Journal of Cardiology 63 (2014) 449–459

Contents lists available at ScienceDirect

Journal of Cardiology

journal homepage: www.elsevier.com/locate/jjcc

Original article

Alcohol stress on cardiac tissue – Ameliorative effects of *Thespesia populnea* leaf extract

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ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form
25 September 2013
Accepted 9 October 2013
Available online 2 December 2013

Keywords:
Alcohol
Free radicals/scavengers
Cardiomyopathies
Antioxidants

ABSTRACT

Background: Cells are naturally equipped with antioxidant defenses to counterbalance free radical production. Overproduction of free radicals is one of the reasons for a variety of diseases. The current investigation was planned to evaluate chronic alcohol- (for 30 days) induced oxidative stress in the cardiac tissue of rat and to explore the effectiveness of *Thespesia populnea* (TP)-induced cardio-protection in rat heart by utilizing an *in vivo* model of cardiac injury by alcohol.
Methods: Ten groups of rats were maintained and were divided into different groups. Alcohol 20% was administered and *Thespesia* leaf extracts (TPE) were administered at a dose of 250 mg/kg to chronic alcoholic rats for 30 days. The heart tissue was isolated and processed for further analysis, and also blood for estimation of blood alcohol level and serum creatine phosphokinase (CPK). The activities/levels of antioxidant enzymes, malondialdehyde (MDA), and protein carbonyls (PC) were estimated using established protocols. Histopathology was performed as evidence for the work and to establish the results.
Results: Activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and reduced glutathione content (GSH) showed a decrease, while glutathione-S-transferase (GST) activity, MDA, and PC recorded an elevation due to alcohol treatment in the cardiac tissue compared to the control rats. Alcohol-induced myocardial injury was observed, indicated by a significant increase in serum CPK, a well-known biomarker of myocardial injury, and histopathological evidence supported these observations by revealing predominantly extensive edema with vacuolization and severe necrosis.
Conclusion: Treatment with TPE confers protection on the heart tissue during alcohol-induced oxidative stress, and thereby minimizes oxidative damage to the cardiac tissue as clearly marked in histopathology.

Introduction

Alcohol, one of the potential causative agents along with cigarette smoke forms the main agent for multifarious disorders in humans. At present the pathogenesis of this damage is unclear, despite much having been published about the deleterious effects of alcohol [1]. Oxidative damage, inflicted by excessive reactive oxygen species (ROS), is considered an important pathophysiological condition, promoting cell injury and death in various disorders [1]. Alcoholic cardiomyopathy develops as a result of chronic high-dose alcohol consumption and nutritional deficiency in the rat model [2]. Acute as well as chronic toxic effects of alcohol may result in irreversible organ damage [3]. Oxidative stress derived from alcohol metabolism has been a major focus in the study of alcohol-induced tissue injury. The metabolism of alcohol produces ROS detrimental to the cellular antioxidant defense system [4], causing cell injury [5]. The response of the body to chronic or acute administration of ethanol has been shown to result in generation of oxygen-derived free radicals to cause alterations in cardiac muscle [6]. Alcohol abuse has been linked as a risk factor to various cardiovascular diseases, digestive tract disorders, and cancers [7]. Ethanol has been shown to interfere with a number of myocardial metabolic steps and cellular mechanisms [8]. The cardiovascular system is a major target for ROS. It has been observed that ROS play an important role in the onset of cardiac toxicity in chronically ethanol-intoxicated animals, causing

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http://dx.doi.org/10.1016/j.jjcc.2013.10.015

alterations in cardiac muscle [6]. Certain chronic diseases such as acute cardiomyopathy (ACM) and other heart diseases [9–11], may develop following chronic alcohol ingestion and contribute to alcoholism-related high morbidity and mortality.

The therapeutic benefit of medicinal plants is often attributed to their antioxidant properties [12]. Many plant species have been investigated for their natural antioxidants [13], novel antioxidants [14], and free radical scavenging properties [15]. However, there is still a demand to find more information concerning the antioxidant potential of plant species. In recent years, considerable attention has been directed toward the identification of plants with antioxidant ability that may be used for human consumption. Therefore, research has focused on the use of antioxidants, with particular emphasis on naturally derived antioxidants, which may inhibit ROS production and may display protective effects [16].

Thespesia populnea (TP) of the Malvaceae family is a large tree found in tropical regions and coastal forests of India. The leaves, flowers, and fruits are useful in treating cutaneous infections such as scabies and psoriasis. The decoction of the leaf is commonly used for the treatment of skin and liver diseases. Oil of the leaf mixed with vegetable oil is useful in urethritis, gonorrhea, as an astringent, and for hepatoprotective and antioxidant activities [17,18]. Many chemical compounds and herbal formulations have been studied for their antioxidant activity in heart tissue by using *in vivo* models such as isoproterenol-induced myocardial infarction [19,20] and cisplatin-induced nephrotoxicity [21].

Although various anti-oxidative dietary supplements have been evaluated for their ability to prevent atherosclerosis, no effective ones have been determined [22]. Whether oxidative stress and abnormalities in the cardiac tissue produced by chronic doses of alcohol can be convalesced with TPE has not been established to date. This study was undertaken to examine the effect of chronic alcohol exposure on the heart and to evaluate the role of oxidative stress in alcohol-induced myocardial injury and the possibility of reversing the effects with TPE.

Methods

Procurement and maintenance of experimental animals

Wistar strain male albino rats ($n = 60$) weighing 180 ± 20 g were obtained from the Indian Institute of Sciences, Bangalore. The rats were housed in clean polypropylene cages, maintained in a temperature-controlled room ($25 \pm 2\,^{\circ}C$) with a photoperiod of 12 h light and 12 h dark cycle. The rats were pair fed with standard pellet diet and water *ad libitum*. This study was approved by the Institutional Animal Ethics Committee (Regd. No. 438/01/a/CPCSEA/dt.17.07.2001) in its resolution number 15/IAEC/SVU/2001/dt. 04.03.2008).

Chemicals

All the chemicals used in the present study were AnalaR grade and obtained from the following scientific companies: Sigma (St. Louis, MO, USA), Fisher Scientific (Pittsburg, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), and Qualigens (Mumbai, India). Vitamin E was used as a reference standard.

Standard antioxidants

The standard antioxidants used in this study were ascorbic acid, α-tocopherol, butylated hydroxy anisole (BHA), and butylated hydroxy toluene (BHT). Ascorbic acid was dissolved in double distilled water. BHA and BHT were dissolved in methanol.

Selection of plant material

Fresh leaf of TP was collected. The plant material was taxonomically identified and authenticated by the Department of Botany, Sri Venkateswara University, Tirupati. A voucher specimen (SVUB-H-No: 728) was deposited in the herbarium of the Department for future reference. Leaf was thoroughly cleaned and dried under shade. Sufficient quantity of dried leaf was crushed, coarsely ground, and powdered in an electric grinder, sieved to obtain 100 g of fine brown powder as the final weight and used for extraction. Accordingly, 95% ethanol (polar), ethyl acetate (intermediate polar), and water for aqueous extracts were selected. All the three solvents, namely ethyl acetate, 95% ethanol, and water were used in cold percolation for 24 h, and the solvent was filtered using layers of moist muslin cloth. The extract was recovered, and ethyl acetate, 95% ethanol, and water were added to its respective leaf powder and the extraction was continued. This process was repeated three or four times until the extract was rendered colorless. The extract was distilled and concentrated under reduced pressure in a Buchi rotovapour (R-114; Buchi, Flawil, Switzerland), yielding a dark color residue, and dried in a vacuum desiccator to remove any remaining water and were kept under refrigeration. Ethyl acetate, ethanol, and water yielded 12.897%; 10.019%; and 12.679% of extracts, respectively. Required quantity of ethyl acetate (EA-E), ethyl alcohol (Et-E), and aqueous extracts (AQ-E) of TP leaf were suspended in 5% gum acacia at a concentration dose equivalent of 250 mg/kg calculated according to weight, and used in all experiments [23].

Determination of TP leaf extract dose concentration

Earlier reports on TP suggested different dosages of TP extracts of different parts for different experimental designs/protocols [24,17,25–28]. We wanted to determine the effective dose of TPE for alcohol-induced toxicity, as this is the first work reporting the effectiveness of TPE on alcohol-induced toxicity in rats. In the current study, dosage-dependent studies were done, to select the effective dose to counter alcohol-induced stress. TPE (50,100, 150, 200, 250, 300 mg/kg body wt.) were tested for their effectiveness by *in vivo* studies in Wistar rats for 30 days, with 6 rats per dosage group. We estimated SOD, CAT, and GPx activities in heart tissue of normal control, alcohol group, TPE control, and alcohol + TPE. Thus, in our study we found that 250 mg/kg of TPE effectively upregulated all the antioxidant enzymes in alcohol-induced groups. Hence we concluded that 250 mg/kg would be an adequate and effective dose for alcoholic subjects. Therefore, the concentration was determined at 250 mg/kg body weight and administered to rats.

Treatment protocol

The rats were divided into 10 groups of 6 animals each, and treatment was given every day perorally (p.o.) using an orogastric tube, for 30 days. Chronic alcohol dosage was administered to the rats and the selection of dose of alcohol is based on the reports of Husain et al. [29]. Alcohol was administered first and then was followed by the extracts for the IV, VI, VIII, and X groups in difference of 10 min.

Group I ($n = 6$): Rats received 5% acacia gum only (5 ml/kg per day p.o.) for 30 days and served as normal controls (NC).
Group II ($n = 6$): Rats received only 20% alcohol (AL) (Hayman, Witham, Essex, UK).
Group III ($n = 6$): Rats were given only ethyl acetate leaf extract (EA-E).

Group IV ($n = 6$): Rats were treated with alcohol and ethyl acetate leaf extract (AL + EA-E).
Group V ($n = 6$): Rats received only ethyl alcohol leaf extract (Et-E).
Group VI ($n = 6$): Rats were treated with alcohol and ethyl alcohol leaf extract (AL + Et-E).
Group VII ($n = 6$): Rats were treated with AQ leaf extract (AQ-E).
Group VIII ($n = 6$): Rats received alcohol and AQ leaf extract (AL + AQ-E).
Group IX ($n = 6$): Rats received drug (vitamin E) and served as drug control or reference control (Drg).
Group X ($n = 6$): Rats received alcohol and vitamin E (25 mg/kg per day p.o.) (AL + Drg).

Isolation of tissues

After the completion of 30 days of treatment, the rats were killed 2½ h after the last dose of ethanol and other extracts, under light ether anesthesia, they were killed by cervical dislocation. The hearts were dissected out immediately, washed with ice-cold saline placed on a chilled glass plate. One part was used to prepare homogenate for further biochemical analysis and the remaining part of heart was fixed in 4% buffered paraformaldehyde and used for histological studies.

Histopathological studies

The heart tissue was fixed for 48 h in 4% paraformaldehyde, dehydrated by passing successively in different mixtures of ethyl alcohol–water, cleaned in xylene, and embedded in paraffin. Sections of heart (5 μm thick) were prepared, stained with hematoxylin and eosin, and mounted using neutral DPX mountant for microscopic observation. Slides mounted with sections were later visualized under Leica DCM 6000 inverted microscope (Leica Microsystems, Wetzlar, Germany), and the histopathological study was done under blinded conditions.

Biochemical assays

Isolation of subcellular organelles such as mitochondria was done using the methods of Cotman and Matthews [30], Dodd et al. [31], and Kodavanti et al. [32]. These fractions were used for the assay of marker enzymes. Activities of all the antioxidant enzymes were estimated in mitochondrial fraction of heart tissue.

Hepatic and renal SOD activity was assayed by the method of Misra and Fridovich [33] at 480 nm for 4 min on a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan). The activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 U per milligram of protein. CAT activity was determined by using the method of Aebi [34] and the absorbance of the sample was measured at 240 nm for 1 min in a UV-spectrophotometer. The activity of GPx was determined by the method of Flohe and Gunzler [35] in the presence of NADPH and the absorbance was measured at 340 nm using cumene hydrogen peroxide. GR enzyme activity was determined according to the method of Carlberg and Mannervik [36]. The concentration of GSH in heart subcellular fractions was measured as described by Akerboom and Sies [37]. The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product MDA by using the method of Ohkawa et al. [38]. All enzyme activities were expressed per mg protein and the tissue protein was estimated according to the method of Lowry et al. [39] using bovine serum albumin (BSA) as the standard. Markers for oxidative stress such as PC in heart tissue were estimated as given by Fagan et al. [40] and Levine et al. [41].

Blood alcohol estimation assay

Blood samples were taken from the retro-orbital plexus. Blood alcohol levels (BALs) were measured on the 1st, 10th, 20th, and 25th day. On the last day (30th day), 2½ h after alcohol administration, the blood samples were collected into heparinized tubes and immediately stored at 4 °C and BALs were measured using the alcohol dehydrogenase kit (Sigma–Aldrich; procedure 332-UV) according to the manufacturer's instructions.

Creatine phosphokinase (CPK) activity

Serum obtained from the blood collected from the retro-orbital plexus was used for the assay. In reference to the assay method as described for CPK activity in serum [42], a kit was used for the estimation of serum CPK (CK-20; Sigma Chemical Co.).

Phytochemical screening

The extract was subjected to preliminary screening for various active phytochemical constituents with possible antioxidant activity such as alkaloids, steroids, tannins, phenols, flavonoids, terpenoids, cardiac glycosides, saponins, tannins, phlobatannins, steroids, and anthraquinones [43,44].

Thinlayer liquid chromatography (TLC) screening for free radical scavenger or antioxidants

Samples of 100 μg (10 μl of a 10 mg/ml solution) of different extracts of TP leaves were spotted on silica coated TLC silica gel plates (Sigma) with a micropipette, and the solvent was allowed to travel for 8–9 cm in a tank containing the eluent (chloroform, ethyl acetate, formic acid, methanol) in a chromatography tank [45]. The plates were removed from the tank, air-dried, and then viewed under UV light at 254 and 366 nm, respectively. The dried TLC plates were then sprayed with a 0.1% (w/v) solution of the stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. Spots with antioxidant activity inhibit free radical effect of DPPH, thus leading to deep yellow color on a purple background on the TLC plates. The antioxidant activity of the various spots was monitored based on the color changes observed with time in seconds on the TLC plates which was used to distinguish components of plant extracts with potential antioxidant or radical-scavenging properties [46–48].

Free radical scavenging assays

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of TP was determined by the method of Halliwell et al. [49]. The reaction mixture consisted of 1 mM EDTA, 10 mM $FeCl_3$, 10 mM H_2O_2, 10 mM deoxyribose, 1 ml of different dilutions of the extract (50–250 lg/ml), 50 mM phosphate buffer (pH 7.4), and 100 μl (1 mM) of ascorbic acid in sequence. The mixture was incubated at 37 °C for 1 h. A 1 ml portion of the incubated mixture was mixed with 1 ml of 10% TCA and 1 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm.

Scavenging of hydrogen peroxide

The ability of TP to scavenge hydrogen peroxide (H_2O_2) was determined according to the method of Ruch et al. [50]. A solution of H_2O_2 (2 mM) was prepared in phosphate buffer (0.1 mM, pH 7.4) at 20 °C. An aliquot of the extracts was dissolved in phosphate buffer (1 ml; 0.1 mM, pH 7.4) at various concentrations (50–250 lg/ml) and mixed with 600 μl of H_2O_2. BHT and ascorbic acid were used as the

reference compounds. The concentration of H_2O_2 was measured by reading the absorbance at 230 nm after 10 min against a blank solution containing phosphate buffer without H_2O_2. The extracts were capable of scavenging H_2O_2 in a concentration-dependent manner.

Superoxide anion scavenging activity

The superoxide anion ($O_2^{\bullet -}$, superoxide) scavenging activity of TP was determined by the method of Nishikimi et al. [51] with slight modifications. The superoxide anion was generated using two non-enzymatic systems. The first one consisted of 630 lM of nitrobluetetrazolium (NBT), 30 lM of phenazine methosulfate, and 156 lM NADH in 0.1 M phosphate buffer, pH 7.4. The product of the reduction of NBT was followed spectrophotometrically at 560 nm as an index of the superoxide anion production.

Statistical analysis

Values are expressed as mean ± SD from 6 animals. All the statistical analyses were carried out using SPSS statistical tool (SPSS for windows, release 17.0.1, 2008, SPSS Inc., Chicago, IL, USA). Dunnett's multiple comparison test and one-way analysis of variance (ANOVA) were used to assess the differences. All p-values of <0.05 were considered statistically significant.

Results

Phytochemical screening

The preliminary phytochemical screening showed the presence of steroids, triterpenoids, tannins, phenols, phlobatannins, flavonoids, in the ethyl acetate and ethanol extract, alkaloids, anthroquinone, glycosides, catechins, fixed oils and fats, furanoids, proteins, phenolics, and saponins in the aqueous extract.

Free radical scavenging analyses

The free radical scavenging effect of EA, Et, and AQ leaf extracts of TP, assessed using hydroxyl radical scavenging activity, superoxide anion scavenging activity, and scavenging of H_2O_2 is shown in Fig. 1. All extracts demonstrated remarkable free radical scavenging effects with considerable activity when compared with standard antioxidants ascorbic acid and butylated hydroxytoluene.

Blood alcohol levels

At 2½ h after the last alcohol dose was given perorally using a gavage, the BALs in the chronic alcohol-treated rats were measured in comparison with controls. A significant rise in the BALs 2½ h after ethanol administration was observed. The effect of TPE, administered post-alcohol dose, showed that the BALs were reduced due to the shielding effect of the leaf extracts, as shown in Fig. 2A.

Creatine phosphokinase (CPK) activity

To estimate the overall heart damage by chronic alcohol exposure and the effect of leaf extracts, CPK activity was measured after the last alcohol dose administration. Chronic alcohol administration caused a significant increase in the serum CPK activity (Fig. 2B) and this elevation was significantly suppressed by TPE. Myocardial damage was evident with increase in the CPK activity in the serum of the alcohol-treated rats. These alcohol-induced changes were all inhibited in the rats treated with TPE prior to alcohol dosage.

Histopathology results on heart tissue for all treatments (Control group to Drg + Al group)

The control tissue showed normal blood vasculature, and absence of myofibrillar necrosis. However, the heart tissue in

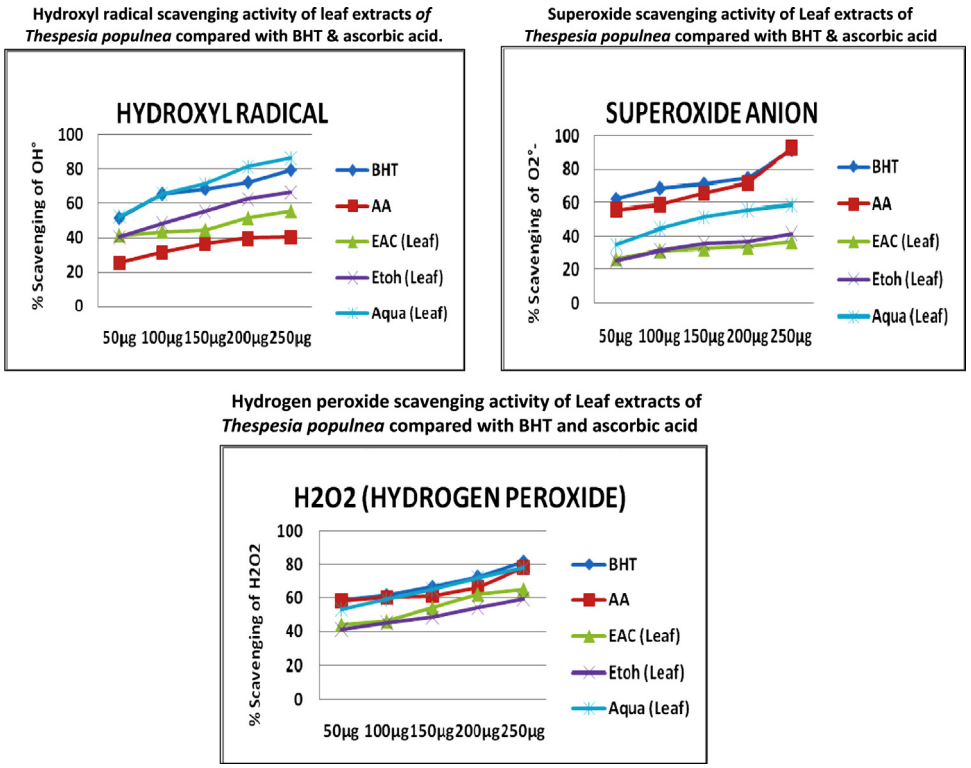

Fig. 1. Free radical scavenging activity – leaf. Free radical scavenging analyses on the ethyl acetate, ethanol, and water extracts with butylated hydroxytoluene and ascorbic acid as standards (data expressed as average [n = 6]).

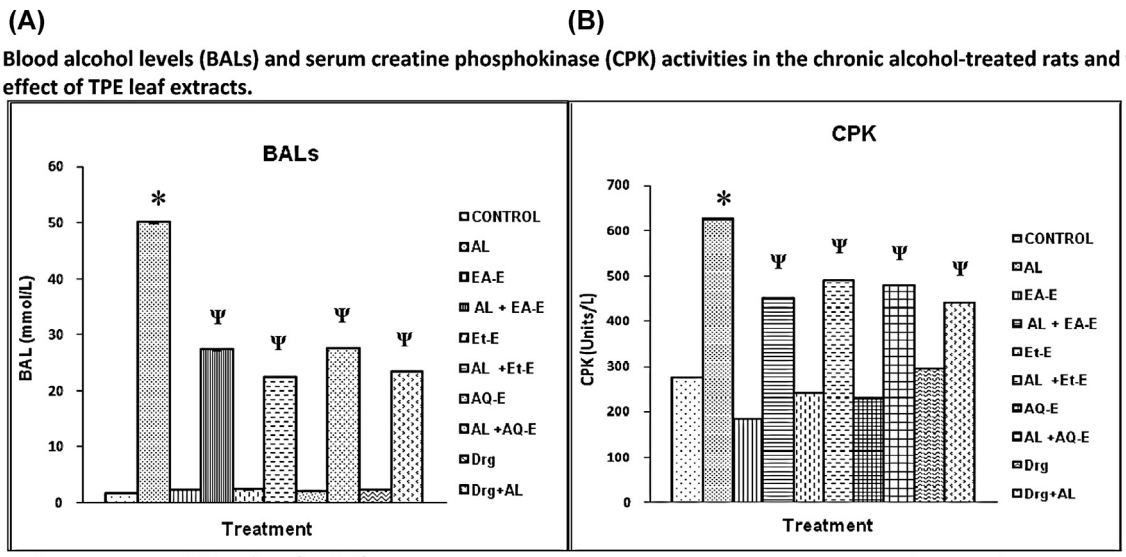

Fig. 2. (A) Blood alcohol levels and (B) serum creatine phosphokinase activities in the chronic alcohol-treated rats and the effect of *Thespesia* leaf extracts.

alcohol-treated rats showed disrupted blood vessels, vacuolization, and severely damaged myofibrils (Fig. 3). Rats treated with EA, Et, AQ leaf extracts showed normal architecture, spindle-shaped nucleus with clear cytoplasm and mild granular appearance of cytoplasmic organelles. Alcohol groups treated with EA, Et, AQ leaf extracts after alcohol administration showed normal architecture except mild areas of congestion, which confirmed the effects of TPE. Only the vitamin E treated group showed normal cardiac architecture and normal myofibrils with clear nuclear details. Vitamin E treatment after alcohol administration showed normal architecture except mild areas of congestion and spindle-shaped nucleus.

Impact of ethyl acetate, ethanol, and water extracts on antioxidant enzymes of heart

Heart antioxidant enzyme activity (SOD, CAT, GPx, GR, and GST) was significantly decreased by alcohol toxicity, compared to controls. The EA leaf extract showed constant effects on all the enzymes when compared to the effects of Et and AQ leaf extracts. The effect of Et extract was higher in SOD and GR when compared with the EA and AQ leaf extracts (Figs. 4–8). The reference drug control showed considerable changes, when treated for alcohol toxicity. SOD, CAT, GR, and GST activities were higher with Al + Dg than with Et and AQ.

Impact of ethyl acetate, ethanol, and water extracts on antioxidant stress markers of heart

With reference to marker enzymes GSH and MDA, the levels of GSH activity significantly decreased while MDA levels increased in rats treated with alcohol when compared with controls (Figs. 9 and 10). The MDA and GSH activity levels were recovered after treatment with extracts. The AQ leaf extract had a slightly higher effect on GSH when compared with EA and Et extracts, with EA slightly higher than AQ. The restoration of the MDA levels was negligible among all the extracts compared to the controls, which reflects amelioration of the antioxidant enzyme system to normalcy. The drug control showed a significant increasing effect in ameliorating GSH and MDA activities in alcohol-treated rats.

Impact of ethyl acetate, ethanol, and water extracts on sensitive marker of oxidative injury (protein carbonyls, PC) in heart

PC, a responsive marker of oxidative injury, was higher when compared to the controls. However, when treated with leaf extracts after treatment with alcohol (AL) dosage, restitution to normal levels was observed. AQ-E extract showed an increase when compared to EA and Et (Fig. 11). Although the drug control had a lower effect compared to the extracts, it also showed a marked difference in reducing the alcohol effects.

Discussion

Oxidant balance in the heart has an important role in protecting the heart and in allowing normal cardiac contractile performance. In general, the amount of antioxidants in the heart is sufficient to protect it from any oxidant production that might occur under normal circumstances [52]. *In vitro*, the overproduction of free radicals or an impaired antioxidant function depresses myocardial contractility, causes myocardial tissue injury, and induces myocyte apoptosis [53]. Reduction of heart anti-oxidative capacity and morphological changes have been observed in animals chronically intoxicated with ethanol [54,55]. Reactive oxygen species, generated from ethanol metabolism, have been indicated to play a role in the onset of heart disturbances in ethanol-intoxicated animals [56,57]. Antioxidants may have a protective role in these conditions. Edés et al. [54] have reported the protective effect of antioxidants against myocardial lipid peroxidation in rats after chronic alcohol ingestion.

Antioxidant supplements, medicinal plants, or foods rich in antioxidants, may be used to help the human body in reducing oxidative damage by free radicals and active oxygen [58]. Many plant extracts and plant products have been shown to have significant antioxidant activity [59], which may be an important property of medicinal plants associated with the treatment of several diseases including cholestasis [60]. Thus, herbal plants are considered a useful means to prevent and/or ameliorate certain disorders, such as cardiomyopathy, diabetes, atherosclerosis, and other complications [43]. The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD,

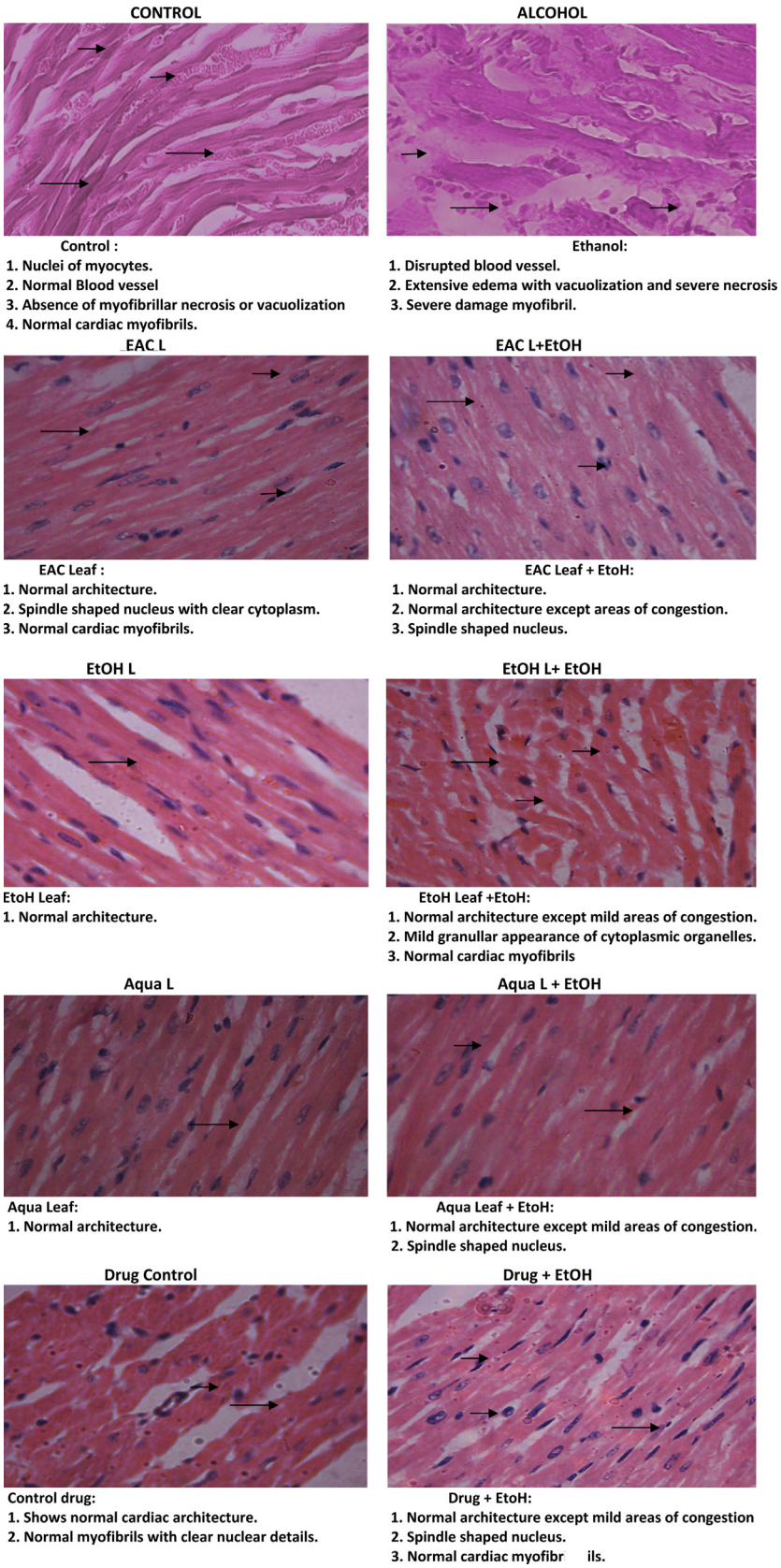

Fig. 3. Leaf-histopathology – heart.

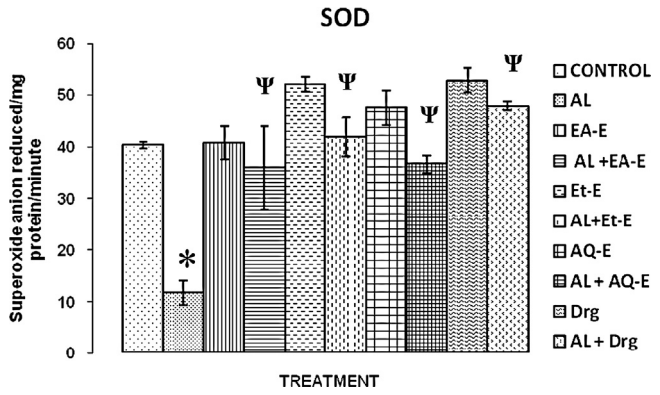

Fig. 4. Result of ethyl acetate, ethanol, and water extracts on superoxide dismutase activity in cardiac tissue of normal and experimental rats.

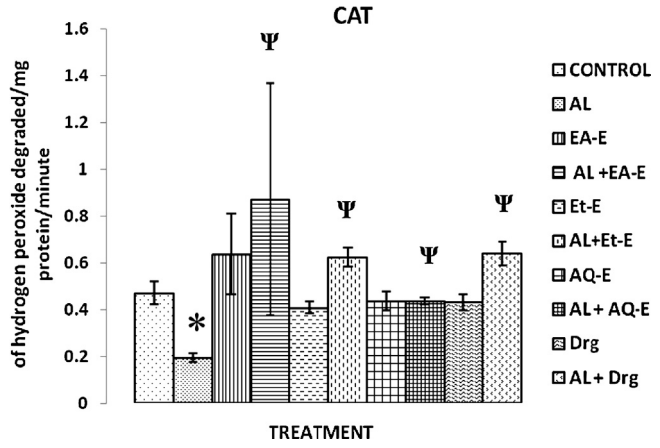

Fig. 5. Result of ethyl acetate, ethanol, and water extracts on catalase activity in cardiac tissue of normal and experimental rats.

CAT, GRD, and GPx. These enzymes constitute a mutually supportive team of defense against ROS [61].

TP leaf extracts were assessed for their potential antioxidant and free radical scavenging activity. It proved to be a noteworthy marker for evaluating the antioxidant value in the leaf extracts. Analysis of free radical scavenging ability of the leaf extracts provided an uncomplicated, quick, and expedient method to assess antioxidants and radical scavengers. Superoxide scavenging activity and H_2O_2 scavenging activity demonstrated BHT and AA to

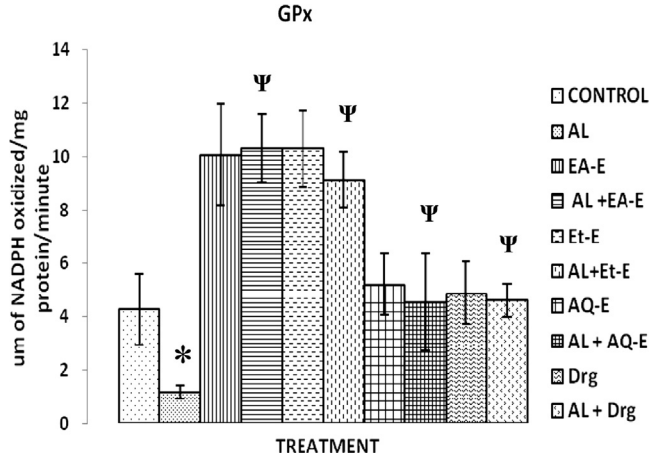

Fig. 6. Impact of ethyl acetate, ethanol, and water extracts on glutathione peroxidase activity in cardiac tissue of normal and experimental rats.

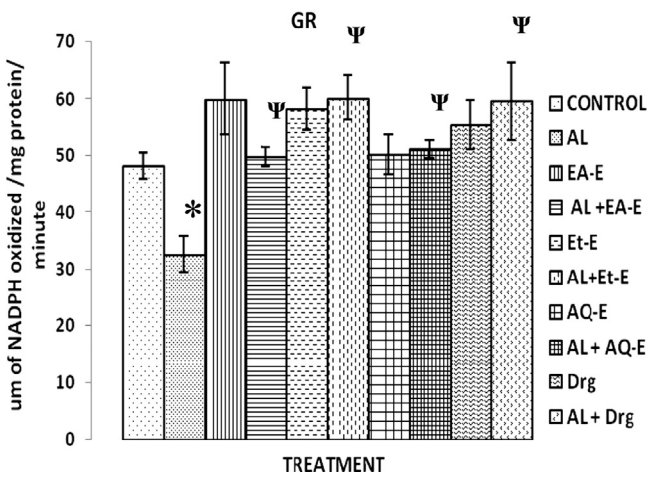

Fig. 7. Impact of ethyl acetate, ethanol, and water extracts on glutathione reductase activity in cardiac tissue of normal and experimental rats.

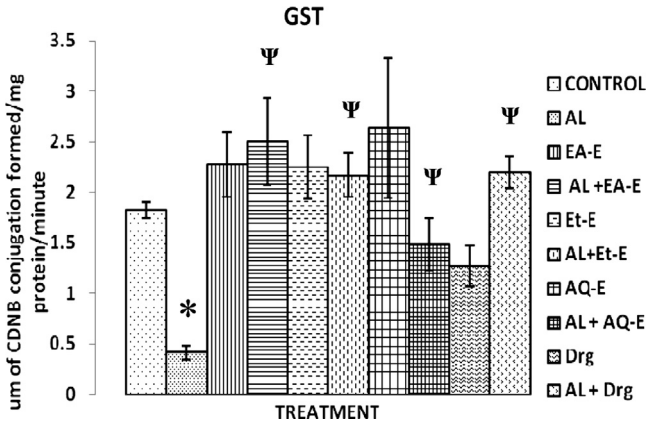

Fig. 8. Impact of ethyl acetate, ethanol, and water extracts on glutathione-S-transferase activity in cardiac tissue of normal and experimental rats.

be higher in activity compared to the effects of extracts. But in hydroxyl radical scavenging activity, the effect of aqueous leaf extract was found to be higher when compared to the other extracts, BHT and AA.

Pathologic changes occurring in cardiac myocytes are an important cause of cardiac remodeling [53]. The histopathological conditions due to chronic alcohol were reversed on treatment

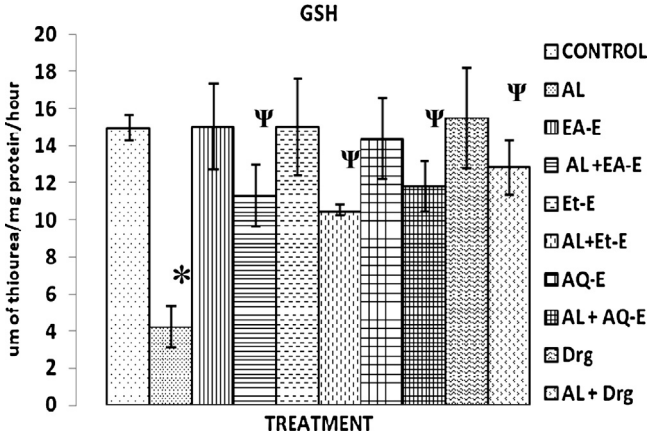

Fig. 9. Outcome of ethyl acetate, ethanol, and water extracts on reduced glutathione levels in cardiac tissue of normal and experimental rats.

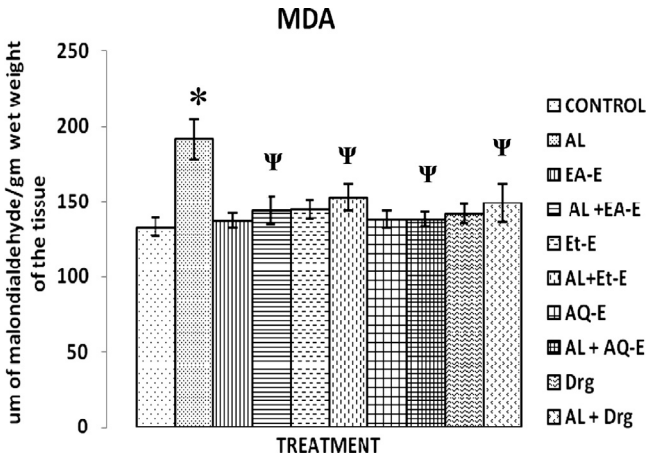

Fig. 10. Outcome of ethyl acetate, ethanol, and water extracts on malondialdehyde activity in cardiac tissues of normal and experimental rats.

with TPE. Alcohol-treatment of the rats treated with TPE aided the cardiac tissue and blood vessels to regain this normal structure. TPE minimized the damage and production of free radicals in alcohol-treated rats, and hence the cardiac tissue morphology and structural functionality were re-established from the free radical damage in these groups (Fig. 3).

In the current research work, EA, Et, and AQ leaf extracts of TP were evaluated for their antioxidant potential against alcohol-induced oxidative stress on heart tissue that have not been studied so far. EA, Et, and AQ extracts exhibited significant antioxidant activity against alcohol-induced oxidative stress in rats and also with the reference drug (vitamin E) (25 mg/kg).

The data obtained from this study demonstrate that chronic ethanol administration in rats caused significant cardiac injury, which was revealed by the elevation of serum CPK activities. This observation was substantiated by histopathological results on the cardiac tissue. Creatine kinase is used as a reliable marker in the assessment of myocardial, muscular, and cerebral damage [62]. Creatine kinase is also a better indicator of heart or muscle damage. Creatine kinases are sensitive to oxidative damage [63]. Wolosker et al. [64] showed that creatine kinase is sensitive to free radicals, especially by the oxidation of the thiol group of its structure. Creatine kinase is a reliable marker for prediction of infarct size and left ventricular function in the acute phase as well as subsequent cardiac events after acute myocardial infarction [65,66]. Oxidative stress has been regarded as one of the most important

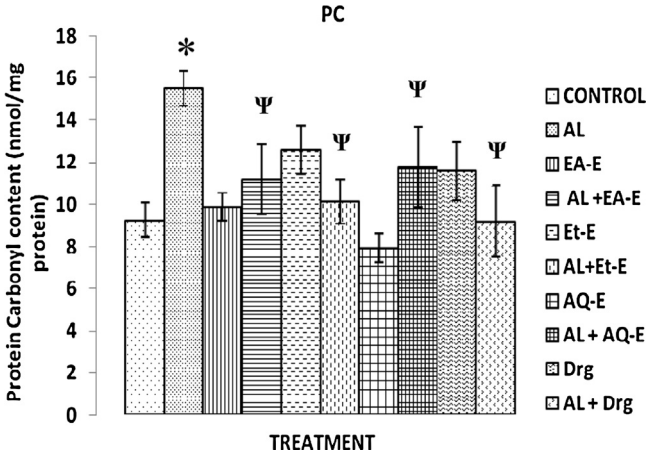

Fig. 11. Significance of ethyl acetate, ethanol, and water extracts on protein carbonyl activity in cardiac tissue of normal and experimental rats.

contributors to the progression of atherosclerosis [67]. As a natural protective mechanism, myocardial antioxidants inhibit or delay the oxidative damage that consequently prevents thrombosis, myocardial damage, and arrhythmias during acute myocardial infarction [68]. However, prolonged oxidative stress due to impaired balance between prooxidant and antioxidant mechanism may lead to lipid peroxidation and tissue damage [69].

Increased activity of serum CPK is a well-known diagnostic marker of myocardial injury and was reported to increase during alcoholic cardiomyopathy [70]. These cytosolic enzymes are released from the heart into blood stream during myocardial membrane damage [71]. However, these enzymes are not restricted to cardiac muscle tissue and their activity increases in the serum in non-cardiac tissue injuries also [72]. CPK is reported to increase in alcoholic liver injury [73] and alcoholic myopathy [74], respectively. Thus, the increased activities of these enzymes observed in ethanol-administered rats not only reflect the alcoholic myocardial injury but may also reflect the alcoholic liver and muscle injury.

SOD is considered the first line of defense against the deleterious effects of oxy-radicals in the cell by catalyzing the dismutation of superoxide radicals to H_2O_2 [75]. SOD is an important defense enzyme which catalyzes the dismutation of superoxide radicals to H_2O_2. There are also several reports that superoxide radicals damage endothelial function, and SOD acts protectively for the endothelial cells [76]. CAT and GPx are considered to be the primary antioxidant enzymes, since they are involved in the direct elimination of ROS. Under normal conditions, scavenging mechanisms operate swiftly to remove excess ROS. Superoxide ions are removed by SOD in mitochondria [77] and the resultant H_2O_2 is removed by CAT, GPx, and peroxiredoxin. In our current study, SOD activity was notably inhibited in alcohol-intoxicated rats, as reported by earlier studies [78]. Catalysis of dismutation of superoxide radicals to H_2O_2 by SOD thereby reduces the likelihood of superoxide anions interacting with NO to form reactive peroxynitrite [79]. Decrease in SOD activity due to ethanol indicates inefficient scavenging of ROS, which might be implicated to oxidative inactivation of enzyme [80]. However, the activity levels of SOD were recovered to normal levels in the TP leaf extract-treated rats with chronic alcohol stress.

CAT is a heme protein, which decomposes H_2O_2 into a nontoxic product and protects the tissues from highly reactive hydroxyl radicals. CAT is one of the most competent enzymes, and it cannot be saturated by H_2O_2 at any concentration [81]. Ethanol administration reduces CAT activity in cardiac, liver, kidney, and other tissues [82]. In consonance with the earlier findings, a similar trend was noticed in rats treated with alcohol. The decrease in the CAT activity may be attributed to the inhibition of SOD and ethanol-related loss of NADPH, or excess of H_2O_2 production, or enhanced lipid peroxidation, or a combination of these factors [83–85]. Our results are in concurrence with the above findings. The CAT activity was restored to normalcy after treatment with TP extracts, which shows the antioxidant property of the extracts against oxygen free radicals (OFR). Thus, the analysis of antioxidant status in our study indicates that enzymatic antioxidants decreased due to alcohol-induced toxicity.

GPx functions along with CAT to scavenge excess of H_2O_2 and lipid peroxides in response to oxidative stress [86]. Unlike CAT activity, GPx activity depends on the balance between the levels of glutathione and glutathione disulfide [87]. GPX activity was considerably reduced after alcohol treatment when compared to control. The observed decrease in GPx activity may also be due to reduced availability of GSH. Thus, decrease in GPx activity may be implicated in both free radical-dependant inactivation of enzyme [80] and depletion of its co-substrates, that is, GSH and NADPH [88]. In the current study the EA and Et extracts showed greater activity compared to the aqueous and vitamin E formulations, which

is in concurrence with the free radical scavenging results. The reversal of GPx activity to normal levels in the cardiac tissue with leaf extract-treatment was due to antioxidant activity by scavenging/detoxifying the endogenous metabolic peroxides generated in the heart tissue after alcohol injury. GST and GR are secondary antioxidant enzymes which help in the detoxification of ROS by decreasing the peroxide levels by GST or by maintaining a steady supply of metabolic intermediates like glutathione as by GR for the primary antioxidant enzymes. In the current investigation with TPE on oxidative stress in cardiac tissue, GR activity showed a significant decrease among heart antioxidant enzymes. GR is responsible for the regeneration of GSH, and the decrease in GR activity in heart tissue may be due to the decline in the production and availability of GSH to overcome H_2O_2 [89]. Decrease in the activity of GR in ethanol treated rats was observed when compared with control animals. A significant decrease in GR activity may be attributed to the impaired conversion of oxidized form of glutathione (GSSG) into GSH, modulating the GSH/GSSG ratio. Ethanol consumption leads to niacin deficiency (vitamin B_3), which has been shown to increase the toxicity caused by ROS [90]. The reversal of the GPx and GR activities to normalcy is apparently due to the antioxidant bioactive compounds and other detoxifying metabolic products in TPE leaf extracts.

GST plays a physiological role in initiating the detoxification of potential alkylating agents. The relatively easy inducibility of this enzyme by ethanol indicates its important role in detoxification of xenobiotics such as ethanol [91]. GST is a detoxifying enzyme that catalyzes the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic forms [92], suggesting that its action is due to oxidative stress [93]. In this study, chronic ethanol consumption resulted in a significantly higher activity of myocardial GST. Increased GSH participation in conjugation reaction mediated by increased GST activity seems to be a plausible model for GSH levels to reduce during chronic alcohol exposure [94]. Therefore, the enhancement of GST activity in the myocardium of ethanol-treated rats could be considered an adaptive response protecting the tissue against ethanol-induced oxidative damage. However, reversal of conditions caused due to alcohol toxicity was observed after treatment with EA, Et, and AQ leaf extracts.

GSH plays an important role in the maintenance of the intracellular redox state. Perturbations in the redox status and the main protective role of glutathione against oxidative stress are well elucidated by previous reports [95]. Among the endogenous antioxidant systems, reduced GSH plays multiple roles in the detoxification of toxic chemicals [96]. The levels of GSH significantly decreased in alcohol-treated rats. The GSH levels and the antioxidant enzyme activities are relatively lower in the heart than in other tissues [97]. As a result, heart tissue may be more susceptible to oxidative damage. The observed decrease in GSH levels in the alcohol-treated rats may be due to diminished activity of GR, which is a crucial enzyme for maintaining GSH/GSSG ratio in the cell. The GSH content changed after alcohol administration depending on the type of extracts used [98]. However, stress condition due to alcohol was observed to be inverted after the treatment with TPE.

Chronic or acute alcohol administration also results in alterations of mitochondrial composition and fluidity, which would impair mitochondrial function [99]. These detrimental changes in mitochondrial structure and function would make significant contributions to the generation of ROS and oxidative stress. Under oxidative stress conditions, macromolecules such as lipids and proteins suffer from oxidative damage leading to the accumulation of the oxidized products in the cell. Oxidative damage to lipids and proteins in the alcohol-treated heart were thus detected by measuring the levels of lipid peroxidation and protein oxidation.

The products of lipid peroxidation such as MDA are toxic to cells [100]. MDA is a major lipid peroxidant end product; increased malondialdehyde content may contribute to increased generation of free radicals and/or decreased activities of antioxidant system [101]. Heart tissue is rich in polyunsaturated fatty acids and is known for its high oxygen consumption. Therefore, it is more susceptible to oxidative stress than other tissues [102]. Administration of alcohol leads to the generation of peroxy radical O_2, which is associated with inactivation of CAT, GSH, and SOD enzymes. In addition, heart has relatively lower levels of antioxidant enzyme activity than the majority of other tissues. Increased lipid peroxidation can negatively affect the membrane function by decreasing membrane fluidity and changing the activity of membrane bound enzymes and receptors [103]. We observed a significant increase in lipid peroxidation during alcohol consumption, as reported by earlier studies [104]. Chronic alcohol treatment clearly provoked toxicity in rats, leading to the increase in MDA levels in heart in rats treated with alcohol [105]. The potential mechanism for increased lipid peroxidation in cardiac tissue may be the increased lipid substrate within the myocardium which can serve as a larger target for oxidation by free radicals [106]. In the present study, increased lipid peroxidation observed after ethanol treatment might have been due to the high polyunsaturated fatty acid content of the diet, which is known to be particularly vulnerable to ROS. TPE co-treatment strongly suppressed the alcohol-induced lipid peroxidation of heart, implicating protection against alcohol-induced toxicity.

Protein oxidation is a useful marker to evaluate oxidative stress *in vivo*. ROS are known to convert amino groups of proteins into carbonyl moieties [107]. PC content is actually the most general indicator and by far the most commonly used marker of protein oxidation [108,109]. Measurement of PC content can be used as a marker for protein damage induced by oxidative stress, which is shown to increase in chronic ethanol consumption [110,111]. The formation of carbonyl derivatives of proteins is suggested to be a useful measure of oxidative damage to proteins [112]. Elevation in PC levels found in the chronic alcohol-treated rats in the present study was also reported by earlier studies [113]. In fact, elevated levels of PC is generally a sign of oxidative stress and also a sign of disease-derived protein dysfunction [114]. However, the levels were restored to normalcy in the rats with alcohol toxicity when treated with TPE. The relationship among protein oxidation, protein dysfunction, and diseases remain largely unclear. However, it is known that oxidative modification of enzymes and structural proteins may play a significant role in the etiology of diseases.

In conclusion, clarifying by the evidence and also by the conclusive results on the effects of leaf extracts of TP on cardiac tissue affected by chronic alcohol through which oxidative stress is induced will help us in the understanding of other aspects associated with the effects of chronic alcohol. The result obtained from the supplementation with TPE showed that the antioxidant significantly inhibited oxidative stress and cardiac injury induced by chronic alcohol exposure. Elucidation of cardiac dysfunction due to alcohol-induced oxidative stress may in addition one day facilitate a better understanding of the more common regular forms of treating alcoholism, which will aid in the treatment of cardiac disorders.

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