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Research Article

AMELIORATIVE EFFECT OF AQUEOUS TULSI LEAF (*Ocimum sanctum*) EXTRACT AGAINST CADMIUM-INDUCED OXIDATIVE STRESS IN RAT LIVER

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ABSTRACT

Objective: The purpose of our study was to examine whether administration of aqueous extract of the leaves of *Ocimum sanctum* L. (Tulsi) do possess a protective effect against cadmium-induced oxidative stress in rat liver.

Methods: Male albino rats were divided into four groups; control, cadmium treated, aqueous extract protected and only aqueous extract treated (positive control). The rats were treated with cadmium chloride subcutaneously every alternate day for a period of fifteen days and the extract was administered orally every day for fifteen days. The alterations in the activity of the different bio-markers of hepatic damage, biomarkers of oxidative stress, activities of the antioxidant, pro-oxidant enzymes and some of the mitochondrial enzymes were studied. Histomorphology and alteration in tissue collagen level was studied through H-E staining and Sirius red staining respectively. Quantification of tissue collagen content was evaluated using confocal microscopy.

Result: The studies revealed that the Tulsi leaf aqueous extract protected the biomarkers of organ damage, oxidative stress, antioxidant enzymes, pro-oxidant enzymes, mitochondrial respiratory chain enzymes, enzymes of electron transport chain and oxidative stress index from getting altered in the rat liver tissue following treatment with cadmium chloride.

Conclusion: The current work suggests that the Tulsi leaf aqueous extract may be useful as a protective antioxidant supplement with promising antioxidant potential to combat cadmium-induced oxidative damages in the hepatic tissue.

Keywords: Antioxidant, Cadmium, Hepatotoxicity, Liver, Oxidative stress, Tulsi leaf extract.

INTRODUCTION

Free radicals play a key role in triggering a number of human diseases, such as atherosclerosis, neurodegenerative disorders, cancer and aging. Free radicals and reactive oxygen species are generated by various endogenous systems in our body due to exposure to different physiochemical conditions or pathological states. An antioxidant is a molecule stable enough to donate an electron to a free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property [1]. The term oxidative stress is the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable, causing damage to a wide range of molecular species including lipids, proteins, and nucleic acids. There are a number of synthetic phenolic antioxidants that have been reported to be widely used as antioxidants in food industry, cosmetics, and therapeutic industry. However, the physical properties such as their high volatility and instability at elevated temperature, strict legislation on the use of synthetic food additives, carcinogenic nature of some synthetic antioxidants, and consumer preferences have shifted the attention of manufacturers from synthetic to natural antioxidants [2]. Therefore, recent researches mainly focus on newer bioactive fractions or molecules from nature with health-promoting antioxidant potential.

The Tulsi plant (*Ocimum sanctum*) is a traditionally important medicinal local herb which grows widely in the Indian subcontinent and many other countries of South-East Asia. The plant is known to contain many potent and useful compounds [3]. The ancient systems of medicines including Ayurveda, Greek, Roman, Siddha and Unani have mentioned its therapeutic applications in various pathological disorders [4]. The hydroalcoholic extract of the leaves of *Ocimum sanctum* has also been shown to ameliorate peptic ulcer induced by ethanol possibly through its antioxidant potential [5].

Cadmium (Cd) is known to humans since ancient times. The heavy metal enters the environment from occupational (mining, smelting operations and electronics manufacturing) and non-occupational exposure [6]. Cadmium is classified by IARC as a human carcinogen. Since, the half-life of cadmium in humans is more than 15 years and therefore environmental risk due to exposure is constantly increasing [6,7,8]. Cadmium has the ability to induce severe alterations in various organs following either acute via inhalation results in pulmonary oedema and respiratory tract irritation or chronic exposure which often leads to renal dysfunction, anaemia, osteoporosis, and bone fractures [9].

The toxic action of cadmium appears to be multifactorial. It is said that cadmium has catalytic activity in the oxidative reactions of biological macromolecules [10]. The toxicity of Cd may be associated with the production of reactive oxygen species (ROS) such as superoxide anion free radical, hydroxyl free radical and hydrogen peroxide [11-13]. ROS generation can overwhelm cells' intrinsic antioxidant defenses resulting in oxidative stress. Cells under oxidative stress display various dysfunctions due to lesions caused by ROS to lipids, proteins and DNA [14].

Herein, we demonstrate that the aqueous Tulsi leaf extract (TLE) exhibited antioxidant properties when tested *in vitro*, and, pre-treatment of the experimental rats with this aqueous extract ameliorated cadmium-induced hepatic injury possibly through its antioxidant mechanism(s). As Tulsi leaves are consumed by the people at this part of the world since ancient times as sacred leaf with broad spectrum disease defending properties and with no reported side-effects, the results of the present studies may have future therapeutic relevance in the areas where humans are exposed to cadmium either occupationally or environmentally.

MATERIALS AND METHODS

Chemicals

Cadmium chloride ($CdCl_2$) was purchased from Merck Limited, Delhi, India. All the other chemicals used including the solvents,

were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

Animals

Male albino Charles-Foster rats, weighing 140–170 g, were obtained from a CPCSEA registered animal supplier. The animals were acclimatized under standard laboratory conditions for 2 weeks prior to dosing. They had free access to standard diet and water *ad libitum*. The animals were maintained in our animal house under controlled conditions of temperature (25±2°C), relative humidity (50±10%) and normal photoperiod (12 h light and 12 h dark) following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Government of India. The experimental protocols had the approval of the Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M. Sc. (Vet.), Ph. D., CPCSEA Nominee to Department of Physiology, University of Calcutta acted as an expert for monitoring care and maintenance of experimental animals.

Collection of Tulsi leaves

The fresh, green Tulsi leaves (*Ocimum sanctum*) were collected from Kolkata and surrounding areas throughout the year during the course of the study. The identity of the plant was confirmed by Mr .P. Venu , Scientist 'F' , the Botanical Survey of India, Central National Herbarium (Government of India, Ministry of Environment and Forests), Botanic Garden, Howrah 711 103,West Bengal. The Herbarium of the plant was deposited in the BSI against voucher specimen no.CNH/I-I/40/2010/Tech.II 231.

Preparation of the aqueous Tulsi leaf extract (TLE)

The method of preparation of aqueous Tulsi leaf extract (TLE) was followed as according to Mitra et al. [15]. The collected Tulsi leaves were shade dried and powdered. The dried Tulsi leaf dusts were soaked overnight in double distilled water (15 g per 100 ml), filtered through loin cloth (fine cotton cloth).The filtrate was centrifuged at 5000 rpm for 10 min (using a REMI cold-centrifuge) .The supernatant, thus obtained, was filtered again through loin cloth and the filtrate collected in sterile polypropylene tubes and frozen at -20 °C. The contents of the tubes were then lyophilized and the resulting lyophilized material therein, herein referred to as the aqueous Tulsi leaf extract (TLE), was stored at -20 °C until further use. A definite amount of the TLE (the lyophilized material) was always freshly dissolved in double distilled water to give a particular concentration and an aliquot of this solution (not more than 0.5 ml) was fed to rats with the help of a feeding needle. Any leftover of this solution was discarded. The yield of TLE was 8.33±0.45% (w/w).

Determination of Total Antioxidant Status (TAS)

Total Antioxidant Status (TAS) was measured spectrophotometrically [20]. The value of TAS of TLE was expressed as mmol Trolox equivalent/l. This assay relies on the ability of antioxidant(s) in the sample to inhibit the formation of $ABTS^{\cdot+}$ from oxidation of ABTS (2.2*0*-azino-di-(3-ethylbenz-thiazoline sulfonate). The stock solutions included 7.4mM $ABTS^{\cdot+}$ solution and 2.6mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. A weighed amount of liver tissue was homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.4. After addition of 100 µL of diluted $ABTS^{\cdot+}$ solution (A734nm = 0.700 ± 0.020) to 1 ml diluted homogenate or Trolox standards in ethanol the absorbance reading was taken at 30°C exactly 1 min after initial mixing and up to 6 min. Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data. The values of TAS in homogenate were expressed as mmol Trolox equivalent/mg tissue protein.

Determination of Total oxidant status (TOS)

Total oxidant status (TOS) was measured using Erel's TOS method [21], which is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidative species in acidic medium and the measurement of the ferric ion by xylenol orange. A weighed amount of liver tissue was homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.4. Briefly the assay mixture contains homogenate, Reagent 1 (xylenol orange 150 µM, NaCl 140 mM and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75) and Reagent 2 (ferrous ion 5 mM and o-dianisidine 10 mM in 25 mM H_2SO_4 solution). The first absorbance is taken before the mixing of R1 and R2 (as sample blank) and the last absorbance is taken when the reaction trace draws a plateau line (about 3–4 min after the mixing) at 560 nm. The oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules, which are abundant in the reaction medium. The ferric ions make a coloured complex with xylenol orange in an acidic medium. The color intensity measured spectrophotometrically, is related to the total quantity of oxidant molecules in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed as the µmol H_2O_2 equivalent/mg tissue protein.

Oxidative stress index (OSI)

The TOS to TAS ratio was regarded as the OSI [21] and was calculated as follows: OSI (arbitrary unit) = TOS/TAS x 100.

Experimental design for *in vivo* studies

The rats were randomly divided into four groups (n=6). The treatment of rats was carried out as per the schedule mentioned below:

Group I: Control rats (**C**) treated with normal saline every alternate day for a period of 15 days.

Group II: Aqueous **TLE** treated rats; dose, 200 mg/kg body weight, administered orally every day for a period of 15 days.

Group III: Cadmium chloride ($CdCl_2$) treated rats (**Cd**); route of administration subcutaneous, dose 0.44 mg/kg body weight every alternate day for a period of 15 days.

Group IV: Aqueous TLE was administered orally at a dose of 200 mg/kg body weight every day at the same time of the day for a period of 15 days. Cadmium chloride was administered subcutaneously to the same group of rats, at a dose of 0.44 mg/kg body weight every alternate day for a period of 15 days **(Cd+TLE)** one hour after administration of the aqueous TLE.

At the end of the treatment period, the animals were kept fasted overnight and were sacrificed through cervical dislocation after subjecting them to mild ether anesthesia. The chest cavity was opened first through a vertical incision and the blood was carefully collected through cardiac puncture for the preparation of serum. Thereafter, the abdomen was opened and the liver were surgically extirpated, collected, rinsed well in saline and soaked properly with a piece of blotting paper and stored in sterile plastic vials at -20°C for further biochemical analyses. For histological studies, a suitable amount of the hepatic tissue was placed immediately after removal in appropriate fixative. Each set of experiment was repeated at least three times.

Assessment of serum specific markers related to hepatic damage

Serum glutamate pyruvate transaminase (SGPT) was measured by the method of Reitman and Frankel [22]. Values are expressed as IU/L. The hepatic specific Type 5 isoform of Lactate Dehydrogenase (LDH5) activity and the total serum activities of lactate dehydrogenase (LDH) were obtained by measuring the oxidation of NADH (0.1mM) to NAD+ at 340nm using 1.0mM sodium pyruvate as substrate. The samples for the measurement of total LDH were prepared by incubating the serum at 37°C for 30 mins. Likewise, the samples for the measurement of LDH5 were prepared by incubating the serum samples at 57°C for 30mins, which destroys the isoform LDH5. The resulting enzyme activity was then subtracted from the

total serum LDH activity to obtain the activity of LDH 5 [27]. The enzyme activity was expressed as IU/L [23].

Histological studies

Studies using tissue sections stained with hematoxylin and eosin (H & E)

A portion of the extirpated rat liver was fixed immediately in 10% formalin and embedded in paraffin following routine histological procedure. Hepatic tissue sections (5 µm thick) were prepared and stained with hematoxylin–eosin (Sigma). The stained tissue sections were examined under Leica microscope and the images were captured with a digital camera attached to it [24].

Quantification of fibrosis by Confocal Microscopy

The rat liver tissue sections (5 µm thick) were stained with Sirius red (Direct Red 80; Sigma Chemical Co, Louis, MO, USA) according to the method of Ghose Roy et al. [25] and imaged with a laser scanning confocal system (Zeiss LSM 510 META, Germany) and the stacked images through multiple slices were captured. Four slides were prepared for each rat from each group and only the representative images are presented. The digitized images were then analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) and the total collagen area fraction of each image was measured and expressed as the % collagen volume.

Measurement of lipid peroxidation (LPO) and reduced GSH level

A weighed amount of the rat hepatic tissue was homogenized (10%) in ice-cold 0.9% saline (pH 7.0) with a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30s and the lipid peroxides in the homogenate were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust, [26] with some modification as adopted by Bandyopadhyay et al. [29]. Briefly, the homogenate was mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 80°C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 12,000 x g for 10 min at room temperature was measured at 532 nm using a UV–VIS spectrophotometer (Bio-Rad, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as standard. The values were expressed as nmoles of TBARS/mg protein.

Protein carbonyl content was estimated by DNPH assay [27]. About 0.1 g of tissue was rinsed in 10 mM PBS buffer (pH 7.4) and homogenized and centrifuged at 10,000xg for 10 min at 4°C. After centrifugation, 0.5 ml of the supernatant was taken in each tube and 0.5 ml DNPH in 2.0 M HCl was added to the tubes. The contents of the tubes were mixed thoroughly every 10 min in the dark for 1 hour using a vortex machine. Proteins were then precipitated with 30 % TCA and centrifuged at 4000 x g for 10 min. The pellet, thus obtained, was washed three times with 1.0 ml of ethanol:ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0 ml of 6.0 M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The absorbance was recorded at 370 nm using a UV / VIS spectrophotometer. The protein carbonyl content was calculated using a molar absorption coefficient of 2.2x104 M-1cm-1. The values were expressed as nmoles of carbonyl/mg protein.

Reduced GSH content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlac and Lindsay, 1968 [28] with some modifications by Bandyopadhyay et al. [29]. A weighed amount of tissue was homogenized (10%) in 2 mM ice-cold ethylenediaminetetraacetic acid (EDTA). The homogenate was mixed with Tris–HCl buffer, pH 9.0, followed by the addition of DTNB for color development. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer to determine the GSH content. The values were expressed as nmoles GSH/mg protein.

Glutathione Disulphide (GSSG) was assessed by the method of Ikediobo et al. [30]. Briefly, the hepatic tissue sample was homogenized in ice-cold phosphate buffer (pH 7.4). After allowing the mixture to stand for 5 min at room temperature, the homogenates were centrifuged (10,000g; 10 min at 40C). The reaction mixture contained the supernatant, NADPH (0.30 mM), DTNB (0.225 mM), and Glutathione Reductase (GR) (1.6 units/ml) in a 100 mM phosphate/1 mM EDTA buffer (pH 7.4), was added later to start the reaction. The change of absorbance was monitored at 412 nm for 3 min with a UV / VIS spectrophotometer. Standard and test samples were run in triplicate for each assay and the measurements were repeated three times. The reaction rate and the standard curves prepared earlier were used to calculate concentrations of GSSG. Redox ratio is determined by the calculating the ratio of GSH to GSSG.

Total sulfhydryl group content was measured following the method as described by Sedlac and Lindsay [28]. The values were expressed as nmoles/mg protein.

Determination of the activities of the antioxidant enzymes

Copper-zinc superoxide dismutase (Cu-Zn SOD or SOD1) activity was measured by hematoxylin autooxidation method of Martin et al., 1987 [31] with some modifications as adopted by Mukherjee et al., 2010 [32]. Briefly, the weighed amount of tissue was homogenized (10%) in ice-cold 50mM phosphate buffer containing 0.1mM EDTA, pH 7.4. The homogenate was centrifuged at 12,000 × g for 15 min and the supernatant collected. Inhibition of haematoxylin autooxidation by the cell free supernatant was measured at 560nm using a UV-VIS spectrophotometer (*BIO-RAD* Smartspec Plus). Two unit enzyme activity is 50% inhibition of the rate of autooxidation of haematoxylin in 1 min/mg protein. The enzyme activity was expressed as units/min/mg of tissue protein.

Manganese superoxide dismutase (Mn-SOD or SOD2) activity was assayed by method of Marklund and Marklund [33] which involves inhibition of pyrogallol autooxidation in the presence of EDTA at pH 8.2. Briefly, a weighed amount of tissue was homogenized (10%) in ice-cold 50mM Tris-HCl buffer containing 0.1mM EDTA, pH 7.4 and centrifuged first, at 2,000 rpm for 5 min, and the supernatant thus obtained, was carefully collected and centrifuged again at 10,000 rpm in cold for 20 min. The supernatant was discarded and the pellet was suspended in 50 mM Tris-Hcl buffer, pH 7.4. To 50 µl of the suspended pellet, 50 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min in a UV / VIS spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The enzyme activity was expressed as units/min/mg of tissue protein.

Catalase was assayed by the method of Beers and Seizer [34] with some modifications as adopted by Chattopadhyay et al. [35]. A weighed amount of tissue was homogenized (5%) in ice-cold 50mM phosphate buffer, pH 7.0. The homogenate was centrifuged in cold at 12,000 × g for 12 min. The supernatant, thus obtained, was then collected and incubated with 0.01ml of absolute ethanol at 4°C for 30 min, after which 10% Triton X-100 was added so as to have a final concentration of 1%. The sample, thus obtained, was used to determine catalase activity by measuring the breakdown of H_2O_2 sphectrophotometrically at 240nm. The enzyme activity was expressed as µmoles of H_2O_2 consumed / min / mg tissue protein.

Determination of the activities of the enzymes of the glutathione metabolizing pathway

Glutathione reductase (GR) assay was carried out according to the method of Krohne-Ehrich et al. [36]. The assay mixture in the final volume of 3 ml contained 50mM phosphate buffer, 200 mM KCl, 1mM EDTA and water. The blank was set with this mixture. Then, 0.1 mM NADPH was added together with suitable amount of homogenate, as source of the enzyme, into the cuvette. The reaction was initiated with 1mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored at 340 nm. The specific activity of the enzyme was calculated as nmol of NADPH oxidized/min/mg protein.

Glutathione peroxidase (GPx) activity was measured according to the method of Paglia and Valentine [37] with some modifications [18]. A weighed amount of hepatic tissue was homogenized (10%) in ice-cold 50mM phosphate buffer containing 2mM EDTA, pH 7.0. The assay

system contained, in a final volume of 1ml, 0.05 M phosphate buffer with 2 mM EDTA, pH 7.0, 0.025 mM sodium azide, 0.15 mM glutathione, and 0.25 mM NADPH. The reaction was started by the addition of 0.36 mM H_2O_2. The linear decrease of absorbance at 340 nm was recorded using a UV /VIS spectrophotometer. The specific activity was expressed as nmol of NADPH oxidized/min/mg tissue protein.

Hepatic glutathione-S-transferase (GST) activity was measured sphectrophotometrically according to Habig et al. [38] by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) at 340 nm. One unit of enzyme will conjugate 10.0 nmol of CDNB with reduced glutathione per minute at 25ºC. The molar extinction of CDNB is 0.0096 µM-1cm-1. The enzyme activity was expressed as nmol of CDNB–GSH conjugate formed/min/mg protein.

Indirect assessment of in vivo generation of superoxide anion free radical (O2•-) by determining the activities of the pro-oxidant enzymes, xanthine oxidase (XO) and xanthine dehydrogenase (XDH)

Xanthine oxidase of the rat hepatic tissue was assayed by measuring the conversion of xanthine to uric acid following the method of Greenlee and Handler [39]. Briefly, the weighed amount of hepatic tissue was homogenized in cold (10%) in 50mM phosphate buffer, pH 7.8. The homogenates were centrifuged at 500 × g for 10 min. The resulting supernatant was further centrifuged at 12,000 × g for 20 min in cold. The supernatant, thus obtained, was collected and used for spectrophotometric assay of the enzyme at 295nm using 0.1mM xanthine in 50mM phosphate buffer, pH 7.8, as the substrate. The enzyme activity was expressed as milliUnits/min/mg tissue protein.

Xanthine dehydrogenase activity was measured by following the reduction of NAD^+ to NADH according to the method of Strittmatter [40] with some modifications [32]. In brief, the weighed amount of rat hepatic tissue was homogenized in cold (10%) in 50mM phosphate buffer with 1mM EDTA, pH 7.2. The homogenates were centrifuged in cold at 500 × g for 10 min. The supernatant, thus obtained, was further centrifuged in cold at 12,000 × g for 20 min. The final supernatant was used as the source of the enzyme, and the activity of the enzyme was measured spectrophotometrically at 340nm with 0.3mM xanthine as the substrate (in 50mM phosphate buffer, pH 7.5) and 0.7mM NAD^+ as an electron donor. The enzyme activity was expressed as milliUnits/min/mg tissue protein.

Measurement of endogenous free hydroxyl radical (•OH)

The •OH generated *in vivo* in hepatic tissue was measured by using dimethyl sulfoxide (DMSO) as a specific •OH radical scavenger following the method of Bandyopadhyay et al. [29]. Dimethyl sulfoxide forms a stable product [methane sulfonic acid (MSA)] on reaction with •OH. Accumulation of MSA was measured to estimate the •OH generated *in vivo* after forming a coloured complex with Fast blue BB salt. Four groups of rats containing four animals in each group were used for each experiment. The cadmium treated group of rats was injected intraperitonially (i.p.) with 0.4 ml of 25% DMSO per 100 gm body weight 30 min before subcutaneous (s.c.) injection of cadmium chloride (0.44 mg/kg body weight) at four days interval. Aqueous TLE (200mg/kg body weight) was administered orally to the rats of TLE and Cd+TLE groups, 30 min prior to DMSO injection, which was followed by subcutaneous (s.c.) injection of cadmium chloride (0.44 mg/kg body weight) 30 mins after DMSO injection. The control group of rats was treated with DMSO (i.p. injection) only. After the treatment period the rats of each group were euthanized by cervical dislocation and liver was collected. The hepatic tissue was then processed in cold for MSA which was allowed to react with Fast blue BB salt to yield a yellow product. This was measured spectrophotometrically at 425 nm using benzenesulfinic acid as the standard. The values obtained were expressed as nm of •OH / g tissue.

Determination of the activities of pyruvate dehydrogenase and some of the mitochondrial Kreb's cycle enzymes

The weighed amount of rat hepatic tissue was homogenized (10%) in ice-cold 50 mM phosphate buffer, pH 7.4 with a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30s. The homogenate was then centrifuged at 500 x g for 10 min. The supernatant, thus obtained, was again centrifuged at 12,000 x g for 15 min to obtain a pellet containing mitochondria. This pellet was re-suspended in the buffer and used for measuring the activities of the mitochondrial enzymes.

Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically according to the method of Chretien et al. [41] with some modifications by following the reduction of NAD+ to NADH at 340nm using 50mM phosphate buffer, pH 7.4, 0.5mM sodium pyruvate as the substrate and 0.5mM NAD+ in addition to the enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan et al. [42] by measuring the reduction of NAD+ to NADH at 340nm with the help of a UV-VIS spectrophotometer. One ml assay volume contained 50mM phosphate buffer, pH 7.4, 0.5mM isocitrate, 0.1mM $MnSO_4$, 0.1mM NAD+ and the suitable amount of enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Alpha-Ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically according to the method of Duncan et al. [42] by measuring the reduction of 0.35mM NAD+ to NADH at 340nm using 50mM phosphate buffer, pH 7.4 as the assay buffer and 0.1mM α-ketoglutarate as the substrate. The enzyme activity was expressed as units/min/mg tissue protein.

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide (K_3FeCN_6) at 420nm according to the method of Veeger et al. [43] with some modifications. One ml assay mixture contained 50mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4mM succinate, 2.5mM K_3FeCN_6 and a suitable aliquot of the enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Determination of the activities of some of the mitochondrial respiratory chain enzymes

NADH-Cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565nm according to the method of Goyal and Srivastava [44]. One ml of assay mixture contained in addition to the enzyme, 50mM phosphate buffer, 0.1mg BSA, 20mM oxidized cytochrome c and 0.5µM NADH. The activity of the enzyme was expressed as units/min/mg tissue protein.

The cytochrome c oxidase activity was determined spectrophotometrically by following the oxidation of reduced cytochrome c at 550nm according to the method of Goyal and Srivastava [44]. One ml of assay mixture contained 50mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome c and a suitable aliquot of the enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

DNA fragmentation assay

The extent of DNA fragmentation has been assayed by electrophoresis of genomic DNA samples, isolated from rat liver, on agarose/EtBr gel by the procedure described by Sellins and Cohen [45]. Briefly, hepatic tissue was collected and washed twice with cold PBS. Then this was suspended in 100 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton X-100). After centrifugation, the supernatant was incubated with proteinase K (100 mg/ml) for 6 h at 37°C. Then the suspension was extracted with 20 ml of 5 M NaCl and 120 ml of isopropyl alcohol at 50°C overnight. After centrifugation, the collected DNA was electrophoresed in 1% agarose gel containing 0.5 mg/ml ethidium bromide and photographed under UV light.

Estimation of the cadmium content in the rat liver tissue by Atomic Absorption Spectrophotometry (AAS)

The hepatic tissue samples were processed and the cadmium content was measured as per the protocol mentioned in the cook book of the Sophisticated Analytical Instrument Facilities' (SAIF), "Thermo Scientific iCE 3000 Series Atomic Absorption Spectrometer" at the Bose Institute, Kolkata. The samples were prepared with nitric acid (65%) for total dissolution as described by Mitra et al. [46]. The cadmium content was expressed in µg/g of rat liver tissue.

Estimation of protein

Protein content was estimated by the method of Lowry et al. [47] using the bovine serum albumin as standard.

Statistical evaluation

Each experiment was repeated at least three times with different rats. The data for various biochemical parameters were expressed as means ± S.E.M. The statistical significance of the data has been determined using one-way analysis of variance (ANOVA) after ascertaining the homogeneity of variances between the treatments and significant difference among treatment groups were evaluated by Scheffes' test. The results were considered statistically significant at $p < 0.05$. All statistical analyses were made using Microcal Origin version 7.0 for Windows.

RESULTS

Status of cadmium concentration of the rat hepatic tissue

Table 1 reveals a highly significant increase in the rat hepatic tissue Cd concentration following treatment of rats with cadmium chloride. The concentration of cadmium in the tissue was partially but significantly reduced (34.69 %, **P ≤ 0.001 vs Cd-treated group) when the animals were pre-treated with 200 mg / kg bw (fed orally) of the aqueous TLE indicating that the extract may possess cadmium chelating activity.

Biomarkers of hepatic damage: SGPT, LDH, LDH5

Table 1 illustrates the level of activity of serum specific markers, namely SGPT, LDH5 and total LDH which were found to be significantly higher in Cd-treated group of rats when compared to control (1.16, 1.87 and 2.11 folds increase respectively, *P ≤ 0.001 vs. control). The activities of these enzymes were found to be significantly decreased in the animals pre-treated with aqueous TLE (48.37%, 38.64%, and 39.47 % decrease respectively, **P ≤ 0.001 vs. Cd-treated group). However, the extract by itself did not inhibit the activities of these enzymes to any significant extent. The results indicate that the aqueous TLE do possess the capability to provide protection against Cd-induced hepatic damage.

Routine H and E staining of the rat hepatic tissue sections

Treatment of rats with cadmium at a dose of 0.44 mg / kg bw sc, caused damage to hepatic tissue morphology. Tissue sections from cadmium-treated rat liver shows maintained lobular architecture. Portal veins are dilated and congested. Portal and periportal lymphocyte infiltration along with periportal hepatocyte necrosis are seen. Mild lobular lymphocyte infiltration and spotty hepatocyte necrosis are noted in cadmium treated liver tissue sections. The damage was found to be protected completely when the rats were pre-treated with aqueous TLE as is evident from the H and E stained tissue sections under 200x magnifications, indicating again a protective effect of this extract against Cd-induced hepatic damage in rats (Fig. 1A).

Table 2: It shows effect of aqueous Tulsi leaf extract against cadmium-induced changes in serum enzymes of hepatic function in rats and cadmium concentration in the tissue.

Treatment	SGPT (IU/L)	LDH5 (IU/L)	LDH (IU/L)	Cadmium concentration (µg/g)
C	8.89±0.611	0.47±0.0056	0.54±0.053	ND
Cd	19.99±1.247*	0.88±0.0146*	1.14±0.028*	56.53±2.407
Cd+TLE	10.32±0.433**	0.54±0.0201**	0.69±0.004**	36.92±1.029**
TLE	8.51±0.385	0.48±0.0092	0.51±0.056	ND

Values are given as mean±S.E.M. of 6 animals in each group.

*P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group

ND: Not detected

Studies on collagen content and quantification of fibrosis

The figure 1B (magnification 200X) reveals that Picrosirius red stained cadmium-treated rat liver tissue sections show that there occurred a deposition of collagen especially around the central vein of the hepatic lobule indicative of tissue fibrosis. However, when the rats were pre-treated with aqueous TLE, the tissue fibrosis was found to be almost completely protected. Figure 1C shows the similar images captured by confocal laser scanning microscope for quantification of fibrosis. The results further indicate a protective effect of the extract against Cd-induced damage in rat hepatic tissue. The images of the tissue sections presented here are at 200x magnification.

Figure 1D shows that the percentage volume of total collagen content in hepatic tissue sections also decreased significantly (3.99 folds decrease, *P≤0.001, n=4) in cadmium chloride treated rat liver which was also found to be protected almost completely from being decreased in aqueous TLE protected rats.

Biomarkers of oxidative damage

Table 3 reveals a significantly higher hepatic tissue LPO level and PCO content following treatment of rats with cadmium (2.71 folds and 2.03 folds increase, *P ≤ 0.001 vs. control). This elevated level of lipid peroxidation products and protein carbonyl content were found to be decreased significantly (55.29% and 53.24% from Cd-treated group, **P ≤ 0.001; reaching almost control level) when the rats were pre-treated with aqueous TLE, orally one hour before cadmium treatment. Table 3 also reveals Cd-induced decrease in the level of reduced GSH and total sulfhydryl (TSH) (49.26% and 35.87% respectively, *P ≤ 0.001 vs. control), increase in GSSG content (53.61% increase, *P ≤ 0.001 vs. control) and decrease in redox status (66.95% decrease, *P ≤ 0.001 vs. control) in rat hepatic tissue were found to be almost completely protected when the animals were pre-treated with aqueous TLE at a dose of 200 mg / kg body weight, fed orally. However, the aqueous extract by itself (positive control) has no effect on these biomarkers. The results indicate the protective ability of the aqueous TLE against Cd-induced oxidative stress in rat liver.

Status of the antioxidant enzymes and enzymes of glutathione metabolizing pathway

Table 4 reveals that aqueous Tulsi leaf extract is also capable of preventing cadmium-induced alteration in cytosolic Cu-Zn-SOD, mitochondrial Mn-SOD and catalase activity in rat hepatic tissue (2.17 folds, 2.15 folds increase and 34.31% decrease respectively, *P ≤ 0.001 vs. control). The extract was found to protect the activity of the enzymes (45.62%, 60.38% decrease and 48.82% respectively from Cd treated group, **P ≤ 0.001) from being altered. Table 4 also illustrates a significant increase in GST and glutathione peroxidase activities of the rat hepatic tissue (2.55 folds and 1.68 folds increase respectively, *P ≤ 0.001 vs. control) and a significant decrease (28.81%, **P ≤ 0.001 vs. $CdCl_2$ treated group) in the activity of GR following exposure of rats to cadmium chloride. However, pre-treatment of rats with the present dose of aqueous TLE significantly protected the GST, GPx and GR activities (51.71%, 24.19% and 1.45 folds **P ≤ 0.001 vs. cadmium) from being altered. The results indicate that the aqueous TLE may have an influence on the GSH biosynthesis.

Effect on TAS, TOS and OSI in cadmium chloride-induced oxidative stress in rat liver: protection by aqueous TLE

The value of Total Antioxidant Status of TLE was found to be 2.98± 0.012 mmol Trolox equivalent/l. Table 5 illustrates a significant

increase in Total oxidant status (TOS) (3.94 folds increase, *P ≤ 0.001 vs. control) and a highly significant decrease (36.76%, *P ≤ 0.001 vs. $CdCl_2$ treated group) in the total antioxidant status following exposure of rats to cadmium chloride. However, pre-treatment of rats with the present dose of aqueous TLE significantly protected TOS level from being increased (60.98 % decrease, **P ≤ 0.001 vs. cadmium) and the total antioxidant status level was protected (56.89% increase, **P ≤ 0.001 vs. cadmium) from being decreased. Table reveals that the aqueous TLE is capable of providing protection against cadmium chloride-induced increase in the oxidative stress index of the rat hepatic tissue (3.82 folds increase respectively, *P ≤ 0.001 vs. control). The results indicate that the aqueous TLE may have a direct influence on the antioxidant level and the status of oxidative stress.

Status of the activity of the hepatic pyruvate dehydrogenase, some of the mitochondrial Kreb's cycle enzymes and the activity of the hepatic mitochondrial respiratory chain enzymes

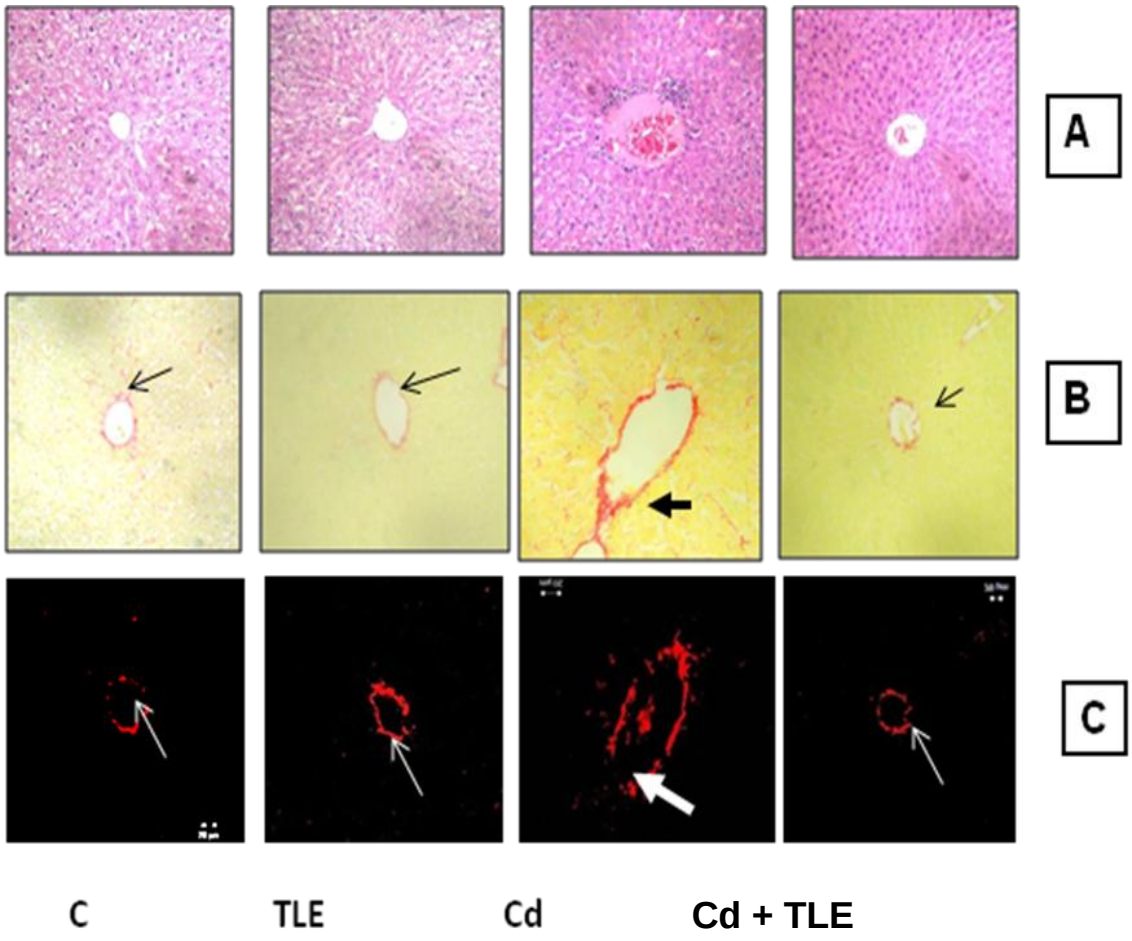
Table 6 reveals that treatment of the rats with cadmium chloride inhibits the activities of rat hepatic pyruvate dehydrogenase, isocitrate dehydrogenase, alpha keto glutarate dehydrogenase and succinate dehydrogenase (65.28%, 53.33%, 44.44% and 65% decrease respectively, *P ≤ 0.001 vs. control). When the rats were pre-treated with 200 mg / kg body weight of the aqueous TLE, the activity of these enzymes, however, was found to be significantly protected compared to the activity observed in the Cd-treated group (2.72 folds, 2.09 folds, 1.73 folds, 2.83 folds increase respectively, **P ≤ 0.001 vs. Cd-treated group). Treatment of rats with cadmium at the present dose inhibits NADH cytochrome c reductase activity and cytochrome oxidase activity (52.92% and 57.94% decrease respectively, *P ≤ 0.001 vs. control). The activity of both the enzymes were found to be protected significantly when the rats were pre-treated with 200 mg / kg bw of the aqueous TLE (2.22 folds increase in NADH cytochrome c reductase activity and 2.42 folds increase in cytochrome oxidase activity, **P ≤ 0.001 vs. Cd-treated group) (**Table 6**).

Status of the endogenous formation of reactive oxygen species (ROS) *in vivo*: an indirect assessment

Treatment of rats with cadmium chloride at a dose of 0.44 mg / kg s.c., every alternate day for a period of 15 days, may generate copious amounts of superoxide anion free radical, an important ROS, in hepatic tissues which is reflected in the elevated levels of activity of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) and xanthine oxidase to xanthine dehydrogenase ratio (5.28 folds, 3.26 folds and 42.50%, *P ≤ 0.001 vs. control). The activities of these pro-oxidant enzymes were found to be protected from being increased when the rats were pre-treated with 200 mg / kg body weight of the aqueous TLE. (Table 7). The results demonstrate that cadmium chloride-induced oxidative stress involves generation of reactive oxygen species like superoxide anion free radical which may cause damage to biomacromolecules and seriously impair tissue antioxidant mechanisms. Table 7 illustrates the effect of aqueous TLE on the scavenging of endogenous •OH generated *in vivo* following treatment of rats with cadmium chloride. Treatment of rats with cadmium chloride caused increase of endogenous generation of •OH in liver (1.70 folds increase, *P ≤ 0.001 vs. control). Pre-treatment of rats with the aqueous TLE decreased the cadmium chloride-induced •OH formation to near basal levels. The TLE by itself, however, did not have a significant effect on the basal level of endogenous hydroxyl radicals.

Protective effect of aqueous TLE on Genomic DNA damage in cadmium chloride-induced oxidative stress in rat liver

Figure 2 illustrates the extent of DNA fragmentation. Pre-treatment of rats with the aqueous TLE partially protected the cadmium chloride-induced genomic DNA damage in liver and in figure (Fig. 2.), the ladder (lane 3) represents DNA degradation in cadmium chloride-treated liver tissue, indicating random DNA degradation.



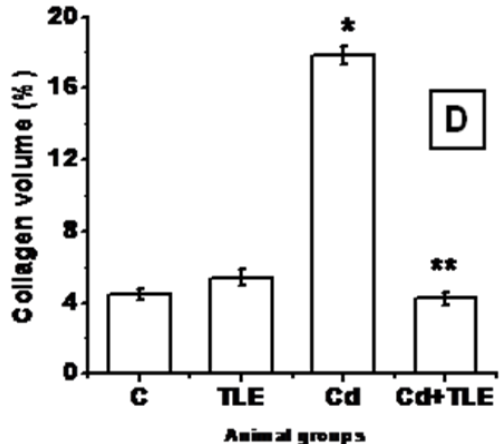


Fig. 1: It shows the protective effect of aqueous Tulsi leaf extract against cadmium-induced changes in the rat hepatic tissue morphology. [A] H and E stained and [B] Sirius red stained sections (200X magnification). [C] Similar images captured by confocal laser scanning microscope for quantification of fibrosis. Arrow heads indicate collagen fibres in B and C. [D] Graph showing collagen volume % of the hepatic tissues.

C = Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected sc; TLE = 200 mg/kg bw of aqueous Tulsi leaf extract; Cd+TLE = 200 mg/kg bw of aqueous Tulsi leaf extract + 0.44 mg/kg bw $CdCl_2$ injected s.c. *P ≤ 0.001 compared to control values using ANOVA. **P ≤ 0.001 vs. compared to cadmium-treated values using ANOVA.

Table 3: It shows the effect of aqueous Tulsi leaf extract against cadmium-induced changes in lipid peroxidation level, protein carbonyl content, reduced glutathione level, oxidized glutathione level, redox ratio and total sulfhydryl content of hepatic tissue.

Treatment	LPO (p moles of TBARS/ mg tissue protein)	PCO (n moles of carbonyl/ mg tissue protein)	GSH (n moles/ mg tissue protein)	GSSG (n moles/ mg tissue protein)	Redox status (GSH/GSSG)	TSH (n moles/ mg tissue protein)
C	5.3±0.172	0.38±0.0222	49.78±2.424	13.99±0.506	3.57±0.303	99.05±4.988
Cd	14.36±0.811*	0.77±0.0324*	25.26±1.394*	21.49±1.394*	1.18±0.009*	63.52±4.346*
Cd+TLE	6.42±0.505**	0.36±0.0335**	50.54±3.383**	15.78±0.343**	3.20±0.203**	102.9±9.424**
TLE	5.57±0.577	0.31±0.0112	53.65±1.818	11.98±0.309	4.37±0.149	103.71±2.760

Values are given as mean±S.E.M. of 6 animals in each group.

*P ≤ 0.001 vs. control. ** P ≤ 0.001 vs. cadmium treated group

Table 4: It shows the effect of aqueous Tulsi leaf extract against cadmium-induced changes in the activities of various antioxidant enzymes in hepatic tissue of rats.

Treatment	Cu-Zn SOD (units/min/mg of tissue protein)	Mn-SOD (units/min/mg of tissue protein)	Catalase (μmoles of H_2O_2 consumed / min / mg tissue protein)	GPx (nmol of NADPH oxidized/min/mg protein)	GR (nmol of NADPH oxidized/min/mg protein)	GST (nmol of CDNB–GSH conjugate formed/min/mg protein)
C	1.74±0.07121	0.74±0.027	122.05±3.303	0.37±0.027	0.59±0.013	1.03±0.028
Cd	3.77±0.215*	1.59±0.151*	80.17±0.235*	0.62±0.025 *	0.42±0.022 *	2.63±0.063 *
Cd+TLE	2.05±0.064**	0.63±0.055**	119.31±0.876**	0.47±0.028 **	0.61±0.016 **	1.27±0.026 **
TLE	1.48±0.157	0.75±0.035	124.39±1.287	0.33±0.020	0.58±0.007	1.19±0.030

Values are given as mean±S.E.M. of 6 animals in each group.

*P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.

Table 5: It shows the effect of aqueous Tulsi leaf extract against cadmium-induced changes in total oxidant status, total antioxidant status and oxidative stress index in hepatic tissue of rats.

Treatment	TOS (μ mol H_2O_2 equivalent/mg tissue protein)	TAS (m mol Trolox equivalent/mg tissue protein)	OSI (arbitrary units)
C	11.9±1.834	8.84±0.061	0.22±0.022
Cd	46.9±0.716*	5.59±0.179*	0.84±0.038*
Cd+TLE	18.3±0.031**	8.77±0.054**	0.21±0.004**
TLE	10.6±0.067	8.73±0.408	0.19±0.004

Values are given as mean±S.E.M. of 6 animals in each group.

*P ≤ 0.001 vs. control. ** P ≤ 0.001 vs. cadmium treated group

Table 6: It shows the effect of aqueous Tulsi leaf extract against cadmium-induced changes in the activities of some of the mitochondrial Kreb's cycle enzymes and enzymes of electron transport chain in hepatic tissue of rats.

Treatment	Pyruvate dehydrogenase (units/min/mg tissue protein)	Isocitrate dehydrogenase (units/min/mg tissue protein)	Alpha ketoglutarate dehydrogenase (units/min/mg tissue protein)	Succinate dehydrogenase (units/min/mg tissue protein)	NADH-cytochrome c oxidoreductase (units/min/mg tissue protein)	Cytochrome oxidase (units/min/mg tissue protein)
C	0.072±0.0067	0.015±6.995E-4	0.027±0.00074	1.00±0.072	9.77±0.738	0.107±0.0067
Cd	0.025±0.00055*	0.007±6.941E-4*	0.015±0.00151*	0.35±0.029*	4.60±0.321*	0.045±0.0013*
Cd+TLE	0.068±0.00405**	0.0146±8.613E-4**	0.026±0.00024**	0.99±0.048**	10.21±0.317**	0.109±0.0083**
TLE	0.081±0.0035	0.017±4.216E-5	0.029±0.00146	1.12±0.095	10.36±0.475	0.103±0.0021

Values are given as mean±S.E.M. of 6 animals in each group.

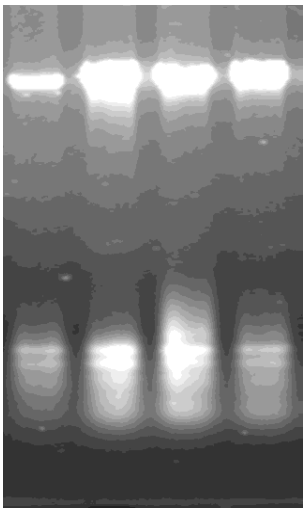
*P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.

Table 7: It shows the effect of aqueous Tulsi leaf extract against cadmium induced-changes in the activities of XO, XDH and XO/XDH ratio in hepatic tissue of rats.

Treatment	Xanthine oxidase (munits/min/mg tissue protein)	Xanthine dehydrogenase (munits/min/mg tissue protein)	Xanthine oxidase/ Xanthine dehydrogenase ratio	Hydroxyl radical (n moles OH radical/mg tissue protein)
C	2.92±0.156	0.312±0.0047	93.24±3.632	32.46±0.601
Cd	15.42±0.379*	1.018±0.0917*	132.87±8.224*	55.24±0.112*
Cd+TLE	3.13±0.253**	0.491±0.0204**	79.97±1.397**	31.07±1.146**
TLE	2.49±0.15	0.357±0.0089	69.75±4.020	26.13±0.092

Values are given as mean±S.E.M. of 6 animals in each group.

*P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.



C, TLE, Cd, Cd+TLE

Fig. 2: It shows the protective effect of aqueous TLE against cadmium-induced DNA damage in rat liver. Lane (1) Control liver, lane (3) cadmium treated liver, lane (4) cadmium+aqueous TLE treated liver, lane (2) aqueous TLE only. C = Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected s.c.; TLE = 200 mg/kg bw of aqueous TLE, fed orally; Cd+TLE = 200 mg/kg bw of aqueous TLE, fed orally + 0.44 mg/kg bw $CdCl_2$ injected s.c.

DISCUSSION

The present study reflects the antioxidant and free radical scavenging activity of aqueous Tulsi leaf extract (TLE). Our result also describes the protective effect of aqueous TLE against cadmium-induced hepatic tissue damage in experimental rats.

In the present studies, cadmium accumulates in considerable amounts in rat liver because it is a highly perfused organ and contains huge amounts of metal binding useful substances. The accumulation of the metal in the organ may be a valid reason leading to damage and dysfunction [48]. The serum levels of these organ specific marker enzymes like, SGPT, LDH5 and total LDH were increased indicating hepatic damage following sub-chronic exposure to cadmium. Leakage of large quantities of intracellular or membrane enzymes into the blood stream indicates a loss of functional integrity of membrane architecture [49]. Oral administration of aqueous TLE at the present dose (i. e., 200 mg/kg bw, fed orally) decreased cadmium accumulation level in the liver and also attenuated the cadmium-induced elevation of the serum levels of these marker enzymes indicating that the extract may have the capacity to provide protection to the rat hepatic tissues. This protection might have been exerted through some phytochemical(s)/ phytonutrient(s) present in the extract which partially chelates the metal ion, rendering it inactive.

Histological examination of hematoxylin-eosin stained hepatic tissue sections following cadmium exposure at the present dose and duration showed portal and periportal lymphocyte infiltration along with periportal hepatocyte necrosis. Picrosirius red stained hepatic tissue sections in the figure revealed that there is deposition of collagen protein in the extracellular matrix following injury of liver tissue due to treatment of rats by cadmium when compared to control. The deposition of collagen was intense around the central vein. This indicates fibrosis of hepatic tissue in cadmium-treated rats. Pre-treatment of rats with aqueous TLE was found to prevent such alterations indicating the protective role of the extract in maintaining the tissue integrity.

In our experiments, the rats exposed to cadmium chloride had increased peroxidation of membrane lipids and protein carbonyl content of liver tissue. Lipid peroxidation is primarily an outcome of free radical generation. Lipids when reacted with free radicals can undergo the highly damaging chain reaction of lipid peroxidation [35]. Cellular proteins are also the targets of oxidation resulting in the formation of aldehyde and ketone residues [50]. Our results clearly indicate that the tissue suffered reactive-oxygen intermediate mediated damage induced by cadmium. The aqueous TLE was found to be effective in decreasing the lipid peroxidation and the protein carbonyl content of liver tissue. Lipid peroxidation is self perpetuating unless terminated by chain breaking antioxidants. The results indicate that the aqueous TLE seems to possess anti-oxidative properties.

Cadmium has high affinity for reduced glutathione (GSH) which is a primary intra-cellular antioxidant and conjugating agent, participating in enzymatic and non-enzymatic detoxification of many oxidative toxicants [51]. Our results also revealed a significant increase in GSSG content in the liver tissue of rats. Studies further showed a decrease in the GSH/GSSG ratio and a significant depletion of total tissue sulfhydryl (TSH). The intracellular GSH/GSSG ratio is highly regulated. However, an increase in ROS levels can decrease the cell's ability to convert GSSG to GSH. Under such conditions, GSSG accumulates in cells and there is a concomitant decrease of the GSH/GSSG ratio. Thus, this ratio is a dynamic indicator of oxidative stress [52]. The present work indicates that disruption of the cellular glutathione system is a key element in the mechanism of Cd-induced oxidative stress in liver. However, pre-treatment of rats with the aqueous TLE prevented the GSH depletion in the tissues. Furthermore, the aqueous extract was also found to restore the total tissue sulfhydryl and the redox status was found to be protected from being altered indicating that the extract have antioxidative potential.

The glutathione level is regulated by the activity of glutathione metabolizing enzymes. The key functional element of glutathione is the cysteinyl moiety, which provides the reactive thiol group. In the presence of GPx, GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by GSSG reductase at the expense of NADPH [55]. In the present studies, the activity of the glutathione dependent enzyme, GR was found to be reduced significantly in hepatic tissues of experimental rats, administered with cadmium chloride. The formation of cadmium sulfhydryl complex with SH groups of the GR might lead to a decrease in the activity of the enzyme [56]. Our data also shows that administration of cadmium chloride to rats elevate GST activity in the liver. The elevated activity of GST may be an attempt by the tissues to counteract the increased peroxides or toxic electrofiles [55]. Our studies demonstrated that following cadmium chloride treatment, the activity of GPx was found to be increased in the rat hepatic tissues. This indicates that GSH metabolizing pathway is disturbed in cadmium chloride-treated rats. It seems clear that cadmium decreases intracellular GSH level not only by binding to its thiol group, but also by decreasing the activity of GR. The intracellular redox status is disturbed due to large fluxes of hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) might result in an imbalance in GSH/GSSG ratio which in turn can affect intracellular GPx and GR activities. When sufficient amount of the thiol decreases in the rat hepatic tissue, the heavy metal divalent cation, like, cadmium (Cd^{2+}) binds to other relevant bio-molecules present in the sub-cellular membranes, endoplasmic reticulum (ER), mitochondria or within the nucleus causing damage. However, when the rats were pre-treated with the aqueous TLE, fed orally, the activities of the glutathione metabolizing enzymes, like, GST, GR and GPx were found to be significantly protected from being altered. This convincingly establishes the potentiality of the aqueous TLE in regulating glutathione biosynthesis that provides an effective antioxidant defence against cadmium chloride-induced oxidative stress mediated tissue damage.

Antioxidant enzymes are key components of the cellular defense mechanisms to battle against oxidative stress [53]. SOD and catalase are two primary enzymes, involved in direct elimination of active oxygen species (hydroxyl radical, superoxide anion free radical and hydrogen peroxide). Catalase catalyze the conversion of hydrogen peroxide to water, thus catalase could reduce the tissue injury by removing the H_2O_2. SOD catalyses the destruction of superoxide anion free radical by dismutation and H_2O_2 formation [54]. The SOD activity was found to be increased in the hepatic tissue with a concomitant decrease in the activity of the catalase enzyme in the hepatic tissue following treatment of rats with cadmium chloride, indicating accumulation of hydrogen peroxide in the tissue. Pre-treatment of rats with aqueous TLE, however, provided protection to these important antioxidant enzymes in the organ from being altered. Therefore, our studies revealed that aqueous TLE has the capability to provide protection against cadmium chloride-induced rat hepatic tissue injury mediated, perhaps, by reactive oxygen species (ROS) and the other related toxicants. Thus, aqueous TLE seems to have the potential to be considered as a beneficial antioxidant. This aqueous extract of the Tulsi leaves may function simply by quenching free radicals and the other related toxic intermediates generated during oxidative stress due to cadmium chloride or may improve the antioxidant enzyme status of the tissue in the face of the oxidative stress.

Mitochondria are the popular site of ROS generation. In our studies, we found that there has been considerable decrease in activities of pyruvate dehydrogenase and the Kreb's cycle enzymes like isocitrate dehydrogenase, alpha-keto glutarate dehydrogenase and succinate dehydrogenase following treatment of rats with cadmium. The activities of all these enzymes were protected from being altered when the rats were pre-treated with the aqueous TLE. Cadmium (Cd^{2+}) produces direct action on mitochondrial function [57,58]. Cadmium has also been reported to decrease the respiratory activity [59]. These enzymes are sensitive to reactive oxygen species (ROS) and inhibition of this enzyme could be critical in the metabolic deficiency induced by oxidative stress [60-62]. The principal site of ROS production by cadmium seems to reside in complex III [63]. The impairment of electron transfer through complex I and complex III may induce superoxide anion free radical formation. The electron transfer chain of mitochondria is also a well-documented source of H_2O_2. Cadmium induces an imbalance in the mitochondrial steady state that allows the induction and effects of oxidative stress [64]. In our present study, cadmium administration inhibits NADH cytochrome c reductase and cytochrome oxidase enzymes of electron transport chain, and succinate dehydrogenase of mitochondria of rat liver. The activities of these enzymes were found to be protected when the rats were pre-treated with aqueous TLE. This strongly indicates that the aqueous TLE possesses either some metal chelating property or is simply able to protect mitochondria from cadmium induced ROS production by itself being a quencher of reactive oxygen species.

Xanthine oxidase (XO) is an important source of free radical generation. Xanthine oxidoreductase, under normal conditions, exists in dehydrogenase form and uses NAD+ and there is no or very little production of superoxide anion. During ischemic conditions, the adenosine nucleotide pool is degraded to hypoxanthine and xanthine, along with conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase acts on xanthine and hypoxanthine with the resultant production of superoxide anion free radical [65]. Our studies reveal that the treatment of rats with $CdCl_2$ may generate copious amounts of superoxide anion free radical in the hepatic tissues which is reflected in elevated levels of activity of xanthine oxidase and xanthine dehydrogenase and their ratio. i.e., XO/XDH, which was found to be almost completely protected when the rats were pre-

treated with 200 mg/kg body weight of the aqueous TLE, fed orally. This protective effect of the aqueous TLE may be due to the presence of phyto-chemicals that protect the thiols from being oxidized. Prevention of intra-molecular disulfide formation within XDH will decrease conversion of the enzyme to XO and decrease the XO/XDH ratio, as seen in our study. Also, the aqueous TLE may be a direct scavenger of superoxide anion free radicals produced *in vivo* due to cadmium or it can prevent superoxide anion free radical production indirectly by chelating cadmium or by enhancing the activities of the antioxidant enzymes.

Treatment of rats with cadmium chloride at the present dose and duration generates copious amounts of hydroxyl radical. Toxicity of superoxide anion free radical and hydrogen peroxide could involve the formation of much more reactive hydroxyl radical (•OH) [66]. Hydroxyl radical is mainly generated via Fenton reaction. Since cadmium does not directly participate in Fenton reaction, thus hydroxyl radical formation *in vivo* may be via indirect pathway. When the rats were pre-treated with aqueous TLE, fed orally, at the present dose, hydroxyl radical generation was found to be protected from being increased, and, the values obtained in the aqueous TLE protected rats were found to be comparable to the values that were obtained in the control animals. The results point again toward the antioxidant potential of the aqueous TLE.

Tulsi leaves have earlier been reported to be rich in phenolics, polyphenols and flavonoids [67,68]. Active principles of *O. sanctum* represent a large group of polyphenolic flavonoids that are helpful in preventing lipid peroxidation [69]. Treatment with ethanolic extract of *O. sanctum* significantly increases the levels of superoxide dismutase, catalase and glutathione in the experimental model [70,71]. The OSI reflects the redox balance between oxidation and anti-oxidation as determined from the total antioxidant status (TAS) and total oxidant status (TOS) [75, 76]. Our results showed that OSI values were increased in the hepatic tissues in cadmium-treated rats, indicating that cadmium treatment changed the redox balancing capability and therefore oxidative stress was inevitable. Pre-treatment of rats with aqueous TLE, however, provided protection by preventing change in OSI and TOS in the organ. Increased oxidative stress index indicates Cd-induced increased intracellular ROS production which reacts with bio-molecules in cells, including DNA resulting oxidative damage to DNA. This in turn results in random fragmentation of genomic DNA leading to the formation of a DNA ladder on agarose gel electrophoresis, suggesting cadmium-induced cell damage via necrotic pathway. Cadmium-induced increase DNA damage was found to be protected only partially, when the rats were pre-treated with the aqueous TLE, indicating that the phyto-chemicals present in the aqueous TLE seems to be capable of providing protection to ROS mediated DNA damage due to cadmium.

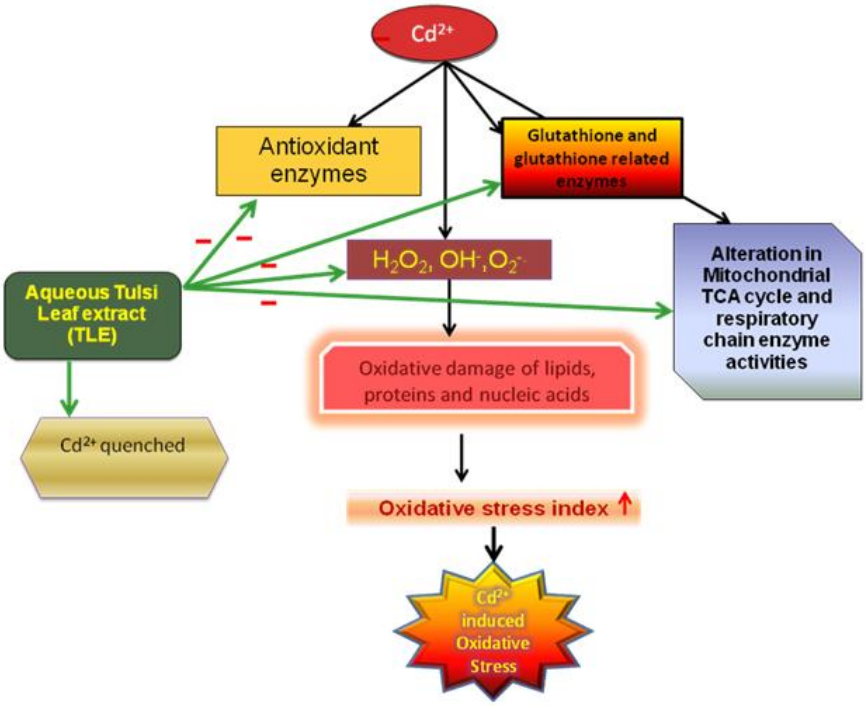


Fig. 3: It shows the possible mechanism of protection of aqueous Tulsi leaf extract against cadmium-induced oxidative damage in liver.

The results of the present studies indicate that aqueous TLE has the potential to provide protection against cadmium-induced oxidative stress in rat liver through its direct as well as indirect antioxidant activity and, also through its possible cadmium chelating properties as shown in the schematic diagram (Fig. 3). The results of the present studies may be of future therapeutic relevance particularly in the areas where humans are chronically exposed to cadmium either occupationally or environmentally. Tulsi leaves are well known for its medicinal importance, can also serve as a possible pharmacological intervention and, the extract itself or the bio-active fractions obtained therefrom may be used also as a future antioxidant supplement to combat oxidative stress-induced tissue damage due to cadmium.

CONCLUSION

It is concluded from the current work that treatment of rats with cadmium chloride at the present dose caused oxidative stress-induced damages in the liver tissue. The aqueous TLE has the capability to provide protection against cadmium-induced oxidative stress possibly through its direct as well as indirect antioxidant activity. The results of the current work reveal that this Tulsi leaf extract may be useful as a protective antioxidant supplement with promising antioxidant potential to combat oxidative stress-induced hepatic damages due to cadmium.

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