2. MATERIALS AND METHODS

2.1 Experimental Animals

In the present experimental studies, healthy adult female Wistar rats, *Rattus norvegicus*, were procured, bred and reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University. Weight matched animals (160-180 g) were selected and housed in polypropylene cages lined with husk and kept in a semi-natural light/dark condition (12 h light/12 h dark). The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India), consisting of protein (22.21%), fat (8.0%), fiber (3.11%), balanced with carbohydrates (>67%), vitamins and minerals. Prior to the experimental period animals were kept in the same condition for acclimatisation. Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University and animals were cared in accordance with the “Guide for the care and use of laboratory animals” (National Institutes of Health, 1985) and “Committee for the purpose of control and supervision on experimental animals” (CPCSEA guidelines, 2011). The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg. No. 816/1999/CPCSEA), Annamalai University, Annamalainagar.

2.1.1 Maintenance of animals

During the experimental period, the animals were kept in PVC cages and maintained in the animal lab for 45 days. These PVC cages were closed by metal grid and kept in the departmental animal hygienic room for the acclimation. The proper areas of animals were maintained by following the instructions given by Behringer (1973).
The size of each PVC cage was 18" × 129" × 16".

* In each cage six rats were housed (Fig. 1) for carrying out the experimental work comfortably.

* The animal room temperature was maintained at 27°C ± 2°C throughout the experimental period.

* The rats were daily fed with rat food pellets and clean drinking water was provided with *ad libitum*.

* To maintain the hygienic conditions, the excess amount of food and excreta was removed daily from the cages.

* The rats were handled gently, without applying more pressure on animals, with gloves and care was taken during the transfer of animals from one cage to another cage.

* The rats were acclimatized to the laboratory conditions for two weeks prior to experimentation.

* Those rats showing symptoms of disease were removed immediately from the cages, to avoid the contaminations.

### 2.1.2 Source of chemicals and reagent kits

Mercury chlorides, Ferulic acid, TNF-α, TGF-β1 and cox-2 antibody were purchased from Sigma Chemical Co., USA and mRNA extraction kit (Genei Bangalore, India). All other chemicals and solvents were of analytical grade and purchased from Fine Chemicals Himedia Laboratories Pvt. Ltd., Mumbai, India.
2.1.3 Metal for toxicity studies

The toxicant of heavy metal, mercuric chloride that was used in the experimental studies possessed in the following characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical name</td>
<td>Mercuric chloride</td>
</tr>
<tr>
<td>Empirical formula</td>
<td>HgCl₂</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>271.50</td>
</tr>
<tr>
<td>Colour</td>
<td>White</td>
</tr>
<tr>
<td>Form</td>
<td>Powder</td>
</tr>
<tr>
<td>Nature</td>
<td>Poison</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>5.44</td>
</tr>
<tr>
<td>Melting point</td>
<td>277°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>302°C</td>
</tr>
<tr>
<td>Solubility in cold water</td>
<td>Sparse</td>
</tr>
<tr>
<td>Solubility in warm water</td>
<td>Freely soluble</td>
</tr>
</tbody>
</table>

2.1.4 Mechanism of action

For most of the toxic properties of the heavy metal toxicity, metal ions are bound with sulphur atoms when sulphur is in the form of sulfhydryl group which is present in the living cells. The formation of stable bond of divalent mercury replaces the hydrogen atom to form mercaptides. Such type of mercaptides /mercury is capable of inactivating sulfhydryl enzymes and they interfere with cellular metabolism and functions.

2.1.5 Ameliorative agents

The following chemicals were used as ameliorative material for mercuric chloride induced toxicity in rats.
2.1.6 Ferulic acid

Chemical formula : $\text{C}_{10}\text{H}_{10}\text{O}_4$

Molecular weight : 194.18g/mol

Purity : 98.0%

Storage : Store in a cool, dry, dark place

Solubility : Freely soluble

Colour : White or light yellowish brown

Function : Antioxidant properties

2.1.7 Structure of Ferulic acid

![Figure 6: Ferulic acid](image)

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a ubiquitous phenolic compound found in plant tissues and thus is a bioactive ingredient in many foods (Choi et al., 2012).

2.1.8 Mechanism of Ferulic acid action

The antioxidant potential of Ferulic acid can usually be attributed to its structural characteristics. Ferulic acid, because of its phenolic nucleus and unsaturated side chain can readily form a resonance stabilized phenoxy radical, which accounts for its potent antioxidant activity (Gohil et al., 2012). Any reactive radical colliding with Ferulic acid easily abstracts a hydrogen atom to form phenoxy radical. This radical is highly resonance stabilized since the unpaired
electron may be present not only on the oxygen but it can be delocalized across the entire molecule. Additional stabilization of the phenoxy radical is provided by the extended conjugation in the unsaturated side chain (Srinivasan et al., 2007). This resonance stabilization accounts for the effective antioxidant potential of Ferulic acid (Kim and Lee, 2007).

Moreover, Ferulic acid is a strong scavenger of free radical and it has approved in certain countries as food additive to prevent lipid peroxidation (Srinivasan et al., 2007). Ferulic acid, 4-hydroxy-3-methoxycinnamic acid, is a ubiquitous phenol in seeds and leaves of various plants (Kim and Lee, 2012).

2.1.9 *Terminalia arjuna* plant

**Scientific classification (or) taxonomy of plant**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td>Myrtales</td>
</tr>
<tr>
<td>Family</td>
<td>Combretaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Terminalia</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>Arjuna</em></td>
</tr>
</tbody>
</table>

*Terminalia arjuna* seed

*Figure -7 Terminalia arjuna seed*
The plant material seed of *Terminalia arjuna* seed was collected from the local areas of Annamalai nagar, Chidamaram, Cuddalore district, Tamilnadu, India. *Terminalia arjuna* is commonly known as arjuna. The *Terminalia arjuna* tree is about 20-25 meters tall. It has oblong, conical leaves which are green on the top and brown below. It has pale yellow flowers, its glabrous, 2.5 to 5 cm fibrous woody fruit, divided into five wings, appears between September and November.

### 2.1.10 Preparation of seed extract

The collected *Terminalia arjuna* seeds were cleaned and shade-dried. The dried seeds were pulverized by a mechanical blender and passed through a 20-mesh sieve. A powdered seed (500g) was successively extracted with methanol solvent using a Soxhlet apparatus. The extraction was carried out for 24 hrs at room temperature. The extracts were filtered and concentrated at 35°C, and the weight of residue was recorded and percentage yield was calculated.

### 2.1.11 Mechanisms of Action of Methanolic fractions of *Terminalia arjuna* seed extract

*Terminalia arjuna* plant is highly medicines improvement of cardiac muscle function and subsequent improved pumping activity of the heart seems to be the primary benefit. It is antioxidant properties thought the saponin glycosides might be responsible for the inotropic effect of methanolic fractions of *Terminalia arjuna* seed extract, while the flavonoids and oligomeric proanthocyanidins (OPCs) provide free radical scanning activity and vascular strengthening. (Yadav *et al.*, 2001).
2.1.12 Mercuric chloride toxicity studies

In present study the heavy metal toxicity test conducted by median-lethal dose value, which is the least dose required to kill the 50 percent of the population of test animals (Cremlyn, 1978). The LD_{50} value of mercuric chloride on rat was already determined by Suresh Kumar (1999) in our lab (LD_{50} value is 13.0 mg of mercury chloride/ kg body weight of the animal for 7 days). The same calculation (LD_{50}) value was also used in the present experimental study for preparing the sub-lethal dose. Ten times dilution of the median-lethal dose is sub-lethal dose (1.30 mg of mercuric chloride/ kg of animal for 45 days). At sub-lethal dose metal toxicity tests were conducted to measure the impact of the toxicant on rat upto 45 days. In this experimental study the renewal technique of sub-chronic static test was also adopted in which the rats were periodically dosed to concentrate of the same composition of mercuric chloride solution usually once in 24 hours.

2.1.13 Ferulic acid and Methanolic fractions of *Terminalia arjuna* seed extract

Dose preparation

Acute toxicity studies of Ferulic acid and methanolic fractions of *Terminalia arjuna* seed extract solvent were carried out on female albino wistar rats. The solution was prepared at particular concentration (5mg/kg body weight of animal 45 days). The test was conducted by allowing six rats after the oral administration of the extract. The rate of mortality was observed at entire periods. The dose at which 100% survival was observed for 45 days was considered as sub-lethal dose.
2.2 Experimental procedure

In present study were at sub-lethal dose of mercury chloride was prepared in distilled water and the required dose was given orally (1.30mg/kg body weight) animal for 45 days. After the 45th day, the animals were orally administered with Ferulic acid and Methanolic fractions of *Terminalia arjuna* seed extract at the dosage rate of 5mg/kg body weight for animal for another 45 days. After the scheduled treatments, the animals were sacrificed and the organs (Heart, Liver and Kidney) were taken for the biochemical, histopathological and bioenzymological purposes.

2.2.1 Experimental protocol

Wistar albino rats were divided into six groups each consisting of six animals

**Experimental design**

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Untreated control</td>
</tr>
<tr>
<td>Group-II</td>
<td>Mercury treatment</td>
</tr>
<tr>
<td>Group-III</td>
<td>Post treatment of Ferulic acid</td>
</tr>
<tr>
<td>Group-IV</td>
<td>Post treatment of <em>Terminalia arjuna</em> seed extract</td>
</tr>
<tr>
<td>Group-V</td>
<td>Ferulic acid alone treatment</td>
</tr>
<tr>
<td>Group-VI</td>
<td><em>Terminalia arjuna</em> seed extract alone treatment</td>
</tr>
</tbody>
</table>
2.2.2 Processing of blood and tissue samples

After completing the scheduled treatment, the animals were anaesthetized using chloroform between 9am and 10am. The animals were sacrificed by cervical dislocation and then the required organs (heart, liver and kidney) and blood was collected and used for both bio-chemical and bio-enzymological analysis.

2.2.2.1 Serum preparation

The blood sample was collected in a dry test tube and allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 300 g for 10 min. The serum sample was used for bio-marker analysis.

2.2.2.2 Tissue homogenate preparation

After cervical dislocation, heart, liver and kidney organs were isolated immediately from the sacrificed animals. The organs (500mg) were sliced into pieces and homogenised in appropriate buffer in cold condition (pH 7.0) to give 10% homogenate (w/v). The homogenate was centrifuged at 120 g for 10 min at 0 °C in a cold centrifuge. The supernatant was separated and used for various biochemical and bio-enzymological estimations.

2.2.3 List of parameters analysed in this study

Seed extract preparation

Methanolic solvent extractions of *Terminalia arjuna* seed extract.
Heart, liver and Kidney tissue

Oxidant, Non-enzymatic antioxidant, enzymatic antioxidants and histology

- LPO content: Lipid peroxidation Thiobarbituric acid reactive substances (TBARS). Reduced glutathione (GSH). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).
- Histology and histopathological changes all treatment groups.

Serum Bio-enzymological markers:

- Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP). Creatine phosphokinase (CPK), Lactate dehydrogenase (LDH) and total Cholestrol (TC).

Heart tissue

- ECG: Electrocardiogram spectrum analysis for all the treatment groups.
- Cardiac inflammatory marker COX-2 antibody protein expression by immunohistochemistry.
- TNF-α protein expression
- TGF-β1mRNA expression

2.3 Histopathology study

In the present experimental study, the following procedure was adopted to prepare the histological and histopathological slides. After completing the experimental duration, the animals were sacrificed with the help of cervical
dislocation. After the cervical dislocation selected vital organs (Heart, liver and Kidney) were quickly isolated and fixed in buffered formalin solution for 3 hours. The tissues were washed in running tap water and processed following the standard technique (Gurr, 1959) for microtomy. The tissues were dehydrated in ascending grades of alcoholic series and then cleaned in xylol and kept in cold in filtration for 24 to 72 hours. After completing the cold infiltration the tissues were transferred in to hot infiltration and then embedded in paraffin wax (58-60°C). From the wax material serial sections were cut at 6-8µ thickness and these were deparaffinished in xylol and then passing through descending glades of alcoholic series and counter stained with aqueous Haematoxylin-Eosin stain. Stained sections were mounted in DPX for microscopical observations.

2.4 Biochemical and bio-enzymolgical estimations in selected organs

2.4.1 Non-Enzymatic Antioxidant

2.4.1.1 Estimation of Lipid peroxidation (LPO/TBARS)

The concentration of LPO/TBARS in the tissues (Heart, Liver and Kidney) was estimated by the method of Nichays Samuelsen (1968).

Reagents

1. Thiobarbituric acid (TBA) – 0.375% in hot distilled water
2. Trichloroacetic acid (TCA) – 15%
3. Hydrochloric acid (HCl) – 0.25N
4. TBA-TCA-HCl reagent: Solution 1 and 3 was mixed in ratio of 1:1:1 freshly prepared prior to use.
**Procedure**

The required amount of selected tissues (Heart, liver, and kidney) homogenate was prepared in Tris-HCL buffer (pH 7.5). After completing the homogenization 1 ml of the tissue (Heart, Liver and Kidney) homogenate was taken in a clean test tube and 2.0 ml of TBA-TCA-HCL reagent was added and then mixed thoroughly. The mixture was kept in a boiling water bath (60°C) for 15 minutes. After completing the incubation period, the tissue mixture was cooled with the help of running tap water. After cooling, the mixture was read the absorbance of the chromophore at 535 nm against the reagent blank in an UV visible spectrophotometer (Spectronic -20, Bausch and Lamb). 1, 1’, 3, 3’ tetra methoxy propane was used to construct the standard graph.

The values were expressed as n mol/mg wet wt. of tissues.

**2.4.1.2 Estimation of reduced glutathione (GSH)**

The reduced glutathione level was determined by the method of Beutler and Kelley (1963).

This method was based on the development of yellow color when 5,5’-dithio-bis-2-nitrobenzoic acid (DTNB) is added to compound containing sulphhydryl groups. The color developed was read at 412nm.

**Reagents**

1. 0.3M Disodium hydrogen phosphate.
2. 0.1% disodium salt of EDTA.
3. Precipitating reagent: 1.67g of Metaphosphoric acid, 0.2g of EDTA
4. Disodium salt, 30g sodium chloride in one liter of distilled water.
5. 5,5’-Dithio(bis)-2-nitrobenzoic acid (DTNB) reagent: 40mg of DTNB in 100ml of 1% sodium citrate.

6. Standard solution: 10mg of reduced glutathione in 100ml distilled water.

**Procedure**

0.2ml of sample (Heart, liver and kidney tissue homogenate) was mixed with 1.8ml of EDTA solution. To this 3.0ml of precipitating reagent was added, mixed thoroughly and kept in the room temperature for 5 minutes before centrifugation. The mixed content was centrifuged at 2500 rpm for 5 minutes. After centrifugation 2.0ml of the supernatant was taken in a clean test tube and added the following reagents. 4.0ml of 0.3M disodium hydrogen phosphate solutions and 1.0ml of DTNB reagent were added to the supernatant the color developed was read at 412nm in an UV spectrophotometer. A set of standard solutions containing 20-100µg of reduced glutathione was treated similarly.

The values were expressed as mg/100mg for tissues.

**2.5 Enzymatic antioxidant**

**2.5.1 Assay of superoxide dismutase (SOD; EC 1.15.1.1)**

Superoxide dismutase activity in the tissues was assayed by the method of Kakkar *et al.* (1984).

The assay of SOD was based on the inhibition of the formation of NADH – phenazine- methosulphate- nitroblue tetrazolium formation. The reaction was initiated by the addition of NADH. After incubation for 90 sec adding glacial acetic acid stops the reaction. The colour developed at the end of the reaction was extracted into n-butanol layer and measured in a spectronic 20 at 520 nm.
Regents

1. Sodium pyrophosphate buffer (0.025M, PH 8.3): 1.115g of tetra sodium pyrophosphate was dissolved in 100 ml of distilled water and the PH was adjusted to 8.3 by using 0.025M Hydrochloric acid.

2. Phenazine methosulphate (186µM): 6mg of phenazine methosulphate was dissolved in 100 ml of distilled water.

3. Nitroblue tetrazolium (300µM): 24.5mg of nitroblue tetrazolium was dissolved in 100 ml of distilled water.

4. NADH (780µM): 51.8 mg of NADH was dissolved in 100 ml water.

5. Glacial acetic acid


7. Chloroform.

8. Ethanol.

Procedure

The selected tissues (Heart, Liver and Kidney) were isolated from the animal immediately and then homogenized in 2 ml of 0.25M sucrose solution. The homogenized content was centrifuged at 10,000 rpm for 30 minutes in cold condition. After completing the centrifugation the supernatant was taken in a test tube and it was dialyzed with Tris-HCL buffer (0.0025M, PH 7.4). The dialyzed supernatant was used for enzyme assay. In a clean test tube 1.2 ml of dialyzed supernatant was taken and 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methisulphate, and 0.3ml of nitroblue tetrazolium reagents were added
and then kept in a water bath at 30°C for 90 seconds. After completing the incubation the contents were made up to 3.0 ml by adding glass distilled water and the 0.2 ml of NADH reagents was added for initiating the reaction. The reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes, and the centrifuged for 15 minutes at 3000 rpm. After centrifugation the butanol layer was separated. The colour intensity of the chromogen was measured at 560 nm in UV spectrophotometer against butanol as blank and the system devoid of enzyme served as control.

One unit of enzyme activity is defined as the enzyme reaction which generates 50% inhibition of NBT reduction in one minute under the assay condition and is expressed as specific activity in units / mg protein.

Values are expressed as unit / min / 100 mg protein. Each unit is the amount of tissue that inhibits 50% reduction of NBT.

2.5.2 Estimation of Catalase (CAT; EC 1.11.1.6)

The activity of catalase in the tissues was determined by the method of Sinha (1972).

Dichromate in acetic acid was converted to perchromic acid and then to chronic acetate when heated in the presence of H\textsubscript{2}O\textsubscript{2}. The chronic acetate formed was measured at 620nm. The catalase preparation was allowed to split H\textsubscript{2}O\textsubscript{2} for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining H\textsubscript{2}O\textsubscript{2} as chronic acetate was determined colorimetrically.
Reagents

1. Phosphate buffer (0.01M, PH 7.0): 120 mg sodium dihydrogen phosphate (NaH$_2$PO$_4$) and 142 mg of disodium hydrogen phosphate (Na$_2$HPO$_4$) were dissolved in 100 ml of distilled water and the PH was adjusted to 7.0.

2. Hydrogen peroxide (H$_2$O$_2$) 0.2N: 0.682 ml of Hydrogen peroxide (1:12Kg/Liter) was dissolved in 100 ml of distilled water.

3. Dichromate acetic acid reagent: 5% potassium dichromate solution was mixed with glacial acetic acid in the ratio of 1:3.

4. Dichromate was mixed with glacial acetic acid. From this 1ml was diluted again with 4ml of acetic acid.

5. Standard hydrogen peroxide: 0.066 ml Hydrogen peroxide was dissolved in 100 ml of H$_2$O$_2$.

Procedure

The isolated whole selected tissues (Heart, liver and kidney) were homogenized in 2.0 ml of phosphate buffer solution and centrifuged for 10minutes at 2000 rpm. In a clean dry test tube 0.9 ml of phosphate buffer, 0.1ml sample (tissue homogenate) and 0.4 ml of hydrogen peroxide were added and then kept the contents in room temperature. After 30 to 60seconds 2ml of dichromate acetic acid mixture was added in the contents and then kept in boiling water bath at 37°C for 10minutes and then the contents were allowed to cool in the room temperature. The colour developed was read at 620 nm in an UV spectrophotometer. Hydrogen peroxide was used to construct the standard graph.

The values are expressed as µ moles H$_2$O$_2$ consumed /min/mg protein.
2.5.3 Estimation of glutathione peroxidase (GPx; EC 1.11.1.19)

The activity of GP\textsubscript{X} in the tissues was measured by the method of Rotruck \textit{et al.} (1973). A known amount of enzyme preparation was allowed to react with H\textsubscript{2}O\textsubscript{2} in the presence of GSH for a specified time period. Then the remaining GSH content was measured.

**Reagents**

1. 0.4 M phosphate buffer: 4.36 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.
2. 10 mm sodium azide was dissolved in 100 ml of distilled water.
3. 10% TCA: 10g TCA was dissolved in 100 ml of distilled water.
4. 0.4 mm EDTA solution: 11.69 mg of ETDA was dissolved in 100 ml of distilled water.
5. 0.2 mm H\textsubscript{2}O\textsubscript{2}: 0.6 ml of hydrogen peroxide was dissolved in 100 ml of distilled water.
6. 2 mM standard glutathione: 61.47 mg of glutathione was dissolved in distilled water.
7. 0.11 mM DTNB: 2.33 mg of DTNB was dissolved in 100 ml of distilled water.

**Procedure**

The selected tissues (Heart, liver and kidney) were homogenized in 2.0 ml of phosphate buffer and centrifuged at 2,500 rpm for 5 minutes. After centrifugation 0.2 ml of clear supernatant was taken in a clean test tube and then the following enzyme mixture was added. The enzyme mixture contained 0.2 ml of
0.4 mM of EDTA and 0.1 ml 10 mM of sodium azide. The reaction mixture was mixed well and kept for two minutes at 37°C in an incubator. After the incubation period, 0.2 ml of reduced glutathione and 0.1 ml of H₂O₂ were again added to the above mixture and incubated at 37°C exactly for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10% TCA. The colour was developed and then read at 412 nm in an UV spectrophotometer. Reduced glutathione was used to construct the standard graph.

The values are expressed as µmoles of GSH utilized / min / mg protein.

2.6 Biochemical and bio-enzymological estimations in serum

2.6.1 Assay of aspartate aminotransferase (AST; EC.2.6.1.1)

Aspartate aminotransferase was assayed by using the method of Reitman and Frankel (1957).

AST is catalyse and react with L-aspartate then transfer of amino group to α-ketoglutarate gives to formation of oxaloacetate and glutamate. The amount of oxaloacetate was measured by converting it into pyruvate by treating with aniline-citrate and then reaction the pyruvate with 2, 4-dinitrophenylhydrazine to form 2, 4-dinitrophenylhydrazone derivative which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is correlated to AST activity.

Reagents

1. Buffered substrate: 2.66g of DL-aspartate and 38 mg of α-ketoglutarate were dissolved in 20.5 ml of 1 N sodium hydroxide, with gentle heating. This was made up to 100 ml with phosphate buffer (0.01 M, pH 7.4).
2. Aniline-citrate reagent: 50 g of citric acid was dissolved in 50 mL of distilled water and mixed with equal volume of redistilled aniline.

3. Dinitrophenylhydrazine (DNPH) reagent: 1.0 mm DNPH in 2.0 N HCl.

4. Sodium hydroxide: 0.4 N

5. Pyruvate standard: 2.0 mm

**Procedure**

0.5 ml of buffered substrate was added to 0.1 ml of serum homogenate and placed in a water bath at 37°C. To the blank tubes, 0.1 ml distilled water was added instead of sample. Exactly an hour later, 2 drops of aniline-citrate reagent and 0.5 ml of DNPH reagent were added and kept at room temperature for 20 min. Finally, 5.0 ml 0.4 N sodium hydroxide was added. A set of standards were also treated in the same manner and read at 520 nm after 10 min.

The results were expressed as IU/L for serum

**2.6.2 Assay of alanine aminotransferase (ALT; EC.2.6.1.2)**

Serum alanine aminotransferase was assayed by using the method of Reitman and Frankel (1957).

ALT is catalyse and react with L-aspartate then transfer of amino group to α-ketoglutarate gives to formation of pyruvate and glutamate. The pyruvate so formed, is allowed to react with 2, 4-dinitrophenylhydrazine to produce 2,4 dinitrophenylhydrazone derivatives which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is correlated to ALT activity.
Reagents

1. Buffered substrate: 1.78 g of DL-alanine and 38 mg of α-ketoglutarate were dissolved in buffer. 0.5 ml of NaOH was added and the volume was made up to 100 ml with phosphate buffer (0.01 M, pH 7.4).

2. All other reagents were same as that used for the assay of aspartate transaminases.

Procedure

1. Procedure was same as that used for the assay of aspartate transaminases except the incubation time which was reduced to 30 min (60 min for AST).

The results were expressed as IU/L serum

2.6.3 Assay Alkaline Phosphates (ALP) activity

An alkaline phosphate was estimated by King and Armstrong method (1988).

Reagents

1. Buffered substrate

A. 0.01M disodium phenyl phosphate-1.09g of disodium phenyl phosphate was dissolved in water and made upto 500ml, boiled and cooled. A little chloroform was added and kept in refrigerated.

B. Sodium carbonate – Sodium bicarbonate buffer [0.1M pH 10]- 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate was dissolved in water and made upto 500ml.

Mix A and B equal volume
2. 0.5N NaOH.
3. 0.5M Sodium bicarbonate.
4. 0.6% amino antipyrine.
5. 2.4% Potassium ferricyanide.

**Procedure**

2.0 ml of each buffered substrate was taken into clean test tubes and it was incubated for 5 minutes at 37°C. Then 0.1 ml of serum was added in the tubes and incubated for 15 minutes. The reaction was arrested by addition of 0.8 ml of 0.5N NaOH and then 1.2ml of 0.5M Sodium carbonate was added. The contents were thoroughly mixed. Again 1ml of amino antipyrine was added and then 1.0ml of potassium ferricyanide was and the colour developed was read at 520 nm against the reagent blank UV – spectrophotometer.

The activities of serum ALP were expressed as IU/ml of serum.

**2.6.4 Assay of lactate dehydrogenase (LDH; EC. 1.1.1.27)**

The activity of lactate dehydrogenase was assayed by the method of King [1965]. LDH following reaction:

\[
\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+
\]

The pyruvate is coupled with 2,4-dinitrophenylhydrazine to give a corresponding hydrazone and this produces a brown colour in alkaline medium that is measured spectrophotometrically at 520 nm.
Reagents

1. Glycine buffer: 0.1 M

2. Buffered substrate: 2.78 g of lithium lactate was dissolved in 125 mL of glycine buffer containing 75 ml of 0.1 N sodium hydroxide solutions. This was prepared just before use.

3. NAD$: 10 \text{mg in 2.0 ml distilled water}

4. 2, 4-dinitro phenyl hydrazine reagent (DNPH): 200 \text{mg of DNPH was dissolved in 1 L of 0.1 N hydrochloric acid}

5. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of buffer.

Procedure

1.0 ml of buffered substance was mixed with 0.05 ml of serum or tissue homogenate (test) and the tubes were incubated at 37 °C for 15 min. 0.2 ml of coenzyme solution (NAD) was then added and again incubated for 15 min. The reaction was stopped by the addition of 1.0 ml of 2, 4-dinitrophenylhydrazine. The tubes were incubated at 37 °C for another 15 min and 5 ml of 0.4 N NaOH was added to each tube and mixed well. The intensity of the colour was measured at 520 nm. A control was performed simultaneously as like a test but serum was added after the addition of DNPH.

The enzyme activity was expressed as IU/L for serum.
2.6.5 Assay of creatine phosphokinase (CPK)

Creatinephospho kinase (CPK) activity in serum was determined by the method of Okinaka et al. (1961).

\[
\text{ATP + Creatine} \xrightarrow{\text{CPK}} \text{ADP + Creatine phosphate}
\]

The creatine phosphate is hydrolyzed by phosphomolybdic acid at room temperature to creatine and inorganic phosphate and the inorganic phosphate reacts with acid molybdate reagent to form phosphomolybdic acid. The hexavalent molybdenum of the phosphomolybdic acid is reduced by 1, 2, 4-aminonapthol sulphonic acid (ANSA) to give a blue colour complex that is estimated spectrophotometrically at 680 nm.

**Reagent**

1. Tris- HCL buffer: 0.1 M, pH 9.0.
2. ATP solution: 0.0185 M in tris- HCL buffer.
3. Magnesium-Cystine hydrochloride solution
4. Creatine : 240 mm
5. TCA : 10%
6. Ammonium mol : 2.5 g of ammonium molybdate was dissolved in 100 ml of sulphuric acid
7. ANSA (1-amino-2-napthol-4-sulfonic acid) : 25 %
8. Standard phosphorus: 35.1 mg of potassium dihydrogen phosphate was accurate weighed, dissolved in 100 ml of double distilled water. 1.0 ml of this solution contains 80 µg of phosphorus.
**Procedure**

The reaction mixture comprised of 0.05 ml of serum, 0.1 ml of substrate, 0.1 ml of ATP solution, and 0.1 ml of cysteine hydrochloride solution. The final volume was made up to 2.0 ml with distilled water and incubated at 37.8°C for 30 min. The reaction was arrested by the addition of 1.0 ml of 10% trichloroacetic acid (TCA) and the contents were subjected to centrifugation. To 0.1 ml of the supernatant, 4.3 ml distilled water and 1.0 ml ammonium molybdate were added and incubated at room temperature for 10 min. 0.4 ml of 1-amino-2-napthol-4-sulfonic acid (ANSA) was added and the color developed was read at 640 nm after 20 min.

The activity of the enzyme was expressed as IU/L for serum.

**2.6.6 Estimation of total cholesterol (TC)**

Total cholesterol in the serum was estimated by the enzymatic method described by Allain *et al.* (1974).

Cholesterol esters were hydrolyzed by cholesterol esterase to free cholesterol and free fatty acids. The free cholesterol produced and pre-existing ones were oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide formed reacts with 4-aminooantipyrene and phenol in the presence of peroxidase to produce red coloured quinoneimine dye. The intensity of colour produced was proportional to the cholesterol concentration.

**Reagents**

1. Enzyme reagent: 4-aminooantipyrene, cholesterol esterase, phenol, cholesterol oxidase and horseradish peroxidase.

2. Cholesterol standard: 200 mg %
**Procedure**

To 10 µL of serum or 10 µL of lipid extract, 1.0 ml of enzyme reagent was added, mixed well and kept at 37 °C for 5 min. 10 µL of cholesterol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.

Cholesterol concentration was expressed as mg/dL of serum.

2.7 **Immunohistochemistry**

Immunohistochemistry was performed as described by Rocha et al. (2009) using super sensitive polymer-HRP detection system kit, from sigma, USA. The super sensitive polymer-HRP detection system is a novel detection system using a non-biotin polymeric technology that makes use of two major components: Super Enhancer™ and a Poly-HRP reagent. As the system is not based on the biotin-avidin system, the problems associated with endogenous biotin are completely eliminated.

The detection of antigens in tissues by immune staining is a two-step process. The first step involves the binding of an antibody to the antigen of interest and the second step involves the detection and visualization of bound antibody by one of a variety of enzyme chromogenic systems. The choice of detection system will dramatically impact the sensitivity, utility and use of the method.

**Procedure**

Paraffin-embedded tissue was cut to obtain sections of about 4 µm thicknesses. The mounted paraffin-embedded slices were deparaffinized in xylene and rehydrated using an ethanol/H₂O gradient. Heat mediated antigen retrieval step
was carried out for 10 min and then the slides were allowed to cool to room temperature for another 20 min. This was followed by peroxidase block treatment (to block endogenous peroxidase enzyme activity) for 10-15 min and then power block treatment (to block non-specific binding of antibodies to highly charged sites) for another 15 min. The sections were incubated with the concerned diluted primary antibodies (COX-2) solution (for 2 h, 1:200) followed by treatment with the super enhancer solution (for 30 min) and super sensitive Poly-HRP solution (for 30 min). After color development with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstaining with haematoxylin, the sections were observed under the light microscope and photographs were taken.

**Immunohistochemical Staining:**

Two or Three serial sections of 4µm thickness were made and taken on APES coated slides. The sections were deparaffinised by heating on the slide warmer at 60°C for 1 hour. The sections were dewaxed in 2 changes of xylene, each of 15 mins and dehydrated in graded alcohol (100%, 90%, 70% and 50%) and then change to water each of 5 mins. The slides were placed in a coplin jar, with citrate buffer solution which in turn was kept in a pressure cooker containing water. The pressure cooker was then closed with the lid and brought to full pressure. Timing was noted down after the full pressure was reached and was kept for 10 mins duration. The pressure cooker was allowed to cool down to room temperature before removal of the slides. All the reagents stored in the refrigerator were brought to room temperature (24-28 °C) prior to immunostaining. All the incubations were performed at room temperature using a humidifying chamber. At no time the tissue sections were allowed to dry during the staining procedure.
After tapping off the excess buffer from the slide, the sections were covered with 3% hydrogen peroxide for 10 mins to block endogenous peroxidise activity followed by treatment with protein block for 10 mins to avoid cross reaction. Primary antibody application: The sections were covered completely with Rabbit Monoclonal antibody against COX-2 receptor (Thermo Scientific, UK). Then the slides were washed gently with PBS and kept in the PBS buffer bath for 5 mins. The slides were washed and treated with poly Horseradish peroxidase enzyme (HRP) (SCYTEK, USA) for 30 mins. The slides were then washed with PBS and immunostaining was carried out by staining with DAB (3, 3-diaminobenzidine tetra hydrochloride) for 5 mins following which it was washed in distilled water to remove excess chromogen. The slides were immersed in Mayer’s hematoxylin for 7 mins and bluing was done in running tap water for 10 mins. The sections were dehydrated in alcohol for 10 mins and air dried thoroughly and mounted using DPX. The immunostained slides were observed for positivity under 4X/10X/40X magnifications and recorded with a high quality photomicrograph.

2.7 Electrocardiogram (ECG) analysis

![ECG spectrum analysis](image)

**Figure 8: ECG spectrum analysis**
2.7.1 Measurement of ECG

Equipment

MP100 data acquisition system

ECG100 C amplifier modules

Two (2) LEAD100S shielded electrode leads

One (1) LEAD100 electrode lead

Procedure

a. Hardware Setup

Snap the ECG100C module to the UIM100

Select channel 1 on the ECG100C amplifier

Place the ECG100C amplifier gain to 500, and the module switch settings to:

35 Hz LPN Filter and 0.05 Hz HP Filter

b. Test system setup

1. Inject Ketamine (100 mg/kg., i.p) to anaesthetize the animal

2. Fix 3 ECG Lead at the appropriate places as mentioned (The leads were attached to right arm, left arm and left leg of rat)

3. Read the ECG of the animal by using Acqknowledge 3.9.0 software.
2.8 Western blot analysis

2.8.1 Western blot analysis of inflammatory marker expression

The Western blot was carried out by the method of Towbin et al. (1979). Immunoblot analysis was carried out for TNF-α protein expression in mercuric chloride treated rat with Ferulic acid and the methanolic fraction of *Terminalia arjuna* seed extract administrated in heart tissue. The results were normalized to β-actin gene expression.

**Principle**

Following the protein estimation, the samples were separated using SDS-PAGE gel electrophoresis and the separated molecules are blotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking, the primary antibody was added and allowed to bind to the protein followed by washing (which removes nonspecifically bound antibody); then an enzyme-labeled secondary antibody was added, to detect the primary antibody. The location of the secondary antibody was determined by adding an appropriate substrate for the enzyme conjugated to the secondary antibody.

**Reagents**

1. Acrylamide stock: 30% acrylamide, 0.8% N,N′-methylene bisacrylamide
2. Separating gel buffer: 2.25 M Tris, 0.6% sodium dodecylsulfate (SDS), pH 8.8
3. Sample buffer: 0.063 M Tris, 2% SDS, 10% sucrose, 0.01% bromophenol blue, pH 6.8
4. 10% ammonium per sulfate
5. N,N,N',N'-tetramethylethylenediamine (TEMED)

6. Running gel buffer (5X): 0.25 M Tris, 0.5% SDS, 1.92 M glycine

   30.3 g Tris 5.0 g SDS and 144.1 g glycine were dissolved in 700 mL of distilled water. pH was adjusted to 8.3 with conc. HCl and diluted to 1 L with distilled water. The working running buffer was prepared by making a 1:5 dilution of the stock 5X buffer with distilled water.

7. Water-saturated isobutanol

8. β-Mercaptoethanol

9. Staining and fixing solution:

   2.5 g coomassie brilliant blue R250 was dissolved in 1 L solution containing methanol, acetic acid and distilled water in the ratio 5:1:4.

10. Destaining solution: 100 mL of absolute methanol and 100 mL of glacial acetic acid were mixed with 800 mL of distilled water.

**Procedure**

The heart tissue was homogenised in an ice-cold RIPA buffer (1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 m mol/L EDTA, 20 m mol/L Tris (pH 7.4), 150 m mol/L NaCl, 10 mmol/L NaF, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) containing a protease inhibitor cocktail). The homogenate was centrifuged at 12,000 rpm/min for 15 min at 4 °C to remove debris and the supernatant was used to determine the protein concentration of the lysates using the Lowry protein assay (Lowry et al., 1951).
Transfer of proteins to membrane

The heart tissue extracts containing 50 μg of proteins were fractionated on 12% SDS–PAGE gel and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA) using Biorad semi-dry apparatus (Biorad, USA). Before assembling the transfer system, soaked PVDF membrane in methanol for 10 min and blotting papers in cold transfer buffer. Prepared sandwich, blotting paper, membrane, gel and blotting paper, were placed in the transfer apparatus and few drops of transfer buffer was added and subjected to an electric current 20 V for 1 h under cold condition. After the transfer, the sandwich was removed from the transfer system. Membrane was stained with 0.5% ponceau in 1% acetic acid to confirm equal loading.

The PVDF membrane were blocked with 5% (w/v) nonfat milk (blocking solution) in TBST (1.5 M NaCl, 20 mM Tris–HCl, 0.05% (v/v) Tween-20) for 6 h and then incubated with primary antibodies (Sigma-Aldrich Saint Louis, Missouri, USA), diluted 1:1000 in blocking solution, for overnight at 37 °C. The membranes were washed with TBST thrice for 10 min interval and then incubated with horseradish peroxidase conjugated secondary antibody (diluted 1:2000) in blocking solution for 2 h at 37 °C. Then the membranes were washed with TBST thrice for 10 min interval, after extensive washes in TBST, the bands were visualized by treating the membranes with 3, 3'-diaminobenzidine tetrahydrochloride (Western blot detection reagent). Densitometry was done using ‘Image J’ analysis software.

2.9 Extraction of total mRNA

Total mRNA was isolated from rat heart tissue using mRNA extraction kit (Genei Bangalore, India), following the manufacturer’s instructions. The mRNA integrity was determined by agarose gel electrophoresis, and the concentration and purity were measured spectrophotometrically (Kingston et al., 1996).
Total mRNA was converted to single stranded cDNA using 2μg of total mRNA as a template. Oligo (dT) 12-18 primer and Moloney murine leukemia virus reverse transcriptase were used as per manufacture’s instruction. The following primers were used for the mRNA expression: TGF-β1 (168 bp; GenBank Accession No: NM_21803): forward primer 5’-TTGCTTCAGCTCCACAGA GA-3’, reverse primer 5’-TGTTGTAGAAGCAGCAAGGAC-3’-GAPDH (132bp) (GenBank Accession No: NM_14433): forward primer 5’-GACCACAGTCCA TGC CATCAC-3’ reverse primer 5’-GCTGTTGAAGTCGACAGGAGAC-3’.

2.9.1 Real Time-PCR analysis

The specific mRNA expressions were performed by real-time PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the Eppendorf (Thermocycler) real-time PCR instrument, software version V1.5.0.39 (Genei Bangalore, India). Twenty-five microliter of reaction mixture contained 0.1μl of 10 μM forward primer and 0.1μl of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μl of RT Mastermix, 11.05 μl of nuclease-free water, and 1.25μl of cDNA sample. The primers used in the current study were chosen from previously published studies and are listed above. Assay controls were incorporated on to the same plate, namely, no template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and anneal/extension at 60 °C for 1 min. Melting (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.
Procedure

In a clean test tube 560µl of lysis buffer to a nuclease free was taken. Then added 5.6 µl of carrier RNA and 140µl of tissue sample. The contents were mixed immediately by inverting and incubated at room temperature for 10 min. After completing the incubation period 560µl of (100%) ethanol was added and then mixed well by vortex for 30 seconds. Pipette 630µl of sample into the PureFast spin column. Centrifuge for 1min. Discard the flow-through and place the column back into the same collection tube. Then adding reaming 630µl of sample and then centrifuge for 1min. Discard flow through and place the column back into the some collection tube. In the same manner the samples were washed with Wash Buffer-I and Wash Buffer II. Then centrifuge the contents for an additional 2 min. This step is essential to avoid residual ethanol. Transfer the PureFast spin column into a fresh 1.5 ml micro-centrifuge tube (not included). Then add 60µl of Elution Buffer to the center of PureFast spin column membrane. Incubate one minute. Again centrifuge for 1 min and discard the PureFast spin column. Centrifuge tube now contains the eluted Viral RNA. Either use the eluted RNA directly in RT-PCR or store the eluted viral RNA at-80˚C for later analysis.

Recommendation for RT-PCR

Use 5-20µl of elute for reverse transcription.

cDNA synthesis

Material Required

RT reaction buffer, RNase Inhibiter, Reverse Transcriptase, dNTPs, Oligo dT)18, Randam Hexamer Primers.
Important Note

Avoid ribonuclease contamination;

- Use nuclease free lab ware or DEPC treated tubes and pipettes tips.
- Wear gloves when handling RNA and all reagents.
- Keep all tubes tightly closed during the reverse transcription reaction.

Template RNA

- Purified RNA must be free of salts, ions, ethanol, and phenol to avoid inhibiting the cDNA synthesis reaction.
- Access RNA quality (integrity) prior to cDNA synthesis. Total eukaryotic RNA can be analysed by agarose gel electrophoresis. Both 18S and 28SrRNA must appear as sharp bands after electrophoresis of total RNA. Any smearing band is an indication of degraded mRNA. If this occurs, a new sample of total RNA should be prepared.

Procedure

Note: Thaw all reagents on ice, after thawing, mix and briefly centrifuge the components of the kit before open.

In a sterile, RNase-free tube on ice the following reagents were added in the indicated order.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA synthesis mix</td>
<td>10µl</td>
</tr>
<tr>
<td>Oligo(dT)</td>
<td>5µl</td>
</tr>
<tr>
<td>Purified RNA</td>
<td>50µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20µl</strong></td>
</tr>
</tbody>
</table>
After completing the mixing the contents were centrifuged, and then incubate the contents for 10min at 25 °C followed by 30 min at 50 °C (Note: For RNA template amount greater than 1µg, prolong the reaction time to 30 min. For RNA templates that are GC rich or have a large amount of secondary structure, the reaction temperature can be increased to 60 °C). Terminate the reaction by heating at 85 °C for 5min. The product of the first cDNA synthesis can be used directly in PCR/QPCR or stored at -20 °C for up to one week.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo master mix</td>
<td>20µl</td>
</tr>
<tr>
<td>TGF-β1 primer mix</td>
<td>7µl</td>
</tr>
<tr>
<td>cDNA- synthesis</td>
<td>3µl</td>
</tr>
<tr>
<td><strong>Total Final volume</strong></td>
<td><strong>30µl</strong></td>
</tr>
</tbody>
</table>

After amplification, load entire PCR product into 2% agarose gel electrophoresis.

**2.10 Statistical analysis**

All quantitative measurements were expressed as means ± SD for control and experimental animals. Values are given as mean ± S.D. for six rats in each group. The data for various biochemical parameters were analyzed using analysis of ‘t’-test and the group means was compared by Duncan’s multiple range test (DMRT) (Duncan, 1955). Values were considered statistically significant at 5% level of confidence limit (p<0.05).