4. Materials and Methods

4.1. Materials

All the materials used in this study were purchased from authorized firms from India. Media, chemicals and reagents were purchased from Hi-Media PVT. LTD. Mumbai - 400 086. Glassware’s were purchased from Borosil Glass Works Limited, Mumbai- 400 018 (India), plastic wares were procured from M/S Tarsons Products PVT LTD. New Delhi - 110001.

4.2. Methods

4.2.1. Plant materials selection, collection and extraction

4.2.1.1. Plant material – Selection and Collection

Seed kernel of *Mangifera indica* was selected to screen its bio potentials based on its traditional usage. Seed kernel of *Mangifera indica* was collected as wild from the thathachariar garden, Thiruvanaikoil, Tiruchirappalli, Tamil Nadu, India during the month of June, 2011. Care was taken to select healthy fruit. Only neelum variety has been selected and screened its biopotential and phytochemical features.

4.2.1.2. Authentication of plant material

The plant material was initially identified by the Gardner (variety Neelum) of thathachariar garden, Thiruvanaikoil, Tiruchirappalli, Tamil Nadu, India. This identity of the plant was confirmed by Dr. John Britto, Professor, Department of Botany and Director, Rapinat herbarium, St. Joseph’s College, Thiruchirapalli, Tamilnadu, India and also a member of doctoral committee Dr. P. Brindha, Associate Dean, SASTRA University, Thanjavur. The voucher specimen of the plant material has been submitted in the M. R. Government Arts College, Mannargudi for future reference.

4.2.1.3. Processing of plant

Fresh *Mangifera indica* fruit was collected from thathachariar garden, Thiruvanaikoil, Tiruchirappalli. Fruit was peeled off using sterile sharp knife. Flesh part of the fruit was consumed and the seed was washed completely. Hard shell of the seed was
removed and the soft embryonic portion was collected and dried completely. The dried seed kernel sample was powdered using electric grinder (Smith mixie, India). Powder was stored in sterile container for further studies.

4.2.1.4. Preparation of Aqueous extract
The powdered plant material (150gm) was mixed with water and extracted completely. The seed kernel powder was mixed with sterile water and kept for 72 hours and filtered with a muslin cloth and it was condensed in hot air oven at 50˚C. The aqueous extracts were stored in a sterile container and refrigerated for future use (Jonathan, 2009).

4.2.1.5. Preparation of Phenolic extract
Phenolic extract was collected by making use of soxhlet extraction. It was performed by placing 50gm plant material with 1:1 ethanol and methanol. Extraction was performed at 90˚C for 12 hours. The extracts were filtered under the vacuum through Whatman filter paper (No. 1) under gravity. Extract was dried under vacuum evaporator for removing the solvent. The remaining residues were stored in refrigerator till further use (Shi et al., 2005).

4.2.2. Anatomical Studies of fruit pulp
4.2.2.1. Processing of plant materials
The fresh seed was taken, cut and removed kernel from the shell and fixed in FAA (Formalin 5ml + Acetic acid 5ml + 70% Ethyl alcohol 90ml). After 24 hrs of fixing, infiltration of the specimens was carried by gradual addition. The specimens were cast into paraffin blocks (Sass, 1940).

4.2.2.2. Sectioning
The paraffin embedded specimen was sectioned with the help of a Rotary Microtome. The thickness of the wax was 10-12 mm. Dewaxing of the sections was done by customary procedure (Johansen, 1940). The sections were stained with toluidine blue as per the method published by O’Brien et al., (1964). Since toluidine blue is a polychromatic
stain, the staining results were remarkably good and some cytochemical reactions were also observed. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. and the necessary sections were also stained with safranin and fast-green and iodine (IKI - for Starch).

For studying the stomatal morphology, venation pattern and trichome distribution paradermal sections (sections taken parallel to the surface) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey’s maceration fluid (Sass, 1940) were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerine after staining. Different cell components were studied and measured.

4.2.2.3. Photomicrographs

Microscopic descriptions of seed kernel are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Microscope. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are noted as given in the standard anatomy books (Easu, 1964).

4.2.3. Pharmacognostic studies

4.2.3.1. Organoleptic Character Evaluation (Kokate, 1994 & Khandelwal, 2005)

*Mangifera indica* seed kernel was subjected to macroscopic studies which comprised of organoleptic characters of the drugs viz., colour, odour, taste, texture etc.
4.2.3.2. Physicochemical parameter analysis

The determination of various physicochemical parameters such as foreign matter, Total ash, acid insoluble ash, water soluble ash, water soluble extractive value, alcohol soluble extractive value were calculated as per Indian Pharmacopoeia (Anonymous, 1996 and Khandelwal et al., 2005).

4.2.3.2.1. Foreign matter

Five grams of powdered seed kernel sample was taken and spread a a thin layer on clean transparent sheeth and the pieces of foreign matter were sorted out by visual inspection. The powder was sieved through a 250 micron sieve. All portions of the foreign matter were pooled and weighed.

4.2.3.2.2. Ash values
4.2.3.2.2.1. Total ash

Two grams of accurately weighed powdered seed kernel was incinerated in a silica dish at a temperature of 400°C until free from carbon. Then it was cooled and weighed accurately by using instrument. The % w/w of ash with reference to the air-dried sample was calculated.

4.2.3.2.2.2. Acid insoluble ash

Five ml of hydrochloric acid was added to the glass beaker containing the total ash. The beaker was then covered with a watch-glass and the mixture was boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot water and this liquid was added in to the beaker. The insoluble matter was collected on an ashless filter paper and washed with hot water until the filterate attains neutral. The filterpaper contains insoluble matter and it was transferred to crucible dish and dried on a hot plate and ignited to get a constant weight. The residue was allowed to cool in desiccators for 30 minutes and then weighed. The content of acid insoluble ash was calculated in mg per g of air dried matter.
4.2.3.2.2.3. Water soluble ash

Total ash was boiled with 25ml of water for 5 minutes. The insoluble matter was collected in a beaker or an ash less filter paper. Then washed with hot water and ignited for 15 minutes at 400°C. Insoluble matter was weighed and subtracted from the total ash, the difference in weight was representing the water soluble ash. Percentage of water soluble was calculated with reference to the air dried sample.

4.2.3.3. Fluorescence analysis

Powdered seed kernel was subjected to analysis under visible as well as ultra violet light after treatment with various solvents like chloroform, hexane, benzene, ethanol, ethyl acetate, acetone, 50% sulphuric acid, 10% sodium hydroxide (aqueous and alcoholic) and dilute hydrochloric acid and observed for colour change (Kokate, 1994 & Chase and Pratt, 1949).

4.2.3.4. Extractive values
4.2.3.4.1. Water soluble extractive value

Four grams of air dried coarsely powdered seed kernel was macerated with 100 ml of distilled water in a closed flask for 24 hours. It was frequently shaken for 6 hours and allowed to stand for 18 hours. It was filtered rapidly and the filtrate was evaporated to dryness in a flat bottomed shallow dish and dried at 105°C to attain constant weight and weighed.

4.2.3.4.2. Alcohol soluble extractive value

Four grams of air dried coarsely powdered seed kernel was macerated with 100 ml of alcohol of the specified strength in a closed flask for 24 hours. Then it was allowed for shaking frequently for 6 hours and allowed to stand for 18 hours. It was filtered rapidly and the filtrate was evaporated to dryness in a flat bottomed shallow dish and dried at 105°C to attain constant weight and weighed.
4.2.3.5. Determination of microbial index

Medicinal plant materials normally carry a greater number of bacteria and moulds when handled by humans for processing. The following methods were adopted to detect microbial content of the raw plant powder.

4.2.3.5.1. Total viable aerobic count

Aerobic count was performed by making use of serial dilution technique cum pour plate method. One gram of plant powder was taken and diluted upto \(10^{-5}\). One ml sample from \(10^{-2}\) and \(10^{-3}\) dilutions were added to sterile petriplates. Then 20 ml of molten nutrient agar was poured and rotated clockwise and anticlockwise direction. Plates were allowed to solidify and incubated for 24 hours at 37ºC.

4.2.3.5.2. Total viable fungal count

Total fungal count was performed by making use of serial dilution technique cum spread plate method. One gram of plant powder was taken and diluted upto \(10^{-3}\). Hundred micro litre sample from \(10^{-1}\) dilution was spread on Rose Bengal Chloramphenicol Agar plates. Plates were allowed to solidify and incubated for 48 hours at 30ºC.

4.2.3.5.3. Test for enteric pathogens

Test for enteric pathogens was performed by making use of serial dilution technique cum spread plate method. 1g of plant powder was taken and diluted upto \(10^{-3}\). 100 micro liter of sample from \(10^{-1}\) dilution was spread on EMB agar plates and Hektoein Enteric Agar plates. Plates were allowed to solidify and incubated for 24 hours at 37ºC.

4.2.4. Antibacterial sensitivity assay

4.2.4.1. Test microorganisms

The test microorganisms except uropathogenic \(E. coli\) were collected from the PG and Research Department of Microbiology, Srimad Andavan Arts and Science College, Thiruvanaikoil, Tiruchirappalli, Tamil Nadu. Uropathogenic \(E. coli\) was obtained from Chromopark Research Centre, Namakkal, Tamil Nadu. Identity of the strains was
confirmed at our laboratory. Identification was done by making use of Bergey’s manual of determinative bacteriology 9th edition. *Salmonella* sp., was isolated from stool samples, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* were isolated from pus samples and Uropathogenic *Escherichia coli* were isolated from urine sample.

### 4.2.4.2. Identification of Test microorganisms

#### 4.2.4.2.1. Identification of *Escherichia coli* (Koneman *et al.*, 1994)

*Escherichia coli* is one of the proteobacteria belongs to the family Enteriobacteriaceae. Enrichment, differential and selective media were used to identify *E.coli*. A loop full of pure culture was taken and inoculated on Hektoein Enteric Agar (HE) and incubated at 37°C for 24 hours under aerobic condition. Salmon colour colony was selected from HE medium and was inoculated on other selective cum differential media like MacConkey agar, EMB agar, XLD medium, Raj Hans medium and SS agar. All of them were incubated aerobically at 37°C for 24 hours and looked for specific colony morphology, which would confirm the isolation of *Escherichia coli*. *Escherichia coli* were identified by making use of biochemical tests in addition to its growth characters on nutrient agar and microscopic analysis. Hi-Chrome UTI agar was also used to differentiate uropathogenic *Escherichia coli*.

#### 4.2.4.2.2. Identification of *Salmonella* sp., (Koneman *et al.*, 1994)

A loop full of inoculum from overnight culture broth was inoculated on selective and differential media like Hektoein enteric agar, XLD agar, Bismuth Sulphite agar, Rajhans medium and SS agar. Microscopy, macroscopy and biochemical tests were also performed to identify *Salmonella* sp.

#### 4.2.4.2.3. Identification of *Pseudomonas aeruginosa* (Koneman *et al.*, 1994)

Overnight grown pure culture was streaked on Mac Conkey agar and was incubated at 37°C for 24 hours. Non Lactose fermenting, colonies were selected for further biochemical tests. It was also inoculated on Cetrimide agar for confirmation. *Pseudomonas aeruginosa* produce bluish green colour colonies.
4.2.4.2.5. Identification of *Staphylococcus aureus* (Koneman *et al.*, 1994)

Blood agar medium was inoculated with the overnight grown pure culture and incubated at 37°C for 24 hours. Mannitol salt agar, Baired Parker agar and Vogel Johnson agar were also used for the confirmation of the *Staphylococcus aureus* identity. Microscopy and biochemical features were assessed for the completion of the identity of *Staphylococcus aureus*.

4.2.4.2.6. Identification of *Streptococcus pyogenes* (Koneman *et al.*, 1994)

Neomycin blood agar and ordinary Blood agar medium were inoculated with the test culture and incubated at 37°C for 24 hours under aerobic as well as microaerobic condition. β haemolytic colonies from the both media were subjected for biochemical tests.

4.2.4.3. Confirmation of clinical isolates (Koneman *et al.*, 1994)

Selected colonies from selective and differential media were subjected to macroscopy, microscopy and biochemical tests for identification.

4.2.4.3.1. Macroscopic observation

**Colony morphology** on agar surface on selective cum differential agar aids to identify the bacterial isolate. Each and every individual species of microorganism form colonies of characteristic shape, size and appearance (Presscott *et al.*, 1999). Characteristic features of the organism were observed by macroscopic observations.

4.2.4.3.2. Microscopic observations

Microscopic observations like size, shape and motility reveal the availability of different morphological characters among microorganisms. Simple staining, gram staining and hanging drop methods were done to look for their shape, grams nature and motility of the isolate respectively (Henry, 1994).
4.2.4.3.3. Shape of the organism
Shape of an isolate was identified by making use of **simple staining** procedure followed by its observation under light microscope. Bacterial smear was stained with methylene blue dye and examined under bright field microscope (Nikon). Microbial cells were observed for their shapes like rod, cocci or spiral.

4.2.4.3.4. Grams nature
Gram staining was performed to look for the grams nature of the isolate. A purple coloured cell retains grams crystal violet and were called gram positive bacterium. Pink coloured cells lost primary stain and picked up safranin colour and were called as gram negative bacterium.

4.2.4.3.5. Motility
Bacteria were motile by their flagella. The number and location of which vary among different species. Motility can be observed directly by hanging drop technique i.e., by placing a drop of culture on a microscopic slide and looked under microscope by keeping them inverted.

4.2.4.3.6. Biochemical tests
Physiological and metabolic characteristics of the microorganisms were assessed through biochemical tests. These characteristics are very useful because they are directly related to the nature and activity of microbial enzymes and transport proteins. Proteins are gene products. Analysis of these characteristics provides an indirect comparison of microbial genomes. The following tests were done to identify the isolates. They are Indole test (I), Methyl red test (MR), Voges Proskauer test (VP), Citrate utilization test (C), Urease production test (U), Nitrate reduction test (N), Decarboxylation of lysine, ornithine and arginine, Phenylalanine deaminase test, Oxidase test, Catalase test, TSI agar test and Carbohydrate fermentation test.
4.2.4.4. Standard strains used for confirming identity and checking antimicrobial sensitivity pattern

*Escherichia coli* (MTCC 723), *Pseudomonas aeruginosa* (MTCC 1034), *Staphylococcus aureus* (MTCC 737), *Streptococcus pyogenes* (MTCC 1928) and *Salmonella enteritidis* (MTCC 125239) were the MTCC strains used for reference purpose.

4.2.4.5. Assessment of antibiotic sensitivity pattern

All isolates were subjected into antibiotic sensitivity test according to Roy *et al.*, 2006 and Bauer *et al.*, 1966. The susceptibility of isolates were examined by a disc diffusion assay.

4.2.4.5.1. Antibiotics used for the assay

- Gentamycin (Gen) - 30µg/disc
- Ciprofloxacin (CF) - 30 µg/disc
- Ampicillin (A) - 10 µg/disc
- Erythromycin (E) - 15 µg/disc
- Co-trimoxazole (Co) - 30 µg/disc
- Cephalosporins (CE) - 10 µg/disc
- Novobiocin (NV) - 05 µg/disc
- Cefpodoxime (CPD) - 30 µg/disc
- Tetracycline (T) - 30 µg/disc

All discs were purchased from Hi-Media, India.

4.2.4.5.2. Media used for the assay

Muller Hinton Agar (Hi-Media). The Muller Hinton Agar was prepared and sterilized at 121°C and inoculated the isolates then incubated at 37°C for 24 hrs.

4.2.4.5.3. Determination of Antibacterial activity

Disc diffusion method was followed (Bauer *et al.*, 1966) to determine the antibacterial activity. Petri plates containing 20 ml of Mueller Hinton agar were seeded with 4
hours old fresh culture of clinical isolates and referral strains. By making use of template drawn discs were dispensed on the solidified Mueller Hinton agar with test organisms. This was incubated at 37°C for 24 hours in an incubator (Rands SBC). The test was performed in triplicates. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - Media). The resistance patterns were interpreted as per CDC recommendations.

4.2.4.6. Antibacterial study of plant extract

4.2.4.6.1. Preparation of disc with plant extracts

Known quantity of extracts of both aqueous and phemolic were dissolved in DMSO: Methanol of 1:1 ratio. This in turn was diluted with equal volume of phosphate buffered saline (PBS pH 7). It was then filter sterilized by making use of sortorious syringe filter of pore size 0.22µm. Sterile discs of 6 mm diameter (Hi-Media) were loaded with 50µg - 250 µg / disc concentration of the extract and were dried. Dried discs were stored in sterile containers till use. Solvent loaded discs were also prepared and used as negative control. Oxytetracycline loaded Hi-Media discs were used as positive control.

4.2.4.6.2. Preparation of inoculums

Bacterial strains were inoculated in nutrient broth and incubated at 37ºC for 4 hours in a shaker (Orbit tech, Scigenics, India) and was used for antibacterial activity test.

4.2.4.6.3. Determination of Antibacterial activity

Disc diffusion method was followed (Bauer et al., 1966) to determine the antibacterial activity of the seed kernel extract of Mangifera indica. Petriplates containing 20 ml of Mueller-Hinton agar were seeded with 4 hours old fresh culture of clinical isolates and referral strains. By making use of template drawn extracts and fractions loaded discs were dispensed on the solidified Mueller Hinton agar with test organisms. Oxytetracycline antibiotic disc (30µg/disc) obtained from M/s Hi-Media laboratories Ltd, Mumbai was used as positive control for bacteria and solvent loaded discs were used as
negative control. The plates were incubated at 37°C for 24 hours in an incubator (Rands SBC). The test was performed in triplicates. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - Media).

4.2.4.6.4. Determination of Minimum Inhibitory Concentration

Agar dilution method was used to find out Minimal Inhibitory Concentration (Anonymous, 1993a and b). Stock concentration of various plant extract was prepared by making use of DMSO : Methanol, in the ratio of 1:1 which in turn was diluted with equal volume of phosphate buffered saline, pH 7. Mueller Hinton agar was prepared, sterilized and kept ready in molten condition. 20ml of the molten media was taken and was mixed with known concentration of different extracts / fractions and were added in different tubes. This mixture was swirled carefully for complete mixing of extract and media and poured on to the plate. After getting solidified it was inoculated with the test organism and standard organism. The plates were incubated at 37°C for 24 hours. MIC was recorded based on the growth of the organisms.

4.2.5. In-vitro antioxidant assay

A great number of In Vitro methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. \( \alpha, \alpha\)-diphenyl-\( \beta\)-picrylhydrazyl radical scavenging assay (DPPH), Ferric reducing antioxidant power (FRAP), Nitric oxide radical scavenging assay, Superoxide anion radical scavenging assay, ABTS radical scavenging assay, Hydroxyl radical scavenging assay, are the In Vitro antioxidant assay methods used to assess the antioxidant activity of the seed kernel extract of *M.indica*.

4.2.5.1. DPPH assay: \( \alpha, \alpha\)-diphenyl-\( \beta\)-picrylhydrazyl

4.2.5.1.1. Antioxidant activity by DPPH staining

An aliquote (3μL) of the sample and standard (Quercetin and Ascorbic acid) were carefully loaded onto a 10cm X 10cm Silica gel plate (silica gel 60 F254; Merck) and
allowed to dry for 3 minutes. Drops of the sample were loaded in an order of decreasing concentration along the row. After 5 minutes, the TLC plate was sprayed with 0.2% DPPH in methanol. Discolouration of DPPH indicates scavenging potential of the compound tested (Polshettiwar et al., 2007).

4.2.5.1.2. DPPH assay by TLC

This preliminary test was performed with a rapid TLC screening method using the \( \alpha, \alpha \)-diphenyl-\( \beta \)-picrylhydrazyl radical (DPPH) as a spray reagent (Chang et al., 2002; McDonald et al., 2001). Analytical TLC silica gel plate (10cm X10cm) was developed using chloroform : methanol : water (61:32:7) after application of 5\( \mu \)L of the test sample solution (1mg/ml), dried and sprayed with DPPH solution (0.2%, MeOH). After 5 minutes, the active compound was appeared as yellow spots against a purple background. The purple stable free radical 2, 2-diphenyl-1-picrylhydrazyl was reduced to yellow diphenylpicryl hydrazine. Quercetin was used as a positive control.

4.2.5.1.3. DPPH Radical Scavenging Activity (Spectrophotometric assay)

The free radical scavenging capacity of the extracts of Mangifera indica aqueous and phenolic extracts was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Extracts of Mangifera indica seed kernel was mixed with 95% methanol to prepare the stock solution (10mg/100ml). The concentration of extract solution was 10mg/100ml or 100\( \mu \)g/ml. From stock solution 2ml, 4ml, 6ml, 8ml and 10ml of the solution were taken in five test tubes and serially diluted, this was made up to final volume of each test tube to 10ml whose concentration was then 20\( \mu \)g/ml, 40\( \mu \)g/ml, 60\( \mu \)g/ml, 80\( \mu \)g/ml and 100\( \mu \)g/ml respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing extracts and after 10 minutes, the absorbance was taken at 517nm using a spectrophotometer (Systronics UV-Visible Spectrophotometer 119, INDIA). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10mg/100ml or 100\( \mu \)g/ml) of extracts. Control sample was prepared containing the same
volume without any extract and reference ascorbic acid. 95% methanol was used as blank (Soler Evans et al., 1997).

4.2.5.2. Ferric reducing power assay

This experiment was carried out as described previously (Cuendet et al., 1997). One ml of the plant extract solution (final concentration 100-500mg/L) was mixed with 2.5ml phosphate buffer (0.2M, pH 6.6) and 2.5ml potassium ferricyanide (K₃Fe (CN₆)) (10g/L), then the mixture was incubated at 50°C for 20 minutes. To this 2.5ml of trichloroacetic acid (100g/L) was added and centrifuged at 3000rpm for 10 minutes. Finally, 2.5ml of the supernatant solution was mixed with 2.5ml of distilled water and 0.5ml FeCl₃ (1g/L) and the absorbance was measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 119, India). Ascorbic acid was used as standard and phosphate buffer as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

\[
\%	ext{increase in Reducing Power} = \frac{A_{test}}{A_{blank}} - 1 \times 100
\]

A_{test} is the absorbance of test solution; A_{blank} is absorbance of blank. The antioxidant activity of the seed kernel extract was expressed as IC₅₀ and compared with standard.

4.2.5.3. Nitric oxide radical scavenging activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduce production of NO. Nitric oxide was generated from sodium nitroprusside, which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO₂⁻) which diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink colour, which can be measured at 546 nm. Sodium nitroprusside (10 mM, 2 ml) in phosphate buffer saline was
incubated with the test compounds in different concentrations at room temperature for 30 minutes. After 30 minutes, 0.5 ml of the incubated solution was added with 1ml of Griess reagent and the absorbance was measured at 546 nm (Polshettiwar et al., 2007).

4.2.5.4. Superoxide radical scavenging activity (PMS-NADH System)

Superoxide anions were generated using PMS / NADH system. The superoxide anions were subsequently made to reduce nitroblue tetrazolium, which yielded a chromogenic product, which was measured at 560 nm. Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitroblue tetrazolium (NBT). About 1ml of nitro blue tetrazolium (156µM), 1ml NADH (468µM) in 100mM phosphate buffer of pH 7.8 and 0.1ml of sample solution of different concentrations were mixed. The reaction was started by adding 100µl PMS (60µM). The reaction mixture was incubated at 25°C for 5 minutes and absorbance of the mixture was measured at 560nm against blank samples. The percentage inhibition was determined by comparing the results of control and test samples (Kumarasamy et al., 2007).

4.2.5.5. ABTS radical scavenging assay

ABTS (2, 2’-azinobis-3-ethylbenzothiozoline- 6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in absorption. The relatively stable ABTS radical was green and it was quantified spectrophotometrically at 734 nm. ABTS radical cations were produced by the reaction of ABTS and APS. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and the percentage inhibition was calculated. The stock solutions included were 7 mM ABTS solution and 2.4 mM potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowed them to react for 14 hrs at room temperature in dark. The solution was then diluted by mixing 1ml ABTS solution with 60ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was
prepared for each assay. Seed kernel extract (1 ml) was allowed to react with 1ml of the ABTS solution and the absorbance was taken at 734 nm after 7 minutes using a spectrophotometer. All determinations were performed in triplicate (n = 3) (Blois et al., 1958).

4.2.5.6. H$_2$O$_2$ scavenging activity

H$_2$O$_2$ scavenging ability of aqueous and phenolic extracts of Mangifera indica seed kernel was determined according to the method of Ali et al., (2009). A solution of H$_2$O$_2$ (40mM) was prepared in phosphate buffer (pH 7.4). The aqueous and phenolic extracts at 30µg/ml concentration in 3.4ml phosphate buffer were added to H$_2$O$_2$ solution (0.6ml, 40mM). The absorbance value of the reaction mixture was recorded at 230nm. Blank solution was containing the phosphate buffer without H$_2$O$_2$.

4.2.5.7. Assessment of % inhibition and IC$_{50}$

Radical scavenging activity of the extract and standard were expressed in terms of % inhibition. It was calculated using the formula \( \left( \frac{A_{Control} - A_{Sample}}{A_{Control}} \right) \times 100 \). Where \( A_{Control} \) is the absorbance of the control and \( A_{Sample} \) is the absorbance in the presence of the sample of aqueous and phenolic extracts. The IC$_{50}$ value is defined as the concentration (in µg/ml) of extracts that produced 50% antioxidant effect. IC$_{50}$ = Concentration of extract / % inhibition X 50.

4.2.6. Antidiarrhoeal Activity

4.2.6.1. Animals and Experimental Design

Swiss albino rat (20-35g) of either sex obtained from Tamil Nadu Veterinary and Animal Sciences University, Chennai and were used. They were housed in polypropylene cages in the departmental animal house at 26±2°C for one week before and during the experiments. The study was approved by the Ethics Committee for Animal Experimentation, Srimad Andavan Arts and Science College, Tiruchirappalli -5 (Ph.D MB-1/2012-13 April-July 2012). Fresh, dry husks were used as bed material. They were
fed commercially with standard pellet diet and distilled water. Food was withdrawn 18-24 h before the experiment, though water was allowed *ad libitum*. Mice were divided into seven groups of 6 animals each. Extracts and vehicles were administered to the animals using feeding tube.

- **Group I**: Normal control
- **Group II**: Castor oil control
- **Group III**: Castor oil + Loperamide (4mg/5ml)
- **Group IV**: Castor oil + received 50mg/Kg bw MISKPE
- **Group V**: Castor oil + received 100mg/Kg bw MISKPE
- **Group VI**: Castor oil + received 200mg/Kg bw MISKPE

### 4.2.6.2. Effect of extract on castor oil induced diarrhoea – Faecal Score Method

The method, described by Shoba *et al.*, (2001), was used for this study. First group served as the control and received saline. All other 6 groups received castor oil at a dose of 0.1 ml per animal orally. The second group served as castor oil control. Sixty minutes after castor oil administration, the third group received loperamide. The fourth group received phenolic extract of *Mangifera indica* seed kernel (MISKPE) (50mg/kg; bw), fifth group received MISKPE at 100mg/kg; bw, sixth groups received MISKPE at 200 mg/kg bw. Following administration, the animals were placed separately in cages with filter paper, which was changed every hour. The total number of faeces and diarrhoeal faeces excreted was recorded for a period of 76h. The total score of diarrhoeal faeces of control group was considered that of 100%. The results were expressed in percentage of inhibition.

### 4.2.6.3. Effect of extract on intestinal transit

This was done according to the method proposed by Sarin and Bafna (2012) using charcoal meal as a diet marker. First group served as the control and received saline. All other 6 groups received castor oil at a dose of 0.1 ml per animal orally. The second group served as castor oil control. Thirty minutes after castor oil administration, the third group received loperamide (3mg/kg, bw). The fourth group received phenolic extract of *Mangifera indica* seed kernel (MISKEE) (100mg/kg; bw), fifth group received MISKEE at
200mg/kg; bw, sixth groups received MISKEE at 400 mg/kg bw. concentrations. Each animal was given 1ml of charcoal meal orally (3% deactivated charcoal in 10% aqueous feed) after 30 minutes of castor oil administration. All animals were sacrificed after 30 minutes of charcoal meal administration and the distance covered by the charcoal meal in the intestine, from the pylorus to the caecum was measured and expressed as a percentage of distance moved.

**Castor oil induced enteropooling**

The albino rats were divided in to six groups and each group comprises of six rats only. They were fasted overnight and allowed the only access of water. Group I was treated with 0.2ml of normal saline, which served as control; Group II is a disease control, Group III received standard drug of Loperamide 3mg/kg bw. Groups IV, V and VI received three doses of the extract such as 50, 100 and 200mg/kg bw respectively. All doses were administered orally. Then after 1 hr of incubation, all the animals were treated with 3ml of castor oil for the diarrhoeal inducement. After two hours, the rats were allowed to sacrificed their lives and the small intestine ligated at both the pyloric sphincter and the ileocaecal junction and dissected. The small intestine was weighed and the milky contents were oozed out into a tube for measuring the volume level of the contents. The emptied intestine was reweighed and calculate the difference between empty and full weights of the intestine (Dicarlo et al., 1993).

**Table 4.1**

**Antidiarrhoeal assay – Treatment model**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Treatment</th>
<th>dose</th>
<th>Route of admin</th>
<th>No. of animals</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>Control - saline</td>
<td>-</td>
<td>Oral</td>
<td>6</td>
<td>1. Faecal Score Method (Shoba et al., 2001)</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>Disease Control</td>
<td>1ml</td>
<td>Oral</td>
<td>6</td>
<td>2. Intestinal transit (Sarin and Bafna, 2012)</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>Standard (Loperamide)</td>
<td>3mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td>3. Enteropooling (Dicarlo et al., 1994)</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>MISKPE</td>
<td>50 mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>MISKPE</td>
<td>100 mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>VI</td>
<td>MISKPE</td>
<td>200 mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
4.2.7. Anti Ulcer Assay

The experimental protocols were subjected to the scrutinization of the Institutional Animals Ethical Committee and were cleared by the same. All experiments were performed during morning according to CPCSEA guidelines for care of laboratory animals and the ethical guideline for investigations of experimental pain in conscious animals. The study was approved by the Ethics Committee for Animal Experimentation, Srimad Andavan Arts and Science College, Tiruchirappalli -5 (SAC-OPh.D/BC/05-13 dated 27.09.2013). Swiss albino mice (20-35 g) of either sex obtained from Tamil Nadu Veterinary and Animal Sciences University, Chennai were used. They were housed in polypropylene cages in the departmental animal house at 26±2ºC for one week before and during the experiments. Fresh dry husks were used as bed material. They were fed commercially with standard pellet diet and distilled water. Food was withdrawn 18-24 h before the experiment, though water was allowed ad libitum.

4.2.7.1. Antiulcer study – acid alcohol induced ulcer model

Ulcer was induced by administering acid ethanol (0.3M hydrochloric acid in 60% ethanol). All the animals were fasted for 36 h before administration of acid alcohol. The animals were divided into six groups, each consisting of six rats. Rats in group I, served as control, received saline (1 ml) orally. Group II rats were given only acid alcohol, which are considered as a disease control, Rats in group III were administered with ranitidine (32mg/kg) as a standard reference drug. Rats in group IV, V and VI received MISKEE at doses of 50, 100 and 200 mg/kg bw, respectively. Gastric ulcers were induced in rats by administrating acid ethanol orally, after 45 min of MISKPE and ranitidine treatment. They were kept in specially constructed cages to prevent coprophagia during and after the experiment. The animals were anaesthetized 1 h later with anaesthetic ether and the stomach was incised along the greater curvature and ulceration was scored (Deshpande et al., 2003).
Table 4.2
Antiulcer assay – Treatment model

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>Route of administration</th>
<th>Number of animals</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>Control</td>
<td>Saline</td>
<td>Oral</td>
<td>6</td>
<td>1. Ulcer Index</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>Disease Control</td>
<td>1ml acid alcohol (0.3M hydrochloric acid in 60% ethanol)</td>
<td>Oral</td>
<td>6</td>
<td>2. Gastric pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3. Gastric volume</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4. Total acidity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5. Antioxidant analysis</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>Standard (Ranitidine)</td>
<td>32mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>MISKPE</td>
<td>100 mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>MISKPE</td>
<td>200 mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>VI</td>
<td>MISKPE</td>
<td>400 mg/kg bw</td>
<td>Oral</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

4.2.7.1. Determination of acid secretary parameters

4.2.7.1a. Determination of total acidity (Antonial et al., 2007)

About 10 ml of gastric juice was transferred in a porcelain evaporating dish. 1-2 ml of topfer’s reagent was added. A colour change was observed, a bright red colour appear if free hydrochloric acid is present. 1-2 drops of phenolphthalein was added to the gastric juice with topfer’s reagent. Titrated it with 0.01N NaOH from a burette, mixing was done after each addition until the last trace of red colour disappeared and was replaced by a canary yellow colour. The numbers of milliliters of NaOH used was read from the burette. This represents the amount of free hydrochloric acid. The titration was continued until the red colour of phenolphthalein appeared (deep red), titrated to the point at which further addition of alkali did not deepen the colour. Reading was taken (ml NaOH) for total acidity.

Calculation: Total acidity (mEq/L)=ml of 0.01N NaOH x 10

4.2.7.1b. Ulcer index (Khayaei and Salehi, 2006)

The stomach was removed and fixed on a cork plate and numbered and severity of ulcers were registered with a stereo microscope using the following scores:

Scoring of ulcer was made as follows.

Normal stomach.......(0)

Red colouration........(0.5)
Spot ulcer.................(1)
Hemorrhagic streak..(1.5)
Ulcers.........................(2)
Perforation...................(3)

Mean ulcer score for each animal was expressed as ulcer index.
Calculation of ulcer index $U_1 = (UN + US + UP) \times 10^{-1}$
Where, $U_1 = \text{Ulcer index}$

$UN = \text{Average number of ulcers per animal}$
$US = \text{Average of severity score}$
$UP = \text{Percentage of animals with ulcer}$

After the experimental period animals were sacrificed by cervical decapitation. Blood was collected. Liver tissue was dissected out and washed in ice-cold saline. Liver tissues were homogenized in 0.1M phosphate buffer (pH 7.4) and used for studying various parameters. Stomach tissues were used for histopathological studies.

4.2.7.1c. pH (Nwinyl et al., 2004)

The stomachs were removed and the contents were drained into a graduated centrifuge tube through a small nick along the greater curvature. The tubes were centrifuged at 3000rpm for 10mins and the centrifuged samples were decanted and analyzed for pH using pH paper.

4.2.7.2. In-Vivo Anti-oxidant Activity

The animals were sacrificed after treatment. Stomach tissue was isolated and washed with normal saline and stored for 12 h for in-vivo antioxidant studies. The separated tissues were homogenized with motor driven Teflon coated homogenizer in ice-cold (10% w/v) 0.1 M Tris-HCl buffer pH 7.4 to get 10% homogenate. The homogenate was centrifuged at 10000 rpm for 10 min at 5°C. The supernatant was collected and used for following in-vivo studies.
4.2.7.2.1. Enzymatic Anti-oxidant Activity


Superoxide dismutase is an endogenous enzymatic antioxidant which catalyzes the dismutation of superoxide free radical. This method is based on the inhibition of the spontaneous oxidation of the adrenaline to adrenochrome by the enzyme superoxide dismutase. It was performed by taking 0.5ml of supernatant tissue homogenate in a test tube. To this 1.5ml of carbonate buffer (pH 10.2), 0.5ml of 0.1Mm EDTA and 0.4ml of epinephrine was added and the OD was taken at 480nm. Epinephrine was added just before taking the OD. The activity of SOD was expressed as units/min/mg protein. One unit of the enzyme is defined as the amount of enzyme, which inhibits the rate of adrenaline auto oxidation by 50%.

4.2.7.2.1b. Estimation of Glutathione peroxidase (GPx) Activity (Hsu and Yen, 2007)

Glutathione was measured by its reaction with DTNB to give a compound that absorbs at 412 nm. 0.32 M Phosphate buffer, pH 7.0, 0.8 mM EDTA, 10 mM Sodium azide, 3 mM reduced glutathione, 2.5 mM H$_2$O$_2$, 10% TCA, 0.3 M Disodium hydrogen phosphate, DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate) and Reduced glutathione were the reagents required. The reaction mixture consisted of 0.2 ml each of EDTA, sodium azide, H$_2$O$_2$, 0.4 ml of phosphate buffer, 0.1 ml homogenate/mitochondria and was incubated at 37°C at different time intervals. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml DTNB were added and the colour developed was read at 420 nm immediately. The activity of GPx was expressed as μmoles of glutathione oxidized/min/mg protein.

4.2.7.2.2. Non-Enzymatic Anti-Oxidant Activity

4.2.7.2.2a. Assay of lipid peroxidation (LPO) (Ohkawa, 1979)

In this method malondialdehyde and other TBARS were estimated by their reactivity with thiobarbituric acid (TBA) in acidic condition to generate a pink coloured chromophore which were read at 535 nm. 0.1 ml of tissue homogenate, Tris-HCl buffer
(pH 7.5) was treated with 2 ml of (1:1:1 ratio-TBA-TCA-HCl reagent, thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. The level of lipid peroxides was expressed as nmoles of MDA formed/mg protein.

4.2.7.3. Histophatological Techniques (Sujai, 1993)

Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

4.2.7.3.1. Collection of materials

Thin pieces of 3 to 5 mm, thickness were collected from tissues showing gross morbid changes along with normal tissue.

4.2.7.3.2. Fixation

Tissue was kept in fixative for 24-48 hours at room temperature. The fixation was useful in the following ways: Serves to harden the tissues by coagulating the cell protein, prevents autolysis, preserves the structure of the tissue and prevents shrinkage.

Common Fixatives: 10% Formalin

4.2.7.3.3. Haematoxylin and eosin method of staining

Deparaffinise the section by xylo1 5 to 10 minutes and remove xylo1 by absolute alcohol. Then cleaned the section in tap water and stained with haematoxylin for 3-4 minutes and again cleaned under tap water. Allow the sections in tap water for few minutes and counter stained with 0.5% eosin until section appears light pink 15 to 30seconds) and then washed in tap water. Blotted and dehydrated in alcohol and cleared with xylo1 (15 to 30 seconds). Mounted on a Canada balsam or DPX Moutant and kept the slide dry and remove air bubbles.
4.2.8. Evaluation of In-Vivo Anticancer Activity

4.2.8.1. Animal used

Inbred female Swiss mice of 2 months age, weighing 20 ± 5 g, were purchased from Govt veterinary College Mannuthi, Thrissur, India, were used for the study. The mice were obtained from the stock in breed colony, which was maintained by mating the mice (brothers and sisters). They were housed at room temperature of 22°C under 12 hr light/12 hr dark cycle in the animal house. Mice were fed with commercial pellet diet and water ad libitum freely throughout the study. All animal procedures were performed after approval from the IAEC (institution of animal ethical committee) and in accordance with the recommendations for the proper care and use of laboratory animals. The study was approved by the Ethics Committee for Animal Experimentation, Srimad Andavan Arts and Science College, Tiruchirappalli -5 (SAC-OPh.D/BC/01-13 dated 27.09.2013).

4.2.8.2. EAC -Induced Ascitic Antitumour Model (Ganguly, 1994)

4.2.8.2a. Adjust cell count to $1 \times 10^6$ cells

0.5ml of 0.4% Tryphan blue, 0.3ml of PBS and 0.2ml of EAC cell suspension were mixed and left aside for 5min and not more than 15min. From this one drop of solution was taken on a neubaur chamber and a cover slip was placed. This was placed on Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells doesn’t take colour and these cells appear in white colour on blue background. Non-viable cells(dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula.

\[
\text{Cell count} = \text{No. of cells} \times \text{Dilution factor} \times \text{volume factor}
\]

4.2.8.2b. EAC-induced ascitic antitumour model

The anti tumour activity of the extracts were determined by injecting EAC cell suspension ($1 \times 10^6$ cells per mouse) into the peritoneal cavity of the animals and treatment was started after 24 hours of the tumour inoculation continued once daily for 14 days and
the antitumour efficacy of test sample was compared with that of 5-Fu (20mg/kg, i.p) and EAC control.

**Table 4.3**

Anticancer assay – Treatment model

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>Route of administration of cell line</th>
<th>Number of animals</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>Control</td>
<td>Saline</td>
<td>Intra Peritoneal</td>
<td>6</td>
<td>1. Body weight</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>Disease Control</td>
<td>1ml</td>
<td>Intra Peritoneal</td>
<td>6</td>
<td>2. Mean Survival Time (MST)</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>Standard (5-Fu)</td>
<td>20mg/kg bw</td>
<td>Intra Peritoneal</td>
<td>6</td>
<td>3. Viable and non-viable cell count</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>MISKP E</td>
<td>50 mg/kg bw</td>
<td>Intra Peritoneal</td>
<td>6</td>
<td>4. Haematological parameters</td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>MISKP E</td>
<td>100 mg/kg bw</td>
<td>Intra Peritoneal</td>
<td>6</td>
<td>5. Antioxidant analysis</td>
</tr>
<tr>
<td>6</td>
<td>VI</td>
<td>MISKP E</td>
<td>200 mg/kg bw</td>
<td>Intra Peritoneal</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

4.2.8.3. Determination of Body Weight and Survival Time

4.2.8.3a. Body weight analysis

All the mice were weighed for every five days, after tumour inoculation. Average gain in body weight was determined and % decreases in body weight were calculated by the formula.

\[
\text{%Decrease in body weight} = \left( \frac{\text{Decrease in body weight/initial body weight}}{} \right) \times 100
\]

4.2.8.3b. Mean Survival Time (MST)

After induction every day checks all the groups for mortality & record how many days the mouse is survived the mean survival time (MST) and percentage increase in life span (ILS %) was calculated by using the formula.

\[
\text{Mean survival time} = \frac{(1\text{st Death} + \text{Last Death})}{2}
\]

\[
\text{ILS} \% = ((\text{Mean survival of treated group/ Mean survival of control group})-1) \times 100
\]
4.2.8.3c. Viable and non-viable cell count (Tryphan blue) (Sheeja et al., 1997) – Dye Exclusion Method

After 14 days of treatment animals are slightly anaesthetized with diethyl ether. The tumour cells were aspirated from the peritoneal cavity of tumour bearing mice using insulin syringe. 0.2ml of the cell suspension was mixed with 0.5ml of 0.4% Tryphan blue, 0.3ml of normal saline or PBS and kept aside for 5min and not more than 15min. From this one drop of solution was taken on a neubar chamber and a cover slip is placed. This is placed on Haemocytometer and the viable and non-viable cells were counted under 10X power. Viable cells doesn’t take colour of tryphan blue and these cells appear in white colour on blue background Non-viable cells(dead cells) take blue colour and give dark blue shading to the cells, cell count was calculated using formula .

\[
\text{Cell count} = \text{No. of cells} \times \text{Dilution factor} \times \text{volume factor}
\]

4.2.8.4. Estimation of Haematological Parameters

4.2.8.4a. Enumeration of Red Blood Cells (Schalm et al., 1975)

Blood was drawn into a red blood cell pipette of haemocytometer along with RBC diluting fluid at 1:200 dilution. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried. Using 45X or high power objective the RBC’s were counted uniformly in the larger corner squares. The cells were expressed as number of cells x10^{12}/l.

4.2.8.4b. Enumeration of WBC (John, 1972)

Blood and Turks fluid were mixed at 1:20 dilution using a white blood cell pipette of haemocytometer. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried. Using 10X or low power objective the WBC’s were counted uniformly in the larger corner squares. The cells were expressed as number of cells/10mm.
4.2.8.4c. Estimation of haemoglobin - Sahli’s acid haematin method (Kachmar, 1970)

0.1 N HCl was added to the Haemoglobinometer up to the lowest marking using HB pipette. 20µl of blood was drawn using sahli’s pipette and added to Haemoglobinometer. Mix the blood and acid carefully without bubbles. In this time, Haemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube. The reaction mixture was stand at room temperature of 10 minutes. Mixed solution was diluted with distilled water by adding few drops of water carefully and by mixing the reaction mixture until the colour matches the colour in the comparator. The lower meniscus of the fluid was noted and reading was noted in g/100ml.

4.2.8.4d. Estimation erythrocyte sedimentation rate

To take the 20ml of the citrated blood (mix 1.6ml blood with 0.4ml of 3.8% of sodium citrate solution) in westerngren tube exactly to the ‘0’ mark. Note the level to which the RBC column has fallen at the end of 3 minutes.

4.2.8.5.1. Enzymatic Anti-Oxidant Activity


It was already described in 4.2.7.2.1a

4.2.8.6.1b. Estimation of Catalase activity (Aebi, 1984)

Catalase causes rapid decomposition of hydrogen peroxide to water. Dichromate in acetic acid was converted to perchloric acid and then to chromic acetate when heated in presence of H₂O₂. The chromic acetate thus produced is measured spectrophotometrically at 610 nm. The catalase preparation is allowed to split H₂O₂ for different periods of time. The reaction is stopped at specific time interval by the addition of dichromate-acetic acid mixture and the remaining H₂O₂ is determined by measuring chromic acetate. Dichromate/acetic acid reagent 5% solution of potassium dichromate in acetic acid at 1:3 ratio, 0.01 M Phosphate buffer, pH 7.0 and 0.2 M Hydrogen peroxide were the reagents
used for this test. The assay mixture contained 4 ml of hydrogen peroxide, 5 ml of phosphate buffer and 1 ml of homogenate. One ml portions of the reaction mixture was withdrawn and blown into 2 ml of dichromate/acetic acid reagent at 1 min intervals. Then the mixture was incubated for 30 min later the OD was measured at 570 nm. The activity of catalase was expressed as μmole of H$_2$O$_2$ consumed/min/mg protein.

4.2.8.6.2. Non-Enzymatic Anti-Oxidant Activity

4.2.8.6.2a. Assay of lipid peroxidation (LPO) (Ohkawa, 1979)

It was already described in 4.2.7.2.2a

4.2.8.6.2b. Estimation of Proteins (Lowry et al., 1951)

This method is a combination of both Folin-ciocalteau and Biuret reaction which involves two steps.

Step: 1-Protein binds with copper in alkaline medium and reduces it to Cu++.  
Step: 2-The Cu++ formed catalyses the oxidation reaction of aromatic amino acid by reducing Phosphomolybdotungstate to heteropolymolybdinum, which leads to the formation of blue colour and absorbance was measured at 640 nm.

Reagents

Alkaline copper reagent

Solution A: 2 % sodium carbonate in 0.1 N NaOH.  
Solution B: 0.5 % copper sulphate in 1 % sodium potassium tartarate 50 ml of solution A was mixed with 1 ml of solution B just before use.  
Folin's phenol reagent (commercial reagent) : 1:2 dilutions  
Bovine serum albumin (BSA).

Procedure

To 0.1 ml of the homogenate, 0.9 ml of water, 4.5 ml of alkaline copper sulphate reagent were added and allowed to stand in the room temperature for 10 minutes .To this 0.5 ml of Folin’s reagent was added. After 20 minutes, the colour developed was measured at 640 nm. The level of protein present was expressed as mg/g tissue or mg/dl.
4.2.9. Phytochemical analysis

4.2.9.1. Qualitative phytochemical screening

Freshly prepared extracts were tested for the presence of phytochemical constituents using standard methods (Anonymous, 2006; Lala, 1981).

4.2.9.1.1. Test for Alkaloids

i) Dragendorff’s test: To 1 ml of the extract, 1 ml of Dragendorff’s reagent (potassium bismuth iodide solution) was added. An orange-red precipitate indicates the presence of alkaloids.

ii) Mayer’s test: To 1 ml of extract, 1 ml of Mayer’s reagent (Potassium mercuric iodide solution) was added. Whitish yellow or cream coloured precipitate indicates the presence of alkaloids.

4.2.9.1.2. Test for steroids

2 ml of acetic anhydride was added to 0.5 g phenolic extract with 2 ml of H₂SO₄. The colour change from violet to blue or green indicates the presence of steroids.

4.2.9.1.3. Test for terpenoids

Salkowski test: To 0.5 g of the extract, 2 ml of chloroform was added and carefully add 3 ml concentrated H₂SO₄ to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

4.2.9.1.4. Test for flavonoids

i) Alkaline reagent test: Few drops of dilute ammonia and Con. HCl were added to the portion of extract. A yellow colouration indicates the presence of flavonoids.

ii) Zinc Hydrochloride test: To few drops of extract, zinc dust and con. HCl was added, the presence of red colouration indicates the presence of flavonoids.

iii) Aluminium test: To few drops of extract, 1 % Aluminium solution was added, yellow colour indicates the presence of flavonoids.
4.2.9.1.5. Test for Saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and again shaken vigorously and observed for the formation of emulsion.

4.2.9.1.6. Test for tannins

i) A little quantity of test solution was taken and mixed with basic lead acetate solution. Formation of white precipitates indicates the presence of tannins.

ii) To 1 ml of the extract, add few drops of ferric chloride solution. Formation of a blue-black or brownish green colour product shows the presence of tannins.

iii) A little quantity of the extract was treated with aqueous ammonia solution. A deep green colour indicates the presence of tannins.

4.2.9.1.7. Test for Coumarins

0.5 g of the moistened plant extract was taken in a test tube. The mouth of the tube was covered with filter paper treated with 1 N NaOH solution. Test tube was placed for few minutes in boiling water and then the filter paper was removed and examined under the UV light for yellow fluorescence indicated the presence of coumarins.

4.2.9.2. Quantification of metabolites

Secondary metabolites like flavonoids, tannins and phenols were quantified employing standard methods (Ayoola et al., 2008). Coumarins was estimated by making use of the method described by Kuster and Rocha (2004).

4.2.9.2.1. Determination of total tannins

The total tannin content in the plant extract was determined by modification of a previous method (Polshettiwar et al., 2007). The water and phenolic extracts (0.1 ml) was mixed with 0.5 ml of Folin- Denis reagent followed by 1ml of Na₂CO₃ (0.5% w/v) solution and made up to 10 ml with distilled water. The absorbance was measured at 755 nm within
30 minutes of the reaction against the reagent blank. Standard curve was prepared using 20, 40, 60, 80 and 100 μl of tannic acid. Total tannins in extracts were expressed as equivalent to tannic acid (g TE/g extract).

4.2.9.2.2. Determination of total flavanoids

Aluminum chloride colorimetric method was used for flavonoids determination (Chang et al., 2002). The seed kernel extract (0.5ml of 1:10g/ml) in ethanol was mixed with 1.5ml of methanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It remained at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415nm with a single beam Systronics UV/Visible spectrophotometer (India). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100g/ml in methanol.

4.2.9.2.3. Determination of total phenols

Total phenols were determined by making use of the method given by McDonald et al., (2001). The diluted seed kernel extract (0.5 ml of 1:10 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na₂CO₃ (4 ml, 1M). The mixtures were allowed to stand for 15 minutes and the total phenols were determined by colourimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L⁻¹ solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g⁻¹ of dry mass), which is a common reference compound.

4.2.9.3. TLC Analysis

TLC was performed by making use of precoated plates supplied by M/S Qualigens PVT. LTD., Mumbai. Chromatogram was developed on silicagel G using methanol and water (4:1) as mobile phase to detech phenolic acid. Similarly MISKPE was separated using chloroform and ethyl acetate (4.5:0.5) for the detection of Tannins (Anonymous, 2006).
4.2.9.4. UV-FTIR analysis

Fourier Transform Infrared Spectroscopy analysis (FTIR) (Kannan et al., 2013)

The infrared (IR) spectra of phenolic extract *M. indica* seed kernel were obtained using Fourier Transform Infrared Spectroscopy (Perkin Elmer Spectrum GX FTIR, U.S.A). Samples discs were made by mixing 5 mg of dry biomass with 150 mg of potassium bromide (KBr), and pressed them into tablet form. Infrared spectra were recorded over 4000 - 500 cm\(^{-1}\) region with a resolution of 0.2 cm.

4.2.9.5. NMR analysis

\(^1\)H and \(^13\)C NMR experiments were performed on a Bruker advance DPX300 spectrometer operating at 300 and 75 MHz respectively. Chemical shift values (\(\delta\)) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants given in hertz.

4.2.9.6. GC-MS

Make : Thermo GC- Trace Ultra ver: 5. Thermo MS DSQ IIII
Column : DB 35 - MS capillary standard non-polar column
Dimension : 30 Mts, ID: 0.25 mm, FILM: 0.25 \(\mu\)m
Carrier Gas : HE, FLOW: 1.0 ML/Min
Temp Prog : Oven temp 70° C raised to 250° C at 10 C/min

4.2.10. Statistical analysis

All the values were expressed as mean ± SD (standard Deviation). Statistical analysis was carried out by using Origin software package (version 6.0). Statistical significance of differences between the control and experimental groups was assessed by One-way ANOVA. The value of probability less than 5% (\(P < 0.05\)) was considered statistically significant (Panse & Sukhatme, 1978).