Materials and Methods

3. MATERIALS AND METHODS

3.1 Ethanobotanical Survey

Field survey was conducted using a questionnaire to find out the medicinal plants which are used by the traditional medical practitioners of Malasar community of Velliangiri hills and to find out the medicinal plants with socioeconomic value.

Data on the following aspects were also collected:

- Availability of the medicinal plants
- Traditional knowledge about the selected herbs
- Utility in rural health care
- Number of formulations prepared by the traditional medical practitioners
- Number of patients treated per year

The data collected were consolidated and tabulated. Different plant specimens were collected with the help of tribal people. They were identified with the help of Gamble volume books. The local names of the plants were gathered from the tribal community. Details about medicinal uses of the plants were collected from the tribal healers.

3.1.1 Selection of three Ethanomedicinal plants by survey in Velliangiri hills

From the above survey, only three important potential medicinal plants such as *Annona muricata* L., *Spermacoce articularis* L. f. and *Rauvolfia tetraphylla* L. were selected to bring out the therapeutic usage. The study area of the present work was the foot hills of Velliangiri Hills which forms the eastern part of Western Ghats in the Tamil Nadu State. Velliangiri hills are known for its indigenous medicines and confined to the area of settlements of tribal people located at a distance of 25km from Coimbatore. The leaves of *Annona muricata* L., *Spermacoce articularis* L.f. and *Rauvolfia tetraphylla* L. were collected in the month of November 2015. The plant specimens were authenticated by Botanical Survey of India, Coimbatore.

The leaves of these plants were thoroughly washed and air dried in shade, and powdered using mixer grinder. The powder was further passed through a 2mm sieve to obtain finer particles. The powdered samples were stored in a clean glassware container for analysis.

3.2 Pharmacognostic Studies

3.2.1 Macroscopic characteristics (Kokate e*t al.*, 2010)

The morphological description of the plant parts such as shape ,size and surface characters were noted.

3.2.2 Microscopic characteristics (Sass, 1998)

Transverse sections of the leaves of *Annona muricata* L., *Spermacoce articularis* L.f., *Rauvolfia tetraphylla* L. were taken stained with safranin and mounted in glycerol. They were studied for different microscopic characters and photographs of different magnifications of the sections were taken with Ultrascope 9.1v.for normal observation ,Bright field microscope was used for the study of crystal and starch grains, Polarized light was employed.

3.3 Organoleptic characters (Khandelwal, 2003)

Organoleptic characters were evaluated which included the parameters like colour, shape, odour, taste, surface characteristics and texture and some specific characteristics of the material were considered as a first step towards establishment of identity and degree of purity of the drug.

3.4 Physico-chemical analysis (The ayurvedic pharmacopoeia, 1996)

3.4.1 Determination of Total ash

Two gram of air dried powder was taken in a tarred silica crucible and incinerated by gradually increasing the temperature to make it dull red hot until free from carbon then it is cooled and weighed and the experiment was repeated until it reached constant value. The percentage of total ash was calculated with reference to the air-dried drug.

3.4.2 Determination of Acid insoluble Ash

The ash obtained in total ash was boiled with 25ml of 2N hydrochloric acid for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water, dried the filter paper, ignited and weighed. Then the percentage of acid insoluble ash was calculated with reference to the air-dried drug.

3.4.3 Determination of Water Soluble Ash

The total ash obtained was boiled with 25ml of water for 5minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15minutes at a temperature not exceeding 450° C. The weight of insoluble matter was substracted from the weight of total ash. The difference in weight represents the water-soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug.

3.4.4 Determination of Sulphated Ash

A silica or platinum crucible was heated to redness for 10 minute, allowed to cool in a desicator and weighed. 2gms of the substance was taken accurately weighed, into the crucible, gently ignited at first, until the substance was thoroughly charred. Moistened the cooled residue with 1ml of sulphuric acid, heated gently until white fumes were no longer evolved and ignited at 800°C ± 25°C until all black particles have disappeared. The ignition was conducted in a place protected from air currents then allowed the crucible to cool and added a few drops of sulphuric acid and heated. Ignited as before, allowed to cool and weighed. The operation was repeated until two successive weighings do not differ by more than 0.5g.

3.4.5 Determination of Moisture content (Loss on Drying)

10g of the powdered drug was taken in a preweighed flat and thin porcelain dish and was dried in the oven at 105ºC for 5 hours. Cooled in a dessicator and weighed. The dry weight was taken (D W). Moisture content was determined by the formula: Moisture content = (I W - D W) X 100.

3.5 Preliminary Phytochemical Analysis

3.5.1 Preparation of extracts

20 g of powdered leaf material of *Annona muricata* L., *Spermacoce articularis* L.f. and *Rauvolfia tetraphylla* L. was dispersed in 250 ml of solvents such as water, ethanol, acetone, chloroform, hexane, petroleum ether and subjected to soxhlet extraction for 6-8hrs. The mixture was evaporated to dryness in a rotary flash evaporator and stored in refrigerator.

The condensed extracts of *Annona muricata* L., *Spermacoce articularis* L.f. and *Rauvolfia tetraphylla* L. were screened for preliminary phytochemical studies.

3.5.2 Qualitative Analysis

i) Test for alkaloids (Kokate *et al*., 2006)

Mayer's reagent: 1ml of extract was added to 2ml of Mayer's reagent and development of cream precipitate indicate the presence of alkaloids.

Wagner's reagent: 1ml of extract was added to 2ml of Wagner's reagent and development of reddish brown precipitate indicate the presence of alkaloids.

ii) Test for flavonoids (Prakash ***et al.*, 2011)**

Alkaline reagent: Extracts were treated with few drops of sodium hydroxide solution which will result in the formation of intense yellow colour, on addition of dilute acid, it becomes colourless which indicate the presence of flavonoids.

Lead acetate solution test: Test solution when treated with few drops of lead acetate solution would result in the formation of yellow precipitate.

iii) Test for Phenols (Prakash *et al*., 2011)

Ferric chloride test: Extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicate the presence of phenols.

iv)Tests for Cardiac Glycosides (Harborne, 1998)

Keller-Kiliani Test: A solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% $FeCl_3$ was mixed with the 10ml aqueous plant extract and 1ml of Conc H_2SO_4. A brown ring was formed between the layers which showed the entity of cardiac steroidal glycosides.

Borntrager's test: Few drops of dilute sulphuric acid were added to 1 ml of each of the extracts. This was boiled and filtered. The filtrate was ex-tracted with chloroform. The chloroform layer was treated with 1 ml of ammonia. The formation of red colour on the ammonical layer showed the presence of anthraquinone glycosides (*Harborne*, 1984; Sofowora, 1993).

v) Test for Steroid (Sofowora, 1993)

3ml of extract was mixed with 2ml of chloroform and conc H_2SO_4 was added side wise. A red colour produced in the lower chloroform layer indicate the presence of steroids.

vi)Test for protein (Prakash *et al.*, 2011)

Xanthoprotein: 3ml of test solution was taken in a test tube. To this 1ml of Conc. Sulphuric acid was added along the sides of the test tube. The formation of yellow precipitate indicated the presence of xanthoprotein.

Biuret test: The extract was treated with 1ml of 10% sodium hydroxide solution and heated. To this a drop of 0.7% copper sulphate was added. Formation of purplish violet colour indicate the presence of proteins.

vii) Test for carbohydrates (Tiwari *et al.*, 2011).

One gram of each of the extracts was dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Benedict's test: To 1 ml of each of the filtrates was added 5 ml Benedict's reagent and heated gently for 2 minutes and cooled. Orange red precipitate indicated the presence of reducing sugars.

Fehling's Test: To 1 ml of each of the filtrates was hydrolyzed with dilute HCl, neutralized with alkali and heated with Fehling's A & B solution. Formation of a red precipitate indicated the presence of reducing sugars.

viii) Test for Saponins (Kokate, 2005)

Foam test : 2ml of extract was taken in a test tube and added 6ml of water.The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

ix) Test for Amino acids (Sawhney & Singh., 2000)

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

x) Test for Coumarin (Sofowra, 1993)

To 1ml of extract, 1ml of 10% Sodium hydroxide was added. Formation of yellow colour indicates the presence of coumarin.

xi) Test for terpenoids (Sofowra, 1993)

2ml of extract was dissolved in 2ml chloroform and evaporated to dryness.To this, 2ml of Conc H_2SO_4 was added and heated for about 2 minutes. A greyish colour indicates the presence of terpenoids.

xii) Test for Naphthoquinones (Kokate *et al*., 2005)

Junglone test: Sample was treated with 2ml of chloroform extract and 2ml of ethyl ether with dilute ammonia solution. Pink colour formation indicates naphthoquinones.

xiii) Test for Anthraquinone glycosides (Trease and Evans, 1989)

10ml of benzene was added to sample in a conical flask and soaked for 10 minutes and then filtered. Further 10ml of 10% ammonia solution was added to the filtrate and shaken vigorously for 30 seconds and pink, violet, or red color indicated the presence of anthraquinones in the ammonia phase.

3.5.3 Quantitative Analysis

3.5.3.1 Determination of total alkaloid content (Ajanal *et al*., 2012)

The total alkaloid content was determined according to UV Spectrophotometer method. This method is based on the reaction between alkaloid and bromocresol green. The part of the plant extract was dissolved in 2 N HCl and then filtered, 1 ml of this solution was transferred to separating funnel and washed with 10 ml of chloroform. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this solution was transferred to a separating funnel and then 5 ml of bromocresol solution along with 5 ml of phosphate buffer was added. The mixture was shaken and the complex formed was fractioned with chloroform by vigorous shaking. The fractions were collected and made up to 10 ml in a volumetric flask using chloroform. The absorbance of the complex in chloroform was measured at 470 nm. All experiments were performed thrice; the results were averaged and reported in the form of mean ± S.E.M.

Based on the results of phytochemical analysis *A.muricata* L. and *S.articularis* L.f. were selected as best and used for the further study.

3.5.3.2 Determination of total flavonoid content (Vábková & Neugebauerova, 2013)

The total flavonoid content was measured using a modified colorimetric method. The appropriate amount of extract was added to a test-tube together with distilled water. Then 5% $NaNO_2$ was added, after 5 minutes 10% $AlCl_3$ and after another 5 minutes 1 M NaOH followed by the addition of distilled water. The absorbance was measured against the blank at 510 nm after 15 minutes. The standard curve was prepared using different concentration of catechin. The flavonoid content was expressed as g catechin equivalents (CE) per 100 g of dry weight (dw)

3.5.3.3 Determination of Total Phenolic Content (Vábková & Neugebauerova, 2013)

The total phenolic content was estimated using the modified Folin - Ciocalteu photometric method. The appropriate amount of filtered methanol extracts were oxidized with Folin-Ciocalteu's reagents and after 5 minutes the reaction mixture was neutralized with saturated sodium carbonate. The solution was then immediately diluted to the volume of 50 ml with distilled water. The absorbance was measured at 750 nm after 90 minutes of incubation at room temperature against the blank, gallic acid. The total phenolic content is expressed as g gallic acid equivalents (GAE) per 100 g of dry weight (dw).

Invitro Studies

3.6 Determination of Antioxidant activity

Antioxidant activity was carried out to analyze, whether the leaf extract of *Annona muricata* L. and *Spermacoce articularis* L.f. contains substance capable to mop up free radicals and prevent them from causing cell damage. Antioxidants are the molecule that slow or prevent oxidation of other molecule.

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the leaf extract of *Annona muricata* L. *and Spermacoce articularis* L.f. was determined by the method of Ruch *et al*., 1989.

Sample preparation

The leaf extracts (4ml) of *Annona muricata* L. *and Spermacoce articularis* L.f. were prepared by using distilled water at different concentration. To the plant extract 0.6ml of 4mM hydrogen peroxide solution which is prepared in 0.1M phosphate buffer (pH -7.4) was added. The mixture was incubated for 10 minutes and absorbance was read at 230 nm in UV visible spectrophotometer against blank solution containing the plant extract without H_2O_2. Non-enzymatic antioxidant Ascorbic acid (control) was used as standard reference.

0.1 M phosphate buffer preparation

Reagent (A): 1.419 g of Na_2HPo_4 was dissolved in 50 ml of distilled water.

Reagent (B): 1.56 g of NaH_2Po_4 was dissolved in 50 ml of distilled water.

0.1 M phosphate buffer was prepared from 40.5 ml of Reagent A and 9.5 ml of Reagent B, were made up to the volume 100ml with distilled water. 4mM H_2O_2 was prepared by 136µl of H_2O_2 in 100ml of distilled water.

Hydrogen peroxide scavenging activity of leaf extracts were calculated using the formula given below,

$$\text{\% Scavenging activity (Hydrogen peroxide)} = \frac{(\text{A Control} - \text{A Sample}) \times 100}{\text{A Control}}$$

Where, A control – Absorbance of control without the extract

A sample – Absorbance of the powder extract.

3.7 Antimicrobial Activity: (Kumari & Gupta 2013).

Media preparation

2gm of Muller hinton agar was dissolved in 100 ml of distilled water and boiled. Agar was then autoclaved for 15 min at 121°C and left to cool at room

temperature. Once the medium was cooled (about 45°C), it was poured into Petri dishes. Each petri dish was left on the flat surface for 30-40 min until completely set. The test microorganisms (*Escherichia coli; Staphylococcus aureus; Pseudomonas aeruginosa* and *Bacillus subtilis)* were seeded into the respective medium by the spread plate method. The surface of the medium was allowed to dry for about 3 min. The wells (10mm) were punched over the agar plates using sterile gel puncher. Various concentration (10, 25 & 50 µl) of extracts were added to the wells. The plates were incubated for 24 hours at 37^0C. After incubation the diameter of inhibitory zones formed around the wells were measured in mm and recorded.

3.8 *Invitro* anti-inflammatory

Invitro Anti-inflammatory Activity by Human Red Blood Cell membrane stabilization method (Azeem *et al.*, 2010).

Human red blood cell membrane method was used for the *Invitro* estimation of anti-inflammatory activity. Blood was collected from healthy volunteers and was mixed with equal volume of sterilized Alsever's solution (equal volume of 2% dextrose, 0.8 % sodium citrate, 0.05% citric acid & 0.42% sodium chloride in water). This blood solution was centrifuged at 4000 rpm and the packed cells were separated. The packed cells were washed with isosaline solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract, reference sample and control were separately mixed with 1ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of HRBC suspension. Instead of hyposaline 2 ml of distilled water was used in the control. All the assay mixtures were incubated at 37^0C for 30 minutes and centrifuged at 4000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by spectrophotometer at 560 nm. The percentage of hemolysis was estimated by assuming the hemolysis produced in the control as 100%. Percentage Protection = 100 – OD of sample X 100 OD of Control

3.9 Thin Layer chromatography (Wagner & Bladt, 1996)

Preparation of plates

The glass plates were cleaned and dried in hot air oven. Silica powder was added to distilled water and mixed continuously using a magnetic stirrer. The slurry

was poured in to a clean dried slide and scattered all over the slide to make a thin flim.The silica plates were activated by heating them in hot air oven at 100ºC for 1hr. After 1hr,the silica plates were allowed to cool at room temperature and marked about 1cm from the bottom. The extracts were loaded in the bottom centre of the slide. Suitable solvent was taken in a beaker. The plate was kept in the beaker without touching the baseline of solvent and left for development. The final solvent front was marked and the plate was dried. The developed TLC plates were dried and visually observed for various bands, using suitable spray.

Methanolic extracts were tested on TLC for presence of bioactive compounds

Pre coated TLC plate (Merck, Germany) was used in this study. Around 50µl of aqueous extracts of *Annona muricata L. and Spermacoce articularis L. f.* were loaded on the plates with the standard (Ascorbic acid and Gallic acid 10mg/ml). They were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and HRF values of these spots were recorded by exposing chromatogram to the iodine vapours.

The retention factor (Rf) value was calculated as follows

Measured and recorded the distance of each spot from the point of its application and calculated the Rf value by dividing the distance travelled by the spots by the distance travelled by the solvent of the mobile phase.

Alkaloids : The test sample was applied on precoated TLC plates developed in chloroform: glacial acetic acid: methanol : water (64:34:12:8) as mobile phase, dried sprayed with the dragendroff's reagent. Appearance of orange red spot at room temperature indicates the presence of alkaloid.

Saponin: The test sample was applied on precoated TLC plates developed in chloroform: glacial acetic acid: methanol : water (64:34:12:8) as mobile phase, dried sprayed with 1% vanillin 5% sulphuric acid reagent and dried at 110ºC for few minutes Glycosides appear as dark bluish to black spot.

Triterpenoids: The detection for triterpenoids was done by applying the samples on TLC plate impregnated with silver nitrate and they were developed in butanol-2M

ammonium hydroxide (1:1) as mobile phase. The detection was done by spraying antimony trichloride. The purplish spot indicates the presence of triterpenoids.

3.9.1 FTIR analysis

This study was carried out to identify the chemical constituents and elucidate the structures of the compound of the leaves such as *Annona muricata* L., *Spermacoce articularis* L.f. It is analysed by FTIR spectrum obtained with 0.2 nm resolutions at 400-4000 nm-1 **(Hazra *et al.*, 2007).** Each and every analysis was repeated twice for the spectrum confirmation.

3.9.2 Synthesis of silver nanoparticles (Paralikar, 2014)

To one mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles, 4ml solution was added to 4ml of each aqueous leaf extracts of *Annona muricata* L., *Spermacoce articularis* L.f. and color changes were recorded. The prepared solution was taken in 100ml elementary flask and covered with aluminium foil air tightly and setup was kept in dark room for 72 hours.

3.9.3 UV-Visible spectra analysis (Chauhan *et al., 2014*)

The reduction of pure AG + ions was monitored by measuring the UV-Visible spectrum using UV-Visible spectrophotometer SL-159 (Elico). The spectra were recorded at room temperature using three centimeter quartz cuvettes.

3.9.4 GC-MS analysis (Hema *et al., 2010)*

GC-MS plays a key role in the analysis of unknown compounds of plant origin. 1µl of aqueous leaf extract of *Annona muricata* L. *Spermacoce articularis* L.f. was employed for analysis of different compounds. GC-MS analysis was performed using Thermo GC - Trace Ultra Ver : 5.0, Thermo MS DSQ II system and Gas Chromatograph interfaced to a mass spectrometer (GC-MS) equipped with a DB 35 - MS Capillary Standard Non - Polar Column For GC/MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium, Flow - 1.0 ML/Min was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 2 ml was employed (Split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature of 70 °C raised to 260°C with 6 °C

/minute Total GC running time was 50 minutes. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was Turbo mass.

Identification of Phytocomponents

Identification of compounds was conducted using the database of Wiley 9 library combined with the National Institute of Standards and Technology (NIST) library. The name, Molecular weight, Molecular formula and area under peak of the components of the test materials were ascertained.

3.10 Heavy metal Analysis : (Cabrera *et al.*, 1994)

Weighed one gram of the sample into a 100ml conical flask.15ml of triacid mixture (Con. HNO_3, Con. H_2SO_4, Con. $HClO_4$ at 9:2:1) was added and the contents were digested on a hot plate at 200ºC until white fumes appear. After cooling the volume was made up to 50ml with double distilled water and filtered through Whatman No.1 filter paper and then fed into the Atomic absorption Spectro photometer (Perkin Elmer Analyst 200, USA) and analysed for Cd, Pb, Zn, Ni and Cr.

3.11 Pharmacology Study (*Invivo* Studies)

3.11.1. Acute toxicity Study

Preparation of extracts

Annona muricata L. and *Spermacoce articularis* L.f. were dried under shade and then coarsely powdered with a mechanical grinder and stored in an airtight container separately. 100gms of powdered leaf material of *A.muricata*.L and *S.articularis* L.f. was macerated in 2L of distilled water for 36 hrs at room temperature with occasional shaking. The macerate was filtered through muslin and the filtrate was concentrated in a rotary evaporator at 40°C. The resulted extract was stored in refrigerator and used for further studies.

Experimental Animals

Male Swiss albino mice (20 – 25 g) and Wistar albino rats (180 – 200 g) of either sex were used for the study. The animals were obtained from animal house,

Nandha College of Pharmacy, Erode. The animals were placed randomly and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24 ± 2°C and relative humidity of 30 – 70 %. A 12:12 light: day cycle was followed. All animals were allowed to free access to water and fed with standard commercial pelleted rat chaw (M/s. Hindustan Lever Ltd, Mumbai).

Method: (Ecobichon, 1997)

Acute toxicity studies were performed according to OECD-423 (Organization of Economic and Cooperation Development) guidelines. Male Swiss mice selected by random sampling technique were employed in this study. The animals were fasted for 4 h with free access to water. The aqueous extract of *Annona muricata* L. and *Spermacoce articularis* L.f. was administered orally at a dose of 5 mg/kg initially and mortality if any was observed for first 24 hrs and after 72 hrs. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one animal out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, then higher (50, 300, 1000 & 2000 mg/kg) doses of the plant extracts were employed for further toxicity studies.

3.11.2 Analgesic activity – Eddy's Hot Plate

Experimental Animals

Swiss Albino mice of either sex weighing between 25 to 30g were used in this study. Mice were maintained in polypropylene cages with husk as bedding at a temperature of 24+ 2ºC and relative humidity 30-70 % with 12:12 light and dark cycle. Animals were given standard diet and water Animals were acclimatized to the laboratory conditions one week before the experiment. Animals were fasted overnight before the experiment. Experimental protocol was approved by the institutional Animal Ethical Committee(AEC) Reg. No. (No.688/PO/Re/S/02/CPCSEA) **Annexure – II.**

Method: (Eddy and Leimbach, 1953)

Animals were divided into six groups of 5 mice each. Group I served as control received the vehicle (distilled water, 10ml/kg). Groups II served as reference control, administered with Pentazocine (10mg/kg, i.p.). Group III and IV animals

were treated with 200 and 400 mg/kg of *Annona muricata* L. respectively. Group V and VI animals were treated with 200 and 400 mg/kg of *Spermacoce articularis* L.f. respectively. The test drugs were administered orally using gastric gavages by dissolving in distilled water.

The Eddy's hot plate test was used to measure response latency time, according to the method described by Eddy and Leimbach. Animals were placed on hot metal plate maintained at 55 ± 1^{o}C. Elapsed time between the placement of the animal on the hot plate and the occurrence of the licking of the hind paws or jump off from the surface was recorded as response latency in seconds. The specificity and sensitivity of the test were increased by measuring the reaction time of the first evoked behavior regardless of whether it is paw licking or jumping. The responses were measured at 30, 60 90 and 120 seconds. Only mice that show initial nonreceptive response within 30 seconds were selected and used for the study. The cut-off time for the hot plate latencies was set at 15 secs. The percentage protection against thermal pain stimulus was calculated according to the following formula:

$$\text{Percentage protection against thermal stimulus} = \frac{\text{Test Mean (Ta)} - \text{Control Mean (Tb)}}{\text{Control Mean (Tb)}} \times 100$$

3.11.3 Antipyretic Study

Experimental Animals

The experiment was carried out on albino rats of either sex with the average weight of 1.5-2.0Kg which were kept in cages, maintained at 23-25ºC and were given standard diet. Food was withdrawn 24 hrs prior to the experiments but had free access to water. Experimental protocol was approved by the institutional Animal Ethics Committee (IAEC). Reg. No. 688/PO/Re/S/02/CPCSEA.

Method: (Hukkeri *et al.*, 2006)

Six groups of 5 rats each were injected subcutaneously with 10ml/kg, of yeast suspension (15% in 0.5% w/v methylcellulose) to induce pyrexia, after measuring the basal rectal temperature of each animal 19 hrs after yeast injection, the rectal temperature was recorded again and animals showing a rise in temperature of < 0.6^{o}C were discarded. Thereafter, treatment was carried out as follows. Group I served as

control received the vehicle (distilled water, 1ml/kg). Groups II served as reference control, administered with Aspirin (100mg/kg). Group III and IV animals were treated with 200 and 400 mg/kg of *Annona muricata* L. respectively. Group V and VI animals were treated with 200 and 400 mg/kg of *Spermacoce articularis* L.f. respectively. The test drugs were administered orally using gastric gavages by dissolving in distilled water.

Rectal temperature was then recorded at 20, 21, 22, 23 and 24 hrs after yeast injection. Percentage reduction in rectal temperature was calculated by the following formula.

$$\% \text{ Reduction} = \frac{B - Cn}{B - A} \times 100.$$

A - Normal temperature.

B - Rectal temperature at 18 h after yeast administration.

Cn - Rectal temperature after drug administration.

3.11.4 Anti-inflammatory activity

Experimental Animals

The experiment was carried out on albino rats of either sex with the average weight of 1.5-2.0 Kg which were kept in cages, maintained at 23-25ºC and were given standard diet. Food was withdrawn 24 hrs prior to the experiments but had free access to water. Experimental protocol was approved by the institutional Animal Ethics Committee (IAEC) Reg. No. 688/PO/Re/S/02/CPCSEA.

Method

Carrageenan-Induced Rat Paw Oedema (Winter *et al*,. 1962)

Albino Wistar rats were used in the study, and the rats were divided into six groups of 5 each. Group I served as control received the vehicle (distilled water, 1ml/kg). Groups II served as reference control, administered with Indomethacin (10mg/kg). Group III and IV animals were treated with 200 and 400 mg/kg of *Annona muricata* L. respectively. Group V and VI animals were treated with 200 and 400

mg/kg of *Spermacoce articularis* L.f. respectively. The test drugs were administered orally using gastric gavages by dissolving in distilled water.

After 30 minutes, acute inflammation was produced in the right hindpaw of each rat by sub plantar injection of 0.05ml freshly prepared carrageenan suspension (1%) in normal saline. The volumes of the oedematous paws were measured using Plethysmometer following oral administration of the test drugs, 0 min (before carrageenan injection) and at every 1 h intervals for 5 h. Oedema was expressed as the increment in paw thickness due to carrageenan administration. The percentage of anti-inflammatory activity was calculated using the formula given below:

$$100 \times \left(1 - \frac{Vt}{Vc}\right)$$

Vc – Control Mean and Vt – Test Mean

3.12 *Invitro* cytotoxicity assay (Denizot & Lang, *et al* .,1986)

3.12.1 Determination of mitochondrial synthesis by MTT assay

Principle

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based. The principle involved is the cleavage of tetrazolium salt MTT (3-(4,5 Dimethylthiazole-2-yl)-2,5-Diphenyltetrazolium bromide) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The numbers of cells were found to be proportional to the extent of formazan production by the cells used (Denizot & Lang 1986).

Materials

1. Cell lines: HELA-Human Cervical Cancer Cell Line (From NCCS, Pune)
2. Cell culture medium: DMEM- High Glucose - (#AL111, Himedia)
3. Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
4. Fetal Bovine Serum (#RM10432, Himedia)

5. MTT Reagent (5 mg/ml) (# 4060 Himedia)
6. DMSO (#PHR1309, Sigma)
7. Camptothecin (#C9911, Sigma)
8. D-PBS (#TL1006, Himedia)
9. 96-well plate for culturing the cells (From Corning,USA)
10. T25 flask (# 12556009, Biolite - Thermo)
11. 50 ml centrifuge tubes (# 546043 TORSON)
12. 1.5 ml centrifuge tubes (TORSON)
13. 10 ml serological pipettes (TORSON)
14. 10 to 1000 ul tips (TORSON)

Equipments

1. Centrifuge (Remi: R-8^{o}C).
2. Pipettes: 2-10µl, 10-100µl, and 100-1000µl.
3. Inverted microscope (Biolink)
4. 37°C incubator with humidified atmosphere of 5% CO_2 (Healforce, China)

Assay controls

1. Medium control (medium without cells)
2. Negative control (medium with cells but without the experimental drug/compound)
3. Positive control (medium with cells and 25µM of Camptothecin)

Note: Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the MTT to formazan. To account for this reduction, it is important to use the same medium in control as well as test wells.

Steps followed

1. Seeded 200µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allowed the cells to grow for about 24 hours.
2. Added appropriate concentrations of the test agent (Mentioned in the results - Excel sheet).
3. Incubated the plate for 24 hrs at 37°C in a 5% CO_2 atmosphere.

4. After the incubation period, the plates were taken out from incubator, and removed spent media and added MTT reagent to a final concentration of 0.5mg/mL of total volume.
5. Wraped the plate with aluminium foil to avoid exposure to light.
6. Returned the plates to the incubator and incubated for 3 hours. (Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.)
7. Removed the MTT reagent and then added 100 µl of solubilisation solution (DMSO).
8. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures.
9. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm and 630nm used as reference wavelength.
10. The IC50 value was determined by using linear regression equation i.e. Y =Mx+C. Here, Y = 50, M and C values were derived from the viability graph.

3.12.2 DNA Fragmentation Assay (Herrmann *et al.*, 1994)

DNA fragmentation analysis was carried out using agarose gel electrophoresis as described by Herrman *et al* with some modifications. The HeLa cells (Human Cervical Cancer Cell Line) were treated with each extract and then incubated for 24 h at 37°C. The treated cells were harvested by trypsinization, washed twice with PBS, and then used for DNA isolation. The cells were centrifuged at 8000 rpm for 5min and then washed with PBS (pH 7.2). After discarding the supernatant, 200 μL of lysis solution (10mM Tris (pH 7.4), 5mM EDTA, 0.2% Triton X-100) and 10 μL of 1mg/mL−1 proteinase K were added. The cells were left in a 56°C water bath 8 µl of RNase (100 μg mL - 1) was added to each tube, and then the incubation continued at 37°C for 45 minutes. 20µl of 1.5M NaCl was added, and then the tubes were inverted several times before being centrifuged at 12000rpm for 10min. The supernatant was added to clean eppendorf tubes. Ice cold isopropyl alcohol (2x the volume) was added. The tubes were inverted several times and left at -80°C for 20 minutes. The tubes were centrifuged at 12000rpm for 15min in a microcentrifuge and the supernatant was then discarded. The isolated DNA was allowed to air-dry before being resuspended in 1XTE buffer (10mM Tris-HCl (pH 7.4) and 0.5mM EDTA.

Electrophoresis was conducted at 100 V for 30 min. The agarose gel was stained with ethidium bromide for 10 min and then rinsed in distilled water for another 10 min. The DNA bands were photographed under Gel Documentation. 1kb DNA marker was used to estimate the size of the DNA fragments.

3.12.3 Statistics

Statistical analysis

Analysis of Variance (ANOVA) was performed on all parameters to compare treatment effects and the influence of different organisms and different solvents. Means were separated using Duncan's Multiple Range Test (DMRT). Student's t-test to compare the heavy metal levels in both the plant species using SPSS (version 16.0).