Research Paper

Hepatoprotective Potential of Green Tea Extract against Experimental Hepatotoxicity in Rats

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An experimental study was conducted to evaluate the hepatoprotective effect of aqueous extract of *Camellia sinensis* or green tea extract and N-acetyl-L-cysteine in acetaminophen-induced hepatotoxicity in rats. Male Wistar rats (n=24) of 3 mon age were equally divided into 4 groups. Group 1 served as normal control. Hepatotoxicity was induced in the remaining three groups with oral administration of 500 mg/kg of acetaminophen from day 1 to day 3. Groups 2, 3 and 4 were subsequently administered orally with distilled water, 300 mg/kg of N-acetyl-L-cysteine and 100 mg/kg green tea extract, respectively for 11 d. Mean body weights and biomarkers of hepatotoxicity were estimated on days 0, 4 (confirmation of toxicity) 15 (at the end of treatment). Hematological parameters were evaluated on 4 and 15 d. Antioxidant profile and ATPase enzymes were assessed at the end of the experiment. Livers were subjected to histopathology and transmission electron microscopy after the sacrifice on day 15. Antioxidant profile, ATPase, haematological and serobiochemical parameters were significantly altered and histopathological changes were noticed in liver of toxic control group. These changes were reversed in groups 3 and 4 that were administered with N-acetyl-L-cysteine and green tea extract, respectively. The results of the present investigation enunciated that green tea extract has potent hepatoprotective activity, though N-acetyl-L-cysteine was found superior in restoring the pathological alterations in acetaminophen-induced hepatotoxicity in Wistar rats.

Key words: Acetaminophen, *Camellia sinensis*, catechins, green tea, hepatotoxicity, N-acetyl-L-cysteine, rats

The liver is one of the vital organs of the body and plays a key role in the metabolism and detoxification process; disorders of this organ remain some of the most serious health problems[1]. Drug-induced hepatic injury is considered as the primary cause of hepatotoxicity[2]. Acetaminophen (paracetamol, N-acetyl-p-aminophenol) via CYP_{450}-mediated N-hydroxylation metabolized to N-acetyl-p-benzoquinoneimine (NAPQI)[3]. N-acetyl-p-benzoquinoneimine (NAPQI), a highly toxic, reactive metabolite of acetaminophen, which causes oxidative stress and glutathione (GSH) depletion plays a key role in dose-dependent hepatotoxicity[4]. N-acetyl-L-cysteine (NAC) is a sulfur-based amino acid and potent antioxidant proved effective as an antidote for hepatotoxicity due to acetaminophen overdose[5]. NAC acts as a precursor for GSH synthesis and was shown to be beneficial against reactive oxygen species (ROS) generation, mitochondrial dysfunctions and in mitochondrial dependent and independent apoptotic cell death in cancer[6].

Herbal alternatives are one of the best ways to minimize liver damage and were used prophylactically, also as antidotes[7]. Green tea is an important dietary source of the plant polyphenols from *Camellia sinensis*[8]. The phenol rings in the compound are comprised of phenyl and hydroxyl group structures that possess antiinflammatory, immunomodulatory and antioxidant properties[9]. Singarvel *et al.*[10] reported that the hepatoprotective effect of *C. sinensis* in CCl_4-treated rats owing to its antioxidant property. Hence, an experimental study was conducted to assess the hepatoprotective effect of *C. sinensis* (green tea) against acetaminophen-induced hepatotoxicity in male Wistar rats.

MATERIALS AND METHODS

Acetaminophen and NAC were purchased from

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Himedia, Mumbai and Sisco Research Laboratories Pvt. Ltd., Mumbai, respectively. Green tea (Lipton) was procured from the local supermarket and green tea extract (GTE) was prepared by brewing in hot water. Male Wistar rats (n=24) of 3 mon age were procured from Sanzyme Pvt. Ltd., Hyderabad, India. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC, Approval No. CPCSEA I/10/2014, Dated: 27.11.2014). All animals were maintained under standard conditions prescribed by CPCSEA.

Experimental design:

The experimental study was carried out on 24 rats that were randomly divided into four groups comprising of 6 rats in each group. Group 1 served as normal control. Group 2 animals were administered acetaminophen (500 mg/kg p.o) once daily for the first 3 d and distilled water (5 ml/kg p.o) once daily for the next 11 d after the last dose of acetaminophen. Group 3 animals received acetaminophen as in group 2 followed by NAC (300 mg/kg p.o) once daily for the next 11 d. Group 4 rats received acetaminophen as in group 2 followed by GTE (100 mg/kg p.o) once daily for the next 11 d. Following a 24 h fasting with access to water *ad libitum,* hepatotoxicity was induced in groups 2, 3 and 4 with 500 mg/kg of acetaminophen per oral administration for 3 consecutive days[11].

Mean body weights of the groups were estimated on days 0, 4 (confirmation of toxicity) and 15 (at the end of treatment). Haematological parameters such as total erythrocyte count, total leukocyte count, haemoglobin, and packed cell volume and prothrombin time were estimated in all the groups on days 4 and 15. On the day 15, blood samples were collected centrifuged; sera were separated and stored at −80° until assayed for alanine transaminase, bilirubin, total protein and glucose. At the end of the experiment, liver tissues were collected for the assay of reduced GSH, glutathione peroxidase (GPX), super oxide dismutase (SOD), catalase (CAT) and thiobarbituric acid reacting substance (TBARS). The liver tissue was collected in ice cold conditions for determining ATPase activity. Histopathological and transmission electron microscopic studies of liver tissues were performed to draw possible conclusions at the end of the experiment.

Tissue samples were homogenized in 1 ml of 10 mM Tris HCl buffer of pH 7.1 and centrifuged at 12 000 g for 10 min. The supernatant was used for the measurement of liver enzymatic and non-enzymatic antioxidants. SOD was estimated by the procedure involving inhibition of superoxide-dependent reduction of tetrazolium dye MTT to formazan[12]. CAT is allowed to split H_2O_2 followed by adding dichromate/acetic acid mixture to stop the reaction, resultant chromic acid measured gives the amount of freely available H_2O_2[13]. Quantitative estimation of GSH was done by the method where reduced GSH was reacted with 5-5' dithiobis-2-nitrobenzoic acid (DTNB) and absorbance was measured at 412 nm[14]. The activity of GPx[15] and TBARS[16] was estimated as prescribed by the previous studies.

Na^+-K^+ATPase and Mg^{2+}ATPase estimation[17]:

Na^+-K^+ATPase and Mg^{2+}ATPase transports Na^+, K^+ and Mg^{2+}, respectively against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). The liberated Pi was estimated as per the micro method determination of Pi reported Chen *et al.*[18]. Ouabain, a Na^+-K^+ATPase inhibitor, was used to assess the Mg^{2+}ATPase in the sample. Na^+-K^+ATPase was determined by difference in the presence and absence of ouabain.

Reaction mixture comprising of 1 M NaCl-1.40 ml, 1 M KCl-140 µl, 0.1 M $MgCl_2$-300 µl, 0.2 M EDTA-10 µl, 0.5 M tris-HCl pH 7.4-400 µl and distilled water 2.75 ml was prepared and stored in a refrigerator. Half milliliter of the above mixture was taken and made to a final volume of 1.0 ml. ATP 30 mM was prepared by dissolving 181.8 mg of disodium ATP in 6 ml of distilled water, pH was adjusted to 7.4 with 0.5 M Tris base, final volume was made up to 10 ml with distilled water and stored in frozen condition. Ouabain (10 mM) solution was prepared by dissolving 14.57 mg of ouabain in 2 ml of distilled water, which was kept frozen in an amber coloured bottle. Trichloroacetic acid (TCA) 10% was prepared and used.

Enzyme preparation and ATP solution were thawed and kept on ice. Liver microsomes were diluted to 3 mg protein/ml. The following solutions were added serially. The reaction was terminated exactly after 30 min of incubation by adding 1 ml of 10% TCA and tubes were immediately transferred to ice, kept for 10 min and centrifuged for 5 min to remove the precipitate. Whole supernatant was taken for phosphate estimation using the micro method reported by Chen *et al.*[18]. Five hundred microlitres of the supernatant and 3.5 ml of distilled water were taken in a test tube and 4 ml of the reagent containing 1 volume of 10% ascorbic acid, 1 volume of 2.5% ammonium molybdate and 2 volumes

of distilled water, was added. All tubes were incubated at 37° for 2 h. The absorbance was read at 820 nm. The enzyme activity was expressed as μg of Pi liberated/mg microsomal protein/30 min.

Hepatic alanine transaminase, total bilirubin, glucose and total protein concentration in serum were estimated as per the instructions given in the Erba diagnostic kit using Erba Chem 7 autoanalyser. At the end of the study, all the animals were sacrificed; liver samples were collected and fixed in 10% neutral buffered formalin. Subsequently, the fixed samples were processed and stained with haematoxylin and eosin (H and E) stain as described by Singh and Sulochana[19]. For microscopic studies, samples were transferred to vials and fixed in 3% glutaraldehyde, stained with saturated aqueous uranyl acetate and counter stained with 4% lead citrate[20] and were observed at different magnifications under transmission electron microscope (Model: Hitachi, H-7500) at Ruska Laboratory, PVNR TVU, College of Veterinary Science, Hyderabad, India.

Statistical analysis:

The experimental data were expressed as mean±standard error of mean. Using one way analysis of variance (ANOVA) followed by Duncan's[21] post hoc analysis. All groups are compared and tested for significance with the help of Statistical Package for Social Sciences (SPSS 21.0). Values were considered significant if ($P<0.05$).

RESULTS AND DISCUSSION

Significant ($P<0.05$) reduction of mean body weights (Table 1) in acetaminophen control group could mainly attributed to oxidative stress and ROS generated in acetaminophen-induced hepatotoxicity and altered metabolism of liver[22]. Weight reduction might also be attributed to hyperglycaemia induced in this study, which adds to the catabolism of proteins, resulting in weight loss. Weight gain observed in the animals treated with NAC and GTE (groups 3 and 4, respectively) might be due to restoration of antioxidant defences and recovery of hepatic histoarchitecture as evident from the findings of this study.

The levels of serum markers like ALT, total bilirubin and glucose presented in Table 2 were significantly ($P<0.05$) increased and total proteins was significantly ($P<0.05$) decreased in this study could be due to loss of functional integrity of cell membrane in liver. This is in agreement with findings of Madhu Kiran *et al.*[23]. Treatment with NAC and GTE significantly ($P<0.05$) decreased the activity of above marker enzyme on 15 d, suggesting their protective effect in this study. These observations supports that the reversal of serum transaminases levels to normal may be due to regeneration of hepatocyte and healing of hepatic parenchyma[24].

The reduced GSH level of liver tissue were significantly ($P<0.05$) increased in groups treated with NAC and

TABLE 1: MEAN BODY WEIGHTS (g) OF DIFFERENT GROUPS OF RATS

Group	Control	APAP control	APAP+NAC	APAP+GTE
Day 0	173.16±1.04^{a}	172.83±0.94^{a}	173.50±0.99^{a}	173.33±0.88^{a}
Day 4	194.16±4.32^{a}	179.33±2.04^{b}	193.33±1.02^{a}	193.5±0.88^{a}
Day 15	239.5±2.18^{a}	192.83±1.13^{d}	233.66±2.41^{b}	226.33±1.85^{c}

APAP is acetaminophen, NAC is N-acetyl-L-cysteine and GTE is green tea extract. Values are expressed mean±standard error (n=6); One way ANOVA (SPSS). Means with different alphabets as superscripts differ significantly ($P<0.05$) among the groups at respective time intervals

TABLE 2: LIVER FUNCTIONAL BIOMARKERS IN DIFFERENT GROUPS OF RATS

Parameter		Control	APAP control	APAP+NAC	APAP+GTE
Total protein	Day 4	7.57±0.07^{a}	4.56±0.06^{b}	4.67±0.02^{b}	4.66±0.02^{b}
	Day 15	7.56±0.08^{a}	4.59±0.05^{a}	6.23±0.12^{b}	5.56±0.03^{c}
Concentration (g/dl)					
Total bilirubin	Day 4	0.7±0.01^{c}	1.42±0.01^{a}	1.41±0.0^{ab}	1.38±0.01^{b}
	Day 15	0.81±0.03^{c}	1.39±0.01^{a}	0.79±0.01^{c}	0.9±0.01^{b}
Concentration (mg/dl)					
Glucose	Day 4	80.85±0.19^{c}	98.87±0.24^{a}	97.86±0.46^{b}	98.48±0.30^{ab}
	Day 15	82.24±0.44^{c}	96.26±0.25a	82.91±0.33^{c}	88.67±0.37^{b}
Concentration (g/dl)					
ALT activity (IU/l)	Day 4	38.86±0.33^{c}	90.96±1.18^{a}	88.03±0.46^{b}	88.16±0.32^{b}
	Day 15	37.36±0.43^{d}	37.36±0.43^{a}	66.40±0.74^{c}	76.31±0.97^{b}

APAP is acetaminophen, NAC is N-acetyl-L-cysteine and GTE is green tea extract. Values are mean±standard error (n=6); One way ANOVA (SPSS). Means with different alphabets as superscripts differ significantly ($P<0.05$) among the groups at respective time intervals

GTE when compared to toxic group. However NAC surpassed GTE, which might be due to the ability of NAC to generate GSH and maintain GSH homeostasis in the body[24]. TBARS levels was significantly ($P<0.05$) increased in acetaminophen-treated control group signified that acetaminophen triggered massive lipid peroxidation[25,26], which was responsible for liver injury. GTE showed free radical scavenging ability that was comparable to NAC. SOD and CAT activities were significantly ($P<0.05$) increased in NAC and GTE treated groups as compared to toxic group 2 and also there was corresponding decrease in TBARS (extend of lipid peroxidation) in liver of treated groups 3 and 4 (Table 3).

The activities of Na^+/K^+ATPase and Mg^{2+}ATPase in liver presented in Table 4 were significantly ($P<0.05$) increased in treatment groups might be due to the membrane stabilizing activity of NAC and GTE extract owing to their antioxidant potential. This may be attributed to the changes in the levels of total cholesterol and triglycerides as the activity of ATPase enzymes is more likely to be changed with the change in the level of these lipids[27].

Acute acetaminophen treatment in group 2 significantly ($P<0.05$) lowered TEC, Hb and PCV levels as compared to all other groups at respective time intervals from day 4-15. It might be due to acetaminophen-treated hepatic damage[28]. Further, significant ($P<0.05$) rise in TLC in acetaminophen-treated control group could possibly be due to the stimulation of immune system against the invading antigens and also to an IL-1β-mediated rise in the respective colony stimulating factors[29]. Treatment with NAC and GTE reversed these alterations in haematological parameters, which could be attributed to the protective effects on tissue repair and deceleration of disease progression.

In the present study, acetaminophen-induced prothrombin time prolongation seen in the group 2 controls might as well be attributed to hepatic injury resulting in a reduction in the production and activation of certain blood clotting factors as the metabolic machinery could have been compromised in the liver[30]. The treatment improved the clotting time owing to the regeneration of hepatic histoarchitecture and its physiology (Table 5).

The histological examination of liver sections from acetaminophen-treated control (group 2) revealed periportal infiltration, moderate to severe necrotic degeneration and fatty changes when compared to normal control group 1 (figs. 1, 2A and 2B). NAC-treated group 3 showed regenerative changes and GTE-treated group 4, revealed mild congestion and moderate degenerative changes as shown in figs. 3 and 4. Quantitative measures of liver histological alterations were presented in Table 6. TEM image of hepatocyte of normal control group 1 (fig. 5) showed normal nucleus, normal mitochondria, rough endoplasmic reticulum and normal euchromatin. TEM

TABLE 3: ANTIOXIDANT AND PEROXIDATION PARAMETERS OF LIVER IN DIFFERENT GROUPS OF RATS

Parameter	Control	APAP control	APAP+NAC	APAP+GTE
TBARS concentration (n moles of MDA released/mg protein)	14.36±0.1^c	17.64±0.09^a	14.41±0.12^c	15.80±0.11^b
GSH concentration (n moles/mg protein)	6.53±0.08^a	1.47±0.01^d	4.08±0.01^b	3.94±0.02^c
GP_x activity (U/mg protein)	6.53±0.08^a	1.47±0.01^d	4.08±0.01^b	3.94±0.02^c
SOD activity (U/mg protein)	8.46±0.07^a	6.67±0.01^d	7.45±0.06^c	7.85±0.03^b
Catalase activity (U/mg protein)	4.94±0.01^a	3.18±0.01^d	4.64±0.02^b	4.19±0.01^c

APAP is acetaminophen, NAC is N-acetyl-L-cysteine and GTE is green tea extract. Values are mean±standard error (n=6); One way ANOVA (SPSS). Means with different alphabets as superscripts differ significantly ($P<0.05$) among the groups

TABLE 4: ATPase ACTIVITY OF LIVER IN DIFFERENT GROUPS OF RATS

Parameter	Control	APAP control	APAP+NAC	APAP+GTE
Mg^{+2}ATPase activity (µg of pi liberated/mg microsomal protein/30 min)	4.48±0.01^a	2.75±0.01^d	3.94±0.01^b	3.72±0.01^c
Na^+-K^+ATPase activity (µg of pi liberated/mg microsomal protein/30 min)	10.32±0.14^a	5.84±0.07^b	10.02±0.22^a	9.89±0.02^a

APAP is acetaminophen, NAC is N-acetyl-L-cysteine and GTE is green tea extract. Values are mean±standard error (n=6); One way ANOVA (SPSS). Means with different alphabets as superscripts differ significantly ($P<0.05$) among the groups

TABLE 5: HEMATOLOGICAL PARAMETERS OF DIFFERENT GROUP OF RATS

Parameter		Control	APAP control	APAP+NAC	APAP+GTE
Total erythrocyte count (×10^6/mm)	Day 4	7.25±0.03^a	6.33±0.02^c	6.47±0.01^b	6.43±0.03^b
	Day 15	7.46±0.05^a	6.28±0.03^c	7.38±0.01^a	6.88±0.03^b
Total leukocyte count (10^3/mm^3)	Day 4	7.86±0.01^d	9.64±0.01^a	9.24±0.01^c	9.58±0.02^b
	Day 15	7.94±0.01^b	9.69±0.01^a	7.89±0.01^c	7.960.01^b
Haemoglobin	Day 4	14.80±0.02^a	10.49±0.01^b	10.52±0.06^b	10.59±0.04^b
concentration (g/dl)	Day 15	14.66±0.04^a	9.64±0.05^d	14.32±0.01^b	12.69±0.02^c
Packed Cell	Day 4	37.46±0.01^a	33.48±0.15^b	33.56±0.03^b	33.41±0.07^b
Volume (%)	Day 15	37.43±0.01^a	28.27±0.16^d	36.72±0.03^b	35.88±0.02^c
	Day 4	15.23±0.02^b	27.42±0.04^a	27.37±0.04^a	27.36±0.06^a
Prothrombin time (s)	Day 15	15.24±0.02^d	29.84±0.02^a	16.25±0.02^c	19.55±0.06^b

APAP is acetaminophen, NAC is N-acetyl-L-cysteine and GTE is green tea extract. Values are mean±standard error (n=6); One way ANOVA (SPSS). Means with different alphabets as superscripts differ significantly (P<0.05) among the groups at respective time intervals

TABLE 6: QUANTITATIVE MEASURES OF LIVER HISTOLOGICAL ALTERATIONS

Groups	Sinusoidal dilatation	Cellular infiltration	Degenerative changes	Necrosis
Control	+	-	-	-
PCM control	+++	+++	++++	++++
PCM+NAC	+	+	++	++
PCM+GTE	++	++	+++	+++

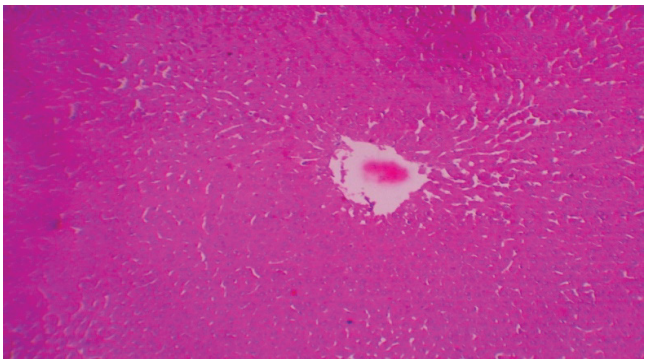

Fig. 1: Photomicrograph of liver of normal control group
Liver sections from normal control group 1 rats stained with HE showed normal to mild sinusoidal dilatation

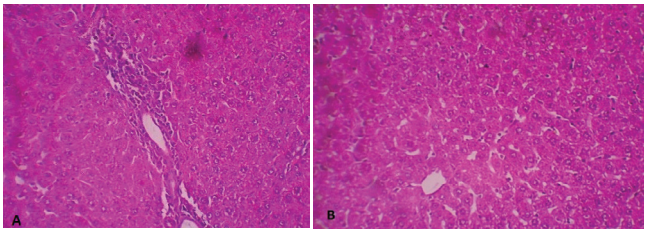

Fig. 2: Photomicrographs of liver of acetaminophen-treated control group
Liver sections from acetaminophen-treated control group 2 rats stained with HE showed A. Periportal infiltration and degenerated hepatocytes and B. Sinusoidal dilatation and few cells of coagulative necrosis

image of hepatocyte of acetaminophen-treated group 2 (fig. 6A and B) revealed Kupffer cell infiltration and mild fatty changes in cytoplasm. NAC-treated group 3 (fig. 7) showed hepatocyte with regenerated mitochondria, rough endoplasmic reticulum, normal nucleus and nucleolus. Similarly, TEM of hepatocyte of GTE-treated group 4 (fig. 8) showed reconstitution of mitochondria and mild margination of chromatin material.

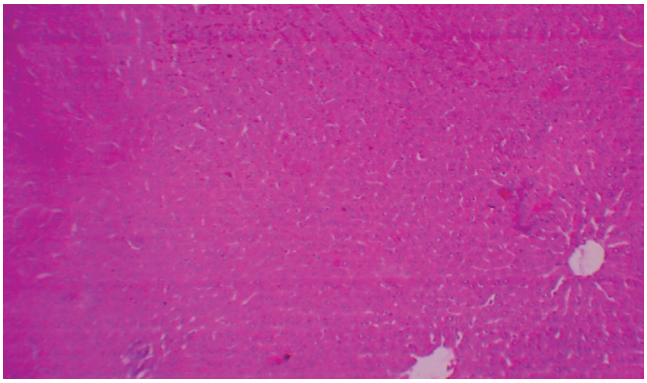

Fig. 3: Photomicrograph of liver from NAC-treated group
Liver sections from N-acetyl-L-cysteine (NAC)-treated group 3 rats stained with HE showed no signs of pathological change except for sinusoidal dilatation

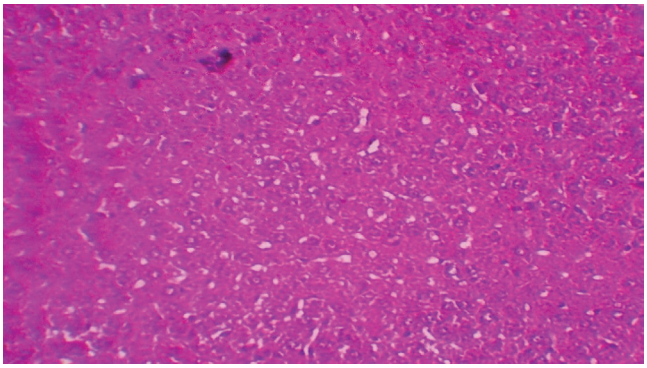

Fig. 4: Photomicrograph of liver from GTE-treated group
Liver sections from green tea extract (GTE)-treated group 4 rats stained with HE showed regenerative changes with mild dilatation of sinusoids and mild congestion

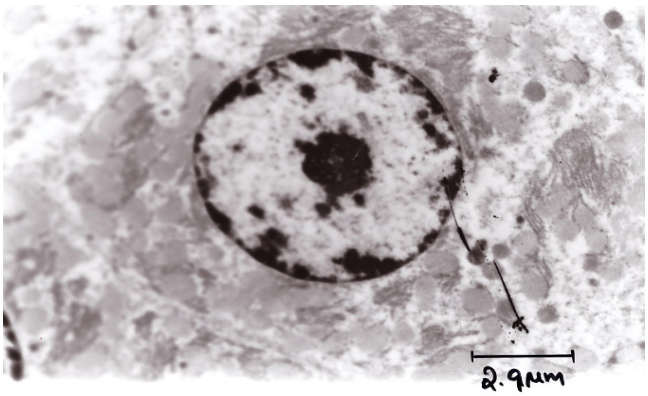

Fig. 5: TEM image of hepatocyte of normal control group
Hepatocyte from normal control group 1 rats displayed normal nucleus, normal mitochondria, rough endoplasmic reticulum and normal euchromatin

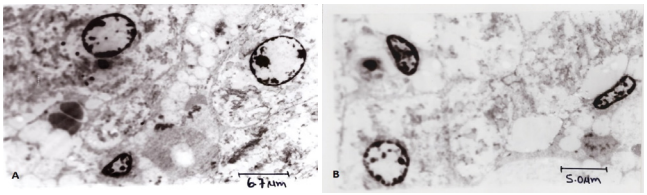

Fig. 6: TEM images of hepatocyte of acetaminophen-treated control group
A. Hepatocyte from acetaminophen-treated group 2 rats showed a distorted mitochondria and rough endoplasmic reticulum, pyknotic and eccentric nucleus, chromatin margination and mild fatty changes; B. Kupffer cell infiltration and mild fatty changes in cytoplasm

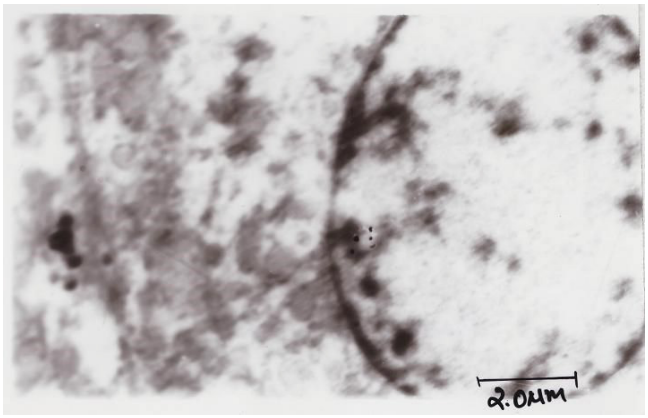

Fig. 7: TEM image of hepatocyte of NAC-treated group
Hepatocyte isolated from N-acetyl-L-cysteine (NAC)-treated group 3 rats showed regenerated mitochondria, rough endoplasmic reticulum, normal nucleus, nucleolus and cytoplasm showing vesicular structures

The present study revealed that NAC and GTE extract protected against acetaminophen-induced hepatotoxicity probably due to inhibiting inflammatory and oxidative biomarkers. The overall beneficial effects of GTE are attributed to antioxidant potential as evident from oxidant-antioxidant markers in this study coupled with hepatoprotective property.

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Conflict of interest:

There are no conflicts of interest.

Financial support and sponsorship:

Nil.

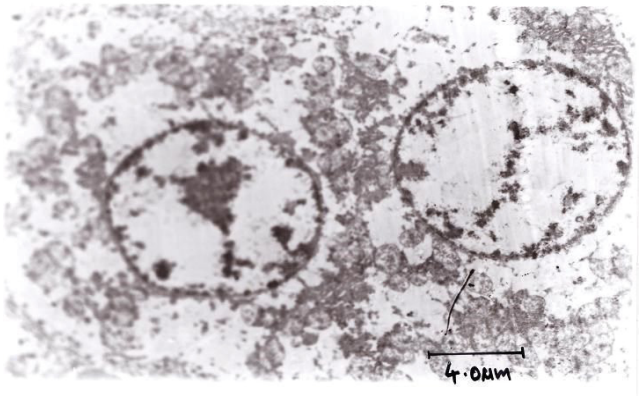

Fig. 8: TEM image of hepatocyte of GTE-treated group
Hepatocyte from green tea extract (GTE) treated group 4 rats, showed clumping of shrunken mitochondria, mild reconstitution of rough endoplasmic reticulum and margination of chromatin

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