Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 2, 2013

Research Article

# AMELIORATIVE EFFECT OF AQUEOUS CURRY LEAF (*MURRAYA KOENIGII*) EXTRACT AGAINST CADMIUM-INDUCED OXIDATIVE STRESS IN RAT LIVER: INVOLVEMENT OF ANTIOXIDANT MECHANISMS

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*Received: 26 Jan 2013, Revised and Accepted: 29 Feb 2013*

**ABSTRACT**

Objective: The present study was intended to examine whether administration of aqueous extract of the Curry leaves (*Murraya koenigii* L.) do possess a protective effect against cadmium-induced oxidative stress in rat liver.

Methods: Male albino rats used for present studies were divided in to 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.

Results: Cadmium-induced liver damage was clearly evident from increased activities of serum glutamate pyruvate transaminase , alkaline phosphatase, lactate dehydrogenase 5, and total LDH. An increased tissue level of lipid peroxidation, and protein carbonyl as well as decreased reduced glutathione and total-thiol (SH) content along with significant changes in the activities of antioxidant enzymes like superoxide dismutase, catalase, glutathione S transferase and glutathione peroxidase indicate development of oxidative stress due to Cd treatment. Besides, increased activities of the pro-oxidant enzymes, enhanced formation of superoxide and hydroxyl radical further confirms elevated levels of oxidative stress following cadmium treatment. Cadmium treatment also altered the activities of mitochondrial Kreb's cycle and respiratory chain enzymes. Tissue histomorphological studies also showed considerable damage following cadmium treatment. All these changes were significantly protected when the rats were pre-treated with aqueous Curry leaf extract.

Conclusion: The present studies suggest that the aqueous Curry leaf extract may be beneficial in ameliorating cadmium-induced oxidative damage in the liver of rats.

**Keywords:** Cadmium, Curry leaf extract, Oxidative stress, *Murraya koenigii*, Hepatotoxicity, Rat liver.

## INTRODUCTION

Cadmium (Cd) is a widespread 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 [1]. It is listed by the U.S. Environmental Protection Agency as one of 126 priority pollutants. Cadmium is carcinogenic for a number of tissues and is classified by IARC as a human carcinogen [2]. Over the past two centuries, anthropogenic and industrial activities have led to high emissions of Cd into the environment at concentrations significantly exceeding those originating from natural sources [3]. Since, the half-life of cadmium in humans is estimated to be between 15 and 20 years [1,3] the risk of environmental exposure is constantly increasing. Cadmium has the ability to induce severe alterations in various organs, including liver 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 [4].

The toxic action of cadmium, although not completely understood, is recognized 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 [5]. 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 [6-8]. 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 [9]. Although formally Cd belongs to the group of transition elements, it almost adopts only one oxidation state, which is 2+. Thus, in most chemical reactions it behaves similar to main-group metals. Cadmium does not induce production of ROS through a Fenton-like reaction [10]. The molecular mechanism for the toxic effects of cadmium involves interferences with the activities of antioxidant, pro-oxidant and some other enzymes, alteration in thiol proteins, inhibition of energy metabolism and alteration in DNA structure. According to recent researches cadmium generates reactive oxygen- and carbon-centered radical species by involvement of both iron mediation through iron-catalyzed reactions and activation of Kupffer cells [11].

Depending on the route of exposure, Cd has differing rates of absorption and varying health effects. Cadmium is a multi target cumulative toxicant. Its level in the body increases over time because of its slow elimination. The liver is one of the target organs of both chronic and acute Cd exposure. While hepatocytes and endothelial cells of the liver sinusoids are supposed to be the primary cellular targets in liver, ultrastructural changes may vary depending upon the exposure type and duration following administration [12]. Since cadmium has numerous adverse effects, researches on cadmium-induced hepatic stress not only focus on its mechanism of toxicity but also on how its toxicity can be counteracted. Amelioration of hepatotoxicity using synthetic drugs causes undesirable side effects [13] and may be inadequate in curing the damage. Hence, in recent decade herbal alternatives have stimulated a new wave of interest in ethnomedicine that would be invaluable especially in view of the minimal side effects [14].

In this context, we have chosen for our studies, a very common plant of medicinal importance, the Curry plant (*Murraya koenigii*), which grows widely in the Indian subcontinent and many other countries of

South-East Asia. The Curry plant belonging to the family Rutaceae is native to India and now distributed in most of southern and south-East Asia. The leaves of this plant are well-known as Curry leaves and have been used as one of the important herbs of southern Indian cooking. The different parts of Curry plant have also been used as a folk medicine. The leaves have been reported to increase digestive secretions and relieve nausea, indigestion, and vomiting [15]. Recent researches have shown that blood parameters/constituents and hepatic tissue morphology in Curry plant fed rats remained unchanged which signifies that curry leaf has no adverse effects. The Curry leaves are used traditionally as antiemetic, antidiarrheal, febrifuge and blood purifier. The whole plant is considered to be tonic and stomachic. The plant has been studied for their various pharmacological activities like antioxidant, antibacterial, anti fungal, antiprotozoal, anti-lipid peroxidative, hypoglycemic and hypolipidemic activity [16]. The traditional use of this plant is based on the oral administration of a decoction prepared from leaves in water. Curry leaf is rich in phenolics, polyphenols and flavonoids [17, 18]. The Curry leaf extract has recently been shown to provide protection against oxidative stress induced in diabetes [19]. Curry leaf has a much higher flavonol profile obtained by LC-MS-MS analysis of curry leaf extracted with different solvents [17]. Various flavonols and carbazoles from curry leaf extract are known to possess antioxidative properties [20].

Herein, we demonstrate that the aqueous Curry leaf extract (CuLE) exhibited antioxidant properties when tested *in vitro*, and, pre-treatment of the experimental rats with this extract ameliorated cadmium-induced hepatic injury possibly through its antioxidant mechanism(s). As Curry leaves are consumed by the people at this part of the world as one of their dietary items since ancient times 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 120–150 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 Curry leaves

The fresh, green Curry leaves (*Murraya koenigii* ( L.) *Spreng*) 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 Curry leaf extract (CuLE)

The method of preparation of aqueous Curry leaf extract (CuLE) was followed as according to Zhang et al. [21] with modification. The collected curry leaves were shade dried and powdered. The dried Curry leaf dusts were soaked overnight in double distilled water (7.5g 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 Curry leaf extract (CuLE), was stored at -20 °C until further use. A definite amount of the CuLE (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 CuLE was 15.59±0.36 % (w/w).

### Assessment of antioxidant property of aqueous CuLE in vitro

### Determination of DPPH radical scavenging activity

The *in vitro* radical scavenging activity of CuLE was determined spectrophotometrically using diphenylpicryl hydrazyl (DPPH) radical according to the method of Ghosh et al. [22]. 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.

### 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 **CuLE** treated rats; dose, 100 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 CuLE was administered orally at a dose of 100 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+CuLE)** one hour after administration of the aqueous CuLE. Before administration of $CdCl_2$ solution to rats, researchers always used clinical masks and rubber hand gloves as a measure of protection.

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 was 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.

### 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. [23]. The cadmium content was expressed in µg/g of rat liver tissue.

**Assessment of serum specific markers related to hepatic damage**

Serum glutamate pyruvate transaminase (SGPT) was measured by the method of Reitman and Frankel [24]. Values are expressed as IU/L . Serum alkaline phosphatase activity was measured by the method of Kind and King [25]. Values are expressed as KA units/dL. The total serum activities of lactate dehydrogenase (LDH) and lactate dehydrogenase 5 (LDH5) were obtained by measuring the oxidation of NADH (0.1mM) to NAD+ at 340nm using 1.0mM sodium pyruvate as substrate according to the method of Strittmatter [26] with some modifications [27]. 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.

**Histological studies**

**Studies using tissue sections stained with hematoxylin and eosin**

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 Olympus microscope and the images were captured with a camera attached to it.

**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. [28] 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, protein carbonyl content, reduced GSH level and total sulfhydryl group content**

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 [29], with some modification as adopted by Bandyopadhyay et al. [30]. 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 (PCO) content was estimated by the method of Levine et al. [31]. About 0.1 g of liver tissue was rinsed in 10 mM phosphate buffered saline (PBS ; [pH 7.4]) and homogenized in cold and centrifuged at 10,000xg for 10 min at 4°C. After centrifugation, 0.5 ml of tissue supernatant was taken in each tube and 0.5 ml DNPH in 2.0 M HCl was added to the tubes. The tubes were vortexed every 10 min in the dark for 1 hour. Proteins were then precipitated with 30 % TCA and centrifuged at 4,000 x g for 10 min. The pellet 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 determined at 370 nm. The protein carbonyl content was calculated using a molar absorption coefficient of $2.2x10^4$ $M^{-1}cm^{-1}$. The values were expressed as nmoles / 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 [32] with some modifications by Bandyopadhyay et al. [30]. A weighed amount of hepatic 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.

Total sulfhydryl group content was measured following the method as described by Sedlac and Lindsay [32]. 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 [33] with some modifications as adopted by Mukherjee et al., 2010 [34]. Briefly, the weighed amount of hepatic 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.

Hepatic Manganese superoxide dismutase (Mn-SOD or SOD2) activity was assayed by method of Marklund and Marklund [35] which involves inhibition of pyrogallol autooxidation in the presence of EDTA at pH 8.2. Briefly, a weighed amount of hepatic 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 [36] with some modifications as adopted by Chattopadhyay et al. [37]. A weighed amount of hepatic 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.

Glutathione reductase assay was carried out according to the method of Krohne-Ehrich et al. [38]. 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 activity was measured according to the method of Paglia and Valentine [39] with some modifications [40]. 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 activity was measured sphectrophotometrically according to Habig et al. [41] 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**

**($O_2^{\cdot-}$) by determining the activities of the pro-oxidant enzymes xanthine oxidase and xanthine dehydrogenase**

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 [42]. 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 [26] with some modifications. 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. [30]. 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 were 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 CuLE (100mg/kg body weight) was administered orally to the rats of CuLE and Cd+CuLE 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 the 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 activity was measured spectrophotometrically according to the method of Chretien et al. [43] 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 activity was measured according to the method of Duncan et al. [44] 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 activity was measured spectrophotometrically according to the method of Duncan et al. [44] 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 activity was measured spectrophotometrically by following the reduction of potassium ferricyanide ($K_3FeCN_6$) at 420nm according to the method of Veeger et al. [45] 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 [46]. 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 [46]. 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.

**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**

**Assessment of DPPH radical scavenging activity of aqueous CuLE** *in vitro*

The DPPH radical scavenging activity of aqueous CuLE is shown in Figure 1. The extract exhibited over 50% scavenging activity of DPPH radical at a concentration of 1.12 µg/mL.

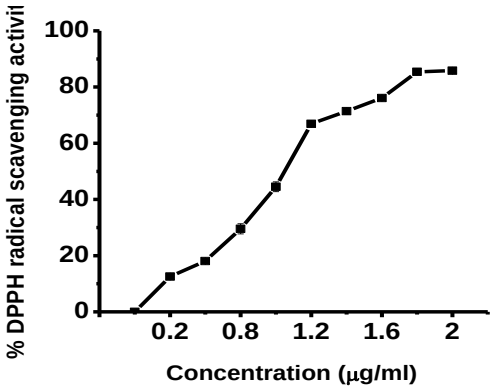



**Fig. 1: Figure shows DPPH radical scavenging activity of aqueous curry leaf extract.**

**Status of cadmium concentration of the rat hepatic tissue**

Figure 2 reveals a highly significant increase in the rat hepatic tissue concentration of Cd following treatment of rats with this heavy metal. The concentration of cadmium in the hepatic tissue was partially but significantly reduced (35.40 %, ** P ≤ 0.001 vs Cd-treated group) when the animals were pre-treated with 100 mg / kg bw (fed orally) of the aqueous CuLE indicating that the extract may possess cadmium chelating activity.

**Biomarkers of hepatic damage: SGPT, LDH, LDH5, ALP**

Table 1 illustrates the level of activity of serum specific markers, namely SGPT, LDH5, total LDH and ALP which were found to be significantly higher in Cd-treated group of rats when compared to control (1.60, 2.15, 1.56 and 2.02 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 CuLE (29.32%, 51.16%, 33.59% and 41.52 % 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 CuLE do possess the capability to provide protection against Cd-induced hepatic damage.

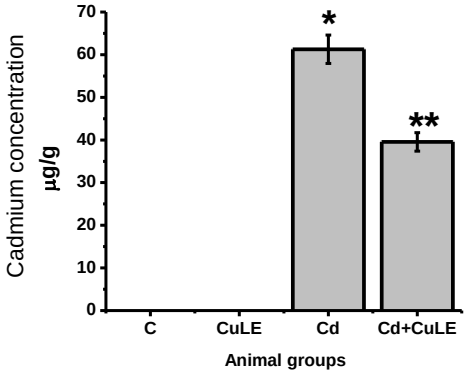



**Fig. 2: Figure shows cadmium concentration of hepatic tissue in presence and absence of Curry leaf extract. C= Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected sc; CuLE = 100 mg/kg bw of *Murraya koenigii*; Cd+CuLE = 0.44 mg/kg bw $CdCl_2$ injected sc + 100 mg/kg bw of *Murraya koenigii* . The values are expressed as Mean ± S.E.M.**

**Table 1: Table shows effect of aqueous Curry leaf extract against cadmium induced changes in serum enzymes of liver function in rats.**

| Treatment | SGPT (IU/L) | Total LDH (IU/L) | LDH5 (IU/L) | ALP (KA units/dL) |
|---|---|---|---|---|
| C | 7.70±0.166 | 0.84±0.040 | 0.20±0.008 | 19.86±0.309 |
| Cd | 12.31±0.253* | 1.31±0.023* | 0.43±0.012 * | 40.17±1.444 * |
| Cd+CuLE | 8.70±0.038** | 0.87±0.032 ** | 0.21±0.028 ** | 23.49±0.195** |
| CuLE | 6.98±0.226 | 0.83±0.022 | 0.18±0.017 | 21.57±0.471 |

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

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

**Histopathology and morphometry**

**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. This damage was found to be protected completely when the rats were pre-treated with aqueous CuLE 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 (Figure 3). Histomorphometric data of liver tissue sections are presented in Table 2. Hepatic degeneration was evaluated by counting 100 cells under 200x magnifications. Significant increase in sinusoidal, central vein and portal triad diameters as well as hepatic degeneration was observed in cadmium exposed rats. Pre-treatment of rats with aqueous CuLE at a dose of 100 mg/kg bw significantly reduced sinusoidal, central vein and portal triad diameters as well as hepatic degeneration.

**Studies on collagen content and quantification of fibrosis**

The figure 4A (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 CuLE, the tissue fibrosis was found to be almost completely protected. Figure 4B 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 4C shows that the percentage volume of total collagen content was also increased significantly (3.99 folds, * P ≤ 0.001 vs. control, n=4) in cadmium-treated rat liver which was decreased to the normal level when the rats were pre-treated with aqueous CuLE indicating again that the extract is capable of preventing cadmium-induced hepatic tissue damage.

**Biomarkers of oxidative damage**

Figure 5A reveals a significantly higher hepatic tissue LPO level following treatment of rats with cadmium (2.33 folds increase, ***P ≤ 0.001** vs. control). This elevated level of lipid peroxidation products was found to be decreased significantly (54.21% from Cd-treated group, **P ≤ 0.001; the LPO level reaching almost control level) when the rats were pre-treated with aqueous CuLE, orally one hour before cadmium treatment, indicating the ability of this aqueous extract to protect the rat hepatic tissue against oxidative stress-induced changes due to cadmium.

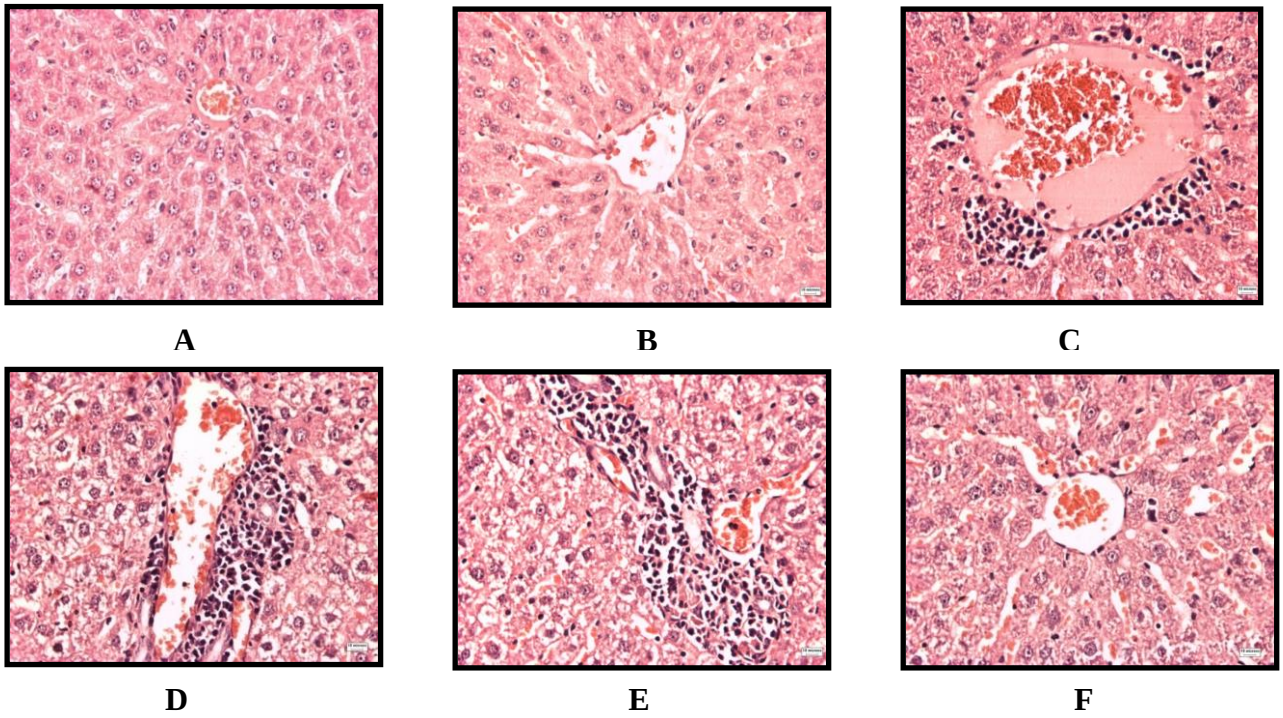



**Fig. 3: Figure shows histology of liver tissue on treatment with Cd, CuLE, Cd-CuLE; H&E staining (magnification 200x). (A) Control liver section with normal hepatocytes. (B) CuLE treated group (positive control). (C,D,E) Liver tissue sections of Cd-treated rats. (C) Demonstrates hepatocyte necrosis (D) Demonstrates dilation and congestion of portal veins (E) Demonstrates portal and periportal lymphocyte infiltration along with periportal hepatocyte necrosis. (F) Cd-CuLE treated group. Demonstrates near normal hepatocytes, mild sinusoidal dilatation around central vein when compared to cadmium treated liver.**

The PCO content of the rat hepatic tissue was found to be significantly increased following treatment of rats with cadmium (1.90 folds increase, *P ≤ 0.001 vs. control). This elevated level of protein oxidation was found to be decreased (57.89%, **P ≤ 0.001 vs. cd-treated group), when the rats were pre-treated with aqueous CuLE, orally one hour before cadmium treatment. The results indicate that the aqueous CuLE appears to have the potential to provide protection against Cd-induced oxidative stress in rat liver (Figure 5B).

Figure 5C reveals that Cd-induced decrease (57.24 % decrease, ***P ≤ 0.001** vs. control) in the level of reduced GSH in rat hepatic tissue was found to be almost completely protected when the animals were pre-treated with aqueous CuLE at a dose of 100 mg / kg body weight, fed orally. However, the aqueous extract by itself has no effect on the tissue GSH level (positive control). The results indicate the protective ability of the aqueous CuLE against Cd-induced oxidative stress in rat liver.

The level of rat hepatic non-enzymatic antioxidant total sulfhydryl group (TSH) in the control and the experimental rats are shown in the figure 3D. A significant depletion of the tissue TSH (37.13 % decrease, ***P ≤ 0.001** vs. control) was noticed in rats treated with cadmium when compared to control. Pre-treatment of rats with the aqueous CuLE significantly protected the tissue TSH level from getting decreased (Figure 5D).

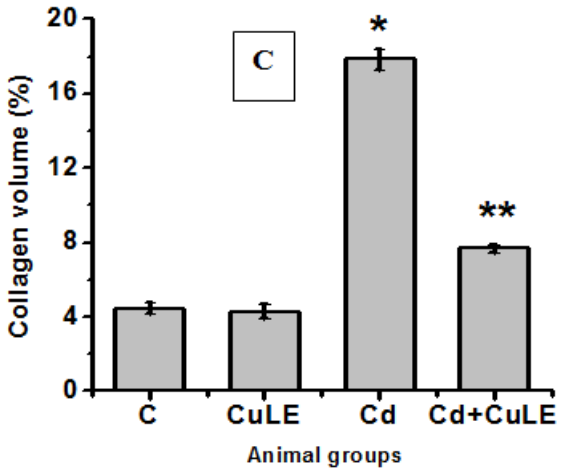



**Fig. 4: Figure shows protective effect of aqueous Curry leaf extract against cadmium-induced changes in the rat hepatic tissue morphology, 200x magnifications. Sirius red stain [A], Red color stretches are collagen depositions. and confocal images of A [B], Arrow heads indicate collagen fibres in A and B. Collagen volume (%) of the confocal images [C]: C= Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected sc; CuLE = 100 mg/kg bw of aqueous Curry leaf extract; Cd+CuLE = 0.44 mg/kg bw $CdCl_2$ injected sc + 100 mg/kg bw of aqueous Curry leaf extract. *P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.**

**Table 2: Table shows histomorphometric analysis of liver in control, M (100 mg/kg), Cd and Cd+M (100 mg/kg) groups.**

| Groups | Sinusoidal diameter (µM) | Portal triad diameter (µM) | Central vein diameter (µM) | Hepatocyte degenerations (in 100 cells) |
|---|---|---|---|---|
| C | 4.18±0.41 | 74.78±4.54 | 32.21±6.55 | 8.4 |
| Cd | 8.51±0.65 * | 119.54±23.6* | 41.52±0.62* | 18.76 * |
| Cd+CuLE | 4.67±0.31** | 78.57±6.94** | 33.32±5.96 | 11.45 ** |
| CuLE | 4.11±0.45 | 74.46±3.23 | 31.66±5.48 | 8.23 |

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

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

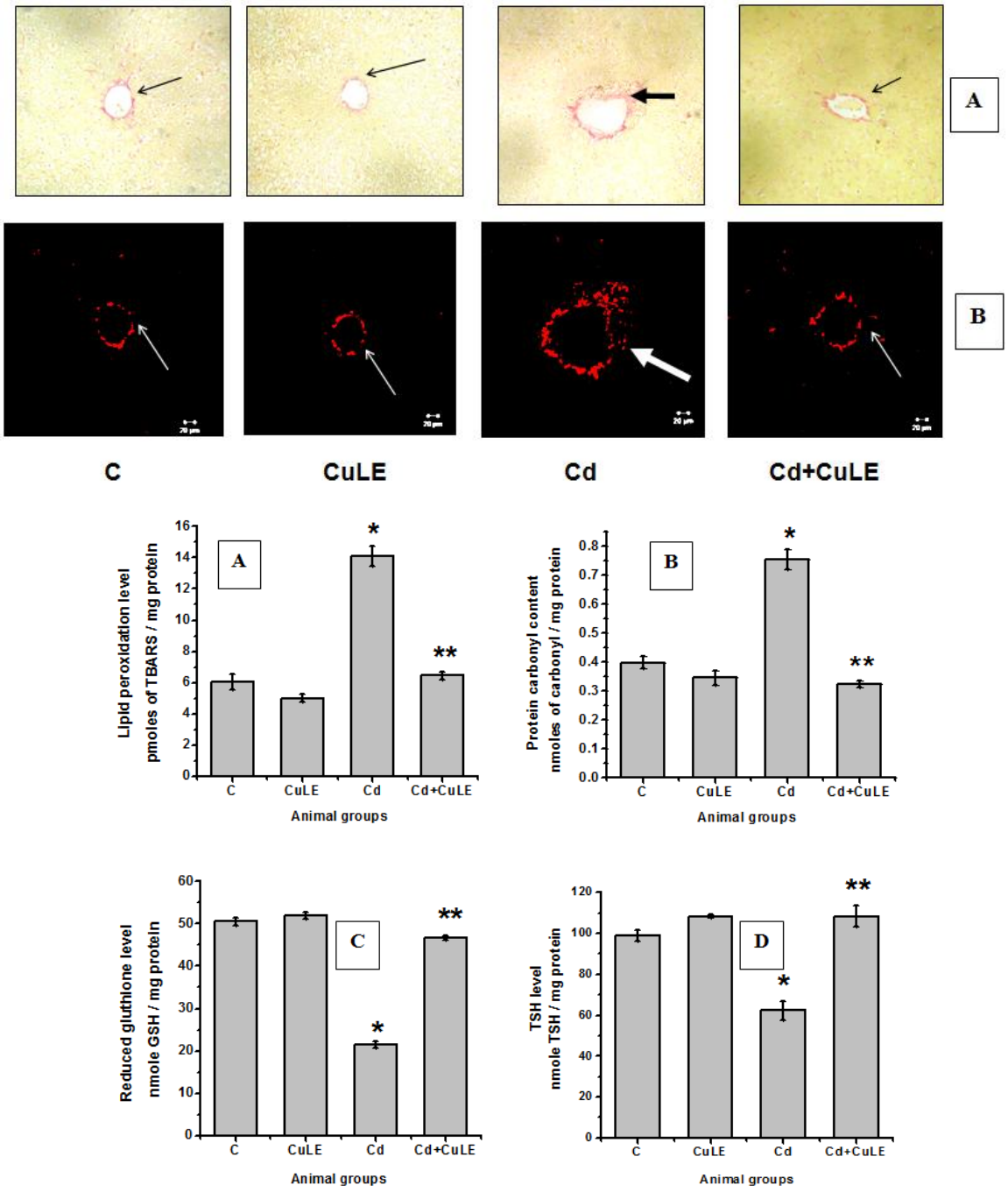



**Fig. 5: Figure shows protective effect of aqueous Curry leaf extract against cadmium-induced increase in the level of lipid peroxidation [A] and protein carbonyl content of rat hepatic tissue[B] and decrease in reduced glutathione level [C] and total sulfhydryl level [D]. . C; Control, injected with vehicle; Cd ; 0.44 mg/kg bw $CdCl_2$ injected sc; CuLE = 100 mg/kg bw of aqueous Curry leaf extract; Cd+CuLE = 0.44 mg/kg bw $CdCl_2$ injected sc + 100 mg/kg bw of aqueous Curry leaf extract. The values are expressed as mean ± S.E.M.; *P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.**

**Status of the antioxidant enzymes**

Table 3 reveals that treatment of rats with cadmium at the present dose caused increase of activities of both cytosolic Cu-Zn SOD and mitochondrial Mn-SOD (1.72 folds and 2.14 folds increase respectively, ***P ≤ 0.001** vs. control). When the rats were pre-treated with aqueous CuLE, the activities of these enzymes were found to be significantly protected from being increased. However, the extract by itself, was found to have no significant effect on the activity of both the enzymes. The results strongly indicate that aqueous CuLE seems to possess antioxidant activity. Table 3 also illustrates the significant increase in GST activity and glutathione peroxidase activity (41.24 % and 1.37 folds increase respectively, ***P ≤ 0.001** vs. control) following exposure of rats to cadmium. However, pre-treatment of rats with the present dose of aqueous CuLE significantly protected the GST and GPx activity (29.38 % and 29.38 decrease respectively, ****P ≤ 0.001** vs. cadmium) from getting altered. Table 3 further reveals a highly significant decrease in the activity of GR and catalase (53.57 % and 28.43 % decrease respectively, ***P ≤ 0.001** vs. control) following treatment of rats with cadmium at the present dose. The GR and catalase activities were protected from getting decreased when the rats were pre-treated with aqueous CuLE at the dose of 100 mg / kg bw (fed orally). The aqueous extract, by itself, has no effect on the activity of these enzymes.

**Table 3: Table shows effect of aqueous Curry leaf extract against cadmium induced changes in various antioxidant enzymes in hepatic tissue of rat.**

| 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 | 2.43±0.078 | 0.84±0.019 | 153.02±4.11 | 0.40±0.006 | 0.84±0.018 | 0.39±0.004 |
| CuLE | 2.30±0.052 | 0.74±0.044 | 154.27±3.67 | 0.30±0.009 | 0.82±0.018 | 0.31±0.004 |
| Cd | 4.19±0.249 * | 1.79±0.080 * | 109.51±7.7* | 0.55±0.003 * | 0.39±0.011 * | 0.55±0.003 * |
| Cd+CuLE | 2.87±0.17** | 0.79±0.028** | 148.49±8.41** | 0.39±0.017** | 0.80±0.028** | 0.35±0.017** |

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

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

**Status of the activity of the hepatic pyruvate dehydrogenase and some of the mitochondrial Kreb's cycle enzymes**

**Table 4** reveals that treatment of the rats with cadmium inhibits the activities of rat hepatic pyruvate dehydrogenase, isocitrate dehydrogenase, alpha keto glutarate dehydrogenase and succinate dehydrogenase (63.09 %, 61.11 %, 48.97% and 52.75% decrease respectively, *P ≤ 0.001 vs. control). When the rats were pre-treated with 100 mg / kg body weight of the aqueous CuLE, the activity of these enzymes, however, was found to be significantly protected compared to the activity observed in the Cd-treated group (2.61 folds, 2.12 folds, 1.62 folds, 88.37 % increase respectively, **P ≤ 0.001 vs. Cd-treated group).

**Status of the activity of the hepatic mitochondrial respiratory chain enzymes**

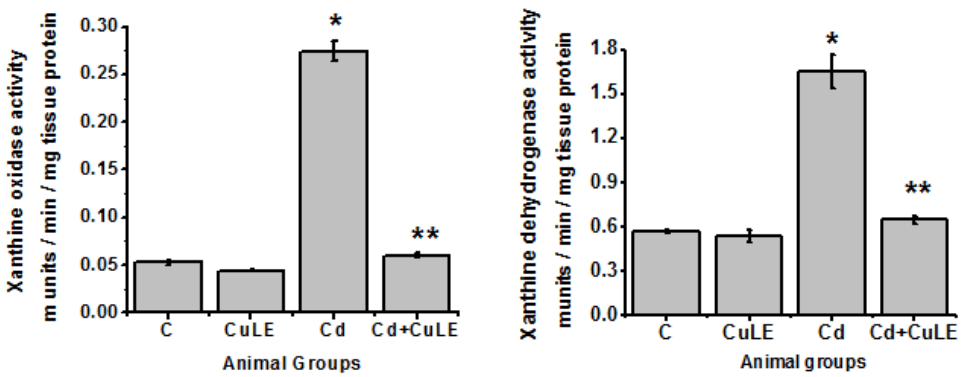
Treatment of rats with cadmium at the present dose inhibits NADH cytochrome c reductase activity (60.09 % decrease, *P≤ 0.001 vs. control) and cytochrome oxidase activity (42.86% decrease, * P ≤ 0.001 vs. control). The activity of both the enzymes were found to be protected significantly when the rats were pre-treated with 100 mg / kg bw of the aqueous CuLE (2.72 folds increase in NADH cytochrome c reductase activity, **P ≤ 0.001 vs. Cd-treated group and 40.28 % increase in cytochrome oxidase activity, **P ≤ 0.001 vs. Cd-treated group) (**Table 4**).

**Table 4: Table shows effect of aqueous Curry leaf extract against cadmium induced changes in the activities of some mitochondrial respiratory chain enzymes and enzymes of electron transport chain in hepatic tissue of rat.**

| 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 | 7.77±0.410 | 1.81±0.056 | 3.27±0.092 | 1.06±0.004 | 11.79±0.522 | 0.21±0.004 |
| CuLE | 8.17±0.208 | 1.78±0.126 | 3.37±0.067 | 1.11±0.018 | 12.12±0.289 | 0.20±0.004 |
| Cd | 2.87±0.228* | 0.74±0.060* | 1.67±0.076* | 0.53±0.023* | 4.47±0.579* | 0.12±0.01* |
| Cd+CuLE | 7.48±0.413** | 1.57±0.030** | 2.70±0.093** | 1.01±0.009** | 12.14±0.514** | 0.17±0.005** |

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

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



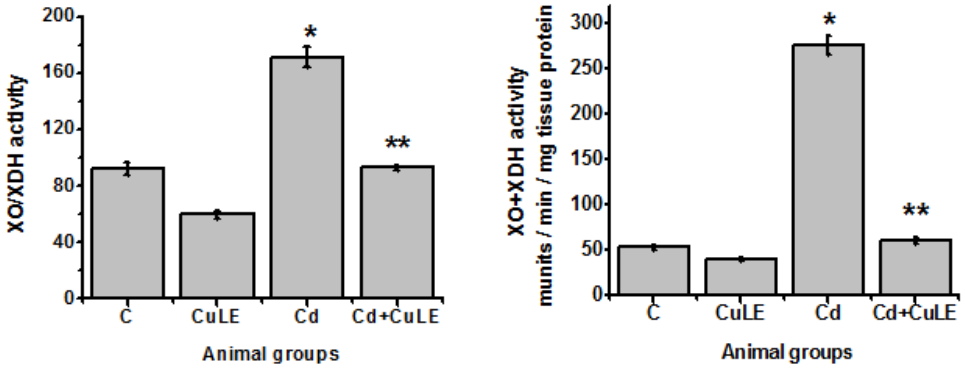



**Fig. 6: Figure shows protective effect of aqueous Curry leaf extract against cadmium induced increase in rat hepatic xanthine oxidase [A], xanthine dehydrogenase activities [B], the total activity of XO and XDH [C] and the ratio of XO/XDH activities [D] C= Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected sc; CuLE = 100 mg/kg bw of *Murraya koenigii*; Cd+CuLE = 0.44 mg/kg bw $CdCl_2$ injected sc + 100 mg/kg bw of *Murraya koenigii*. The values are expressed as Mean ± S.E.M.; *P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.**

Figure 7 illustrates that treatment of rats with the present dose of cadmium caused nearly 3 fold increase of endogenous generation of •OH compared to control (*P ≤ 0.001 vs. control). However, pre-treatment of rats with aqueous CuLE decreased the cadmium induced •OH formation to near basal levels. The CuLE by itself, however, did not have a significant effect on the basal level of endogenous hydroxyl radicals.

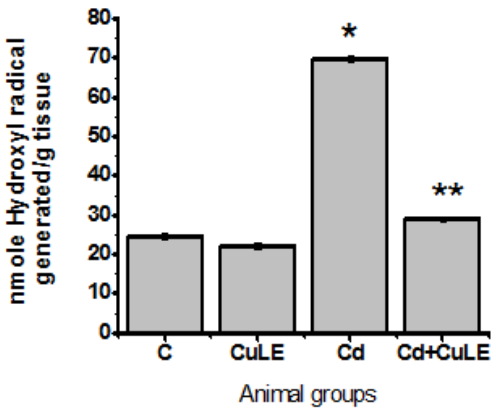



**Fig. 7: Figure shows protective effect of aqueous Curry leaf extract against cadmium induced increase in hydroxyl radical generation *in vivo* in rat liver tissue C= Control, injected with vehicle; Cd = 0.44 mg/kg bw $CdCl_2$ injected sc; CuLE = 100 mg/kg bw of *Murraya koenigii*; Cd+CuLE = 0.44 mg/kg bw $CdCl_2$ injected sc + 100 mg/kg bw of *Murraya koenigii*. The values are expressed as Mean ± S.E.M.; *P ≤ 0.001 vs. control. **P ≤ 0.001 vs. cadmium treated group.**

The aqueous CuLE seems to possess antioxidant activity which may be exerted through the scavenging of the reactive oxygen species (direct antioxidant activity) generated *in vivo* following treatment of rats with cadmium and, via protecting the activities of the antioxidant enzymes from being altered and or influencing the biosynthesis of the cellular antioxidants (indirect antioxidant activity). Thus, the results of the present studies strongly indicate that this aqueous CuLE appears to have the ability to provide protection against cadmium-induced oxidative stress in the hepatic tissue of the experimental rats.

## DISCUSSION

The present studies describe the protective effect of aqueous CuLE against cadmium-induced hepatic tissue damage in experimental rats.

In the present studies, cadmium accumulates in considerable amounts in rat liver. The liver is a major site for the biotransformation, accumulation and excretion of exogenous chemicals. The liver is a highly perfused organ and a seat of all enzymatic reactions including production of metal binding useful substances like metallothionein; the choice of the body's metal accumulation site becomes liver. Cadmium accumulation in liver was previously reported by Stohs and Bagchi [49] who considered that the accumulation of the metal in the organ to be an important mechanism leading to hepatic damage and dysfunction. When liver fails to detoxify the harmful effects by simply forming Cd-metallothionein complex or other detoxifying processes, unbound cadmium reaches other organs, where the active metal again binds to membrane or –SH containing groups [9]. Our results show that the pre-treatment of experimental rats with aqueous CuLE shows significant difference in cadmium accumulation in the liver which signifies that the leaf extract protects against cadmium induced damage not only by antioxidative property but also by partially chelating the metal ion, rendering it inactive.

In our present studies, the serum levels of these organ specific marker enzymes were increased indicating hepatic damage following sub-chronic exposure to cadmium. The present dose of cadmium (i.e., 0.44 mg/kg bw every alternate day, for a period of fifteen days) not only produced significant changes in the parameters studied in comparison to control animals but also there was no animal mortality during the entire treatment period. High levels of activities of serum glutamate pyruvate transaminase (SGPT), total lactate dehydrogenase (LDH), lactate dehydrogenase 5 (LDH 5) isoenzyme and alkaline phosphatase (ALP) critically point toward hepatic damage. Leakage of large quantities of intracellular or membrane enzymes into the blood stream indicates a loss of functional integrity of membrane architecture [50]. Oral administration of aqueous CuLE at the present dose (i. e., 100 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 hepatic tissue. This hepatoprotection might have been exerted through some phytochemical(s)/ phytonutrient(s) present in the extract.

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. However, the hepatic tissue sections from the rats pre-treated with aqueous CuLE maintained normal lobular architecture. The results indicate the ability of the aqueous extract to provide protection against cadmium-induced tissue injury. 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 CuLE was found to prevent the deposition of the collagen 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 ($p<0.001$, $n=6$).

In our experiments, the rats exposed to cadmium had increased peroxidation of membrane lipids of liver 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 [37]. Cadmium may induce oxidative stress by enhancing LPO and thus may cause damage to cellular components. The aqueous CuLE is found to be effective in decreasing the lipid peroxidation level of liver tissue. Lipid peroxidation is self perpetuating unless terminated by chain breaking antioxidants. The results indicate that the aqueous CuLE 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 [51]. 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 CuLE seems to possess an ability to reduce the protein carbonyl content of the hepatic tissue possibly through scavenging the reactive oxygen species or by chelating the heavy metal or by both.

Cadmium is a redox-stable metal; therefore, radical production by Cd must be mediated through some indirect mechanism(s). One proposed mechanism by which Cd may generate free radicals is disruption of the cellular antioxidant defense systems. Reduced glutathione (GSH) is an important antioxidant, participating in enzymatic and non-enzymatic detoxification of many oxidative toxicants. It is worthwhile to investigate whether the cadmium-induced elevation of GSH concentration is associated with alterations in antioxidant enzyme activities [52]. GSH is abundant in the liver and is thought to be the first line of defense against Cd-induced hepatotoxicity as Cd binds tightly to thiol groups [11]. Metabolites containing thiol groups have a strong antioxidant capacity through their ability to oxidize and form disulfide bridges that in their turn can be recycled to the reduced state. Glutathione is a primary intracellular antioxidant and conjugating agent that accounts for up to 90% of the total low molecular weight cellular thiols. Cadmium shows a high affinity for GSH. Glutathione acts by scavenging Cd to prevent its interaction with critical cellular targets [3]. Both GSH and total sulfhydryl group (TSH) 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 hepatic 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 liver. However, pre-treatment of rats with the aqueous CuLE prevented the GSH depletion in the liver tissue. 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.

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. Glutathione is the predominant defense against reactive oxygen species (ROS), which are reduced by GSH in the presence of GSH peroxidase. As a result, 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 [53]. The GSH redox cycle consist of GSH, GPx, GR and GST, which are the major components of the antioxidant defense system. Coordinated activities of these enzymes maintain intracellular thiol status. GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferases (GST) and glutathione peroxidases (GPx). The GST has an important role in detoxification of the lipid hydroperoxides and toxic electrofiles by catalyzing the conjugation of these electrofiles with GSH, thus, contributing to the protection of the cell integrity [54]. Our data shows that cadmium administration elevates the activity of GST in the rat hepatic tissue. The role of glutathione reductase (GR) is the reduction of oxidized glutathione (GSSG) to biologically active GSH using NADPH as a cofactor. In the present investigation, the activities of the glutathione dependent enzyme, GR, was reduced significantly in the liver of experimental rats administered with cadmium. The formation of cadmium sulfhydryl complex with SH groups of the enzyme might lead to a decrease in the activities of GR and depletion of GSH level [55]. In our studies, the GPx activity is also increased in rat liver. GPx detoxifies peroxides with GSH acting as an electron donor in the reduction reaction, producing GSSG as an end product. This indicates that GSH pathway is disturbed by cadmium. Cadmium decreases intracellular GSH level not only by binding to its thiol group, but also by decreasing the activity of GR thereby GSH formation from GSSG is reduced. Cadmium also stimulates the activity of GST which further aids in decreasing the level of GSH in the organ. When sufficient amount of thiol decreases in the organ, the metal binds to other relevant biomolecules present in sub-cellular membranes, ER, mitochondria or inside nucleus causing damage.

Antioxidant defense system protects the aerobic organisms from the deleterious effects of reactive oxygen metabolites [50]. In stress conditions, normal capacities of these mechanisms are insufficient, triggering cells to increase and expand their antioxidative network. SOD, catalase, and glutathione peroxidase are three primary enzymes, involved in direct elimination of active oxygen species (hydroxyl radical, superoxide anion free radical and hydrogen peroxide). Catalase and glutathione peroxidase catalyze the conversion of hydrogen peroxide to water, thus catalase and GPx 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 [56]. Our results demonstrated an increase in SOD activity with a concomitant decrease in the activity of catalase in the cadmium-treated rats. This indicates that $H_2O_2$ accumulated in the hepatic tissue, contributes partly to the damage caused by cadmium to this organ. Geret et al. [57] have also observed a dramatic increase in cytosolic SOD activity upon prolonged exposure of the gills of the clam, *Ruditapes decussates* to Cd. Ikediobi et al. [58] observed increases in SOD activity in rat liver cells treated with cadmium and explained it as a response to accumulation of ROS (particularly $H_2O_2$ and $O_2^{\cdot-}$) in the cytosol induced by Cd. According to Ikediobi et al. [58], cells have the mechanism to sense ROS and induce specific responses. Time-dependent leakage of MnSOD from the mitochondria into the cytosol and a possible non-specific interaction with Cd provide rational explanations for a dramatic increase in SOD activity following prolonged exposure of cells to Cd. Catalase contains iron in its active site. The decreased activity of catalase may be due to the direct binding of the metal to the active site of the enzymes, or cadmium may decrease iron availability to the enzyme, or due to the increased usage of the enzyme in scavenging free radicals induced by the metal. GPx is a Se dependent oxido-reductase which protects tissues from toxin induced oxidative damage. Increase in GPx activity could be a protective mechanism of the tissue to remove accumulated $H_2O_2$. Administration of aqueous CuLE protected the activities of SOD, CAT and GPx from getting altered in the hepatic tissue of cadmium-treated rats. The protection afforded by the aqueous CuLE may be due to the ability of the extract to reduce the accumulation of free radicals generated following cadmium treatment. The phyto-constituents in the extract may scavenge the ROS or may inhibit their formation. They may also reduce the levels of the pro-oxidants by up-regulation of the expression of the antioxidant enzymes and needs further investigation.

Xanthine oxidase (XO) is an important source of free radical generation. Xanthine oxidoreductase, under normal conditions, exists in dehyrogenase 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 [59]. Thus, XO in oxidative stress conditions may play an important role in contributing free radical mediated damage. A significant increase in the activities of xanthine oxidase and xanthine dehydrogenase (XDH) as well as an increase in XO/XDH ratio in the tissue confirms generation of superoxide anion free radical following cadmium treatment in our experiments. When the rats were pre-treated with aqueous CuLE in our experiment, at the present dose, the activities of these enzymes were protected from being altered and found comparable to the activities observed in the control animals, indicating again toward the antioxidant potential of the extract.

Treatment of rats with cadmium at the present dose and duration generates copious amounts of hydroxyl radical. Toxicity of superoxide anion radical and hydrogen peroxide could involve the formation of much more reactive hydroxyl radical [60]. Hydroxyl radical is mainly generated via Fenton or Haber-Weiss reaction. Since cadmium does not directly participate in Fenton reaction, thus hydroxyl radical formation *in vivo* may be via an indirect pathway. When the rats were pre-treated with aqueous CuLE, at the present dose, hydroxyl radical generation was found to be decreased and comparable to the level observed in the control animals, indicating again toward the antioxidant potential of the extract.

Mitochondria are the major organelles that produce reactive oxygen species (ROS) and the main target of ROS-induced damage as observed in various pathological states. Cadmium penetration of the mitochondria has been identified with the outcome of significant inhibition of mitochondrial function, increased ROS production, and eventual apoptotic cell death [3]. 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 CuLE. 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 [61, 62]. Cadmium has also been reported to decrease the respiratory activity [63]. Heavy metals are also known to affect respiratory chain complexes and there is clear cut substrate specificity [64]. Isocitrate dehydrogenase is a major NADPH producer in the mitochondria and thus plays a key role in cellular defense against oxidative stress-induced damage. Decreased activity of isocitrate dehydrogenase indicates marked elevation in ROS generation, DNA fragmentation, lipid peroxidation, and concurrent mitochondrial damage with a reduction in ATP level [65]. 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 [48]. 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 [66]. Pyruvate dehydrogenase (PDH) has been demonstrated to be sensitive to insults that induce oxygen free radicals [67], which might be a cause of decrease of the enzyme activity.

Regarding effects on the mitochondrial electron transport chain, heavy metals in mitochondria most likely inhibit the activity of complexes II and III more than that of the other complexes. The principal site of ROS production by cadmium seems to reside in complex III [7]. Earlier researchers have reported that cadmium markedly inhibits uncoupler-stimulated oxidation on various NADH-linked substrates as well as that of succinate [68]. 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 $O2\bullet-$. An additional source of free radicals is located on the outer mitochondrial membrane, where the oxidative deamination of amines by monoamine oxidases is associated with reduction of $O_2$ to $H_2O_2$. 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 [69]. 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 CuLE. This strongly indicates that the aqueous CuLE 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.

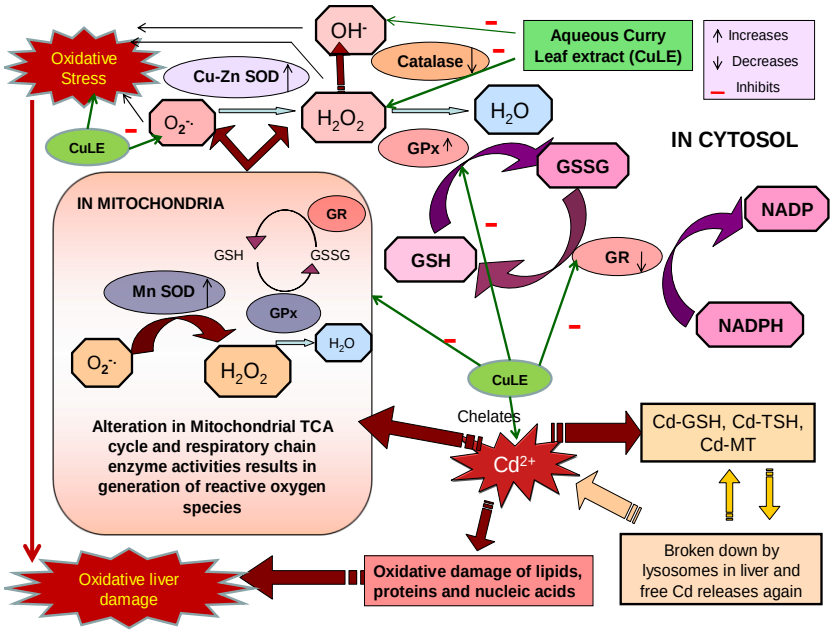



**Fig. 8: Figure shows possible mechanism of protection by curry leaf aqueous extract against cadmium induced oxidative stress in rat liver.**

Herbal plants are known to contain a variety of antioxidants. Quantitative analysis of aqueous CuLE reveals the presence of various phytochemical antioxidants, like polyphenols, flavonoids, tannins, alkaloids, etc [26]. In our experiments we found that aqueous CuLE was able to scavenge free radicals as evident from the percentage inhibition of DPPH radicals which indicates significant radical scavenging activity of the extract. 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 [70]. DPPH free radical-scavenging by antioxidants is due to their hydrogen-donating ability. In the present work, we found that the aqueous CuLE was effective in ameliorating cadmium induced hepatic damage. Recently, herbal medicines are being increasingly utilized to treat a wide variety of clinical diseases, including liver diseases [71] with relatively little knowledge regarding their modes of action. *In vitro* experiments reveal that the extracts of leaves of the Curry plant do possess radical scavenging activity [18]. Very recently, the aqueous extract and the isolates of Curry plant have been shown to reduce lipid peroxidation and decreased cellular damage thereby protecting the liver from ethanol-induced toxicity [72]. Ghosh et al. also showed that aqueous extract of curry leaves possesses hepato-protective potential tested *in vivo* against lead induced damage [73]. The results of these as well as our studies indicate that the aqueous CuLE contains antioxidant phytonutrients which seem highly effective and, in our experiments, probably functions synergistically *in vivo*, providing a promising prevention against oxidative damage.

The results of the present studies indicate that aqueous CuLE 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 (Figure 8). 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. As Curry leaves are part of a regular diet in India and many parts of the world since ancient times with no reported toxicity, it may also serve as a possible nutritional intervention and, the extract itself or the bio-active fractions obtained therefore may be used also as a future nutritional supplement to combat oxidative stress-induced tissue damage in the people exposed 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 CuLE 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 Curry leaf extract may be useful as a protective nutritional supplement with promising antioxidant potential to combat oxidative stress-induced tissue damages in the areas where humans are exposed to cadmium occupationally or environmentally.

## ACKNOWLEDGEMENT

This work is supported from the funds available to Dr. DB under a UGC Major Research Project, Govt. of India [F. No. 37-396/2009(SR)]. Elina Mitra is a Project Fellow under the UGC Major Research Project awarded to Dr. DB. AKG is a Senior Research Fellow (SRF) of the RFSMS program of UGC under University of Calcutta. DM is a Senior Research Fellow (SRF) of CSIR, Govt. of India. DG is a DST-INSPIRE Junior Research Fellow (JRF), Govt. of India. SBF is a University Research Fellow (URF) under UGC programme. Dr. AC is supported from the funds available to her from a UGC minor research project. Dr. SKP and Dr. SD are supported from the funds available to them from their respective institutes. The authors are also thankful to the Director, Indian Institute of Chemical Biology, Kolkata for extending their help to use their central instrumentation facility and Director, Bose institute, Kolkata for their assistance.

## REFERENCES

1. Goering PL, Waalkes MP, Klaassen CD. Toxicology of cadmium. In: Goyer RA, Cherian MG, eds. Handbook of experimental pharmacology: toxicology of metals. 1964. Springer-Verlag, New York: p. 189-214.
2. IARC (International Agency for Research on Cancer) Cadmium and cadmium compounds. IARC Monogr Eval Carcinog Risk Hum 1993; 58:119–237.
3. Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, et al. Cadmium stress: an oxidative challenge. Biometals 2010; 23:927-940.
4. Agency for Toxic Substances and Disease Registry (ATSDR) Agency for Toxic Substance and Disease Registry, U.S. Toxicological Profile for Cadmium, Department of Health and Humans Services, Public Health Service, Centers for Disease Control, Atlanta, GA. 2005.
5. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem 2005; 12:1161-208.
6. Szuster-Ciesielska A, Stachura A, Slotwinska M, Kaminska T, Sniezko R, Paduch R, Abramczyk D, Filar J, Kandefer-Szerszen M. The inhibitory effect of zinc on cadmium-induced cell apoptosis and reactive oxygen species (ROS) production in cell cultures. Toxicology 2000; 145:159–171.
7. Wang Y, Fang J, Leonard SS, Rao KMK. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic Biol Med 2004; 36:1434–1443.
8. Liu J, Kadiiska MB, Corton JC, Qu W, Waalkes MP, Liu Y, Klaassen CD. Acute cadmium exposure induces stress-related gene expression in wild-type and metallothionein-I/II null mice. Free Radic Biol Med 2002; 32:525–535.
9. Ercal N, Gurer-Orhan H, Aykin-Burns N. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr Top Med Chem 2001; 1:529-539.
10. Stohs SJ, Bagchi D, Hassoun E, Bagchi M. Oxidative mechanisms in the toxicity of chromium and cadmium ions. J Environ Pathol Toxicol Oncol 2001; 20:77-88.
11. Liu J, Qian SY, Guo Q, Jiang J, Waalkes MP, Mason RP, Kadiiska MB. Cadmium generates reactive oxygen and carbon-centered radical species in rats: insights from in vivo spin-trapping studies. Free Radic Biol Med 2008; 45:475–481.
12. Kayama F, Yoshida T, Elwell MR, Luster MI. Role of tumor necrosis factor-alpha in cadmium-induced hepatotoxicity. Toxicol Appl Pharmacol 1995; 131:224-34.
13. Rao YK, Lu SC, Liu BL, Tzeng YM. Enhanced production of an extracellular protease from Beauveria bassiana by optimization of cultivation processes. Biochem Eng J 2006; 28:s57–s66.
14. Desai SN, Patel DK, Devkar RV, Patel PV, Ramachandran AV. Hepatoprotective potential of polyphenol rich extract of Murraya koenigii L.:An in vivo study. Food Chem Toxicon 2012; 50:310-4.
15. Tachibana Y, Kikuzaki H, Lajis NH, Nakatani N. Antioxidative activity of carbazoles from Murraya koenigii leaves. J Agric Food Chem 2001; 49:5589-5594.
16. Khan BA, Abraham A, Leelamma S. Haematological & histological studies after curry leaf (Murraya koenigii) & mustard (Brassica juncea) feeding in rats. Ind J Med Res 1995; 102:184–186.
17. Singh AP, Wilson T, Vorsa V, Luthria D, Freeman MR, Scott RM, Bilenker D, Shah S, Somasundaram S, Vorsa N. LC-MS-MS characterization of curry leaf flavonols and antioxidant activity. Food Chem 2011; 127:80–85.
18. Ningappa MB, Dinesha R, Srinivas L. Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (Murraya koenigii L.) extracts. Food Chem 2008; 106: 720-728.
19. Arulselvan P, Subramanian SP. Beneficial effects of *Murraya koenigii* leaves on antioxidant defense system and ultrastructural changes of pancreatic β-cell in experimental diabetes. Chem Biol Interact 2007; 165:155-164.
20. Tachibana Y, Kikuzaki H, Lajis NH, Nakatani N. Comparison of antioxidative properties of carbazole alkaloids from *Murraya koenigii* leaves. J Agric Food Chem 2003; 51:6461- 6467.
21. Zhang Q, Piao XL, Piao XS, Lu T, Wang D, Kim SW. Preventive effect of Coptis chinensis and berberine on intestinal injury in rats challenged with lipopolysaccharides. Food Chem Toxicol 2011; 49:61–69.
22. Ghosh J, Das J, Manna P, Sil PC. Protective effect of the fruits of Terminalia arjuna against cadmium-induced oxidant stress and

hepatic cell injury via MAPK activation and mitochondria dependent pathway. Food Chem 2010; 123:1062–1075.
23. Mitra E, Ghosh AK, Ghosh D, Mukherjee D, Chattopadhyay A, Dutta S, Pattari SK, Bandyopadhyay D. Protective effect of aqueous Curry leaf (Murraya koenigii) extract against cadmium-induced oxidative stress in rat heart. Food Chem Toxicol 2012; 50:1340-53.
24. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957; 28:56-63.
25. Kind PRN, King EJ. Estimation of plasma phosphatase by determination of hydrolysed phenol with anti-pyrine. J Clin Pathol 1954; 7:322–326.
26. Strittmatter CF. Studies on avian xanthine dehydrogenases: Properties and patterns of appearance during development. J Biol Chem 1965; 240:2557–2564.
27. Varcoe JS. Clinical Biochemistry: Techniques and Instrumentation-A practical approach, first ed. World Scientific Publishing Company; 2001. p. 40-43.
28. Roy SG, De P, Mukherjee D, Chander V, Konar A, Bandyopadhyay D, Bandyopadhyay A. Excess of glucocorticoid induces cardiac dysfunction via activating angiotensin II pathway. Cell Physiol Biochem 2009; 24:1–10.
29. Buege JA, Aust SG. Microsomal Lipid Peroxidation. Methods Enzymol 1978; 52:302-310.
30. Bandyopadhyay D, Ghosh G, Bandyopadhyay A, Reiter RJ. Melatonin protects against piroxicam-induced gastric ulceration. J Pineal Res 2004; 36:195–203.
31. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994; 233:346-357.
32. Sedlak J, Lindsay RH. Estimation of total, protein-bound, nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968; 25:192-205.
33. Martin Jr. JP, Daily M, Sugarman E. Negative and Positive assays of superoxide dismutase based on hematoxylin autooxidation. Arch Biochem Biophysi 1987; 255:329-326.
34. Mukherjee D, Roy SG, Bandyopadhyay A, Chatyopadhyay A, Basu A, Mitra E, Ghosh AK, Reiter R, Bandyopadhyay D. Melatonin protects against isoproterenol-induced myocardial injury in the rat: antioxidative mechanisms. J Pineal Res 2010; 48:251–262.
35. Markund S, Markund G. Involvement of the superoxide anion radical in the autoxidation of pyragallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47:469-474.
36. Beers Jr. RF, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952; 195:133-140.
37. Chattopadhyay A, Biswas S, Bandyopadhyay D, Sarkar C, Datta AG. Effect of isoproterenol on lipid peroxidation and antioxidant enzymes of myocardial tissue of mice and protection by quinidine. Mol Cell Biochem 2003; 245:43-49.
38. Krohne-Ehrich G, Schirmer RH, Untucht-Grau R. Glutathione reductase from human erythrocytes. Isolation of the enzyme and sequence analysis of the redox-active peptide. Eur J Biochem 1977; 80:65-71.
39. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70:158-169.
40. Chattopadhyay A, Choudhury TD, Bandyopadhyay D, Datta AG. Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. Biochem Pharmacol 2000; 59:419-425.
41. Habig WH, Pabst MJ, Jakoby WB. Glutathione -S- transferases, the first enzymatic step in mercapturic acid formation. J Biol Chem 1974; 249:7130 -7139.
42. Greenlee L, Handler P. Xanthine oxidase. IV. Influence of pH on substrate specificity. J Biol Chem 1964; 239:1090-1095.
43. Chretien D, Pourrier M, Bourgeron T, Séné M, Rötig A, Munnich A, Rustin P. An improved spectrophotometric assay of pyruvate dehydrogenase in lactate dehydrogenase contaminated mitochondrial preparations from human skeletal muscles. Clin Chim Acta 1995; 240:129-136.
44. Duncan MJ, Fraenkel DG. Alpha-ketoglutarate dehydrogenase mutant of Rhizobium meliloti. J Bacteriol 1979; 137:415-419.
45. Veeger C, DerVartanian DV, Zeylemaker WP. Succinate dehydrogenase. Methods Enzymol 1969; 13:81-90.
46. Goyal N, Srivastava VM. Oxidation and reduction of cytochrome c by mitochondrial enzymes of Setaria cervi. J Helminthol 1995; 69:13-17.
47. Lowry OH, Rosebrough NJ, Farr AL. Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-275.
48. Kil SI, Shin SW, Yeo HS, Lee YS, Park JW. Mitochondrial NADP-Dependent Isocitarte Dehydrogenase protects cadmium-induced apoptosis. Mol Pharmacol 2006; 70:1053-1061.
49. Stohs SJ, Bagchi D. Oxidative mechanism in the toxicity of metal ions. Free Radic Biol Med 1995; 18:321–336.
50. Renugadevi J, Prabu SM. Cadmium-induced hepatotoxicity in rats and the protective effect of naringenin. Exp Toxicol Pathol 2010; 62:171-181.
51. Gong P, Chen FX, Ma GF, Feng Y, Zhao Q, Wang R. Endomorphin 1 effectively protects cadmium chloride-induced hepatic damage in mice. Toxicology 2008; 251:35-44.
52. Hatcher El, Chen Y, Kang YJ. Cadmium resistance in A549 cells correlates with elevated glutathione content but not antioxidant enzymatic activities. Free Radic Biol Med 1995; 19:805-12.
53. Butterfielda DA, Castegnaa A, Pocernicha CB, Drakea J, Scapagninib G, Calabrese V. Nutritional approaches to combat oxidative stress in Alzheimer's disease. J Nutr Biochem 2002; 13:444–461.
54. Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene 2003; 22:7369-737.
55. Gerson RJ, Shaikh ZA. Difference in the uptake of cadmium and mercury by the rat hepatocyte primary cultures. Role of sulfhydryl carrier. Biochem Pharmacol 1984; 33:199-203.
56. McCord JM, Keele Jr. BB, Fridovich I. An enzyme based theory of obligate anaerobiosis: the physiological functions of superoxide dismutase. Proc Natl Acad Sci USA 1976; 68:1024-7.
57. Geret E, Serafim A, Barreira L, Bebianno MJ. Effect of cadmium on antioxidant enzyme activities and lipid peroxidation in the gills of the clam *Ruditapes decussates*. Biomarkers 2002; 7:242-56.
58. Ikediobo C, Badisa VL, Takem LA, Latinwo LM, West J. Response of antioxidant enzymes and redox metabolites to cadmium-induced oxidative stress in CRL-1439 normal rat liver cells. Int J of Mol Med 2004; 14:87-92.
59. Reghuvanshi R, Kaul A, Bhakuni P, Mishra A, Mishra MK. Xanthine oxidase as a marker of myocardial infarction. Ind J Clin Biochem 2007; 22:90-92.
60. Halliwell B, Gutteridge JM. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett 1992; 307:108-12.
61. Belyaeva EA, Glazunov VV, Nikitina ER, Korotkov SM. Bivalent metal ions modulate Cd2+ effects on isolated rat liver mitochondria. J Bioenerg Biomembr 2001; 33:303-318.
62. Belyaeva EA, Glazunov VV, Korotkov SM. Cyclosporin A-sensitive permeability transition pore is involved in Cd(2+)-induced dysfunction of isolated rat liver mitochondria: doubts no more. Arch Biochem Biophys 2002; 405:252-264.
63. Modi HR, Katyare SS. Effect of Treatment with Cadmium on Structure-Function Relationships in Rat Liver Mitochondria: Studies on Oxidative Energy Metabolism and Lipid/Phospholipids Profiles. J Membr Biol 2009; 232:47-57.
64. Belyaeva EA, Korotkov SM, Saris NE. In vitro modulation of heavy metal-induced rat liver mitochondria dysfunction: a comparison of copper and mercury with cadmium. J Trace Elem Med Biol 2010; 25S:S63-S73.
65. Jo SH, Son MK, Koh HJ, Lee SM, Song IH, Kim YO, et al. Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial NADP+-dependent isocitrate dehydrogenase. J Biol Chem 2001; 276:16168-76.
66. Tretter L, Adam-Vizi V. Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress. Philos Trans R Soc Lond B Biol Sci 2005; 360:2335-45.

67. Sheline CT, Wei L. Free radical-mediated neurotoxicity may be caused by inhibition of mitochondrial dehydrogenases in vitro and in vivo. Neuroscience 2006; 140:235-246.
68. Miccadei S, Floridi A. Sites of inhibition of mitochondrial electron transport by cadmium. Chem Biol Interact 1993; 89:159-67.
69. Gobe G, Crane D. Mitochondria, reactive oxygen species and cadmium toxicity in the kidney. Toxicol Lett 2010; 198:49-55.
70. Anusuya N, Sellamuthu M. Antioxidant and free radical scavenging potential of different solvent extracts of Indigifera tinctoria L. leaves. Int J Pharm Pharm Sci. 2013; 5 (1):142-147.
71. Mathews LA, Dhanyaraj D, Prathibhakumari P V, Prasad G. Hepatoprotective and antioxidant potential of Sphaeranthus indicus [Linn] on liver damage in Wistar rats. Int J Pharm Pharm Sci. 2012; 4 (3):222-225.
72. Sathaye S, Bagul Y, Gupta S, Kaur H, Redkar R. Hepatoprotective effects of aqueous leaf extract and crude isolates of Murraya koenigii in vitro ethanol-induced hepatotoxicity model. Exp Toxic Pathol 2011; 63:587-91.
73. Ghosh D, Firdaus SB, Mitra E, Dey M, Bandyopadhyay D. Protective effect of aqueous leaf extract of Murraya koenigii against lead induced oxidative stress in rat liver, heart and kidney: a dose response study. Asian J Pharm Clin Res. 2012; 5 (4):54-58.