Oleic acid, one of the major components of ethyl acetate partitioned fraction of aqueous extract of bark of *Terminalia arjuna*, protects against adrenaline induced myocardial injury in male albino rats

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ABSTRACT

**Background:** Our earlier studies demonstrate the protective role of aqueous extract of bark of *Terminalia arjuna* against adrenaline-bitartrate induced myocardial injury in male albino rats. In the present study, GCMS analysis of the ethyl acetate partitioned fraction of this aqueous extract revealed the presence of oleic acid as one of the major components. Hence, in the present study, we also investigated the protective role of oleic acid against adrenaline induced myocardial injury in male albino rats and tried to elucidate the underlying mechanism of such protection. **Methods:** In this set of experiments a total number of 40 adult healthy male albino rats were divided into 10 groups comprising of 4 animals each. First group constituted of the vehicle-treated control. The rats of the second group were treated with sub-cutaneous (s.c.) injection of adrenaline-bitartrate at the dose of 0.3mg/kg body weight. The rats of the third, fourth, fifth and sixth groups were orally fed, respectively, with different doses of oleic acid (2.5, 5, 10, 20 mg/kg body weight) where water was used as the vehicle. The rats of seventh, eighth, ninth and tenth groups were orally fed with different doses of oleic acid (2.5, 5, 10, 20 mg/kg body weight) and subjected to sub-cutaneous (s.c.) injection of adrenaline-bitartrate at the dose of 0.3mg/kg body weight. **Results:** Treatment of rats in this set of experiment with adrenaline-bitartrate altered the activities of serum lactate dehydrogenase total (LDH-T), lactate dehydrogenase-1 (LDH-1), serum glutamate pyruvate transaminase (SGPT) and elevated the level of lipid peroxidation and protein carbonylation, decreased the glutathione content as well as altered the activities of antioxidant enzymes and the enzymes of Kreb’s cycle and respiratory chain. Tissue histo-morphological studies also showed considerable damage following adrenaline-bitartrate treatment. Pre-treatment of rats with different doses of oleic acid significantly protected against these myocardial damages in a dose dependent manner. **Conclusion:** Oleic acid, one of the major components of the ethyl acetate partitioned fraction of aqueous extract of bark of *Terminalia arjuna*, significantly protected against adrenaline induced myocardial injury in male albino rats in a dose dependent manner.

Keywords: Adrenaline, myocardial ischemia, oleic acid.

1. INTRODUCTION

Adrenaline, produced and secreted from medullary region of adrenal gland, is generally considered as a hormone involved in “fight or flight” mechanism1. It is considered as a potent factor in bringing about myocardial ischemia as well as myocardial infarction. In early phase of myocardial infarction systemic circulatory catecholamine level is vigorously increased2 and it is reported to be secreted from the ischemic region of myocardium3. In addition, auto-oxidation of catecholamine results in generation of cytotoxic free radicals4. Endogenous plasma adrenaline concentrations in resting adults have been reported normally to be less than 10 ng/L, but may increase by 10-fold during exercise and by 50-fold or more during time of stress5. So, it can be recognized that adrenaline is an endogenous stress inducer in higher doses. In our earlier studies, oral administration of aqueous extract of bark of *Terminalia arjuna* (TA) were shown to protect against adrenaline induced myocardial injury in male albino rats. TA is a very important medicinal plant widely used in the preparation of ayurvedic formulations for many years, primarily as a cardio tonic preparation in India6,7,8. Oral administration of crude
bark of *Terminalia arjuna* augments endogenous antioxidants of rat heart and also prevents oxidative stress associated with in vitro ischemic-reperfusion (IR) injury of the heart \(^9,10,11,12\). Therefore it can be said that aqueous bark extract of *Terminalia arjuna* consists of one or more compounds that act as a cardio protective agent either solely or in conjugate. GCMS analysis of the ethyl acetate partitioned fraction of the aqueous extract of bark of *Terminalia arjuna* revealed the presence of oleic acid, an 18 carbon monounsaturated omega 9 fatty acid, as one of its major components. From this viewpoint oleic acid draws attention in this investigation. Various studies have identified that oleate, a fatty acid ester obtained from the condensation of oleic acid; possess several effects on cardiovascular system \(^13,14\). Oleic acid can improve lipid profile status \(^15\), helps maintain a balanced body weight \(^16\), and prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory responses in neuronal cells \(^17\) and skeletal muscle \(^18\). However, the underlying molecular mechanisms of the protective role of oleic acid in cardiovascular cells are poorly known. The primary oleic (C18:1, n-9) acids can act on the endothelium by stimulating several ion channels through interaction with endothelium-derived hyperpolarizing factors, thereby altering cardiac excitability \(^19,20\) and also can alter the activity of both sensory nerves and vascular smooth muscle cells. 2-Hydroxyleoleic acid (2-OHOA) is a compound derived from oleic acid. New research has revealed the potent hypotensive activity of 2-OHOA that indicates its ability to lower systolic blood pressure \(^21-23\). Thus, the aim of our present study is to elucidate the protective role of oleic acid against adrenaline induced myocardial injury in male albino rats and its underlying mechanism of protection.

In this study, we provided evidences that oral treatment of oleic acid has potential to protect against adrenaline-induced oxidative stress mediated damages in rat cardiac tissue and this protection either may be exerted through antioxidant mechanism(s) or any other unknown underlying endogenous mechanism(s).

### 2. MATERIALS AND METHODS

#### 2.1. Animal

Male albino rats of Wister strain, weighing 150-200g 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 forests, Government of India. All the experimental protocols had the approval of the Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta (approval no IAEC/proposal-Ph.D. /DB-01, 2013 dated 23.03.2013). Prof. P.K. Samanta, M.Sc. (Vet.), Ph.D, CPCSEA nominee to IAEC, Department of Physiology, University of Calcutta, monitored care and experiments on animals.

#### 2.2. Chemicals and reagents

Oleic acid was purchased from Sigma Chemicals in liquid form. Sodium pyruvate, isocitrate, succinate, a-ketoglutarate and bovine serum albumin (BSA) were purchased from SRL Chemicals, Mumbai, India. Adrenaline bitartrate was procured from Vulcan laboratories, India. Thiobarbituric acid (TBA) was procured from Spectro Chem. All the other chemicals used including the solvents, were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India. All other chemicals used were of analytical grade.

#### 2.3. Treatment and collection of tissue samples

For our present study, the animals were housed in galvanized wire cages, in well ventilated, air conditioned rooms of our animal house with 12 hours light/dark cycle, at about 18°C temperature for 7 days to get adapted to laboratory conditions. All rats had been given a standard diet containing 18% protein, 71% carbohydrate and vitamins which are considered to be an adequate (normal) dietary protein level. The animals were released from quarantine and after that, treatment of rats was carried out as per the schedule mentioned below.

Total number of animals is 40. They were divided into 10 groups as mentioned below:

- **GROUP I**: Control group (CON). Rats were injected with vehicle.
- **GROUP II**: Adrenaline treated group (ADR 0.3). Rats were treated with adrenaline-bitartrate at the dose of 0.3mg/kg of body weight by sub-cutaneous (s.c.) injection.
- **GROUP III**: Positive control group 1 (OA 2.5). Rats treated with oleic acid at the dose of 2.5 mg/kg of body weight.
- **GROUP IV**: Positive control group 2 (OA 5). Rats treated with oleic acid at the dose of 5 mg/kg of body weight.
- **GROUP V**: Positive control group 3 (OA 10). Rats treated with oleic acid at the dose of 10 mg/kg of body weight.
- **GROUP VI**: Positive control group 4 (OA 20). Rats treated with oleic acid at the dose of 20 mg/kg of body weight.
- **GROUP VII**: Protection group 1 (ADR 0.3 + OA 2.5). Rats co-treated with oleic acid at the dose of 2.5 mg/kg of body weight and with adrenaline at the dose of 0.3 mg/kg of body weight.
- **GROUP VIII**: Protection group 2 (ADR 0.3 + OA 5). Rats co-treated with oleic acid at the dose of 5 mg/kg of body weight and with adrenaline at the dose of 0.3 mg/kg of body weight.
- **GROUP IX**: Protection group 3 (ADR 0.3 + OA 10). Rats co-treated with oleic acid at the dose of 10 mg/kg of body weight and with adrenaline at the dose of 0.3 mg/kg of body weight.
- **GROUP X**: Protection group 4 (ADR 0.3 + OA 20). Rats co-treated with oleic acid at the dose of 20mg/kg of body weight and with adrenaline at the dose of 0.3mg/kg of body weight.
At the end of treatment, the animals were sacrificed by cervical dislocation following light ether anaesthesia. The abdomen and the chest region of each rat were surgically cut to open the heart. After opening the heart blood was collected to obtain serum. Then the heart tissue was collected. Those tissues were kept in sterile plastic vial at -20°C for further biochemical analysis. For histological studies, an appropriate portion of the ventricular part of the heart was placed immediately in formalin fixative.

2.4. Preparation of tissue homogenate
A 10% tissue homogenate of heart was prepared in ice cold 0.1M phosphate buffer (pH 7.4) using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 sec. The homogenate was kept in cold and processed for biochemical analyses within 30 minutes of preparation.

2.5. Measurement of serum glutamate oxaloacetate transaminase (SGOT), total lactate dehydrogenase (LDH-T), lactate dehydrogenase 1 (LDH-I) activities
Serum GOT activity was measured by standard methods. Non-hemolyzed serum was mixed with glutamate pyruvate transaminase substrate and incubated for 30 min at 37°C. Then, 2, 4-dinitrophenyl hydrazine (DNPH) solution was added, mixed and kept for 20 min at room temperature. Thereafter, 0.4 (N) NaOH was added, mixed and kept at room temperature for 10 min. The intensity of the developed color was noted at 540 nm after setting the UV/VIS spectrophotometer to zero with water (Bio-Rad, Hercules, CA, USA) 24.

The total serum lactate dehydrogenase (LDH-T) activity was obtained by measuring the oxidation of NADH (0.1 mM) to NAD+ at 340 nm according to the method of Strittmatter (1965)25 with some modifications 26.

The serum lactate dehydrogenase 1 (LDH-I) activity was obtained by measuring the oxidation of NADH (0.1 mM) to NAD+ at 340 nm using 1.0 mM sodium pyruvate as substrate, after incubating the serum samples at 65°C which destroys all isoenzymes except LDH-1 for 30 min according to the method of Strittmatter (1965)25 with some modifications 26.

2.6. Measurement of nitric oxide (NO) concentration in the rat cardiac tissues
Nitric oxide concentration in the cardiac tissues were measured spectrophotometrically at 548nm according to the method of Fiddler (1977) 27 by using Griess reagent (1879) 28.

2.7. Measurement of cardiac tissue lipid peroxidation (LPO) level, reduced glutathione (GSH) content, total glutathione (TSH) content and protein carbonyl (PCO) content
The lipid peroxides in the cardiac tissue homogenates were determined separately as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust 29 with some modifications as adopted by Bandyopadhyay et al 30.

The reduced glutathione (GSH) content (as acid soluble sulfhydryl) and total glutathione content of the cardiac tissue homogenates were estimated separately by its reaction with DTNB (Ellman’s reagent) following the method of Sedlak et al 31 with some modifications by Dutta et al, 2014 32. Protein carbonyl content was estimated by DNPH 33 assay. The values were expressed as nmoles / mg protein.

2.8. Measurement of the activities of Cu-Zn superoxide dismutase (Cu-Zn SOD), Catalase, Mn superoxide dismutase (Mn SOD), Glutathione reductase (GR), Glutathione peroxidase (GPx), Glutathione-S-transferase (GST) and Oxidized glutathione (GSSG) content of rat cardiac tissue
Copper-zinc superoxide dismutase (Cu-Zn-SOD) activity was measured by hematoxylin auto-oxidation method of Martin et al 34 with some modifications as adopted by Mishra et al 35. The enzyme activity was expressed as units /mg protein.

Catalase activity was assayed by the method of Beers et al, 1952 36 with some modifications as adopted by Chattopadhyay et al 37. The enzyme activity was expressed as μmoles of H2O2 consumed/ mg protein.

Manganese superoxide dismutase (Mn-SOD) activity was measured by pyrogallol autooxidation method 38 as modified by Rudra et al 39. The glutathione reductase activity was measured according to the method of Krohne- Ehrich et al. 40. The specific activity of the enzyme was calculated as units/ mg protein.

The glutathione peroxidase activity was measured according to the method of Paglia and Valentine, 1967 41 with some modifications as adopted by Chattopadhyay et al. 37.

The glutathione-S-transferase activity of the rat cardiac tissue was measured spectrophotometrically according to Habig et al 42. GSSG was measured by the method of Sedlak and Lindsay 31 with some modification of Bandyopadhyay et al. 30. Tissues were homogenized (10%) in 2 millimolar ice-cold ethylenediaminetetraacetic acid (EDTA). The reaction mixture contained 0.1 millimolar sodium phosphate buffer, EDTA, NADPH and 0.14 units per ml glutathione reductase. The absorbance was measured at 340 nm using a UV-VIS spectrophotometer to determine the GSSG content. The values were expressed as nmoles GSSG/mg protein.
2.9. Indirect assessment of the generation of superoxide anion free radical \( (O_2^-) \) by xanthine oxidase (XO) and xanthine dehydrogenase (XDH)

Xanthine oxidase activity of rat cardiac tissue was assayed by measuring the conversion of xanthine to uric acid following the method of Greenlee et al. Xanthine dehydrogenase (XDH) activity was measured by following the reduction of NAD\(^+\) to NADH according to the method of Strittmatter with some modifications. The enzyme activity was expressed as milliunits/ mg protein.

2.10. Measurement of the activities of pyruvate dehydrogenase and Kreb’s cycle enzymes

Pyruvate dehydrogenase (PDH) activity of rat cardiac tissue was measured spectrophotometrically according to the method of Chretien et al. with some modifications as adopted by Mishra et al.

Isocitrate dehydrogenase (ICDH) activity of rat cardiac tissue was measured according to the method of Duncan et al. by measuring the reduction of NAD\(^+\) to NADH at 340 nm with the help of a UV–VIS spectrophotometer.

Alpha-Ketoglutarate dehydrogenase (α-KGDH) activity of rat cardiac tissue was measured spectrophotometrically according to the method of Duncan et al.

Likewise, succinate dehydrogenase (SDH) activity of rat cardiac tissue was measured spectrophotometrically by following the reduction of potassium ferricyanide \([K_3Fe(CN)_6]\) at 420 nm according to the method of Veeger et al. with some modifications as adopted by Mishra et al.

2.11. Measurement of the activities of some of the mitochondrial respiratory chain enzymes

NADH-Cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565 nm according to the method of Goyal et al.

Cytochrome c oxidase activity was determined spectrophotometrically by following the oxidation of reduced cytochrome c at 550 nm according to the method of Goyal et al.

2.12. Isolation of mitochondria from heart tissues

The mitochondria from heart tissues were isolated according to the procedure of Dutta et al. A portion of the heart tissue were cleaned and cut into small pieces. Five hundred mg of both the tissues were placed separately in 10ml of sucrose buffer \([0.25(M)\text{ sucrose, 0.001(M EDTA, 0.05(M Tris-HCl (pH 7.8)]}\) at 25°C for 5min. The tissues were then homogenized separately in cold for 1 minute at low speed by using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA). The homogenates were centrifuged at 1500rpm for 10 minutes at 4°C. The supernatant was poured through several layers of cheesecloth and kept in ice. This filtered supernatant was centrifuged at 4000rpm for 5 minutes at 4°C. The supernatant, thus, obtained, was further centrifuged at 14000rpm for 20 minutes at 4°C. The final supernatant was discarded and the pellet was re-suspended in sucrose buffer and stored at -20°C for further analysis. However, most of the enzymatic assays were carried out with freshly prepared mitochondria.

2.13. Estimation of proteins

Proteins of the different samples were determined by the method of Lowry et al.

2.14. Tissue morphological and histochemical studies

2.14.1. Staining of tissue sections using hematoxylin-eosin (H & E), and periodic acid Schiff (PAS) stains

A portion of the extirpated rat heart were fixed immediately in 10% formalin and embedded in paraffin following routine procedure as used earlier by Dutta et al. Sections of heart tissues (5 μm thick) were prepared. The cardiac tissue sections were stained with hematoxylin-eosin stain and periodic acid Schiff (PAS) stain. The stained tissue sections were examined under Leica microscope and the images were captured with a digital camera attached to it.

2.14.2. Quantification of fibrosis by confocal microscopy

A portion of the extirpated rat cardiac (left ventricular portion) tissue were fixed immediately in 10% formalin and embedded in paraffin following routine procedure. Additionally, the left ventricular tissue sections (5 μm thick) were stained with Sirius red (Direct Red 40; Sigma Chemical Co) and imaged with laser scanning confocal system (Zeiss LSM 510 META, Carl Zeiss Micro Imaging GmbH, Jena, Germany), and the stacked images through multiple slices were captured.

2.14.3. Scanning electron microscopy (SEM)

Small pieces of heart tissues were fixed overnight with 2.5% glutaraldehyde. After washing three times with PBS, the pieces were dehydrated for 10 min at each concentration with a graded ethanol series (50, 70, 80, 90, 95 and 100%). The dehydrated pieces were immersed in pure tert-butyl alcohol and were then placed into a 4°C refrigerator until the tert-butyl alcohol solidified. The frozen tissue pieces were dried by placing them into a vacuum bottle. The cardiac tissue surface morphology was evaluated by scanning electron microscopy (SEM; Zeiss Evo 18 model EDS 8100).
2.14.4. Scanning electron microscopy of mitochondria of cardiac tissues

The incubated mitochondrial suspension was centrifuged, and the supernatant was removed. The pellet was fixed overnight with 2.5% glutaraldehyde. After washing three times with PBS, the pellet was dehydrated for 10 min at each concentration with a graded ethanol series (50, 70, 80, 90, 95 and 100%). The pellet was immersed in pure tert-butyl alcohol and was then placed into a 4°C refrigerator until the tert-butyl alcohol solidified. The frozen samples were dried by placing them into a vacuum bottle. Mitochondrial morphology was evaluated by scanning electron microscopy (SEM; Zeiss Evo 18 model 8100).

2.15. Gas chromatography-mass spectroscopy (GC-MS) protocol

The aqueous extract of bark of Terminalia arjuna in the form of ethyl acetate partitioned fraction (EAPF) was subjected to GC-MS analysis after necessary procedure.

The phytochemicals in the samples were identified using an Agilent Technologies 6890 N Network GC system & interfaced to Agilent Technologies 5973 Inert Mass Selective Detector employing the following conditions: column DB-1 ms fused silica capillary column (30mmX0.25mm I.D.X 0.10μMdf , composed of 100% Di-methylpolysiloxane), For GC-MS detection, an electron ionization system with ionizing energy of 70eV was used; helium(He) gas (99.99%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 2μl was employed (split ratio of 10:1). The injector, MS Source & MS Quadrapole temperature were fixed at 250°C & ion source temperature 280°C and turbo speed of the pump was 100%. The oven temperature was programmed from 50°C (isothermal for 2 minutes), with an increase of 10°C/min to 100°C (isothermal for 5 minutes), then 10°C/min to 280°C (isothermal for 5 minutes). For tuning of the MSD in EI mode Perfluorotributylamine (PFTBA) was used as tuning compound. Mass spectra were taken at 70Ev; a scan interval of 0.5 seconds and fragments from 69 to 502Da. Total GC running time was 30 minutes. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass.

The identification of phytochemicals was accomplished by comparison of retention time and fragmentation pattern with mass spectra in the National Institute Standard and Technology (NIST) which consists of more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known component inherent in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

2.16. In vitro assessment of antioxidant capacity of oleic acid

(a) Assessment of DPPH free radical scavenging activity: The DPPH scavenging activity of oleic acid was determined by the method of Chen and Yeh et al.43
(b) Assessment of hydroxyl radical (•OH) scavenging activity by using DMSO as a probe: Dimethyl sulfoxide forms a stable product [methane sulfonic acid (MSA)] on reaction with •OH during incubation which was measured by the method of Babbs and Steiner64 as modified by Bandyopadhyay et al.58
(c) Assessment of hydroxyl radical (•OH) scavenging activity by using deoxyribose as a probe: Hydroxyl radical scavenging activity was performed according to the method of Halliwell and Gutteridge 56 as modified by Chattopadhyay et al.57
(d) Assessment of reducing power: Potassium ferriicyanide reducing activity of oleic acid was measured by the method of Oyaizu et al.58
(e) Assessment of superoxide anion (O_2^-) free radical scavenging activity: Superoxide anion (O_2^-) free radical scavenging activity of oleic acid was studied by the method of Misra and Fridovich et al.60
(f) Determination of hydrogen peroxide (H_2O_2) scavenging activity and metal chelating activity: The hydrogen peroxide (H_2O_2) scavenging activity was measured by studying the breakdown of H_2O_2 at 240 nm by using a UV / VIS spectrophotometer 60 and metal chelating property of oleic acid was estimated by the method of Ningappa et al.61

2.17. Statistical evaluation

Each experiment was repeated at least three times with different rats. Data are presented as mean ± S.E.M. Significance of mean values of different parameters between the treated groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.

3. RESULTS

The first part of this study is to elucidate the components present in aqueous extract of Terminalia arjuna bark ethyl-acetate partitioned fraction (EAPF).

3.1. UV-Visible spectral scan of ethyl acetate partitioned fraction (EAPF) of Terminalia arjuna (1:100 ratio)

The result represented in figure 1 demonstrates that a single peak was observed after UV-Visible spectral scanning of EAPF of Terminalia arjuna 1:100 ratio. This indicate that the absorption maximum of the compounds present in this EAPF of Terminalia arjuna.
Table 1. Lists of important phyto-components with their activities

<table>
<thead>
<tr>
<th>Peak serial no</th>
<th>Retention time (minutes)</th>
<th>Area %</th>
<th>Resolved compounds</th>
<th>Molecular weight (gm/mol)</th>
<th>Antioxidant/ Antimicrobial/ Anti ulcerative/ others</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.042</td>
<td>65.43</td>
<td>Benzoic Acid</td>
<td>122.12</td>
<td>Antioxidant and antimicrobial</td>
</tr>
<tr>
<td>2</td>
<td>9.332</td>
<td>0.7</td>
<td>1,2-Benzenediol</td>
<td>110.11</td>
<td>Antiseptic</td>
</tr>
<tr>
<td>3</td>
<td>12.534</td>
<td>0.45</td>
<td>D-Glucuronolactone</td>
<td>176.12</td>
<td>Antioxidant, anti-inflammatory and antitumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nonanoic acid</td>
<td>158.23</td>
<td>Antioxidant and antimicrobial</td>
</tr>
<tr>
<td>4</td>
<td>13.276</td>
<td>0.48</td>
<td>Glutethimide</td>
<td>217.26</td>
<td>Antioxidant and antimicrobial</td>
</tr>
<tr>
<td>5</td>
<td>14.729</td>
<td>0.26</td>
<td>Azelaic Acid</td>
<td>188.22</td>
<td>Serves as a distressor and anti ulcerative</td>
</tr>
<tr>
<td>6</td>
<td>17.354</td>
<td>3.59</td>
<td>Hexadecanoic acid</td>
<td>256.42</td>
<td>Antioxidant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tridecanoic acid</td>
<td>214.34</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>7</td>
<td>18.864</td>
<td>4.69</td>
<td>Oleic Acid</td>
<td>282.46</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>8</td>
<td>21.951</td>
<td>5.62</td>
<td>Cyclopropanonanoic acid</td>
<td>370.12</td>
<td>Antioxidant and antimicrobial</td>
</tr>
</tbody>
</table>

3.3. The GC-MS pattern of Oleic acid

Although benzoic acid is the major component of ethyl acetate partitioned fraction (EAPF) of *Terminalia arjuna* bark, but pure benzoic acid and its esters are very much toxic and carcinogenic. In TA it may remain in a conjugate form that masks its toxicity and exert its beneficiary action. Since oleic acid is also one of the major components of the EAPF of TA and no adverse effects of oleic acid are reported till date, we can assume that oleic acid may play a major role in the cardio protective effect of *Terminalia arjuna* and it can be anticipated that pure oleic acid may exert its antioxidant and cardio protective property more prominently. Hence, the rest of the study was done to evaluate the efficacy of oleic acid as a cardio protective agent.

![Figure 1. UV-Visible spectral scan of ethyl acetate partitioned fraction (EAPF) of *Terminalia arjuna*](image)

![Figure 2. Gas chromatogram (GC) of the major fraction of ethyl acetate partitioned fraction (EAPF-) of *Terminalia arjuna* bark without retention time.](image)

![Figure 3. Gas chromatogram (GC) of the major fraction of ethyl acetate partitioned fraction (EAPF-) of *Terminalia arjuna* bark with retention time.](image)
3.4. Studies on bio-marker enzymes of cardiac damage

The results presented in Figure 5 and Figure 6 reveal that adrenaline bi-tartrate induced elevation (37.99%, 116%, 115%, 68.75% and 75% increase respectively, *P < 0.001 vs control group) in the activities of serum glutamate oxaloacetate transaminase (SGOT), serum total lactate dehydrogenase (LDH-TOTAL) and lactate dehydrogenase 1(LDH-1), serum nitric oxide content and heart tissue nitric oxide content (NO). These parameters were found to be dose dependently restored to near control value (20.85%, 48.14%, 44.64%, 33.33% and 32.64% decrease respectively, **P<0.001 vs. adrenaline- treated group) when the rats were pre-treated with increasing doses of oleic acid the maximum restoration being at a dose of 10mg/kg fed orally (ADR 0.3 + OA 10) followed by adrenaline treatment. However, the degree of protection did not increase further significantly with a further increase in the dose of oleic acid. Moreover, oleic acid alone did not exhibit any significant effect on the above parameters.

Figure 4. Mass spectra of oleic acid in TA

Figure 5. Bar graphs represent the protective effect of oleic acid, administered to different groups of rats in different doses, against adrenaline-induced increase in SGOT (A), LDH-1 (B), LDH-TOTAL(C).

Figure 6. Bar graphs represent the protective effect of oleic acid, administered to different groups of rats in different doses, against adrenaline-induced increase in NO of serum (A) and tissues (B).
3.5. Studies on biomarkers of oxidative stress and anti-oxidant enzymes of cardiac tissues

Figure 7 showed a significant increase in cardiac LPO level, protein carbonyl and total sulfhydryl group content (TSH) following treatment of rats with adrenaline bitartrate (0.3mg/kg bw by s.c injection) *(ADR 0.3)* 74.64%, 87.27% and 17.84% respectively (*P < 0.001 vs. control). This elevated level of lipid peroxidation products and protein carbonyl level and TSH were found to be protected significantly dose- dependently (35.50%, 41.74% and 12.26% **P < 0.001) when the rats were pre-treated with increasing doses of oleic acid the maximum protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw, by s.c injection) *(ADR 0.3 + OA 10)*, indicating the ability of oleic acid to protect the cardiac tissue against oxidative stress-induced changes due to adrenaline. On the other hand, a significant decrease was observed in reduced GSH content *(figure. 7)* of cardiac tissue following treatment of rats with adrenaline bitartrate (37.73%, *P < 0.001 vs. control) *(ADR 0.3)*. This decreased level of reduced GSH content was found to be protected from being decreased significantly (42.42%, **P < 0.001) in a dose-dependent manner when the rats were pre-treated with increasing doses of oleic acid the maximum protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate 0.3 mg/kg bw *(ADR 0.3 + OA 10)*. However, treatment with oleic acid alone did not show any significant effect on any of the above parameters.

**Figure. 7. Protective effect of oleic acid, administered to different groups of rats in different doses, against adrenaline-induced alteration in LPO (A), GSH (B), PCO (C), TSH (D).**

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Adrenaline(mg/kg)bw.+Oleic acid(mg/kg)bw.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid peroxidation</strong> (n moles TBARS/mg protein)</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>ADR 0.3</td>
</tr>
<tr>
<td>OA 2.5</td>
<td>OA 5</td>
</tr>
<tr>
<td>OA 10</td>
<td>OA 20</td>
</tr>
<tr>
<td>ADR 0.3+ OA 2.5</td>
<td>ADR 0.3+ OA 5</td>
</tr>
<tr>
<td>ADR 0.3+ OA 10</td>
<td>ADR 0.3+ OA 20</td>
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</table>

| **Protein Carbonyl Content** (n moles of carbonyl/mg protein) |
| CON           | ADR 0.3                                |
| OA 2.5        | OA 5                                   |
| OA 10         | OA 20                                  |
| ADR 0.3+ OA 2.5 | ADR 0.3+ OA 5                           |
| ADR 0.3+ OA 10 | ADR 0.3+ OA 20                           |

| **GSH (B)** |
| CON           | ADR 0.3                                |
| OA 2.5        | OA 5                                   |
| OA 10         | OA 20                                  |
| ADR 0.3+ OA 2.5 | ADR 0.3+ OA 5                           |
| ADR 0.3+ OA 10 | ADR 0.3+ OA 20                           |

| **TSH (D)** |
| CON           | ADR 0.3                                |
| OA 2.5        | OA 5                                   |
| OA 10         | OA 20                                  |
| ADR 0.3+ OA 2.5 | ADR 0.3+ OA 5                           |
| ADR 0.3+ OA 10 | ADR 0.3+ OA 20                           |

The values are expressed as Mean ± S.E.; * P < 0.001; compared to control values using ANOVA; ** P<0.001 compared to adrenaline-induced values using ANOVA.

*The values are expressed as Mean ± S.E.; * P < 0.001; compared to control values using ANOVA; ** P<0.001 compared to adrenaline-induced values using ANOVA.*
Figure 8 further showed a significant increase in cardiac antioxidant enzyme activities both Cu-Zn SOD and Mn-SOD following the treatment of rats with adrenaline bitartrate (138.84%, 134.04% *P < 0.001 vs. control) (ADR 0.3) and decrease in catalase activity (42.08% *P < 0.001 vs. control) (ADR 0.3). These alterations in antioxidant enzyme activities in cardiac tissue were found to be protected significantly in a dose-dependent manner (47.50%, 52.79% **P < 0.001 in case of Cu-Zn SOD and Mn-SOD respectively and 48.04% (**P < 0.001) in case of catalase activity) when the rats were pre-treated with increased doses of oleic acid the maximum protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw) (ADR 0.3 + OA 10) indicating the ability of oleic acid to protect the cardiac tissue against oxidative stress-induced changes due to adrenaline. However, oleic acid alone did not possess any effect on the activities of these antioxidant enzymes.

On the other hand, figure 9 showed significantly decreased activities of GPx, GR and GST and decreased GSSG content of cardiac tissue following treatment of rats with adrenaline bitartrate (43.58%, 52%, 57.97%, 37.73%, *P < 0.001 vs. control) (ADR 0.3). These alterations in antioxidant enzyme activities and GSSG content in cardiac tissue were found to be protected significantly (45.45%, 83.33%, 72%, 42.42%, **P < 0.001) in a dose dependent manner when the rats were pre-treated with increasing concentrations of oleic acid the best possible protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg, s.c) (ADR 0.3 + OA 10).

The values are expressed as Mean ± S.E.; * P < 0.001; compared to control values using ANOVA; **P<0.001 compared to adrenaline-induced values using ANOVA.
3.6. Studies on the activities of mitochondrial tri-carboxyl acid cycle (i.e. Krebs’s cycle) enzymes and Status of mitochondrial respiratory chain enzyme of cardiac tissues

Figure 10 reveals that treatment of rats with adrenaline bi-tartrate at a dose 0.3mg/kg bw, s.c., every day for a period of 17 days maximally decreased PDH, α-KGDH, ICDH and SDH, the mitochondrial tri-carboxyl acid cycle enzymes activity by 65%, 42.36%, 57.20% and 65.06% respectively (*P < 0.001 vs. Control group) (ADR 0.3). All of these enzyme activities were found to be significantly protected from being altered (153.45%, 69.48%, 92.30% and 161.76% increases **P<0.001 compared to adrenaline-induced values using ANOVA) in a dose-dependent manner when the rats were pre-treated with increasing doses of oleic acid the maximum protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw), half an hour before adrenaline bitartrate treatment in rats (ADR 0.3 + OA 10). The degree of protection did not increase further significantly with further increases in the doses of oleic acid. Thus, oleic acid appears to have the potential to provide protection against adrenaline induced oxidative stress in cardiac tissues of rats. However, oleic acid alone did not possess any significant effect on the mitochondrial Kreb’s cycle enzymes.

The values are expressed as Mean ± S.E.; *P < 0.001; compared to control values using ANOVA; ** P<0.001 compared to adrenaline-induced values using ANOVA.
Figure 11 further clearly shows that treatment of rats with adrenaline bitartrate at a dose 0.3mg/kg b.w. s.c., every day for a period of 17 days significantly decreased both cytochrome-c-oxidase and NADH cytochrome-c-oxidoreductase activity (57.53% and 75.33% decreased,*P<0.001 vs. control group) (ADR 0.3) in the cardiac tissues of rats. Both of these enzymes activities were found to be significantly protected from being altered (107.52% and 270% increases **P<0.001 vs. adrenaline- treated group) when the rats were pre-treated with increasing doses of oleic acid, the maximum protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw) half an hour before adrenaline treatment in rats (ADR 0.3 + OA 10). Thus, oleic acid appears to have the potential to provide protection against adrenaline induced oxidative stress in cardiac tissues of rats. However, it did not possess any significant effect alone on the activities of respiratory chain enzymes.

Figure 11. Protective effect of oleic acid, administered to different groups of rats in different doses, against adrenaline-induced alteration in cytochrome c oxidase (A) and NADH-cytochrome c oxidoreductase (B).

The values are expressed as Mean ± S.E.; * P < 0.001; compared to control values using ANOVA; ** P<0.001 compared to adrenaline-induced values using ANOVA.

3.7. Indirect assessment of generation of superoxide anion free radicals in adrenaline induces oxidative stress in rat heart tissues.

Figure 12 shows that treatment of rats with adrenaline bitartrate at a dose of 0.3mg/kg bw. s.c. every day for a period of 17 days generated copious amounts of superoxide anion free radicals in cardiac tissues of rats which was reflected in elevated levels of activities of xanthine oxidase (XO), xanthine dehydrogenase (XDH), XO+XDH, XO/XDH and XO/(XO+XDH) respectively (123.76%, 71.90%, 93.75%, 30.95% and 13.04% increases,*P<0.001 vs. Control) (ADR 0.3). These activities were found to be significantly (43.80%, 36.53%, 40.09%, 14.54% and 5.76% decrease **P<0.001 vs. adrenaline-treated group) protected from being altered when the rats were pre-treated with increasing doses of oleic acid the maximum protection being provided at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw) half an hour before adrenaline treatment in rats (ADR 0.3 + OA 10). The results reveal that there occurred generation of superoxide anion radical in rat heart tissues.

Figure 12. Protective effect of oleic acid, administered to different groups of rats in different doses, against adrenaline-induced alteration in XO (A), XDH (B), XO+XDH ratio (C), XO/XDH ratio (D), XO/(XO+XDH) ratio (E).
following treatment of rats with adrenaline bitartrate (0.3mg/kg bw. s.c.) which was found to reduced by pre-treatment with oleic acid which indicates that oleic acid may possess antioxidant potential. The maximum protection is found at the dose of 10mg/kg bw (ADR 0.3 + OA 10). However, the degree of protection did not increase further significantly with a further increase in doses of oleic acid. However, oleic acid treatment alone did not possess any significant effect on the above parameters.

3.8. Histological studies

3.8.1. Routine H and E staining of the rat cardiac tissue

Figure 13 (upper panel) depicts haematoxylin-eosin stained left ventricular longitudinal sections of rat heart tissues (magnifications 400X). The sections of adrenaline bi-tartrate treated (0.3mg/kg bw. s.c.) rat heart showed necrosis of muscle fibre and also lysed fibre (ADR 0.3). But these damages of cardiac tissues were found to be gradually protected from being altered in rats pre-treated with increasing doses of oleic acid fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw). Best possible protection was found at a dose of 10mg/kg oleic acid fed orally followed by adrenaline treatment. No significant alteration was observed with oleic acid treatment alone.

3.8.2. PAS stain of cardiac tissues

In the figure 14, PAS stained light microscopy of the tissue sections of control groups showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. But in adrenaline bitartrate (0.3mg/kg bw.s.c.) group light
microscopy of the tissue sections showed edema, with contraction of bands, disruption of myofibrillar structures, hemorrhage and polymorphonuclear leukocyte infiltration (ADR 0.3). The cardiac muscle fibres showed vascular changes with fragmentation suggestive of focal necrosis. But these damages of cardiac tissues were found to be gradually protected with increasing doses of oleic acid with complete protection being effected when the rats were pre-treated with oleic acid at a dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw) But there are no significant changes found in only oleic acid treatment groups treated with oleic acid alone in different doses (2.5mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively).

3.8.3. Acid Sirius stain of cardiac tissues

Figure 15 demonstrates images of Sirius red-stained left ventricular longitudinal sections of rat heart tissues (magnifications 400X). Red colour stretches are collagen depositions. The depletion of collagen in the cardiac tissues of rats following treatment with adrenaline bitartrate in dose of 0.3mg/kg bw (ADR 0.3) was observed. This was found to be only partially ameliorated when the rats were pre-treated with oleic acid at a dose of 10mg/kg fed orally the best effective dose followed by treatment with adrenaline bitartrate (0.3 mg/kg bw). There are no significant changes found in groups treated with oleic acid alone in different doses (2.5mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively).

3.8.4. SEM of cardiac tissue

Figure 16 demonstrates images of scanning electron microscopy (SEM) of left ventricular longitudinal sections of rat heart tissues. Adrenaline bi-tartrate (0.3mg/kg bw. s.c.) treated sections showed irregular manner of cardiac muscle fiber, damages of branching of cardiac muscle fibers and also necrosis of fibers (ADR 0.3). But this was found to be partially ameliorated when the rats were pre-treated with oleic acid at the best effective dose of 10mg/kg fed orally followed by treatment with adrenaline bitartrate (0.3 mg/kg bw). There are no significant changes found in groups treated with oleic acid alone in different doses (2.5mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively).

Figure 14. PAS staining of the rat cardiac tissue. Black arrows indicate the damaged portion of the cardiac tissue in ADR 0.3. But no such changes were found in ADR 0.3+OA 20 group.

Figure 15. Acid sirius stain of the rat cardiac tissue. Panel A represents the light photomicrographs of the cardiac tissue section. Panel B represents the black and white pictures of the tissue section captured by confocal microscope. Panel C represent the merged picture of cardiac tissue. Panel D represent the representative figures captured by red filter of confocal microscope. Black, red and blue arrow arrows indicate the damaged portion of the cardiac tissue where collagen content is much less than control. Oleic acid pretreatment at a dose of 10 mg/kg bw protected those changes (ADR 0.3 + OA 10).

3.8.5. SEM of cardiac mitochondria

Figure 17 depicts images of scanning electron microscopy (SEM) of mitochondria isolated from left ventricular longitudinal sections of rat heart tissues. Mitochondria obtained from adrenaline bi-tartrate (0.3mg/kg bw. s.c.) treated cardiac tissue sections were found to be leaky, with damages of outer membrane and loss of intactness in their architecture (ADR 0.3). When the rats were pre-treated with oleic acid at a dose of 10 mg/kg bw i.e. the best effective dose, half an hour before adrenaline treatment (ADR 0.3 + OA 10) for 17 days, the leakiness, damages of outer membrane and loss of intactness was almost completely protected. But there are no significant changes found in groups treated with oleic acid alone in different doses (2.5mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively).
3.8.6 Studies on the antioxidant properties of Oleic acid in vitro

Figure 18(A-G) demonstrates the scavenging property of oleic acid, *in vitro*. The use of DPPH radicals provides an easy and rapid and convenient method to evaluate the antioxidant and radical scavenging activity of various extracts and molecules. Figure 18.A demonstrates an almost linear DPPH radical scavenging activity of oleic acid which is indicative of a strong radical scavenging potential of this molecule. The oleic acid exhibited maximum DPPH radical scavenging activity at a concentration of 10 µg/mL. Figure 18.B shows that oleic acid scavenges the hydroxyl radical, generated in a pure chemical system, in vitro, in a concentration-dependent manner. Dimethyl sulfoxide (DMSO) [1 µg/ml] was used as a specific hydroxyl radical scavenger (probe) and the assay was carried out in presence of a fixed concentration of DMSO and increasing concentrations of oleic acid. Figure 18.C reveals that oleic acid protects against deoxyribose degradation in a dose-dependent manner indicating hydroxyl radical (·OH) scavenging capability of oleic acid. The figure.18.D reveals a concentration-dependent increase of reducing power of oleic acid. Figure.18.E reveals a concentration-dependent superoxide anion free radical scavenging activity by oleic acid which shows that oleic acid does possess antioxidant potential indicating that oleic acid is capable of acting as an antioxidant by scavenging ROS.

Figure 18.F clearly demonstrates that oleic acid has no significant \( \text{H}_2\text{O}_2 \) scavenging activity. However, figure.18.G also reveals that oleic acid was found to have no metal chelating activity with increasing concentration, suggesting that oleic acid do not bear the potential of chelating metals, *in vitro*.
myocytes cells, circulating blood cells (e.g., leukocytes, platelets), and cardiac interaction of a number of cell types, including coronary endothelial tissues respectively. The cellular mechanisms involved in the pathogenesis of myocardial injury are complex and involve the interaction of a number of cell types, including coronary endothelial cells, circulating blood cells (e.g., leukocytes, platelets), and cardiac myocytes all of which are capable of generating ROS. Free radicals which are generated due to chronic subcutaneous administration of adrenaline bitartrate causes oxidative stress which have the potential to injure cardiac myocytes directly, and can initiate a series of cascade chain reactions in cardiac myocytes membrane. As a result, genetic alterations occur that ultimately result in an amplification of the initial ROS-mediated cardiomyocyte dysfunction, damage and/or cytotoxicity. In adrenaline treated rats increased serum level of LDH-1 and LDH-TOTAL is probably due to damage or leakage of cardiac myocytes membrane by free radical or ROS. But a significant decrease near to control value of these enzyme activities was observed in a dose-dependent manner when they were pre-treated with different doses of oleic acid (2.5 mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively). So, it may be assumed that oleic acid can protect membrane damage of cardiac myocytes.

4. DISCUSSION

Chronic stress is considered as one of the major causes of myocardial infarction, which occurs due to elevation of the blood levels of adrenaline, a naturally occurring catecholamine, synthesized by adrenal medulla of human body. An increased circulating catecholamine level has been reported in the early phase of myocardial ischemia. Oleic acid was selected as an anti-ischemic agent because oleic acid is a common mono unsaturated fatty acid (MUFA) concerned with different parameters of cardiovascular disorders like high blood pressure. It can regulate the activity of adrenoreceptor signaling pathways which stimulate the adrenergic receptors (α- and β-adrenoceptors) that helps to regulate blood pressure. Diet rich in oleic acid for 4 weeks can reduce blood pressure while increasing good HDL cholesterol in women. Oleic acid normalizes or increases fat oxidation (burning). Lim et al. (2013) found that oleic acid increases the expression of genes involved in fat burning. This means that human body is more efficient at using fat as fuel. In addition oleic acid accelerates the rates of complete fat oxidation in muscle cells. It can replace other omega fatty acids in cell membranes. Since oleic acid is less susceptible to oxidation damage than omega-6 and omega-3 fatty acids, replacing these fatty acids with oleic acid protects cardiac myocytes cell membranes from free radicals and other oxidative stressors.

This oleic acid treatment study clearly indicates that chronic oral administration of oleic acid is capable of mitigating adrenaline bitartrate-induced cardiac myopathy through probably antioxidant mechanism(s) or any other alternative mechanism(s). The subcutaneous administration of pharmacological dose of adrenaline bitartrate is able to cause myocardial infarction as is evidenced by the significant increase in major serum parameter like serum glutamate oxaloacetate transaminase (SGOT) levels, lactate dehydrogenase -1(LDH-1), lactate dehydrogenase total (LDH-TOTAL) and concentration of nitric oxide (NO) in both serum as well as cardiac tissues respectively. The cellular mechanisms involved in the pathogenesis of myocardial injury are complex and involve the interaction of a number of cell types, including coronary endothelial cells, circulating blood cells (e.g., leukocytes, platelets), and cardiac myocytes all of which are capable of generating ROS. Free radicals which are generated due to chronic subcutaneous administration of adrenaline bitartrate causes oxidative stress which have the potential to injure cardiac myocytes directly, and can initiate a series of cascade chain reactions in cardiac myocytes membrane. As a result, genetic alterations occur that ultimately result in an amplification of the initial ROS-mediated cardiomyocyte dysfunction, damage and/or cytotoxicity. In adrenaline treated rats increased serum level of LDH-1 and LDH-TOTAL is probably due to damage or leakage of cardiac myocytes membrane by free radical or ROS. But a significant decrease near to control value of these enzyme activities was observed in a dose-dependent manner when they were pre-treated with different doses of oleic acid (2.5 mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively). So, it may be assumed that oleic acid can protect membrane damage of cardiac myocytes.

A significantly increase in LPO level, which is an important biomarker of oxidative stress; indicate a possible ROS mediated damage of the myocardial membrane. Catecholamine readily forms chelate complexes with metal ions such as iron, copper and manganese. Both iron and copper ions are present in the coronary flow fraction in a redox active form that supports free radical mediated deleterious reactions. Oleic acid in different doses (2.5mg/kg,5mg/kg, 10mg/kg,20mg/kg fed orally respectively) reduce LPO level dose dependently in heart tissues by interfering with any of the steps in catecholamine metabolism or by chelating the free radicals or arrest the free radicals that are generated due to redox-active transition metals like copper or iron. Oleic acid is able to afford adequate protection against peroxidation of cell membrane lipid. 2-Hydroxyoleic acid (2-OHOA), a compound derived from oleic acid possesses potent hypotensive activity. In the presence of hypertension the plasma lipid membrane undergoes structural modifications which results in alterations of signaling proteins, reduced capability of signal transduction systems and cation transport malfunction. All these alterations lead to the loss of blood pressure control. An important class of proteins which helps regulate blood pressure is guanidine nucleotide regulatory proteins (G-proteins). These G-proteins in turn aid in the modulation of signal transduction pathways, including the adenyl cyclase (AC)/cAMP signaling pathway that promotes vascular functions such as vasodilation, vascular permeability, and heart contractibility by protecting membrane lipid of cardiac myocytes. 2-OHOA can regulate blood pressure through several mechanisms involving regulation, modification and control of G-proteins, and cardiac cell signaling pathways to promote vaso-relaxation as well as membrane lipid and protein protection.
Protein carbonyl content is another important index of oxygen radical-mediated protein damage under various pathophysiological conditions. Oxidation of cardiac myocyte membrane proteins can generate stable as well as reactive products that can generate additional radicals on reaction with transition metal ions. Though some oxidized proteins like peripheral and integral protein of cell membrane gradually accumulate and contribute to damage but most oxidized proteins are functionally inactive and are rapidly removed. Our experimental results demonstrate that the cardiac tissues suffered from oxygen mediated protein damage as evident from increased protein carbonyl content due to chronic subcutaneously administered adrenaline bitartrate. Oleic acid is able to protect the membrane proteins of cardiac tissues by either scavenging or probably neutralizing the toxic free radicals by donating its electron (e\(^-\)) from its OH group thereby protecting the critical cellular proteins from getting oxidized\(^{79}\).

Reduced glutathione (GSH) is an important bio-molecule and it functions as free radical scavenger and in the repair of radical caused biological damage. Significant reduction in cardiac tissue GSH content following adrenaline bitartrate treatment strongly indicates generation of oxidative stress induced myocardial ischemia. However, pretreatment of rats with oleic acid dose-dependently protected the cardiac tissues from these alterations. Probably oleic acid can increase the synthesis of glutathione or maintain the endogenous antioxidant status by stimulating transcription factor of such antioxidant enzymes\(^{79}\).

Superoxide dismutase (SOD) is another important antioxidant defense enzyme which catalyzes the dismutation of superoxide radicals\(^{63,80}\). The increase in both cytosolic and mitochondrial SOD activity in cardiac tissues of adrenaline bitartrate treated animals may be an adaptive response towards oxidative stress. Many studies indicate over-expression of various SODs which confers significant protection against ischemia-reperfusion injury\(^{63,80}\).

The decrease in catalase activity after adrenaline bitartrate administration may be due to over production of H\(_2\)O\(_2\) in cardiac myocytes or excessive generation of O\(_2\)- leading to the inactivation of the enzyme. Superoxide anion free radical is small enough to gain access to the heme cavity of catalase and might convert the resting enzyme to ferro-oxy state (compound III) which is known to be inactive\(^{81}\). Interestingly, when the rats were pre-treated with oleic acid at the different pharmacological dose (i.e., 2.5mg/kg,5mg/kg, 10mg/kg,20mg/kg fed orally respectively), the activities of the key antioxidant enzymes were found to be significantly protected near control from being altered pointing toward the protective role of oleic acid against adrenaline bitartrate-induced chronic stress to the rat myocardium. It is probably due to free radical scavenging activity of oleic acid; its open OH group (both cis and trans form) can neutralize free electrons in cardiac myocytes membrane and can stop cascade chain reaction of cell damage by free radical\(^{72}\).

Intracellular thiol status is generally maintained by the co-ordinated activities of the enzymes like GR, GPx and GST. Glutathione-S-transferases are a group of multi-functional isoenzymes which play an important role in the detoxification of toxic electrophiles by catalyzing the conjugation of these electrophiles with GSH.

Our data shows that administration of adrenaline bitartrate to rats reduces GST activity in cardiac tissues. Glutathione reductase is the enzyme responsible for the reduction of oxidized glutathione (GSSG) to GSH. In the present investigation, the activity of the GSH dependent enzyme, GR was reduced significantly in the cardiac tissues of experimental rats administered with adrenaline bitartrate. The formation of adrenaline sulphydryl complex with SH groups of GR might lead to a decrease in the activity of the enzyme. Our studies further demonstrated that following treatment with adrenaline bitartrate, the activity of GPx is decreased. The decreased activity of GPx probably further aggravate the situation of oxidative stress. This indicates that GSH metabolizing pathway is disturbed in adrenaline bitartrate-treated rats. It seems clear that adrenaline decreases intracellular GSH level not only by binding to its thiol group, but also by decreasing the activity of GR. Therefore, the rate of GSH formation from GSSG is reduced significantly than control level.

In our experiments, treatment of rats with adrenaline bitartrate also stimulated the activity of GST which may further aid in decreasing the level of GSH in rat heart. Pre-treatment of oleic acid protected the activities of all these enzymes from being altered. Oleic acid can increase the synthesis of glutathione or endogenous antioxidant status by stimulating transcription factors of such antioxidant enzymes\(^{79}\).

As mitochondria are currently recognized as a principal target of oxidative stress, we have studied the effect of adrenaline bitartrate on some of the mitochondrial enzymes related to energy metabolism. Our current studies have investigated the status of activity of PDH and some of the TCA cycle enzymes, particularly, ICDH, \(\alpha\)-KGDH, and SDH which are related to ATP production in mitochondria through oxidative phosphorylation. In each case, the activities of the enzymes are significantly inhibited after adrenaline bitartrate administration. Pre-treatment of rats with oleic acid in different doses
(2.5mg/kg, 5mg/kg, 10mg/kg, 20mg/kg fed orally respectively) significantly protected the activities of these crucial enzymes, in the cardiac tissues, from being altered, probably due to reduced oxygen consumption by cardiac myocytes mitochondria and increased post ischemic recovery.

In normal conditions, xanthine oxidoreductase exists in dehydrogenase form and uses NAD\(^+\) and there is no or very little production of \(\text{O}_2^\cdot\). Under ischemic conditions, there is depletion of ATP and subsequent loss of membrane Ca\(^{2+}\) gradient. Increased Ca\(^{2+}\) levels activates Ca\(^{2+}\) dependent proteases which cause selective proteolysis of the dehydrogenase to convert it into XO which acts both on hypoxanthine and xanthine at the expense of molecular oxygen to produce \(\text{O}_2^\cdot\). Thus, XO in oxidative stress conditions may play an important role in contributing free radical mediated damage. A significant increase in the activity of XO and XDH as well as an increase in XO to XDH ratio, XO/XDH ratio and XO/XO+XDH ratio in the cardiac tissues, in our experiments, confirms generation of ROS following treatment of rats with adrenaline bitartrate. Earlier workers have also indicated the involvement of XO in free radical production. However, when the rats were pre-treated with different doses of oleic acid the activities of these enzymes were protected from being altered indicating again toward the antioxidant potential of oleic acid.

In our model H & E stained light microscopy tissue sections (Figure 13) of control group showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils in the control group. But in adrenaline bitartrate group light microscopy of the tissue sections showed edema, focal hemorrhage and leukocyte infiltration. The muscle fibres showed vascular changes with fragmentation and hydrophobic changes in the myofibrillar structures. PAS stained light microscopy of the tissue sections of control groups (figure. 14) showed normal myofibrillar structure and a considerably good amount of glycogen content. But in adrenaline bitartrate group, those tissue sections showed significantly low level of glycogen content indicating a malfunction in cardiac tissue metabolism.

Acid Sirius stain of cardiac tissue sections clearly indicates altered tissue architecture and decreased collagen content of the cardiac tissues following treatment of rats with the 0.3 mg/kg bw dose of adrenaline bitartrate (figure. 15). Moreover, decreased amount of collagen fibers in the tissue framework gives rise to loose tissue indicating towards a pathological condition. In such conditions, the cardiac tissue loses its normal contractility and indignity. In, our model the scanning electron microscopy (SEM) of left ventricular portion of cardiac tissues (figure. 16) in the adrenaline bitartrate treated rats showed irregular arrangement of cardiac myofilament, some myofilaments are damaged and ruptured respectively.

The heart needs a continuous supply of ATP to fulfill its energy need. Mitochondria are the primary ATP provider via the conversion of food fuels to usable energy with the process of oxidative phophorylation by its ETC in inner membrane. The mitochondria of cardiac tissues of the adrenaline bitartrate treated group, showed a perforated surface with convoluted membranes when viewed under scanning electron microscopy (SEM). The mitochondria were markedly contracted with large membrane blebs covering the mitochondrial surface and leakage was also found in the outer surface. These changes in adrenaline bitartrate treated mitochondrial surface were found to be significantly protected from being taken place when pre-treated with oleic acid dose dependently.

Our in vitro experiments reveal that oleic acid does possess radical scavenging activity such as reducing power, DPPH radical scavenging activity, superoxide anion free radical scavenging activity, hydroxyl radical (•OH) scavenging activity. DPPH is one of the compounds that possess a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers. Further, it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. However, oleic acid did not exhibit any \(\text{H}_2\text{O}_2\) scavenging activity and metal ion chelating effect with increasing concentrations of oleic acid. Figure.19 depicts a schematic diagram to explain the generation of myocardial injury as well as the protective effect of oleic acid against this injury.

Thus, from our dose-response study, it may be concluded that pre-treatment of rats with oleic acid at a dose of 10 mg/kg body weight maximally protected all the biochemical and histo-pathological alterations in the cardiac tissues from being taken place when the animals were challenged with adrenaline-bitartrate at a dose of 0.3 mg/kg bw in vivo. Further increase of the dose of oleic acid did not demonstrate better results.

From our in vitro studies it can be concluded that oleic acid is capable of scavenging reactive oxygen species and do possess potentially strong reducing power with no metal chelating activity, indicating that the protective effect of oleic acid observed in in vivo model seems to be exerted through its antioxidant activity.
5. CONCLUSION
The present study addressed the question as to which component(s) of the aqueous TA bark extract is responsible for its cardio-protective potential in rat model. The results of the current investigations revealed oleic acid, one of the major components of the aqueous TA bark extract, has tremendous potential to provide protection against adrenaline-induced oxidative stress mediated cardiac injury in rat model and antioxidant mechanisms seems to be the mechanism responsible behind such protection. So it can be suggested that pure oleic acid possess full potential to be used in drugs as a cardio-protective component or can be used as an anti-ischemic agent.

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Conflict of Interests
The authors declare no conflict of interest.

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