3. Materials and methods

3.1. Geographical location of Silent Valley

Silent Valley occupies Palakad districts of Kerala (Southern western Ghats) and cover an area of 236.74 km² and lies between 11°03’ to 11°13’N latitude and 76°2’ to 76°3’E longitude (Fig.1). The vegetation is floristically rich compared to other regions of Southern-Western Ghats and represents several unique habitats. The local communities adjust to the forest have access right over the forest as stipulated in the village forest management plan by-laws.

3.2. The name ‘Silent Valley

Silent Valley forests locally known as Sairandhrivanam, one linked to the mythological character, Droupadi in Mahabharata. Also the river Kunthipuzha named after Kunthi Devi, mother of Pandavas of Mahabharata, runs through the Silent Valley in north-south direction. It is popularly believed that Pandavas lived here with their consort Droupadi. It is also believed that Silent Valley has been given the name due to the absence of cicada insects which usually produce a distinct sound in tropical forests. However, cicadas have started to inhabit in these forests.

3.3. Physiographic features and Climate

Silent Valley is roughly a rectangular table land, located at the south-western corner of Nilgiris. It is closed on all sides with high and continuous ridges along the entire north, northeast and east with steep escarpments along the western and southern border. The whole is thus shielded from the extremes of climate as well as anthropogenic intervention and also it remains an ecological Island with a special microclimate. The Palakad district is experience dry climate with high temperature. In contrast to this, Silent Valley receives maximum rain, enjoys mist and low temperature. Both south west and north east monsoons are active in Silent Valley with a precipitation of 3180 mm per annum and the highest rain fall is recorded during the month of July (885.8mm). Average minimum
temperature ranges from 8-14 °C and average maximum from 23-29°C. The highest temperature is experienced during May (30°C) and the lowest during January (7°C). Maximum precipitation during south-west monsoon brings over 500mm annually.

3.4. Study area

The tribal regions are the remote area of Palakad district, Kerala where the people have no urgent access to modern medicinal facilities. Therefore, the traditional medicines are the preferred for such people. There is no hospital facilities for intimate treatment of people in the remote area of Silent Valley, and people rely on indigenous medicinal plant for basic health care treatment. The majority of tribal peoples are speaking in Tamil but only a limited number of people are speaking Malayalam at study areas. But, the tribal are speaking their mother tongue (Aadhivaasi paasai) during the conversation within the tribal communities. Life styles of people are very poor and they are economically depends on cattle grazing, agriculture and natural resources. Before this ethnobotanical investigation, we got research permission from the Principal Chief Conservator of Forests (Wild life) and Chief Wildlife Warden and The Director, Scheduled Tribes Development Department, Thiruvananathapuram, Kerala. The Research Permission order No. D3.199/2014.

3.5. Data collection

The study areas were investigated to get information from the local communities of Silent Valleys. Because, they are having good practical knowledge about medicinal plants were interviewed in 5 villages (Agali, Kottathara, Padavayal, Sholayur and Mannarkad in palakkad district) (Fig.2). During the course of the study, twenty four field trips were carried out in the study area totally 90 days were spent with their local people. Methods of selecting informants depended upon the distribution of local people having sound knowledge. They were requested to collect specimens of the plants they know or to show the plant species on site. (Fig. 3 & Fig.4). These informants were traditional healers themselves or had tradition of healing in their families and had knowledge of the medicinal use of the plants. The wealth of medicinal plant knowledge among the people of
this district is based on hundreds of years of beliefs and observations. This knowledge has been transmitted orally from generation to generation. However it seems that it is vanishing from the modern society since younger people are not interested to carry on this tradition.

3.6. Interview with tribals

In the total of eight informants, six men and two women were identified between the ages of 42 to 75 to get the ethno-medicinal information through direct interviews or oral conservations (Appendix A). They were selected based on their knowledge of medicinal plants within their families and neighbours. The questionnaires were used to obtain information on medicinal plants with their local names, parts used any other plants/agents used as ingredients mode of preparation and administration etc, were recorded for each collected ethnomedicinal plants. A field data sheet has been prepared to record the plant details with ethno-medicinal information gathered from the traditional healers.

**Questionnaires (Appendix A)**
1. Participant’s name and surname.
2. Age and gender of the participant.
3. Pen telephone and address of the participant.
4. Educational qualification of the participant.
5. Date of interview.
6. Name of the participant’s residential address place.
7. How long do you live in the residential place?
8. Name the used local plant.
9. Mention the disease cured by the plant.
10. Mention the part of the plant would you use.
11. How can you make the plant for use?
12. Do you know how and when will you use the plant?
13. Name the dose do you use approximately.
14. Mention the duration taken by the convalescence period.
15. Is there any complication occurred from the plants used by you?
3.7. Preservation of plant specimens

Standard method was followed with record to collection of plant materials, drying, mounting, preparation and preservation of plant specimens (Jain, 1964). Voucher specimens of medicinal plants in triplicate were collected prepared and identified. Plants with their correct nomenclature were arranged alphabetically by family name, vernacular name ethnomedical uses. The identification and nomenclature of the listed plants were based on the Flora of Presidency of Madras (Gamble, 1935) and the Flora of Tamil Nadu Carnatic (Matthew, 1983). These specimens were later verified at Rapinat herbarium, St. Joseph College Trichy (Fig. 5). All the preserved specimens were deposited at A.V.V.M. Sri Pushpam College (Pushpam Herbarium Cabinet (PHC)), Poondi, Thanjavur, India.

3.8. Ailment categories

Based on the information obtained from the traditional healers in the study area, all the reported ailments were categorized into 15 categories (Table 1) viz. Gastro intestinal ailments (GIA), dermatological infections/diseases (DID), respiratory systems diseases (RSD), genito-urinary ailments (GUA), fever (FVR), skeletonmuscular system disorders (SMSD), poisonous bites (PB), circulatory system/cardiovascular diseases (CSCD), endocrinal disorders (ED), liver problems (LP), dental care (DC), hair care (HC), ear, nose, throat problems (ENT), cooling agents (CA) and general health (GH). Several diseases were placed in one ailment category based on the body systems treated.

3.9. Data analysis

3.9.1. Informant consensus factor (Fic)

The informant consensus factor (Fic) was used to see if there was agreement in the use of plants in the ailment categories between the plant users in the study area. The Fic was calculated using the following formula (Heinrich et al., 1998)

$$F_{ic} = \frac{N_{ur} - N_t}{N_{ur} - 1}$$
Where Nur refers to the number of use-reports for a particular ailment category and Nt refers to the number of taxa used for a particular ailment category by all informants. The product of this Factor ranges from 0 to 1. A high value (close to 1.0) indicates that relatively few taxa are used by a large proportion of the informants. A low value indicates that the informants disagree on the taxa to be used in the treatment within a category of illness.

3.9.2. Use value (UV)

The relative importance of each plant species known locally to be used as herbal remedy is reported as use value (UV) and it was calculated using the following formula (Phillips et al., 1994).

\[ UV = \frac{\sum U}{n} \]

where UV is the use value of a species, U is the number of use reports cited by each informant for a given plant species and n is the total number of informants interviewed for a given plant. The UV is helpful in determining the plants with the highest use (most frequently indicated) in the treatment of an ailment. UVs are high when there are many use-reports for a plant and low when there are few reports related to its use.

3.9.3. Fidelity level (FL)

To determine the most frequently used plant species for treating a particular ailment category by the informants of the study area, we calculated the fidelity level (FL). The FL was calculated using the following formula (Friedmen et al., 1986). Where Np is the number of use-reports cited for a given species for a particular ailment category and N is the total number of use reports cited for any given species. Generally, high FLs are obtained for plants for which almost all use-reports refer to the same way of using it,
whereas low FLs are obtained for plants that are used for many different purposes (Srithi et al., 2009).

\[
FL \ (\%) = \frac{N_E}{N} \times 100
\]

3.10. Antimicrobial activity

All the plant extracts were tested for antimicrobial activity against five bacterial strains and a fungus.

3.10.1. Preparation of plant extracts

To 500g of each sample powder, 1500 ml of each solvent, viz. chloroform, acetone, methanol and water was added serially for preparing the extracts in increasing solvent polarity, and in reverse order for decreasing solvent polarity. Extraction with each solvent was done for 24 h at room temperature, after which the supernatant of each solvent was recovered by filtering through Whatmann filter paper. This process was repeated thrice and the respective solvent from the supernatant was evaporated in a rotary vacuum evaporator to obtain the crude extract. These extracts (both increasing and decreasing polarity) were stored at 4° C until used for the evaluation of antimicrobial activity. The method was followed by Sharma et al, (2012).

3.10.2. Microbial strains

Antimicrobial activities were tested against five bacterial and a fungal strain includes two Gram-positive strains like Bacillus subtilis (ATCC6633) and Staphylococcus aureus (ATCC6538), three Gram-negative bacterial strains like Neisseria gonorrhoeae (ATCC43070), Chlamydia trachomatis (ATCC VR1477) and Treponema pallidum (ATCC35580) and a fungus Candida albicans (ATCC10231) were obtained from American Type Culture Collection (ATCC) at Manassas, USA through Alpha Omega Hi-Tech Bio Research Centre at Salem, Tamil Nadu, India.
3.10.3. Inoculums preparation

Bacterial strains were sub cultured on overnight at 35°C in Muller- Hinton agar slants. The colonies were transferred using 5ml of sterile saline water, standardized at 350nm (equivalent to harf McFarland). This gave a stock suspension of microorganisms equal to 1×10^6 CFM/ml saline. Sabouraud dextrose agar (SDA) was used for subculturing C. albicans at 30°C for 24hr. As previously mentioned bacterial pathogens a suspension of 1×10^5 CFU/ml saline was prepared (Parakh and Chanda, 2008).

3.10.4. Antimicrobial Assay

Agar well diffusion was used to evaluate the antimicrobial activity of each plant extract as reported by Valgas et al, (2007). The same procedure was also used in disk diffusion method; the agar plate surface is inoculated by spreading a volume of the microbial inoculums over the entire agar surface. Then, the wells were dispensed with the respective well (20-100ml) at preferred concentrations. Chloramphenicol (40µg/mL), Cefotaxime, Azithromycin, Doxycycline and Clotrimazole (5µg/mL are used as positive controls. Then, the agar plates were incubated in 24 h at 37±2°C for bacterial strains and 30±2°C 5n 72-96 h for fungal strain. The assay was carried out in triplicates. The measurements of zone inhibition were taken from the top of the well to the clear zone in millimeter (mm).

3.10.5. Statistical analysis

The Simple Arithmetic mean was used for data analysis and Standard Error (SE) used for compare the data with controls.

3.11. GC-MS (Gas Chromatography-Mass Spectrometry) analysis
The phytochemical investigation of extract was performed on a GC-MS equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra ver.:2.2, Thermo TSQ QUANTUM XLS Experimental conditions of GC-MS system were as follows: DB 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25μm. Flow rate of mobile phase (carrier gas: He) was set at 1.0 ml/min. In the gas chromatography part, temperature programme (oven temperature) was raised 40 °C to 290 °C at 5°C/min and injection volume was 1.0 μl. A scan interval of 0.5 seconds with scan range of 40-600 m/z. Total GC running time was 35min and the results were compared by using Wiley Spectral library search program.

3.11.1. Extraction and fractionation of sample

Hibiscus rosa-sinensis flowers were dried under shadow conditions for identify the chemical components of the extracts. Then, the 50gm of powdered flower was successively extracted with 500ml of methanol by using a soxhlet apparatus. The methanol extract was collected after complete filtration and then evaporated the solvents for dryness of the sample under reduced pressure in Rota vapor. The paste form of the extracts was used for identifying the phytochemicals by GC-MS. It was carried out in the solvent of methanol flower sample.

3.12. Computational studies

Now a day’s computational analysis one of the prominent studies for research. Additionally, the computational tools are helps to identify new drug molecule for Pharmaceutical sectors.

3.12.1. Biological database source

GC-MS identified Hibiscus rosa-sinensis flower molecules were retrieved from the chemical database (www.chemspider.com). The macromolecule of N. Gommorhea enzyme was retrieved from protein data bank (www.rcsb.com). The protein data bank albeta numeric ID is 4M98.
3.12.2. Computational tools

Pharmacoinformatic analysis was carried out in Schrodinger computational suite of Maestro 10.2 version packages like ligprep, sitemap, glide SP and grid generation (Schrodinger, LLC, New York, 2015). This software put together in DELL PRECISION T1700 workstation machine running on Intel (R) Core (TM) i5-4590 CPU processor with 8GB RAM and 240 GB hard disk with centos Linux as the operating system.

3.12.3. Protein preparation

The target was prepared by using Protein Preparation Wizard tool in Maestro 10.2 version. It is very important step in this study, because that are solved lot of target molecule problems includes missing side chains, back chains are added and also updated the molecule missed residues. After this preparation, there the workspace was analyzed for found the target problems. It was displayed the target heteromers and water molecules. Generally, X-ray crystallography of the structure is very difficult to binding with the water molecules. Through this process, we evacuated those water molecules from target and it also increases the entropy of target.

3.12.4. Validation of binding site and Grid generation

Binding site validation is a vital role on the route of molecular docking. It is displays the active sites in target molecule. This process was covered entire protein molecule for validating their active sites by using sitemap tool. It also showed the binding cavity active residues, volume of site and site score. Then, the suitable site was taken for grid generation which is used to fix the target site for docking. Thereafter, the known molecules are docked with *N. gonnorhea* protein in order to find out the docking parameters with the help of Grid-based ligand docking (Glide, module 4.4. module, Schrodinger, LLC, New York, 2012). Centroid of the target was selected from the target.
Docking at the centroid of binding cavity grid box is generated with X: 5.3; Y: 3.71; Z:-20.86 coordination. This site was explained with a 10 Åo radius around the ligand binding cavity on the target. Besides, ligand placed at various grid positions and then rotated it around three Euler angles.

3.12.5. Ligand preparation

Ligand molecules of the phytocompounds were converted into the 3D structure by using ligprep tool. The ligand was drawn geometry optimized via Optimized Potentials for Liquid Simulations 62005 (OPLS62005) force field (Ligprep, Version 2.2, 2015, Schrodinger suite Maestro 10.2 version). Partial atomic charges are also computed by the OPLS62005 force field. Ligprep tool was used the purpose of 3D structures generating from 1D (Smiles) to 2D (SDF) representation, probing for tautomers and steric isomers and geometry minimization of ligands.

3.12.6. Molecular docking

Molecular docking performs rigid flexible docking for predicting the ligand binding affinities, efficiency and inhibitory constant. All the ligands were docked with a active site of target using Glide Standard precision docking mode (SP). There, the active compounds only will have available posses that avoid these penalties and also get receive favourable scores with accurate hydrophobic contacts between protein and ligand.

3.13. Plant material

The flowers of *Hibiscus rosa-sinensis* were collected from Silent Valley, Kerala. The plant was authentically identified at Rapinat herbarium centre, St. Josephs College, Trichy. The RHC identification number is JEM001. Then, the voucher specimen (PHC67) was renamed before deposition at Pushpam Herbarium Cabinet (PHC), Department of Botany, A.V.V.M Sri Pushpam College (Autonomous), Poondi, Thanjavur.

3.13.1. Extract preparation
The collected flowers were washed under running tap water for remove the unwanted particles on sample. Then, the sample was dried under shadow condition and it was ground fine powder with the help of Bajaj mixer. The powdered sample is filled in air tight container prior to the extraction. Based on the antimicrobial study, the efficient solvent extract was taken as further study.

3.13.2. Chromatographic techniques

Chromatography has been developed into a new method of separation of mixture of substances mainly when they are available in small amounts. This method is very useful when the components of a mixture have almost the same physical and chemical properties.

3.13.2.1. Preparation of Thin Layer Chromatography Plates (PTLCP)

25g of Merck silica gel was suspended in 50mL of deionised water and shaken vigorously for 45 seconds in rubber-stopper Erlenmeyer flask (500mL). The thickened slurry was poured into the glass and pulled with a ruler in two sides at 1 mm trailing edge to prepare similar plates. Then, the plates were allowed to air dry 30 minutes in the oven at 50°C (until they turn white). After preparation of the plates, a few drops of the methanol extracts were applied (using a capillary tube) to the bottom of each of the pre-coated and pre-heated (50°C for 30 minutes) glass plates. After 5 minutes of drying, each of the plates was placed in the separate glass chamber with solvent system Hexane: acetone (9:1 v/v) as the mobile phase. The different solvent systems were tried as mobile phase, of which Hexane: acetone (9:1) and methanol: chloroform (9:1) gave clearly visible spots hence tried with that mobile phase.

After the movement of solvent at the top of the plates, each plate was removed from the glass chamber and separately air-dried. After air-drying, the produced spots were located by their fluorescence under long and short wave UV light (254 and 366 nm respectively).

3.13.2.2. Column chromatography (CC)

The extract residue was applied onto a silica gel column (200 g, 100cm x 3.5 cm),
to isolate the active compound from the crude extracts using hexane as a solvent and the polarity was increased by hexane then chloroform and fractions (100 mL) each were collected. The obtained fractions were concentrated and monitored by TLC using hexane, acetone and methanol (97:2:1%) as mobile phase. Fraction A₁ eluted with chloroform: methanol (9:1), showed spots in solvent system chloroform and methanol (98:2%). Fraction A₁ was re-chromatographer on silica gel column (25 g, 2cm x 100cm), elution was carried out beginning with chloroform and methanol independently. No spots were found in hexane: acetone. To separate the compounds, it was tried to increase the polarity of the element. For this purpose, polarity was increased gradually with successive addition of acetone. At 0.5% addition of acetone, the compound 1 was started to coming out to yield 60 fractions (20 mL each). The solvent evaporated and the fractions were TLC monitored. The Rf value was found to be 0.57. They were combined and re-crystallized from acetone to pale yellow colour. The collected fractions were concentrated using a Rotavapour. TLC was used to analyze the active compound, and those with similar chemical components were pooled together. The pure compound was dried in pre-weighed pill vial and its mass (g/ml) determined.

3.13.2.3. Thin layer chromatography (TLC)

TLC plates were supplied by Merck, Germany (TLC Silica gel 60 F254) was used to observe the separation of individual compounds as a single spot were trimmed and the position of the origin marked by a straight line. The column fractions of sample was spotted on the origin and put in a lidded tank containing a solvent system. The procedure was followed with other plates and various solvent-solvent ratios (chloroform: methanol in the ratio 8:2) until good resolution was noticed. The level of solvent in the tank was about 1 cm beneath the origin. The solvent travelled up the plate by capillary action till it reached the solvent front (also marked by a straight line across). The lid was lifted off and the plate is dried before it was viewed by spraying with silver nitrate and iodine vapour and
visualized under UV light to identify and confirm the compounds eluted through column chromatography.

\[
R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent front}}
\]


Methanol flower extract of *Hibiscus rosa-sinensis* was chromatographed on a silica gel column, eluting with solvent mixtures of increasing polarity composed of methanol and chloroform, (90%:10%). Then, the fractions were collected. The purity of all the fractions were collected and analysed by thin layer chromatography on silica gel with methanol and petroleum ether (9:1) the solvent mixture. Spots were visualized by spraying the plates with 20% antimony chloride in chloroform (spray reagent). The fractions collected with methanol: petroleum ether (increasing polarity of methanol up to 90%), were pooled together, after showing a single spot of same Rf value.

3.15. Spectral analysis
3.15.1. Fourier-transform infrared spectroscopy (FTIR)

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract (Eberhardt et al., 2007; Hazra et al., 2007). In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to and then compressed into a thin pellet which can be analyzed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution then placed onto a single salt plate. The solvent is
then evaporated off, leaving a thin film of the original material on the plate. The PTLC leaf isolates was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000-450 cm\(^{-1}\) in FT-IR spectroscopy (Perkin Elmer FT-IR Spectrometer, USA).

### 3.15.2. Mass spectrometry

The electron-impact (EI) mass spectra of many hundreds of sesquiterpene hydrocarbons and oxygenated derivatives of known structures are available as mass spectral libraries (Eschenmoser and Chimia, 1990). The first step in examining a mass spectrum is usually the determination of the molecular ion peak. Knowing the accurate mass of the molecular ion, it is then easy to obtain the molecular formula and from it the number of double bond equivalents of the molecule. It can be helpful as next to note the major fragment ions and attempt to elucidate the main fragmentation pathways. The best way in checking such fragmentation pathways is to look for metastable ions. Knowing the molecular weight and the main features of fragmentation of a compound, it may be possible to make a tentative structural assignment.

### 3.15.3. NMR spectroscopy

NMR-Spectra are spectra of the precession frequency of nuclei with a magnetic moment in a static field. The position of the NMR signal (i.e. the resonance frequency) is called the chemical shift \(\delta\). The values of the chemical shift \(\delta_H\) and \(\delta_C\) in \(^1H\)NMR and \(^{13}C\)NMR spectra give informations on partial structures and functional groups contained in the studied molecule. Coupling constants between protons which can be obtained from the fine structure of signals help to identify neighbour protons in building up of partial structures. The resolution of one dimensional \(^1H\)-NMR spectra is rarely good enough to enable the calculation of all the important coupling constants (Martin and Crouch, 1991; Sanders and Hunter, 1990).

### 3.16. Agar-well diffusion method
Identification and characterization of antimicrobial compounds from selected ethnomedicinal plants of Silent Valley (Western Ghats, Kerala) with emphasis on Venereal diseases

Antimicrobial activity was determined by agar-well diffusion method (Wiswanathan et al., 2012) with modifications according to the present experimental conditions. Different concentrations of *Hibiscus rosa-sinensis* flower extracts (100, 75, 50 and 25 µg/ml) and isolated compound (10, 7.5, 5 and 2.5 µg/ml) were prepared by two-fold dilution method and tested.