2. MATERIARLS AND METHODS

2.1 Selection of plant for the study

Cissampelos pareira L var. hirsuta belongs to the family Menispermaceae was selected for the study based on the ethno botanical information compiled through interviews from siddha and ayurveda medical practitioners, traditional healers and old experienced people which form authentic and first hand source of references. It has been used since the ancient times as a cure for menstrual problems, hormonal imbalance, and to ease childbirth, postpartum pain, prevent miscarriage, and control uterine hemorrhages, controls hormonal acne and premenstrual syndrome (Amresh et al., 2008).

2.2 Collection and authentication of plant materials

The plant C. pareira L var. hirsuta for the proposed study was collected during April - May 2014 from different ecotypic regions such as Ambasamudram (AC1), Ooty (AC2), Peermade (AC3), Kumaracoil (AC4), Kozhikode (AC5), Yelahanka (AC6) and Ariyankuppam (AC7). The plant samples were identified and authenticated by Dr. A.G. Pandurangan, Director and Head, Plant Systematic and Evolutionary Division, JNTBGRI (Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, India (Collection number :76876). The voucher specimens (JNTBGRI/PS/173/2016) have been deposited at TBGT, JNTBGRI, Palode, Thiruvananthapuram, Kerala, India, for future reference.
2.2.1 Systematic classification of *Cissampelos pareira* (Scientific classification, 2012)

- **Domain**: Eukaryota
- **Kingdom**: Plantae
- **Subkingdom**: Tracheobionta
- **Division**: Magnoliophyta
- **Phylum**: Tracheophyta
- **Class**: Magnoliopsida
- **Sub class**: Magnolidae
- **Order**: Ranunculales
- **Family**: Menispermaceae
- **Genus**: Cissampelos
- **Botanical name**: *Cissampelos pareira* L var. hirsuta

2.2.2 Plant description

*C. pareira* is a perennial climbing shrub with small greenish yellow flowers; leaves are peltate or orbicular-reniform, ovate with truncate or cordata base, glabrous and hairy above up to 3-12 cm long. Flowers unisexual; pedicel up to 2 mm long; male flowers clustered in the axil of a small leaf; sepals are 4 in numbers, ovate, hairy outside, greenish or yellowish; corolla cup-shaped; filaments of stamens completely fused; female flowers in bracteate racemes, 3-8 together in the axial of each bract; bracts to 1 x 1 cm, orbicular, pubescent; sepals 2; petals absent; ovary densely hairy; styles 3; ovary superior, hairy; 3-lobed stigma. Fruit short, hairy, orange to red drupe, one seeded. Horseshoe-shaped; embryo elongate, narrow, embedded in endosperm, cotyledons flattened. The stem is woody, flexible, and slender up to 1 cm and twines for support. The root system consists of flexible, light brown, lateral roots with sinkers and moderately abundant fine roots. The plant is common in orchards, hedges, park and gardens of moist soils, ether creeping or twining around other plants, also
common on the hilly tracts along water courses. It can also be propagated from root cuttings, planted at the beginning of monsoon. Sometimes it dies back in hot water. *C. pareira* is mostly collected from the wild. It is very widespread and locally common (*Cissampelos pareira*, 2012).

### 2.3. Molecular characterization of *Cissampelos pareira*

#### 2.3.1 Extraction of genomic DNA

The genomic DNA was isolated from seven samples of *Cissampelos pareira* using modified Cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987).

#### 2.3.2 Reagents and Chemicals

- An extraction buffer consisting of Tris HCL 1.214 g, NaCl 8.18 g, EDTA 0.75 g, CTAB 2 g and 800 µl of β-mercaptoethanol.
- Chloroform: isoamylalcohol (24:1)
- Wash solution: 70% ethanol and 90% ethanol.
- TE buffer: 10 mM Tris HCL (pH8).
- 0.3 v of isopropanol and 0.2 v of ammonium acetate

#### 2.3.3 Protocol

About 100 mg of the plant leaf tissue was homogenized using liquid nitrogen and the powdered tissue was transferred to a microcentrifuge tube. One thousand microlitre of extraction buffer was added. It was then incubated at 55 °C for 3-5 hours in water bath and then kept at room temperature for 10-15 minutes. After incubation 500 µl of (24:1) chloroform: Isoamyl alcohol was added and mixed well and centrifuged for 10 minutes at 10000 rpm at 4 °C. The aqueous phase was pipette
out in a new labelled microcentrifuge tube and 200 µl of ammonium acetate and 300 µl of isopropanol were added and the contents were mixed. It was kept in refrigerator for overnight and the precipitated DNA was pelleted by centrifugation at 10000 rpm for 2 minutes. The pellet obtained was again washed with 500 µl of 90% cold ethanol, kept undisturbed for 5 minutes and centrifuged at 10000 rpm for 2 minutes. The pellet was dried and then dissolved in 20 µl of TE buffer and was stored at 4 °C.

### 2.3.4 Agarose gel electrophoresis for DNA quality check.

The quality of the DNA isolated was checked using agarose gel electrophoresis (Maniatis *et al.*, 1982). 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 Volt until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using gel documentation system (Bio-Rad). The isolated DNA was used as template for PCR analysis.

### 2.3.5 PCR amplification with universal primers *rbcL* and *matK*

Seven collected plant samples of *C. pareira* from different ecotypic regions were barcoded using *matK* and *rbcL* primers. PCR was performed to amplify the specific DNA sequence using respective barcode candidate specific primers. Details of the primers are given in table 2.1. PCR amplification reaction was carried out in a 20 µl reaction volume, which contained 1X Phire PCR buffer (contains 1.5 mM
MgCl$_2$), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5 M Betaine, 5 pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). *matK* – PCR amplification profile and *rbcL* – PCR amplification profile were given in tables, 2.2, 2.3).

### Table 2.1: Primers used for DNA Barcoding of plant samples collected from different ecotypic regions

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Name</th>
<th>Direction</th>
<th>Primer Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>matK</em></td>
<td>390F</td>
<td>Forward</td>
<td>CGATCTATTCAATATTTC</td>
</tr>
<tr>
<td></td>
<td>1326R</td>
<td>Reverse</td>
<td>TCTAGCACAGAAAGTCGAAGT</td>
</tr>
<tr>
<td><em>rbcL</em></td>
<td>rbcLa_F</td>
<td>Forward</td>
<td>ATGTCACCACAAGAGACTAAAGC</td>
</tr>
<tr>
<td></td>
<td>rbcL724_R</td>
<td>Reverse</td>
<td>GTAAATCAAGTCCACCRCG</td>
</tr>
</tbody>
</table>

### Table 2.2: *matK*-PCR amplification profile for the DNA barcoding of plant samples collected from different ecotypic regions

<table>
<thead>
<tr>
<th>S.No</th>
<th>Activity</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>98°C</td>
<td>30 sec</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>98°C</td>
<td>5 sec</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>50°C</td>
<td>10 sec</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72°C</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72°C</td>
<td>60 sec</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Storage</td>
<td>4°C</td>
<td>infinite</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3: *rbcL* PCR amplification profile for the DNA barcoding of plant samples collected from different ecotypic regions

<table>
<thead>
<tr>
<th>S.No</th>
<th>Activity</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>98°C</td>
<td>30 sec</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>98°C</td>
<td>5 sec</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>60°C</td>
<td>10 sec</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72°C</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
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<td>60 sec</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Storage</td>
<td>4°C</td>
<td>infinite</td>
<td>1</td>
</tr>
</tbody>
</table>

2.3.6 Agarose gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75 V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder New England Biolabs (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.3.7 ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37 °C for 15 minutes followed by enzyme inactivation at 37 °C for 15 minutes.
2.3.8 Sequencing using bigdye terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the bigdye terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Product(ExoSAP treated)</td>
<td>10-20 ng</td>
</tr>
<tr>
<td>Primer</td>
<td>3.2 pM</td>
</tr>
<tr>
<td>Sequencing Mix</td>
<td>0.28 µl</td>
</tr>
<tr>
<td>5x Reaction buffer</td>
<td>1.86 µl</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>make up to 10µl</td>
</tr>
</tbody>
</table>

The sequencing PCR temperature profile consisted of a 1st cycle at 96 °C for 2 minutes followed by 30 cycles at 96 °C for 30 seconds, 50 °C for 40 seconds and 60 °C for 4 minutes for all the primers.

2.3.9 Post sequencing PCR clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction were prepared.
2. 12 µl of master mix I was added to each reaction containing 10µl of reaction contents and mixed properly.
3. Master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction were made.
4. Added 52 µl of master mix II was added to each reaction.
5. Contents were mixed by inverting.
6. Incubated at room temperature for 30 minutes
7. Spinned at 14,000 rpm for 30 minutes
8. The supernatant was decanted and 100 µl of 70% ethanol was added.
9. Spinned at 14,000 rpm for 20 minutes
10. The supernatant was decanted and 100 µl of 70% ethanol was added
11. Spinned at 14,000 rpm for 10 minutes
12. The supernatant was decanted and air dried the pellet

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

2.3.10 Sequence alignment and data analysis

The DNA barcode sequences from the GenBank were acquired and aligned along with the laboratory derived barcode sequences. The alignment was performed using multiple sequence alignment algorithms ClustalW (Thompson, 2002). The genetic distance and variance were analyzed using MEGA7, according to the Kimura 2 - parameter (K2P) model (Tamura et al., 2007), while the genetic distance was determined by calculating the average values of their intra-specific distances, theta and minimum intra-specific distances. The species-specific divergence was analyzed based on the intra-specific distances (Selvaraj et al., 2015). DNA sequences of various samples of *Cissampelos pareira* were identified using Basic Local Alignment Search Tool (BLAST) and Distance based method (Bruno et al., 2000; Kent, 2002).

2.3.11 Phylogenetic analysis

Phylogenetic analysis is used to determine the evolutionary relationships between species. The results of the analysis can be drawn in a hierarchical diagram called a cladogram or phylogram (phylogenetic tree). The branches of a tree denote the hypothesized evolutionary relationships (phylogeny). Each member in a branch, also known as a monophyletic group assumed to be descended from a common
ancestor. Originally, phylogenetic trees were created using morphology and now it is carried out using the DNA sequences. The pattern of the evolution of seven samples of *Cissampelos pareira* was studied by phylogenetic analysis using MEGA 7 and the phylogeny was attained by Maximum likelihood method. The estimation was accomplished using bootstrapping approach. The translation expression profile of DNA sequences were also estimated using MEGA 7 software (Tamura *et al.*, 2004).

2.4 Plant extraction

2.4.1 Preparation of extract using hot percolation process

The collected fresh plant parts such as leaf, root and stem of *C. pareira* were rinsed with distilled water and air dried in shade. The dried plant material was homogenized by electric mixer grinder to obtain coarse powder and stored in air-tight bottles for further analysis. The shade dried, powdered leaf root and stem were extracted separately (Mukherjee, 2002) with different solvents in increasing order of polarity such as hexane, chloroform, ethyl acetate, ethanol and aqueous by hot percolation method using soxhlet apparatus. The powdered materials were evenly packed in a soxhlet extractor for about 36 hours with different solvents. The temperature was maintained (25 °C - 100 °C) on an electric heating mantle with thermostat control. The extracts were evaporated in a rotary vacuum evaporator at 40 °C to dryness and stored at 4 °C in an air tight bottle for further analysis (Raghavendra, 2011). The percentage yield was calculated and then subjected to phytochemical screening, Thin layer chromatographic profiling studies etc. The dried extract was properly stored in the desiccators for further experiment and analysis.
2.5 Preliminary phytochemical characterization of *Cissampelos pareira* L. var. *hirsuta*

Qualitative phytochemical analysis gives general idea regarding the nature of constituents present in the crude extracts. The phytochemical screening of constituents alkaloids, flavonoids, steroids, tannins, terpenoids, phenols, saponins, cardiacglycosides, anthroquinones,volatile oils, aminoacids, and reducing sugars) in the leaf, root and stem of *C. pareira* in various extracts (hexane, chloroform, ethylacetate, ethanol and aqueous) were carried out by the following standard procedures (Sofowora 1993; Trease and Evans,1989; Harborne, 1998 ).
### Table 2.4: Preliminary phytochemical screening of extracts

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mayer’s test: Test solution + one or two drops of Mayer’s reagent.</td>
<td>A white or yellow or cream coloured precipitate</td>
<td>Presence of alkaloid.</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline reagent test: Test solution + sodium hydroxide solution + dil. HCl</td>
<td>Intensity of yellow colour increases which become colourless on addition of dil. HCl</td>
<td>Presence of Flavonoid</td>
</tr>
<tr>
<td>3</td>
<td>Test solution + H₂O + lead acetate</td>
<td>White precipitate</td>
<td>Presence of tannin</td>
</tr>
<tr>
<td>4</td>
<td>Test solution + one drop of 5% ferric chloride.</td>
<td>Dark green colour</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>5</td>
<td>Libermann-Burchard test: Test Solution + 3-4 drops chloroform + few drops of acetic acid, acetic anhydride + 2 drops of Conc. H₂SO₄ and heated gently.</td>
<td>Blue or green colour</td>
<td>Presence of steroid</td>
</tr>
<tr>
<td>6</td>
<td>Fehling’s test: Test solution + 2 ml of fehling’s reagent (Soln A + Soln B) and heated</td>
<td>Red orange colour develops</td>
<td>Presence of reducing sugar</td>
</tr>
<tr>
<td>7</td>
<td>Test solution + 2 ml of chloroform + 2 ml Conc. H₂SO₄ and heated gently.</td>
<td>Grayish colour</td>
<td>Presence of terpenoid</td>
</tr>
<tr>
<td>8</td>
<td>Test Solution + H₂O</td>
<td>Foamy layer develops</td>
<td>Presence of saponin</td>
</tr>
<tr>
<td>9</td>
<td>Test solution + Conc. H₂SO₄ and shaken well</td>
<td>Red colour</td>
<td>Presence of Anthraquinone</td>
</tr>
<tr>
<td>10</td>
<td>Biuret test: Test solution + 10% sodium hydroxide solution + few drops of 0.1% copper sulphate solution</td>
<td>Violet or pink Colour</td>
<td>Presence of protein and aminoacid</td>
</tr>
<tr>
<td>11</td>
<td>Keller-Kiliani test: Test solution + 0.2 ml glacial acetic acid + a drop of FeCl₃ + 1 ml of Conc. H₂SO₄</td>
<td>A brown ring at the interface</td>
<td>Presence of Cardiac Glycosides</td>
</tr>
<tr>
<td>12</td>
<td>Extract + 1 ml of 90% ethanol + few drops of FeCl₃</td>
<td>Green colour</td>
<td>Presence of volatile oil</td>
</tr>
</tbody>
</table>
2.6 Thin layer chromatography

Thin Layer Chromatography (TLC) is an analytical method used for the separation, identification and estimation of different classes of natural products. Hexane, chloroform, ethyl acetate, ethanol and aqueous extracts of leaf, root and stem of *C. pareira* were subjected to thin layer chromatographic analysis to find out the phyto constituents by using the method of Sherma and Fried (2003). The thin layer chromatography was performed in an aluminium sheet, 60 F254 (Merck), which was coated with a thin layer of adsorbent material, usually silica gel, as per conventional one dimensional ascending method using the solvent system, hexane : ethyl acetate : acetic acid (5 : 4 : 1) for all the extracts. Spots of two microliters (2 µl) of each extract was carefully spotted using a micropipette at about 1 cm from the bottom of the TLC sheet and were carefully placed in the solvent system at an angle 45°, taking care that the solvent system does not come in contact with the spot. The chromatogram was allowed to develop and after the development of the chromatogram, the solvent front was marked and the plate was finally allowed to dry at room temperature. The coloured substances were visualized on the chromatogram while the colourless components were detected using visualizing agent UV radiation at 365 nm. The qualitative evaluation of the plate was done by determining the migration behaviour of the separated substances given in the form of \( R_f \) value. The colours of the spots and \( R_f \) values were recorded.

\[
R_f = \frac{\text{Distance travelled by the solute from the origin}}{\text{Distance travelled by the solvent from the origin}}
\]
2.7 Quantitative estimation of secondary metabolites

Quantitative estimation of secondary metabolites like total phenol and total flavonoid present in various solvent leaf extracts of *C. pareira* were carried out using the standard procedures.

2.7.1 Determination of total phenol content

Total phenolic content was estimated by Folin Ciocalteau reagent based assay by McDonald *et al.* (2001) with little modifications. 1 ml each of extract and standard gallic acid (20, 40, 60, 80, 100 µg/ml) was taken in the test tubes and 5 ml of distilled water and 0.5 ml of Folin Ciocalteu’s reagent was mixed and shaken. After 5 minutes, 1.5 ml of 20 % sodium carbonate was added and the volume was made up to 10 ml with distilled water and was allowed to incubated for 2 hours at room temperature which resulted in intense blue colour. After incubation, absorbance was measured at 750 nm using UV-visible spectrophotometer. The experiments were performed in triplicates. The standard calibration curve of gallic acid was plotted to get the $R^2$ value and linear equation of gallic acid. Total phenolic content of extracts were expressed as mg gallic acid equivalents (GAE).

2.7.2 Determination of total flavonoid content

The total flavonoid content of crude extract was determined by aluminum chloride colorimetric method (Jia *et al.*, 1999). 1ml of extract and 1ml of standard solution of quercetin (20, 40, 60, 80, and 100 µg/ml) was taken in the test tubes and 4 ml of distilled water and 0.3 ml 5% sodium nitrite solution was added. After 5 minutes, 0.3 ml 10% aluminium chloride was added followed by the addition of 2 ml 1 M NaOH and the total volume was made up to 10 ml with distilled water and
mixed well which resulted in orange yellowish colour. The absorbance was measured at 510 nm with distilled water as blank. The experiments were performed in triplicates. The standard calibration curve of quercetin was plotted to get the $R^2$ value and linear equation of quercetin. The total flavonoid content of extracts were expressed as mg quercetin equivalents (QE).

2.8 Gas Chromatography-Mass Spectrometry analysis (GC-MS)

GC-MS analysis of the crude leaf extract of *C.pareira* was carried out on a GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Column Elite-1 fused silica capillary column (30 mm X 0.25 mm) composed of 100% Dimethyl poly siloxane operating in electron impact mode gas at a constant flow of 1ml/min and an injection volume of 2µl was employed (split ratio of 10:1); Injector temperature 250 °C; Ion-source temperature 280 °C. The oven temperature was programmed from 110°C (isothermal for 2 min.), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with a 9min, isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5seconds and fragments from 45 to 450 Da (Merlin *et al*., 2009).

2.8.1 Identification of components

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the phytochemical constituents of the ethyl acetate leaf extract was ascertained (Nezhadali and Parsa 2010).
2.9 Pharmacological analysis of *Cissampelos pareira*

2.9.1 Antimicrobial activity of plant extracts

The antimicrobial activities of various solvent extracts of leaf, root and stem of *C. pareira* were carried out by agar-well diffusion and disc-diffusion methods.

a) Preparation of sterile disc: Filter paper discs (6 mm diameter; Oxoid) were placed on the inoculated agar surfaces and impregnated with required quantity of plant extracts using micropipette and they were allowed to dry in air.

b) Culture media preparation and sterilization: The media used for antibacterial and antifungal test was Nutrient agar (NA, Himedia, India) and potato dextrose agar (PDA, Himedia, India) respectively. The media prepared was then sterilized by autoclaving at 121 °C for 20 minutes.

c) Inoculum preparation: The pathogenic bacteria were maintained as pure cultures in nutrient agar slants with periodic sub culturing of every 4-5 days. The bacteria were inoculated into nutrient broth and incubated at 37 °C for 4 hours. Fungi were maintained as pure culture in potato dextrose agar with periodic subculturing of every 7-8 days and were inoculated in potato dextrose broth for 6 hours.

d) Microorganisms Used: The microorganisms used for antimicrobial studies were obtained from Microbial Type Culture Collection (MTCC), Chandigarh. The different bacterial strains (Gram positive and Gram negative) used in the present study were *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC 96), *Streptococcus pyogenes* (MTCC 442), *Vibrio cholerae* (MTCC 3904), *Klebsiella pneumoniae* (MTCC 7407) and *Salmonella typhimurium* (MTCC 733). The different fungal strains used were *Aspergillus niger* (MTCC 961), *Aspergillus fumigates* (MTCC 4333), and *Penicillium chrysogenum* (MTCC 5108).
2.9.2 Antibacterial activity - Agar-well diffusion assay

The antibacterial activity of hexane, chloroform, ethyl acetate, ethanol and aqueous extracts of different parts of *C. pareira* was performed by agar well diffusion assay by the method (Adegoke *et al.*, 2010). Different bacteria were inoculated separately on the solidified Muller Hinton agar (MHA) in separate Petri dishes by streaking with sterilized cotton swabs and allowed to set wells of 5 mm diameter and 5 mm depth in the solidified agar using a sterile borer. About 10 µl of test samples (1000 mg/ml) were dispensed into the wells and allowed to stand about 15 minutes for pre-diffusion of samples. 10 µl at a concentration of 100 µg/ml was loaded into respective wells for each agar plates as references (positive control) for bacterial species, whereas 99.5 % oof DMSO was used as negative control. The plates were then incubated at 37 °C for 24 hours and the sensitivity of the test bacteria to the extracts were determined by measuring the diameters of the zone of inhibition surrounding the wells. The entire tests were performed in triplicates.

2.9.3 Disc diffusion assay

The antibacterial activity of different leaf extracts such as hexane, ethyl acetate and ethanol showed highest activity in agar-well diffusion assay were selected for disc-diffusion assay (Kirby *et al.*, 1996). 20 ml of sterile Muller-Hinton agar (Hi-media, Mumbai) were poured in to Petri plates and the test culture (100 µl of suspension containing $10^8$ CFU/ml bacteria) were swabbed on the top of the solidified media and allowed to dry for 10 minutes. The disc were impregnated with 10 µl of the selected extracts at three different concentrations such as 1.25, 2.5 and 5.0 mg /disc and placed on the surface of the medium and kept it as such for 30 minutes at room temperature for compound diffusion. Filter discs impregnated with 10 µl of DMSO
was used as a negative control, while streptomycin (10 μg/disc Hi Media) was used as positive control. The plates were incubated for 24 hours at 37 °C. Zone of inhibition were recorded in millimetres (mm). Each antimicrobial assay was performed in triplicate and mean values were reported.

2.9.4 Antifungal activity

The antifungal activities of solvent extracts (hexane, chloroform, ethyl acetate, ethanol and aqueous) of leaf, root and stem of *C. pareira* were carried out in potato dextrose agar (PDA) plates by agar well diffusion method (Adegoke *et al*., 2010). Pure fungal strain were swabbed over the surface of PDA plates using sterile cotton swab and then wells of 6 mm diameter were made over agar plates. Each well was then loaded with 100 μl of sample containing 500 mg of extract which was prepared using DMSO. 10 μl of fluconazole at a concentration of 100 μg/ml was loaded into respective wells for each agar plates as references (positive control) and 99.5% of DMSO was used as negative control. The plates were incubated for 72 hours at 37°C. The antifungal activity was determined in triplicates by measuring the diameter of the inhibition zone around the well.

2.9.5 Determination of minimum inhibitory concentration (MIC) via micro dilution method

The ethyl acetate leaf extract was selected to carryout MIC, since the antimicrobial activity was proved to be high in the same. MIC was assessed in 96 well polystyrene plates. About 100 ml of different concentrations of ethyl acetate leaf extract were prepared for test bacterial strains (50, 90, 130, 170, 210, and 250 μg/ml) and fungal strains (50,100,150, 200,250 and 300 μg/ml), whereas 100 ml of different
concentrations of streptomycin (0.1, 1, 5, 10, 15 and 20 µg/ml) and fluconazole (1, 5, 10, 20, 40, and 60 µg/ml) were prepared and used as positive controls. Then leaf extracts and control samples (positive) were loaded individually in the six wells of 96 well plates. On the other hand, six wells were free of extract and antibiotics which served as negative control. 100ml each of test bacteria (2×10⁸ cells/ml) and fungal cultures (1×10⁷ cells/ml) were added to the respective wells and incubated at 37°C and the growth was observed after 24 hours and 72 hours respectively. The least concentration of extract and positive controls, where no bacterial and fungal growth observed were recorded as MIC (Wiegand et al., 2008).

2.9.6 Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

MBC and MFC of ethyl acetate leaf extract and positive control were tested against bacterial and fungal strains by the method of Hasson et al. (2011). A loopful of culture from each MIC test dilutions were streaked in to freshly prepared MHA and PDA plates for bacterial and fungal strains individually and incubated at 37°C for 24 hours and 72 hours respectively. The concentration of the extract and positive control, which did not show any notable growth of bacterial and fungal strains were considered as MBC and MFC respectively.

2.10 In vitro antioxidant activity of potent plant extracts of Cissampelos pareira

Based on phytochemical screening and antimicrobial activity of various parts of C. pareira in different solvents, the leaf extracts such as ethyl acetate, ethanol and hexane showed to be highly potent and were selected for antioxidant study. The antioxidant properties of leaf extracts of C. pareira were determined with reference to DPPH assay, hydrogen peroxide radical scavenging assay, nitric oxide radical scavenging assay, superoxide radical scavenging assay and reducing power assay.
2.10.1 1, 1 diphenyl 2, picryl hydrazyl (DPPH) free radical scavenging assay

The scavenging activity of the extracts were estimated by using DPPH as free radical scavenging assay (Madaan et al., 2011). Solution of DPPH (0.1 mM) in methanol was prepared by dissolving 1.9 mg of DPPH in methanol and the volume was made upto 100 ml with methanol. The solution was kept in darkness for 30 minutes to complete the reaction. 1 ml of DPPH solution was added to 1 ml of different (20, 40, 60, 80 and 100 µg/ml) concentrations of extract and allowed to stand at room temperature for 30 minutes. The mixture was measured spectrophotometrically (UV-1800, UV-VIS spectrophotometer, Shimadzu) at 517 nm. The free radical scavenging activity was calculated as using following formula,

\[
\% \text{ Inhibition} = \frac{A_0 \text { blank} - A_1 \text { sample}}{A_0 \text { blank}} \times 100
\]

Where ‘\(A_0 \text { blank}\)’ is the absorbance of the control and ‘\(A_1 \text { sample}\)’ is the absorbance of the sample of test sample. Ascorbic acid was used as standard which has the same concentrations as that of the extract. The antioxidant activity of the sample was expressed as IC\(_{50}\) value, which was defined as the concentration (in µg/ml) of sample that inhibits the formation of DPPH radicals by 50%.

2.10.2 Hydrogen peroxide (H\(_2\)O\(_2\)) radical scavenging assay

Hydrogen peroxide (H\(_2\)O\(_2\)) is a biologically important oxidant because of its ability to generate the hydroxyl radical which is extremely potent. The ability of the hydroxyl radical to remove or add hydrogen molecules to unsaturated hydrogen bonds of organic lipids makes it potentially one of the most reactive oxidants in biological systems. It has very short half-life, however, restricts its diffusion capability and its potency. A solution of H\(_2\)O\(_2\) (40 mM) was prepared in phosphate buffer (pH 7.4).
Different concentration of plant extracts (20-100 μg/ml) in phosphate buffer were added to H$_2$O$_2$ solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution containing phosphate buffer without H$_2$O$_2$. The ability to scavenge the H$_2$O$_2$ radical was calculated using the following equation:

\[
\% \text{ Inhibition} = \frac{A_0^{\text{blank}} - A_1^{\text{sample}}}{A_0^{\text{blank}}} \times 100
\]

Where ‘$A_0^{\text{blank}}$’ is the absorbance of the control and ‘$A_1^{\text{sample}}$’ is the absorbance in the presence of extract sample. Ascorbic acid was used as standard which has the same concentrations as that of the extract. The antioxidant activity of the sample was expressed as IC$_{50}$ value, which was defined as the concentration (in μg/ml) of sample that inhibits the formation of H$_2$O$_2$ radicals by 50% (Halliwell et al., 1987).

2.10.3 Nitric oxide scavenging assay

Nitric oxide radicals generated from sodium nitroprusside (SNP) was measured using this method (Marcocci et al., 1994). The reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25 °C for 180 minutes in front of a visible polychromatic light source (25W tungsten lamp). The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion which was assayed at 30 minute intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylene-diamine dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide.
and subsequent coupling with naphthyl ethylene – diamine dihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the plant extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations. The experiments were carried out in triplicates and percentage scavenging activity was calculated as follows,

\[
\% \text{ Inhibition} = \frac{A_{0 \text{ blank}} - A_{1 \text{ sample}}}{A_{0 \text{ blank}}} \times 100
\]

Where ‘\(A_{0 \text{ blank}}\)’ is the absorbance of the control and ‘\(A_{1 \text{ sample}}\)’ is the absorbance in the presence of extract sample. Ascorbic acid was used as standard which has the same concentrations as that of the extract. The antioxidant activity of the sample was expressed as IC\(_{50}\) value, which was defined as the concentration (in \(\mu\)g/ml) of sample that inhibits the formation of nitric oxide radicals by 50%.

2.10.4 Superoxide radical scavenging assay

The ability of the extract sample to scavenge superoxide was determined by superoxide anion scavenging activity (Srinivasan et al., 2007). The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 ml of Nitroblue tetrazolium (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml plant extracts of different concentrations (20, 40, 60, 80, and 100\(\mu\)g/ml), and 0.5 ml Tris – HCl buffer (16 mM, PH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25\(^\circ\)C for 5 minutes and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The experiments were performed in triplicate and percentage scavenging activity was calculated as follows,

\[
\% \text{ Inhibition} = \frac{A_{0 \text{ blank}} - A_{1 \text{ sample}}}{A_{0 \text{ blank}}} \times 100
\]
Where ‘$A_0$ blank’ is the absorbance of the control and ‘$A_1$ sample’ is the absorbance in the presence of extract sample. Ascorbic acid was used as standard which has the same concentrations as that of the extract. The antioxidant activity of the sample was expressed as IC$_{50}$ value, which was defined as the concentration (in $\mu g/ml$) of sample that inhibits the formation of superoxide radicals by 50%.

2.10.5 Reducing power assay

The reducing power of the crude plant extracts and standard (Ascorbic acid) were determined according to the method of Yen and Chen (1995). The plant extracts (20, 40, 60, 80 and 100 $\mu g/ml$) were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 minutes. A portion (2.5 ml) of 1% TCA was added to the mixture and then centrifuged at 5,000 g for 10 minutes. The upper layer of the solution was mixed with distilled water and 0.1% FeCl$_3$ with a ratio of 1: 1: 2 and the absorbance were measured at 700 nm. Increased absorbance of the reaction mixture indicates an increase in reducing power. The experiments were performed in triplicate.

2.11 Statistical Analysis

The results obtained in the present study were expressed as mean ±SD and were analyzed using One-way ANOVA at 5% significance level. Further a multiple comparison test was conducted to compare the significant differences amongst the parameters by using SPSS 16.0 (SPSS Inc., Chicago, USA). Pearson’s correlation was used to determine the correlation of data between DPPH free radical-scavenging assay, H$_2$O$_2$ radical scavenging assay, nitric oxide scavenging assay, superoxide radical scavenging assay and reducing power assay on total phenolic and flavonoid content. The values for p<0.05 were regarded as significant and p<0.01 as highly significant.
2.12 Isolation and structure elucidation of bioactive compound from *Cissampelos pareira* by silica gel column chromatography

Based on the antimicrobial and the antioxidant activity of various leaf extracts of *C. pareira*, it was observed that the ethyl acetate extract exhibited significant antimicrobial and antioxidant activity. Hence ethyl acetate leaf extract was focused for the isolation and characterization of bioactive compounds.

2.12.1 Separation of phytoconstituents using silica gel column chromatography

2.12.1.1 Extraction of plant material

The fresh leaves of *C. pareira* were collected and cut into small pieces and air dried for several days. The plant materials were then ground into coarse powder. The dried and ground plant powder (500 g) was extracted with ethyl acetate (2.5 liters) in an air tight clean flat bottomed container for 7 days at room temperature with occasional stirring and shaking. The extract was then filtered first through a fresh cotton plug and finally with a Whatman filter papers. The filtrate was concentrated using a rotary evaporator (Heidolph, UK) at low temperature and pressure for further investigation and separation of phytoconstituents.

2.12.1.2 Column packing and fractionation of plant material

In order to isolate the bioactive compound from the crude extracts, they were further fractionated using column chromatography (Furniss and Vogel, 1989). A column of 450 x 40 mm was washed well, dried and was aligned in a vertical position. The lower end of the column was plugged with absorbant cotton wool. The column was clamped and fitted in vertical position on a stand. Activated silica gel (60 – 120 mesh) was packed onto the glass column using n-hexane solvent and allowed to settle gently, until the necessary length of the column was obtained. For
the isolation of bioactive compound, 15 g of crude ethyl acetate leaf extract was loaded on the top of silica gel. The column was eluted with different polarity of solvents stepwise at a flow rate of 1 ml/minute, first with 500 ml of equilibration solvent (n-hexane), 2000 ml of hexane : chloroform (75 : 25 to 10 : 100 V/V), 2000 ml of chloroform : ethyl acetate (75 : 25 to 0 : 100 V/V), 2000 ml of ethyl acetate : methanol (75 : 25 to 10 : 100 V/V). The fractions measuring 10 ml each were collected and concentrated by using rotary evaporator and were analysed by thin layer chromatography.

2.12.1.3 Thin layer chromatography

Thin layer chromatography was performed based on the method of Stahl, (1969). An aliquot of all the concentrated fractions were loaded in activated silica gel TLC plates (20 x 20 cm). The plates are developed using hexane: chloroform (80: 20), chloroform: ethyl acetate (90 : 10) and ethyl acetate : methanol (90 : 10) as the solvents. The spots were located by exposing the plates to iodine fumes. Fractions having same number of spots with similar $R_f$ values on the TLC plates were pooled. The pooled fractions were numbered.

2.12.1.4 Antioxidant activity of fractions

The antioxidant activities of collected fractions were tested using DPPH assay (Madaan et al., 2011) and hydrogen peroxide ($H_2O_2$) radical scavenging assay (Halliwell et al., 1987) as mentioned earlier.
2.13 Identification and characterization of the isolated phytoconstituents using NMR, FT-IR spectral analysis and Mass spectrometry

Based on invitro antioxidant activity of major fractions, the purified active isolated compound was subjected to spectral analysis and mass spectrometry.

2.13.1 $^1$H and $^{13}$C NMR spectral Analysis

The $^1$H NMR and $^{13}$C NMR spectrum was recorded on Bruker’s 300MHz instrument using CDCl$_3$ as the solvent at National Institute for Interdisciplinary Science and Technology (NIIST), Pappanamcode, Trivandrum. The test compound nucleus can be revealed and the number of protons predicted the structure of bioactive isolate with the help of $^1$H and $^{13}$C NMR spectrum analysis (Fischer et al., 2010).

2.13.2 Fourier Transform Infra-Red spectral analysis

The Fourier Transform Infra-Red spectrum was recorded on Perkin Elmer 1310 model at National Institute for Interdisciplinary Science and Technology (NIIST), Pappanamcode, Trivandrum. The isolated active compound was grinded with KBr and pellets were made and FT-IR spectrum was recorded in Shimadzu FT-IR spectrometer by KBr pellet method. FT-IR gives a strong absorption pattern at a particular frequency for a particular functional group. It is a powerful tool for structure elucidation (Silverstein et al., 2005).

2.13.3 Mass spectral analysis

The electrospray mass spectrum for the isolated compound was recorded on a Thermo Finnigan LCQ Advantage max ion trap mass spectrometer at National Institute for Interdisciplinary Science and Technology (NIIST), Pappanamcode, Trivandrum. The 10 µl sample (dissolved in solvent such as methanol / acetonitrile /
water) was introduced into the ESI source through Finnigan Surveyor autosampler. The mobile phase (90:10 MeOH/ACN: H2O) flowed at the rate of 250 µl/minute by Ms pump. Ion spray voltage was set at 5.3 KV and capillary voltage 34V. The MS scan runs up to 2.5 minutes and the spectra print outs are averaged of over 10 scan at peak top in total ion chromatogram. The mass spectrum gives information on various types of peaks and determining the molecular formula for the isolated compound after successful interpretation (Gross, 2004).

2.14 *In vitro* anticancer and cytotoxicity evaluation of isolated compound from ethyl acetate leaf extract of *Cissampelos pareira* using MTT assay

Anticancer and cytotoxicity activity of the isolated compound was determined through MTT cytotoxicity assay (Mosmann, 1983).

2.14.1 Cell lines and culture conditions

Human epithelial ovarian cancer cells, PA-1 and OWA-42 were used as cancer cell lines and L929 (Fibroblast cells was used as normal cell line (control). Both cell lines were obtained from National Centre for Cell Sciences, Pune, India. The cells were cultured in 75 cm² flask containing Dulbecco's Modified Eagle Medium (DMEM medium), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1.5 g/l sodium bicarbonate (Gibco), 10,000 U/ml penicillin (Gibco), 10 mg/ml streptomycin (Gibco), amphotericin B (0.25 mg/ml), Cells were cultured as monolayers in culture flasks at 37 °C in a humidified atmosphere containing 5 % CO₂ at 37 °C. Cells were regularly passaged and maintained before the start-up of experiment. Upon reaching 80-90% of confluence, the cells were trypsinized and sub cultured in healthy condition and exponentially growing cells were used for experiments.
2.14.2 Cytotoxicity assay

For cytotoxicity assays, human epithelial ovarian cancer cell lines PA-1 and OWA-42 and L929 (normal fibroblast cell lines) were seeded in 96 well microliter tissue culture plates and allowed to attach for 24 hrs. After 24 hrs of incubation, 100 µl of various concentrations of the isolated compound was added to the well to obtain a final concentrations of 0.62, 1.25, 2.5, 5 and 10 µg/ml respectively and then the cells were further incubated for 72 hours at 37 °C. The solvent DMSO treated cells served as the control. After 24 and 72 hrs of treatment the cell viability was determined by MTT assay. The proliferation test is based on the colour reaction of mitochondrial dehydrogenase in living cells by MTT [3-(4, 5-Dimethylthiazol-2-Yl) -2, 5-Diphenyltetrazolium Bromide] (Sigma, M-5655). At the end of the treatment period, 15 µl of MTT solution (5 mg MTT/ml of (PBS) phosphate buffered saline, pH 7.2) was added to each well, which was then further incubated at 37 °C in 5% CO₂ for 2-4 hrs. After incubation, MTT containing medium was gently replaced by inverting the plate and the coloured crystals of produced formazan were solubilized by adding 150 µl of DMSO. The assay was done in triplicates. The absorbance was measured at 540 nm on micro plate reader (Biotek, ELx800-MS). Doxorubicin hydrochloride, an anticancer drug was used as a positive control. DMSO was used as control (vehicle control). After treatment, cells were observed under microscope for morphological changes. The cytotoxicity of the isolated compound was expressed as IC₅₀ value (concentration of extract inhibiting cell growth by 50%). IC₅₀, the concentration of compound required to inhibit 50 % cell growth, was determined by using GraphPad Prism Software (GraphPad Prism version 5.0, GraphPad Software, San Diego California USA Anonim-c). The assay
was carried out in triplicates. Percentage viability of isolated compound against all cell lines was calculated using the formula:

\[
\% \text{ of cell viability} = \frac{\text{Absorbance of isolated compound treated cells}}{\text{Absorbance of control cells}} \times 100
\]

Results obtained in the present study were expressed as mean ± SD and were analyzed using one-way ANOVA at 5% significant level. Further a multiple comparison test was conducted to compare the significant differences amongst the parameters by using SPSS 16.0 (SPSS Inc USA).