Chapter-3

MATERIALS AND METHODS

This chapter discusses the analytical techniques used in the study and experimental design for various pharmacological experiments.

3.1 DRUGS AND CHEMICALS
Alloxan, streptozotocin, pentylenetetrazole (PTZ), bisphenol A diglycidyl ether (BADGE), L-arginine, imperatorin, N-nitro-L-arginine methyl ester hydrochloride (L-NAME) were procured from Sigma Aldrich. Pioglitazone was procured from Panacea Biotech. Glucose estimation, total cholesterol, lactate dehydrogenase (LDH) and creatinine kinase (CK-MB) and protein estimation kits were procured from Span Diagnostics. Diazepam and phenytoin were supplied by National Medicos. Misoprostol and naloxone were obtained from Zydus Cadila and Smarth Pharmaceuticals respectively. Other chemicals used in the study were procured from local suppliers and were of analytical grade.

3.2 PLANT MATERIAL
The plant material comprising of the leaves of *Aegle marmelos* Corr. was collected from the Botanical garden of Guru Nanak Dev University, Amritsar and authenticated by Dr. Amarjit Singh Soodan. A specimen sample (SR./Bot.Sci./0350) has been deposited in the herbarium of the department of Botanical and Environmental Sciences of the same university. The clean leaves were shade dried and powdered.

3.3 PHARMACOGNOSTIC STUDIES

3.3.1 MICROSCOPY
Fresh leaves were cut, cleaned and fixed in a solution of formalin, acetic acid and ethanol (formalin: acetic acid: 70% ethanol:: 0.5:0.5:9). This was followed by dehydration of specimens with tertiary butyl alcohol in a graded manner after 24h. Thereafter tertiary butyl alcohol was supersaturated by addition of paraffin wax slowly. 10-12 µm thick sections were cut from paraffin blocks of
specimens using rotary microtome (Johnson, 1940; Sass, 1940). Toluidine blue, safrarin, fast green and iodine-rich potassium iodide staining of the sections was undertaken after dewaxing (Palanisamy et al., 2009; Wallis, 1985). Paradermal sections of leaf were cleared with 5% sodium hydroxide and subjected to epidermal peeling employing Jeffery’s maceration fluid (Palanisamy et al., 2009; Bhaskar and Balakrishnan, 2010) for studying the morphology of stomata, pattern of venation and trichomes. Temporary preparations mounted in glycerine were used. Powder microscopy was carried out after clearing the material with NaOH, staining and then mounting in glycerine. Nikon Labphoto-2 microscopic unit was used for obtaining photomicrographs. Bright field was employed for normal observations and polarized light was employed for studying structures such as lignified cells, which are visible due to their brightness against dark background. Scale bars were used to indicate magnifications.

3.3.2 PHYSICOCHEMICAL PARAMETERS

Ash and extractive values

Various ash values including the total, acid insoluble and water soluble ash as well as the water soluble and ethanol extractive extractive values of the dried powdered leaves were determined according to the standard methods (Indian Pharmacopoeia 2010; Wallis, 1985). Total ash was estimated by incinerating accurately weighed (2-3 g each) air dried leaves in six tared silica crucibles at 450°C to a constant weight. The total ash in first three crucibles was boiled separately in 25 ml of 2 M hydrochloric acid for a period of 5 min. Ashless filter paper was used to collect the residue. It was washed with warm water and further ignited at 450°C to a constant weight to yield acid insoluble ash. The total ash in the remaining three crucibles was boiled separately with 25 ml distilled water for 5 minutes, washed and incinerated at 450°C till constant weight. The residue so obtained was subtracted from total ash to obtain water soluble ash. The powdered leaves of A. marmelos were taken in a quantity of 5 g each in six closed flasks. To the first three flasks, 100 ml of ethanol (95% v/v) and to the next three flasks, 100 ml of distilled water were added. The flasks were shaken at regular intervals for the first 6 h and kept undisturbed for further 18 h, after which, the contents were filtered; 25 ml of the filtrates were
evaporated separately in tared china dishes to dryness till a constant weight was achieved at 105°C.

* Determination of toxic residues, heavy metals, arsenic, pesticide residues and microbial content*

The powdered plant material was tested for the presence of aflatoxins, heavy metals including lead, cadmium, mercury, arsenic, pesticide residue and microbial contamination as recommended by the World Health Organization guidelines (1998) by International Testing Centre, Panchkula (Certificate no. AY-28-07-0110).

* Phytochemical screening*

Preliminary phytochemical screening of the crude extracts was carried out in accordance with the standard phytochemical tests for the various phytoconstituents such as alkaloids, anthraquinones, flavonoids, glycosides, proteins, reducing sugars, steroids, tannins and terpenoids (Farnsworth, 1966; Khandelwal, 2003).

3.3.3 PREPARATION OF EXTRACTS

The extracts were made by taking 100 g of the powder and extraction with hexane, petroleum ether, chloroform and ethanol by soxhelation for 16-18 h. Column chromatography of ethanol extract was carried out with silica gel and hexane: ethyl acetate in varying ratios as mobile phase to yield various fractions. Extracts were subjected to preliminary pharmacological screening for identifying the most active extract, which was further subjected to detailed phytochemical and pharmacological screening.

3.3.4 THIN LAYER CHROMATOGRAPHY OF EHANOL EXTRACT

The most active extract was dissolved in ethanol. TLC was obtained for the ethanol extract and the standard by loading on TLC plates using 2µL CAMAG capillary tubes. The plates were sprayed with 0.5% anisaldehyde in sulfuric acid and heated at a temperature of 105°C for a period of 2 min and then observed.
3.4 HPTLC STANDARDIZATION OF ETHANOL EXTRACT
The HPTLC unit (CAMAG, Switzerland) comprising of sample applicator (Linomat V) and scanner (TLC Scanner III) was used for the standardization of ethanol extract. Precoated TLC plates (Silica gel 60) of 200 µm (E. Merck, Germany) were employed as the stationary phase. The development of chromatograms was carried out in linear ascending mode in a twin trough glass development chamber (CAMAG, Switzerland) pre-saturated with vapour for a duration of 10 min. Solutions of the standard and sample were loaded in band form (band length 8 mm, sample application rate 80 nl s\(^{-1}\) and track distance 10 mm). Development of the plates was done at room temperature (25 ± 2°C), 55 ± 5% relative humidity and the mobile phase consisted of toluene-ethyl acetate-formic acid (5: 3.5: 0.1, v/v/v). Thereafter the plates so developed were dried and scanned for qualitative analysis. The inspection of chromatogram was done at 254 and 365 nm using TLC Scanner III. A reflectance mode of 350 nm was used for densitometric determinations with optical filter K 540, slit dimension 6.00 mm × 0.30 mm, scanning speed 100 mm s\(^{-1}\), data resolution 25 µm step\(^{-1}\). Imperatorin was employed as the standard.

3.5 ISOLATION AND CHARACTERIZATION OF IMPERATORIN
Pure imperatorin was isolated using column chromatography and characterized by \(^1\)H NMR and LCMS. Ethanol extract was charged on a column using silica gel (60-120) as the stationary phase. Elution of the column with hexane: ethyl acetate (9:1) yielded an impure solid which was rechromatographed over silica gel to obtain a pure solid. The isolated molecule was characterized by determining the melting point and spectral analysis using \(^1\)H NMR and LC-MS (Fig. 6).

3.6 MOLECULAR DOCKING STUDIES
ArgusLab 4.0.1 software was used to built imperatorin and semi-empirical quantum mechanical method PM3 was used to minimize energy. Protein data bank (pdb) was used to download the crystal coordinates of PPAR-β (pdb ID 1GWX) and PPAR-γ (pdb ID 1K74). The active sites of PPAR receptors were pasted in the work space and imperatorin was docked on it. The grid based
docking algorithm is instrumental in carrying out conformational studies and geometric optimizations of ligands. A score and lowest energy values were used to determine the ligand orientation and position. H-bonds and hydrophobic interactions of the molecule with the receptors give an approximation of ligand binding.

3.7 PHARMACOLOGICAL EVALUATION
The various extracts were investigated for in vitro antiproliferative activity in cancer cell lines. Preliminary studies revealed the ethanol extract to be the most effective therefore the ethanol extract was used for evaluation of analgesic activity, ameliorative effect in diabetic nephropathy and cardiomyopathy and antiepileptic activity.

3.7.1 IN VITRO STUDIES
EVALUATION OF ANTIPROLIFERATIVE ACTIVITY
In vitro cytotoxicity evaluation of the various extracts and the various fractions was carried out using six different human cancer cell lines including lung (A-549), colon (CoLo- 205), ovary (IGR-OV-1), prostrate (PC-3), leukemia (THP- 1) and breast (MCF- 7) cancer cell lines. These were provided by National Cancer Institute, Frederick, U.S.A. Briefly, RPMI-1640 medium with 2 mM glutamine at a pH of 7.4 was used as a culture medium to grow the cells. The medium was also inoculated with fetal bovine serum (10%), streptomycin (100 µg/ml) and penicillin (100 units/ml) and in a carbon dioxide incubator (37°C, 5% CO₂, 90% RH). The harvest of cells at subconfluent stage was done by treating with trypsin (0.05%) in phosphate buffered saline (pH 7.4) containing 0.2 % EDTA. Cell viability was determined using tryptan blue exclusion and cells with a viability of more than 98% were used for cytotoxicity studies. Complete growth medium was used to prepare cell suspension (1 x 10⁵ cells/ml). The extracts were used in a concentration of 100 µg/ml prepared in DMSO. Tissue culture plates with 96-wells were used and aliquots (100 µl) of cell suspension were put into each well. After 24 h of growth in CO₂ incubator, extracts to be tested were added to complete growth medium. This was followed by incubation for 48 h more. Thereafter, gentle trichloroacetic acid layering was done to inhibit the growth. Further incubation
of the plates was carried out for 1 h at a temperature of 4°C. The supernatant was discarded and plates washed with distilled water and air dried. Sulforhodamine B staining was done to quantify the cell growth (Skehan et al., 1990). The absorbance of the adsorbed dye dissolved in the Tris-HCl buffer was measured at 540 nm on ELISA reader. Positive controls included paclitaxel, adriamycin and mitomycin.

3.7.2 IN VIVO STUDIES

Animals

Animal studies were conducted on wistar rats (150-200 g) and Swiss albino mice (25-35 g) of either sex. The animals were kept in central animal house facility of Guru Nanak Dev University on straw bedding in cages under natural light and dark cycle and were acclimatized to the laboratory conditions for 7 days prior to initiation of the experiments. Mice were fed with standard rodent diet [composition: crude fibre (12%), crude protein (20%), crude fat (2.5%), acid insoluble ash (4%), salt (2%), calcium (0.5%), and phosphorus (0.5%), vitamin (5000 IU/Kg)]. The experimental protocol was approved by the institutional animal ethics committee and experiments were carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

3.7.2.1 Analgesic Activity

Analgesic activity was studied using thermal and capsaicin induced hyperalgesia. For thermal hyperalgesia tail, immersion and hot plate models were used (Sharma et al., 2006). Group detailed protocol is given in table 2 and each group comprised of 6 animals.

In hot plate method, the animals were administered respective treatments by i.p. injection and placed on Eddy’s hot plate heated to a temperature of 55 ± 2°C. The licking of paws, jumping or rearing was taken as the end point and a cut off time of 16 s was taken to avoid damage to the paws. In tail immersion method, the distal portion of the tail was suspended in water heated to a
temperature of 55 ± 2°C. The flicking of the tail was taken as the end point and a cut off time of 14 s was observed to avoid damage to the tail. Readings were taken in both these models at 15 min, 30 min, 45 min, 60 min, 90 min and 120 min intervals.

Table 1 Experimental protocol for thermal hyperalgesia

<table>
<thead>
<tr>
<th>Group</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control: Mice were injected 0.5 % carboxy methyl cellulose (0.1 ml per 10 g body weight).</td>
</tr>
<tr>
<td>Group II</td>
<td>Diclofenac treated: Mice were injected diclofenac (25 mg Kg⁻¹; i.p.).</td>
</tr>
<tr>
<td>Group III</td>
<td>AME treated (50 mg Kg⁻¹): Mice were injected with AME (50 mg Kg⁻¹; i.p.).</td>
</tr>
<tr>
<td>Group IV</td>
<td>AME treated (100 mg Kg⁻¹): Mice were injected with AME; i.p.).</td>
</tr>
<tr>
<td>Group V</td>
<td>AME treated (200 mg Kg⁻¹): Mice were injected with AME (200 mg Kg⁻¹; i.p.).</td>
</tr>
</tbody>
</table>

In capsaicin induced hyperalgesia, 10 groups each comprising of six animals was used. Capsaicin was injected into the plantar surface of the hind paws and licking of the paws was observed for 10 min (Piovezan et al., 2000). The protocol is given in table 3.

Table 3 Experimental protocol for capsaicin induced hyperalgesia

<table>
<thead>
<tr>
<th>Group</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group VI</td>
<td>Capsaicin treated control: Mice were injected 20 µl of capsaicin (0.1 µg in 20 µl dissolved in 0.9 % saline) in the plantar surface of the right hind paw and observed for licking of the right hind paw for the next 10 min.</td>
</tr>
<tr>
<td>Group VII</td>
<td>Pentazocine treated: Mice were injected with pentazocine (45 mg Kg⁻¹; i.p.) followed by capsaicin in the right hind paw after 30 min and observed for the hind paw licking.</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Diclofenac treated: Mice were injected with diclofenac (25 mg Kg⁻¹; i.p.) followed by capsaicin in the right hind paw after 30 min and observed for the hind paw licking.</td>
</tr>
<tr>
<td>Group IX</td>
<td>AME treated: Mice were injected with AME (100 mg</td>
</tr>
<tr>
<td><strong>Group</strong></td>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
</tr>
<tr>
<td>X</td>
<td>Misoprostol + AME</td>
</tr>
<tr>
<td>XI</td>
<td>Naloxone + AME</td>
</tr>
<tr>
<td>XII</td>
<td>L-arginine + AME</td>
</tr>
<tr>
<td>XIII</td>
<td>L-NAME + AME</td>
</tr>
</tbody>
</table>

3.7.2.2 Early type-1 diabetic nephropathy and cardiomyopathy

The rats were acclimatized for 2 weeks before starting the experiment. Diabetes was induced by a single intraperitoneal injection of alloxan monohydrate (150 mg Kg⁻¹) to overnight fasted rats to induce diabetes. The treatments were given for a period of 14 days as described by Mishra et al. (2010). The extract was suspended in 0.5 % carboxymethyl cellulose and the suspension thoroughly mixed using a vortex mixer. The protocol is given in table 4.

*Biochemical parameters*

On the 14th day blood collection was done by retro-orbital puncture under ether anaesthesia from overnight fasted rats. Blood was centrifuged at 3000 rpm for 10 min for isolation of serum. Thereafter, the animals were sacrificed;
hearts and kidneys were dissected out for further biochemical and histological examination. For histological studies the tissues were preserved in formalin.

Table 4 Experimental protocol for early type-I diabetic nephropathy and cardiomyopathy

<table>
<thead>
<tr>
<th>Group I</th>
<th>Normal non diabetic: the animals were normal saline at a dose of 1 ml Kg(^{-1}) i.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>Diabetic vehicle treated (0.5% CMC): the animals were administered vehicle (carboxymethylcellulose 0.5%) at a dose of 1 ml Kg(^{-1}) i.p.</td>
</tr>
<tr>
<td>Group III</td>
<td>Diabetic tolbutamide treated (100 mg Kg(^{-1})): the animals were administered tolbutamide at a dose of 100 mg Kg(^{-1}); i.p.</td>
</tr>
<tr>
<td>Group IV</td>
<td>Diabetic AME treated (AME 100 mg Kg(^{-1})): the animals received a daily dose (100 mg Kg(^{-1}); i.p.) of ethanol extract suspended in 0.5% CMC.</td>
</tr>
<tr>
<td>Group V</td>
<td>Diabetic AME treated (AME 200 mg Kg(^{-1})): the animals received a daily dose (200 mg Kg(^{-1}); i.p.) of ethanol extract suspended in 0.5% CMC.</td>
</tr>
<tr>
<td>Group VI</td>
<td>Diabetic AME treated (AME 400 mg Kg(^{-1})): the animals received a daily dose (400 mg Kg(^{-1}); i.p.) of ethanol extract suspended in 0.5% CMC.</td>
</tr>
</tbody>
</table>

For the estimation of the renal and cardiac parameters, the left kidney and the heart were immediately perfused with ice cold saline and homogenized in chilled (1.15\%) potassium chloride (Mishra et al., 2010). The centrifugation of the tissue homogenates was carried out at a temperature of 4°C at 800 g for a period of 5 min. The supernatant was further centrifuged at 4000 g at 4°C for 60 min for determining the catalase levels. Further processing was done according to the methods described below.

*Estimation of fasting blood glucose*

Determination of fasting blood glucose (FBG) was done by glucooxidase/peroxidase assay using Span diagnostic kit. Glucose is oxidized to gluconic
acid and hydrogen peroxide which reacts with phenol and 4-aminoantipyrine (4-AAP) to form quinoneimine dye, the absorbance of which is directly proportional to the glucose concentration in the sample and measured at 505 nm. Briefly, serum (10 µL) was added to the GOD/POD reagent (1 ml) followed by addition of 2 ml of distilled water and incubation for 10 min at a temperature of 37°C. The absorbance was taken at 505 nm. The absorbance of the standard was noted by using standard reagent provided in the kit instead of serum and calculations were done using the following formula:

$$\text{FBG (mg dL}^{-1}) = \frac{A_{\text{test}}}{A_{\text{std}}} \times 100$$

where $A_{\text{test}}$ = Absorbance of test, $A_{\text{std}}$ = Absorbance of standard

**Estimation of total cholesterol**

Cholesterol esterase hydrolyses cholesterol esters to free cholesterol and fatty acids. Subsequently, cholesterol oxidase brings about the oxidation of the 3 hydroxyl group of free to cholest-4-en-3-one and hydrogen peroxide. This hydrogen peroxide reacts with phenol and 4-amino antipyrine to form quinoneimine dye. The absorbance of this dye is directly proportional to the concentration of cholesterol and is measured at 505 nm. Briefly, serum (10 µL) was added to enzyme reagent provided in the kit (1 ml) proceeded further by addition of 2 ml of distilled water and incubated at a temperature of 37°C for a period of 10 min. The absorbance was taken at 505 nm. The absorbance of the standard was noted by using standard reagent provided in the kit instead of serum and calculations were done using the following formula:

$$\text{TC (mg dL}^{-1}) = \frac{A_{\text{test}}}{A_{\text{std}}} \times 100$$

where, $A_{\text{test}}$ = Absorbance of test, $A_{\text{std}}$ = Absorbance of standard

**Estimation of serum creatinine level**

The levels of creatinine in the serum were estimated using Jaffe’s method (Jaffe et al., 1886). Briefly, 0.2 ml of serum was taken and 3 ml of working standard solution was added to it, mixed and left undisturbed for 30 min and
thereafter absorbance noted at 505 nm. The sample absorbance was extrapolated from the standard curve.

**Estimation of blood urea nitrogen**

The level of blood urea nitrogen (BUN) was estimated using the method of Wybenga *et al.* (1971). Diluted serum (0.1 ml) was mixed with a solution containing acid reagent, colour reagent and distilled water in equal proportion. The contents were homogenized and kept on boiling water bath for 15 min followed by cooling for 5 min and absorbance measured at 540 nm.

**Preparation of reagents**

*Preparation of stock acid reagent:* For the preparation of the reagent ferric chloride (1.0 g) hexahydrate was dissolved in distilled water (30 ml) followed by addition of orthophosphoric acid (20 ml).

*Preparation of acid reagent:* 100 ml of concentrated sulphuric acid was added to 400 ml of distilled water. To this 0.3 ml of stock acid reagent was added and volume made up to 1 L with distilled water.

*Preparation of stock colour reagent A:* For the preparation of the reagent, 2.0 g of diacetyl monoxime was dissolved in 100 ml of distilled water.

*Preparation of stock colour reagent B:* For the preparation of the reagent, 500 mg of thiosemicarbazide was dissolved in 100 ml of distilled water.

*Preparation of colour reagent:* For the preparation of the reagent, 35 ml of reagent A was mixed with 35 ml reagent B and volume made up to 500 ml with distilled water.

*Preparation of urea standard:* For the preparation of the reagent, 1.0 g of urea was dissolved in 100 ml distilled water and further dilutions were made to achieve concentrations range up to 200 mg dL⁻¹.
Estimation of total proteins

Lowry’s method was used to determine the level of total proteins was (Lowry et al., 1951) based on the following principle. The peptide bonds in proteins react with cupric ions in alkaline solution to form a coloured complex, the absorbance of which is measured at 750 nm. Bovine serum albumin was used as standard. 5 ml of the Lowry’s reagent was taken and to it supernatant was added (0.3 ml diluted to 1 ml). The resultant mixture was thoroughly homogenized using vortex mixer and kept undisturbed for a period of 5 min at room temperature. This was followed by addition of Folin- Ciocalteu reagent (0.5 ml), mixing the contents and further incubation for a period of 30 min at room temperature and absorbance noted at 750 nm. The protein contents of the tissue were estimated by extrapolating from the standard curve plotted using BSA. In the case of the tissue homogenate, to 100 µL of the diluted supernatant, distilled water was added to make the volume up to 1 ml. The samples were further similarly processed and the protein content calculated by extrapolating from the standard curve.

Preparation of reagents:

Preparation of Lowry’s reagent: 1% copper sulphate solution was mixed with 2% w/v sodium- potassium tartrate and 2% w/v sodium carbonate in 0.1 M sodium hydroxide, in the ratio of 1:1:98.

Preparation of 0.1 M sodium hydroxide solution: 4 g of sodium hydroxide was dissolved in distilled water and volume was made up to 1 L with distilled water to give 0.1 M sodium hydroxide.

Preparation of 1 % copper sulphate solution: Copper sulphate (1.0 g) was dissolved in 0.1 M sodium hydroxide and volume made up to 100 ml with sodium hydroxide to give 1 % copper sulphate solution.

Preparation of 2 % sodium potassium tartrate solution: Sodium potassium tartrate (2 %) solution was prepared by dissolving 2 g of
sodium potassium tartrate in 0.1 M sodium hydroxide and making up the volume to 100 ml with sodium hydroxide.

**Preparation of 2 % sodium carbonate solution:** The solution was prepared by dissolving 2 g of sodium carbonate in 0.1 M sodium hydroxide and making up the volume to 100 ml.

**Estimation of lipid peroxidation**

Lipid peroxidation is an index of oxidative stress. Lipids are highly susceptible to peroxidation when exposed to free radicals such as hydroxyl and superoxide free radicals. Upon peroxidation, the lipids yield malondialdehyde, which on reaction with thiobarbituric acid forms a pink coloured complex. Estimation of the thiobarbituric acid reactive substances (TBARS) has been studied as an index of lipid peroxidation and determined by the procedure of Okhawa *et al.* (1979). Briefly, tissue homogenate was taken in a volume of 0.2 ml and to it addition of 0.2 ml of 8.1 % sodium lauryl sulphate, 1.5 ml of 30 % acetic acid (pH 3.5). 1.5 ml of 0.8% thiobarbituric acid solution was done. Further, the volume made up to 4 ml using distilled water. After this, the incubation of the mixture was done at 95 °C for a period of 1 h. This was followed by cooling and addition of 1ml of distilled water and 5 ml of butanol- pyridine mixture (5% v/v) followed by centrifugation at 4000 rpm for 10 min. After this the absorbance was observed at 532 nM. A standard curve was plotted using 1,1,3,3-tetramethoxy propane. The samples TBARS content calculated by extrapolating from the standard curve.

**Preparation of reagents:**

**Preparation of 8.1 % sodium dodecyl sulphate solution:** The solution was prepared by dissolving 810 mg of sodium dodecyl sulphate in 10 ml of distilled water.

**Preparation of 30 % acetic acid solution:** The solution was prepared by diluting 30 ml of glacial acetic acid to 100 ml with distilled water and adjusting the pH of the resultant solution to 3.5 with saturated solution of sodium hydroxide.
Preparation of 0.8% thiobarbituric acid (TBA) solution: The TBA solution was prepared by dissolving 400 mg of TBA in 50 ml of warm water.

Preparation of 15:1 v/v n-butanol- pyridine mixture: The reagent was prepared by mixing 90 ml of n- butanol with 6 ml of pyridine.

Preparation of 1 nM 1,1,3,3 tetramethoxy propane: The reagent was prepared by diluting 0.82 ml of 1,1,3,3-tetramethoxy propane to 5 ml with distilled water to obtain a 1 M solution and further diluting 1 ml of this solution to 10 ml with distilled water and then carrying out dilutions of this solution eight times to get 1 nM 1,1,3,3-tetramethoxy propane.

Estimation of reduced glutathione
Determination of reduced glutathione (GSH) was carried out by the method described by Ellman (1959). The method involves reduction of 5’,5’-dithiobis-2-nitrobenzoic acid [DTNB] by sulphydryl group present in reduced glutathione (GSH) which leads to yellow coloured 2-nitro-5-mercaptobenzoic acid. Briefly, 1 ml of 10 % trichloroacetic acid was added to 1ml of tissue homogenate. This was followed by centrifugation for a period of 10 min at 4 °C and 5000 rpm. Thereafter, 0.5 ml of the supernatant was taken and to it 2 ml of 0.3 M disodium hydrogen phosphate buffer (pH 8.4) and 0.4 ml of distilled water were added followed by the addition of 0.25 ml of freshly prepared DTNB (0.001 M). Absorbance was recorded at a wavelength of 412 nm after 10 min incubation of the test mixture. A standard curve was plotted with 10- 100 µM of GSH. The samples GSH content calculated by extrapolating from the standard curve.

Preparation of reagents:

Preparation of 10% trichloroacetic acid: The reagent was prepared by dissolving 10 g of trichloroacetic acid in 100 ml of distilled water.
Preparation of 0.3 M disodium hydrogen phosphate: The reagent was prepared by dissolving 4.26 g of anhydrous disodium hydrogen phosphate in 100 ml distilled water.

Preparation of DTNB: The reagent was prepared by dissolving 7.92 mg of DTNB in 20 ml of 1% sodium citrate.

Preparation of 100 µM GSH: The reagent was prepared by dissolving 6.14 mg of reduced glutathione (GSH) in 200 ml distilled water.

Estimation of catalase
Determination of catalase (CAT) activity was done using the method described by Aebi (1974). Briefly, 50 µL of the tissue homogenate was added to the cuvette containing 1.95 ml of 50 mM phosphate buffer (pH 7) and to this 1 ml of 30 mM hydrogen peroxide was added. The change in the absorbance was noted at intervals of 30 s for 3 min at 240 nm. Calculations were made using the following formula.

\[
\text{CAT activity} = \frac{A}{E \times V \times P}
\]

Where \(A\) = Change in absorbance, \(E\) = extinction coefficient of \(\text{H}_2\text{O}_2\) i.e. 0.071 mmol cm\(^{-1}\), \(V\) = Sample volume and \(P\) = Protein

Preparation of reagents:

Preparation of 50 mM phosphate buffer: Potassium dihydrogen phosphate (680 mg) was dissolved in 100 ml of distilled water. Sodium hydroxide (200 mg) was dissolved in 100 ml of distilled water. Phosphate buffer was prepared by adding 50 ml of potassium dihydrogen phosphate solution to 29.1 ml of sodium hydroxide solution.

Preparation of 30 mM \(\text{H}_2\text{O}_2\): For this 102 mg of \(\text{H}_2\text{O}_2\) solution was taken and volume made up to 100 ml with distilled water.
Estimation of lactate dehydrogenase

Lactate dehydrogenase (LDH) was estimated using Span Diagnostics kits and is based on the method described by King (1959). The method involves reaction of lactate with nicotinamide adenine dinucleotide (NAD), which results in the formation of pyruvate and NADH. The rate of NADH formation is measured spectrophotometrically. To 50 µL of serum, 1.0 ml of working reagent given in the kit was added, mixed well and change in absorbance was read at intervals of 30 seconds for a duration of 2 min at 340 nm.

Estimation of creatinine kinase

The creatinine kinase (CK-MB) estimation was done using Span Diagnostics kits. The kit is based on the method originally described by Hughes (1962). The basic principle of the approach is that CK catalyses the conversion of creatine phosphate to creatine and ATP. Glucose is converted to glucose-6-phosphate by the ATP formed in the previous step. The enzyme glucose-6-phosphate dehydrogenase catalyses the conversion of glucose-6-phosphate to gluconate and NADPH. The rate of formation of NADPH is measured spectrophotometrically at a wavelength of 340 nm. To 20 µL of serum, 1.0 ml of working reagent given in the kit was added, mixed well and change in absorbance at 37°C was read at intervals of 30 seconds for a duration of 2 min at 340 nm.

Histopathological studies

The right kidney and hearts were preserved in formalin for histopathological studies. Sections (4-5 µm) were prepared with microtome and stained for 15 min with haematoxylin followed by counter stain with eosin for 2 min. The changes were studied through photomicrographs taken with digital camera (Olympus E-520) attached to pathological microscope (Magnus MLXi).

3.7.2.3 Early type-II diabetic nephropathy and cardiomyopathy

Six groups of animals were used (n=6 per group). In the normal group (group XXI), rats received a daily dose of the vehicle. In the rest of the groups, rats were fed on high fat diet (HFD) containing powdered normal pallet diet (365 g Kg⁻¹), lard (310 g Kg⁻¹), casein (250 g Kg⁻¹), vitamin mineral mix (60 g Kg⁻¹),
cholesterol (10 g Kg⁻¹), DL- methionine (3 g Kg⁻¹), yeast powder (1 g Kg⁻¹) and sodium chloride (1 g Kg⁻¹), for two weeks before induction of diabetes with streptozotocin (STZ) to overnight fasted rats at a dose of 35 mg Kg⁻¹. HFD and low dose of STZ is known to induce diabetes similar to the metabolic derangements of type II diabetes (Srinivasan et al., 2005). All the remaining groups received a daily dose of the respective treatments for 2 weeks as described table 5.

Table 5 Experimental protocol for early type-II diabetic nephropathy and cardiomyopathy

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Diabetic control: The animals received 0.5% carboxymethyl cellulose (1 ml) daily orally for 2 weeks.</td>
</tr>
<tr>
<td>Group II</td>
<td>Diabetic control: The animals received 0.5% carboxymethyl cellulose (1 ml) daily orally for 2 weeks.</td>
</tr>
<tr>
<td>Group III</td>
<td>Pioglitazone treated: The animals received pioglitazone at a dose of 40 mg Kg⁻¹ daily orally for 2 weeks.</td>
</tr>
<tr>
<td>Group IV</td>
<td>AME treated: The animals received AME at a dose of 100 mg Kg⁻¹ daily orally for 2 weeks.</td>
</tr>
<tr>
<td>Group V</td>
<td>BADGE + pioglitazone treated: The animals received BADGE at a dose of 30 mg Kg⁻¹ intraperitoneally followed after 30 min by pioglitazone at a dose of 40 mg Kg⁻¹ daily orally for 2 weeks.</td>
</tr>
<tr>
<td>Group VI</td>
<td>BADGE + AME: The animals received BADGE at a dose of 30 mg Kg⁻¹ intraperitoneally followed after 30 min by AME at a dose of 100 mg Kg⁻¹ daily orally for 2 weeks.</td>
</tr>
</tbody>
</table>

**Biochemical parameters**

At the end of four weeks, blood collection was done as mentioned earlier. Thereafter, the animals were sacrificed and the hearts and the kidneys were isolated. The right kidney and heart of one animal were preserved in formalin
for histopathological studies and the left kidney and the heart were processed for biochemical estimations. The analyses were carried out for various blood parameters including fasting blood glucose (FBG), total cholesterol (TC), blood urea nitrogen (BUN), creatinine, lactate dehydrogenase and creatinine kinase using the methods described earlier. For the estimation of the renal and cardiac parameters, the left kidney and heart were immediately perfused with ice cold saline and homogenized in chilled (1.15%) KCl (Mishra et al., 2010). The centrifugation of the tissue homogenates was carried out at a temperature of 4°C at 800 g for a period of 5 min. The supernatant was further centrifuged at 4000 g at 4°C for 60 min for determining the catalase levels. Thereafter, the various estimations were done as described earlier.

3.7.2.4 Antiepileptic activity
AME, phenytoin and diazepam were dissolved in 0.5% carboxymethylcellulose (CMC). Pentylenetetrazole (PTZ) was dissolved in distilled water and administered intraperitoneally (i.p.) in all the groups.

PTZ induced convulsions
Chemically induced convulsions were studied using the method described by Singh and Goel (2009). In all the groups the animals were given the treatments as described in table 6.

Table 6 Experimental protocol for PTZ induced convulsions

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CMC: Animals were injected with 0.5% CMC (0.1 ml per 10 g body weight) followed by PTZ at a dose of 80 mg Kg⁻¹ i.p. after 30 min and observed for 30 min for various stages of convulsions.</td>
</tr>
<tr>
<td>II</td>
<td>Diazepam (3 mg Kg⁻¹): Animals were injected with diazepam at a dose of 3 mg Kg⁻¹ followed by PTZ at a dose of 80 mg Kg⁻¹ i.p. after 30 min and observed for 30 min for various stages of convulsions.</td>
</tr>
<tr>
<td>III</td>
<td>Phenytoin (25 mg Kg⁻¹): Animals were injected with phenytoin at a dose of 25 mg Kg⁻¹ followed by PTZ</td>
</tr>
</tbody>
</table>
at a dose of 80 mg Kg\(^{-1}\) i.p. after 30 min and observed for 30 min for various stages of convulsions.

<table>
<thead>
<tr>
<th>Group IV</th>
<th>AME (100 mg Kg(^{-1})): Animals were injected with AME at a dose of 100 mg Kg(^{-1}) followed by PTZ at a dose of 80 mg Kg(^{-1}) i.p. after 30 min and observed for 30 min for various stages of convulsions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group V</td>
<td>AME (200 mg Kg(^{-1})): Animals were injected with AME at a dose of 200 mg Kg(^{-1}) followed by PTZ at a dose of 80 mg Kg(^{-1}) i.p. after 30 min and observed for 30 min for various stages of convulsions.</td>
</tr>
</tbody>
</table>

**MES induced convulsions**

In all the groups, the animals were given the treatments i.p. and 30 min later an electric shock of 50 mA and 0.2 s duration was applied using corneal electrodes (Singh and Goel, 2009) and the animals were observed for 30 min for various stages of convulsions as described in table 3.6.

<table>
<thead>
<tr>
<th>Group VI</th>
<th>Vehicle treated: Animals were injected with 0.5% CMC (0.1 ml per 10 g body weight) and after 30 min an electric shock of 50 mA and 0.2 s duration was applied using corneal electrodes and the animals were observed for 30 min for various stages of convulsions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group VII</td>
<td>Phenytoin treated: Animals were injected with phenytoin at a dose of 25 mg Kg(^{-1}) and after 30 min an electric shock of 50 mA and 0.2 s duration was applied using corneal electrodes and the animals were observed for 30 min for various stages of convulsions.</td>
</tr>
<tr>
<td>Group VIII</td>
<td>AME treated: Animals were injected with AME at a dose of 100 mg Kg(^{-1}) and after 30 min an electric shock of 50 mA and 0.2 s duration was applied using corneal electrodes and the animals were observed for 30 min</td>
</tr>
</tbody>
</table>
for various stages of convulsions.

| Group IX | AME treated: Animals were injected with AME at a dose of 200 mg Kg\(^{-1}\) and after 30 min an electric shock of 50 mA and 0.2 s duration was applied using corneal electrodes and the animals were observed for 30 min for various stages of convulsions. |

Mechanistic groups
In all the groups the animals were given the respective treatments i.p. 30 min before AME (100 mg Kg\(^{-1}\)) followed by PTZ (80 mg Kg\(^{-1}\)) and observed for 30 min for various stages of convulsions as given in table 8.

Chronic PTZ treated groups
In all the groups, the animals were given the respective treatments daily from day 1 to day 15. Submaximal doses of PTZ (50 mg Kg\(^{-1}\)) were injected to the animals intraperitoneally on the first, fifth, tenth and the fifteenth days after 30 min of the respective treatments and animals were observed for severity of convulsions and on the final day, animals were subjected to forced swimming test and test for locomotor activity after recovery from convulsions. After this, the animals were sacrificed, brains isolated and perfused with normal saline and homogenized with KCl for biochemical estimations. The protocols are described in table 9.

Forced swimming test
On the 15\(^{th}\) day animals were subjected to forced swimming test as described by Singh et al. (2012). Briefly, mice were forced to swim individually in glass jar (25 x 12 x 25 cm\(^3\)) containing water (25°C) for 6 min and observed for immobility.

Locomotor activity
Locomotor activity was recorded by placing the animals individually in actophotometer after recovery from convulsions as described by Singh et al. (2012). Mice were individually in actophotometer for a period of 5 min and the activity count recorded.

Biochemical estimations
Brains were isolated and homogenized in phosphate buffer (pH 7.4, 10% w/v). The homogenates were subjected to assay for TBARS, GSH and CAT as described above.

Table 8 Experimental protocol for mechanistic groups in PTZ induced convulsions

<table>
<thead>
<tr>
<th>Group</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group X</strong></td>
<td>BADGE treated group: BADGE was administered at a dose of 30 mg Kg(^{-1}) i.p. followed by AME at a dose of 100 mg Kg(^{-1}). After this PTZ was administered at a dose of 80 mg Kg(^{-1}) and the animals were observed for 30 min for various stages of convulsions.</td>
</tr>
<tr>
<td><strong>Group XI</strong></td>
<td>L-arginine treated group: L-arginine was administered at a dose of 40 mg Kg(^{-1}) i.p. followed by AME at a dose of 100 mg Kg(^{-1}). After this PTZ was administered at a dose of 80 mg Kg(^{-1}) and the animals were observed for 30 min for various stages of convulsions.</td>
</tr>
<tr>
<td><strong>Group XII</strong></td>
<td>L-NAME treated group: L-NAME was administered at a dose of 40 mg Kg(^{-1}) i.p. followed by AME at a dose of 100 mg Kg(^{-1}). After this PTZ was administered at a dose of 80 mg Kg(^{-1}) and the animals were observed for 30 min for various stages of convulsions.</td>
</tr>
</tbody>
</table>

Table 9 Experimental protocol for chronic PTZ induced convulsions

| Group XIII | Vehicle treated control: Animals were injected with 0.5% CMC (0.1 ml per 10 g body weight) for 15 days. On days 1, 5, 10 and 15 the animals were injected with a sub maximal dose of PTZ (50 mg Kg\(^{-1}\)) after 30 min of the treatments and observed for severity of convulsions. On the 15\(^{th}\) day after recovery of the animals, the severity of depression was studied using forced swimming test and locomotor activity was studied using actophotometer. |
| Group XV | Diazepam treated: Animals were injected with diazepam at a dose of 3 mg Kg\(^{-1}\) for 15 days. On days |
1, 5, 10 and 15 the animals were injected with a submaximal dose of PTZ (50 mg Kg\(^{-1}\)) after 30 min of the treatments and observed for severity of convulsions. On the 15th day after recovery of the animals, the severity of depression was studied using forced swimming test and locomotor activity was studied using actophotometer.

| Group XVI | AME treated: Animals were injected with AME at a dose of 100 mg Kg\(^{-1}\) for 15 days. On days 1, 5, 10 and 15 the animals were injected with a sub maximal dose of PTZ (50 mg Kg\(^{-1}\)) after 30 min of the treatments and observed for severity of convulsions. On the 15th day after recovery of the animals, the severity of depression was studied using forced swimming test and locomotor activity was studied using actophotometer. |

### 3.8 ACUTE TOXICITY STUDIES

Acute toxicity studies were carried out using OECD guidelines (2002). Briefly, overnight fasted animals were divided into four groups with three animals per group. Varying doses of the extract, 300, 500, 1000 and 2000 mg Kg\(^{-1}\) were administered. Thereafter, the animals were observed continuously for any behavioural alterations for initial 4 h and mortality up to 24 h. The animals were further observed for behavioral alterations and mortality for the next 14 days.

### 3.9 STATISTICAL ANALYSIS

All data is expressed as mean ± S.E.M. One way ANOVA with Tukey test was used for evaluating statistical significance between different groups and values with p<0.05 were considered to be statistically significant. All statistical analyses have been carried out with the help of Instat software (Graph Pad Software Inc., San Deigo).