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Aqueous bark extract of *Terminalia arjuna* protects against cadmium-induced hepatic and cardiac injuries in male Wistar rats through antioxidative mechanisms

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ABSTRACT

Cadmium (Cd) is one of the most ubiquitous toxic heavy metal in the environment. The present study was conducted to evaluate the protective role of aqueous bark extract of *Terminalia arjuna* (TA) against Cd induced oxidative damage in hepatic and cardiac tissues as the TA bark extract has folkloric medicinal use in the treatment of various hepatic and cardiac disorders. Male Wistar rats were divided into 4 groups. The control group was treated with normal saline as the vehicle; the second group orally administered with TA (20 mg/kg bw) daily for 15 days; the third group injected with Cd-acetate (0.44 mg/kg bw, s.c.) every alternate day for a period of 15 days; and the fourth group was administered with TA, 60 min prior to Cd treatment. The biomarkers of organ damage were significantly increased in the Cd treated groups. Besides, a significant alteration in the tissue levels of biomarkers of oxidative stress, the activities and the levels of antioxidant enzymes was observed following treatment with Cd. Additionally, some of the enzymes were found to be inhibited uncompetitively by Cd when tested in an *in vitro* system. Furthermore, evidence gathered from studies on the histological parameters and mitochondrial membrane potential in both the tissues argue in favour of the possible protective role of TA against Cd induced damage. Finally, gas chromatography–mass spectrometry revealed the presence of eight major bioactive phytochemicals in aqueous bark extract of TA having potent free radical scavenging property. The results indicate that the extract could protect hepatic and cardiac tissues against Cd-induced oxidative stress mediated damages through antioxidant mechanism(s).

1. Introduction

Cadmium has been increasingly recognized as a highly toxic environmental pollutant for several decades and is considered as one of the most dangerous heavy metal due to its non-degradable characteristic, toxicity and long half-life. Cd has been classified as a type 1 carcinogen by the International Agency for Research on Cancer of USA (IARC, 1993). It is mainly released from mining, smelting, burning of fossil fuels, refining of metals, sewage sludge, electronics manufacturing and cigarette smoking, eventually contaminating water, air and soil (Bauman et al., 1993). Extensive studies on the effects of cadmium toxicity on different aquatic organisms, such as *Photobacterium phosphoreum* (Qu et al., 2013b), *Daphnia magna* (Qu et al., 2013a; Wang et al., 2016) and *Carassius auratus* (Qu et al., 2013a) clearly indicated that Cd may imperil the growth and development of the aquatic life under any alkaline environment rather than in acidic ones. Notably, the problem becomes much severe since humans are maximally exposed to cadmium through inhalation and ingestion (Mead, 2010). After entering the body, Cd is transported and taken up by the cell through ionic mimicry because of its similar physico-chemical property to essential metals, such as iron (Fe), zinc (Zn), or calcium (Ca) (Vesey, 2010). Once the organs of the body are exposed to Cd, they are absorbed and irreversibly accumulated, particularly in liver and heart, since the biological half-life of cadmium is more than

Abbreviations: Cd, Cadmium; GSH, Reduced Glutathione; TSH, Total thiol content; Mn-SOD, Manganese-superoxide dismutase; Cu-Zn SOD, Copper Zinc superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxide; GR, Glutathione reductase; GSSG, Glutathione disulphide; $O_2^{\cdot-}$, Superoxide anion radical; H_2O_2, Hydrogen peroxide; $^{\cdot}OH$, Hydroxyl radical; H^+, Hydrogen ion; NADP, Nicotinamide adenine dinucleotide phosphate; NADPH, Nicotinamide adenine dinucleotide hydrogen phosphate; Δ;Ψ;m, Membrane potential; TA, aqueous bark extract of *Terminalia arjuna*

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20 years due to its low ratio of excretion (ATSDR, 2005). Among these organs, Cd is profoundly distributed in the hepatic tissue causing varying degrees of toxicity (Pope and Rall, 1995).

Based on available literature, Cd is found to be a hepatic as well as cardio-toxic heavy metal (Casalino et al., 2002; Menke et al., 2009; Maitra et al., 2012) that causes severe interruptions in their functionality. Acute hepatotoxicity may lead to ischemia due to endothelial cell injury, histopathological changes such as irregular architecture of parenchymatous tissue, congestion of blood vessel, disruption of mitochondria, enhanced depletion of glycogen and collagen fibre formation, cellular degeneration and necrosis; whereas chronic exposure to cadmium generally leads to dysfunction of renal tissue, anaemia, osteoporosis and bone fractures (EI-Sokkary et al., 2010; ATSDR, 2005). However, in context of cardiovascular system, Cd exposure was evidenced to be associated with numerous cardiovascular pathologies, such as hypertension (Varoni et al., 2003) and hemorrhagic injury (Nolan and Shaikh, 1986) in human. Several mammalian studies demonstrated a possible connection between cadmium exposure and development of atherosclerosis (Messner et al., 2009) where Cd may induce such disease severity by acting on the vascular endothelium (Kaji, 2004).

Being a non-Fenton metal, Cd indirectly induces oxidative stress by the generation of excessive reactive oxygen species (ROS). Several mammalian studies demonstrated the ability of Cd to replace Fe (a redox active metal), thereby increasing the availability of free Fe in the cell leading to induction of oxidative stress through the Fenton reaction (Casalino et al., 1997). Cd-induced ROS mainly interact with different intra-cellular macromolecules, resulting in oxidative deterioration of membrane lipids, proteins and DNA, and ultimately developing various pathological conditions in humans and other animals. Furthermore, being a thiol-attractive metal, Cd initially targets GSH- a first line defense antioxidant. Such interaction may lead to disruption of the intracellular pool of ROS scavengers promoting an imbalance in the cellular redox level causing an emergence of oxidative stress (Cuypers et al., 2010).

Several medicinal plant products rich in different kind of antioxidant phyto-constituents have been found to be beneficial against Cd induced oxidative stress mediated damages to hepatic and cardiac tissues (Dwivedi and Jauhar, 1997). Therefore, the current study was designed to evaluate the efficacy of the aqueous bark extract of *Terminalia arjuna* (TA) against Cd-induced oxidative stress mediated damages in liver and heart of male Wistar rats. *Terminalia arjuna* (commonly known as 'Arjuna'; family- Combretaceae) was first described by Ayurvedic sage Vagbhatta as a cardio-tonic and is being implied profoundly in India for thousands of years, since it contains several bioactive compounds which provide various health benefits, such as many specific phyto-constituents including arjunoglucoside, triterpene glycosides-arjunetin, arjunolitin, arjunoside, terminolitin; arjunolic acid, triterpinesaponins-arjunic acid, falvonoids-arjunone, arjungenin; arjunolone, and some non-specific phytocompounds including phytosyerols i.e., proanthocyanidines, β-sitosterol, and minerals-Ca, Mg, Zn, and Cu (Bhattacharjee et al., 2016; Dwivedi, 2007). It is abundantly distributed throughout Indo-sub Himalayan tract of Uttar Pradesh, Madhya Pradesh, West Bengal and Deccan regions. Clinical evaluation of various preparations from TA demonstrated its beneficial role in the treatment of coronary artery disease (Dwivedi and Jauhar, 1997) and severe refractory heart failure (Bhharani et al., 1995) in human as well as hyperlipidemia in rat (Taha et al., 2014) models. In addition, the TA bark powder was also found to possess diverse beneficial properties, such as-anti-carcinogenic in human and mice (Ahmad et al., 2014), antidyslipidaemic in rat (Chander et al., 2004), anti-hypocholesterolaemia in rabbit (Gupta et al., 2001), anti-inflammatory and antioxidant in rat (Chander et al., 2004) and antibacterial (Kumar et al., 2013) as well.

Since, Cd does not affect the cardiovascular system as its prime target, therefore the exact scenario of its toxicity on such organ remains a topic of argument. However, the controversy remains as scarcity of information regarding the underlying mode of action of Cd induced cardio as well as hepatotoxicity is felt. On the other hand, in Indian system of traditional medicine, aqueous bark extract of TA has been used by people, as there was no reported side effect of TA till date. Therefore, the present study is an attempt to demonstrate the beneficial role of aqueous bark extract of TA in protecting the rat liver and heart tissues against Cd-induced damage. Accordingly, an in vivo study was performed and the observed results were further supported by an *in vitro* study. The results reveal that the extract provides protection possibly through its antioxidant mechanism(s) which may be of future therapeutic relevance.

2. Materials and methods

2.1. Chemicals and reagents

Cadmium acetate [$(CH3COO)_2Cd.2H_2O$] was purchased from Qualigens Limited, Mumbai, India. Powder of bark of *Terminalia arjuna* was purchased from Herby House, Kolkata, India. All the other chemicals used in this study including the solvents, were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India; Qualigens Limited, Mumbai, India; SD Fine Chemicals, India; and Merck Limited, Delhi, India. JC-1(5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'tetraethylbenzimidazolcarbocyanine iodide) was purchased from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. Different primary [Cu-Zn SOD (ab16831); Mn-SOD (ab13534) and catalase (ab16731)] and secondary [Goat secondary Ab to Rabbit IgG] antibodies were purchased from Abcam Biotechnology Company, Abcam, USA.

2.2. Animals

Male Wistar rats, weighing 180–220 g were used for the present investigation. All rats were allowed to acclimate for at least 7 days and kept under standard laboratory conditions with water *ad libitum* and sufficient food throughout the investigation period. Rats were handled as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, with the approval of the Institutional Animal Ethics Committee [IAEC- IV/Proposal/DB-06/2014/Dt. 13/03/2014) of Department of Physiology, University of Calcutta.

2.3. Preparation of aqueous bark extract of TA

Aqueous bark extract of *Terminalia arjuna* was prepared with the help of the well established method of Saha et al. (2012) with some modifications (Bhattacharjee et al., 2016). Briefly, the bark powder of TA was soaked for 4 h in double distilled water (1:4) (5 g per 20 ml), filtered through a fine cotton cloth followed by a centrifugation (REMI cold-centrifuge) at 3000 rpm for 15 min at 4 °C. The supernatant was then filtered again through loin cloth. The filtrate was then concentrated by a vacuum evaporator (REMI, India) and the resulting concentrated material was stored in sterile polypropylene tubes and frozen at − 20 °C for further use. The yield of TA extract was approx. 10% (w/w).

2.4. Gas chromatography–mass spectroscopy analysis (GC-MS) of aqueous bark extract of TA

In order to identify the phyto-constituents present in the aqueous bark extract of TA, GC-MS profiling was performed at National Test House (NTH, Salt Lake City, Kolkata, West Bengal). GC-MS analysis was carried out on an Agilent Technologies 6890 N Network GC system and interfaced to Agilent Technologies 5973 Inert Mass Selective Detector employing the following conditions: column DB-1 ms fused silica

capillary column (30 mm x 0.25 mm I.D. x 0.10μMdf, composed of 100% Di-methylpolysiloxane), operating an electron impact mode at 70eV; helium(He) gas (99.99%) was used as carrier gas at a constant flow of 1 mL/min and an injection volume of 2 μl was employed (split ratio of 10:1). The injector, MS Source & MS Quadrapole temperature was maintained at 250 °C (constant) and ion source temperature at 280 °C (constant) and turbo speed of the pump was 100%. The oven temperature was monitored at 50 °C (isothermal for 2 min), with an increase of 10 °C/min to 100 °C (isothermal for 5 min), then 10 °C/min to 280 °C (isothermal for 5 min). Mass spectra were taken at 70eV; a scan interval of 0.5 s and fragments from 69 to 502Da.The mass spectral fragmentation pattern of the unknown compounds were identified (NBS75K.L Library ID) after comparing with that of available known mass spectral databases of NIST library (Paranthaman et al., 2012).

3. Experimental design

Male Wistar rats (n = 30) were used for two parallel experiments-an in vivo and an *in vitro* experiment.

3.1. In vivo experiment

Male Wistar rats (n = 24) were equally divided into four groups. The rats in the first group were subcutaneously injected with vehicle (normal saline [0.9%]) and served as the control (C) for the experimental groups. While rats in the second group (TA) were treated with aqueous bark extract of TA (20 mg/kg bw, orally once daily). Animals in the third group (Cd) were injected with cadmium (cadmium acetate) subcutaneously at a dose of 0.44 mg/kg bw every alternate day for a period of 15 days. Rats belonging to the fourth group (Cd + TA) were administered with TA (20 mg/kg bw, fed orally), 60 min prior to subcutaneous injection of cadmium acetate (0.44 mg/kg body weight). The experimental doses of TA and Cd employed in this study were the outcome of a dose-dependent experiment in our earlier study in the same species (Bhattacharjee et al., 2016).

3.1.1. Animal sacrifice, collection of blood and tissue samples

Following completion of the experiment, animals were kept fasted over night at room temperature and were sacrificed through cervical dislocation after subjecting them to mild ether anaesthesia. Prior to the collection of heart and liver, blood was collected from the animals by cardiac puncture for the preparation of serum. Thereafter, the abdomen and thoracic cavity was opened and the liver and heart were surgically extirpated and dipped immediately in ice-cold saline (0.9% NaCl). The weight of individual liver and heart were noted and the degree of hypertrophy in both the tissues were calculated in terms of liver weight to body weight ratio (mg/g) and heart weight to body weight ratio (mg/g), respectively. A small piece of each of the hepatic and cardiac tissue were immediately stored in appropriate fixative for microscopic studies. The rest of the tissues were stored in separate sterile vials at −20 °C for further analysis.

3.1.2. Determination of liver weight-to-body weight and heart-weight-to body weight ratio

Pre-experimental (i.e., before initiation of the treatment) and post-experimental (i.e., at the end of the treatment) body weights of all the groups of rats were measured. Following sacrifice, weight of liver and heart of individual animal was noted. Liver to body weight and heart to body weight ratio were also determined.

3.1.3. Estimation of the cadmium content in the liver and heart tissues

The cadmium content in the hepatic and cardiac tissues were (Hilliard and Smith, 1979) estimated (Capar et al., 1978) as per the instrument manual guidelines of the “Agilent Technologies AA280” Atomic Absorption Spectrophotometer with hydride vapor generator available at the National Test House (NTH), Kolkata. The content of cadmium was expressed as μg/g of hepatic and cardiac tissues.

3.1.4. Preparation of tissue homogenates

The liver and heart tissue homogenates (10%) were prepared freshly in 50 mM potassium phosphate buffer (pH 7.4), using a Potter–Elvehjem glass homogenizer (Bhattacharjee et al., 2016; Mukherjee et al., 2010). One portion of the homogenate was used for the measurement of enzyme activities and the other portion for Western blot analysis.

3.1.5. Biomarkers of organ damage: serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT)

Activities of SGPT and SGOT were measured according to the method of Reitman and Frankel (1957), respectively. The enzyme activities were expressed as IU/L.

3.1.6. Estimation of the levels of serum lactate

The activities of total lactate dehydrogenase (TLDH) and lactate dehydrogenase5 (LDH5) in serum were also measured following the method described by Levine et al. (1994). The enzyme activities were expressed as IU/L. Moreover, the activity of lactate dehydrogenase1 (LDH1) in serum was measured following the method of Strittmatter (1965), with some minor modifications (Varcoe, 2001). The enzyme activity was expressed as IU/L.

3.1.7. Estimation of the levels of creatine kinase-MB (CK-MB) and alkaline phosphatase (ALP) in serum

Myocardial enzyme leakage was also estimated by measuring the levels of serum specific marker enzyme CK-MB using the standard kit. The results were expressed as U/L. Besides, the serum specific hepatic damage marker ALP was measured following the method of IFCC (Tiet et al., 1983). The levels were expressed as U/L.

3.1.8. Measurement of the biomarkers of oxidative stress: levels of lipid peroxidation (LPO), protein carbonyl (PCO) content, reduced glutathione (GSH) and total thiol content (TSH)

Hepatic and cardiac tissue homogenates (10%) were prepared in chilled saline (0.9%, pH 7.0) with a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) and lipid peroxides in both the tissue homogenates were determined as thiobarbituric acid reactive substances (TBARS) following the method of Buege and Aust (1978) with some modifications as adopted by Bandyopadhyay et al. (2004). Levels were expressed as nmoles of TBARS/mg of tissue protein.

The PCO content of liver and heart tissues were estimated by DNPH assay following the method of Levine et al. (1994). The values were expressed as nmoles/mg tissue protein.

The GSH and TSH contents in the hepatic and cardiac tissues were estimated following the method of Sedlak and Lindsey (1968), with some modifications (Bandyopadhyay et al., 2004). The values were expressed as nmoles/mg tissue protein.

3.1.9. Measurement of activities of antioxidant enzymes: superoxide dismutases [SOD1 [(CuZnSOD) and SOD2 (MnSOD)], catalase (CAT), glutathione reductase (GR) and glutathione peroxidise (GPx)

The activities of SOD1 and SOD2 were measured by pyrogallol autoxidation method of Marklund and Marklund (1974). The enzyme activities were expressed as units/mg of tissue protein.

The activity of CAT was determined following the method described by Beers and Sizer (1952), with some modifications as adopted by Chattopadhyay et al. (2003). The enzyme activity was expressed as nmoles H_2O_2 consumed/mg of tissue protein.

The method of Krohne-Ehrich et al. (1977) was used to determine the activity of GR. On the other hand, activity of GPx was determined following the method as described by Paglia and Valentine (1967), with some modifications as adopted by Chattopadhyay et al. (2003). The

enzyme activities were expressed as nmoles/mg tissue protein.

3.1.10. Determination of the tissue levels of different proteins by western blot analysis

Western blot analysis was performed with liver and cardiac tissue homogenates which were prepared as described earlier by Bandyopadhyay et al. (2004). The samples were subjected to SDS–PAGE (10%) according to the method of Laemmli (1970). Eighty microgram proteins were loaded in each lane for immunodetection of SOD1, SOD2, CAT and GAPDH. The relative density of the bands obtained was quantified using ImageJ software (NIH, Bethesda, MD, USA).

3.1.11. Determination of the activities of pro-oxidant enzymes: xanthine oxidase (XO) and xanthine dehydrogenase (XDH)

The method of Greenlee and Handler (1964) along with some modifications (Mukherjee et al., 2010) was used to determine the activity of XO, by measuring spectrophotometrically the conversion of xanthine to uric acid. The activity of XDH was also measured spectrophotometrically following the method of Mukherjee et al. (2010). The enzyme activities were expressed as milli Units/mg tissue protein.

3.1.12. Determination of the activities of pyruvate dehydrogenase (PDH) and some of the Krebs cycle enzymes [isocitratedehydrogenase (ICDH), alpha-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH)]

The hepatic and cardiac tissue samples were homogenized (10%) in chilled phosphate buffer (50 mM, pH 7.4) with a Potter Elvenjem glass homogenizer (Belco Glass, Inc., Vineland, NJ, USA). The homogenates were centrifuged at 5000 × *g* for10 min and the supernatant was again centrifuged at 12000 × *g* for 15 min in order to obtain the mitochondrial fraction. The pellet thus obtained was resuspended in the buffer and used for measuring the activities of mitochondrial enzymes. The activity of PDH was determined spectrophotometrically at 340 nm following the method of Chretien et al. (1995). The activity of ICDH and α-KGDH were determined spectrophotometrically following the method as described by Duncan et al. (1979). The method of Veeger et al. (1969) was used to measure the activity of succinate dehydrogenase (SDH). The enzyme activity was recorded spectrophotometrically at 420 nm.The enzyme activities were expressed as units/mg tissue protein.

3.1.13. Determination of the activities of respiratory chain enzymes

The activities of NADH-Cytochrome c oxidoreductase and cytochrome c oxidase were determined following the method of Goyal and Srivastava (1995) spectrophotometrically at 565 and 550 nm, respectively. The activities of the enzymes were expressed as units/mg tissue protein.

3.1.14. Evaluation of DNA fragmentation

The method of Yamada et al. (2002) was used for the isolation of DNA from liver and heart tissues. In order to confirm the extent of DNA damage, we performed electrophoresis of extracted genomic DNA samples; resolved in agarose/EtBr gel. 2 μg DNA from each sample was loaded on to 1% agarose gel made with TAE and electrophoresed following the method of Garner et al. (1981). The gel was stained by 1 mg/mL ethidium bromide solution and fluorescence of DNA bands were detected by Bio Rad gel doc.

3.1.15. Evaluation of apoptosis using DAPI staining

Formalin fixed paraffin embedded 5 μM thick hepatic and cardiac left ventricular tissue sections were processed and stained according to a standard procedure (Suzuki et al., 1997). The samples were then equilibrated to pH 7.0 using a neutral pH buffer. The tissue sections were stained with DAPI staining solution and observed under an Olympus IX81confocal laser scanning microscope (Fluoview software, version 4.1) and the images were captured under 20x magnification (Excitation wavelength 358 nm, emission wavelength 461 nm). The digitized images were then analyzed using ImageJ analysis system (ImageJ, NIH software) and total fluorescence of each image measured and expressed as fluorescence intensity (FI) (arbitrary unit).

3.1.16. Tissue morphological and histochemical studies

3.1.16.1. Studies using tissue sections stained with haematoxylin-eosin (HE). The extirpated rat liver and heart tissues were fixed immediately in 10% formalin and embedded in paraffin following routine histological procedure. Five μm thick hepatic and cardiac tissue sections were prepared and stained with haematoxylin–eosin (H-E). The stained tissue sections were examined under a light microscope (Leica) at 40x magnification (Roy et al., 2009).

3.1.17. Quantification of fibrosis by confocal microscopy

The method of Roy et al. (2009) was used to stain the liver and heart tissue sections (5 μm thick) with Sirius Red (Direct Red 80; Sigma Chemical Co., St. Louis, MO, USA) and imaged with a confocal laser scanning microscope (Olympus IX81Software Fluoview, version 4.1) and the images were captured at 20x magnification. The digitized images were then analyzed using image analysis system (Image J, NIH Software) and the total collagen area fraction of each image was measured and expressed as the percent (%) collagen volume.

3.1.18. Evaluation of tissue architecture through scanning electron microscopy (SEM)

Both hepatic and cardiac tissues as well as the mitochondria isolated from them were fixed in 2.5% cold glutaraldehyde for 24–48 h immediately after dissection of the animals for SEM study following the method of Watanabe et al. (1988). The prepared samples were evaluated by scanning electron microscopy (SEM; Zeiss Evo 18 model EDS 8100).

3.1.19. Determination of the mitochondrial membrane potential (Δψm)

Mitochondrial membrane potential was assessed by using the mitochondria-specific lipophilic cationic fluorescent dye JC-1 following a well calibrated method of Cossarizza et al. (1993). Mitochondria from both the tissues were prepared as described above and were stained with JC-1 dye (0.2 μg/mL) and then incubated in dark at 37 °C for 20 min. The flow cytometer (BDFACS Versa, USA), with the excitation wavelength 488 nm and an emission wavelength of band pass filter 586/42 nm was used for the detection of mitochondrial membrane potential (Δψm) and was expressed in percentage (%).

3.1.20. Estimation of protein

Protein content of different samples was estimated by the method of Lowry et al. (1951) using the bovine serum albumin as the standard.

3.2. In vitro experiment

3.2.1. Isolation of hepatic and cardiac mitochondria and cytosol

Mitochondria was isolated from the liver and heart tissues following the method of Hare et al. (1980) with minor modifications (Chattopadhyay et al., 1992**).** Liver and heart tissues of rat (n = 6) were collected immediately following sacrifice, and were homogenized in 50 mM phosphate buffer (pH 7.4) at 4 °C to prepare 10% tissue homogenate. Now, the homogenates were centrifuged at 600 g at 4 °C for 10 min and the supernatant was collected which was then centrifuged again at 16000 g for 45 min at 4 °C. Both the supernatant containing cytosolic fraction (for the estimation of Cu-Zn SOD activity) and the pellet containing mitochondrial fraction (for the estimation of PDH, ICDH, α-KG, and SDH activity) were collected. The pellet was then resuspended in 50 mM Tris-sucrose buffer (pH 7.8) and preserved at −20 °C which was used as crude mitochondrial samples for further biochemical assays.

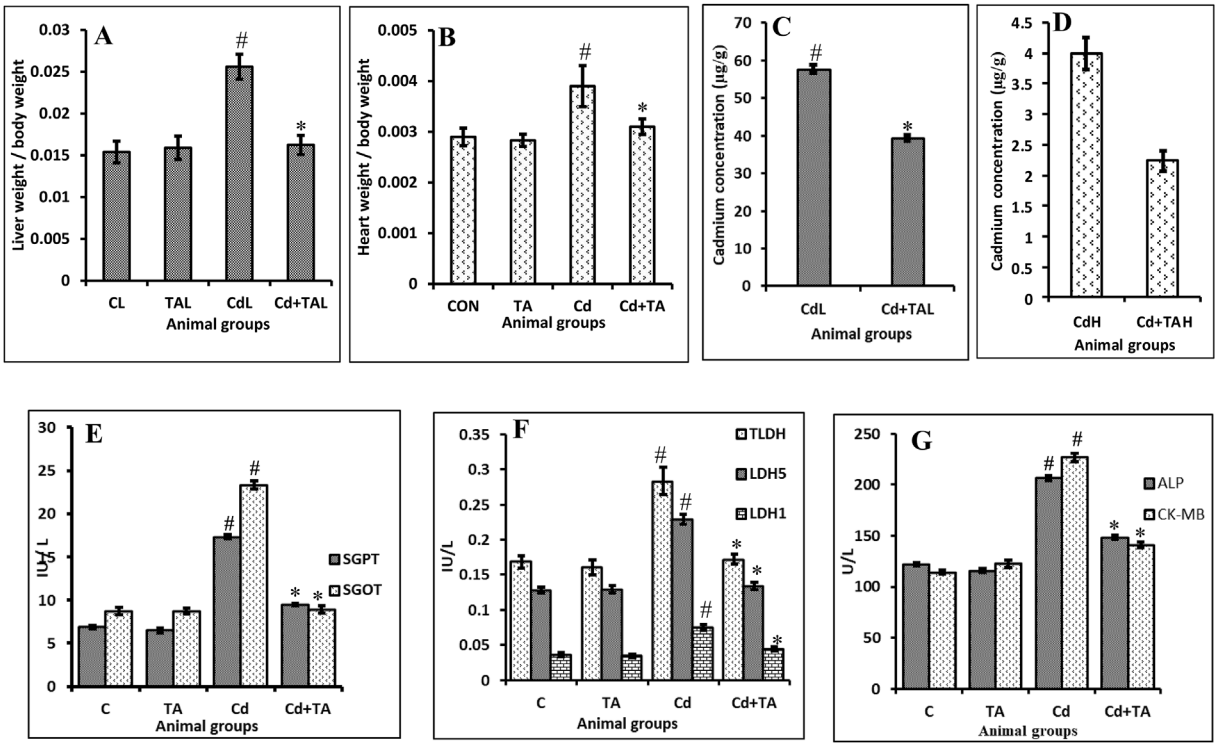

Fig. 1. Protective effect of aqueous bark extract of TA against Cd induced changes in liver weight to body weight ratio(1A), heart weight to body weight ratio (1B), cadmium concentrations in liver (1C) and heart (1D), SGPT, SGOT, TLDH, LDH5, LDH1, ALP, CK-MB, (1E, 1F and 1G respectively)were measured in control (C), positive control (TA), Cd-acetate treated (Cd) and *Terminalia arjuna* protected (Cd + TA) rats. The values are expressed as Mean ± S.E.M.; #$p < 0.001$; versus C; *$p < 0.001$ versus Cd using one way ANOVA.

3.2.2. Isolation of peroxisomes

Five percent (5%) hepatic and cardiac tissue homogenates were prepared in 50 mM phosphate buffer (pH 7.0) and centrifuged at 7000 rpm for 25 min at 4 °C and the supernatant was collected. 0.9 mL of the supernatant was incubated with 0.01 mL absolute ethanol at 4 °C for 30 min. After which 100 µL of 10% Triton-X-100 was added to the ethanol incubated supernatant and shaken vigorously for 5 min to rupture peroxisomal membrane. The sample, thus obtained was used to determine the activity of catalase (Beers and Sizer, 1952).

3.2.3. Incubation of cytosolic, mitochondrial and peroxisomal suspension with cadmium and Terminalia arjuna

Fifty percent (50%) cytosolic, mitochondrial and peroxisomal suspension were incubated in 50 mM phosphate buffer (pH 7.4) in four different groups: (i) Control (without any treatment) (ii) TA (120 µg) alone (iii) Cadmium (0.1 mM) alone (iv) TA (120 µg) in presence of 0.1 mM Cd at 37 °C for 60 min. In either case the reaction was terminated following addition of 0.02 mL 35 mM EDTA after completion of 1 h incubation.

3.2.4. Terminalia arjuna against cadmium-induced hepatic and cardiac cytosolic and mitochondrial injury: a dose–response study

Approximately 6–8 mg/mL of mitochondrial protein was incubated with 12.5, 25, 50, 100, and 200 µM of Cd respectively in lysis buffer for 60 min at 37 °C. A portion of the cytosol and mitochondria was then assayed for LPO by the method of Buege and Aust (1978) with some modifications as adopted by Bandyopadhyay et al. (2004) to determine the most effective dose of Cd. Levels were expressed as nmoles of TBARS/mg of tissue protein.

Isolated cytosolic and mitochondrial samples of liver and heart tissues of rat were incubated with 100 µM Cd and different concentrations of TA (15, 30, 60, 120 and 240 µg) for 60 min at 37 °C. Samples were then assayed for LPO levels to determine the minimum effective dose of TA.

Furthermore, activities of catalase in liver tissues, and both isoforms of SOD (Cu-Zn SOD and Mn-SOD) in heart tissues were measured following well calibrated spectrophotometric methods as described earlier (Marklund and Marklund, 1974). Moreover, activities of PDH and different Krebs cycle enzymes (ICDH, α-KGDH, SDH) were also measured in both the tissues following the methods as mentioned earlier (Chretien et al., 1995; Duncan and Fraenkel, 1979; Veeger et al., 1969).

3.3. Statistical analysis

The values are presented as mean ± S.E.M of triplicate observations. The statistical significance of the data has been determined using one-way analysis of variance (ANOVA) and significant difference among treatment groups were evaluated by Tukey test. The results were considered as statistically significant at the level of $p < 0.001$. All statistical analyses and data presentation are performed using Microcal Origin version 7.0 and Windows Excel.

4. Results

4.1. GC-MS profiling of aqueous bark extract of TA

GC-MS analysis of the aqueous bark extract of TA revealed the occurrence of total 37 chemical compounds as the phyto-constituents, among which mainly seven were identified as biologically potent compounds because of their hydroxyl group and/or double bonds that could have possibly contributed to free radical scavenging efficacy of the aqueous bark extract of TA. The identified prevailing bioactive components are Benzoic acid (14.20%), Hexadecanoic acid methyl ester (0.26%), Tetradecanoic acid (0.62%), 9,12-Octadecadienoic acid (Z,Z)-,methyl ester (0.33%), 9Octadecenoic acid (Z)-, methyle (0.67%) and Oleic Acid (2.98%). The other compounds found in GC-MS profiling

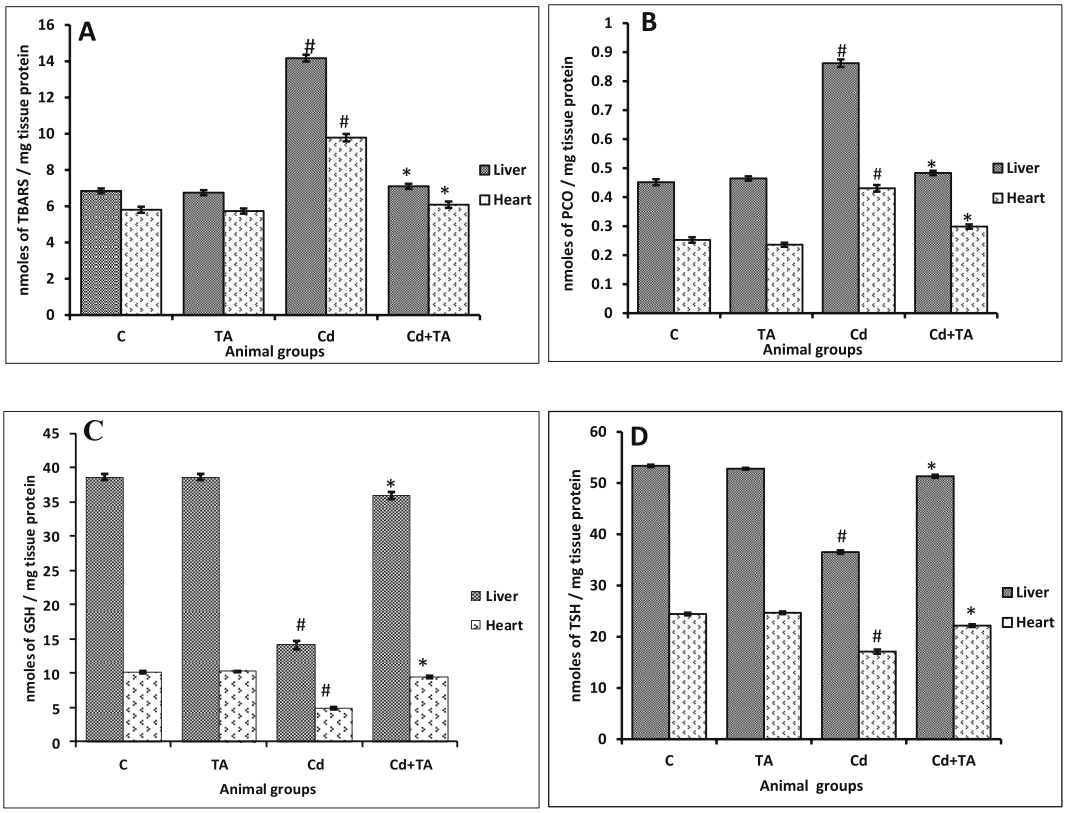

Fig. 2. Ameliorative effect of aqueous bark extract of TA (20 mg/kg bw, orally) against Cd (0.44 mg/kg bw, s.c.,) induced alterations in the levels of LPO, PCO, GSH and TSH (2A, 2B, 2C, and 2D, respectively) in both liver and heart tissues were measured in control (C), positive control (TA), Cd-acetate treated (Cd) and *Terminalia arjuna* protected (Cd + TA) rats. The values are expressed as Mean ± S.E.M.; #$p < 0.001$; versus C; *$p < 0.001$ versus Cd using one way ANOVA.

of the aqueous bark extract of TA are: Sparteine, 14.alpha.-hydroxy-17-oxo- (0.40%), Cyclopentanone, 3-methyl- (0.40%), Akton (0.35%), Ethane, 1,1,2,2-tetrachloro- (0.35%), 2-Nonadecanol (0.22%), Ethanone, 1-(2-furanyl)- (0.13%), Benzene, 1-fluoro-3-methyl- (0.25%), 2-Hexene, 3-methyl-, (Z)- (0.04%), 11-Tricosene (0.31%), Diphenylacetylpyrrolidine (0.99%), 1-Octadecanol (0.31%), 2-Pyrimidinamine (1.24%), Cholestan-3-ol, 4,4-dimethyl-, (0.09%), Astemizole (2.04%), Chloroform (1.72%), Tricosanoic acid, 2-methoxy-, methyl ester (7.78%), Fluoxymesterone (3.44%) (Supplementary Fig. 1).

5. In vivo experiments

5.1. Liver weight/body weight and heart weight/body weight ratio

Administration of cadmium acetate caused significant elevation in the weight of liver (Fig. 1A) as well as heart (Fig. 1B) by 60% and 30% respectively, compared to control values. The organ/body weight ratio in Cd + TA group was found to be significantly protected from being altered when compared to the Cd treated group. The TA only group did not show any change in the organ to body weight ratio.

5.2. Levels of cadmium in hepatic and cardiac tissues

A highly significant increase in the level of Cd in both the tissues of the Cd treated group was noted when compared to control values. The concentration of cadmium in the liver and heart (Fig. 1C and D) tissues were significantly reduced (31.87% and 43.89%, respectively) in Cd + TA group when compared to the Cd treated group.

5.3. Biomarkers of organ damage: SGPT and SGOT

The administration of Cd caused significant elevation in the serum level of SGPT and SGOT (1.52 and 1.68 folds, respectively) when compared to control values. The serum levels of both SGPT and SGOT were found to be protected (45.21% and 61.87%, respectively) significantly from being altered in the Cd + TA group when compared to the Cd treated group (Fig. 1E). The animals treated with TA only, however, did not show any change in the levels of these marker enzymes.

5.4. Activities of TLDH, LDH-5 and LDH-1

The administration of Cd caused a significant increase in the activities of T-LDH, LDH-5 and LDH-1 (88.75%, 76.9% and 1.08 fold, respectively) compared to the control. But the activities of all the enzymes were found to be significantly protected, from being altered, by 39.44%, 41.48% and 41.33% respectively, in the Cd + TA group compared to the Cd treated group (Fig. 1F). The animals treated with TA only did not show any change in the activities of these enzymes.

5.5. Activities of CK-MB and alkaline phosphatase (ALP) in serum

The treatment with cadmium acetate significantly increased the activities of CK-MB and ALP by 98.26% and 68.87%, respectively in the serum in comparison to the control (Fig. 1G). The activities of both the enzymes were found to be protected significantly by 37.8% and 28.19%, respectively, in Cd + TA group when compared to the Cd treated group. The TA alone group did not show any change in the enzyme activity.

5.6. Biomarkers of oxidative stress- LPO, PCO, GSH and TSH

The administration of Cd caused a significant elevation in the levels of LPO (1.06 fold in liver and 68.5% in heart) and PCO (91.11% in liver and 72% in heart) compared to the control. However, in the CD + TA group, LPO and PCO were found to be reduced significantly [(LPO by 49.89% in liver and 37.81% in heart) and (PCO by 44.19% in liver and 30% in heart)] in both liver and heart tissues when compared to the Cd treated group (Fig. 2A and B). These parameters in TA only group did not differ from the values observed in control group.

Treatment of rats with Cd caused a significant reduction in the levels of GSH and TSH in liver and heart tissues (GSH, 63.35% in liver and 50.84% in heart; TSH, (31.55% in liver and 30.02% in heart) when compared to the control values. However, in the CD + TA group, GSH and TSH were found to be protected from being reduced significantly [(GSH by 1.53 fold in liver and 90.78% in heart) and (TSH by 40.53% in liver and 35.54% in heart)] in both hepatic and cardiac tissues when compared to the Cd treated group (Fig. 2C and D). However, TA alone group did not show any change when compared to control.

5.7. Activities of antioxidant enzymes

Treatment of rats with Cd caused a significant increase in the activities of Cu-Zn SOD (29.46%), Mn-SOD (90.74%) and a decrease in the activity of catalase (44.54%) in hepatic tissue when compared to the control values (Fig. 3) whereas a decrease in the activities of Cu-Zn SOD (46.32%), Mn-SOD (26.1%) and increase in the activity of catalase (34.57%) was observed in cardiac tissue when compared to the respective control values. But in Cd + TA group, the activities of antioxidant enzymes were found to be significantly ($p < 0.001$) protected from being altered [Cu-Zn SOD (16.29% in liver and 64.55% in heart Mn-SOD (44.67% in liver and 32.41% in heart), catalase (72.91% in liver and 23.53% in heart)] when compared to the Cd treated group. Moreover, compared to control, there occurred a significant alteration in the antioxidant enzyme protein levels following treatment of rats with Cd. However, such alterations in protein level were found to be significantly protected from being taken place in the rats pre-treated with aqueous extract of TA (Supplementary Fig. 2).

Moreover, a highly significant elevation in the activity of GPx (1.94 fold in liver and 1.68 fold in heart) a highly significant ($p < 0.001$) reduction in the activity of GR (56.01% in liver and 66.12% in heart) in both tissues were observed following the treatment of rats with Cd when compared to the control values. However, in the Cd + TA group, a significant protection of the enzyme activities (GPx; 43.61% in liver and 32.97% in heart) (GR; 76.04% in liver and 25.29% in heart) were found when compared to the Cd treated group (Fig. 4A and B). TA alone group did not show any change in the enzyme activity.

5.8. Activities of pro-oxidant enzymes; XO and XDH

The administration of Cd caused a significant elevation in the activities of xanthine oxidase (55.56% in liver and 70.78% in heart) and xanthine dehydrogenase (2.39 fold in liver and 2.03 fold in heart) when compared to control values. The activities of both the enzymes, however, in the hepatic and the cardiac tissues were found to be significantly protected from being elevated [(XO; 74.7% in liver and 70.69% in heart) and (XDH; 58.97% in liver and 61.7% in heart)] when compared to the Cd treated group (Fig. 4C and D). Here also, TA alone group did not show any change in the enzyme activity.

5.9. Activities of pyruvate dehydrogenase and some of the Krebs cycle enzymes

The treatment of rats with Cd caused a significant decrease in the activity of pyruvate dehydrogenase (PDH) (27.6% in liver and 53.93% in heart) and also a significant decrease ($p < 0.001$) in the activities of some of the Krebs cycle enzymes, such as PDH (53.93% in heart and 27.6% in liver), ICDH (50.1% in liver and 43.39% in heart), α-KGDH (45.45% in liver and 56.67% in heart), and SDH (73.79% in liver and 59.81% in heart) in both the tissues when compared to the control values. But the activities of all the enzymes were found to be significantly protected from being altered in the Cd + TA group when compared to the Cd treated group (PDH, 2.80 fold in liver and 1.46 fold in heart; ICDH, 84.81% in liver and 52.34% in heart; α-KGDH, 77.78% in liver and 76.92% in heart; SDH, 1.78 fold in liver and 1.16 fold in heart) [Fig. 5 (A-D)]. Compared to control, the enzyme activities in the TA alone group did not show significant alteration.

5.10. Activities of respiratory chain enzymes

A highly significant decrease in the activities of cytochrome c oxidase (53.6% in liver and 57.45% in heart) and cytochrome c oxidoreductase (72.89% in liver and 54.32% in heart) in both tissues were observed following the treatment of rats with Cd. However, a significant protection in the activities of both cytochrome c oxidase (1 fold in liver and 1.17 fold in heart) and cytochrome c oxidoreductase (2.31 fold in liver and 1.11 fold in heart) were found when compared to the Cd treated group (Fig. 5E and F). The TA alone group did not show any change in the enzyme activities.

5.11. Evaluation of DNA fragmentation

Agarose gel electrophoretic pattern of DNA fragmentation and DNA band density of liver (Fig. 6A) and heart (Fig. 6B) tissues revealed a significant increase in the levels of DNA fragmentation in Cd treated

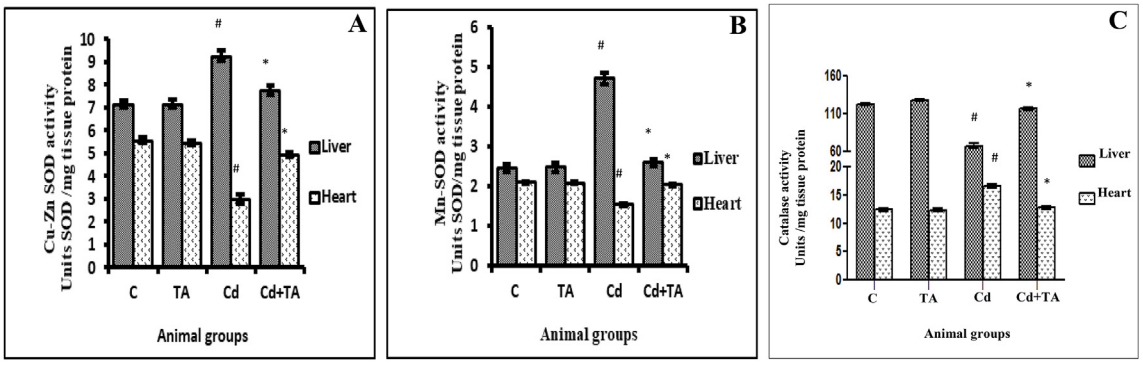

Fig. 3. Protective effect of aqueous bark extract of TA against Cd-induced increase in theenzyme activity of Cu-ZnSOD in liver tissue and decrease in the heart tissue (3A), increase in the enzyme activity of MnSOD in liver tissue and decrease in heart tissue (3B), decrease in the activity of catalase in liver tissueand increase inthe heart tissue (3C) in control (C), positive control (TA), Cd-acetate treated (Cd) and TA protected (Cd + TA) rats. The values are expressed as Mean ± S.E.M.; #$p < 0.001$; versus C; *$p < 0.001$ versus Cd values using one way ANOVA.

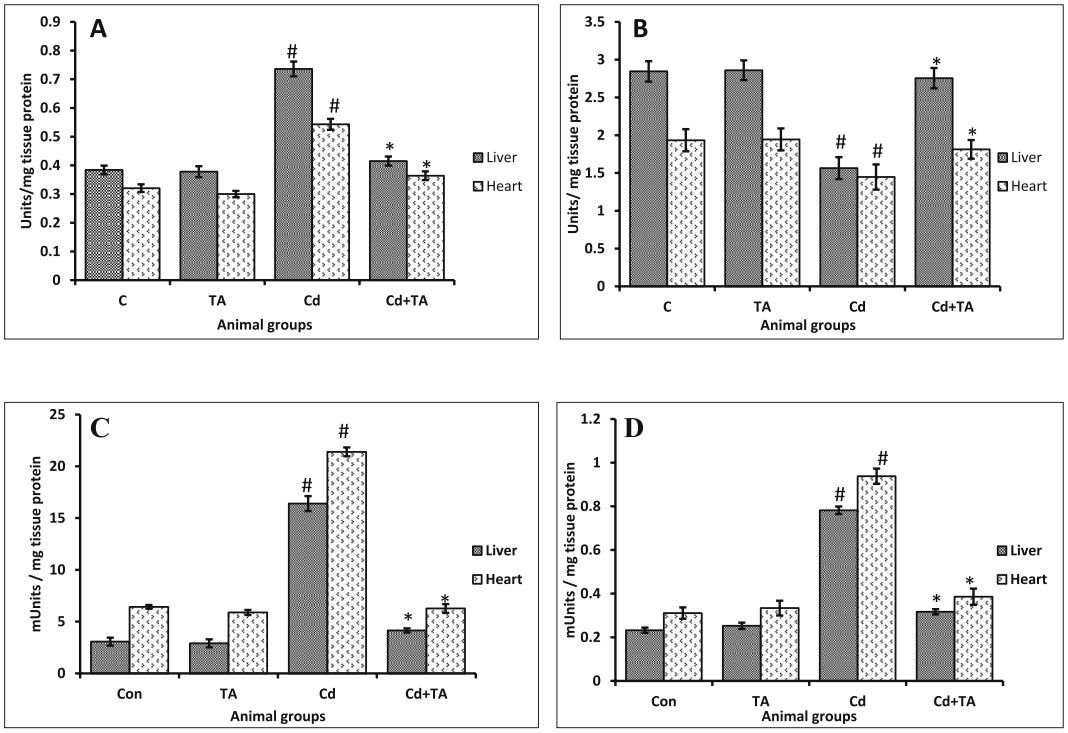

Fig. 4. Ameliorative effect of aqueous bark extract of TA against Cd-induced alterations in the levels of GPx, GR, XO and XDH (4A, 4B, 4C, and 4D, respectively) in both liver and heart tissues in control (C), positive control (TA), Cd-acetate treated (Cd) and TA protected (Cd + TA) rats. The values are expressed as Mean ± S.E.M.; #$p < 0.001$; versus C; *$p < 0.001$ versus Cd values using one way ANOVA.

rats when compared to control. However, treatment of rats with aqueous bark extract of TA was found to decrease the cadmium induced DNA fragmentation significantly in both the tissues. The band intensity did not differ from control in case of liver tissue in TA only group although it is significantly different in case of heart tissue.

5.12. Evaluation of apoptosis using DAPI staining

The DAPI staining of the liver and heart tissue sections showed no evidence of nuclear staining in control and positive control (TA) groups of rats. On the other hand, the cadmium-treated liver and heart tissues showed nuclear staining with DAPI since an increase in the number of mitotic nuclei was observed from the increased level of mean fluorescence intensity. Notably, pre-treatment of rats with aqueous bark extract of TA was found to protect against cadmium induced enormous nuclear fragmentation (Fig. 7). TA alone has no significant effect on nuclear DNA fragmentation in both the tissues.

5.13. Tissue morphological and histo-chemical studies

5.13.1. Studies using tissue sections stained with HE

The liver tissue sections of Cd treated group revealed a marked dilatation of the central vein, portal vein as well as sinusoidal congestion, mild inflammatory cell infiltration in the portal tract, enlarged nuclei, light stained cytoplasm with enlarged vacuoles and necrotic hepatocytes. Similarly, the cardiac tissue sections showed profound degeneration along with capillary dilatation, vascular congestion, and myocardial fibre necrosis. However, all the cadmium mediated damages in both hepatic and cardiac tissues were found to be protected from being altered when the rats were pre-treated with aqueous bark extract of TA. TA alone group, however, did not show any change in tissue morphology in both the organs compared to controls (Fig. 8A).

5.14. Quantification of fibrosis by confocal microscopy

In the Cd treated rats, the staining through picrosirius red showed a significant deposition of collagen, especially around the central vein of the hepatic lobule (Fig. 8B) and a depletion of collagen fibre in the cardiac tissue sections (Fig. 8C) indicating tissue fibrosis. Such alterations were found to be protected from being taken place when the rats were pre-treated with aqueous bark extract of TA in comparison to Cd treated group. Compared to controls, the TA alone group did not show any change in collagen content.

5.15. Evaluation of tissue architecture through SEM

In hepatic tissue sections, the dilated central veins, distortion in the polyhedral arrangement of hepatocytes with profound tissue necrosis were also observed in Cd treated group (Fig. 9A). However, in cardiac tissue sections, the surface architectural alterations in Cd treated rats were confirmed by the irregularities in the branching pattern of the cardiac muscle fibres where the myofibrillar collagen fibres became crosslinked to each other and formed a complicated matrix network (Fig. 9B). On the other hand, ultrastructural changes observed in the mitochondria isolated from the hepatic tissue of Cd treated rats showed small membrane blebs with disrupted cristae with vacuolation, rough and ruptured surface [Fig. 10 A (I)]. Similarly, the mitochondria isolated from cardiac tissue also displayed perforated surface and convoluted membranes with surface covering blebs due to markedly contracted cells [Fig. 10 B (I)]. However, both the tissues as well as isolated mitochondrial samples of Cd + TA group, were found to be protected from any alteration in tissue as well as mitochondrial morphology when compared to the Cd treated group.

5.16. Mitochondrial membrane potential ($\Delta\Psi m$)

In the mitochondrial samples of both liver and heart tissues of control rats, the cationic dye (JC1) accumulated in the matrix and red

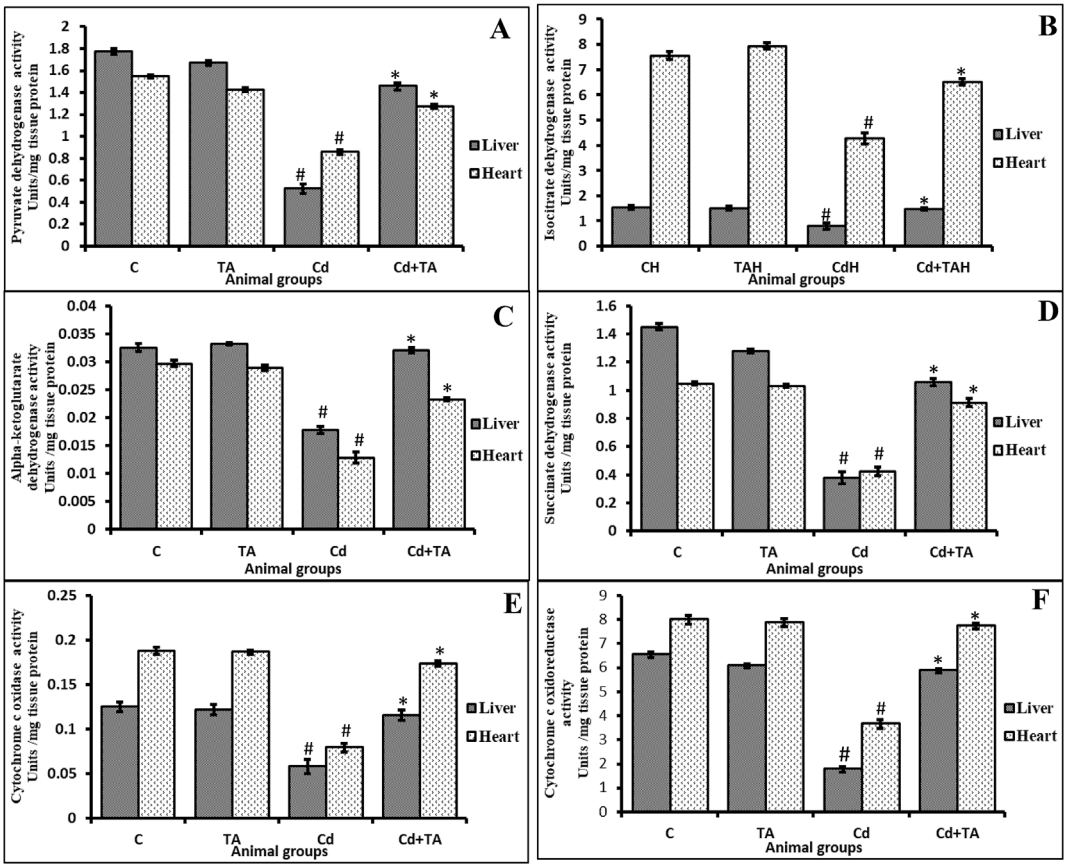

Fig. 5. Protective effect of aqueous bark extract of TA against Cd-induced decrease in the activities of (5A) pyruvate dehydrogenase,(5B) isocitrate dehydrogenase, (5C) alpha-ketoglutarate dehydrogenase, (5D) succinate dehydrogenase, (5E) cytochrome c oxidase and (5F) cytochrome c oxidoreductase in both liver and heart tissues in control (C),only TA administered (TA), Cd-treated (Cd), and TA-protected (Cd + TA) rats. The values are expressed as Mean ± S.E.M.; #$p < 0.001$; versus C; *$p < 0.001$ versus Cd using one way ANOVA.

fluorescence was observed (9.78 ± 0.04 in liver and 2.73 ± 0.05 in heart); whereas in the cadmium treated groups, a significant fluorescence shift (in percentage) from red to green (32.81 ± 0.05 and 21.15 ± 0.06 liver and heart, respectively) was observed. However, the mitochondria isolated from liver and heart tissues of Cd + TA group revealed a significant protection of the inner mitochondrial membrane potential from being altered (11.66 ± 0.05 and 1.69 ± 0.04 in liver and heart, respectively) when compared to the values obtained from the mitochondria of the control rats [Fig. 10 A (II), B (II)].

6. *In vitro* experiments

6.1. Terminalia arjuna against cadmium-induced cytosolic and mitochondrial injury of hepatic and cardiac tissues: a concentration-response study

Incubation of isolated cytosol and mitochondria of rat liver and heart tissues with 100 μM of Cd significantly increased the levels of MDA, which were prevented from being altered upon co-incubation of cytosol and mitochondria of both the tissues with TA in a concentration dependent manner, but the maximum protection was observed to be provided at a concentration of 120 μg (0.12 mg)/mL incubation mixture. Therefore, the subsequent *in vitro* experiments were carried out following the incubation of isolated cytosol and mitochondria of rat liver and heart tissues with Cd at a concentration of 100 μM or, TA at a concentration of 120μg/mL incubation mixture (data not shown).

6.2. Kinetic analysis of the inhibition pattern of different antioxidant enzymes (catalase, Cu-Zn SOD and Mn-SOD)

In order to investigate the nature of inhibition pattern of the activity of catalase in the liver tissue after an *in vitro* incubation of cytosolic suspension with Cd, a Lineweaver Burk Double Reciprocal (LBDR) plot was conducted using increasing concentrations of substrate for catalase, i.e., H_2O_2 (6.125–53 μM). Similarly, in case of Cu-Zn SOD and Mn-SOD activities in cardiac tissue samples following *in vitro* incubation of cytosolic fraction with Cd, LBDR was performed using increasing concentrations of substrate, i.e., pyrogallol (0.05–0.4 mM), for both the SOD isoforms. It was observed that cadmium uncompetitively inhibited catalase, Cu-Zn SOD as well as Mn-SOD, as evident from decreased Vmax and Km values of Cd treated groups and such uncompetitive inhibition pattern was protected in all the cases after co-incubation of TA for every increasing concentration of substrate [Supplementary Fig. 3 (a-c)].

6.3. In vitro study regarding the inhibition pattern of PDH and some of the Krebs cycle enzymes (ICDH, α-KGDH, SDH)

LBDR was conducted to determine the nature of inhibition pattern of PDH, ICDH, α-KGDH, SDH activities in both hepatic and cardiac tissue samples after *in vitro* incubation of mitochondrial samples with Cd, in presence of increasing concentrations of substrate for PDH, i.e., sodium pyruvate (0.0625–0.5 mM); or for ICDH i.e. Isocitrate (0.0625–0.5 mM); or for α-KGDH i.e. α-KG (0.0625–0.5 mM); or for SDH i.e. Succinate (0.5–4 mM) [Supplementary Fig. 3d-k]. It was observed that Cd inhibited all the studied Krebs cycle enzymes

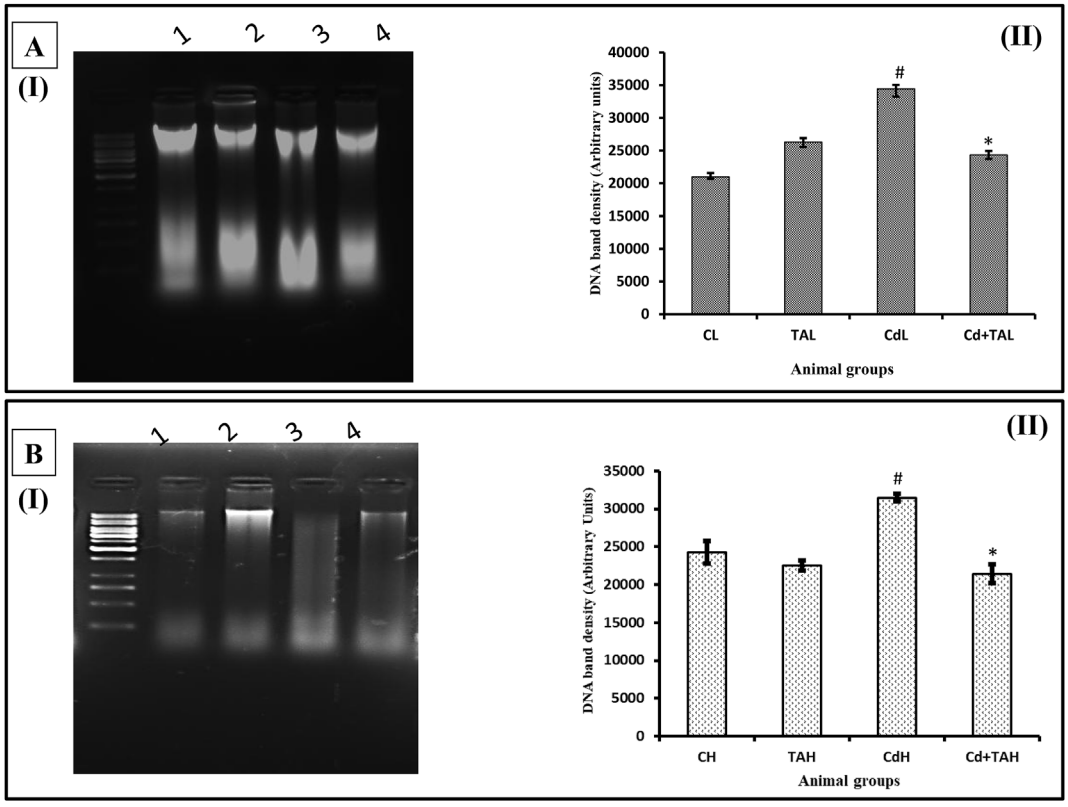

Fig. 6. Analysis of DNA degradation using agarose gel electrophoresis of genomic DNA of both liver [6A (I)] and heart [6B (I)] tissues in control (1), only TA administered (2), Cd-treated (3), and TA-protected (4) rats. The extraction of genomic DNA and agarose gel electrophoresis was repeated at least 3 times. Histogram showing DNA band intensity (arbitrary units) of liver and heart tissue sections [6A (II) and6B (II), respectively]. Values are expressed as means ± S.E.M. #p < 0.001 vs. C; *p < 0.001 vs. Cd; using one way ANOVA.

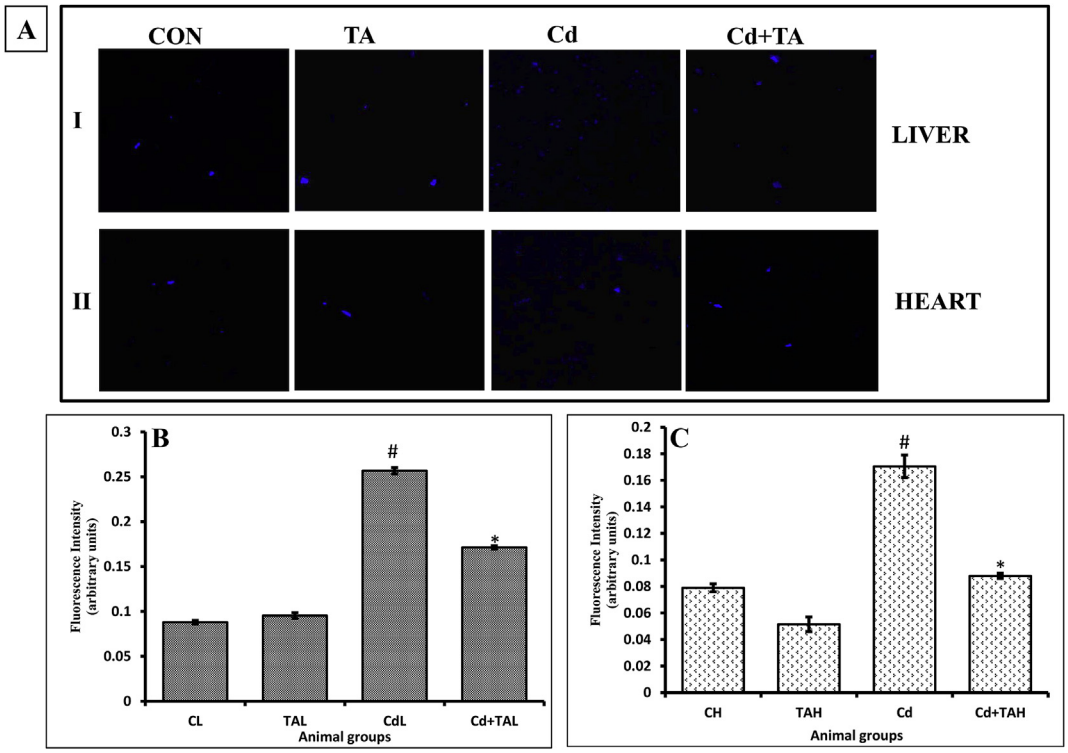

Fig. 7. Representative images (20x magnifications) of DAPI stained liver [Panel A (I)] and heart [Panel A (II)] tissue sections of rat in control (C), only TA administered (TA), Cd-treated (Cd), and TA-protected (Cd + TA) groups. The images were captured by confocal laser scanning microscope. Histogram showing fluorescence intensity (FI) (Arbitrary Units) of the DAPI stained liver and heart tissue sections (7B and 7C, respectively). Values are expressed as means ± S.E.M. #p < 0.001 vs. C; *p < 0.001 vs. Cd; using one way ANOVA.

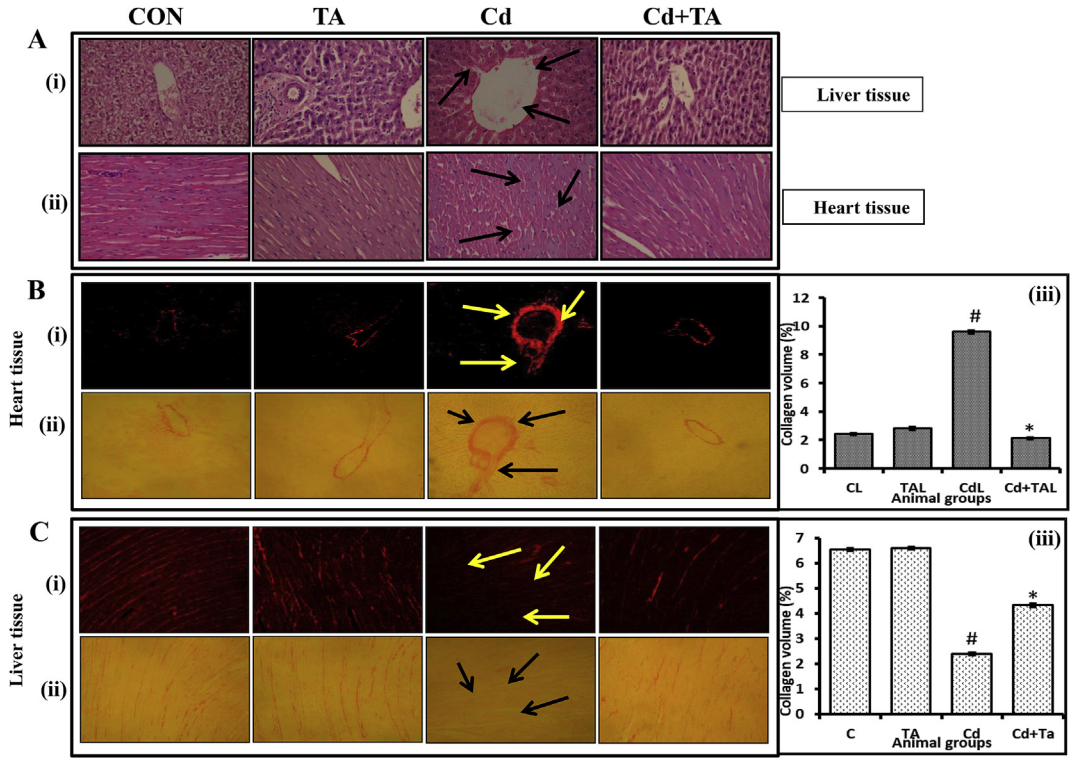

Fig. 8. Representative images (40X magnification) of haematoxylin-eosin stained liver [Panel A (i)] and heart [Panel A (ii)] tissue sections, and also confocal and bright field images of picrisirius red stained liver [8B (i) and 8B (ii)] and heart [8C (i) and 8C (ii)] tissue sections of control (C), TA protected (TA), Cd treated (Cd) and TA protected (Cd + TA) groups. Red colour stretches are the area of collagen. Arrow heads indicate the collagen fibers. Histogram showing collagen volume (%) of the sirius stained liver and heart tissue sections [8B (iii) and 8C (iii), respectively]. Values are expressed as means ± S.E.M. #$p < 0.001$ vs. C; *$p < 0.001$ vs. Cd; using one way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

uncompetitively, as evident from different intercepts in the y-axis and also decreased Vmax and Km values of Cd treated groups and such uncompetitive inhibition pattern of all the krebs cycle enzymes was protected after co-incubation with TA for every increasing concentrations of substrate (Supplementary Table 1).

7. Discussion

Cadmium (Cd), a well-known environmental biohazardous toxicant, may cause acute and chronic intoxications by ingestion or inhalation that ultimately may lead to disruption of a number of biological systems even at a very low exposure level (Bhattacharjee et al., 2016). There are

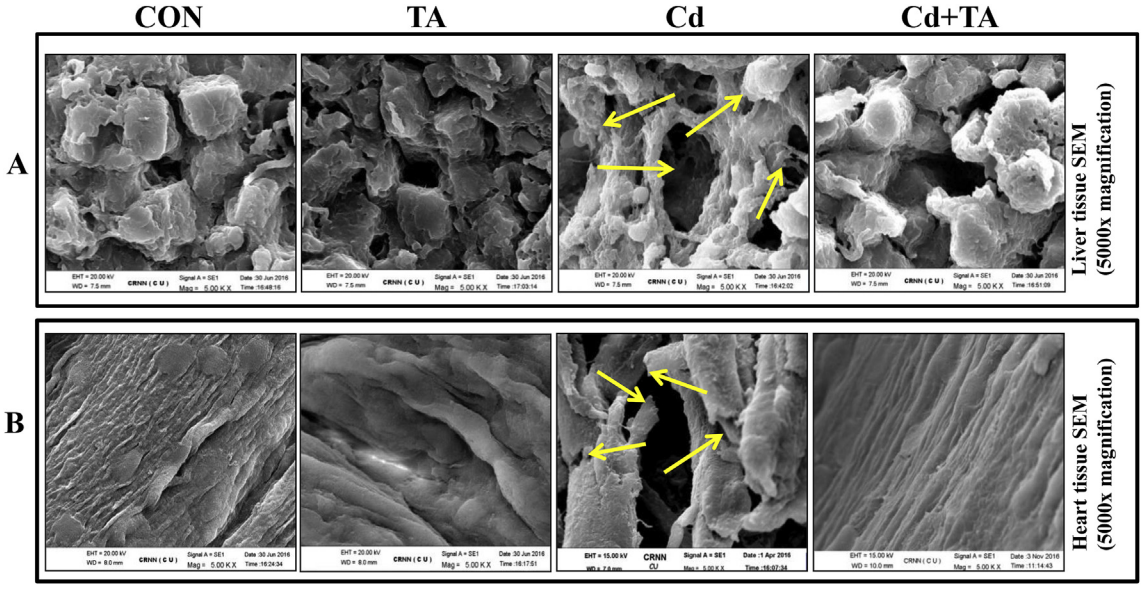

Fig. 9. Representative images of scanning electron micrograph (5000x magnifications) of rat hepatic and cardiac tissue sections in control (C), only TA administered (TA), Cd-treated (Cd), and TA-protected (Cd + TA) groups. Yellow and black arrow heads indicate the damaged portion of hepatic and cardiac tissue sections of cadmium treated rats (0.44 mg/kg BW) (9A, 9B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

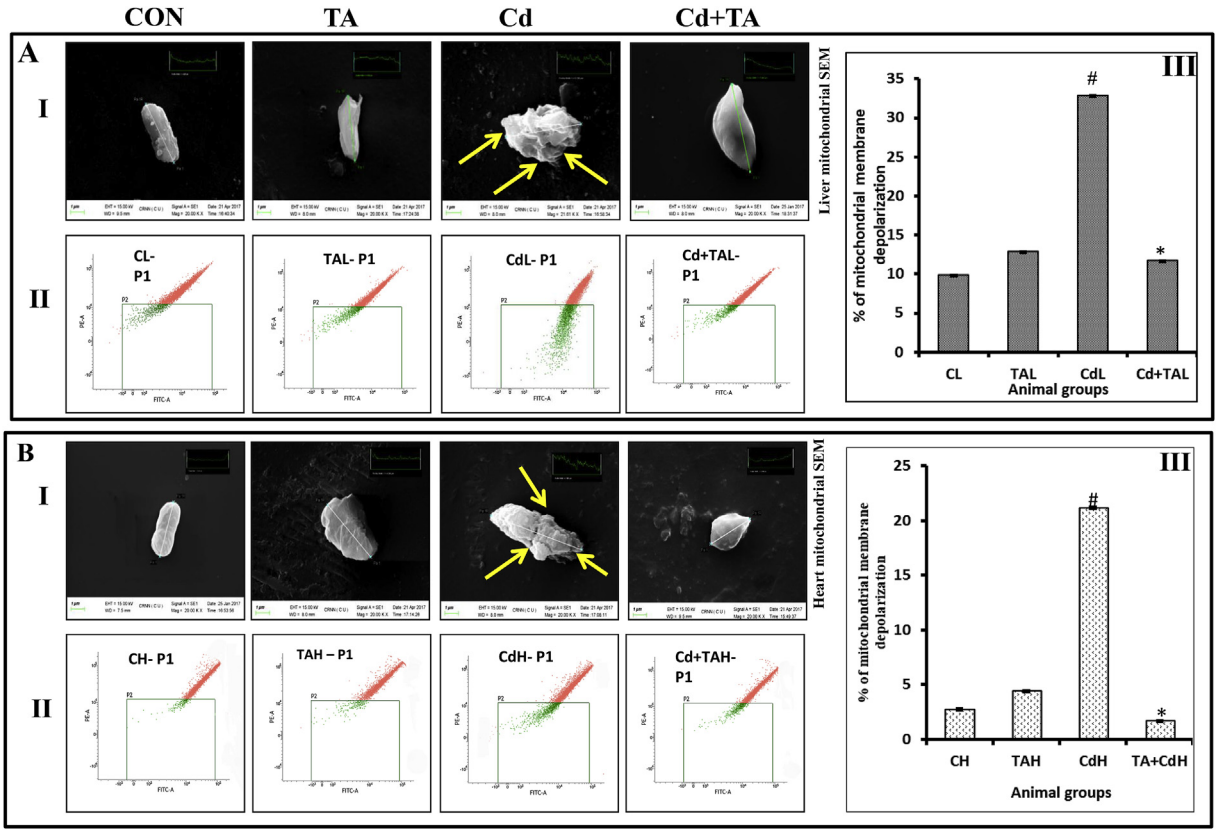

Fig. 10. Representative images of scanning electron micrograph (5000x magnifications) of rat hepatic and cardiac mitochondria in control (C), only TA administered (TA), Cd-treated (Cd), and TA-protected (Cd + TA) groups. Yellow and black arrow heads indicate thedamaged and blebbed portion of hepatic and cardiac mitochondria of cadmium treated rats (10 A-panel I and 10 B- panel I). Cadmium induced changes in liver and heart mitochondrial samples by fluorescence-activated cell sorting (FACS) analysis after JC1 stainingin control (C), positive control (TA), Cd-acetate treated (Cd) and TA protected (Cd + TA) groups (A-panel II and B-panel II, respectively). Bar diagram showing percent of mitochondrial membrane depolarization of the JC1 stained liver (AIII) and heart mitochondrial samples (n = 3 independent experiments) (10 AIII and 10 BIII). The values are expressed as Mean ± S.E.M.; #$p < 0.001$; versus C; *$p < 0.001$ versus Cd using one way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reports available demonstrating the adverse effects of Cd-induced intoxication resulting in cellular alterations caused due to maximum accumulation of Cd primarily in the hepatocytes and to some extent in myocardial cells also (Yiin et al., 2000). It is well proclaimed that cadmium may cause an increase in the production of reactive oxygen species (ROS) such as hydroxyl radical ($HO^{\cdot}$), superoxide anion free radical ($O2^{\cdot-}$), nitric oxide radical, and hydrogen peroxide (H_2O_2) etc.via the Fenton reaction, inducing oxidative stress (Ercal and Galan, 2001). The present study is designed to evaluate the role of exogenous aqueous bark extract of TA (20 mg/kg bw, fed orally) in protecting the liver and heart tissues of male Wistar rats against Cd-induced oxidative stress.

GCMS profiling of aqueous bark extract of TA reveals the presence of seven bioactive phyto-constituents which are known to possess antioxidant, anticancer, anti-inflammatory, antiarthritic, and antimicrobial properties. Among them, benzoic acid (an aromatic compound) is widely used as food preservative and is known to have antimicrobial activities (Chipley, 1993). Similarly, hexadecanoic acid methyl ester compound, reported to have anti-inflammatory activity (Singh et al., 2008), is identified in the extracts of Tulsi leaves (*Ocimum sanctum*) and seeds of Linseed *(Linum usitatissimum*). Moreover, 9, 12-octadecadienoic acid (Z, Z)-, methyl ester, is found in seed extract of *Croton tiglium*, and is documented to possess anti-inflammatory, anti-arthritic, antioxidant and anticancer activities (Mangunwidjaja et al., 2006). Similarly, root extract of *Phyllanthus vasukii* possess 9-octadecenoic acid (Z)-, methyle which is reported for its anti-inflammatory and anticancer properties (Jemimma et al., 2017). Notably, both oleic acid and tetradecanoic acid are identified in the stem bark extract of *Daniellia oliveri*, where oleic acid is reported to have anti-inflammatory properties (Carrillo et al., 2012); while tetradecanoic acid has lipid anchorage characteristics in membrane (Ojochenemi et al., 2017). Thus, it will not be unwise to assume that the antioxidative and anti-inflammatory properties of these bioactive phyto-constituents may play a crucial role in protecting the liver and heart tissues by counteracting the free radicals produced by the treatment of Cd.

In our study, administration of Cd (II) acetate to rats at every alternate day for a period of 15 days is found to elevate the weight of the liver and heart tissues leading to organomegaly or, hypertrophy. Similar observations were reported in earlier studies suggesting inflammation as the possible cause of organomagaly (Donpunha et al., 2011; Dudley et al., 1985). Pre-treatment of rats with aqueous bark extract of TA provided protection against organ enlargement with consequent reduction and eventually restoration of their weight to the control values.

Studies employing AAS revealed that Cd is accumulated highly in the liver and to some extent in the heart tissues (Yiin et al., 2000) of Cd-treated rats which may be due to its low excretion and long half-life (more than 20–30 years) (ASTDR, 2005). Such a speculation can be supported because Cd is known to accumulate in the tissues either by forming a cadmium-metallothionin (Cd-MT) complex (Bodar et al., 1990) or, by binding with –SH group containing compounds (Waalkes and Klaassen, 1985; Ercal et al., 2001). Interestingly, daily oral administration of aqueous bark extract of TA for 15 days prevented Cd accumulation in both the studied tissues and maintained the Cd levels near to control values. But the underlying mechanism of such response to TA remained an excellent area for future research.

Any sort of organ damage may lead to a significant elevation in the level of tissue specific marker enzymes in the serum which may be due to a massive loss of functional integrity leading to their intense leakage into the blood stream (Mitra et al., 2012; Renugadevi and Prabu, 2010). In the present investigation, a profound increase in the serum levels of hepatic and cardiac marker enzymes (SGPT, SGOT, TLDH, LDH5, LDH1, ALP, and CK-MB) in the Cd treated rats indicated the development of respective tissue injury. However, rats pre-treated with aqueous bark extract of TA significantly protected the concerned tissues, since the levels of hepatic and cardiac marker enzymes in the serum were prevented from increasing and were maintained near to the control levels. Such protective effects of TA may be due to the free radical scavenging and antioxidant properties of several phyto-constituents present in the extract. Such contention is further supported by the evidences gathered from the histopathological and histochemical studies of the hepatic and cardiac tissues. Haematoxylin-eosin stained liver tissue sections of Cd intoxicated rats showed a marked dilatation of the central vein, portal vein as well as sinusoidal congestion, mild inflammatory cell infiltration in the portal tract, enlarged nuclei, light stained cytoplasm with enlarged vacuoles and necrotic hepatocytes. Similarly, the cardiac tissue sections showed significant degeneration along with capillary dilatation, vascular congestion, and myocardial fibernecrosis. On the other hand, Cd-treatment resulted in significant deposition of collagen proteins in the hepatic tissue sections and depletion in the cardiac tissue sections following picrosirius red staining, clearly indicating the induction of fibrosis in both the tissues. However, cadmium induced morphological and architectural damages in both the tissues were found to be restored compared to their control following the pretreatment of aqueous bark extract of TA, possibly through the scavenging of Cd-induced free radicals.

Glutathione is a tripeptide, ubiquitous and cysteine rich non-protein thiol compound which mainly protects the cell against heavy metal induced oxidative stress (Meister, 1988). It is well documented that Cd can bind to sulfhydral (-SH) group containing antioxidants, leading to down regulation of such proteins (Klug et al., 1988). Our results revealed a significant reduction in both GSH and total thiol content in the liver and heart tissues of Cd-treated rats, possibly due to formation of cadmium-glutathione complex (Ranas and Verma, 1996). However, Cd induced reduction in the levels of GSH and TSH were found to be prevented following treatment with aqueous bark extract of TA thereby helping to overcome the intracellular oxidative stress.

Cd-induced ROS mainly inhibits the mitochondrial electron transport chain due to the accumulation of various ROS in variable amounts such as hydrogen peroxide, hydroxyl radical, superoxide anions, singlet oxygen and peroxides, etc. (Wang et al., 2004). Our results also revealed a profound increase in the levels of LPO and PCO (bio-markers of oxidative stress) in both hepatic and cardiac tissues following Cd treatment, which might be due to the inhibition of several antioxidant enzymes. The levels of both oxidative stress biomarkers (LPO and PCO) were found to be significantly protected from being altered when administered with aqueous bark extract of TA, possibly due to its free radical scavenging property.

The antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) actively participate in antioxidant defense mechanisms to protect the cell against oxidative stress (Wang et al., 2004). The metalloenzyme SOD mainly catalyzes the dismutation of superoxide anion radicals (McCord, 1987). Catalase-a hemeprotein, protects the cell by removing H_2O_2 from oxidative tissue damage by catalyzing the conversion of hydrogen peroxide to water (Chance et al., 1952). In the present study, the SOD activity (both Cu-Zn SOD and Mn-SOD) revealed a profound increase in the hepatic tissues and a significant reduction in the cardiac tissues following the treatment of Cd. The reduced activities of both the SODs in the cardiac tissues may be due to an inactivation of SODs, either by Cd induced lipid peroxidation or, directly through Cd-enzyme interaction resulting in disruption of enzyme structure which is important for its catalytic function (Obioha et al., 2009). The decreased SOD protein level as evident from the Western blot analysis demonstrates that decreased activity of this key antioxidant enzyme may be the result of Cd induced downregulation of the SOD protein (Yalin et al., 2006). Noteworthy, the excessive accumulation of Cd may have induced the level of H_2O_2 within the hepatic tissue. In order to combat such stressful environment within the hepatocytes, protein levels of both the isoforms of SOD might have been upregulated, resulting in an enhancement of their activities (Ognjanović et al., 2008). In our study, an increase in the catalase activity in heart tissue following Cd administration may be due to generation of excessive superoxide anion radicals resulting in the formation of H_2O_2, which may have inactivated the activity of catalase (Shaikh et al., 1999). On the other hand, decreased catalase activity in the liver tissues of Cd treated rats may be due to inactivation of catalase through antagonistic effect of cadmium with iron (Fe), which is an important cofactor for its activity (Obioha et al., 2009). Cd induced reduction in the activity of catalase might be due to the downregulation at its protein level in the liver tissues. Similarly, upregulation of the protein level of catalase might be the possible cause of its increased activity in the heart tissue following the treatment of Cd. Interestingly, in the present in vivo study, implication of aqueous bark extract of TA at a dose of 20 mg/kg bw restored the activities of the antioxidant enzymes to its normal level. We have also found that when cytosolic and mitochondrial samples of hepatic and cardiac tissues were incubated with Cd at a concentration of 100 μM in an *in vitro* system, an uncompetitive inhibition in the activities of catalase in liver tissue samples and both the isoforms of SOD (Cu-Zn SOD and Mn-SOD) in cardiac tissue samples were observed. Thus, it can be suggested that the observed decrease in the enzyme activities in the in vivo experiment is supported by the results of our *in vitro* experiment.

GPx oxidizes GSH to GSSG by utilizing H_2O_2 as its substrate. In our present study, GPx activity was found to be increased in both liver and heart tissues in Cd intoxicated rats which may be due to decrease in the level of GSH either by binding with -SH group or by decrease in the activity of GR. Another antioxidant enzyme, GR, mainly reduces GSSG to GSH and thus serves as an important antioxidant in defending the cells from harmful free radicals. In our study, GR activity was found to be reduced significantly in hepatic and cardiac tissues in Cd treated rats which may be due to the formation of Cd-SH complex (Gerson and Shaikh, 1984). The activities of all these antioxidant enzymes were found to be restored to their control levels when the rats were pretreated with aqueous bark extract of TA.

Mitochondria are the crucial intracellular target for heavy metal induced ROS (Nigam et al., 1999). The mitochondrial electron transfer chain is also a well-known source of H_2O_2 (Cadenas and Davies, 2000; Wang et al., 2004). In present study, the reduced activities of PDH and some of the Krebs cycle enzymes (ICDH, α-KGDH and SDH) in Cd treated group were found which may be due to the generation of hydrogen peroxide in both hepatic and cardiac tissues. We have also found that when mitochondrial samples of hepatic and cardiac tissues were incubated with Cd at a concentration of 100 μM in an *in vitro* system, an uncompetitive inhibition in the activities of PDH, ICDH, α-KGDH and SDH in both the tissues were observed. Thus, it can be suggested that the decrease in the enzyme activities in our in vivo experiments were supported by the observations of our *in vitro* experiments. Earlier researchers have also reported that Cd significantly inhibits uncoupler-stimulated oxidation ofvarious NADH-linked substrates like succinate (Miccadei and Floridi, 1993). Cd markedly inhibits the activity of complex II and complex III more than that of the other complexes in the mitochondrial electron transport chain by disturbing the mitochondrial phospholipid membrane and thereby retarding its effects by the generation of superoxide anion radical (Wang et al., 2004). Moreover, mitochondrial complex III is considered as the principle site for the generation of ROS in the presence of Cd. Thus, in our present study, the activities of NADH cytochrome c reductase and cytochrome c oxidase were also found to be reduced significantly in both liver and heart tissues following the treatment of Cd. The activities of all these ETC

enzymes in liver and heart tissues were found to be restored to their control levels when the rats were pre-treated with aqueous bark extract of TA.

Reports are available showing that mitochondrial membrane potential depends primarily on the ETC generated proton gradient in the inner mitochondrial membrane (Abe and Itokama, 1973). Therefore, Cd induced alterations in the activity of the enzymes of citric acid cycle and respiratory chain enzymes may affect the permeability of proton (H^+) in the inner mitochondrial membrane, which causes a breakdown in its electrochemical gradient. In our present study, Cd-induced elevation of membrane depolarization was observed in the mitochondrial samples of both isolated liver and heart tissues. The Cd-induced alterations in the membrane potential in liver and heart tissues were found to be protected from being altered when the rats were pre-treated with aqueous bark extract of TA which may be attributed to its free radical quenching and antioxidant capacity.

Overall oxidative damage could explain to a large extent Cd cytotoxicity on mitochondrial architecture and function. Moreover, SEM studies of the mitochondrial membrane surface of both liver and heart tissues were confirmed by observing Cd-induced membrane blebbing which is an indication of initiation of apoptosis (Mukherjee et al., 2012). On the other hand, administration of aqueous bark extract of TA protected the mitochondrial surface from being damaged, which may be due to the reduction in the level of apoptosis by its cytoprotective efficacy (Mittal et al., 2017). However, membrane potential and membrane architectural changes observed in Cd treated liver and heart mitochondrial samples also supported our biochemical findings at tissue level.

Xanthine dehydrogenase (XDH) and xanthine oxidase (XO), both are single-gene product but exist separately as inter-convertible forms. Under normal condition, both XO and XDH act on the same substrate-hypoxanthine or, xanthine, by using molecular oxygen to produce superoxide and uric acid (Hae et al., 1997). Xanthine oxidoreductase (XOD), another form of XDH uses NAD^+ to produce NADH instead of superoxide. However, XDH under ischemic condition is converted into XO due to the accumulation of hypoxanthine and reoxygenation; thereby playing an important role in contributing free radical mediated damage by stimulating the production of superoxide anion free radical (Hae et al., 1997). Our studies reveal that Cd administration may have produced considerably large amounts of superoxide anion free radical which is reflected in the observed increased levels of XO and XDH activities in both the hepatic and the cardiac tissues of rat. Increased activities of both the enzymes were restored to the control levels, when the animals were pre-treated with aqueous bark extract of TA.

In mammals, Cd interrupts the DNA repair system by inhibiting the expression of vital antioxidant enzymes and thereby increasing intracellular ROS production (Pruskiand Dixon, 2002). In our study, the increased levels of DNA strand breaks were observed in both liver and heart tissues in Cd treated rats. However, aqueous bark extract of TA protects from Cd induced DNA damage, whichmay be due to the detoxification of toxic radicals through the antioxidant and free radical quenching properties of TA.

Therefore, our results strongly suggest that pre-treatment of rats with aqueous bark extract of TA at a dose of 20 mg/kg bw (orally) significantly ameliorated Cd (0.44 mg/kg bw, s.c.,) induced oxidative stress mediated tissue damages through its antioxidant mechanisms; the various bioactive constituents may act as direct as well as an indirect antioxidant in addition to their possible role as metal chelators. The findings may have future therapeutic relevance.

Conflicts of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.008.

Transparency document

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