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Research Article

AQUEOUS TULSI LEAF (*Ocimum sanctum*) EXTRACT POSSESSES ANTIOXIDANT PROPERTIES AND PROTECTS AGAINST CADMIUM-INDUCED OXIDATIVE STRESS IN RAT HEART

ELINA MITRA[1], DEBOSREE GHOSH[1], ARNAB KUMAR GHOSH[1], ANJALI BASU[1], AINDRILA CHATTOPADHYAY[2], SANJIB KUMAR PATTARI[3], SANTANU DATTA[4], DEBASISH BANDYOPADHYAY[1]*#

[1]Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, # Centre with Potential for Excellence in a Particaular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 70009, [2]Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700006, [3]R. N. Tagore International Institute of Cardiac Sciences, Kolkata, [4]Department of Cardiothoracic Surgery, IPGMER and SSKM Hospital, 244 AJC Bose Road, Kolkata 700020, India. Email: debasish63@gmail.com

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ABSTRACT

Objective: The present study was intended 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 heart.

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 cardiac 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 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 being altered in the rat tissue following treatment with cadmium chloride.

Conclusion: It can be concluded from the current work that this Tulsi leaf aqueous extract may be useful as a protective antioxidant supplement with promising antioxidant potential to combat oxidative stress-induced tissue damages in the areas where humans are exposed to cadmium occupationally or environmentally.

Keywords: Antioxidant, Cadmium, Heart, Oxidative stress, Tulsi leaf extract.

INTRODUCTION

Oxidative stress is currently being implicated in the initiation and progression of many diseases. Free radicals are implicated in the etiology of several diseases, such as atherosclerosis, neurodegenerative disorders, some forms of cancer and aging. Antioxidants prevent the damage to macromolecules and cells by interfering with the free radicals. Currently there is a great deal of interest in newer bioactive molecules from nature with health-promoting potential. Natural products containing antioxidants from plants are believed to modulate oxidative stress [1]. In this context, we have chosen for our studies, a very common plant of medicinal importance, the Tulsi plant (*Ocimum sanctum*), which grows widely in the Indian subcontinent and many other countries of South-East Asia. *Ocimum sanctum* L., commonly known as Tulsi in India, is a traditionally important medicinal local herb containing many potent and useful compounds [2]. The ancient systems of medicines including Ayurveda, Greek, Roman, Siddha and Unani have mentioned its therapeutic applications in cardiovascular disorders [3]. In one of the studies, it was concluded that *Ocimum sanctum* leaf extract may be of therapeutic and prophylactic value in the treatment of myocardial infarction [4]. Recently, a hydroalcoholic extract of the leaves of *Ocimum sanctum* has been shown to ameliorate peptic ulcer induced by ethanol possibly through its antioxidant potential [5].

Cadmium (Cd) is a well known toxic environmental and industrial pollutant. Human exposures include occupational (mining, smelting operations and electronics manufacturing) and non-occupational exposure (mainly from cigarette smoke that contains high concentrations of cadmium) as well as bioaccumulation in the food chain [6]. Cadmium is also carcinogenic for a number of tissues and is classified by IARC as a human carcinogen [7]. Over the past two centuries, high industrial use of the metal led to high emissions of Cd into the environment at concentrations significantly exceeding those originating from natural sources [8]. Since, the half-life of cadmium in humans is more than 15 years [6,8] and therefore environmental risk due to exposure is constantly increasing. Cadmium has the ability to induce severe alterations in various organs following either acute or chronic exposure. Cadmium exposure is linked with various chronic diseases. Acute Cd exposure via inhalation results in pulmonary oedema and respiratory tract irritation, whereas chronic exposure to Cd often leads to renal dysfunction, anaemia, osteoporosis, and bone fractures [9].

The toxic action of cadmium is said to be multifactorial. It has been suggested that cadmium acts as a catalyst in the oxidative reactions of biological macromolecules, and therefore, the toxicities associated with the metal might be due to the oxidative tissue damage [10]. There is an increasing body of evidence that 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]. An enhanced production of ROS can overwhelm cells' intrinsic antioxidant defenses, and result 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 extract ameliorated cadmium-induced cardiac 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).

Quantitative determination of phytoconstituents of aqueous Tulsi leaves extracts

Determination of total phenolic content

Total phenol content of the extract was determined by the modified Folin-Ciocalteu method [16]. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The contents of the tubes were mixed well for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using a UV / VIS spectrophotometer. Total phenolic content was expressed as gallic acid equivalent (mg GAE/g extract).

Determination of total flavonoid content

Total flavonoids were estimated using the method of Sefi *et al*. [16]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour at room temperature, the absorbance was measured at 420 nm using a UV / VIS spectrophotometer. A yellow color indicated the presence of flavonoids. Total flavonoids were expressed as catechin equivalents (mg/g extract).

Determination of condensed tannin

The estimation of condensed tannin was carried out according to the method of Sefi *et al*. [16]. A 2 mL sample of the extract was mixed with 4 mL of vanillin (1% in 7 M sulfuric acid [H_2SO_4]), and then incubated at 25^0C for 15 min. After the incubation, the absorbance was noted spectrophotometrically at 500 nm. Condensed tannins were expressed as mg tannic acid equivalents (TAE/g extract).

Studies on the antioxidant properties of the aqueous Tulsi leaf extract

Reducing power ability

The reducing power of the extract were investigated separately by the Fe^{3+}-Fe^{2+} transformation in the presence of the extract as described by Ningappa *et al*., [17]. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm using a UV /VIS. One ml of the extract in 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000xg. About 2.5 ml of the supernatant, thus obtained, was diluted with 2.5 ml of water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was monitored at 700 nm using a UV / VIS spectrophotometer.

Assay of superoxide anion ($O_2^{\cdot-}$) free radical scavenging activity

Superoxide anion (O2.-) free radical scavenging activity of TLE were studied separately by following the rate of epinephrine oxidation in alkaline pH at 480 nm using a UV / VIS spectrophotometer [18]. The reaction mixture had in a volume of 1 ml, 50 mM Tris-HCl buffer (pH 10), 0.6 mM epinephrine and different concentrations of the extract(s). The increase in absorbance due to the formation of the adrenochrome was followed for 7 min and the activity was calculated from the linear part of the assay in absence and presence of the extract(s).

Hydroxyl radical scavenging assay

The hydroxyl radical (•OH) scavenging activity of TLE *in vitro* were measured using the Fe3+-ascorbate-EDTA- H_2O_2 system (Fenton reaction) [19]. The assay was based on the quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe3+-ascorbate-EDTA system. The reaction mixture contained, in a final volume of 1 ml, 2 deoxyribose (2.8 mM), phosphate buffer (20 mM, pH 7.4), $FeCl_3$ (100 µM), EDTA (100 µM), ascorbic acid (100 µM) and various concentrations (0-100 µg/ml) of the diluted sample extract. The free radical damage imposed on the substrate was measured using the thiobarbituric acid (TBA) test. After incubation for 30 minutes at 37 °C, 0.5 ml of the reaction mixture was added to 1 ml 2.8 % TCA, 1 ml of 1 % TBA in 0.25 (N) HCl and the mixture was incubated at 90 °C for 30 minutes to develop the colour. After cooling the contents of the tubes to room temperature, the absorbance was measured at 532 nm using a UV / VIS spectrophotometer against appropriate blank. Percentage inhibition was evaluated by comparing the absorbance of the test and the blank solution.

Determination of Hydrogen peroxide (H_2O_2) scavenging activity

The hydrogen peroxide (H_2O_2) scavenging activity of TLE was measured by studying the breakdown of H_2O_2 at 240 nm using a UV / VIS spectrophotometer. The reaction mixture, in a final volume of 3 ml, contained 50 mM phosphate buffer (pH 7.4), 30 mM H_2O_2 and various concentrations of the extract [18].

Determination of DPPH radical scavenging activity

The *in vitro* radical scavenging activity of TLE was determined spectrophotometrically using diphenylpicryl hydrazyl (DPPH) radical according to the method of Ningappa et al. [17]. Aqueous leaf extract (0.1ml) of different concentrations were added to 3 ml of 0.001M DPPH solution in methanol. The solution was shaken and incubated at 37°C for 30 min in the dark. The decrease in absorbance of DPPH was measured against a blank at 517 nm.

Percent (%) inhibition was calculated by comparing the absorbance values with and without extract.

Metal chelating activity

The ferrous ion chelating property of TLE was estimated by the method of Ningappa *et al*., [17]. Briefly, the extract (0–10 mg/ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 0.2ml of 5 mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine– Fe2+ complex formation was calculated as [(Ac-As)/Ac] x 100, where Ac was the absorbance of the control, and As was the absorbance of the extract or standard.

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 heart tissue was homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.4. After addition of 1.0 ml of diluted $ABTS^{\cdot+}$ solution (A734nm = 0.700 ± 0.020) to 10 ml of antioxidant compounds or Trolox standards (final concentration 0–15 mM) 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 heart 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 H2SO4 solution, pH 1.75) and Reagent 2 (ferrous ion 5 mM and o-dianisidine 10 mM in 25 mM H2SO4 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 colored complex with xylenol orange in an acidic medium. The color intensity, which can be 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 heart 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 cardiac 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 cardiac damage

Serum glutamate oxaloacetate transaminase (SGOT) was measured by the method of Reitman and Frankel [22]. Values are expressed as IU/L. The cardiac specific Type 1 isoform of Lactate Dehydrogenase (LDH1) activity was obtained by incubating the serum samples at 65°C for 30mins which destroys all isoforms except LDH1 [23]. The enzyme activity was then determined as before by measuring the NADH oxidation. Values were expressed as Units/ml.

Histological studies

Studies using tissue sections stained with hematoxylin and eosin

A portion of the extirpated rat heart was fixed immediately in 10% formalin and embedded in paraffin following routine histological procedure. Cardiac 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 heart 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 glutathione (GSH) level

A weighed amount of the rat cardiac 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 (PCO) 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.

The 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 cardiac 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 TSH/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 560 nm 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 (CAT) 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 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 50 mM 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 cardiac tissue was homogenized (10%) in ice-cold 50 mM phosphate buffer containing 2 mM 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.

Cardiac 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 $\mu M^{-1}cm^{-1}$. 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

(O_2^-) by determining the activities of the pro-oxidant enzymes, like, xanthine oxidase and xanthine dehydrogenase

Xanthine oxidase (XO) of the rat cardiac 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 cardiac 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 (XDH) 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 cardiac 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 cardiac 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 heart was collected. The cardiac 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 (PDH) and some of the mitochondrial Kreb's cycle enzymes

The weighed amount of rat cardiac 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 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 electrophoresing genomic DNA samples, isolated from rat heart, on agarose/EtBr gel by the procedure described by Sellins and Cohen [45]. Briefly, cardiac 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 by phenol, chloroform and isoamyl alcohol extraction and precipitated by 100% ethanol. 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 heart tissue by Atomic Absorption Spectrophotometry (AAS)

The cardiac 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 heart 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.

Table 1: It shows extraction yield and the content of total antioxidant status, total phenols, total flavonoid and condensed tannins in aqueous extract of the leaves of *Ocimum sanctum* L. (i.e., aqueous TLE)

Sample	Extraction yield[a]	Content			
		Total antioxidant status	Total phenols (mg GAE[b]/g extract)	Total flavonoid (mg CE[c]/g extract)	Condensed tannins (mg TAE[d] /g extract)
Tulsi Leaf aqueous extracts (i.e., aqueous TLE)	8.33±0.45%	2.98± 0.012	47.1 ± 0.023	7.625± 0.018	1.125 ± 0.009

Values represent the means of four separate extractions ± S.E.M.

[a] Extraction yield (%) = (sample extract weight/ sample weight) x 100; [b] GAE, Gallic acid equivalent; [c] CE, Catechin equivalent; [d] TAE, Tannic acid equivalent

RESULTS

Status of phyto-constituents in the aqueous TLE

The results of our chemical analysis of the aqueous TLE (Table 1) have shown the presence of phenols, flavonoids and condensed tannins as major phyto-constituents. The extraction yield was found to be 8.33 ± 0.45%.

Assessment of radical scavenging activity of aqueous TLE *in vitro*

DPPH radical scavenging activity

The DPPH radical scavenging activity of aqueous TLE is shown in Figure 1(A). The aqueous TLE exhibited over 59.94 % scavenging activity of DPPH radical at a concentration of 1 µg/mL.

Reducing power

Figure 1 (B) shows the dose–response curve for the reducing power of aqueous TLE. The amount of Fe2+ complex was monitored spectrophotometrically by measuring the formation of Perl's Prussian blue at 700 nm. The figure reveals a concentration-dependent increase of reducing power of aqueous TLE.

Superoxide anion free radical scavenging activity

Figure 1 (C) reveals that the superoxide anion free radical scavenging activity of the aqueous TLE increased markedly with increasing concentrations of the extract. At a concentration of 30 µg / ml, 47.71 % superoxide anion free radical scavenging activity was observed. The results indicate that the aqueous TLE is capable of scavenging the superoxide anion free radicals *in vitro*.

Protection of deoxyribose degradation in vitro by aqueous TLE

Figure 1 (D) reveals that aqueous TLE protects against deoxyribose degradation in a dose- dependent manner indicating hydroxyl radical (˙OH) scavenging capability of the aqueous TLE. At 100 µg/ml of the aqueous TLE, there occurred 75.78 % protection of deoxyribose degradation compared to control reaction.

Hydroxyl radical (˙OH) scavenging activity of aqueous TLE in Cu-Ascorbate system in vitro

Figure 1 (E) further demonstrates that aqueous TLE scavenges the hydroxyl radical, generated *in vitro*, in a concentration-dependent manner, exhibiting 52.26 % scavenging activity at a concentration of 40 µg/mL.

Metal chelating activity of aqueous TLE in vitro

Aqueous TLE exhibited 28.09 % inhibition of Fe2+–ferrozin complex formation at the dose of 6.75 mg/ml concentration (Fig.1F).

Status of cadmium concentration of the rat cardiac tissue

Table 2 reveals a highly significant increase in the rat cardiac tissue Cd concentration following treatment of rats with cadmium chloride. The concentration of cadmium in the tissue was partially but significantly reduced (34.67 %, **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.

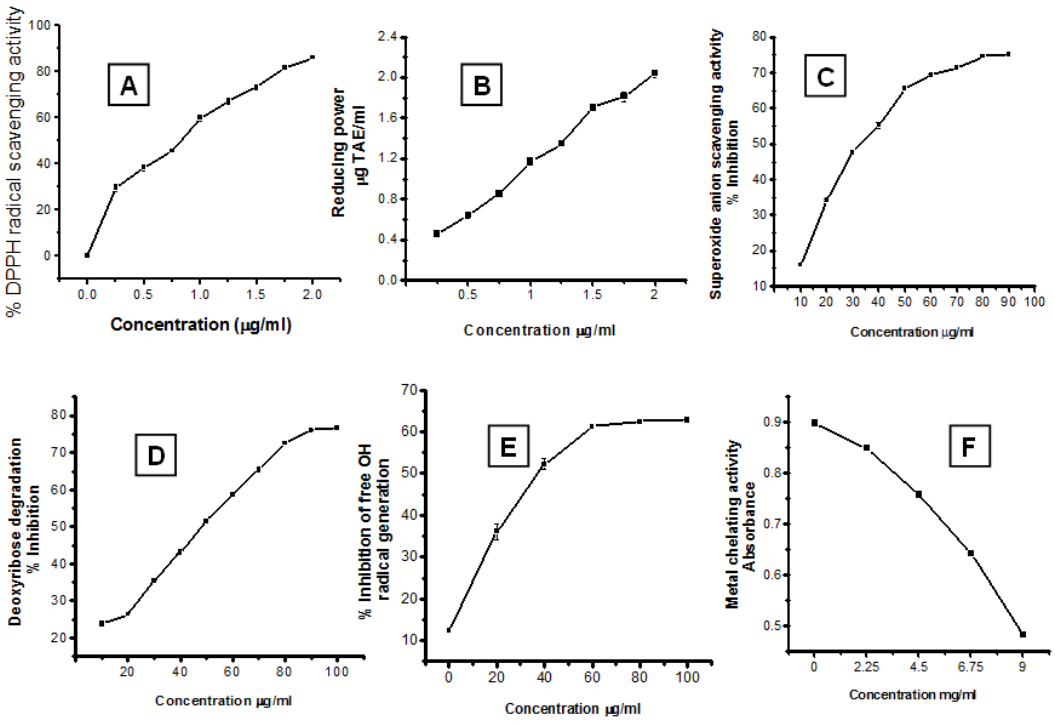

Fig. 1: It shows *in vitro* DPPH radical scavenging activity [A], reducing power [B], superoxide anion free radical scavenging activity [C], protection of deoxyribose degradation [D], hydroxyl radical scavenging ability [E] and metal chelating activity [F] of the aqueous TLE.

Biomarkers of organ damage: SGOT and LDH1

Table 2 reveals the level of activity of serum specific cardiac damage markers, SGOT and LDH1 were found to be significantly higher in Cd-treated group of rats compared to control (3.47 and 2.41 folds increase, * P < 0.001 vs. control). The activities of the enzymes were found to be significantly decreased in the animals pre-treated with aqueous TLE (69.96 % and 55.69 % 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 cardiac damage.

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

Treatment	SGOT (IU/L)	LDH1 (IU/L)	Cadmium concentration (µg/g)
C	7.86±0.424	0.0106±0.00044	ND
Cd	27.26±2.093*	0.0255±0.00189*	3.23±0.053
Cd+TLE	8.19±0.248**	0.0113±0.00038**	2.11±0.097**
TLE	7.03±0.522	0.0087±0.00038	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

Routine H and E staining of the rat cardiac tissue sections

Treatment of rats with cadmium at a dose of 0.44 mg / kg bw sc, caused damage to cardiac tissue morphology. There were clear signs of degenerative changes along with myocardial fibre necrosis in the cardiac tissue sections of cadmium chloride-treated rats. 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 cardiac damage in rats (Figure 2A).

Studies on collagen content and quantification of fibrosis

The figure 2B (magnification 200X) reveals that Picrosirius red stained cadmium-treated rat cardiac tissue sections showed a depletion of collagen following cadmium treatment, an 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 2C 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 cardiac tissue. The images of the tissue sections presented here are at 200x magnification.

Figure 2D shows that the percentage volume of total collagen content in cardiac tissue sections also decreased significantly (7.91 folds decrease, *P<0.001, n=4) in cadmium chloride treated rat heart which was also found to be protected almost completely from being decreased in aqueous TLE protected rats.

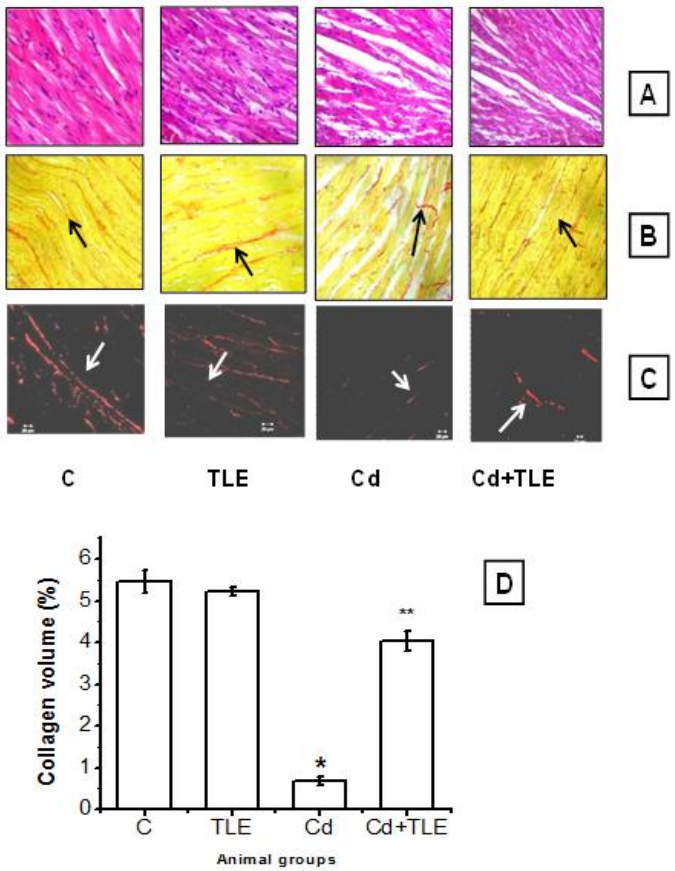

Fig. 2: It shows protective effect of aqueous Tulsi leaf extract against cadmium-induced changes in the rat cardiac tissue morphology. [A] H and E stained and [B] Sirius red stained sections (200X magnification). [C] Similar images captured by confocal laser scanning microscope for quantification of fibrosis. Arrow heads indicate collagen fibres in B and C. [D] Graph showing collagen volume % of the cardiac 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 sc. *P < 0.001 compared to control values using ANOVA. **P < 0.001 vs. compared to cadmium-treated values using ANOVA.

Biomarkers of oxidative damage

Table 3 reveals a significantly higher in cardiac tissue LPO level and PCO content following treatment of rats with cadmium (1.89 folds and 1.96 folds increase, **P < 0.001 vs. control). This elevated level of lipid peroxidation products and protein carbonyl content were found to be decreased significantly (46.26% and 41.93% 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 that Cd-induced decrease in the level of reduced GSH and total sulfhydryl (TSH) (56.93% and 33.96 % respectively, * P < 0.001 vs. control), increase in GSSG content (57.37% increase, * P < 0.001 vs. control) and decrease in redox status (62.5 % decrease, * P < 0.001 vs. control) in rat cardiac 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 heart.

Table 3: It shows 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 cardiac 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	7.01±0.230	0.243±0.0056	30.25±0.902	14.10±0.662	2.16±0.206	88.13±1.486
Cd	13.23±0.255*	0.477±0.0076*	13.03±0.799*	22.19±0.410*	0.81±0.069*	58.20±0.356*
Cd+TLE	7.11±0.211**	0.277±0.0187**	30.14±0.049**	12.30±0.179**	2.49±0.004**	86.72±1.479**
TLE	5.42±0.655	0.220±0.0132	30.08±0.296	13.24±0.936	2.02±0.020	82.12±1.886

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

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 increase in the activity of catalase in rat cardiac tissue (55.87%, 52.83% decrease and 44.93% increase respectively, * P < 0.001 vs. control). The extract was found to restore the activity of the enzymes (2.32 folds, 1.84 folds increase and 24.27% decrease respectively from Cd treated group, **P < 0.001) to near control levels. Table 4 also illustrates a significant increase in GST and glutathione peroxidase activities of the rat cardiac tissue (61.54 % and 64.10% increase respectively, * P < 0.001 vs. control) and a highly significant decrease (64.71 %, *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 (30.95 %, 37.5 %, 2.66 folds, **P < 0.001 vs. cadmium) from being altered. The results indicate that the aqueous TLE may have an influence on the GSH biosynthesis.

Table 4: It shows effect of aqueous Tulsi leaf extract against cadmium induced changes in various antioxidant enzymes in cardiac 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	1.013±0.0742	0.53±0.007	12.91±0.422	0.39±0.009	0.34±0.027	0.26±0.016
Cd	0.447±0.0223*	0.25±0.013*	18.71±0.708*	0.64±0.033 *	0.12±0.002 *	0.42±0.016 *
Cd+TLE	1.043±0.0373**	0.46±0.031**	14.17±0.478**	0.40±0.020 **	0.32±0.008 **	0.29±0.009 **
TLE	1.103±0.0274	0.52±0.004	13.24±0.375	0.27±0.011	0.33±0.016	0.26±0.013

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.

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

Table 5 illustrates a significant increase in Total oxidant status (TOS) (3.09 folds increase, *P < 0.001 vs. control) and a highly significant decrease (39.30%, *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 (64.03 % decrease, **P < 0.001 vs. cadmium) and the total antioxidant status level was protected (1.73 folds, **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 cardiac tissue (4.44 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.

Table 5: It shows effect of aqueous Tulsi leaf extract against cadmium induced changes in total oxidant status, total antioxidant status and oxidative stress index in cardiac 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	14.3±1.834	8.31±0.061	0.17±0.022
Cd	44.2±0.716*	5.05±0.179*	0.76±0.038*
Cd+TLE	15.9±0.004**	8.74±0.054**	0.21±0.004**
TLE	12.53±0.004	8.52±0.408	0.18±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

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

Table 6 reveals that treatment of the rats with cadmium inhibits the activities of rat cardiac pyruvate dehydrogenase, isocitrate dehydrogenase, alpha keto glutarate dehydrogenase and succinate dehydrogenase (48.07 %, 59.21%, 56.52% and 53.19% 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 (1.80 folds, 2.52 folds, 1.88 folds, 2.61 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 (43.98% and 53.33% 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 (2.10 folds increase in NADH cytochrome c reductase activity and 2.13 folds increase in cytochrome oxidase activity, **P < 0.001 vs. Cd-treated group) (**Table 6**).

Table 6: It shows effect of aqueous Tulsi leaf extract against cadmium induced changes in the activities of some mitochondrial respiratory chain enzymes and enzymes of electron transport chain in cardiac tissue of rats.

Treatment	Pyruvate dehydrogenase (units/min/mg tissue protein)	Isocitrate dehydrogenase (munits/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	0.0360±0.00159	7.6±0.43	0.0161±0.00014	0.94±0.061	10.73±0.516	0.15±0.008
Cd	0.0187±0.00235*	3.1±0.38*	0.070±0.00051*	0.44±0.072*	6.01±0.429*	0.07±0.01*
Cd+TLE	0.0337±0.00165**	7.8±0.92**	0.0132±0.00084**	1.15±0.147**	12.6±0.874**	0.149±0.011**
TLE	0.0393±0.00259	8.8±0.73	0.0162±0.00036	1.10±0.091	12.04±0.776	0.154±0.009

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.

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 cardiac 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 (3.47 folds, 2.65 folds and 29.31%, *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 heart (1.53 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 Gemonic DNA damage in cadmium chloride-induced oxidative stress in rat heart

Figure 3 illustrates the extent of DNA fragmentation. Pre-treatment of rats with the aqueous TLE almost completely protected the cadmium chloride-induced genomic DNA damage in heart and in figure, the smear, in the lane 3 on agarose gel has been observed in cadmium chloride-treated heart tissue, indicating random DNA degradation.

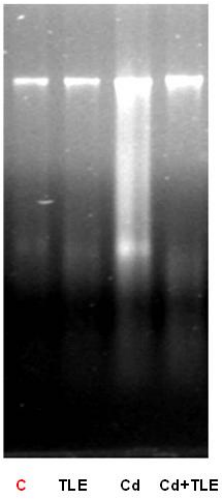

Fig. 3: It shows protective effect of aqueous TLE against cadmium induced DNA damage in rat heart. Lane (1) Control heart, lane (3) cadmium treated heart, lane (4) cadmium+aqueous TLE treated heart, lane (2) aqueous TLE only. C= Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected s.c.;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

Table 7: It shows effect of aqueous Tulsi leaf extract against cadmium induced changes in the activities of XO, XDH, XO/XDH ratio and hydroxyl radical in cardiac 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	6.62±0.296	0.383±0.029	1.74±0.053	6.97±0.060
Cd	22.97±1.976*	1.016±0.031*	2.25±0.164*	10.63±0.092*
Cd+TLE	6.20±0.457**	0.382±0.017**	1.62±0.110**	7.19±0.027**
TLE	6.29±0.264	0.426±0.029	1.49±0.067	7.49±0.159

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.

DISCUSSION

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

In the present studies, cadmium accumulates in considerable amounts in rat heart 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]. Our results show that the pre-treatment of experimental rats with aqueous TLE shows significant difference in cadmium accumulation in the heart which signifies that the leaf extract protects against cadmium induced damage by partially chelating the metal ion, rendering it inactive. This correlates with our *in vitro* studies where we found that the leaf extract has metal chelating property.

In our present studies, the serum levels of these organ specific marker enzymes SGOT and LDH1 were increased indicating cardiac damage respectively 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) 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 cardiac tissues. This protection might have been exerted through some phytochemical(s)/ phytonutrient(s) present in the extract. Our in vitro studies showed that aqueous tulsi leaf extract contains considerable amounts of phenolics and flavonoids which may be responsible for its antioxidant activity.

Histological examination of hematoxylin-eosin stained cardiac tissue sections following cadmium exposure at the present dose and duration showed myocardial fibre necrosis in heart tissue sections. However, the tissue sections from the rats pre-treated with aqueous TLE did not show any such changes. The results indicate the ability of the aqueous extract to provide protection against cadmium-induced tissue injury. Picrosirius stain of cardiac tissue sections show that there was depletion in collagen content in cadmium-treated group, indicating fibrosis. Pre-treatment of rats with aqueous TLE was found to prevent the change in collagen volume in the tissue indicating the protective role of the extract in maintaining the tissue integrity. Collagen volume calculated from confocal microscopic picture of the same tissue sections showed similar results.

In our experiments, the rats exposed to cadmium had increased peroxidation of membrane lipids of heart tissue. Membrane lipids are highly susceptible to free radical damage. 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]. Cadmium may induce oxidative stress by enhancing LPO and thus may cause damage to cellular components. The aqueous TLE is found to be effective in decreasing the lipid peroxidation level of heart 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.

Cellular proteins are the main targets of oxidation resulting in the formation of aldehyde and ketone residues. The carbonyl content in proteins is an indicator of oxidative stress. Most oxidized proteins are functionally inactive and are rapidly removed; some gradually accumulate and contribute to damage. Carbonyl group formations are considered as an early and stable marker for protein oxidation [50]. Our results clearly indicate that the tissue suffered reactive-oxygen intermediate mediated protein damage. This confirms that functionally active oxidized proteins that are capable of causing damage are produced following exposure of the experimental rats to cadmium. However, the aqueous TLE seems to possess an ability to reduce the protein carbonyl content of the cardiac tissue possibly through scavenging the reactive oxygen species or by chelating the heavy metal or by both.

Cadmium has high affinity for reduced glutathione (GSH) which is a primary intacellular antioxidant and conjugating agent, participating in enzymatic and non-enzymatic detoxification of many oxidative toxicants [51]. GSH level was found to be decreased in our studies which indicate generation of oxidative stress following sub-chronic exposure of experimental rats to cadmium that may be responsible for the oxidative damage of bio-macromolecules of the tissue. The present work indicates that disruption of the cellular glutathione system is a key element in the mechanism of Cd-induced oxidative stress in the organ. 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 that additionally indicate toward its antioxidant potential. The results point toward the possibility that the aqueous extract may deliver effective antioxidant phytochemical molecules in the tissues or in blood which probably helps in defending against overall oxidative damage or has specific cadmium chelating effects within the tissues.

Our results also revealed a significant increase in GSSG content in the heart tissue of rats. Studies further showed a decrease in the GSH/GSSG ratio. Reduced glutathione (GSH) is a substrate of glutathione peroxidase (GPx). Two molecules of GSH are required to produce a molecule of oxidized glutathione (GSSG). The GSSG, thus, formed is then either expelled from the cell or reconverted in to GSH under physiological conditions by the glutathione reductase. The intracellular GSH/GSSG ratio is highly regulated [52]. However, an increase in ROS levels (or other factors limiting GSSG reduction) 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. When the rats were pre-treated with the aqueous TLE, the redox status was found to be protected from being altered indicating that the extract have antioxidative potential.

The present study clearly shows that exposure of rats to cadmium chloride caused significant depletion of total tissue sulfhydryl (TSH). However, when the rats were pre-treated with aqueous TLE, the TSH level in the tissue was found to be protected from being altered indicating that the aqueous TLE have antioxidative potential.

Antioxidant enzymes are key components of the cellular defense mechanisms to battle against oxidative stress. Antioxidant enzymes activity can be differentially modified by Cd2+ toxicity [53]. In stress conditions, normal capacities of these mechanisms are insufficient, triggering cells to increase and expand their antioxidative network. 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 decreased in the cardiac tissue with a concomitant increase in the activity of the catalase enzyme in the cardiac tissue following treatment of rats with cadmium chloride, indicating accumulation of superoxide anion 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 cardiac 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.

Glutathione and glutathione-related enzymes play a key role in protecting the cell against the effects of reactive oxygen species. 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. The thiol-disulfide redox

cycle also aids in maintaining reduced protein and enzyme thiols. Without a process to reduce protein disulfides, vulnerable cysteinyl residues of essential enzymes might remain oxidized, leading to changes in catalytic activity [55]. GR and GPx are FAD-dependent and Se-dependent oxido-reductases, respectively, which are supposed to protect the cellular biomolecules and structures from oxidative damage induced due to heavy-metal cations, like, cadmium (Cd2+). In the present studies, the activity of the glutathione dependent enzyme, GR was found to be reduced significantly in cardiac 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 heart. 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 cardiac 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 cardiac tissue, the heavy metal divalent cation, like, cadmium (Cd^{2+}) binds to other relevant biomolecules 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 defense against cadmium chloride-induced oxidative stress mediated tissue damage.

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, i.e., this heavy metal inhibits both mitochondrial electron transfer, disturbing the respiratory complexes, and increases mitochondrial membrane permeability via the induction of MPT pore opening [57,58]. Cadmium has also been reported to decrease the respiratory activity [59]. Isocitrate dehydrogenase is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria, which is needed for the regeneration of mitochondrial GSH or thioredoxin [60]. Alpha keto glutarate dehydrogenase (α-KGDH) is sensitive to reactive oxygen species (ROS) and inhibition of this enzyme could be critical in the metabolic deficiency induced by oxidative stress. Alpha -KGDH is also able to generate ROS during its catalytic function, which is regulated by the NADH/NAD+ ratio [61]. Pyruvate dehydrogenase (PDH) has been demonstrated to be sensitive to insults that induce oxygen free radicals [62], which might be a cause of decrease of the enzyme activity. The principal site of ROS production by cadmium seems to reside in complex III. Earlier researchers have reported that cadmium markedly inhibits uncoupler-stimulated oxidation on various NADH-linked substrates as well as that of succinate [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 a well-documented source of H_2O_2. Several components of complex I, II and III exhibit properties that would allow the reduction of O_2 to $O2^{\bullet-}$. Intracellular and intra-mitochondrial ROS production and elimination is well balanced and such a steady state is attained by various antioxidant enzymes. Cadmium induces an imbalance in the 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 heart. 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 prevent mitochondria from cadmium induced ROS production by itself being a quencher of reactive oxygen species.

Xanthine dehydrogenase (XDH) is a molybdopterin-containing flavoprotein that oxidizes hypoxanthine to xanthine, and xanthine to uric acid. It has two identical subunits containing FAD, molybdenum, and Fe-S clusters that facilitate electron transfer from substrate to the electron acceptor NAD+ [65]. Xanthine dehydrogenase can be converted to xanthine oxidase (XO) by either proteolytic cleavage of the amino terminus or more rapidly by thiol oxidation leading to intra-molecular disulfide formation. Only the dehydrogenase form of the enzyme can reduce NAD^+ and form NADH and, XO remains a major source for superoxide anion free radical ($O^{2\bullet-}$) and hydrogen peroxide (H_2O_2) production [65]. Our studies reveal that the treatment of rats with $CdCl_2$ may generate copious amounts of superoxide anion free radical in the cardiac 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 ($^{\bullet}OH$) [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.

In vitro experiments reveal that the extracts of leaves of the Tulsi plant do possess radical scavenging activity. 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 results of our *in vitro* experiments reveal that the aqueous TLE possesses DPPH radical scavenging activity, reducing power, superoxide anion free radical scavenging activity, hydroxyl radical ($^{\bullet}OH$) scavenging activity and metal chelating activity. DPPH is one of the compounds that possess a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers. Further, it is well accepted that the DPPH free radical-scavenging by antioxidants is due to their hydrogen-donating ability [72]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [73]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of the aqueous TLE suggest that it is likely to contribute significantly towards the observed antioxidant effect. In the present study, the aqueous TLE effectively scavenged the superoxide anion free radical in a concentration-dependent

manner. Although superoxide anion free radical is a relatively weak oxidant, it decomposes to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids [74]. Hydroxyl radicals (•OH) are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage *in vivo* [26]. In our study, we found that the aqueous TLE directly scavenges hydroxyl radical *in vitro* in a concentration-dependent manner. The results also indicate that the aqueous TLE has metal chelating capability and acts as an antioxidant by directly scavenging oxygen free radicals and other reactive oxygen intermediates.

The OSI reflects the redox balance between oxidation and antioxidation 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 cardiac 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 biomolecules in cells, including DNA resulting oxidative damage to DNA [77]. This in turn results in random fragmentation of genomic DNA leading to the formation of a DNA smear on agarose gel electrophoresis, suggesting Cadmium induced cell damage via necrotic pathway. Cadmium induced increase DNA damage was found to be significantly protected from being increased in the rats pre-treated with 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.

The results of the present studies indicate that aqueous TLE has the potential to provide protection against cadmium-induced oxidative stress in rat heart through its direct as well as indirect antioxidant activity and, also through its possible cadmium chelating properties as shown in the schematic diagram (Fig. 4). 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 from the extract may be used also as a future antioxidant supplement to combat oxidative stress-induced tissue damage in the people exposed to cadmium.

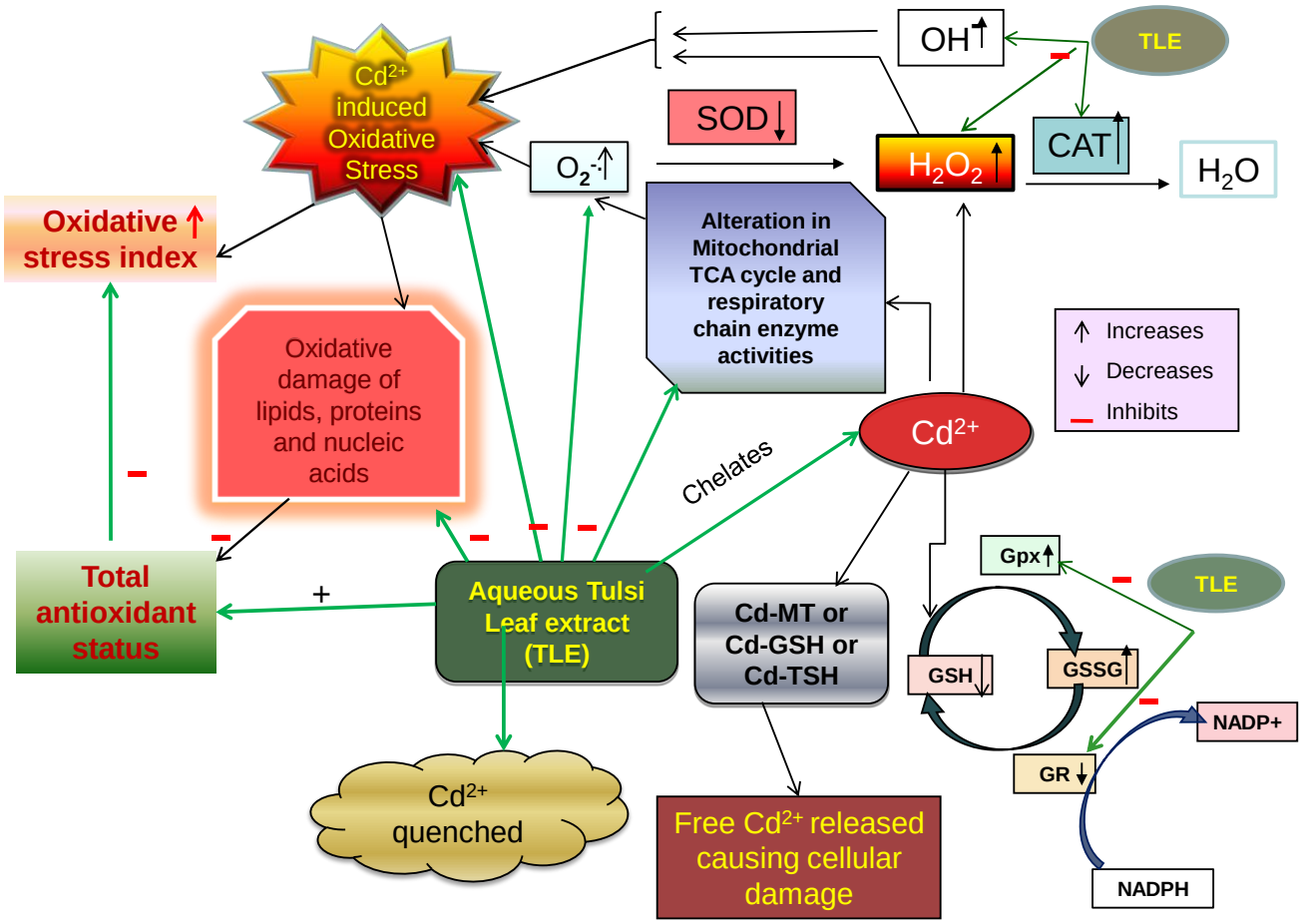

Fig. 4: It shows the possible mechanism of protection of aqueous Tulsi leaf extract against cadmium induced oxidative damage in heart.

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 heart 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 tissue damages in the areas where humans are exposed to cadmium occupationally or environmentally.

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