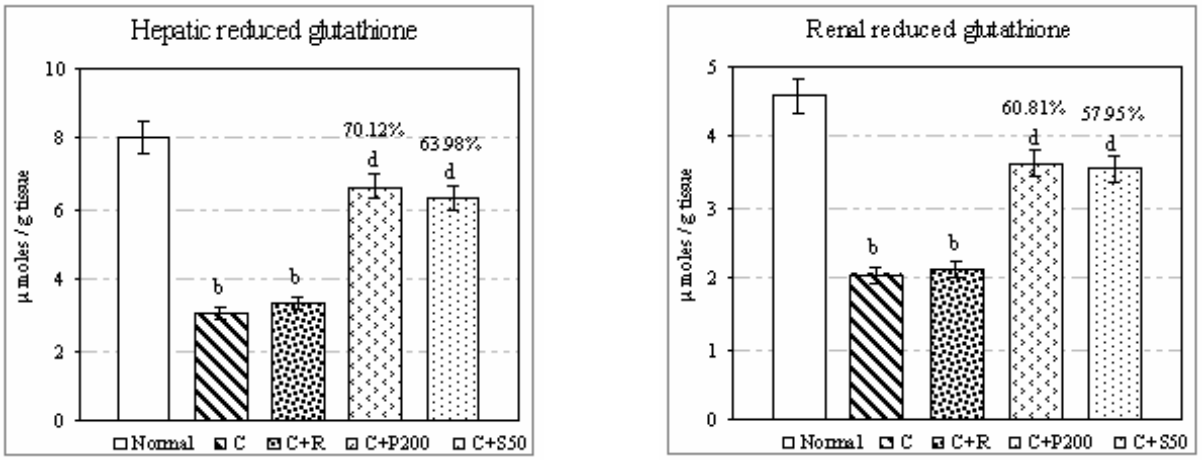


Fig 4. Hepatic and renal GSH of groups: normal, CCl_4 without rest (C), CCl_4 with rest (C+Rest), CCl_4+Propolis (C+P200) and CCl_4+Silymarin (C+S50). For each group n=6. ^{b}p values ≤0.01 for CCl4-treated vs control; ^{d}p values ≤0.01 for Drugs- vs CCl_4-treated. *Significant for ANOVA at 5% using df value 4, 25; hepatic GSH =56.169*, renal GSH =41.356*. Percent protection by propolis and silymarin treatment is represented on their related bars. group 4 (400 X).

KCl (0.15M) and the amount of malondialdehyde (MDA) formed was quantitated by reaction of thiobarbituric acid (TBA) and used as an index of LPO. The homogenate (1 ml) was incubated at 37°C for 30 min and proteins were precipitated by adding 1 ml of TCA (10%) and then centrifuged at 2,000 rpm for 15 minutes. One ml supernatant was taken with 1 ml of TBA solution (0.67%) and kept in boiling water bath at 100ºC for 10 minutes. After cooling, optical density was recorded at λ535 nm against blank. The levels of LPO were expressed as n moles of MDA formed /mg protein using an extinction coefficient of 1.56×10^5/M/cm.

Homogenates of liver and kidney were prepared in 1% sucrose solution for measuring GSH. Briefly, 0.1 ml of homogenate and 0.9 ml of distilled water was added with 1.0 ml sulfosalicylic acid then centrifuged at 3,000 rpm for 10 min. Blank and standards were prepared by taking 0.5 ml of distilled water and 0.5 ml of GSH standard respectively. About 0.5 ml of supernatant was added with 4.5 ml of tris buffer (*p*H 8.23) and finally color was developed by adding 0.5 ml of 5, 5-dithiobis-2-nitro-benzoic acid solution (DTNB) and optical density was recorded at λ412 nm.

Histopathological study

For histopathological study, liver and kidney were fixed immediately in Bouin's fixative and paraffin sections of 5µm thickness were cut. Hematoxylin-eosin (H-E) stained slides were observed under light microscope [32].

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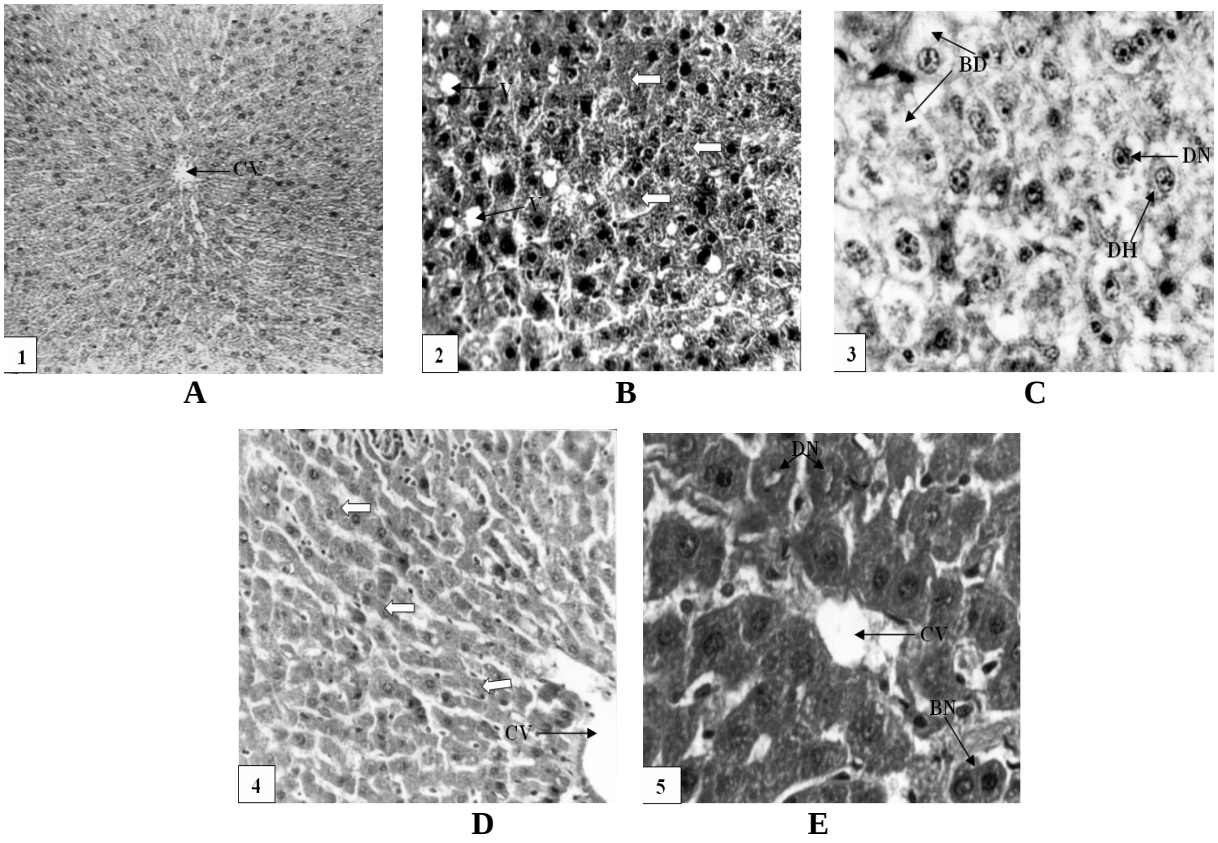


Fig 5. **A**- Liver histoarchitecture of control rat showing normal appearance of central vein (CV), well formed cord arrangement of hepatocytes having conspicuous nucleus in group 1 (140X). **B**- High degree of vacuolation (V), Massive hepatic necrosis (*white arrow*) showing a few surviving hepatocytes, neutrophil polymorph infiltration and mild fatty changes after CCl_4 administration in group 3 (140X). **C**- Ballooning degeneration of hepatocytes (BD), degenerated nucleus (DN) and degenerated hepatocytes (DH) showing injury due to CCl_4 in group 3 (400X). **D**- Clear central vein (CV) with well formed cord arrangement of hepatocytes (*white arrows*) showing recovery in liver after propolis therapy in group 4 (140X). **E**- Propolis Treatment showed well formed polygonal hepatocytes with conspicuous nucleus, some are binucleated hepatocytes (BN) while some has degenerated nucleus (DN) in group 4 (400X).

Statistical analysis

Data were subjected to statistical analysis using one way analysis of variance (ANOVA) and student's-t test [33].

Comparison of all the groups was made by ANOVA at $p \leq 0.05$. Student's t-test was used to compare the mean of two specific groups to observe any significance between groups. p value ≤ 0.01 and 0.05 were considered significant.

RESULTS

Blood biochemistry

Blood biochemical studies represented hepatoprotective efficacy of propolis extract against subchronic injury induced by CCl_4. Propolis treatment for five days reversed varying degree of changes in blood biochemistry. Significant decrease was observed in the level of blood sugar ($p \leq 0.01$), whereas, increase was found in serum protein content ($p \leq 0.01$) after CCl_4 administration. Administration of propolis extract and silymarin shifted blood sugar level and serum protein content towards control values (Fig 1). Propolis treatment showed 60.26% and 35.15% protection in blood sugar level and serum protein contents respectively. CCl_4 elicited toxic response, thereby; a significant enhanced release of AST, ALT, SALP and LDH was observed (Fig 2). Propolis extract protected against altered enzymatic activities and prevented their leakage, conferring its hepatoprotective efficacy ($p \leq 0.01$). On the basis of % protection, propolis showed more than 80% recoupment in diagnostic enzymes of liver dysfunction. Propolis exhibited its better hepatoprotective effect over silymarin in recouping activities of ALP and LDH. These parameters were not recovered significantly in group 3, which were allowed to recover without any treatment and received 1 week rest in comparison to group 2.

Tissue biochemistry

Table 1 show significantly decreased hepatic total protein contents ($p \leq 0.01$) after CCl_4 intoxication and propolis therapy recovered it very close to control ($p \leq 0.05$). No significant fall was observed in renal total protein contents. Three weeks exposure to CCl_4 depleted hepatorenal glycogen contents ($p \leq 0.01$). Significant recoupment was found with propolis extract in liver ($p \leq 0.01$) and kidney ($p \leq 0.05$) when compared to CCl_4-administered animals (Table 1). More than 90% protection was observed in hepatic glycogen content with propolis therapy. Damage in lysosomal integrity was evidenced by sharp increase in the activities of ACPase on

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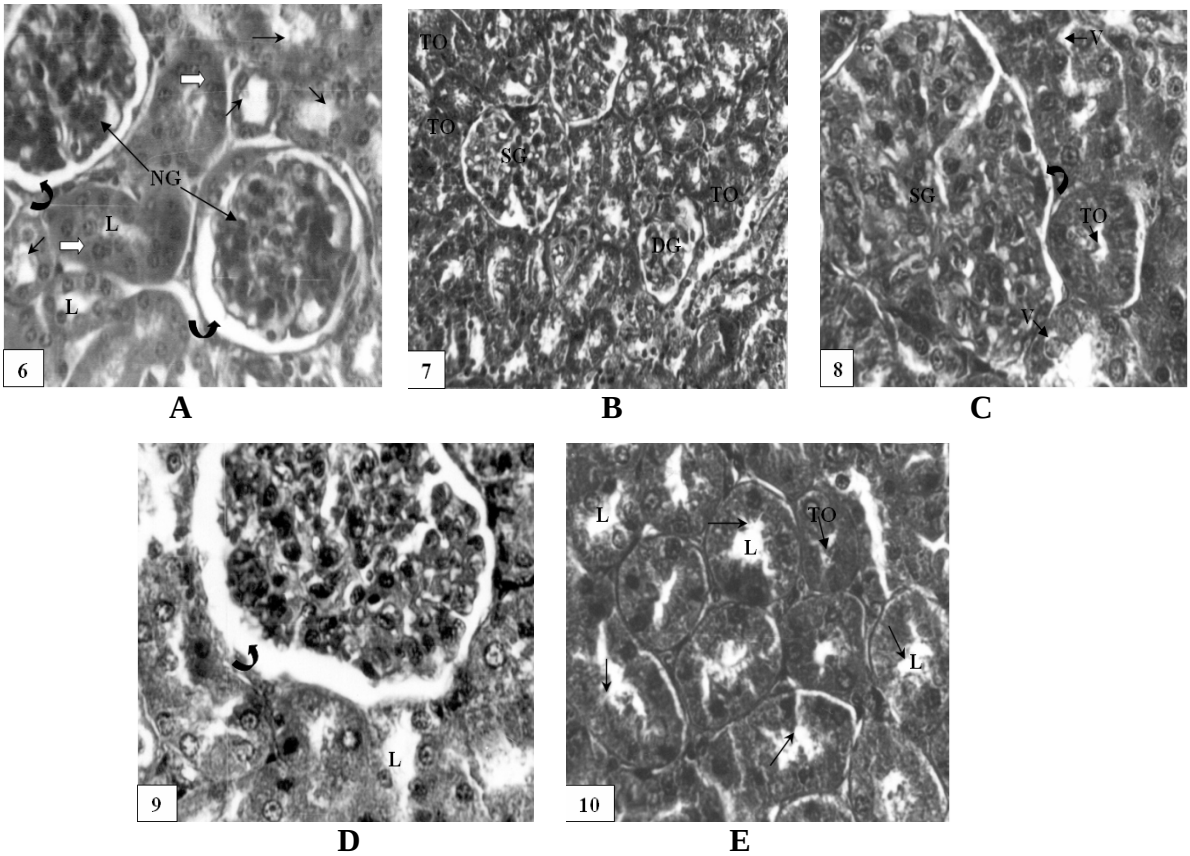


Fig 6. **A**- Kidney histoarchitecture of control rat showing well formed Bowman's capsule with normal glomeruli (NG), uniform space between glomerulus and capsule wall (thick arrows). Uriniferous tubules (white arrows) with both basal and apical nuclei along with normal lumen (L) and brush like edges were present in tubules (thin arrows) in group 1 (400X). **B**- Tubular obstruction in most of the tubular lumens (TO), losing brush like edges of tubular cells, swelling in glomeruli (SG), degenerated glomerulus (DG) and lymphocytic infiltrations due to CCl4 administration in group 3 (140X). **C**- Swelling in glomerulus (SG) showing irregular shape of Bowman's capsule with loss of space between glomerulus and capsule wall (thick arrow), tubular obstruction (TO), vacuolation in tubular cells (V) and losing brush like edges of tubular cells were observed after CCl4 administration in group 3 (400X). **D**- Well formed Bowman's capsule with proper glomerulus and uniform space between glomerulus and capsule wall (thick arrow) along with wider lumen of tubules (L) after propolis treatment in group 4 (400X). **E**- Tubules are normal with wider lumen (L), however some tubules still have tubular obstruction (TO), brush like edges were observed in many areas (thin arrows) after propolis therapy in.

CCl_4 in both organs when compared to control group ($p \leq 0.05$; Table 2). Treatment of propolis extract for 5 consecutive days significantly decreased ACPase activity as compared to CCl_4-intoxicated groups ($p \leq 0.01$). Table 2 demonstrates sharp fall in activities of hepatorenal ALPase and ATPase after subchronic exposure to CCl_4 ($p \leq 0.01$). Propolis therapy proved to be effective in these variables and significantly recovered the activity of hepatorenal ALPase ($p \leq 0.01$), renal ATPase ($p \leq 0.01$) and hepatic ATPase ($p \leq 0.05$). Analysis of variance showed significantly improved activities of ACPase, ALPase and ATPase with propolis therapy ($p \leq 0.01$).

Study of oxidative stress

Fig 3 depicts hepatoprotective effect of propolis extract over CCl_4-induced hepatorenal oxidative stress. Formations of MDA were increased in liver and kidney more than 4 and 3 times respectively after CCl_4 administration ($p \leq 0.01$). Therapy of propolis extract and silymarin significantly prevented formation of MDA and showed values almost very near to control ($p \leq 0.01$). Propolis treatment showed 78.16% and 72.11% protection in hepatic and renal LPO respectively. Fig 4a and 4b present influence of propolis extract on tissue non enzymatic antioxidative status. CCl_4 exposure significantly lowered the hepatorenal GSH level ($p \leq 0.01$). Propolis treatment increased GSH status in target organs showing better recoupment as compared to silymarin-treated positive control ($p \leq 0.01$). Hepatorenal % protection showed 70.12% and 60.81% recovery in antioxidant status of both organs with propolis treatment.

Histopathological observations

Fig 5 represents the influence of CCl_4 and therapeutic agents on liver histopathology. Fig 5-A shows control liver histoarchitecture with normal appearance of central vein, well-formed cord arrangement of hepatocytes having conspicuous nucleus. Subchronic administration of CCl_4 caused degeneration and disintegration of liver cell architecture. Hepatic lesions were characterized by massive hepatic necrosis showing a few surviving hepatocytes and large vacuolation. Cell membrane of many hepatocytes was collapsed and neutrophil polymorph infiltration and mild fatty changes were observed (Fig 5-B and 5-C). Therapy with propolis extract

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for 5 days showed normal lobular pattern with well-formed polygonal hepatocytes having conspicuous nucleus, some were binucleated hepatocytes without neutrophil polymorph infiltration and fatty changes (Figs 5-D and 5-E), thus conferred significant hepatoprotective effect.

Fig 6 represents the effect of CCl_4 and propolis on renal histopathology. Kidney of control group showing well-formed Bowman's capsule with normal glomeruli, uniform space between glomerulus and capsule wall. Uriniferous tubules with both basal and apical nuclei along with wider lumen and brush like edges in tubules were present (Fig 6-A). Administration of CCl_4 for 21 days showed considerable pathological lesions. Bowman's capsules were deformed due to considerable increase in the size of glomeruli occupying space between glomerulus and capsule wall. Degeneration of glomerulus, tubular obstructions with loss of brush like edges, pyknotic nuclei and hyperchromatia of nuclear membrane were seen (Figs 6-B and 6-C). Propolis treatment recouped renal histoarchitecture as proximal and distal convoluted tubules showed normal features with proper Bowman's capsule, wider lumen and brush like edges in well formed tubules (Figs 6-D and 6-E).

DISCUSSION

Carbon-tetrachloride-induced hepatic injury is associated with a variety of biochemical abnormalities and attributed to the release of intracellular constituents into circulation, such as AST, ALT, SALP and LDH. Their estimations are useful quantitative marker for the extent of hepatocellular damage [34]. Elevated activities of AST and ALT in serum are indicative of cellular leakage and loss of functional integrity of cell membranes in liver [35]. In the present study, subchronic exposure to CCl_4 increased activities of AST and ALT more than 4 and 6 times respectively. Alkaline phosphatase mainly arises from the lining of canaliculi in hepatocytes and also brush border of the renal tubules. It is excreted normally via bile through liver and involves in active transport across the capillary wall. Increased activity of alkaline phosphatase, which occurs due to *de novo* synthesis by liver cells, is a reliable marker of hepatobiliary dysfunction due to damage [36]. More than 10 fold increase in the activity of this enzyme after CCl_4 intoxication clearly indicated severity of damage in liver and kidney. LDH is an intracellular enzyme and also is used as a marker of liver injury. In this investigation, leakage of LDH from injured hepatocytes was obvious after subchronic administration of CCl_4. Propolis therapy attenuated increased level of these enzymes and caused a subsequent recovery towards normalization that might be due to recoupment in cell membrane. Stabilization of AST, ALT, SALP and LDH activities by propolis treatment clearly indicated improvement in the functional status of liver cells, which may be due to free radical scavenging action of propolis. Investigators have previously demonstrated antioxidative and hepatoprotective effect of the extract of *Bauhinia racemosa* [37] and *Emblica officinalis* [38] against CCl_4-induced toxicity. Recovery in these variables could be further corroborated by histopathological studies. The histopathological examination revealed that the centrilobular necrosis, damaged hepatic cells, central vein and portal triad tends towards normal by propolis therapy in contrast to CCl_4 intoxicated animals of group 3**.** Improvement in functional status indicates accelerated regeneration in parenchymal cells by propolis treatment, which provided protection against membrane fragility and subsequently leakage of liver marker enzymes into circulation.

Proteins are synthesized in liver. Inhibition of protein synthesis indicates disruption and dissociation of polyribosmes from endoplasmic reticulum [39]. Treatment with propolis extract protected protein depletion induced by toxicant in this study. The presence of various flavonoids and esters might be responsible for stimulation of protein biosynthesis. Disturbance in carbohydrate metabolism is one of the biochemical lesions involved in CCl_4 toxicity. Subchronic exposure to CCl_4 resulted into significant loss of blood glucose level producing hypoglycemia, which might be due to evident decline in hepatic glycogen. Disruption in glycogen storage is associated with the dystrophic changes in organs due to inhibition of key enzymes in carbohydrate metabolism such as hexokinase, glucokinase and phosphoglucomutase [40]. Five days propolis treatment preserved hepatorenal glycogen store dramatically, which is also in agreement with the treatment of *Terminalia belerica* fruit extract and its active principle [41].

ATPase is a mitochondrial lipid-dependent membrane-bound enzyme. Any alteration in membrane lipid leads to change in membrane fluidity, which in turn alters ATPase activity and subsequently energy-dependent cellular function. CCl_4 exposure provoked significant loss of ATPase activity in liver and kidney, which might be due to dysfunctional changes in mitochondria and cell membrane permeability. Inhibition of ATPase after CCl_4 exposure has also been confirmed in other studies [42]. Propolis prevented membrane lesion to a large extent with concomitant recovery in enzymatic activity by maintaining cell membrane permeability. ALPase is a membrane-bound enzyme, which involves in transphosphorylation reactions and mediates membrane transport. Proximal tubules of kidney are rich in ALPase and play an important role in reabsorption of glucose from renal tubules. In the present investigation, administration of CCl_4 caused decrease in hepatorenal activity of alkaline phosphatase. Kidney lesions due to toxic damage were largely confined to tubular epithelium, resulting in the suppression of tubular reabsorbing function as evidenced by structural alterations in tubules. These observations are similar to the reports published on the effect of proprietary herbal formulation [43]. Propolis treatment recovered ALPase activity after subchronic toxicity substantiating regenerative property [19]. ACPase is a lysosomal enzyme and its liberation include ionic imbalance followed by mitochondrial damage and stimulation of lysosomes leading to enzyme release. It is evident that lysosomal enzymes play important role in pathogenesis of CCl_4-induced hepatic

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and renal damage [44]. Present investigation demonstrated increased activity of ACPase after CCl_4 administration, which might be due to altered properties of cell membrane that permit rapid leaching of enzyme. Propolis therapy conferred its protective effect by stabilizing lysosomal membrane. Propolis may have the ability to exert protective effect by modifying lysosomal membrane to prevent the release of lysosomal enzymes as in case of *Withania somnifera* [45].

Increased LPO is a highly destructive process that induces a plethora of structural and functional alterations of cellular membranes and involves oxidation of fatty acids [2]. Increased TBARS level as seen in the present study suggests enhanced LPO leading to hepatorenal injury due to failure of antioxidant defense mechanism. Increased accumulation of LPO products might well be the consequences of a progressive degradation of necrotic tissue. Many natural and synthetic antioxidants are in the use to prevent LPO [46]. Propolis treatment prevented hepatorenal LPO and accelerated repair mechanism of damaged cell membranes. Caffeic acid phenylethyl ester (CAPE), an active component of ethanolic extract of propolis exhibits free radical scavenging property that may block the production of free oxygen radical [7]. The flavonoids act at the initiation stage of LPO as scavengers by reacting with peroxy radicals of polyunsaturated fatty acids (PUFA), breaking the chain reactions.

Glutathione is recognized as a protective compound within the body for removal of potentially toxic electrophilic compounds to afford protection against organ dysfunction. In the present study, CCl_4 induced depletion in hepatorenal glutathione has been constructed as evidence supporting the hypothesis that generated reactive oxygen intermediates leads to glutathione oxidation. Studies suggest that an inverse relationship exists between peroxidative decomposition of membrane PUFA and GSH levels [47]. This fact was also evidenced in our study where GSH contents were depleted with concomitant rise in LPO. Propolis treatment may hinder CCl_4-induced reactive oxygen species due to its antioxidant nature and shows beneficial protective effect in GSH-dependent defense system. Histological observations basically supported the results obtained from biochemical assays.

Possible mechanisms of hepatoprotective action of propolis extract may be due to its free radical scavenging activity as indicated by decrease in lipid peroxidation and increase in glutathione contents. Improved enzymatic biochemical parameters and histopathological observations also indicated recovered structural and functional integrity of the hepatorenal cells and provided further support to the proposed protective mechanism of action by propolis. Thus, it can be concluded that propolis extract possesses therapeutic potential against hepatorenal damage.

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