Materials and Methods

I. Collection of plant materials

The leaves of *Eupatorium glandulosum*, *Eupatorium odoratum* and *Eupatorium triplinerve* were collected from Nilgiri Hills of Western Ghats, Coimbatore and Kanjikode (Kerala) respectively.

Preparation of powder

The leaves were shade dried, kept in an hot air oven at 40^{o}C, powdered mechanically and stored in an air tight container for further studies

Extraction procedure

The powder of selected plant materials were successively extracted with different solvents such as Petroleum ether, Benzene, Chloroform, Acetone, Ethanol and Water in a soxhlet apparatus.

II. Physico Chemical analysis (Kokate *et al.*, 2005)

Analytical method

The powders of selected plant materials were used for calculating the physico-chemical parameters like loss on drying, total ash, acid insoluble ash, water soluble ash and percentage of extractive value.

Loss on Drying

Freshly collected and pre-weighed samples were dried in Hot air oven at 40^{o}C until it reaches constant weight.

Total ash

3 gm of plant powder was ignited in an electric muffle furnace at 450^{o}C in silica crucible until the sample reaches a constant weight.

Acid –insoluble ash values

Total ash obtained was heated by adding 25 ml of diluted hydrochloric acid for 10 minutes. It was then filtered in ashless filter paper (Whatmann No.1) and the residue was incinerated in the furnace to get a constant weight.

Water soluble ash

The same procedure was adapted for the water soluble ash.

Solubility percentage

Alcohol

1gm of powdered material was mixed with 20ml ethyl alcohol and shaken frequently for 6 hours and kept undisturbed overnight. The extract was concentrated and the solubility percentage was calculated on dry weight basis.

Water

The procedure adopted for solubility percentage of alcohol, is used to calculate the solubility percentage of water.

Extractive values

The powdered materials were extracted with different solvents like petroleum ether, benzene, chloroform, acetone, methanol and water in a soxhlet apparatus. The extracts were concentrated and the extractive values were calculated on dry weight basis.

Fluorescent analysis

The fluorescent behavior of the plant materials were analyzed by Chase and Pratt (1949) method. Powdered plant materials treated with different solvents and their illuminations were observed under ordinary and Ultra-violet light conditions.

III. Qualitative phytochemical analysis

Qualitative phytochemical analysis was done by using the procedure of Kokate *et al*., (2005). Presence of alkaloids, flavonoids, glycosides, terpenoids, tannins, phenols, fixed oils, fats, gums, mucilage and saponins were analyzed qualitatively.

Alkaloids

The selected plant powders were extracted with different solvent, and dissolved in diluted sulphuric acid and filtered. The filtrate was then treated with Mayer's and Wagner's reagents separately. Appearance of cream and reddish brown precipitate respectively indicates the presence of alkaloids.

Glycosides

Plant extracts treated with Fehling's reagent and Benedict's reagent separately, appearance of brownish red colour and green precipitate respectively indicates the presence of glycosides.

Phenols and Tannins

Plant extracts were taken in two separate test tubes and treated with 5% ferric chloride and 10% lead acetate. Appearance of blue colour with ferric chloride or precipitation with lead acetate indicates the presence of phenols and tannins respectively.

Flavonoids

Plant extracts treated with ferric chloride leads to the formation of brown precipitate indicates the presence of flavonoids. The extracts treated with 1 gm of magnesium powder and 1ml of concentrated hydrochloric acid, formation of orange colour indicates the presence of flavonoids.

Phytosterols

Plant extracts were treated with few drops of chloroform and filtered. The filtrates were treated with few drops of Concentrated Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of phytosterols.

Saponins

1ml of the plant extract is diluted with distilled water and made up to 20ml and shaken in a test tube for 15 minutes; formation of foamy layer indicates the presence of saponins.

Fixed oils and Fat

Plant extracts treated with 0.5N alcoholic potassium hydroxide along with a drop of phenolphthalein and heated in a water bath for few minutes; formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

IV. Gas Chromatography- Mass Spectrometry analysis (GC-MS)

The GCMS study was performed in ethanol extract of selected plants and their callus using Thermo GC Trace Ultra Version 5.0 equipment with runing time of 35:32 min and the Mass Spectrometry (MS) was achieved by using Thermo MS DSQ II equipment. Auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column TR 5-MS capillary standard non-polar column (30 Mts, ID : 0.25mM, FILM : 0.25 μm) composed of 5% Phenyl polysilphenylene-siloxane and its operating in electron impact mode at 80 eV; helium (99.999%) was used as carrier gas at a constant flow of 1mL/min and an injection volume of 1μl was employed (split ratio of 10:1) under injector temperature 250ºC; ion-source temperature 280ºC.

The oven temperature was programmed from oven temp 80ºC raised to 250ºC at 8ºC /min up to 450ºC. Mass spectra were taken at 80 eV; a scan interval of 0.5 second and fragments from 40 to 550 Da. Essential compounds were identified by their retention times and mass fragmentation patterns using data of standards at NIST (National Institute of Standard Technology) library.

V. HPTLC studies

Preparation of standard solution

Standard solution (Quercetin) was prepared by dissolving 1 mg of quercetin in 10 ml of methanol in a standard flask.

Preparation of plant extracts

The leaf powder was defatted with petroleum ether and extracted with ethanol and water using soxhlet apparatus. The extract was dried and dissolved in methanol according to the required volume.

Chromatography and Detection

Chromatography was performed on a 10x20cm precoated HPTLC Silica gel 60 F_{254} plates (E-Merck, Mumbai, India). Aliquots of each of the extracts were separately applied (Samples and standard) to the plate as 6 mm wide band with an automatic TLC applicator Linomat-5 applicator (CAMAG, Switzerland), 5 mm from the bottom. The plates were developed with the mobile phase of Toluene: Ethyl acetate: Formic acid: methanol (5.5:4:1:0.5). The twin glass chamber was saturated with the mobile phase for about 30 minutes. The plate was developed in twin glass horizontal developing chamber at the room temperature up to 8 cm. Plates were air dried and scanning was performed on a Camag TLC Scanner in absorbance at 254 nm.

Calibration curve of Quercetin

The quercetin compound was determined by using a calibration curve established with a standard concentration range from 40 to 320 ng/spot. A stock solution of standard quercetin (mg/ml) was prepared in methanol. The different volumes of stock solution 0.4, 0.8, 1.2 , 1.6, 2.0, 2.4, 2.8 and 3.2 µl were spotted on HPTLC plate to obtained concentration 40, 80, 120, 160, 200, 240, 280 and 320 ng/spot, respectively (band width 6mm, distance between tracks 7mm) using automatic sample spotter. Peak areas were recorded for quercetin and the calibration curve was prepared by plotting peak area against the concentration of Quercetin.

VI. Heavy Metal studies

The selected three plant materials collected from the wild along with the soil adhere to the root were used for heavy metals analysis. The digested samples were used to analyze the heavy metals like lead, chromium, nickel and cadmium using Atomic Absorption Spectrometer (Model - Perkin Elmer Analyst 200).

Sample digestion

Preparation of Triacid mixture

Conc. Nitric acid, Sulphuric acid and Perchloric acid were mixed together at the ratio of 9:2:1.

Digestion process

0.5 gm of the samples was taken in a conical flask and 10-15 ml of triacid mixture was added and the mouth of the conical flask was covered with a funnel and kept overnight. Next day, the digested sample was heated in a hot plate until yellow fumes disappeared and clear solution obtained. After that, the solution was made upto 50ml with distilled water and filtered using Whatmann No. 1 filter paper and the filtrate was used for heavy metal analysis.

VII. Antimicrobial studies

Well diffusion method was employed to study the antimicrobial properties of the plant extracts. Ethanol and Water extracts of the plants were tested against the human pathogens.

Source and maintenance of microbes

Pure cultures of Bacterial and fungal human pathogens were collected from PSG Institute of Medical Science and Research, Coimbatore and maintained in the microbiology laboratory in Botany department of PSGR Krishnammal College for Women Coimbatore.

Microorganisms used

Bacteria

1. *Staphylococcus aureus*
2. *Bacillus subtilis*
3. *Escherchia coli*
4. *Bacillus cereus*
5. *Klebsiella pneumoniae*
6. *Streptococcus pyogens*

Fungi

1. *Microsporum canis*
2. *Candida tropicalis*
3. *Candida albicans*
4. *Trichophyton rubrum*
5. *Aspergillus niger*
6. *Aspergillus flavus*

Media used

Nutrient Agar Medium and Potato Dextrose Agar Medium were used for culturing bacteria and fungi respectively.

Nutrient Agar Medium

Ingredients gm /litre

Yeast extract	- 2gm
Beef extract	- 1gm
Peptone	- 5gm
Sodium chloride	- 5gm
Agar	- 15gm
pH	- 7.4

Potato Dextrose Agar medium (PDA)

Ingredients gm /litre

Potato	-250
Dextrose	-15gm
Agar	-20gm
pH	- 6.2

Sterilization

All glass wares were well sterilized in hot air oven at 180^{o}C for 3 hours. Instruments like forceps, inoculation needle were sterilized over flame after dipping in alcohol. Media were sterilized in pressure cooker for 20 minutes.

Source of inoculum

For bacteria, 24 hours old culture was used as an inoculum. A loopful of each bacterial culture was suspended in 5ml of sterile Nutrient Broth and shaken vigorously to get the uniform suspension.

For fungi, spore suspension was used. A loopful of sporulating culture of each fungus was suspended in 5ml of sterile distilled water and shaken vigorously.

Method of inoculation

Sterilized petriplates containing sterile medium was swabbed with selected microorganisms in such a way so as to get a thorough coverage of uniform thick lawn of growth of the inoculum.

Well diffusion method

After swabbing the cultures, wells of 6 mm diameter were created with a sterile cork borer. To this, 20-100µl (µg / µl) of plant extracts were dispensed and the petriplates were carefully sealed and incubated.

Method of incubation

Fungal cultures were incubated at 25^{o}C for 48 hours and bacteria at 37^{o}C for 24 hours. Triplicates were maintained for each experiment. Fresh cultures were used for the analysis. The performance of plant extracts were determined by the zone of inhibition.

Minimum Inhibitory Concentration

MIC was studied by tube dilution method (Savitha and Rathnavijaya, 2011). Serial dilutions of methanol extracts of three plants ranging from 1-100 µg were made in the fungal and bacterial broth. The pathogens were inoculated to these broths and incubated for 24 hrs and 48 hrs for bacteria and fungi respectively. The tubes were observed for growth of microbes.

VIII. Antioxidant activity

Preparation of standard solution

The standard solution was prepared by dissolving 1mg of Ascorbic acid in 1ml of methanol to obtain various concentrations such as 20, 40, 60, 80 and 100 µg/ml.

Preparation of test sample

The ethanol and water extracts were dried and about 10mg of dried extract were dissolved in 10ml of methanol to give concentration of mg/ml.

1. DPPH free radical scavenging assay (Williams *et al.*, 1995)

A solution of the radical is prepared by dissolving 2.4 mg DPPH in 100ml methanol. A test solution of various concentrations such as 20, 40, 60, 80 and 100µl were added to 3.98, 3.96, 3.94, 3.92 and 3 mlof DPPH respectively. The mixture was shaken vigorously and kept at room temperature for 20 min under dark condition. All the testes were performed in triplicate. The DPPH reagent itself served as control. After 20 minutes the absorbance was measured at 515nm in spectrophotometer (Deep vision-2305). The scavenging percentage of the extract was calculated using the following formula:

$$\% \text{ of scavenging} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

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

The leaf powder extracts (4ml) were prepared using distilled water at various concentrations. To the plant extract, 0.6 ml 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. Ascorbic acid (control) was used as standard reference.

Hydrogen peroxide scavenging activity of powder extracts were calculated using the following formula,

$$\% \text{ scavenged } (H_2O_2) = \frac{(A_{control} - A_{sample})}{(A_{control})} \times 100$$

Where,

$A_{control}$ - Absorbance of standard reference (Ascorbic acid)

A_{sample} - Absorbance of the powder extract.

IX. Genetic variation studies

Isolation of genomic DNA

DNA was extracted from fresh leaves of selected *Eupatorium* species. DNA extraction was done with the protocol of Doyle and Doyle, 1994. One gram of leaf material was ground into a fine powder using Liquid Nitrogen by sterile mortar and pestle. The ground tissue was transferred into centrifuge tube and 6.5 ml of 2% CTAB and mixed thoroughly. Then ice cold Chloroform: isoamyl alcohol (24: 1) was added to that and centrifuged at 12,000 rpm for 15 min, pellet was discarded and the supernatant was transferred into a new tube. To the sample 0.7 volume of ice cold Isopropanol was added to precipitate the DNA. The precipitated DNA was pelleted down by centrifuging at 10,000 rpm for 10min. The DNA was washed twice with 70% ethanol and the pellet was air dried and suspended in Tris EDTA buffer (TE buffer) and the DNA samples were stored at -20^{o}C at deep freezer.

Purification of DNA

10 µl of RNase was added to the DNA sample and the mixture was initiated at 37^{o}C for 30 min. Equal volume of Chloroform: phenol (1:1) was added and centrifuged at 12,000 rpm for 10 min and the pellet was collected.

RAPD-PCR analysis

Selection of primers:

The following are the primers used for amplification of 10-mer oligonucleotides with arbitrary sequences namely

Primer - 1: OPA 1 (5'-CAGGCCCTTC- 3')

Primer – 2: OPA 4 (5'-AATCGGGCTG- 3')

Primer – 3: OPA 12 (5'-TCGGCGATAG- 3')

Primer – 4: OPA 13(5'-CAGCACCCAC- 3')

Primer - 5: OPA 18 (5'-AGGTGACCGT- 3')

Primer - 6: OPA 19 (5'- CAAACGTCGG-3')

Primer – 7: OPB 07 (5'-GGTGACGCAG- 3')

Primer – 8: OPB 10 (5'-CTGCTGGGAC- 3')

Primer – 9: OPB 17 (5'-AGGGAACGAG- 3')

The PCR amplification was performed with 1µl of genomic DNA. Amplifications were carried out in 20 µl reaction mixture with the following contents:

- Template DNA -1.0 µl
- 10X Taq polymerase buffer - 2.0 µl
- 10 mMdNTPs - 2.0 µl
- Primer- F, 10 mM - 0.5 µl
- Taq DNA polymerase -0.5 µl
- Deionized water - 13.0 µl
- Total volume 20.0 µl

The reaction mixture was carried out in a thermal cycler. The PCR conditions were as follows:

Step I - Initial denaturation 94^{o}C 5 min

Step II - Denaturation 94^{o}C 1min

Step III - Annealing 50^{o}C 1min

Step IV - Extension 72^{o}C 2min

Step V - Repeat step II 35 times

Step VI - Final elongation 72^{o}C 7 min

Step VII - Hold 4^{o}C

Agarose gel electrophoresis

The PCR amplification products were analysed in 2% agarose gels using 0.5 X TBE buffer. The gels were stained with ethidium bromide and visualized under UV light (Sambrook *et al*., 2001). A 100 bp DNA ladder was used as the molecular standard in order to confirm the appropriate RAPD markers. These markers were named by primer origin, followed with the primer number and size of amplified products in base pairs.

X. *In vitro* cytotoxic activity

Maintenance of cell line

Human colon adenocarcinoma (HT-29) was procured from American Type Culture Collection (ATCC) USA. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated Fetal Bovine Serum (FBS), and 1% penicillin solution. Cells were incubated at 37ºC in a humidified, 5% CO_2 atmosphere.

1. MTT Assay (Arung *et al.*, 2009)

Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT dye enters the cells and passes into the mitochondria, where it is reduced to an insoluble, coloured

(dark purple) formazan product. The cells are solubilised with an organic solvent such as DMSO or isopropanol and the released solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Materials & Reagents required

1. Cell line - Human colon adenocarcinoma cells, HT-29 cell line
2. MTT
3. DMSO
4. CO_2 incubator
5. 96-well clear flat-bottom polystyrene tissue-culture plates
6. Tecan Plate reader

Procedure

The MTT assay was used to measure the rate of cancer cell death. A quantity of 1.0 x 10^5 cells/ml was seeded into a 96-well plate and after 24 hours the cells were washed and maintained with different concentration of samples (10-320 µg/ml) and incubated for 45 hours at 37^{o}C under 5% CO_2 atmosphere. The concentration of samples prepared in DMSO, which were serially diluted in complete culture medium were added to this cells in triplicate. After 48 hours incubation, the medium in each well was replaced with MTT (3-[4,5-dimethylthiazol-2-yl]-2,3- diphenyltetrazodium bromide). After 4 hours, DMSO was added to the dye to dissolve the formed violet formazan crystals. The formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. The plates were well shaked for 20 min and the optical density was measured at 590 nm. The percentage of inhibition of the cells are measured using the formula,

$$\% \text{ Inhibition} = 100-(\text{OD of sample}/\text{OD of Control}) \times 100$$

2. SRB Assay (Kasinski *et al.*, 2015)

Principle

Sulforhodamine B (SRB) assay is an anionic bright pink aminoxanthene protein dye with two sulfonic groups. The SRB assay is based on the ability of the SRB dye to bind to protein basic amino acid residues of trichloroacetic acid (TCA) fixed cells. It can be quantitatively extracted from cells and solubilized for optical density (OD) measurement by weak bases such as Tris base. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell number.

Materials and Reagents

1. 96-well clear flat-bottom polystyrene tissue-culture plates
2. 2.5% Trypsin solution
3. Trichloroacetic acid (TCA)
4. Sulforhodamine B sodium salt (SRB) dissolved in 1% acetic acid
5. 10 mM unbuffered Tris base solution
6. CO_2 incubator

Procedure

SRB assay was used to determine the effect of the compounds on cells. The cell cultures were seeded in flat-bottomed 96-well plates. The cells were incubated at 37^{o}C for 24 h in a humidified atmosphere containing 5% CO_2 and then media containing 100 µl of various concentrations (10-320 µg/ml) of test samples were added to each well. The plates were incubated for the next for 24 hours. After incubation, the cells were fixed by adding 25 µl per well of 50% ice-cold TCA to each well and incubated at 4°C for 1 h. The plates were washed 3-4 times in running tap water and stained with 50 µl of SRB reagent (0.4% SRB in 1% acetic acid) for 30 min. The plates were washed five times in 1% acetic acid and allowed to dry overnight. SRB was solubilised with 100 µl

per well by 10mM Tris-base, shaken for 5 min and the OD was measured at 570nm.

% Inhibition= 100-(OD of sample/OD of Control) × 100

3. Lactate dehydrogenase (LDH) Assay (Kuznetsov and Gnaiger, 2006)

Principle

When target cells are treated with a cytotoxic agent, cytoplasmic LDH is released into the culture supernatant due to plasma membrane damage. The LDH activity in the culture supernatant is measured by a substrate reaction and quantitated. The assay quantitatively measures LDH, which is released upon cell lysis. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (INT) into a red coloured formazan. The LDH activity is determined as NADH oxidation or INT reduction over a defined time period. The resulting formazan absorbs maximally at 492nm and can be measured quantitatively at 490nm using a microplate reader or spectrophotometer.

$$\text{Pyruate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^{+}$$

Materials and Reagents

1. LDH assay kit (Cytoscan, India)

Procedure

Cells were incubated with various concentrations (10-320 µg/ml) of test samples. Where cell culture supernatant was incubated with assay reagents at 37^{o}C for 1 minute. The Changes in absorbance / minute (OD / Min) during 3 minutes were measured. The following formula was used to calculate the LDH activity.

LDH activity (U/L) = (OD/ Min) X 16030

IX. Tissue culture studies

Collection of Explants

Leaf and stem (nodal and inter nodal) explants were excised in different periods of a year. First fully expanded leaf, first stem nodal and first internodal regions were collected from garden-grown plants.

Surface Sterilization of explants

Well grown leaves, nodes and internodes were collected and washed thoroughly under running tap water for 10 min without damage to the tissues. In order to avoid the interaction of microbes, explants were first washed thoroughly with sterile distilled water and treated with 75% alcohol for one minute and rinsed 3 to 4 times in sterile distilled water. Then, the explants were treated with 0.1% mercuric chloride for 2-3 min and washed 3 times with sterile distilled water. The excess water on the explants was removed by using sterile tissue paper before culture. The explants were cut into small pieces (0.5-0.8cm) barring the cut ends and transferred to semi-solid culture medium under aseptic conditions in a laminar flow chamber.

Preparation of culture Medium

Murashige and Skoog's (1962) basal medium was used throughout this study. Medium was prepared by using various stock solutions containing major and minor elements including vitamins.

S.no	Constituents	Conc. in stock solution mg/L
1	NH_4NO_3	1650
2	KNO_3	1900
3	$CaCl_2$. $2H_2O$	440
4	$MgSO_4$. $7H_2O$	370
5	KH_2PO_4	170
6	H_3BO_3	6.2

7	KI	0.83
8	$NaMoO_4.2H_2O$	0.25
9	$CoCl_2.5H_2O$	0.025
10	$MnSO_4.4H_2O$	22.3
11	$ZnSO_4.5H_2O$	8.6
12	$CuSO_4.5H_2O$	0.025
13	Na_2EDTA	37.3
14	$FeSO_4.7H_2O$	27.3
15	Thiamine HCl	0.1
16	Nicotinic Acid	0.5
17	Pyridoxine HCl	0.5
18	Glycine	2
19	Myoinositol	100
20	Ascorbic acid	100
21	Sucrose	30 gm/l
22	Agar	10 gm/l
pH		6.5

- Appropriate quantities of the various stock solutions, sucrose, myo-inositol, ascorbic acid and growth regulators (IAA, IBA, 2,4-D, kinetin and BA) were added and total volume of the medium was made up to the required quantity using sterilized distilled water.
- After mixing thoroughly, the pH of all medium is adjusted and appropriate quantity of agar is added. The medium was heated until the agar gets dissolved. Then the medium was distributed into culture tubes (15 ml of the medium in 25mm x 150 mm culture tube) and the tubes were tightly plugged with non-adsorbent cotton. The plugged culture tubes were sterilized in a pressure cooker at 120^{o}C for 20 minutes and cooled at room temperature in slanting position.

Culture condition

The cultures were incubated in culture room at 25±2^{o}C with relative humidity of 65-70 %. The cultures were kept under the light intensity of 2000 LUX at the level of culture tubes, supplied from white fluorescent lamps with 12hrs photoperiod. Six replicates per treatment were kept and the experiments were repeated at least once to confirm the results.

Subculture

Explants and calli were subcultured every 4 weeks. The tubes containing culture material were externally sterilized with 50% alcohol. The materials were cut into required amount and transferred to fresh medium with the help of sterile forceps inside the inoculation chamber. After subculture they were transferred to the culture room.

Growth measurement

Growth of the callus was measured on fresh weight basis and that of multiple shoots on number and height of shoots.

Statistical analysis

All the experiments were conducted with a minimum of three to six replicates per treatment and statistically analyzed using (SPSS) one-way or two way analysis of variance (ANOVA). The significance of the differences among means was contrasted using Duncan's multiple range test at $P \leq 0.05$.