3. MATERIALS AND METHODS

Collection and identification of plant material

The fresh leaf and stem bark of *Chionanthus mala-elengi* were collected from Nilgiri Biosphere Nature Park, Anaikatti, Coimbatore. The Plant material was identified and authenticated by Botanical Survey of India, Coimbatore.

The leaf and stem bark were cleaned to removing any unwanted debris, washed under running tap water and air dried in shade and powdered using mixer grinder. The powder was further passed through a 2 mm sieve to obtain finer particles. The powdered samples were stored in a clean glassware container for further studies.

3.1 Pharmacognostic studies

3.1.1 Morphological characteristics (Kokate *et al.,* 2010)

The macroscopic study is the morphological description of the plant parts which could be seen by naked eye or magnifying lens. Macroscopic observation of the plant parts characters such as shape, size, texture and organoleptic characters (odour and taste) were recorded.

3.1.2 Microscopical characteristics (Sass, 1998)

Free hand transverse sections of the leaf of *C. mala-elengi* 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 and polarized light was employed.

3.1.2.1 Determination of stomatal number and stomatal index (Khandelwal, 2006)

The stomatal index is the percentage of the number of stomata formed by the total number of epidermal cells, including the stomata, each stoma being counted as one cell.

Fragments of leaf from the middle of lamina were cleared by boiling with chlorinated soda. Upper and lower epidermis were peeled separately by means of forceps. Mounts of lower and upper epidermis were prepared separately and focused under the 10X

magnification and the epidermal cells and stomata were traced by looking through the microscope. When a super imposed image of the leaf is seen at the same time, number of epidermal cells were counted and stomata (the two guard cells and ostiole being considered as unit) within the microscopic field where a cell is counted if at least half of its area lies within the field provided by two adjacent sides that are considered for purpose of circulation. Successive adjacent fields until about 400 cells are counted and stomatal number is calculated *i.e*. number of stomata per sq.mm of leaf preparation. The stomatal index was calculated using the formula

$$\textbf{Stomatal index} = \frac{S \times 100}{E+S}$$

Where,

S= the number of stomata in a given area of leaf and

E=the number of epidermal cells (including trichomes) in the same area of leaf.

For each sample of leaf three determinations were made and calculated the average index.

3.1.3 Organoleptic evaluation (Trease, 1996)

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

3.1.4 Powder study (Johansen, 1940)

The shade dried leaf and stem bark of the *C. mala-elengi* plant were powdered and sieved to obtain coarse powder. A small amount of powder was taken onto a microscope slide cleared from chlorophyll by heating with chloral hydrate solution and was mounted in 50% v/v glycerol in water. This was then observed under microscope to study the characteristic features.

3.2 Physicochemical analysis (Anonymous, 2001)

3.2.1 Determination of total ash

Accurately weighed about 2 gram of air-dried powdered drug was taken in a tarred grouch silica crucible and incinerated by gradually increasing the temperature to make it dull red hot until free from carbon, then it is cooled, weighed and the experiment was repeated until it reached the constant value. The percentage of total ash was calculated with reference to the air-dried drug.

3.2.2 Determination of water soluble ash

Total ash was boiled for 5 minutes by adding 25 ml of water. The insoluble matter was collected in a Gooch crucible and washed with hot water, ignited for 15 minutes at a temperature not exceeding 450˚C. The weight of the insoluble matter was subtracted from the weight of the total ash, the difference in weight indicates the water soluble ash. The percentage of soluble ash was calculated.

3.2.3 Determination of acid insoluble ash

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

3.2.4 Determination of sulphated ash

A silica or platinum crucible was heated to redness for 10 minutes, allowed to cool in a desiccator and weighed. Two grams of the substance was taken accurately weighed into the crucible, gently ignited at first until the substance was thoroughly charred. The cooled residue was moistened with one ml of sulphuric acid, heated gently until white fumes were no longer evolved and ignited at 800±25˚C until all black particles have disappeared. The ignition was conducted in a place protected from air currents and then the crucible is allowed to cool and a few drops of sulphuric acid was added and heated, ignited similarly, allowed to cool and weighed. The operation was repeated until two successive weighing do not differ by more than 0.5 gram.

3.2.5 Determination of moisture content (Loss on drying)

Ten gram of powdered drug was taken in a preweighed flat and thin porcelain dish and dried in the hot air oven at 105˚C for 5 hours. Cooled in a desiccator, continued drying and weighed at one-hour intervals until difference between two successive weighing corresponded to not more than 0.25 percent. The loss in weight is usually recorded as moisture.

3.2.6 Determination of extractive value (Khandelwal, 2002)

The dry powdered material (leaf and stem bark) of *C. mala-elengi* was extracted with petroleum ether, chloroform, ethyl acetate, ethanol and water using maceration process. 2 gm of coarsely powdered plant material was accurately weighed and transferred into a dry conical flask. Then, each conical flask was filled with various solvents separately. The flasks were corked and place aside for 24 hours at room temperature shaking frequently. The mixture were filtered carefully using whatmann No.1 filter paper. After the filtrate was collected, it was transferred into a weighed petri plates. The acquired extracts were concentrated to dryness by allowing filtrate for complete evaporation of solvent.

The Percentage of extractive value was calculated by

$$\text{Extractive value (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of plant material}} \times 100$$

3.2.2.1 Fluorescence analysis (Chase Jr and Pratt, 1949)

The small quantity of dried and finely powdered leaf and stem bark samples were placed on a grease free microscope slide and 1-2 drops of freshly prepared solution such as Concentrated HNO_3, Concentrated HCl, Concentrated H_2SO_4, Ammonia solution, $FeCl_3$, Acetic acid, NaOH, Distilled water, Chloroform and Iodine were added.

Then, the slide was placed inside the UV viewer chamber and viewed in daylight and ultra violet radiations. The colours observed by application of different reagents were recorded.

3.3 Preliminary phytochemical analysis

3.3.1 Preparation of extracts

20 gram of dry powdered material (leaf and stem bark) of *C. mala-elengi* was dispersed in 250 ml of solvents such as petroleum ether, chloroform, ethyl acetate, ethanol and water were subjected to soxhlet extraction for 6-8 hours. The mixture was evaporated to dryness in rotary evaporator and stored in refrigerator. The condensed extracts were screened for preliminary phytochemical studies.

3.3.2 Qualitative phytochemical analysis (Harborne, 1984; Kokate, 1994)

Processing of plant materials

The leaf and stem bark of *C. mala-elengi* were washed in running water and cut into small bits to facilitate drying. The pieces of plant materials were dried for 12 hours in a hot air oven at 600˚C. The dried plant materials were powdered using mixer grinder to obtain a fine powder. The powder was further passed through a 2 mm sieve to obtain finer particles. The powdered samples were stored in a clean glassware container until needed for analysis.

Preliminary phytochemical screening

One gram of each of the extract dissolved individually in 5 ml of distilled water and filtered. The filtrates were used for further phytochemical analysis.

(i) Test for alkaloids (Kokate, 2005)

Mayer's test: One ml of filtrate was added to 2 ml of Mayer's reagent and development of cream precipitate indicated the presence of alkaloids.

Dragendorff's test: One ml of filtrate was added to 2 ml of Dragendorff's reagent and appearance of orange brown precipitate indicated the presence of alkaloids.

Wagner's test: One ml of filtrate was added to 2 ml of Wagner's reagent and development of reddish brown precipitate indicated the presence of alkaloids.

(ii) Test for carbohydrates (Kokate, 2005)

Molisch's test: To one ml of each of the filtrate, one drop of Molisch's reagent (10% α-naphthol in ethanol was added), Then, 1-2 drop of Concentrated H_2SO_4 was poured down the side of the test tube, it forms a layer at the bottom of the tube. Formation of a violet coloured ring at the junction of the two liquids indicated the presence of carbohydrates.

Fehling's test: One ml of the filtrate was hydrolyzed with diluted HCl, neutralized with alkali and heated with Fehling's A and B solution. Formation of a brick red precipitate indicated the presence of reducing sugars.

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

(iii) Test for proteins & aminoacids (Prakash *et al.*, 2011)

Biuret test: One ml of each of filtrate was treated with one ml of 10% sodium hydroxide and heated. To this a drop of 0.7% copper sulphate was added. Formation of purplish violet colour indicated the presence of proteins.

Ninhydrin test: One ml of each filtrates was treated with two ml of sample in a test tube and added 3-4 drops of ninhydrin solution and boiled for few minutes. Formation of deep blue colour indicated the presence of amino acids.

Xanthoproteic test: Three ml of the test solution was taken in a test tube. To this one ml of concentrated nitric acid was heated, solution was added for 2 minutes and cooled under tap water, 40% sodium hydroxide solution was added drop by drop. The formation of yellow precipitate indicated the presence of xanthoproteins.

Millon's test: Two ml of each of the filtrates was treated with 2 ml of Millon's reagent, boiled and cooled. To the mixture a few drops of sodium nitrite solution were added. Formation of red precipitate indicated the presence of proteins.

(iv) Test for tannins and phenolic compounds

Ferric chloride test: Each test extract was mixed with 1% ferric chloride solution. Formation of brown/green/blue colour indicated the presence of tannins and phenolic compounds.

(v) Test for steroids and sterols

Liebermann-Burchard's test (LB test): Two mg of dried extract was dissolved in acetic anhydride, heated to boiling, cooled and then one ml of Concentrated H_2SO_4 was added along the side of the test tube. Formation of a reddish brown ring at the junction of two layer indicated the presence of sterols and steroids.

Salkowski's test: Two mg of dried extract was shaken with chloroform. To the chloroform layer concentrated sulphuric acid was added slowly in the sides of the test tubes. Formation of red colour indicates the presence of steroids.

(vi) Test for triterpenoids

Liebermann-Burchard (LB test): 2 mg of dried extract was dissolved in acetic anhydride, heated to boiling, cooled and then one ml of Concentrated Sulphuric acid was added along the side of the test tube. The formation of red colour confirms the presence of triterpenoids.

(vii) Test for saponins

Foam test: In a test tube containing about 5 ml of extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicated the presence of saponins.

(viii) Test for flavonoids

Shinoda test: In a test tube containing 0.5 ml of the extract 10 drops of Concentrated hydrochloric acid followed by a small piece of magnesium was added. Formation of pink, reddish colour indicated the presence of flavonoids.

Ferric chloride test: Test solution of each extract was treated with 2-3 drops of ferric chloride solution. Formation of a blackish green colour indicated the presence of flavonoids.

Lead acetate test: Test solution of each test extract was mixed with few drops of 10% Lead acetate solution. Formation of yellow precipitate confirmed the presence of flavonoids.

(ix) Test for glycosides

Borntrager's test: In one ml of each test extract solution, add 1 ml of 5% H_2SO_4. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with

equal volume of chloroform and kept to stand for 5 minutes. Then, lower layer of chloroform was shaken with half of its volume of dilute ammonia. The formation of rose pink to red colour of the ammoniacal layer indicated the presence of glycosides.

Keller-killiani test (Ayoola *et al*., 2008): In one ml of extract solution, add 2 ml of glacial acetic acid. In the mixture, few dropps of ferric chloride was added. Then, 1 ml of H_2SO_4 was added along the side of the test tube. The formation of brown ring at the interface indicated the presence of glycosides.

x) Test for phlobatannins (Harborne, 1998)

1 ml of each extract was treated with few drops of 1% HCL and boiled in the water bath. The formation of reddish precipitate confirmed the presence of phlobatannins.

(xi) Test for balsams

Four ml of each test extract was treated with 3 drops of alcoholic ferric chloride. The mixture was warmed. The development of dark green colouration indicated the presence of balsams.

(xii) Test for volatile oil

A small quantity of each extract was mixed with dilute NaOH and added 0.1M HCl was added. The appearance of white precipitate confirmed the presence of volatile oils.

(xiii) Test for resins

Acetone-water test: Two ml of each extract was treated with acetone and small amount of water was added and shaken. Appearance of turbidity indicated the presence of resins.

3.3.3 Quantitative phytochemical analysis

3.3.3.1 Estimation of total phenolic content (Vabkova and Neugebauerova, 2013)

The total phenolic content was estimated using the modified Folin-ciocalteu photometric method. The appropriate amount of filtered plant 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 Gallic Acid (GA) Equivalents per gram of dry weight.

3.3.3.2 Estimation of total flavonoid content (Vabkova and Neugebauerova, 2013)

Total flavonoid content was measured using a modified colorimetric method. The appropriate amount of plant 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 Quercetin. The flavonoid content was expressed as mg Quercetin Equivalents (QE) per gram of dry weight (dw).

3.3.3.3 Estimation of total tannin content (Schanderi, 1970)

Estimation of total tannin content in leaf and stem bark of *C. mala-elengi* was determined by Folin-Denis method with minor modification. Stock solution of plant extract was prepared to the concentrated of 1mg/ml. To 0.1 ml of each extract, one ml of distilled water was added and then mixed with 0.5 ml of Folin-Denis reagent. The reaction mixture was alkalinized by the addition of 1 ml of 15% (w/v) sodium carbonate solution and kept in dark for 30 minutes at room temperature. The absorbance of the solution was read at 700 nm using spectrophotometer, and the concentration of tannin in the extract was determined using pure tannic acid as standard (1 mg/ml). A calibration curve was generated using various concentrations of tannic acid (10-50 µg/ml) was obtained. Blank consisting of all the reagents, except for the extract of standard solution is substituted with 0.1 ml of water.

3.3.3.4 Estimation 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 Bromo Cresol Green (BCG). 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. The values were expressed as Atropine equivalents (mg AE/gm extract) derived from standard curve. All experiments were performed thrice; the results were averaged and reported in the form of mean ± SEM.

3.3.3.5 Estimation of total saponin content (Senguttuvan *et al*., 2014)

Estimation of total saponin content was determined by the method described based on vanilla-sulphuric acid colorimetric reaction. About 50 µl of plant extract was added with 250 µl of distilled water. To this, about 250 µl of vanilla reagent (800 mg of vanillin in 10 ml of 99.5% ethanol) was added. Then, 2.5 ml of 72% sulphuric acid was added and it was mixed well. This solution was kept in a water bath at 60°C for 10 minutes. After 10 minutes, it was cooled in ice cold water and the absorbance was read at 544 nm. The values are expressed as Diosgenin Equivalents (mg DE/gm extract) derived from a standard curve.

3.4 Antioxidant activity

Oxidation is one of the most important processes, which produce free radicals in food, chemicals and even in living systems. Free radicals have an important role in processes such as food spoilage, chemical materials degradation and also contribute to more than one hundred disorders in humans.

Antioxidants are defined as substances that even at low concentration significantly delay or prevent oxidation of easily oxidizable substrates. The applications of antioxidants are industrially widespread in order to prevent polymers oxidative degradation, auto oxidation of fats, synthetic and natural pigments discoloration, etc. There is an increased interest of using antioxidants for medicinal purposes in the recent years.

3.4.1 DPPH free radical scavenging activity (Patel Rajesh and Patel Natvar, 2011)

Preparation of standard solution

Required quantity of ascorbic acid was dissolved in petroleum ether, chloroform, ethyl acetate, ethanol and water to give the concentration of 10, 20, 30, 40 and 50 µg/ml.

Preparation of test sample

Stock solution of samples were prepared by dissolving 10 mg of dried leaf and stem bark extract in 10 ml of petroleum ether, chloroform, ethyl acetate, ethanol and water to give concentration of 1mg/ml.

Preparation of DPPH solution

4.3 mg of DPPH was dissolved in 3.3 ml petroleum ether, chloroform, ethyl acetate, ethanol and water and it was protected from light by covering the test tubes with aluminium foil.

Protocol for estimation of DPPH scavenging activity

- 150 µl DPPH solution was added to 3 ml of petroleum ether, chloroform, ethyl acetate, ethanol and water extracts and absorbance was taken immediately at 516 nm for control reading.
- Different volume levels of test sample (10, 20, 30, 40 and 50 µl) were screened and made 200 µl of each dose level by dilution with 3 ml petroleum ether, chloroform, ethyl acetate, ethanol and water
- 150 µl DPPH solution was added to each test tube.
- Absorbance was taken at 516 nm in UV-visible spectrophotometer (Shimadzu, UV-1700, Japan) after 15 min using methanol as a blank.
- The % reduction and IC_{50} were calculated as follows
- The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation:

$$\% \text{ antiradical activity} = \frac{\text{Control absorbance - Sample absorbance}}{\text{Control absorbance}} \times 100$$

3.4.2 Nitric oxide scavenging activity (Sakat *et al*., 2010)

50 µl of each of the concentrations of petroleum ether, chloroform, ethyl acetate, ethanol and water of leaf and stem bark extracts dissolved in dimethyl sulfoxide, as well as ascorbic acid (standard compound) were taken in separate tubes and the volume was uniformly made up to 150 µl with petroleum ether, chloroform, ethyl acetate, ethanol and water extracts. To each tube 5 ml of sodium nitro prusside (5 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 minutes.

The similar procedure was repeated with petroleum ether, chloroform, ethyl acetate, ethanol and water as blank which served as control. After the incubation, 5 ml of griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrophotometer. Ascorbic acid was used as reference standard. The IC_{50} value for each test compounds as well as standard preparation were calculated.

$$\textbf{\% Scavenging / Reduction} = \frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

3.4.3 Hydrogen peroxide scavenging activity (Ruch *et al*., 1989)

Sample extract at concentrations of 10-50 µg/ml was determined using a Hydrogen peroxide solution. Hydrogen peroxide solution (2 mmol/L) was prepared in phosphate buffer (pH-7.4). The various concentrations of extract (petroleum ether, chloroform, ethyl acetate, ethanol and water)/ascorbic acid (10, 20, 30, 40 and 50 µg/ml) was mixed with hydrogen peroxide solution (0.6 ml). The absorbance of hydrogen peroxide was measured at 230 nm was estimated exactly after 10 minutes against a reagent blank solution containing phosphate buffer (3.3 ml) except hydrogen peroxide solution and compared to ascorbic acid which was the reference standard.

3.4.4 Superoxide radical scavenging activity (Nishimiki *et al*., 1972)

One ml of Nitro Blue Tetrazolium (NBT) solution (100 µM) NBT in 100 mM Phosphate buffer (pH 7.5), Add 1 ml NADH solution (300 µM in 100 mM phosphate buffer, pH 7.5) and 1 ml of petroleum ether, chloroform, ethyl acetate, ethanol and water extracts of leaf and stem bark extract of *C. mala-elengi* dissolved in respective solvents (10-50 µg/ml). Then, the reaction was started by adding 100 µl of Phenazine Metho Sulphate (PMS solution) (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the reaction mixture. Then, the reaction mixture was incubated at 25˚C for 5 min and the absorbance was measured at 560 nm against blank. Ascorbic acid was used as reference standard.

3.4.5 Total antioxidant capacity (Prieto *et al*., 1999)

Total antioxidant activity was estimated by Phosphomolybdenum assay. Preparation of Molybdate reagent solution 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were added in 20 ml of distilled water and made up volume to 50 ml by adding distilled water. Petroleum ether, chloroform, ethyl acetate, ethanol and water extracts of leaf and stem bark of *C. mala-elengi* in different concentration ranging from 10 – 50 µl were added to each test tube individually containing 3 ml of distilled water and 1 ml of Molybdate reagent solution. These tubes were kept incubated at 95˚C for 90 minutes. After incubation, these tubes were normalized to room temperature for 20-30 minutes and the absorbance of the reaction mixture was measured at 695 nm. Mean values from three independent samples were calculated for each extract. Ascorbic acid was used as standard.

3.4.2.1 Antimicrobial activity

In recent years**,** there has been an increasing awareness about the importance of medicinal plants. Drugs from these plants are easily available, inexpensive, safe, efficient and rarely accompanied by side effects. Plants which have been selected for medical use over thousands of years constitute the most obvious starting point for new therapeutically effective drugs such as anticancer drugs and antimicrobial drugs. Recently, medicinal plants usage has increased in spite of the advance made in the field of chemotherapy. The medicinal plants have been used as materials for the extraction of active pharmacological agents.

Screening of antibacterial activity

Bacteria tested

Antimicrobial activity of extracts were tested against three gram-positive bacteria such as *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus*, two gram negative bacteria such as *Citrobacter freundii* and *Klebsiella pneumoniae*. Antifungal activity was determined against three fungal pathogens *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. These isolates were obtained from the Microbiology laboratory, PSG Institute of Medical Sciences & Research, Coimbatore, Tamilnadu, India. The stock

cultures of bacteria were maintained on Nutrient Agar slants and fungi on Potato Dextrose Agar slants and stored at 4˚C.

Preparation of inoculum

Stock cultures were maintained at 4˚C on slopes of Nutrient agar. Composition of Nutrient agar medium for bacterial culture are Peptone – 5 gram, beef extract - 3 gram, Sodium chloride - 5 gram, Agar- 15 gram, Distilled water- 1000 ml, pH (at 25˚C) at 7.4± 0.2. Active cultures of experiments were prepared by transferring a loopful of cells from the stock cultures to test tube of Nutrient Agar medium for bacteria that were incubated without agitation for 24 hours at 37˚C. Potato Dextrose Agar medium was used for fungal cultures. Composition of PDA medium for fungal culture are Potato- 200 gram, Dextrose- 20 gram, Agar- 20 gram and Distilled water- 1000 ml, pH 5.6. Active cultures of experiments were prepared by transferring a loopful of cells from the stock cultures to test tube of PDA medium for fungal that were incubated. Chloramphenicol acts as a selective agent to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

Agar well diffusion method (Olurinola, 1996)

Agar well diffusion method was used to screen the antibacterial and antifungal activities of leaf and stem bark of *C. mala-elengi* using different solvent extracts such as petroleum ether, chloroform, ethyl acetate, ethanol and water. One ml of fresh bacterial or fungi culture was pipetted in the center of sterile petri dish. NA for bacteria and PDA for fungi poured into the petri dish containing the inoculum and mixed well. Upon solidification, well were made using a sterile cork borer into agar plates containing inoculums. Then, 100µl of each extract (20% w/v) was added to respective wells. The concentration of extracts (20% w/v) has been selected based on our previous literature. The plates were placed in the refrigerator for 30 minutes to let the extracts diffusion well into the agar. Then, the plates were incubated at 37˚C for 18-24 hours for bacterial pathogens and 28˚C for 48 hours for fungal pathogens. The diameter (mm) clear zone of inhibition was recorded and the experiment was repeated thrice for each replicates the reading were taken in three different fixed direction and average values were observed. Standard fluconazole was used as positive control for fungi. Ampicillin was used as positive control for bacteria. DMSO at a concentration of 10% was employed as a negative control.

3.5.1 FT-IR analysis (Cakmak *et al.,* 2006)

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional group) present in compounds. The wavelength of light absorbed is the characteristic of the chemical bond which can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Ethanol leaf and stem bark extract of *C. mala-elengi* were used for FTIR analysis. 0.2 grams of the dried extract was passed through optical area of ATR (Attenuated Total Reflectance) in FTIR Spectrophotometer by KBR disk method. The extract was grounded, mixed well with KBR and compressed using IR compression machine and then scanned the spectra range between 4000 to 400 cm^{-1}

3.5.2 GC-MS analysis (Ganesh and Vennila, 2011)

GC-MS plays a key role in the unknown components of plant origin. 1 µl of the ethanol extracts of the leaves and stem bark of *C. mala-elengi* was determined Shimadzu GC-MS with QP2010S model equipped with RXi-5sil MS Column (30 meter length× 250 µm in diameter × 0.25 µm in thickness film). Spectroscopic detection by GC-MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with the flow rate of 1ml/min, sampling time - 2.00 min. The initial temperature was set at 70˚C to 280˚C with increasing rate of 30˚C/min and holding time of about 15 min, Pressure-61.3 kpa, injection volume-0.20 µl column over temperature 70.0˚C, Column flow -1.00 ml/min, purge flow - 3.0 ml/min, Total flow - 54.0 ml/min, Flow control mode was linear velocity (36.7˚ cm/sec) was used. The ethanol leaf and stem bark extracts, diluted with ethanol was injected in asplit less injection mode. Relative quantity of the chemical constituents present in the ethanol leaf and stem bark extract of *C. mala-elengi* was expressed as percentage based on peak area produced in the chromatogram. The eluted component is detected in the mass detector and the components were compared with components stored in the library.

Identification of phytocomponents

Identification of chemical constituents was determined based on GC retention time on RXi-5silMS Column and matching of the spectra with computer software data of standards compared with NIST11 & WILLE8 Library. The GC-MS analysis was determined

at Kerala Forest Research Institute, Thrissur District, Kerala (State). The name, molecular weight, molecular formula and area under peak of the components of the test materials, Retention time (minutes), nature of the compound and chemical structure of the constituents were ascertained.

3.6 *In vitro* antidiabetic activity

Traditional anti-diabetic plants might provide new oral anti-diabetic compounds, which can counter the high cost and poor availability of the current medicines for many rural populations in developing countries. Plant drugs are frequently considered to be less toxic and free from side effects than synthetic ones. In India, indigenous remedies have been used in the treatment of diabetes mellitus since the time of Charaka and Sushruta. The World Health Organization (WHO) has listed 21,000 plant species which are used for medicinal purposes around the world. Among these, 2500 species are in India. India is the largest producer of medicinal herbs endowed with a wide diversity of agro climatic conditions and is called as botanical garden of the world. Pharmacological and clinical trials of medicinal plants have shown anti-diabetic effects and repair of β-cells of islets of Langerhans.

3.6.1 α-amylase inhibitory activity (Worthington, 1993)

Reagents

1. 1.0.02M Sodium phosphate buffer (pH 6.9)
2. 2.0.006M NaCl
3. 3.1% Starch
4. 4.Dinitrosalicylic acid

Procedure

Different concentrations of the leaf extract (25-200 µg/ml) and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic α-amylase enzyme (0.5 mg/ml) were incubated at 25°C for 10 min. After the incubation, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to the reaction mixture. Subsequently, the reaction mixture was incubated

25°C for 10 minutes, followed by addition of 1.0 ml of Di Nitro Salicylic Acid (DNSA). Finally the reaction was stopped by incubation in boiling water for 5 minutes and cooled to room temperature. The reaction mixture was diluted with 10 ml distilled water and the absorbance was measured at 540 nm in a spectrophotometer. The mixture of all other reagents and the enzyme except the sample was used as a control. The α-amylase inhibitory activity was expressed as percentage of inhibition.

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{(\text{Control})} - \text{Abs}_{(\text{Sample})}}{\text{Abs}_{(\text{Control})}} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of α-amylase activity under assay condition.

3.6.2 α-glucosidase inhibitory activity (Apostolidis *et al*., 2007)

Reagents

1) α-glucosidase (0.5 mg/ml)
2) 0.1M phosphate buffer (pH 6.9)
3) 5 M p-nitrophenyl-α-D-glucopyranoside

Procedure

Various amounts of leaf extracts (25-200 µg/ml) and 100 µl of α-glucosidase (0.5 mg/ml) in 0.1 M phosphate buffer (pH 6.9) solution were incubated at 25°C for 10 minutes. Then, 50 µl of 5 M p-nitrophenyl-α-D-glucopyranoside in 0.1 M Phosphate buffer (pH-6.9) solution was added. Reaction mixture was incubated at 25°C for 5 minutes and the absorbance was taken at 405 nm by a spectrophotometer. The mixture of all other reagents and the enzyme except the sample was used as control and the results of α-glucosidase inhibitory activity were expressed in terms of inhibition. The percentage α-glucosidase inhibitory activity is calculated by the following formula

$$\text{Inhibition (\%)} = \frac{(\text{AbS}_{\text{control}} - \text{AbS}_{\text{sample}})}{\text{AbS}_{\text{control}}} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of α-glucosidase activity under assay condition.

3.7 *In vitro* cytotoxicity assessment

Determination of mitochondrial synthesis by MTT assay (Denizot and Lang *et al*., 1986)

Cell culture

The Human liver cancer (Hep G_2) was procured from the National Center for Cell Sciences (NCCS), Pune, India. The selected cancer cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM 1-glutamine and Balanced Salt Solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential aminoacid, 1 mM sodium pyruvate, 2 mM 1-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum. Penicillin and streptomycin (100 IU/100 µg) were adjusted to 1 ml/L. The cells were maintained at 37˚C with 5% CO_2 in a humidified CO_2 atmosphere.

Morphological study

The selected Human cancer cells that were grown on cover slip (1×10^5 cells/cover slip) were incubated with extract at different concentration and they were then fixed in an ethanol: acetic acid solution (3:1, v/v). The cover slip was gently mounted on glass slides for morphometric analysis. Three monolayers per experimental group were micrographed. The morphological changes of the cells were analysed using Nikon (Japan) bright field inverted light microscopy at 40 x magnification.

Materials

1. Cell lines: HELA Human cervical cancer cell line (From NCCS, Pune)
2. Cell culture medium: DMEM High Glucose
3. Adjustable multichannel pipettes and a pipettor

4. Fetal Bovine Serum
5. MTT Reagent (5 mg/ml)
6. Dimethylsulfoxide
7. Camptothecin
8. Dulbecco's phosphate buffered saline
9. 96-well plate for culturing the cells
10. T25 flask
11. 50 ml centrifuge tubes
12. 1.5 ml centrifuge tubes
13. 10 ml serological pipettes
14. 10 to 1000 µl tips

Equipements:

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

Assay controls

1. 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 25 µM of Camptothecin

Steps followed

1. Seeded 200 µl 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 2 hours
2. Added appropriate concentrations of the test agent
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 incubation and removed spent media and added MTT reagent to a final concentration of 0.5 mg/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 culture
9. Read the absorbance on a spectrophotometer or an ELISA at 570 nm and 630 nm used as reference wavelength
10. The IC_{50} value was determined by using linear regression equation i.e y=mx+c. HereY=50,

M and C values were derived from the viability graph.

$$\text{Cell Viability (\%)} = \frac{\text{OD value of experimental sample}}{\text{OD value of experimental control}} \times 100$$

3.8 *In vivo* pharmacological study

3.8.1 Acute toxicity study (Seth *et al*., 1972)

Experimental animals

Swiss albino mice (20-25 gm) were obtained from Animal house, IRT (Information Resources and Technology) Perundurai Medical College, Perundurai, Erode (District), Tamilnadu (State), India. The mice were randomly grouped in polypropylene cages with paddy husks as bedding in a proper environmental situation at a temperature of 24±2˚C and relative humidity 30-70%. 12:12 light: day cycle was followed. All animals fed with

standard commercial pelleted rat chaw (M/S Hindustan Lever Ltd, Mumbai) and allowed free access to purified drinking water.

Method

Acute toxicity studies were determined as per Organization of Economic and Cooperative Development (OECD) guidelines No 423, six groups such as 5, 30, 100, 300, 1000 and 2000 mg/kg of fasted healthy mice (Three per group) was administered by random sampling technique. Swiss mice were kept fasting for 4h. They were also provided with purified drinking water. The ethanol leaf extract of *C. mala-elengi* was orally administered as a single dose at the concentration of 5 mg/kg initially and mortality if any was recorded for 3 days. If mortality was noticed in two out of three animals the dose administered was assigned as toxic dose. However, if the mortality was recorded in one animal out of three animals then the same dose was repeated again to confirm the harmful effect. If no mortality was noticed at the higher doses such as 300, 1000, 2000 mg/kg of the ethanol leaf extract of C. *mala-elengi* were employed for further toxicity studies.

3.8.2 Wound healing activity (Kiranmai *et al*., 2011)

Preparation of formulation

10 g and 20 g of dried extract was mixed with simple ointment base to obtain 10% and 20% of ethanol leaf extract of *C. mala-elengi* ointment respectively.

Experimental animals

Male wistar albino rats weighing between 150-220 g were used for this study. On arrival animals were placed randomly and the animals were maintained in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2˚C, humidity of 30-70% and 12:12 light: dark cycle was followed. Animals were allowed to standard diet and water. Animals were acclimatized to the laboratory conditions one week before the study. Animals were fasted overnight before the experiment. Experimental procedure was reviewed by the Institutional Animal Ethical Committee (IAEC) Reg. No. (NCP/IAEC/2019-20/08).

Excision wound

An excision wound was perpetrated by cutting away approximately 500 mm^2 full thickness of assigned area on the anterior – dorsal side of each rats under pentabarbitone (30 mg/kg i.p) anaesthesia. The rats wounds were left undressed to the open environment. This model was used to observed wound contraction and epithelialization time. Animals were closely monitored for any infection and were separated or excluded from the study and replaced. Totally 24 animals were used in this study. The rats were divided into 4 groups of 6 animals each. Excission wound was inflicted in all the rats of 5 groups.

Group I - Animals were treated with simple ointment (negative control)

Group II - Animals were treated with standard Povidone iodine (Positive control) ointment

Group III and Group IV- Animals were treated with 10% and 20% of *C. mala-elengi* ethanolic leaf extract simple ointment base respectively.

All the test drugs were applied topically on the inflicted wound, twice everyday for 12 days. The percentage of wound closure rate was calculated by following formula

$$\% \text{ of Wound closure} = \frac{\text{Wound area on day zero} - \text{Wound area on day } n^{th}}{\text{Wound area on day Zero}}$$

3.8.3 Hepatoprotective activity (Sreedevi *et al*., 2009)

Experimental animals

Male wistar albino rats weighing between 150-220 gram were used in this study. They were maintained in polypropylene cages with paddy husk as bedding at a temperature of 24±2˚C and relative humidity of 30-70% with 12:12 light and dark cycle. Animals were allowed to standard diet and water *ad libitum*. Animals were acclimatized to the laboratory conditions one week before the study. Animals were fasted overnight before the experiment. Experimental procedure was reviewed by the Institutional Animal Ethical Committee (IAEC) Reg. No. (NCP/IAEC/2019-20/08).

Induction of paracetamol induced hepatotoxicity

The animals were divided into five groups of 6 animals each. They should be treated for fourteen days as follows:

Group I - Normal control rats (normal saline 5 ml/kg)

Group II - Distilled water + paracetamol 2 g/kg bw orally (negative control)

Group III - Standard drug silymarin (50 mg/kg day p.o) positive control

Group IV - Ethanol leaf extract (250 mg/kg/day respectively + paracetamol (2 g/kg bw, orally)

Group V - Ethanol leaf extract (500 mg/kg/day respectively+ paracetamol (2 g/kg bw, orally)

All the wistar rat animals were treated as shown above for a period of 14 days. Saline, silymarin and ethanol leaf extract were orally administrated once a day. During this time of study rats were maintained under normal diet and water. On the 15^{th} day, Paracetamol (2 g/kg p.o) was orally administrated to all the groups except group I. The blood samples were collected directly through retro orbital plexus under ether anesthesia for estimation of biochemical parameters such as SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase), ALP (Alkaline Phosphatase), Bilirubin, Urea and Creatinine were determined by using Merck analyzer kit.

3.8.4 Histopathological studies (Luna, 1999)

Isolation of liver

The animals sacrificed were carotid bleeding and the livers were dissected out. The isolated liver was cut into small pieces of about 5 mm in size. Then, the livers were washed in normal saline and fixed in neutral formalin (10% solution) for 3 days. Standard method for the fixation of liver sections were followed. Liver pieces were cleaned in running water for about 12 hours. This was followed by dehydration with alcohol of increasing strength (70%, 80% and 90%) for 12 hours each. Final dehydration was done using absolute alcohol with about 3 changes at 12 hours interval. Cleansing was carried out

by using xylene with changes at 15-20 minutes interval. After washing, the pieces were subjected to paraffin infiltration in automatic tissue processing unit. The pieces were cleaned with running water to remove formalin completely.

Embedding in paraffin

Hard paraffin was melted and poured into L-shaped blocks. The liver pieces were then dropped into the liquid paraffin quickly and allowed to cool.

Sectioning

The blocks were cut with the help of microtome to get sections of thickness of 5 microns. The sections were fixed on a glass slide using egg albumin and allowed to dry.

Staining

Eosin and hematoxylin, the basic stains were used for staining the liver sections.

Experimental procedure

- The sections were deparaffinized by washing with xylene for about 15 minutes.
- The sections were dehydrated by washing in alcohol of decreasing strengths (100%, 90%, 80% and 70%)
- The sections were stained with hematoxylin for 15 minutes and were rinsed in tap water
- The sections observed under microscope showed that the nuclei were distinct and the background was very light or colourless.
- The slide was washed in tap water
- The section was then dipped in ammonia water until it became bright blue by 3 to 5 dips
- The slide was washed in running tap water for 10 to 20 minutes.
- The slide was then stained with eosin for 15 sec to 2 min, depending on the percentage of the eosin and depth of the counter stain desired. For even staining,

the slides were dipped several times in ammonium water before allowing them to set in the eosin for the desired time.

Statistical analysis

The results of all the *in vitro* experiments are expressed as mean ±SE. Statistical significance of one way analysis of variance (One way ANOVA) using SPSS (Statistical Package for Social Sciences) version 17.0 and the individual comparison was done by DMRT (Duncan's Multiple Range Test. Significant difference between the groups were represented by the value $p<0.05$. All the results on *in vivo* studies were expressed as Mean ± Standard Deviation (SD). Data was analyzed using One way ANOVA followed by Dunnett's t-test. The analysis was carried out using Graph pad software. Significant difference between the groups were represented by the value $p<0.05$.