MATERIALS AND METHODS

Materials

The leaves and roots of the *Embelia ribes* Burm.f. and *Chonemorpha fragrans* was collected from Kakkayam, Kozhikode, Kerala and the plant material was identified by the experts at M. S. Swaminathan Research Foundation, Wayanad, Kerala and also by literature survey (Plate 1 & Plate 2).

Fixing of Plant Material

The fresh leaf and root of the *Embelia ribes* and *Chonemorpha fragrans* was cut and fixed in FAE (Formalin – 5 ml+Acetic acid 5 ml + 70% Ethyl alcohol 90 ml). *Chonemorpha* roots were boiled before the process as it was very hard. After 24 hrs. of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning and Staining

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the section was 10-12 μm. The sections were stained with Fluroglucinol. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc.

Photomicrograph

Photographs of different magnifications were taken with Photo microscopic unit. For normal observation, 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.
Preparation of Plant Powder

The aerial part of *Embelia ribes* (Leaf) and the underground part of *Chonemorpha fragrans* (Root) was collected and washed thoroughly. After that the leaf and root was cut into pieces and shade dried at room temperature (25-30°C), for about two weeks. The dried material was ground to fine powder for further analyses.

Fluorescence analysis

The fluorescence analysis of the powdered drug was done according to the methods described by Chase and Pratt (1949). Fine powder and their extracts were examined under visible light and UV light. These powdered materials were also treated with various reagents such as 50% HNO₃, acetone, ethyl alcohol, 50%, H₂SO₄ 1N HCl and 1N aqueous NaOH and changes in color were recorded.

Quantitative Determination of Physico-Chemical Characteristics

The percentage of loss of weight on drying, total ash, water soluble ash, acid insoluble ash, water soluble extractive value, sulphated ash, residue on ignition, moisture content were obtained by employing standard methods of analysis as described in *Pharmacopoeia of India* (Anonymous, 1996).

Determination of total ash

5 gm of dried leaf and root powder was taken in a previously weighed silica crucible and ignited carefully not exceeding dull red heat, until the ash was free from carbon. The crucible was cooled and weighed. The percentage of ash with reference to the air dried plant was calculated.
Determination of water soluble ash

A known weight of the ash was boiled with 25 ml of distilled water. The insoluble matter was collected in a previously weighed sintered crucible. The crucible was washed with water, dried to constant weight and weighed. The percentage of water insoluble ash with reference to the air dried leaf and root was calculated.

Determination of acid – insoluble ash

A known weight of the ash was boiled in 25 ml of dilute HCl. The insoluble matter was collected in a previously weighed sintered crucible, washed with hot water, dried to attain constant weight. The percentage of acid insoluble ash with reference to air dried leaf and root was calculated.

Water soluble extractive value

500 mg of accurately weighed coarsely powdered leaf and root was separately macerated with 45 ml of chloroform and 5 ml of water in Stoppard flask for 24 hours. The filtrate is evaporated in 25 ml of alcoholic extract to dryness at 105°C and weigh. Percentage of water with reference to air dried sample was calculated.

Determination of Sulphated ash

1 gm of dried leaf and root powder was taken in a previously weighed silica crucible and moistened with H₂SO₄. It was ignited gently again. Again it was moistened with H₂SO₄, reignited, cooled and weighed. The percentage of sulphated ash was calculated with reference to the air dried drug.

Phytochemical analysis

Extraction

Mature and healthy plants were collected and washed thoroughly. Both part of the plant (leaf and root) was cut into pieces and was shade dried at room temperature (25-30°C), for about two weeks. The dried plant was ground to fine powder. About 30 gm of plant
powder was taken in a digestion flask fitted to the Soxhlet apparatus and extracts were obtained successively with petroleum ether, benzene, chloroform and ethanol. The aqueous extract was prepared directly by boiling the powder with distilled H₂O. These extracts were concentrated and kept in brown bottles for further use.

All the solvents were used based upon their increasing polarity index. The extracts were evaporated to dryness on a water-bath. The plant extracts were off with distillation apparatus and yielded quantities of (leaves and root) extracts in different solvents were obtained and were further taken to evaluate the Phytochemical studies. (Greenlee, 2007).

**Phytochemical Screening**

The plant extracts were tested for the presence of bioactive compounds such as terpenoids, alkaloids, glycosides, steroids, phenols, tannins, flavonoids and saponins by standard methods of Yadav and Agarwala, 2011 and Imron et al., 2012 (Table 1).

**Quantitative Estimation of Phytochemicals**

**Estimation of Total Carbohydrates by Anthrone Method** (Arnon, 1949)

**Procedure**

1. 100 mg of the plant sample was hydrolyzed in a boiling water bath for 30 min with 5 ml of 2.5 N HCl.
2. After that it was cooled at room temperature and it was neutralized with solid sodium carbonate, until effervescence cease.
3. The volume was made up to 10 ml and centrifuged at 3000 rpm for 10 min.
4. The supernatant was collected, from this 0.5 ml and 1 ml aliquots were taken.
5. The tubes were cooled in ice and 4 ml of anthrone reagent was added and the tubes were kept in a boiling water bath for 8 min.
6. The tubes were cooled at room temperature and also the blank was prepared.
7. The O.D was read at 630 nm and the standard graph was constructed and the amount of carbohydrates present in the given sample was calculated using standard graphs.

**Estimation of Total Protein**

The procedure of Oliver *et al.*, (1951) was followed.

**Reagents**

- Phosphate Buffer (pH 7.2)
  a) KH$_2$PO$_4$ = 13.6 gm dissolved in 500 ml dis. H$_2$O
  b) KOH = 5.6 gm dissolved in 100 ml dis. H$_2$O
  Mix (a) and (b) and adjusting the pH to 7.2
- 10% TCA (Trichloroacetic acid)
  10 g TCA dissolved in 100 ml dis. H$_2$O
- 0.2 N NaOH: 800 mg NaOH dissolved in 100 ml dis. H$_2$O
- Alkaline Copper Reagent
  A - Alkaline Sodium Carbonate
    (2 g Na$_2$CO$_3$ dissolved in 100 ml of 0.1N NaOH)
  B - Copper Sulphate and Sodium Potassium Tartarate
    (500 mg CuSO$_4$ dissolved in 100 ml of 1% Sodium Potassium Tartarate)
- Folin - Phenol
  Commercially available Folin-Phenol was used. It was diluted to 50% using distilled water just prior to use.

**Procedure**

1. 100 mg of the plant materials were taken and homogenized in a mortar separately with ice-cold phosphate buffer (pH 7.2) in an ice bath.
2. The homogenate was filtered through three layered muslin cloth and centrifuged at 3000 rpm for 5 minutes.
3. To the supernatant, 10% ice-cold TCA was added in the ratio 1:1 and left for 30 minutes in ice bath. Precipitated protein was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded.

**Estimation**

The protein precipitate was dissolved in 2 ml of 0.2 N NaOH. Form that 0.2 ml of aliquot was taken along with 2.5 ml of alkaline reagent and 0.5 ml Folin phenol reagent. The final volume was made up into 5 ml using distilled water. After the development purple colour, the O.D was taken at 650 nm and the total protein was estimated using standard graph.

**Estimation of Lipids**

**Procedure**

1. 5 gm of leaf and root are weighted and homogenized using a mortar and pestle with 10 ml of methanol: Chloroform mixture.

2. The homogenate was centrifuged at 5000 rpm for 10 minutes. The supernatant was collected.

3. To the supernatant 2 drops of 0.005 N KCl was added. It was thoroughly mixed and allowed to stand for 15 minutes.

4. The bottom lipid layer was collected in a preweighed beaker and the solvent was evaporated by keeping it on water bath.

5. After the chloroform odor was completely removed the beaker was cooled and reweighted with lipid.

6. The percentage of the lipid was calculated using the following formula,

\[
\text{Percentage of Lipids} = \left( \frac{\text{Weight of the lipid}}{\text{Weight of material}} \right) \times 100
\]
Estimation of Chlorophyll

The method of Arnon (1949) was followed.

Reagents

Acetone – 80%

Procedure

1. 50mg of plant material was ground in a mortar and pestle with a pinch of CaCO₃. 10 ml of 80% ice cold acetone was added and once again ground till the last chlorophylls came out from the tissues completely.

2. The homogenate was centrifuged at 3000 rpm for 10 min. The clear green solution was made up to 5 ml using 80% acetone.

3. Absorbance of the extract was measured at 663 nm and 645 nm respectively.

4. The amount of pigments was calculated using following formula.

   \[
   \text{Chl-a (mg/g)} = (12.7 \times A_{663}) - (2.69 \times A_{645}) \frac{V}{1000} \times W
   \]

   \[
   \text{Chl-ab (mg/g)} = (22.9 \times A_{645}) - (4.68 \times A_{663}) \frac{V}{1000} \times W
   \]

   \[
   \text{Total Chl (mg/g)} = (20.2 \times A_{645}) - (2.69 \times A_{645}) \frac{V}{1000} \times W
   \]

   Where, \( V \) = Volume of the extract (ml)

   \( A \) = Absorbance at respective wavelength

   \( W \) = Weight of the material in gram

   The results were expressed in mg/g tissue on fresh weight basis.

Estimation of Carotenoids

The method of Mancinelli et al., (1975) was followed

Reagents

Acetone – 80%
Procedure

50mg of fresh plant materials (leaf and root) of *E. ribes* and *C. fragrans* were ground separately in a mortar with 80% ice-cold acetone, till the residue become pale green in colour. The extract was centrifuged at 3000 rpm for 10 minutes. Absorbance of the supernatant was measured at 480 nm, 645 nm and 663 nm and the carotenoids content was calculated using the following formula.

\[
\text{Carotenoids mg/g/fw} = (A_{480} + 0.1145) \times (A_{645} - 0.6377) \times A_{663}
\]

Estimation of Total Phenols

The procedure of Farkas and Kiraly (1962) was followed

Reagents

80% ethanol, Folin phenol, 20% Na$_2$Co$_3$

Preparation of Reagents

Folin Phenol

Folin - phenol has freshly prepared with equal amount of water.

20% Na$_2$Co$_3$ (20g Na$_2$Co$_3$ dissolved in 100 ml of water)

Extraction

1. 100 mg of plant material was homogenated with 10 ml of 80% ethanol.
2. The homogenate was centrifuged at 3000 rpm for 5mins and the collected supernatant was made up to known volume.
3. The aliquot was taken from the supernatant and 0.5 ml Folin- phenol reagent and 1 ml of 20 % of Na$_2$Co$_3$ were added. Final volume was made up to 5 ml with distilled water.
4. The mixer was shaken well and kept in a boiling water bath for a minute. Then the tubes were cooled in a running tap water and the resulting blue coloured was measured at 630 nm against blank.
Estimation of Total Free Amino acids (Moore and Stein, 1948)

Reagents

0.2 M Sodium Acetate Buffer (pH 5.5)

(Sodium acetate: 0.2M – 16.4g of anhydrous Sodium acetate in 100 ml of distilled water. 0.2M Acetic acid: 11.55 ml amino acid/1000 ml of distilled water, adjust pH to 5.5).

Ninhydrin Reagent

(2gms of Ninhydrin in 25 ml Methyl cellosolve). Add 25 ml of 0.2M Acetate buffer (pH 5.5) store in a brown bottle).

80% Ethanol

50% Ethanol

Extraction

1. 500 mg of the plant materials were taken and homogenized in a mortar separately with 5 to 10 ml of 80% ethanol.

2. The homogenate was filtered through muslin cloth and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected.

Estimation

1. 0.1 ml of aliquot was taken in a test tube and 1 ml of Ninhydrin solution was added.

2. The final volume of the test tube was made up to 2 ml and kept in a boiling water bath for 20 min.

3. The solution was diluted with 5 ml using distilled water. After 15 min the intensity of the purple color was measured against blank at 540 nm.
Estimation of Total Flavonoids

The procedure of Boham and Kocipal-Abyazan (1974) was followed.

Reagents

Methanol – 80%

Procedure

1. 10 gm of the powered plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature.

2. The whole solution was filtered through Whatman filter paper No.42 (125 nm).

3. The filtrate was later transferred into a previously weighed China dish and evaporated to dryness over a water bath.

4. The final residue was weighed.

Calculation:  Total Flavonoid = Final weight – Initial weight

Estimation of Total Alkaloids

The procedure of Harbone (1973) was followed.

Regents

Acetic Acid – 80%

Conc. Ammonium Hydroxide

Ethanol

Procedure

1. 5 gm the powered plant sample was taken in a 250 ml beaker and 200 ml of 10% acetic acid and ethanol was added.

2. The beaker was covered and allowed to stand for 4h.

3. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume.
4. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete.
5. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered.
6. The final residue was dried and weighed.

**Estimation of Total Oxalate**

The procedure of Day and Underwood (1986) was followed

**Reagents**

1.5 N H$_2$SO$_4$

**Procedure**

1. 3g of powdered sample was taken in a boiling tube.
2. To this 10ml of 1.5 N H$_2$SO$_4$ was added, the mixture was filtered and dried in a china dish.
3. The dried sample was weighted
4. Total oxalate was calculated from weight differences of china dish with dried sample and without sample.

**Estimation of Tannin**

**Reagents**

Folin Denis reagent, Sodium Carbonate solution

**Procedure**

1. 0.5g of powdered material was taken and transferred to a conical flask containing 75ml water. The flask was gently heated and boiled for 30 min.
2. The extract was centrifuged at 2000 rpm for 20 min and the supernatant was collected and made up to known volume.
3. 1 ml of sample extract was transferred to a 100ml volumetric flask containing 75ml water. 5ml Folin Denis reagent and 10ml of sodium Carbonate solution were added and diluted to 100ml with distilled water.

4. It was mixed well and the absorbance was read at 700 nm after 30 min. A blank was prepared with water instead of the sample.

**FT-IR Spectrophotometer Analysis**

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Dried power of different solvent extracts of each plant materials were used for FTIR analysis. 10mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FTIR spectroscope (SJASCO FTIR 410 Spectrophotometer), with a Scan range from 400 to 4000 cm with a resolution of 2 cm⁻¹.

**Thin layer Chromatography**

**Preparation of Plates**

1. 30 gm silica gel was mixed with100ml of distilled water. Uniform suspension was prepared by continuous stirring.

2. The clean, dried glass slides were taken for the silica gel plate preparation and thin coating of silica gel was prepared in slides.

**Developing solvent system**

A number of developing solvent systems were tried (Stahl, 1969; Mukherjee, 2002) to standardize developing solvent for getting, round, legible and satisfactory resolution. The chambers were saturated overnight with the respective mobile phases.
Visualization and documentation

After air drying the TLC plate was visualized in visible light, UV light and iodine chamber. The image of the chromatogram thus developed was recorded immediately. The Rf of each of the spots was computed as the ratio of the distance traveled by the spot to the distance traveled by the developing solvent on the chromatogram. The developed plates were photo documented and photographs taken using 254 nm, 366 nm and visible light recorded.

Data analysis

The Rf values of different spots were calculated using the formula

\[
\text{Rf value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}
\]

Solvent used for TLC

- Petroleum ether extract – Benzene: Chloroform – 1:4
- Benzene extract – Chloroform: Ethyl Alcohol – 4.75 : 0.25
- Chloroform Extract – Chloroform: Ethyl Alcohol – 4.5:0.5 (OR) 4:1
- Ethyl Alcohol Extract – Chloroform: Ethyl Alcohol – 2:3 (OR) 1:4

HPLC Analysis

The HPLC method was performed on a Shimadzu LC-10AT VP HPLC system, equipped with a model LC-10AT pump, UV-Vis detector SPD-10AT, a Rheodyne injector fitted with a 20μl loop and an auto injector SIL-10AT. A Hypersil® BDS C-18 column (4.6 × 250mm, 5μm size) with a C-18 guard column was used. The elution was carried out with gradient solvent systems with a flow rate of 1ml/min at ambient temperature (25-28°C). The mobile phase consisted of 0.1% v/v methanol (solvent A) and water (solvent B). The mobile phase was prepared daily, filtered through a 0.45μm and sonicated before use. Total running time was 15min. The sample injection volume was 20μl while the wavelength of the UV-Vis detector was set at 254nm (Sharanabasappa et al., 2007; Mallikharjuna et al., 2007).
**Instrumentation**

An isocratic HPLC (Shimadzu HPLC Class VP series) with two LC-0 AT VP pumps (Shimadzu), a variable wave length programmable photo diode array detector SPD-M10A VP (Shimadzu), a CTO-10AS VP column oven (Shimadzu), a SCL-10A VP system controller (Shimadzu), a reverse phase Luna 5C18 (2) and Phenomenex column (250 mm X 4.6mm) were used. The mobile phase components Methanol:water (45:55) were filtered through a 0.2μ membrane filter before use and were pumped from the solvent reservoir at a flow rate of 1ml/min which yielded column backup pressure of 260-270kgf/cm². The column temperature was maintained at 27°C. 20μl of the respective sample and was injected by using a Rheodyne syringe (Model 7202, Hamilton).

**Gas Chromatography-Mass Spectrometry Analysis**

The GC-MS analyses were carried out in a shimadzu GC-MS-QP 2010 gas chromatograph fitted with a DBI (Methylphenylsiloxane, 30 m x0.25 mm id.d) capillary column. Carrier gas, helium with a flow rate of 0.7 ml/min; column oven temperature 70°C, 5 min in 180°C, 180-260°C at 3°C/min, 5 min in 60°C, 260-280°C at 0.2°C/min and finally 5 min in 280°C; injector temperature, 280°C detector temperature 290°C, volume injected, 1 μL of TMS ether derivatives in n-hexane (2%); Split ratio, 3:0. The MS operating parameters were as follows: ionization potential 70 eV; ion source temperature 200°C; quadrupole 100°C amu, eV voltage 3000 volts.

The concentrated extract is injected into the GC/MS instrument (Hewlett Packard 5890 GC/MS with Mass Selective Detector with an HP-1 glass capillary column). The sample is volatilized at the injection port and eluted through a capillary column under increasing temperature. As the sample moves through the column, various components are separated due to their affinity for the stationary phase of the column and can be identified by retention time (the time it takes for a compound to pass through the column and gas
chromatograph system). Each chemical component in a sample has a distinct retention time measured in minutes, shown in a peak on a graph which measures abundance on the ordinate against retention time on the abscissa. The integrated peak is correlated to the concentration of the chemical. A mass selective detector breaks up each chromatographic component into fragment ions, which are shown by their abundance, with each ion represented as a vertical line in increasing molecular weight. The height of each line corresponds to the abundance of that ion. The resulting mass spectrum is unique to that chemical. This mass spectrum forms a "Fingerprint" that can identify the compound by a computer search of mass spectra. A computer search of the mass spectra corresponding to all the chromatographic peaks for a sample should yield a statistical match for nicotine at a 12.9 min retention time value if they were present two modes of GC/MS were possible with this instrumental method. First, there is a "Scan" mode which observes at all the constituents of a sample, listing whatever chemical components are preset.

**Compound Identification**

Components of the ethnolic extracts were identified by comparison of their mass spectra and retention indices with those published in the literature and contained in the NIST '98 MS computer library (Wiley).

**Antimicrobial Activity**

The antibacterial activity of isolated plant extraction pellets were tested by agar disc diffusion method. The test organisms used for assay are *Staphylococcus aureus, Shigella sonnei, Vibrio cholerae, Klebsiella pneunmoniae* and *Streptococcus pyogenes*. The antibacterial activity of the extracts evaluated by measuring the zone of inhibition method. The samples for each bacterial strain were sub cultured in individual agar slants.
**Maintenance of Microbial Strains**

Microbial strains to be tested were streaked in agar plates to get the pure cultures. The pure cultures were streaked on Muller Hinton agar slants and stored at 4°C to keep the microbial strains viable.

**Preparation of Muller Hinton Agar Medium:**

Mullar Hinton Agar – 35 g

Distilled water – 100ml

35g of Mullar Hinton Agar was dissolved in distilled water. The content was boiled for complete dissolution and ingredients. The medium was sterilized by autoclaving at 121°C for 15-20 minutes at 15 Lbs pressure.

**Muller Hinton Broth**

Mullar Hinton Agar – 120 g

Distilled water – 100ml

Mullar Hinton Agar with was dissolved in distilled water. The medium was filtered through the cheese and then autoclaved it.

**Preparation of Sterile Antibiotic Discs**

Antibacterial activity was assayed by filter paper disc diffusion method. (Bauer et al., 1996). Whatman No.1 filter paper of 5mm diameter was used. These discs were sterilized before use.

The extracts of the plants were added to the sterile discs. Each sterile disc incorporated individually with 200-500 micro liter of extract of nanoparticles solution using micropipette. Precautions were taken to prevent the flow of the solvent extract from the outer surface of the discs. The condensed extracts were applied in small quantities and the discs were allowed for air-drying. Then another dose of the extract was applied.
Plating

Already prepared Muller-Hinton Agar medium was sterilized well as $120^\circ$ at 15 lbs for 20 minutes. After the temperature got reduced up to the level of handing the medium was poured in already sterilized Petri plates. After solidification it was used to streak the microbes.

Assay of Antibacterial Activity

Muller-Hinton Agar broth was prepared in ten test tubes. These were cotton plugged and autoclaved. The test tubes were labeled according to the type of the bacterial cultures to be inoculated. The Muller-Hinton Agar broth was incubated at $37 \pm 1.5^\circ$ for 18 hrs. After incubation period the microbial strains were smeared on sterile Muller-Hinton agar plates. The plates were incubated at $37^\circ$C for 18 hrs. After the incubation period the inhibition zones around the discs were measured and recorded. Three replicates for each concentration were carried out.

Antifungal Study

The standard strain used for the study is *Candida albicans, Candida tropicalis, Candida parapsilosis, Aspergillus niger* and *Aspergillus flavus*. This was grown on Sabourauds dextrose agar (SDA) (Himedia Laboratories, Mumbai) over night at $37^\circ$C for 24 hours and 48 hours.

3 to 5 colonies of standard strains of *Candida albicans, Candida tropicalis, Candida parapsilosis, Aspergillus niger* and *Aspergillus flavus* were suspended in 2 ml of sterile normal saline and vortexed. The turbidity of the homogenous suspension was adjusted to approximately 0.5 McFarland standards. The sterile swab was dipped on dried plates of Sabouraud’s dextrose agar to get lawn culture.

6 mm of sterile filter paper discs were purchased and sterilized. These were placed and inoculated on SDA plates. 10µl of the extraction was placed on the disc. These plates were incubated at $37^\circ$C. Zone of inhibition was noted around the disc at 24 hours and 48
hours. These experiments were repeated for three times and average diameter was recorded. The negative control used in the study was respective solvents and the positive control was Flucanazole.

**In-vitro assay for Cytotoxicity studies (MTT assay)**

The Cytotoxicity of samples U87 (brain cancer cell line), Hep G2 (human liver cancer cell line) and MCF7 (breast cancer cell line) were determined by the MTT assay. Cells (1 × 10^6 /well) were plated in 1ml of medium/well in 24-well plates. After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations (0.4 µg/ml, 2 µg/ml, 10 µg/ml, 50 µg/ml and 250 µg/ml) of the leaf and root extract of *Embelia ribes* and *Chonemorpha fragrans* in 0.5% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells(MTT) phosphate- buffered saline solution was added. After 4h incubation, 0.04M HCl/isopropanol were added. The absorbance at 570 nm was measured with a UV-Spectrophotometer using wells without sample containing cells as blanks. MTT assay is a quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the ability of living cells. The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl) - 2, 5-diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. Triplicate analysis of in vitro cytotoxicity of leaf and root extracts of *Embelia ribes* and *Chonemorpha fragrans* was carried out with various concentrations. The effect of the samples on the proliferation of cell lines were expressed as the % cell viability, using the following formula: % cell viability = A570 of treated cells / A570 of control cells × 100%.