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Research Article

HEPATOPROTECTIVE EFFECTS OF TANNIC ACID AGAINST CADMIUM-INDUCED OXIDATIVE STRESS: A MECHANISTIC INSIGHT

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ABSTRACT

Objectives: This study explores the potential hepatoprotective effects of tannic acid (TA), a polyphenolic antioxidant, in mitigating cadmium (Cd)-induced oxidative stress in male Wistar rats.

Methods: Cd was administered to induce oxidative stress-induced damage in male Wistar rats, and TA was orally fed to assess its protective role. Following the completion of the 15-day treatment period, serum organ damage markers, biomarkers of oxidative stress, activities of the Krebs' cycle enzymes, and respiratory chain enzymes were measured. Alteration of glycogen and collagen content were histologically studied to evaluate the state of inflammation and tissue damage. Changes in mitochondrial morphology were also studied. In addition, the interaction of Cd with an important antioxidant enzyme, catalase (CAT), was also studied.

Results: Rats pre-treated with TA showed significant protection against the harmful alterations caused by Cd by reducing endogenous reactive oxygen species through the control of the antioxidant defense system, inflammatory responses, and metabolic enzyme activities. Moreover, TA was also found to protect the tissue morphology and collagen deposition of the hepatic tissue and mitochondrial morphology, possibly by increasing mitochondrial viability. From isothermal titration calorimetry and circular dichroism spectroscopy studies, it is evident that TA can protect the secondary structure of CAT from being altered in the presence of Cd.

Conclusion: Our results systematically demonstrate that TA provided protection against Cd-induced oxidative stress-mediated damage in rat liver tissues through its antioxidant and metal chelating mechanism(s). The results suggest the possibility of using TA alone or alongside dietary interventions in instances of oxidative stress triggered by Cd, emphasizing its potential therapeutic relevance.

Keywords: Antioxidant, Cadmium, Liver, Oxidative stress, Tannic acid.

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INTRODUCTION

In the modern era, rapid industrialization and its association with heavy metal toxicity pose a dire threat to the human race at large. A large share of cropland and water bodies around the world is contaminated with heavy metal pollution, which in turn affects the health of people around the globe [1,2]. One of the most common heavy metals responsible for polluting the environment and causing severe health issues in humans is cadmium (Cd) [3,4].

Cd is a naturally occurring heavy metal with the atomic number 48, and it belongs to group 12 of the periodic table. It was first discovered in 1817 by Friedrich Stromeyer and was characterized as a soft, ductile, bluish-white metal with no odor or taste [5]. The possibility that this heavy metal could cause chronic toxic effects in humans was recognized much later with the first reports of pulmonary, bone, and renal lesions in industrial workers published in the late 1930s–1940s [6,7]. The widespread use of this element in various industrial sectors leads to an estimated 22,000 tons of Cd accumulation in the soils globally every year. While Cd has the ability to affect multiple vital organs, including the kidneys and bones, one of its primary target organs is the liver [8]. The long half-life (15–30 years) of this element makes way for its easy accumulation in the hepatic tissue, which causes severe damage, progressing toward hepatic fibrosis, cirrhosis, and ultimately cancer [9,10].

One of the key mechanisms through which Cd mediates its damaging effects on the human body is oxidative stress [11]. Cd is known to induce the generation of reactive oxygen species (ROS) such as superoxide anion free radicals ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), and thus aggravate oxidative stress by disrupting the intracellular redox homeostasis [12]. The generation of these ROS occurs within the mitochondria, which disrupts the inner mitochondrial environment [13]. This, consequently, leads to lipid peroxidation (LPO), protein oxidation, and DNA damage [14]. Thus, Cd-induced oxidative damage has a profound impact on the vital organs of the human body.

Occupational and environmental exposure to Cd plays a major role in the development of various hepatic diseases, and the conventional means of treatment majorly include chelation therapy. The use of agents such as EDTA, DMSA, and DMPS facilitates the easy and rapid excretion of Cd, with EDTA having a superior potential in mobilizing intracellular Cd than the others [15]. However, chelation therapy is associated with certain risks and adverse effects that include gastrointestinal distress and severe complications such as renal failure, and can even cause cardiovascular collapse [16]. Moreover, the administration of chelators may inadvertently cause the depletion of essential trace elements, which demands very careful dosing and adjunctive therapeutic support; that include the administration of powerful antioxidants [17].

Antioxidants are well known for their ability to protect against oxidative stress and can thus prevent damage to vital organs [18]. In recent times, tannic acid (TA), a natural polyphenol, has received widespread attention in the field of antioxidant therapy, due to its potent antioxidant capacity [19]. It is composed of a central glucose molecule derivatized at its hydroxyl groups, with one or more galloyl residues, and is found in several fruits and herbs, and in wine and tea as well [20]. It is also known to have metal chelating properties, which can prove to be extremely beneficial in treating cases of heavy metal toxicity.

The antioxidant, anti-inflammatory, and anti-apoptotic abilities of TA make it an extremely suitable molecule in mitigating heavy metal-induced pathophysiological alterations. Hence, this study explores the protective effects of TA against Cd-induced oxidative damage in rat hepatic tissue. The results indicate that administration of TA to experimental rats provides hepatoprotection against Cd-induced oxidative stress-mediated damage and reveals a novel mechanism of protection by TA through its molecular interaction with Cd.

METHODS

Chemicals

Cd chloride was purchased from Sisco Research Laboratories, Mumbai, India, and TA was purchased from Sigma Aldrich Merck (St. Louis, Missouri, USA). The pure catalase (CAT) enzyme was procured from Sigma Aldrich (St Louis, MO, USA). The kits used in the study were obtained from Arkray Healthcare Pvt. Ltd. (Mumbai, India). 2',7'-dichlorofluorescein (DCF) diacetate (DCFDA) was purchased from Abcam Biotechnology Company (Abcam, USA). All the other chemicals used were of analytical grade and were purchased from Sigma, St Louis, MO, USA, and Sisco Research Laboratories, Mumbai, India.

Animals and experimental design

All the animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta (Proposal Number – IAEC-IV/Proposal/DB-05/2014/DT. March 13, 2014). Adult male Wistar rats were obtained from Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) registered suppliers, operating under the regulatory framework of the Government of India. The body weights of the animals were between 180 g and 220 g. Before the commencement of the treatments, all the animals were acclimatized for 7 days in standardized environmental conditions prevailing at the animal house facility of the Department of Physiology, University of Calcutta. To determine the minimum effective dose of TA, the animals were divided into five groups: Control, Cd, TA12.5+ Cd, TA25+ Cd, and TA50+ Cd. To induce Cd-mediated oxidative stress in the rats, previously established [21] acute dose of Cd, i.e., 0.44 mg/kg b.w., was injected subcutaneously on every alternate day for a period of 15 days. Such a pre-established subcutaneous dose of Cd was chosen to induce toxicity while simulating a controlled, progressive, and sustained systemic exposure. The dose for oral administration of TA for the groups TA12.5+ Cd, TA25+ Cd, and TA50+ Cd was 12.5 mg, 25 mg, and 50 mg/kg b.w., respectively, for a period of 15 days. Following the determination of the minimal effective dose of TA (i.e., 25 mg/kg b.w.), all further experiments were carried out with four different groups of animals: Control (vehicle-treated), TA (25 mg/kg b.w.), Cd (0.44 mg/kg b.w.), TA+Cd (25 mg/kg b.w. + 0.44 mg/kg b.w.). In all experiments, each group contained six animals. TA was administered orally at 12.5, 25, and 50 mg/kg to assess dose-dependent protection, based on prior literature and rat studies demonstrating reduced Cd accumulation in heart/lungs/brain at equivalent intakes (~10–50 mg/kg from 2% solutions) without toxicity [22]. These doses align with Cd hepatotoxicity models, where polyphenols counteract ROS-mediated damage. Low-end threshold, extending safe antioxidant activation seen at ~17.5 mg/kg [23], mid-range chosen from 20 mg/kg efficacy in Nrf2-mediated protection [24], upper effective dose near 40 mg/kg, safe for dose-response in Cd toxicity models [25].

Animal sacrifice, collection of blood and tissue samples

Following the treatment period, the animals of each group were kept fasted overnight. Each animal was euthanized following CPCSEA guidelines, and ventricular blood was collected and allowed to clot for serum to separate out, and then centrifuged at 2500 rpm for 15 min. Serum was collected and stored at −20°C. The hepatic tissues were carefully collected from the animals by surgically opening the abdomen, and were washed in ice-cold saline, and immediately stored at −20°C for further analysis.

Preparation of tissue homogenate, and isolation of cytosolic and mitochondrial fractions

A 10% tissue homogenate of hepatic tissues was prepared in ice-cold phosphate buffer saline (PBS) (pH 7.4) using a Potter–Elvehjem glass homogenizer. The cytosolic and mitochondrial fractions were prepared by the method of Majumder *et al.* [26] with some minor modifications. The homogenate was centrifuged at 3500 rpm for 10 min to eliminate the nuclear debris. The resulting supernatant was carefully collected, with a portion allocated for various assays. The remaining supernatant was subjected to further centrifugation at 14,000 rpm for 45 min, yielding mitochondrial and soluble cytosolic fractions. The final supernatant was stored at −20°C as the cytoplasmic fraction, whereas the pellet was re-suspended in tris-sucrose buffer (pH 7.4) and also stored at −20°C as the mitochondrial fraction for further analysis.

Histological studies of the liver tissue

Liver tissues were fixed in 10% formaldehyde solution, and the fixed tissue portions were embedded in paraffin wax following the routine procedure as described by Roy *et al.* [27]. Thereafter, 5 µm tissue sections were prepared using a microtome, and the morphological analysis of the hepatic tissues was carried out by staining the tissue sections with hematoxylin-eosin (H&E) stain. The glycogen depletion was assessed by staining the sections with periodic acid schiff stain, and for studying the fibrotic alteration in the collagen content of the hepatic tissues, Masson's trichrome and Picrosirius Red stains were used [28,29]. The assessment was semi-quantitatively scored (0–4 scale) by a double-blinded observer. The Olympus BX-53/DP 80 microscope was used to capture the images of the H&E and periodic acid schiff-stained tissue sections at ×50 magnification. An Olympus confocal microscope model FV 3000, using the fluorescein isothiocyanate (FITC) and TRITC channels, was used to capture the images of the Picrosirius Red-stained tissue sections.

Surface topology study of the isolated mitochondria under the scanning electron microscopy (SEM)

Isolated liver mitochondria were fixed in 1.5% (w/v) glutaraldehyde at 4°C for 24–48 h. Following the dehydration of mitochondria with graded ethanol and isoamyl alcohol, the ultrastructural images were captured at ×25,000 magnification using a Carl Zeiss Scanning Electron Microscope [30].

Determination of the activities of serum glutamate pyruvate transaminase (SGPT), lactate dehydrogenase (LDH) (total), LDH 5, and alkaline phosphatase (ALP)

The activities of SGPT and ALP in the serum were determined according to the methods as described in the respective kits. The total activity of LDH and the activity of LDH 5 were measured according to the method of Strittmatter [31].

Measurement of the biomarker of oxidative stress

The levels of LPO in the hepatic tissues were determined according to the method of Buege and Aust [32]. The absorbance of the final supernatant was measured spectrophotometrically at 532 nm, and the data were represented as nmoles of TBARS/mg protein. The protein carbonyl (PCO) content in the hepatic tissue was determined by DNPH assay following the method of Levine *et al.* [33], and the absorbance of the samples was measured spectrophotometrically at 375 nm. The levels of protein carbonylation were expressed as nmoles/mg tissue protein. The levels of GSH in the hepatic tissues were estimated by the

method of Lindsay and Sedlak [34], and the absorbance of the samples was measured at 412 nm using a UV/VIS spectrophotometer. The GSH levels were expressed as nmoles/mg protein.

Assessment of generation of hydroxyl radical (•OH)

To determine the content of •OH generated, the mitochondria were isolated from the liver tissues following the method of Hare *et al.* [35] with minor modifications [36], and the levels of •OH were determined by the method of Babbs and Steiner [37], using DMSO as a •OH scavenger. The values obtained were expressed as nmol of •OH/mg protein.

Determination of the activities of cytosolic (Cu-Zn SOD) and mitochondrial (Mn-SOD) superoxide dismutase (SOD), CAT, glutathione reductase (GR), and glutathione peroxidase (GPx)

The activities of SOD were estimated by the method of Marklund and Marklund [38]. The autoxidation of pyrogallol was set at ΔOD 0.02. The changes in absorbances were noted for 5 min at an interval of 30 s at 420 nm using a UV/VIS spectrophotometer. The method of Beers and Sizer was followed for estimating the activity of CAT [39]. The enzyme activity was expressed as nmoles of H_2O_2 consumed/mg protein. The decrease in absorbance was noted spectrophotometrically for 90 s at an interval of 10 s at 240 nm. Glutathione reductase activity was measured by the method of Krohne-Ehrich *et al.* [40]. At 340 nm, the decrease in absorbance was noted spectrophotometrically at an interval of 15 s for 180 s. The activity of GPx was measured according to the method of Paglia and Valentine [41]. The decrease in absorbance was noted spectrophotometrically at 340 nm at an interval of 10 s for 90 s. The activities of SODs, GR, and GPx were expressed in U/mg protein.

Determination of the activities xanthine oxidase (XO) and xanthine dehydrogenase (XDH)

Xanthine oxidase activity of the rat hepatic tissues was determined by measuring the conversion of xanthine to uric acid following the method of Greenlee and Handler [42]. The auto-oxidation of xanthine was recorded spectrophotometrically at 295 nm. The activity of XDH was determined as described by Mukherjee *et al.* [43]. The changes in absorbance were noted at 340 nm using a UV/VIS spectrophotometer.

Determination of the activities of the pyruvate dehydrogenase (PDH) and some of the enzymes of the Krebs' cycle

The activity of PDH was determined spectrophotometrically according to the method of Chretien *et al.* [44]. Isocitrate dehydrogenase activity was measured according to the method of Duncan *et al.* [45]. The α-ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically according to the method of Duncan *et al.* [45]. The activities of succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) were determined following the method of Veeger *et al.* [46] and Mehler *et al.* [47], respectively.

Determination of the activities of glycolytic enzymes and the enzymes of pentose phosphate pathway

The activity of HK, the first-rate limiting enzyme of the glycolytic pathway, in hepatic cytosolic samples, was assayed spectrophotometrically by reduction of NAD^+ to nicotinamide adenine dinucleotide (NADH) according to the protocol of Abdel-Hamid *et al.* [48]. The activity of another rate-limiting enzyme of the glycolytic pathway, namely, phosphofructokinase (PFK), was recorded spectrophotometrically by the oxidation of NADH [49]. The activity of glucose-6-phosphate dehydrogenase (G-6-PDH) was determined spectrophotometrically by reduction of NADP to NADPH [50].

Determination of the activities of respiratory chain enzymes

The activities of cytochrome C oxidase and cytochrome C oxidoreductase were determined spectrophotometrically following the method of Goyal and Srivastava [51]. The enzyme activities were expressed as U/mg protein.

Measurement of the levels of ROS

To measure the levels of ROS, hepatic tissue samples were stained with DCFDA, followed by washing in PBS. In the presence of ROS in the samples, the incubated non-fluorescent DCFDA gets oxidized into a highly fluorescent DCF [52]. The concentration of ROS was monitored using a flow-cytometer (BDFACS Versa, USA), and the data were processed using FlowJo software and expressed as DCF fluorescence intensity (FITC-A Median).

Isothermal titration calorimetry (ITC) study to determine the binding of Cd with CAT and TA

The binding of Cd with CAT and the binding of Cd with TA were determined by ITC using a MICROCAL PEAQ-ITC (Malvern) [53]. Forty microliters of 0.24 mM Cd was titrated by injecting 300 μL of 0.0147 mM TA into the system at 25°C. Further, the binding propensity of Cd with CAT was determined by titrating 40μL of 0.24 mM Cd against 150 μL of 4×10^{-6} mM of pure CAT.

Circular dichroism (CD) spectroscopy

The CD spectra for CAT in the presence or absence of Cd, TA, or both were recorded from 190 nm to 300 nm at a medium scan rate using a JASCO J-1500 CD spectrometer. Each spectrum was the average of three individual scans. The baseline was corrected using 50 mM potassium phosphate buffer (pH 7.8) as a blank. The spectra were analyzed statistically with OriginPro data analysis software.

Protein estimation

Protein content of the tissue samples was estimated by the method of Lowry *et al.* [54].

Statistical analysis

Data have been presented as the mean±standard error of the mean. To find out the level of significance between groups in different parameters measured, one-way analysis of variance (ANOVA) followed by *post hoc* multiple comparisons using Tukey's honestly significant difference (HSD) test was performed. Normality of the dependent variable within each group was tested using the Shapiro–Wilk test ($p>0.05$). Homogeneity of variances was confirmed using Levene's test ($p>0.05$ across variants) before one-way ANOVA. The Statistical Package for the Social Sciences version 25.0 (IBM) software was used to check the level of significance, and the results were considered statistically significant at the level of $p<0.05$. The graphs were prepared using GraphPad Prism 6 software.

RESULTS

TA dose-dependently ameliorated the Cd-induced alteration of the biomarkers of oxidative stress and hepatic damage

Treatment of rats with $CdCl_2$ at a dose of 0.44 mg/Kg body weight every alternate day for a period of 15 days revealed significant alterations in the histological architecture of the hepatic tissues of the rats. Hematoxylin-eosin stained sections of Cd-treated hepatic tissues revealed significant alterations, such as venous congestion, congestion of sinusoids, accompanied by inflammatory cell infiltration. However, on pre-treatment of the rats with TA, such changes were found to be protected from taking place (Fig. 1a). The results indicate the ability of TA to protect against Cd-induced tissue injury. Periodic acid-Schiff stain of the hepatic tissue sections exhibited a steady depletion of glycogen in the hepatic tissue of Cd-treated animals. Pre-treatment of rats with TA was found to protect against the depletion of glycogen content in a dose-dependent manner, indicating a protective role of TA in maintaining the tissue glycogen (Fig. 1b). Histological examination of Masson's trichrome-stained sections of hepatic tissues of Cd-treated animals demonstrated a significant increase in the collagen content. However, the hepatic tissue sections of the rats pre-treated with TA were significantly protected. The results indicate the ability of TA to provide protection against Cd-induced deposition of tissue collagen (Fig. 1c). Treatment of rats with $CdCl_2$ caused a significant elevation in the activities of total LDH (2.1 fold, *$p<0.05$ vs. control) and LDH5 (2 fold, *$p<0.05$ vs. control), a specific marker of hepatic damage. However, when the rats were pre-treated with TA in three different doses, the activities of total LDH and LDH5 in the serum were found to be significantly protected from being increased (1.44 fold and 1.53 fold, respectively, **$p<0.05$ vs. Cd-treated group) (Fig. 2a).

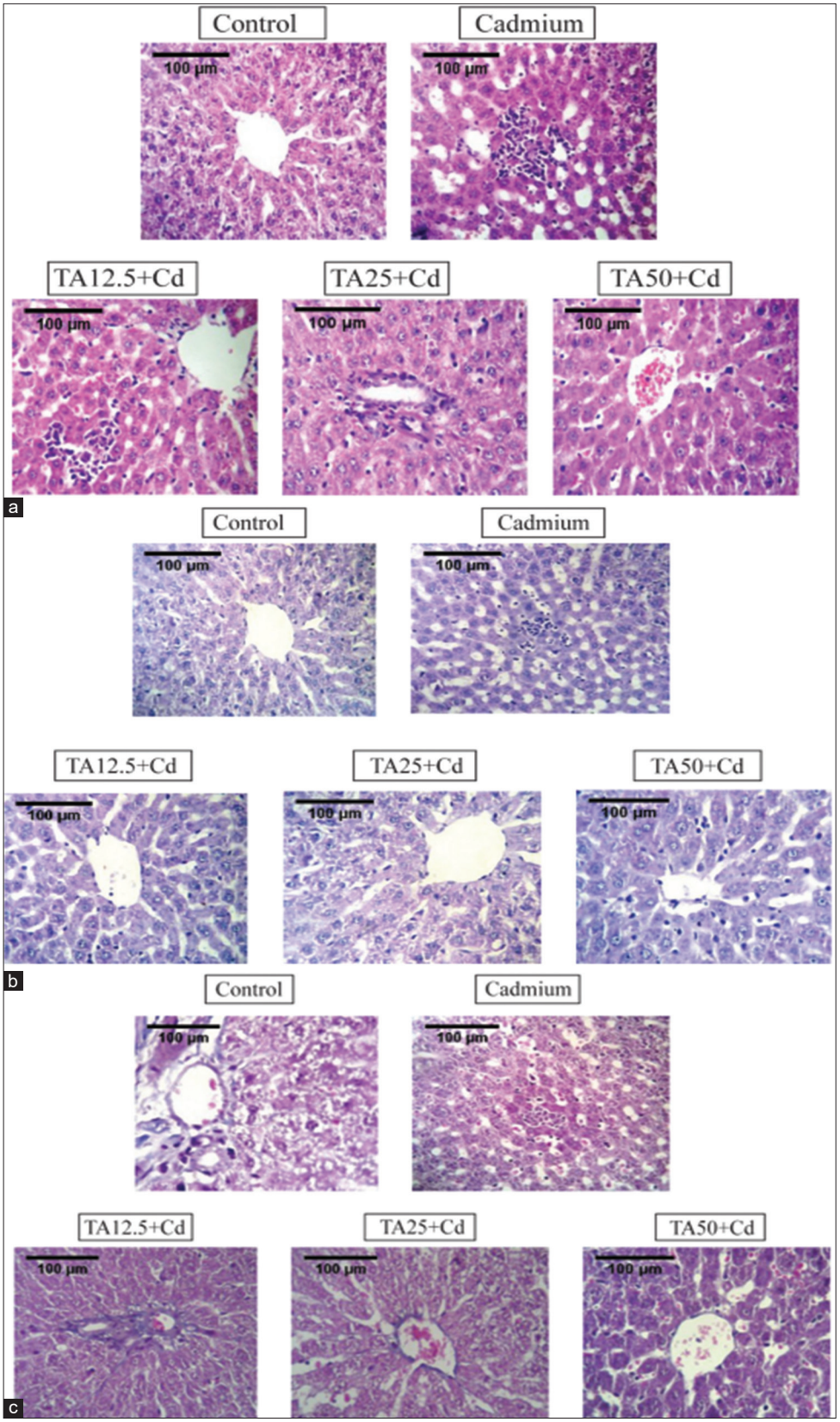

Fig. 1: (a) Protective effects of tannic acid against $CdCl_2$-induced hepatic damage. Microscopic images of hematoxylin and eosin-stained (×40 magnification) hepatic tissue sections demonstrating the morphological alterations in different groups of rats. Scale bar in "100 µm" mentioned in the upper left corner of each micrograph. (b) Periodic acid-Schiff stained (×40 magnification) hepatic tissue sections depicting the content of glycogen in different groups of rats. Scale bar in "100 µm" mentioned in the upper left corner of each micrograph. (c) Masson trichrome-stained hepatic sections exhibiting the collagen contents in different groups of rats. Scale bar in "100 µm" mentioned in the upper left corner of each micrograph

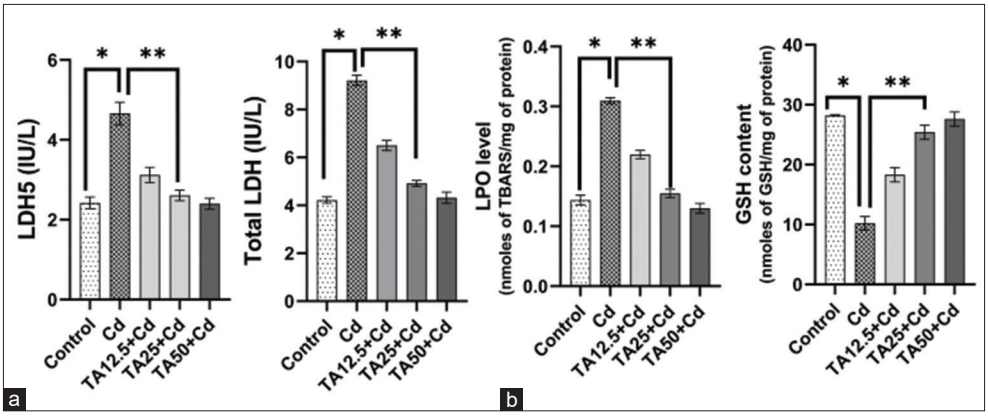

Fig. 2: (a) Effect of increasing doses (12.5, 25, and 50 mg/kg body weight) of tannic acid on biomarkers of hepatic damage in serum, namely, lactate dehydrogenase 5 (LDH5) and total LDH. *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance (ANOVA) with Tukey honestly significant difference (HSD) *post hoc*. (b) Represents the levels of lipid peroxidation and reduced glutathione level in hepatic tissue homogenates of the studied groups of rats. *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way ANOVA with Tukey HSD *post hoc*

The increased activities of these enzymes in the serum indicated the occurrence of damage in the hepatic tissue membrane, which is caused by the generation of oxidative stress. This was evident from a significantly increased level of LPO compared to control (2.15 fold, *p<0.05 vs. control group). Pre-treatment of rats with TA at three different doses significantly protected the LPO levels from being altered (0.29 fold, 0.5 fold, and 0.58 fold **p<0.05 vs. Cd treated group). On measuring the levels of GSH, it was observed that in the Cd-treated group, there was a 0.69-fold increase in the level of GSH (*p<0.05 vs. the control group), while pre-treatment of rats with TA at three different doses was found to protect the levels of GSH from being altered (1.79, 2.49, and 2.70 fold, respectively, **p<0.05 vs. Cd-treated group) (Fig. 2b).

Fig. 3a reveals that Cd significantly increased the activities of cytosolic Cu–Zn–SOD, the mitochondrial Mn–SOD, and CAT in the rat hepatic tissues (1.99 fold, 2.22 fold increase, and 0.61 fold decrease, respectively, versus control, *p<0.05 vs. control). Pre-treatment of rats with TA in three different doses was found to protect the activities of these antioxidant enzymes from being increased (for Cu–Zn–SOD, 0.17 fold, 0.42 fold, and 0.5 fold decrease compared to $CdCl_2$-treated group; for Mn–SOD, 0.13 fold, 0.53 fold and 0.54 fold decrease; for CAT, 1.42 fold, 1.60 fold and 1.62 fold increase compared to $CdCl_2$-treated group, respectively, **p<0.05 versus Cd treated group.

Fig. 3b depicts that the activities of hepatic XO, XDH, the total enzyme activity, i.e., XO plus XDH, XO: XDH ratio, and XO/(XO+XDH) were all increased significantly following the treatment of rats with $CdCl_2$ (4.24-fold, 2.73-fold, 2.84-fold, 1.55-fold, and 1.49-fold increase, respectively, vs. control; *p<0.05 vs. control). All these parameters were significantly protected from being increased when the rats were pre-treated with TA, indicating the ability of TA to influence the activities of these pro-oxidant enzymes (0.78, 0.64, 0.63, 0.33, and 0.35 fold decrease compared to Cd-treated group, respectively, in hepatic tissue, **p<0.05 vs. Cd-treated group).

TA protected the hepatic tissue against Cd-mediated histopathological alterations

In a separate experiment using the best effective dose of TA (i.e., 25 mg/kg b.w.), it was observed (Fig. 4) that this antioxidant was capable of protecting the hepatic tissue by reducing the instances of venous congestion, congestion of sinusoids, hepatocyte necrosis with inflammatory cell infiltration, when compared to the Cd-treated group. The diameter of the central vein, which was significantly increased on administration of $CdCl_2$, was found to be significantly decreased in the presence of TA. Furthermore, no significant change was observed in the TA-only group when compared to the control group, thus indicating that TA, by itself, has no toxic effect on the hepatic tissue.

TA alleviated the Cd-induced increased tissue collagen content

On estimating the collagen content in hepatic tissue, a significant increase (p<0.05) was observed in Cd-treated hepatic tissue sections with a concomitant increase of the MFI, whereas pre-treatment of rats with TA at the dose of 25 mg/kg b.w. protects against the abrupt increase in tissue collagen and improves the hepatic tissue morphology (Fig. 5).

TA alleviated hepatic toxicity, evidenced through improved serum hepatic damage markers

The significantly reduced levels of SGPT, ALP, LDH5, and total LDH activities upon TA pre-treatment imply TA-mediated successful protection of hepatic damage, induced by Cd. In the TA+Cd group, the levels of activities of SGPT, ALP, LDH5, and total LDH were found to be less than by 0.59 fold, 0.35 fold, 0.5 fold, and 0.46 fold, respectively, when compared to the Cd-treated group (p<0.05) (Fig. 6a-d, respectively).

TA protects against Cd-induced alterations in oxidative stress biomarkers and hydroxyl radical generation

To determine the oxidative stress status of the hepatic tissue, the levels of different biomarkers of oxidative stress were assessed. Fig. 7aA-C reveals that TA, at a dose of 25 mg/kg b.w., was capable of significantly lowering the level of TBARS (0.52 fold), preventing protein carbonylation (0.42 fold), and preserving the levels of reduced glutathione at physiological levels when compared to the Cd-treated groups. TA, alone, however, has no significant effect on the biomarkers of oxidative stress.

A similar trend was observed in Fig. 7b, where it is observed that Cd treatment increases the generation of hydroxyl radical within rat hepatic tissue by 1.48 fold, which, however, was found to be prevented from being increased when the rats were pre-treated with TA ($^{\bullet}OH$ generation was only reduced by 0.13 fold compared to the Cd-treated group; p<0.05). TA alone, however, has no effect on $^{\bullet}OH$ generation.

TA ameliorates Cd-induced alterations in the activities of antioxidant enzymes

Cd treatment significantly altered the activities of the antioxidant enzymes such as SOD1, SOD2, mitochondrial peroxidase, GPx, CAT, and GR. Pre-treatment of rats with TA significantly prevented such alterations and protected the activities of all these enzymes (Fig. 8a-f, respectively). Here also, TA alone exhibited no effect on the enzyme activities.

TA prevented the Cd-induced alterations in the status of the pro-oxidant enzymes

Fig. 9a and b shows that there was a significant rise in the activities of both the pro-oxidant enzymes, namely, xanthine oxidase (XO) and

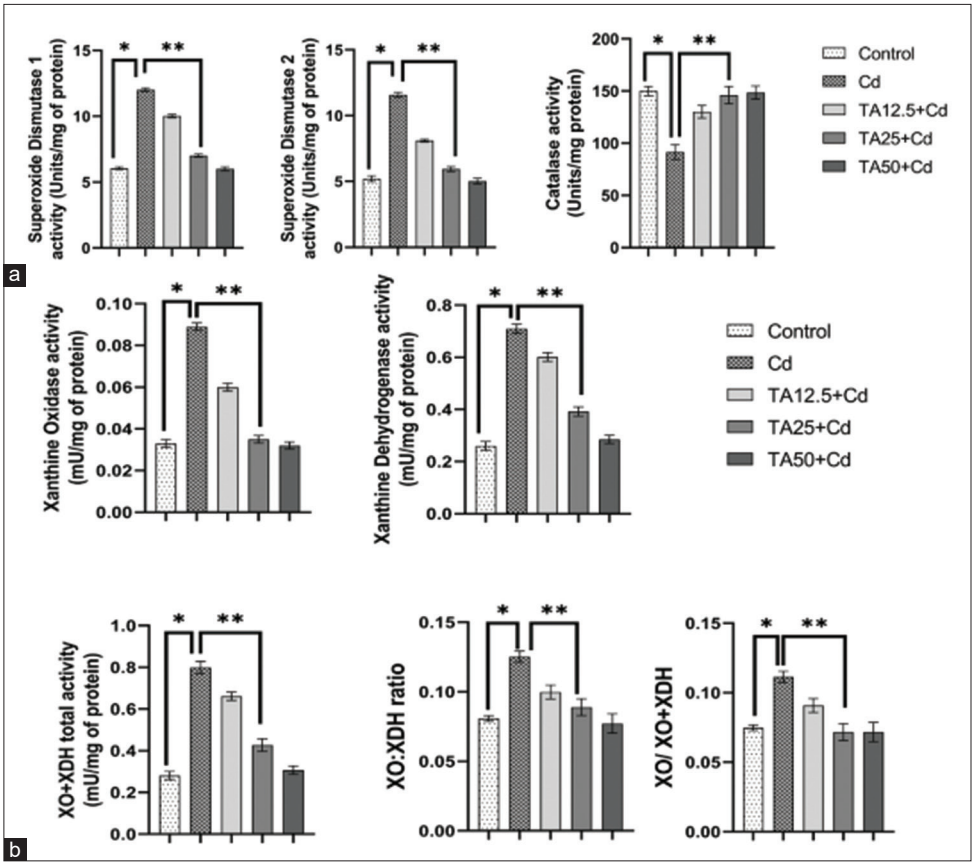

Fig. 3: (a) Increasing doses of tannic acid (TA) protect against the cadmium-induced alterations in antioxidant enzyme activities, superoxide dismutase 1 (SOD 1), SOD 2, and catalase in various subcellular fractions of rat hepatic tissue. The values are expressed as mean±standard error of the mean (SEM) (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis (ANOVA) of variance with Tukey honestly significant difference (HSD) *post hoc*. (b) Increasing doses of TA protect against the cadmium-induced alterations in and pro-oxidant enzyme activities xanthine oxidase (XO), xanthine dehydrogenase (XDH), XO+XDH total activity, XO: XDH ratio, and XO/XO+XDH in various sub-cellular fractions of rat hepatic tissue. The values are expressed as mean±SEM (n = 5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way ANOVA with Tukey HSD *post hoc*

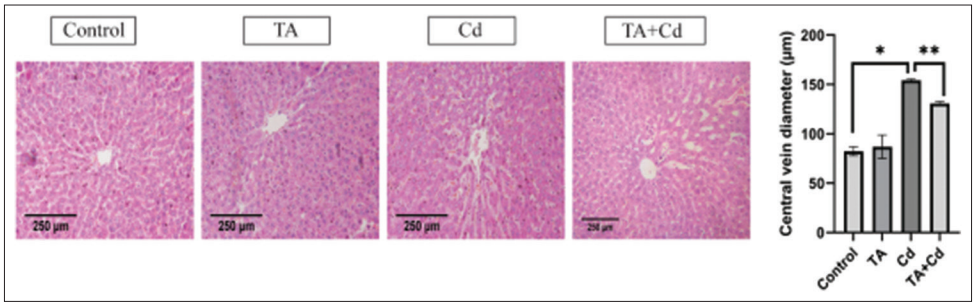

Fig. 4: Photomicrographs of hematoxylin-eosin-stained hepatic tissue sections (×40 magnification) of different groups of rats treated with tannic acid and/or cadmium, showing histo-morphological changes in hepatic tissue when visualized using an Olympus microscope. Scale bar in "250 µm" mentioned in the lower left corner of each micrograph

xanthine dehydrogenase (XDH), were observed in the Cd-treated groups ($p<0.05$). TA, however, at a dose of 25 mg/kg b.w., was able to significantly prevent the change in the activity of both the enzymes when compared to the control at a significance level $p<0.05$, respectively. TA alone, however, has no effect on the activities of these enzymes. In Fig. 9c, a similar trend was also observed for the XO: XDH ratio. The total activities of XO and XDH, and the XO/XO+XDH ratio were found to be significantly increased on treatment with Cd, which were significantly protected from being altered following pre-treatment with TA, thus further validating the protective properties of TA against Cd-mediated toxicities (Fig. 9d and e). Here also, TA alone has no effect on the parameters studied.

TA provided protection to liver tissue by reducing hepatic ROS levels

For further validation, the DCFH-DA probe was used to detect total hepatic ROS concentration. Our findings revealed that Cd significantly increased the fluorescence intensity produced from the DCFH-DA probe. In contrast, TA at 25 mg/kg b.w. prevented the increased fluorescence from being produced due to ROS, as evidenced by the flow-cytometric

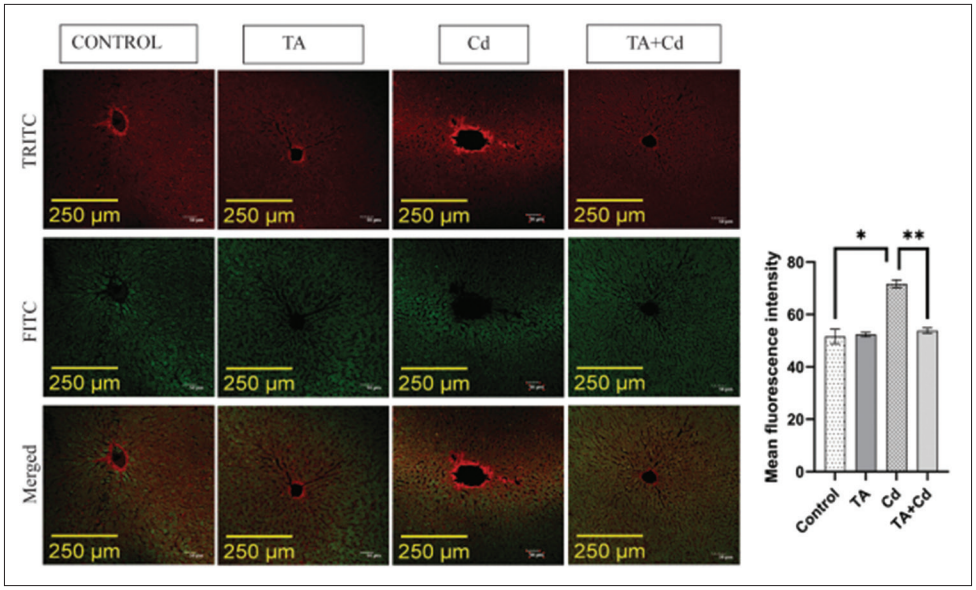

Fig. 5: Picrosirius-red-stained hepatic tissue sections (×100 magnification) captured using an Olympus confocal microscope, with column diagrams in the inset, representing the quantification of hepatic collagen content as mean fluorescence Intensity of tissue sections of different groups of rats. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*. Scale bar in "250 µm" mentioned in the lower left corner of each micrograph

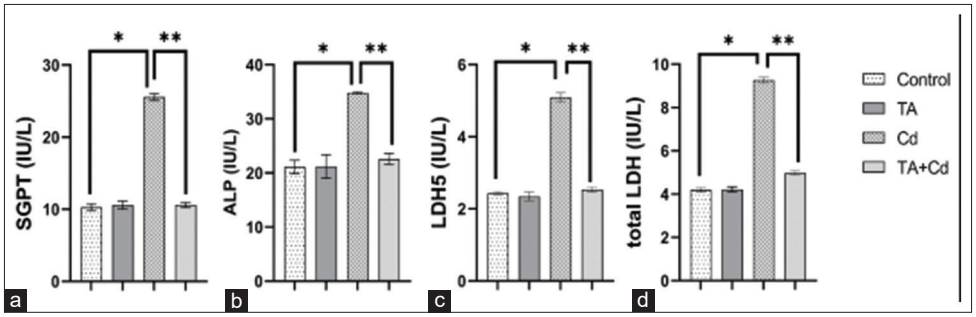

Fig. 6: Graphical representation of the alterations in the levels of biomarkers of hepatic damage (a) serum glutamic pyruvic transaminase, (b) alkaline phosphatase, (c) lactate dehydrogenase isoenzyme 5, and (d) total lactate dehydrogenase in serum of experimental rats. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*

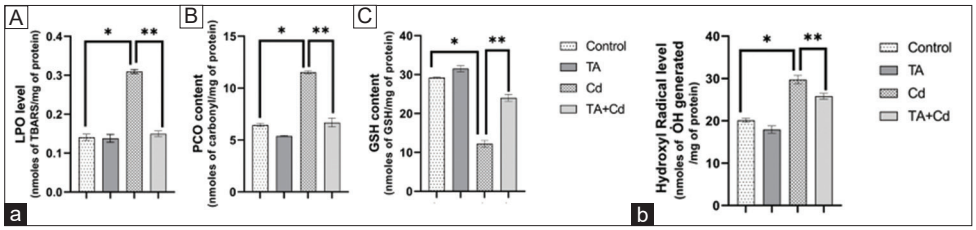

Fig. 7: (a) Column diagrams depicting the alterations in the levels of biomarkers of oxidative stress (A) lipid peroxidation, (B) protein carbonylation, and (C) glutathione in the hepatic tissue of rats treated with tannic acid (TA) and/or cadmium. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance (ANOVA) with Tukey honestly significant difference (HSD) *post hoc*. (b) Column diagram depicting the alterations in the levels of hydroxyl radicals generated in the hepatic tissue of rats treated with cadmium, cadmium and TA alone. The values are expressed as mean±SEM (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way ANOVA with Tukey HSD *post hoc*

overlay diagram (Fig. 10). Here again, TA alone was found to have no significant effect on the increment of fluorescence due to ROS.

Studies on binding of TA with Cd and CAT using ITC

Titration of TA against Cd and CAT through ITC yielded very strong sequential binding matrices with K_d and negative ΔH values, suggesting favorable interactions (Fig. 11).

TA modulated the oxidative homeostasis by protecting against Cd-mediated inhibition of hepatic CAT: An *in vitro* study

The results of our studies, as shown above, indicated that TA has been successful in restoring the activity of CAT, along with several other enzymes associated with maintaining the oxidative balance within the hepatic tissue that were significantly diminished following treatment of rats with Cd. To understand the mechanism of this protection, further

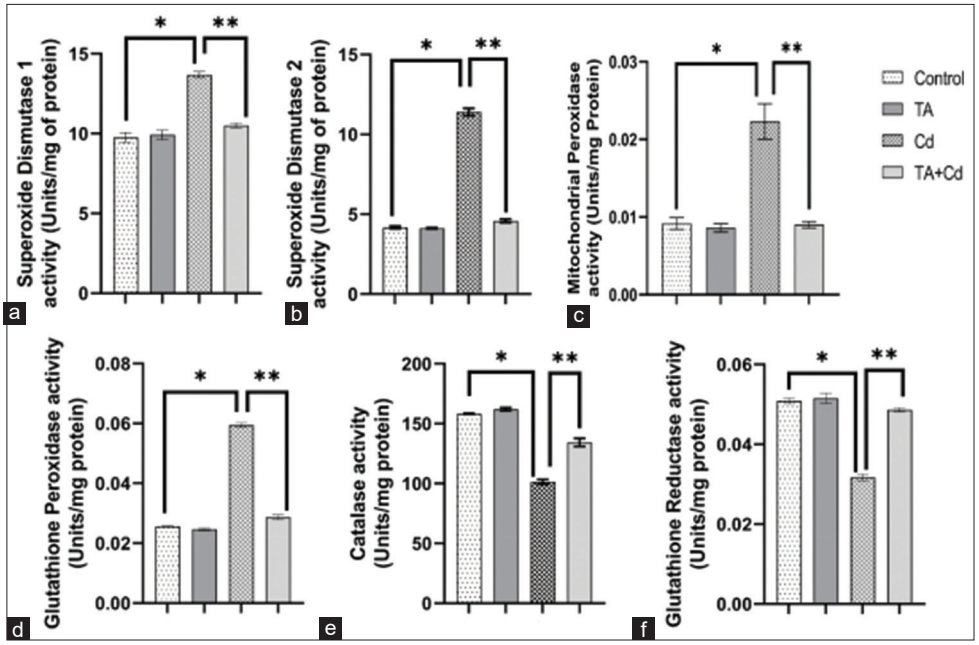

Fig. 8: Column diagrams indicating the alterations in the activities of the antioxidant enzymes (a) superoxide dismutase 1 (SOD 1), (b) SOD 2, (c) mitochondrial peroxidase, (d) glutathione peroxidase, (e) catalase, and (f) glutathione reductase in the hepatic tissue of experimental rats. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*

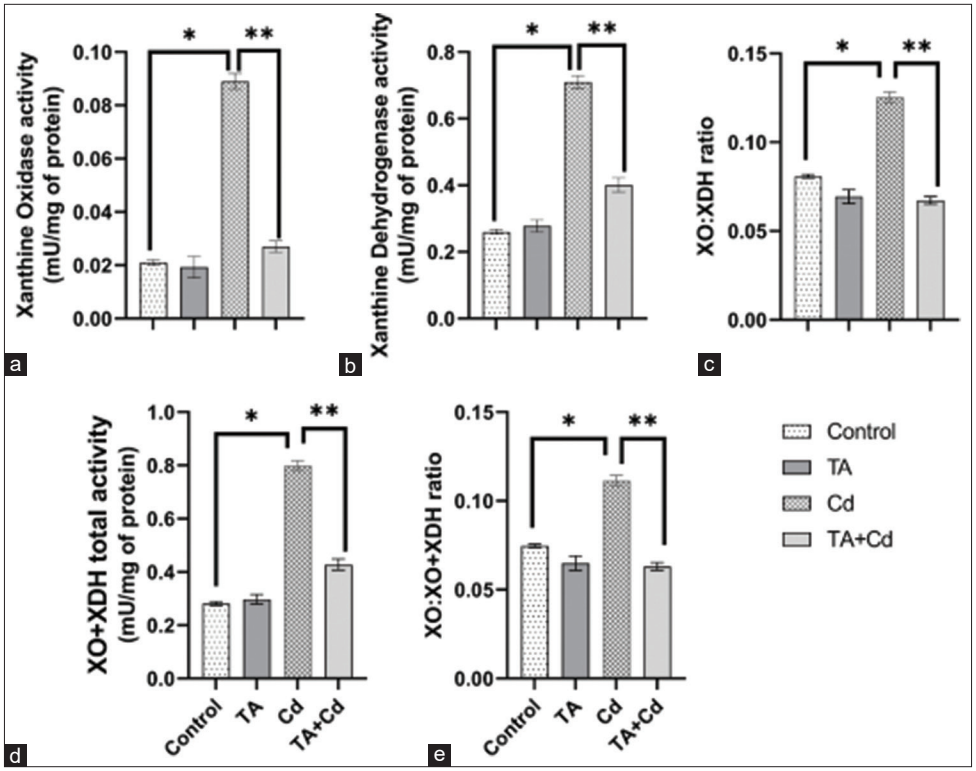

Fig. 9: Column diagrams depicting the alterations in the activities of pro-oxidant enzymes (a) xanthine oxidase, (b) xanthine dehydrogenase, (c) xanthine oxidase (XO):xanthine dehydrogenase (XDH) ratio, (d) XO+XDH total activity and (e) XO: XO+XDH ratio in the hepatic tissues of animals treated with cadmium, cadmium and TA alone. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*

experiments were carried out with pure CAT enzymes. The activity of pure CAT in the presence of increasing concentrations of Cd revealed a significant decline in the activity of the enzyme with a maximal decrease in the presence of Cd at 29.09×10^{-2} μM. CD spectroscopy was performed to elucidate the detrimental effects of Cd on the secondary structure of CAT. Fig. 12 demonstrates that Cd at a concentration of 1 μM is capable of disrupting both the alpha helix and beta sheet structures of the enzyme CAT. These changes were greatly protected

when Cd was introduced in the presence of TA. Furthermore, the non-parametric two-tailed Spearman's Rho correlation studies between CAT activity and the generation of ROS revealed a negative correlation (r=−0.818), which clearly indicates that the decrease in the activity of CAT is a key factor that leads to the excess accumulation of the total ROS in the hepatic tissue.

TA prevented the Cd-mediated inhibition of activities of vital glycolytic and pentose phosphate pathway enzymes

Fig. 13a-c demonstrates a significant decline in the activities of some of the enzymes of the glycolytic pathway, namely, HK (0.43 fold), PFK (0.58 fold), and ALD (0.77 fold) ($p<0.05$) following treatment of rats with Cd. Furthermore, a significant decline in the activity of G6PDH, a key enzyme in the pentose phosphate pathway, was observed, and the results are presented in Fig. 13d. However, such inhibitions were significantly prevented from being altered in the TA+Cd treated groups, thus indicative of TA's protective actions. TA alone has no effect on the activity of these enzymes.

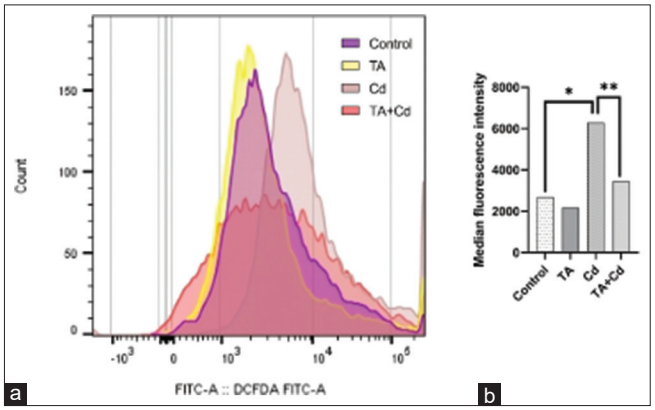

Fig. 10: Staggered overlay presentation of the effects of tannic acid on levels of reactive oxygen species induced by cadmium in hepatic tissue, via flow-cytometric analysis following DCFDA staining (a), and the column diagram (b) represents the median fluorescence intensity of DCFDA as measured in fluorescein isothiocyanate-A channel.

Protective effect of TA against inhibitory actions of Cd on PDH and Krebs' cycle enzymes

As depicted in Fig. 14aA-E, the activities of all five enzymes, i.e., PDH, isocitrate dehydrogenase (ICDH), α-KGDH, SDH, and MDH, were found to be decreased significantly in the Cd-treated groups when compared to their respective controls ($p<0.05$). Whereas the activities of all these enzymes in the TA+Cd groups were found to be protected, when compared to the Cd groups ($p<0.05$). The Michaelis–Menten plots and the Lineweaver–Burk plots of the activities of all of these enzymes reveal an uncompetitive mode of inhibition by Cd. Here, we also observed that TA alone has no significant effect on the activities of these enzymes.

TA protects against Cd-induced inhibition of the activity of enzymes of electron transport chain (ETC)

Fig. 15a and b depicts a significant decrease in the activity of cytochrome c oxidase and cytochrome c oxidoreductase, following treatment of rats with Cd, by 0.42 fold and 0.33 fold, respectively, when compared to their respective control groups ($p<0.05$). The activities of these enzymes, however, were significantly protected when the animals were pre-treated with TA ($p<0.05$). TA alone, however, has no effect on the activities of these enzymes.

TA protected against Cd-mediated structural injury of the mitochondrial surface

The status of the rat hepatic mitochondria was evaluated through SEM studies (Fig. 16). The results revealed that treatment of rats with Cd causes swelling and blebbing of mitochondria, leading to disruption of mitochondrial surface morphology and mitochondrial damage. However, when the animals were pre-treated with TA, the mitochondrial morphology was found to be effectively protected from being altered. Both the values of mitochondrial surface roughness (Rq and Ra) were significantly increased in the presence of Cd. However,

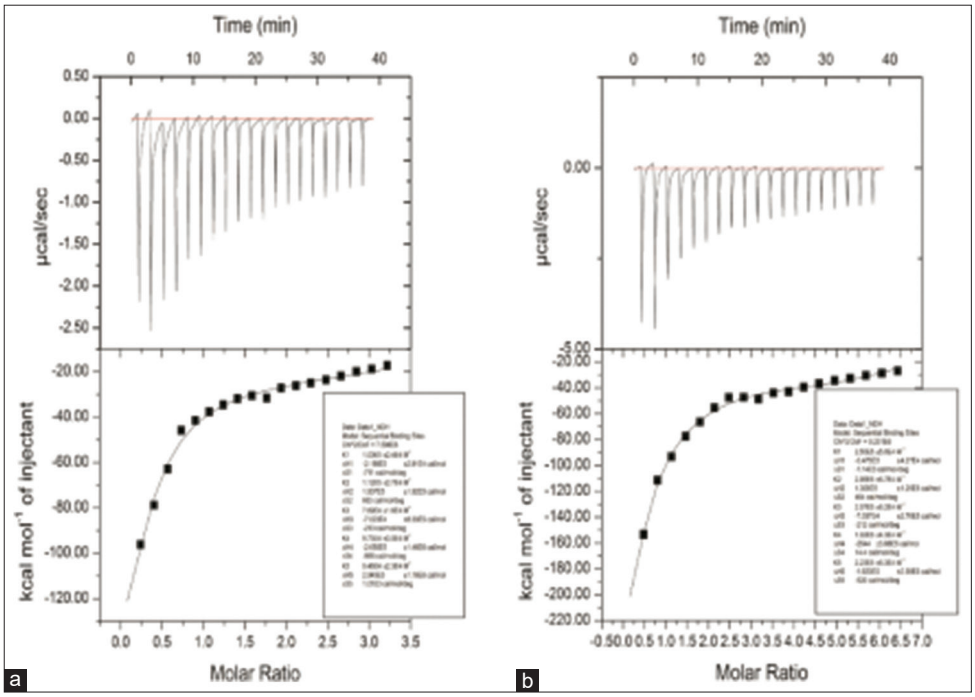

Fig. 11: (a and b) Recordings and graphs depicting the binding of tannic acid with cadmium and catalase, individually, as obtained through PEAQ-ITC (MicroCal Inc., Northampton, MA)

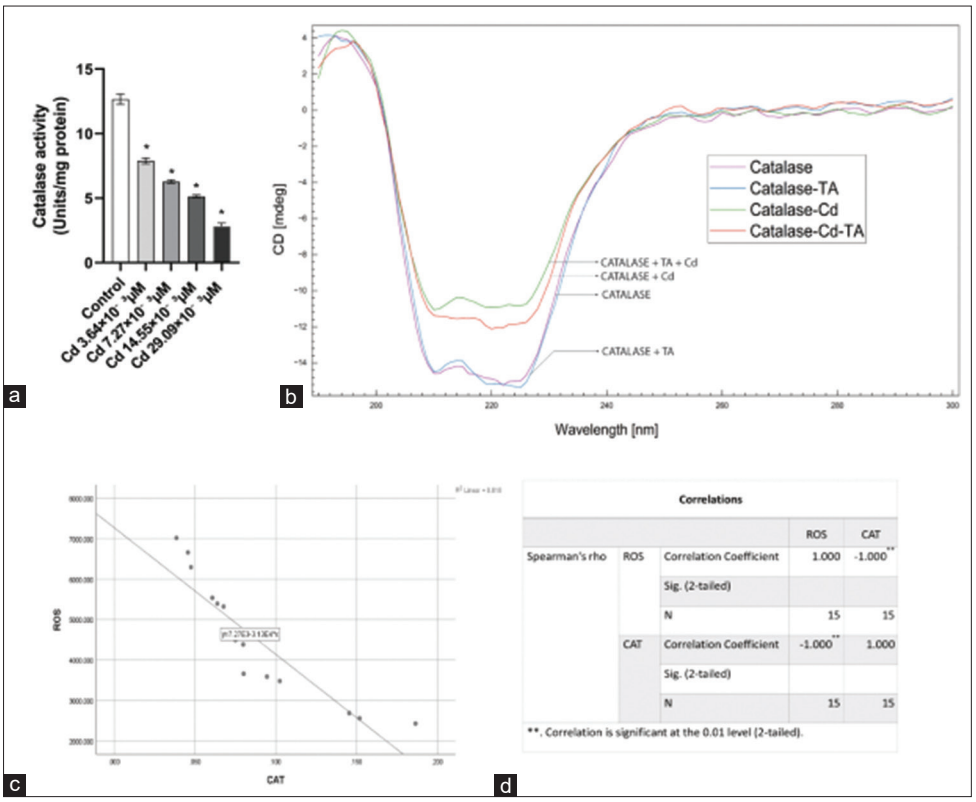

Fig. 12: (a) Depicts activity of pure catalase (CAT) enzyme in presence of different concentrations of cadmium, (b) circular dichroism spectroscopic scan of pure CAT enzyme in presence of cadmium, cadmium and tannic acid alone, delineating the ellipticity of the pure enzyme, (c) representative scatter plot and (Tabular representation of correlation between reactive oxygen species level and CAT (significant [p≤0.01] correlation [r=−0.818])

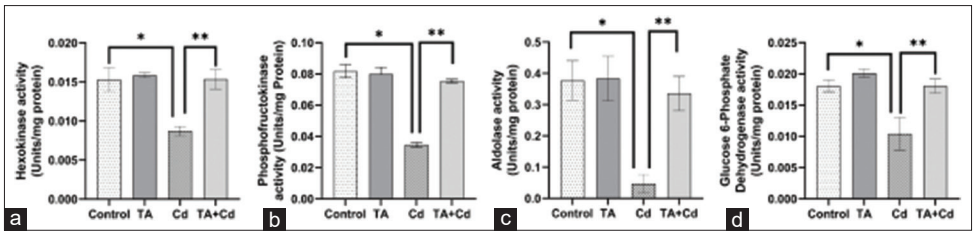

Fig. 13: Graphical depiction of the alterations in the activities of glycolytic enzymes and the enzyme associated with the pentose phosphate pathway (a) hexokinase, (b) phosphofructokinase, (c) aldolase, and (d) glucose-6-phosphate dehydrogenase. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*

pre-treatment of rats with TA significantly decreased these values, indicating the protective effects of TA in maintaining mitochondrial structural integrity. It is interesting to note that here also, TA alone has no disruptive effect on mitochondrial morphology.

DISCUSSION

The objective of the present study was to assess the hepato-protective effects of TA against Cd-induced hepatic damage. This study extensively explores the antioxidant role of TA in ameliorating Cd-induced oxidative damage and demonstrates how TA preserves the catalytic efficiency of vital enzymes and prevents structural damage at both cellular and mitochondrial levels.

In this study, Cd metal (in the form of a Cd-salt) was used as an inducer of oxidative stress in rats, consistent with numerous studies that have established its toxic properties, free radical generation, and consequent role in promoting oxidative stress in biological systems [55-58]. It is known that Cd, being a heavy metal, accumulates and subsequently induces oxidative stress in different organs of an organism [21,28]. From the results of our study, it was seen that administration of Cd caused a significant increase in levels of LPO and a decrease in GSH levels of hepatic tissue of the Cd-treated rats [59]. Our histopathological analyses demonstrate that Cd exposure led to extensive liver injury, including venous congestion, necrosis of hepatocytes, and inflammatory infiltration. However, pre-treatment with TA significantly mitigated these effects, indicating its ability to preserve hepatic architecture and reduce tissue injury. Another critical finding is the protective effect of TA on hepatic collagen content. Cd significantly increased collagen deposition and fluorescence intensity, suggesting fibrotic changes. TA pre-treatment countered this effect, indicating its potential in preventing fibrosis and preserving normal liver histoarchitecture [60].

Biochemical assays further validate the protective role of TA. The significant elevation of serum hepatic damage markers (SGPT, ALP, LDH5, and total LDH) following Cd exposure was notably protected from being altered with TA pre-treatment. These findings suggest TA's

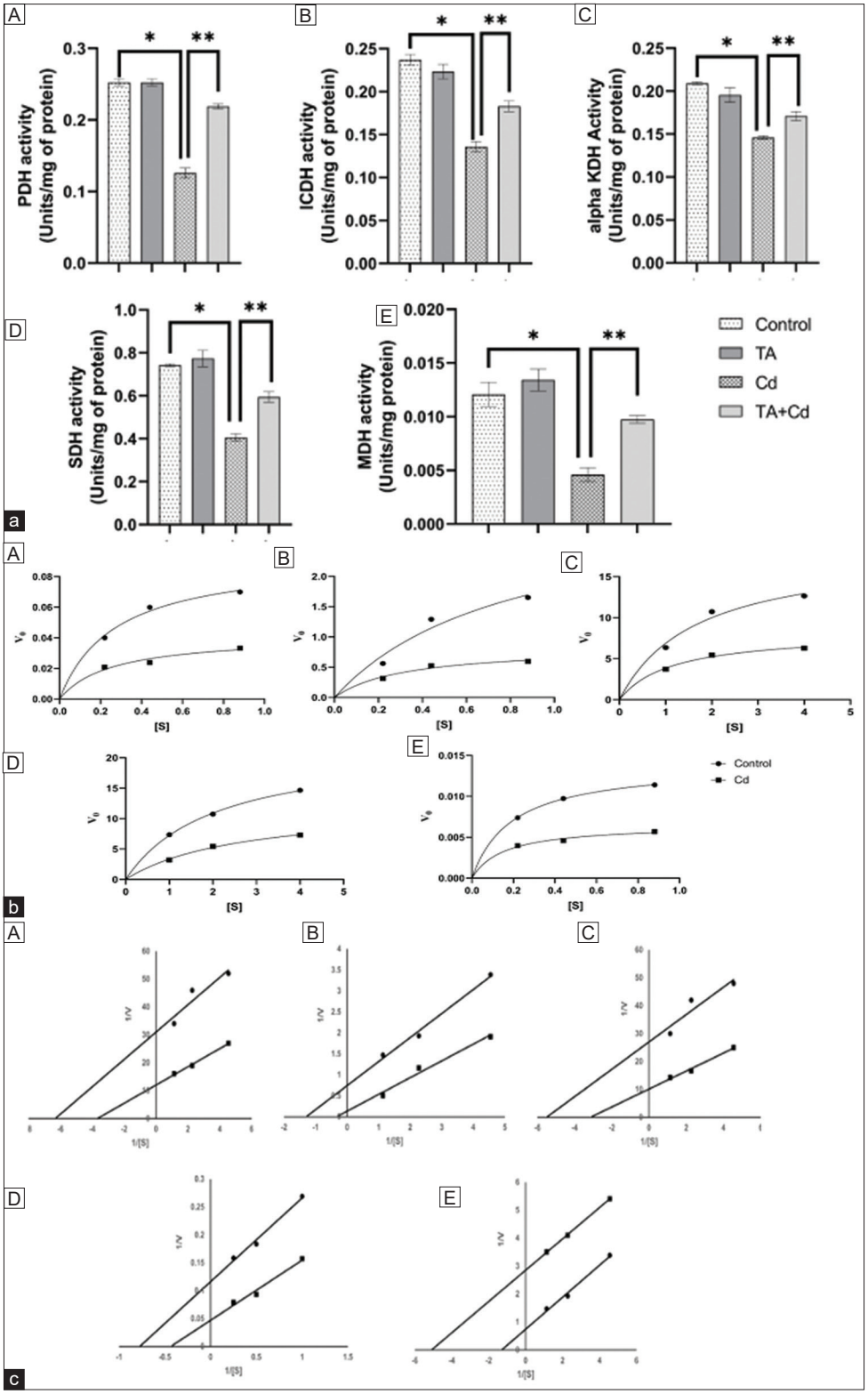

Fig. 14: (a) Histograms representing the alterations in the activities of (A) pyruvate dehydrogenase (PDH) and some Krebs' cycle enzymes (B) isocitrate dehydrogenase (ICDH), (C) alpha ketoglutarate dehydrogenase (α-KGDH), (D) succinate dehydrogenase (SDH), and (E) malate dehydrogenase (MDH) following treatment of rats with Cd. The studies indicated that tannic acid has protective effects on the activities of these enzymes. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*. (b) Michaelis-Menten plots representing the alterations in the activities of (A) PDH and some of the Krebs' cycle enzymes (B) ICDH, (C) alpha KGDH, (D) SDH, and (E) MDH. (c) Lineweaver-Burk plots representing the alterations in the activities of (A) PDH and some of the Krebs' cycle enzymes (B) ICDH, (C) alpha KGDH, (D) SDH, and (E) MDH

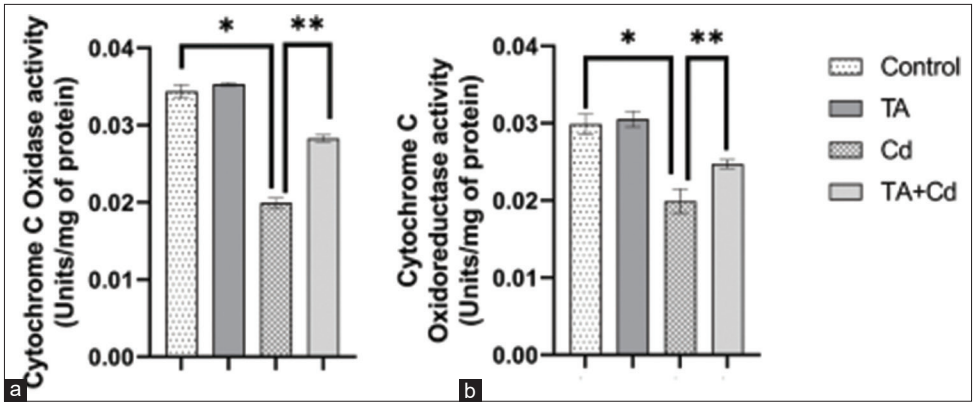

Fig. 15: Graphical representation of the alteration in the activities of the enzymes of the respiratory chain following treatment of rats with Cd. Tannic acid was found to protect the activities of these enzymes. (a) Cytochrome c oxidase and (b) cytochrome c oxidoreductase. The values are expressed as mean±standard error of the mean (n=5). *p<0.05 versus control group, **p<0.05 versus Cd-treated group, using one-way analysis of variance with Tukey honestly significant difference *post hoc*

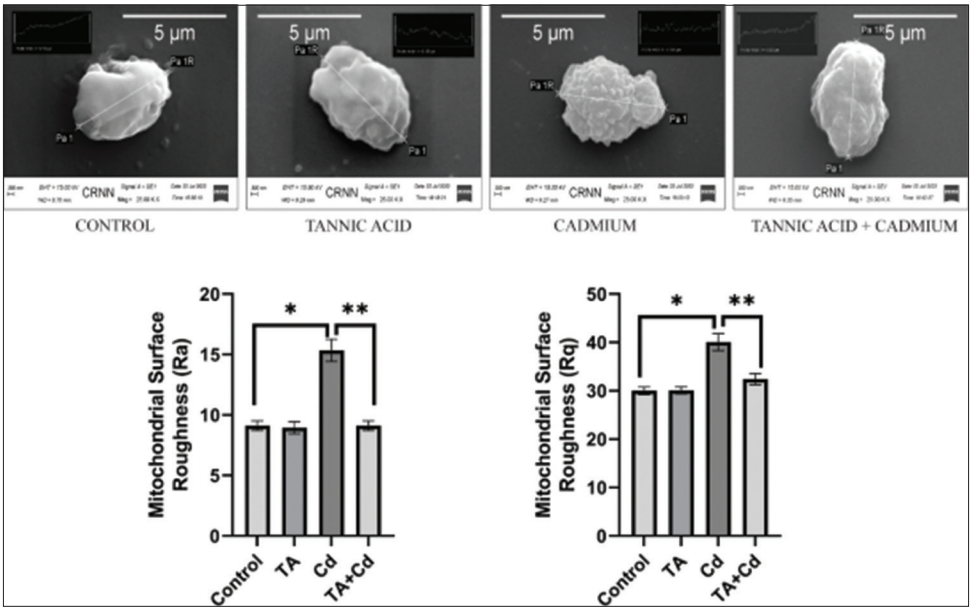

Fig. 16: Representative scanning electron microscopic images (×25,000 magnification) of isolated rat hepatic mitochondria from groups of rats, namely, control, Cd-treated, Cd+TA-treated, and TA alone-treated, indicating changes in mitochondrial surface topology. Scale bar in "5 µm" mentioned in the upper corner of each micrograph

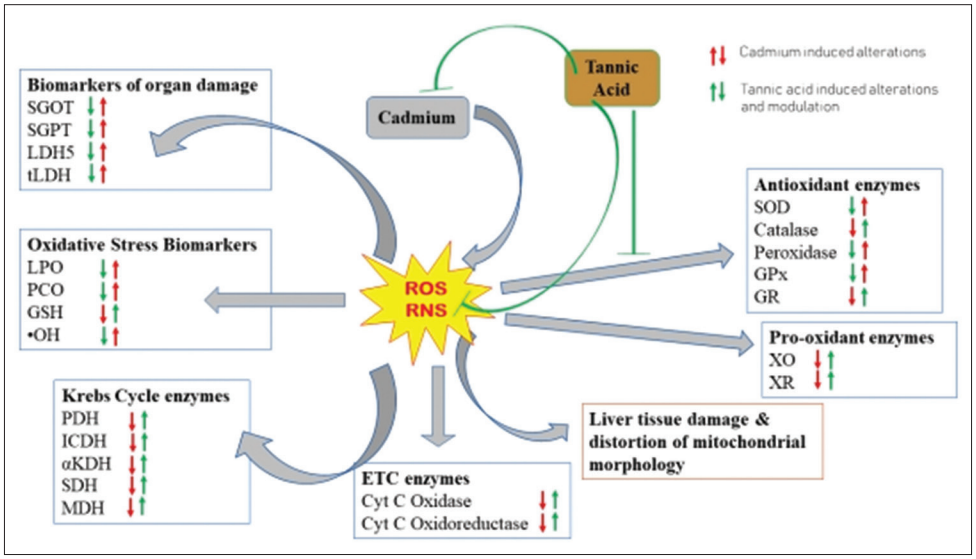

Fig. 17: Schematic representation of the protective role of TA against Cd-induced oxidative damage in rat hepatic tissue

potent role in ameliorating liver injury, likely due to its antioxidative and membrane-stabilizing properties [28].

As oxidative stress plays a central role in Cd-mediated toxicity, it was evidenced by increased LPO, TBARS, protein carbonylation, and hydroxyl radical production, along with depleted glutathione (GSH) levels. Pre-treatment with TA counteracted these changes effectively, reinforcing its antioxidative potential. Moreover, TA significantly protected the activity of critical antioxidant enzymes such as SOD1, SOD2, CAT, GPx, GR, and mitochondrial peroxidase, all of which were suppressed under the influence of Cd-treatment. These findings suggest that TA not only scavenges ROS but also enhances the intrinsic antioxidant defense machinery.

Furthermore, the present study highlights the modulation of pro-oxidant enzymes (XO and XDH) by TA. Cd exposure resulted in elevated activity of these enzymes and increased total activities of XO and XDH, along with significantly altered ratios of XO: XDH and XO: XO+XDH, both of which were significantly attenuated by TA. This regulation is crucial as XO and XDH contribute substantially to ROS generation in pathological conditions.

Further, TA demonstrated a capacity to modulate hepatic energy metabolism. Cd-induced inhibition of key glycolytic enzymes (HK, PFK, and ALD) and pentose phosphate pathway enzyme (G6PDH) was markedly prevented by TA. This indicates that TA helps to maintain cellular energy balance and redox state, which are critical for liver cell survival and function.

Cd exposure also impaired mitochondrial function by reducing the activity of PDH and Krebs' cycle enzymes (ICDH, α-KGDH, SDH, and MDH). Further kinetic studies revealed that Cd uncompetitively inhibited the activities of all these enzymes. TA not only preserved the activity of these enzymes but also maintained ETC function, as evidenced by the protection of the activity of cytochrome c oxidase and cytochrome c oxidoreductase. Given that mitochondria are the primary sites of ROS generation, and our previous experiments indicated excessive oxidative stress and impaired mitochondrial metabolism following Cd exposure, we investigated potential morphological alterations in hepatic mitochondria. In the TA-treated group, mitochondrial architecture appeared well-preserved, exhibiting smooth surfaces and normal morphology. In contrast, mitochondria from Cd-exposed rats displayed pronounced structural disruptions, including surface roughness, extensive blebbing, and abnormal shapes. Notably, pre-treatment of rats with TA effectively preserved mitochondrial integrity, supporting our hypothesis that TA mitigates Cd-induced mitochondrial damage.

To further elucidate the mechanism underlying TA's protective effect, we adopted a dual investigative approach. First, we explored the potential direct interaction between TA and Cd. ITC experiments supported the hypothesis of a metal-chelating action of TA, suggesting that TA may sequester Cd ions and thereby reduce their bioavailability. Second, we examined the protective interaction between TA and CAT. Our ITC data revealed a favorable binding affinity of TA with CAT, which likely prevents Cd from interacting with the enzyme. This protective binding appears to shield CAT from Cd-induced toxic effects. Importantly, TA's interaction with CAT did not compromise the enzyme's activity, nor did it induce any detectable alterations in its secondary structure, as confirmed by CD spectroscopy. These findings collectively support the notion that TA exerts a dual protective role – both by chelating Cd and by stabilizing CAT – thereby preserving antioxidant defense mechanisms in hepatic tissue.

Our findings clearly demonstrate that Cd plays a pivotal role in disrupting oxidative homeostasis within hepatic tissue, primarily by diminishing the activity of key antioxidant enzymes. In this study, we focused on CAT, a crucial enzyme responsible for the catalytic decomposition of H_2O_2 into water and oxygen. It was observed that Cd significantly inhibited CAT activity, *in vitro*, in a concentration-dependent manner, likely through alterations in its secondary structure, as evidenced by CD spectroscopic analysis. Notably, this structural and functional impairment was markedly prevented when TA was administered before Cd exposure.

Hence, this study suggests the presence of a dual mechanism, where TA is capable of protecting the enzymatic properties of CAT by directly scavenging Cd and shielding it in a non-toxic manner, thus preventing Cd from binding with it (Fig. 17).

CONCLUSION

Cd is a persistent environmental pollutant that, when it accumulates in the body, induces free radical generation and oxidative stress in the organism. Pre-treatment with TA, a polyphenol found in several known indigenous Indian plants, ameliorated Cd-induced oxidative stress conditions in rats. Moreover, our *in vitro* studies have demonstrated that TA has the capacity to scavenge Cd and protects CAT from getting damaged, protecting the tissues and biomolecules apart from its antioxidant activity. Thus, it can be concluded that TA protects against Cd-induced hepatotoxicity through multifaceted mechanisms: acting as a potent antioxidant providing protection to the metabolic pathways, and potentially chelating Cd in liver tissue in experimental rats, which may have future therapeutic relevance.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Krishnendu Dalui: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, and Conceptualization. Adrita Banerjee: Methodology, Investigation, Formal analysis, and Data curation. Romit Majumder: Writing – review and editing, Methodology, Investigation, Formal analysis. Madhuri Datta: Writing – review and editing, Methodology. Sanatan Mishra: Methodology, Investigation. Aindrila Chattopadhyay: Writing – review and editing, Conceptualization. Debasish Bandyopadhyay: Writing – review and editing, Supervision, Formal analysis, Conceptualization.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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