Materials & Methods

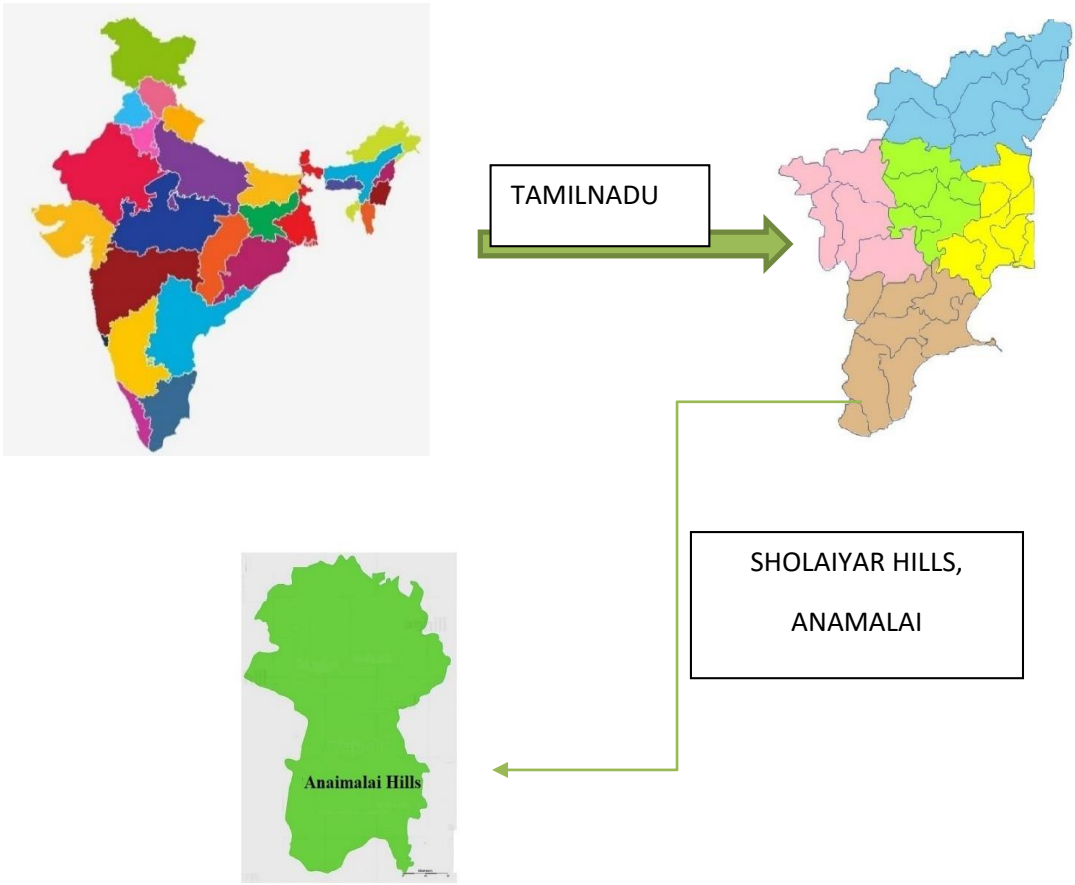
MATERIALS AND METHODS

STUDY AREA

The study was conducted in the surrounding area of Sholaiyar (Fig. 1), Anaimalai hills, Valparai, Coimbatore District, Tamilnadu, India. The Study area Sholaiyar is situated at 3500 feet altitude, 076^{O} 44' 07" E longitude and 10^{O}19 ' 01'N latitude respectively. The Anaimalai hills are one of the largest continuous rain forests in the Western Ghats. Plantations occur in this area include tea, coffee, teak and Eucalyptus.

Anaimalai hills also called as *Elephant Hills*, is a range of mountains in the Coimbatore District of Tamilnadu. The Anaimalai hills are also the tallest of the hills in South India that rises up to 8850 ft. The valuable teak and other timber are in the lower slopes. The coffee plantations are a common sight in some areas. The area was announced as Anaimalai Wildlife Sanctuary in 1974 and established in 1976. There is a 30 Km wide gap between the Nilgiri Hills to the north and the Anaimalai Hills to the South. This gap serves as a major communication route between Kerala and Tamilnadu.

Fig. 1: Map showing the location of the study area Sholaiyar, Anaimalai hills.



Collection and identification of lichen material

Lichen was collected from Sholaiyar, Valparai, Coimbatore District, Tamilnadu, India. It was collected from the bark of silver tree with the help of knife and chisel, in the month of June 2019 without damaging tree trunks. The lichen was authenticated by Dr. Sanjeeva Nayaka, Principal Scientist, Taxonomy division, CSIR—National Botanical Research Institute, Lucknow, India. Herbarium specimen (36008) was prepared and deposited at the herbarium of the Institute.

Identification of lichen species

The lichen was identified on the basis of morphology, anatomy and chemistry with the help of published flora (Divakar and Upreti, 2005; Awasthi, 2007; Singh and Sinha, 2010).

Morphological identification

The external morphology of the lichen was studied under Trinocular microscope.

Anatomical identification

To analyze the anatomical features of lichen and its fruiting bodies, thin hand cut sections were made under Trinocular microscope. The thin dry sections of the thallus and fruiting bodies were immersed in 90% ethyl alcohol to drive off the intercellular or inter-hyphal air bubbles. Then the sections were mounted in lactophenol cotton blue. The following features were observed such as type of algae (green algae or blue green algae); color of epithecium, hymenium, hypothecium; presence of oil globules; type of ascus and its reaction with Iodine; types and color of spores; type of pycnidia and nature of conidia, etc. (Manish Tripathi, 2016).

Chemical identification: (Manish Tripathi, 2016)

Spot test and Thin Layer Chromatography were performed to identify the lichens and to know their chemical constituents.

Spot test:

Spot test is being performed by applying some chemical reagents (K, C, KC, and P) on thethallus and medulla which may lead to change in color. Any change in color is denoted by a positive (+) sign followed by the color produced while no change in color is denoted by a negative (–) sign. The chemical reagents used were as follows.

K test (Potassium hydroxide):

The solution was prepared by dissolving 10 gm potassium hydroxide pellets in 100 ml of distilled water.

C test (Calcium hypochlorite):

It is a freshly prepared smooth solution of calcium hypochlorite or bleaching powder or modern commercial bleaching fluid containing active chlorine. For this test, calcium hypochlorite or bleaching powder or modern commercial bleaching is freshly prepared. In distilled water, Calcium hypochlorite was dissolved in 1:2 ratio or on the other hand,fresh solution of sodium hypochlorite could be used.

KC test (Potassium hydroxide followed by Calcium hypochlorite):

Potassium hydroxide was applied first which was immediately followed by calcium hypochloride on the particular place of thallus.

P test (Para-phenylenediamine):

Solution of para-phenylenediamine was prepared in ethanol ina small quantity because it is unstable and cannot be used for the next day. Steiner's P was prepared by dissolving 1 gm of paraphenylenediamine and 10 gm of sodium sulphate in 100 ml of distilled water with 1 ml of a liquid detergent. It is a more stable solution which keeps the reagent well for about a month.

I test (Iodine):

It is prepared by dissolving 2-5 gm of iodine in water with 0.5 gm of potassium iodide. To study the type of ascus this test is being used.

EXTRACTION

Preparation of lichen extracts

For extraction, lichen thallus was shade dried for about one to two weeks. After drying, it was grinded and stored in an airtight container. The air-dried lichen powdered material (10g) was transferred into 250 ml quick fit flask and extracted in the soxhlet extractor for 48 hours (Vaghasiya *et al*., 2008; Aiyelaagbe and Osamudiamen, 2009) using different Polar (Ethyl acetate, Water and Methanol) and non-polar (Ethanol and Chloroform) organic solvents. The extracts were filtered using Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure to pasty mass (Yilmaz *et al*., 2004) for further studies.

Qualitative chemical evaluation

The different solvent lichen extracts thus obtained were qualitatively tested for the presenceof various phytochemical constituents following the methodologies of (Auwal *et al*., 2014; Wilberforce and Olivia,2017; Kumar Bargah, 2015; De Silva *et al.*, 2017; Roghini and Vijayalakshmi, 2018).

Test for tannins

Ferric chloride Test: 2ml of extract was treated with 0.1% of ferric chloride. Appearance of blue-black coloration indicates the presence of tannins.

Gelatin Test: Five drops of 1% gelatin containing 10% sodium chloride were added to 1 ml of the extract. Formation of white precipitate indicates the presence of tannins.

Test for Flavonoids

Lead acetate Test: 3 ml of extract was added to 10ml of distilled water and then added 1 ml of 10 Sodium hydroxide (NaOH). The change in the color of the mixture to yellow indicates the presence of flavonoids.

Alkaline reagent Test: 5 drops of 5% Sodium hydroxide (NaOH) was added to 1 ml of the extract. Increase in the intensity of yellow color which became colorless after addition of a few drops of 2M hydrochloric acid indicates the presence of flavonoids.

Test for terpenoids

2 ml of the extract was dissolved in 2ml of Chloroform and evaporated to dryness. 2 ml of concentrated Sulphuric acid was then added and heated for about 2 minutes. Appearance of grayish color indicates the presence of terpenoids.

Test for saponins

Foam Test: 5 ml of extract was mixed with water, shaken well and then heated. Formation of froth indicates the presence of saponins.

Olive oil Test: A few drops of olive oil was added to 2 ml of the extract and shaken well. Formation of a soluble emulsion indicates the presence of saponins.

Test for steroids

Salkowski Test: 2 ml of extract was mixed with 2 ml of chloroform. Then, 2

ml of concentrated sulphuric acid was added to the mixture. Formation of reddish-brown coloration at the interface indicates the presence of steroids.

Test for carbohydrates

Molisch's Test: To 2 ml of aqueous extract was added few drops of Ethanolic α - naphthol solution and then added concentrated sulphuric acid from side of the test tube. Formation of violet ring at the junction of the two liquids indicates the presence of carbohydrates.

Fehling's Test: An equal volume of Fehling solution A and B were added to equal volume of extract and heated on a water bath for 10 minutes. Formation of brick red precipitate indicates the presence of carbohydrates.

Test for glycosides

Keller –killiani Test: 2 ml of extract was mixed with 3 ml of chloroform and 10% ammonia solution was added. Appearance of pink color indicates the presence of glycosides.

Test for alkaloids

Hager's Test: To 2 ml of crude extract was added few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids.

Dragendorff's Test: To 2 ml of extract was added 1 ml of Dragendorff's reagent along the side of the test tube. Formation of orange or orange reddish brown precipitate indicates the presence of alkaloids.

Mayer's Test: A drop or two drops of the Mayer's reagent was added to 1 ml of the extract along the sides of the test tube. Formation of white or creamy precipitate indicates the presence of alkaloids.

Wagner's Test: To 1 ml of the extract was added two drops of Wagner's reagent along the side of the test tube. Formation of yellow or brown precipitate indicates the presence of alkaloids.

Test for proteins

Xanthoproteic Test: To 3 ml of extract was added 1 ml of concentrated sulphuric acid. A white precipitate is formed and after boiling turns yellow. Change of the precipitate color to orange on adding ammonium hydroxide indicates the presence of proteins.

Biuret Test: 5 drops of 1% Copper sulphate solution and 2 ml of 10% NaOH were added to 2 ml of the extract and mixed thoroughly. Formation of purple or violet

color indicates the presence of proteins.

Test for phenols

1 ml of extract was added with 1 ml of distilled water and 3-4 drops of 5% NaOH. Appearance of orange coloration indicates the presence of phenols.

Quantitative chemical evaluation

Depending on the results obtained from the qualitative analysis, the quantitative analysis was carried out for Tannins, Flavonoids, Phenols and Saponins.

Total Tannin content

Total tannin content was measured by Folin-Denis method (Oyaizu, 1986). 50 µl of extract was made up to 7.5 ml by adding double distilled water. Then 0.5 ml of Folin-Denis reagent and 1 ml of sodium carbonate were mixed with it. Again, volume was made upto 10 ml by adding double distilled water. Absorption was recorded at 700 nm. The vanillin reagent will react with any phenols that has an un-substituted resorcinol or phlorglucinol nucleus and forms a coloured substitution product which is measured at 700 nm.

Total phenol content

Total phenolics were quantified and expressed as gallic acid equivalents according to a method proposed by Singleton *et al.* (1999). About 3.9 ml of distilled water and 0.5 ml of Folin- ciocalteau reagent were added to 0.1 ml of sample extract in a test tube and incubated at room temperature for 3 minutes after which 2 ml of 20% sodium carbonate was added to this and kept at boiling water bath for 1 minute. Phenols react with phosphomolybdic acid in the Folin- ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated calorimetrically at 650 nm.

Total Flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric method (Zhishen *et al.*, 1999). 1 ml of extract and standard solution of catechin (100 mg/ml) was addedto 10 ml volumetric flask containing 4 ml of distilled water. To this, 0.3 ml of 5% sodium nitrate was added. After 5 minutes 0.3 ml of 10% aluminium chloride was added. Then after 1 minute, 2 ml of 1M sodium hydroxide was added and the total volume was made up to 10 ml with distilled water. The solution was mixed

and the absorbance was measured against prepared reagent blank at 510 nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

Total saponin content

Total saponin content was measured by vanillin-sulphuric acid TSC assay (Le *et al*., 2018). 0.25 ml of extract, standard, and reagent (blank) was added to 0.25 ml of 8% vanillin (8%vanillin was prepared by using ethanol). Then 2.5 ml of 72% sulphuric acid was added. After adding, volumetric flask was kept in water bath for 15 minutes at 60℃ in water bath, with the standards and the reagent blank made up with the solvent used for extracting the lichen samples. After cooling in water at the ambient temperature for 5 min, the absorbance of the standards and extracts are measured at 560 nm using UV–VIS spectrophotometer.

Gas Chromatography Mass Spectrometry analysis

GC-MS analysis was performed at the TUV SUD (South Asia Pvt Ltd Tirupur, India).5 ml of Ethyl acetate extract was evaporated to desiccation and made into 2 ml ethyl acetate.Then the extract was subjected to Gas chromatography – Mass Spectrometry analysis. The Chromatographic separation was finished with CE GC 8000 top MSMD 8000 Fyson instrument with Db 35mr column ($10x10^3$mm x 0.5 mm, 0.25 mm film thickness). In split mode (1:50) with a flow rate of1 mL/min heating programs were executed from 100-250ºC at 3 minutes by using helium as carrier gas. 2 ml of aliquot oil was injected into the column with the injector heater at 250ºC.

Analytical conditions

The following temperatures were maintained for the analysis: Injection temperature - 250ºC, interface temperature - 200ºC, quadruple temperature - 150ºC and ion source temperature - 230ºC. In split-less mode, the injection was performed.

Identification of components (Data analysis)

The mass spectra of compounds in sample were obtained by electron ionization (EI) at 70 eV and the detector operated in scan mode from 20 to 600 atomic mass units (amu). Identification was based on the molecular mass, calculated fragmentations and molecular structure. By using the standard mass spectral database of WILEY and NIST, resolved spectrum was identified for phytochemicals (Ganesh and Vennila, 2011).

Identification of components:

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The name, molecular weight and structure of the components of the test material was determined. By comparing its average peak area to the total area, the relative percentage amount of each component was calculated. The unknown component's spectrum was compared with the spectrum of the component stored in the NIST library version, software, Turbomas 5.2

Isolation of endolichenic fungi from lichen species

The macrolichen species *Hypotrachyna infirma* was selected in the present study for isolating endolichenic fungi because of its dominance in the study area Sholaiyar hills. The selected lichen is a foliose lichen, belonging to the family Parmeliaceae.

Surface sterilization of Lichen thalli

Different types of protocols are available for surface sterilization of leaves or barks of higher plants but very few protocols are there for lichens. In the current study, modified protocol of Suryanarayanan *et al.* (2005) was followed:

Fresh, healthy looking macro lichen thalli were washed in running tap water to remove all debris. After washing the samples were placed in the petriplates. These petriplates contain sterile double distilled water to remove bryophytes/mosses. Then the samples were again washedin sterile double distilled water 20 times until all the other visible contamination get removed.

Then the washed samples were subjected to chemical surface sterilization by dipping them in 30% Hydrogen peroxide (H_2O_2) for 30 seconds, followed by 4% Sodium hypochlorite (NaOCl) for 30 seconds and finally immersing them in 75% ethanol for 30 seconds.

After chemical surface sterilization, the samples were rinsed in sterile double distilled water twice and dried under aseptic conditions and were cut into small segments (0.5 × 0.5 cm).

Isolation of endolichenic fungi

The surface sterilized lichen samples were placed on petriplates containing

different media such as Potato Dextrose Agar (PDA), Sabordaud Dextrose Agar (SDA) and Malt Yeast Extract Agar (MYEA). The petriplates were sealed using parafilm and used.

Composition of Potato Dextrose Agar Medium (PDA)

S.No.	Ingredients	Quantity (gm/litre)
1.	Potato	200 g
2.	Dextrose	20 g
3.	Agar	20 g
4.	Distilled water	1000 ml
5.	pH	6.5

Composition of Sabouraud Dextrose Agar Medium (SDA)

S.No.	Ingredients	Quantity (gm/litre)
1.	Dextrose (Glucose)	40 g
2.	Peptone	10 g
3.	Agar	15 g
4.	Distilled water	1000 ml
5.	pH	5.6

Composition of Malt Yeast Extract Agar (MYEA)

S.No.	Ingredients	Quantity (gm/litre)
1.	Dextrose	10 g
2.	Peptone	5 g
3.	Malt extract	3 g
4.	Yeast extract	3 g
5.	Agar	20 g
6.	Distilled water	1000 ml
7.	pH	6.2

Standardization of surface sterilization was tested by plating out 500 µl of the lastrinsing water from the sterilization procedure and tissue imprint on fresh PDA, SDA, MYEA plates (Schulz *et al.*, 1993). These Plates were incubated in culture room at 25±1°C until fungal growth was initiated. After initiation, the growing fungal mycelia tips were transferred to new PDA plates for obtaining pure cultures and these pure cultures were then examined periodically. After 15 days, those endophytes that had grown on the PDA media were identified and the ones which did not sporulate (sterile cultures) were termed as mycelia sterilia.

Identification of endolichenic fungi:

Endolichenic fungi were identified based on their colonial morphology, microscopic observations and nature of the spores. Using Scotch Tape technique (Harris, 2000), temporary mounts of the fungi were made in lacto phenol cotton blue. The isolates which did not sporulate in culture were treated as mycelia sterilia. The fungi were identified using relevant keys and taxonomic notes (Gilman, 1967; Ellis 1971, 1976; Sutton, 1980; Chowdhary *et al*., 2000) at National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, Maharastra.

Preliminary phytochemical studies of endolichenic fungi

Fungal extraction

A small part of fungal isolates was transferred into Potato Dextrose Broth (PDB) by aseptically scraping using inoculation loop. The isolates were taken into 250 ml conical flasks containing 50 ml of the PDB medium. These conical flasks were incubated in room temperature over a period of time. The inoculum was periodically tested at every 24 hours. After 25 days culture liquid (fungal mycelia and their extract) was filtered with the help of Whatman No. 1 filter paper. The filtrates were used for further phytochemical studies.

Solvent extraction

To the filtrate equal volume of solvents (ethyl acetate, chloroform) were added, mixed well for 10 minutes and kept for 5 minutes. After a while, two clear immiscible layers formed were formed. The upper layer of solvent containing the extracted compounds was separated using separating funnel. Solvent was evaporated and the compound was dried using rotary vacuum evaporator to yield the crude metabolite

(Bhardwaj *et al*., 2015). The crude extract was then dissolved in Dimethylsulphoxide at 1 mg/ml of concentration and the extract kept was at 4°C.

Antimicrobial activity of lichen and endolichenic fungi

Both the Disc diffusion (Bauer *et al*., 1966) and Agar well diffusion methods (Olurinola, 1996) were employed to study the antimicrobial properties of the lichen and endolichenic fungi extracts since some microorganism showed results only in any one of the above-mentioned methods. Methanol extracts of lichen and Ethyl acetate, chloroform extracts of endolichenic fungi were tested against the human pathogens. The antimicrobial activity of lichen and endolichenic fungi against bacterial and fungal human pathogens was carried out at PSG Institute of Medical Science and Research, Coimbatore.

Microorganism used for Disc diffusion method

S.No.	**Name of the organism**
Fungi	
1.	*Aspergillus niger*

Microorganisms used for Agar well diffusion method

S.No.	**Name of the organism**
Gram positive bacteria	
1.	*Staphylococcus aureus*
2.	*Streptococcus sp.,*
Gram negative bacteria	
1.	*Escherichia coli*
Fungi	
1.	*Candida albicans*

Media used

Nutrient Agar Medium (NA) and Potato Dextrose Agar (PDA) Medium were used for bacteria and fungi respectively.

Composition of Nutrient Agar Medium (NA)

S.No.	Ingredients	Quantity (gm/litre)
1.	Yeast extract	2 gm
2.	Beef extract	1 gm
3.	Peptone	5 gm
4.	Sodium chloride	5 gm
5.	Agar	20 gm
6.	Distilled water	1000 ml
7.	pH	7.4

Composition of Potato Dextrose Agar Medium (PDA)

S.No.	Ingredients	Quantity (gm/litre)
1.	Potato	200 g
2.	Dextrose	20 g
3.	Agar	20 g
4.	Distilled water	1000 ml
5.	pH	6.5

Sterilization

Media were sterilized in an autoclave for 20 minutes. In hot air oven, all glasswares were sterilized at 180°C for 3 hours. Instruments like forceps, inoculation loop were sterilized using alcohol and flame.

Source of inoculum

For bacteria 24 hours old bacterial culture, for fungi 24 hours old spore suspension were used. A loopful of each bacterial culture and each sporulating culture were suspended in 5 ml of sterile Nutrient Broth and Potato Dextrose Broth respectively. Then it had shaken vigorously to get the uniform suspension.

Method of inoculation – Disc diffusion method

Preparation of Discs

Circular discs of 6 mm diameter were prepared from Whatman No.1 filter paper and sterilized in an autoclave. From the lichen and endolichenic fungal extracts, 100

mg of crude extract was dissolved in the respective solvent and was loaded onto the filter paper discs to get 100mg/disc concentration for overnight and allowed to dry at room temperature in laminar air flow chamber (Agnese *et al*., 2001; Acar and Goldstein, 1998; National Committee for Clinical Laboratory Standards NCCLS, 2002).

Experiment

Potato dextrose agar medium was prepared and the petriplates and the media were sterilized for 20 minutes at 120ºC in an autoclave and approximately 20 ml of each of the medium was poured into the sterile petriplates under aseptic condition and allowed to get solidify for 15-20 minutes. It was cooled at room temperature. After cooling, the fungal culture was taken (24 h old) and using an inoculation needle, the culture was applied on the surface of the medium in the form of parallel streaks using cotton swabs (Koduru *et al*., 2006; Meyer and Afolayan, 1995). The prepared discs containing the test material were impregnated on the agar medium with framed forceps and gently pressed down to ensure complete contact of the disc with the agar surface uniformly seeded with the test microorganisms and the plates were incubated at 37ºC for 24 hours. Control plates without the lichen and endolichenic fungal extracts i.e., a standard antibiotic Fluconazole (10µg/disc) as positive control and DMSO as negative control (Parekh and Chanda, 2007) were also maintained for reference (Johnson *et al*., 1986) to determine the sensitivity of the tested microbial strains (Shashi *et al*., 2008).

There was a gradual change in concentration in the media surrounding discs. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. After 24 h, antimicrobial activity was determined by measurement of diameter zones of inhibition (mm) (against the test organisms) around each of the extracts and the antibiotics (Lino and Deogracios, 2006). The experiment was carried out three times and the mean of the reading is required to ensure the reliability of the result. All tests were performed in triplicate (Evans *et al*., 2002).

Method of inoculation – Agar well diffusion method

Well diffusion method (Olurinola, 1996) is used similarly to that of disc-diffusion method. Sterile corkborer or a tip is used to make wells in each and every plate. Sterilized petriplates containing sterile Nutrient Agar and Potato Dextrose Agar

medium were swabbed (sterile cotton swabs) with 28 days old broth culture of selected bacteria and fungi in such a way so as to get a thorough coverage of uniform thick lawn of growth of the inoculum. To this, different concentration (20µl, 40µl, 60µl, 80µl, 100µl) of fungal extracts were added using sterile syringe into the wells and the petriplates were carefully sealed and incubated. Control plates without the lichen and endolichenic fungal extracts i.e., standard antibiotics Ampicillin for bacteria (10µg/disc) and Fluconazole for fungi (10µg/disc) as positive controls and DMSO as negative control were also maintained for reference to determine the sensitivity of the tested microbial strains.

Method of incubation

Fungal pathogens were incubated at 28°C for 48 hours and bacterial pathogens at 37°C for 24 hours (Sen and Batra, 2012). For each experiment triplicates were maintained. The activity of fungal extracts was determined by zone of inhibition.

Antioxidant activity

DPPH radical scavenging assay (Silva *et al.*, 2004)

The Free radical scavenging activity of different concentration of extracts (Lichen, endolichenic fungi) (20µl, 40µl, 60µl, 80µl, 100µl) was determined by 1,1-diphenyl-2- picrylhydrazyl (DPPH) method. The corresponding solvents (W/V) with ascorbic acid as the standard, was tested. 0.004% DPPH solution (2ml) was mixed with 2 ml extracts. The intensity of DPPH-purple decolourization changed into DPPH-yellow specifies the scavenging ability of the extract. The mixtures were incubated in the dark condition for 30 minutes at room temperature and their optical density was measured using UV-Vis spectrophotometer at 517 nm. In the result, the absorbance value of the reaction mixture reduced. It indicates the higher free radical- scavenging assay. The inhibition percentage for scavenging activity against DPPH was calculated using the following formula:

$$\textbf{Scavenging activity (\%)} = A_0 - A_1 * 100/A_c$$

Where,

A_0 - absorbance of control (DPPH Solution without the sample),

A_1 - absorbance of DPPH Solution in the presence of the sample (extract/ascorbic acid).

The percentage of scavenging of the extract was compared with positive control.

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

Hydrogen peroxide scavenging (H_2O_2) activity was determined according to the method of (Ruch *et al.*,1989). Hydrogen peroxide solution (40mM) was prepared in phosphate buffer (pH 7.4). Different concentrations (20µl, 40µl, 60µl, 80 µl, 100µl) of extracts were added to 0.6 ml of hydrogen peroxide solution. Phosphate buffer without Hydrogen peroxide was used as a blank solution. The absorbance was measured at 560 nm. The experiment was repeated in triplicate. The percentage of Hydrogen peroxide scavenging of extracts and standard compounds werecalculated as:

$$\text{Percentage scavenged } [H_2O_2] = (Ac - As)*100/\ Ac$$

Where,

A_C - absorbance of the control

A_S - absorbance of the sample (lichen and endolichenic fungi)

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

Total antioxidant capacity was determined by Phosphomolybdenum method. 0.1 ml of different concentrations of (20µl, 40µl, 60µl, 80 µl, 100µl) extracts (Lichen, endolichenic fungi) were added with 1 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mm ammonium molybdate). These were kept in water bath at 95°C for 90 minutes and cooled to normal room temperature. The absorbance was measured at 695 nm. Antioxidant capacity of each sample was designated as ascorbic acid equivalent. The IC_{50} value for each test extracts as well as standard preparation were calculated.

$$\%\text{Scavenging/Reduction} = = (Ac - As)*100/\ Ac$$

Where,

A_C - absorbance of the control

A_S - absorbance of the sample (lichen and endolichenic fungi)

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

5ml of the reaction mixture combined with sodium nitro prusside (5mM) in phosphate – buffered saline (pH -7.3). The mixture with or without the extracts at

various concentrations, was incubated at 25ºC for 3 hours. The nitric oxide radical thus formed contained with oxygen to create the nitrite ion which was determined at 30-minute intermissions by dissolving 1 ml of incubation mixture with the same volume of Griess reagent. The absorbance of the chromophere (purple azo dye) established through the diazotization of nitrite ions with sulfanilamide and successive coupling with naphthyl ethylene diamine dihydro chloride was measured spectrophotometrically at 546 nm. In this assay, ascorbic acid was used as the standard. Nitrogen oxide scavenging of extracts and standard compounds werecalculated as:

$$\text{Percentage scavenged} = (Ac - As)*100/ Ac$$

Where,

A_C - absorbance of the control

A_S - absorbance of the sample (lichen and endolichenic fungi)

Superoxide radical scavenging assay (Beauchamp and Fridovich, 1971)

Superoxide radical created from the photo reduction of riboflavin was discovered byNBT reduction method (Beauchamp and Fridovich, 1971). The reaction mixture (each 3 ml) was prepared by using 1 ml of sample solution and 50mM phosphate buffer (pH 7.8), 13 mM methionine, 21M riboflavin, 100 1M EDTA, NBT (75 1M). For 15 min the tubes were illuminated under incandescent lamp. After 10 min of illumination, there is an increase in the absorbance at 560 nm which was visualized by the formation of blue formazan from a fluorescent lamp. The inhibition of superoxide radicals was determined by comparing the absorbance values of the control with that of the treatments. Ascorbic acid was used as the standard. Superoxide scavenging of extracts and standard compounds werecalculated as:

$$\text{Percentage scavenged} = (Ac - As)*100/ Ac$$

Where,

A_C - absorbance of the control

A_S - absorbance of the sample (lichen and endolichenic fungi)

Anti-inflammatory activity

Principle

The principle concerned in this method is stabilization of human red blood cell

membraneby hypo tonicity induced membrane lysis. Since HRBC (human red blood cell) membrane is similar to lysosomal membrane; the study was undertaken to check the stability of HRBC membrane by the extracts to predict the *in vitro* anti-inflammatory activity. The methanolic extract of lichen and ethyl acetate extract of endolichenic fungi atthe concentration of 50, 100, 200, 500 and 1000 μg/ml respectively, were incubated separately with HRBC solution.

1) Inhibition of albumin denaturation (Mizushima and Kobayashi, 1968; Sakat *et al*., 2010)

The anti-inflammatory activity of the lichen and endolichenic fungal extracts was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima and Kobayashi (1968) and Sakat *et al*. (2010) followed with minor modifications.

Reagents

1. 5% Bovine serum albumin
2. 1N HCl
3. Phosphate buffer saline

Procedure

The reaction mixture (0.5 ml; pH 6.3) consisted of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of distilled water. pH was adjusted at 6.3 using a small amount of 1 N HCl. Different concentrations of the lichen and endolichenic fungal extracts were added to the reaction mixture and were incubated at 37ºC for 20 min and then heated at 57º C for 5 min. After cooling the samples, 2.5 ml of phosphate buffer saline was added. Turbidity was measured spectrophotometrically at 600nm. The percentage inhibition of protein denaturation was calculated as follows:

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

2) HRBC membrane stabilization method (Kumar *et al*., 2011)

The lysosomal enzyme released during inflammation produces a variety of disorders. Theextracellular activity of these enzymes is said to be related to acute or chronic inflammation. The nonsteroidal drugs act either by inhibiting these lysosomal

enzymes or by stabilizing the lysosomal membrane.

Reagents

1.Alsevers solution

- 2% dextrose
- 0.8% sodium citrate
- 0.5% citric acid
- 0.42% NaCl

1. 0.15M Phosphate buffer (pH 7.4)
2. 0.36% Hypo saline
3. HRBC suspension (10% v/v)

Procedure

Blood was collected (2 mL) from healthy volunteers and was mixed with equal volume ofsterilized Alsevers solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl in distilled water) and centrifuged at 3000 rpm. The packed cells were washed with isosaline solution, and a 10 % v/v suspension was prepared with normal saline and kept at 4ºC undisturbed before use. Different concentrations of methanol extract of lichen and ethyl acetate extract of endolichenic fungi (50, 100, 200, 400 and 800 µg /0.5 ml) in normal saline, Aspirin as standard (50, 100, 200, 400 and 800 µg / 0.5 ml) and control(distilled water instead of hypo saline to produce 100 % hemolysis) were separately mixed with 1ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of 10% HRBC suspension. All the assay mixtures were incubated at 37º C for 30 min and centrifuged at 3000 rpm for 20 min and haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. The percentage of HRBC membrane stabilization or protection was calculated by using the following formula:

$$\text{Percentage stabilization} = \frac{(\text{Abs Control} - \text{Abs sample})}{\text{Abs Control}} \times 100$$

Molecular docking studies

The chemical structures for the ligand molecules identified from the lichen extract were created in Mol format using ACD Lab's Chemsketch (www.ACDlabs.com). The 3D structure of the ligands was created using CORINA (Sadowski *et al*., 1994; Schwab, 2010). The receptors for the docking were retrieved from Protein Data Bank (Berman *et al.*, 2000). The ligands in the crystal structure of the retrieved receptors were removed so as to enable binding of the required ligands. Molecular Docking of the identified receptor and ligands was performed using Autodock Tools (ADT) 1.5.6 (Morris *et al.*, 2009) by employing the following method.

Preparation of Receptor and Ligand files

Autodock entails both the receptor and ligand in PDBQT format for assessing the bindingaffinity between them. PDBQT format restrains the atomic coordinates, partial charges and atom types. Initially, the receptor file in PDB format obtained from Protein Databank was accessed in Autodock Workspace. The water molecules in the receptor file were removed and implicitHydrogen atoms were added. Finally, partial charges were added and the receptor file was saved in PDBQT format. Similarly, the ligand files in PDB format were retrieved by Autodock and saved in PDBQT format.

Preparation of Grid and Dock Parameter files

Autogrid 4.2 program in ADT was used to perform the grid computation. The grips maps with a dimension of 90X90X90 and spacing of 0.375 A^{o} were centered along the ligand binding site. The receptor and ligand files in PDBQT format along with the grid maps were saved as the grid parameter file to execute the Autogrid program. After the autogrid calculation, autodock parameter file was created with the receptor, ligand and selection of autodock parameters.

Docking and Visualization

Docking was performed using Lamarckian Genetic Algorithm with 10 independent runs per ligand with an initial population of 150 randomly placed ligand on the receptor binding site.A maximum of 2.5X10^{5} evaluations on the energy will be carried out for 27X10^{3} generations with a mutation rate of 0.02 and a cross over rate of 0.80. The local-energy-minimizationalgorithm was limited to 100 steps for 6% of the population. To explore the conformational space of ligands, the overall translation steps was set to 0.2 Å, and the overall rotation and torsion rotation step were

set to 5 in the docking studies. The autodock 4.0 program in ADT was executed and the docking scores were reported using binding free energy energies in kcal/mol. The bound complex with the receptor and ligand was visualized using Pymol.

***In vitro* cytotoxic activityMaintenance of cell line**

Human colon liver cancer cell Hep G2 was obtained from the National Center for Cell Sciences (NCCS) Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FCS and 2mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Disodium carbonate (Na_2CO_3), 0.1 mm nonessential amino acids, 1 mm sodium pyruvate, 2 mm l-glutamine, 1.5 g/L glucose, 10 mm (4-(2-hydroxyethyl)-1- piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100µg) were adjusted to 1mL/L. The cells were maintained at 37°C with 5% Carbon Dioxide atmosphere.

MTT assay

Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethylhiazol- 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 were solubilized with organic solvents such as DMSO or isopropanol and the released solubilized formazan reagent was measured spectro photometrically. Since reduction of MTT can only occur in metabolically active cells, the levelof activity is a measure of the viability of the cells.

Materials and Reagents required

1. Cell line – Human colon liver cancer cells, HepG2 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

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Kavithaa and Reddy, 2016) was used to evaluate the cytotoxic activity of the extracts. Cells were grown (1×10^4cells/well) in a 96-well plate for 48 h into 80% confluence. The medium was replaced with fresh medium containing serially diluted samples, and the cells were further incubated for 48 h. The culture medium was removed, and 100µL of the MTT [3-(4, 5-dimethylthiozol-2-yl)-3, 5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37^{o}C for 4 h. After removal of the supernatant, 50 µL of DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at620 nm in an ELISA multi well plate reader (Thermo Multiskan EX, USA). The OD value was used to calculate the percentage of viability using the following formula:

% of viability = OD value of experimental sample/OD value of experimental control*100

Bionanotechnology

During the present research, synthesis of AgNP's was done expending the protocol of (Jaidev and Narasimha, 2010). 10 ml of lichen extract and fungal filtrate was prepared using the method declared in "fungal biomass production" was mixed with 90 ml of $AgNO_3$ solution (1.0 mM – 5.0 mM) and incubated in orbital shaker at 50 rpm for 24h in dark conditions. The colour of the solution transformed to brown was noticed by visual observations. After the color change, brown colored solution was centrifuged at 12,000 rpm for 15 min. Further, supernatant was leftover, and pellet was formed which was then treated with sterile deionized water and washed 3-4 times to take away miscellaneous. Then, the pellet was dried and used for further characterization. Silver nitrate solution was treated as negative control and the extracts without $AgNO_3$ solution were treated as positive control. Each experiment was repeated thrice.

UV-Vis Spectroscopy

Synthesis of nanoparticles were observed by specific peak using UV-Vis Spectroscopy (Spectrophotometer Shimadzu, 2450). This technique proves the presence of silver nanoparticles by measuring the absorbance of bioreduced solution at wavelengths between 200 -800 nm. De-ionized water used as a blank.

Fourier transform infrared (FT-IR) spectrometry

FT-IR analysis of the extracts was done at Avinashilingam University, Coimbatore. Infrared spectroscopy can be used for both i.e qualitative analysis and quantitative analysis. IR spectra are strictly created by changes in electronic, rotational and vibrational energy.Commonly solution and, indeed, almost any kind of sample can be held by latest technique knownas attenuated internal reflectance, that is based on total internal reflectance**.** The capability of the interface between two media as a mirror is based on the distinction in refractive indices of the media (Trease and Evans, 1994). The FT-IR spectrum of the solid sample was obtained from ethyl acetate extract of *Nigrospora oryzae* treated with silver nitrate and methanolic extract of *Hypotrachyna infirma* treated with silver nitrate. The spectrometer Shimadzu FTIR- 4200 was used this purpose. The solution was run by placing a drop of the extract on a salt plate highly polished with 100mg of dried potassium bromide KBr and then a second plate was positioned on the top of the first plate so as to enable liquid spread in the thin layer between the plates and clamp the plates together in suitable fashion. Then the spectrum was attained with the help of spectrophotometer and computer. software attached with it (Idrees *et al*., 2004). The frequencies of distinct components present in the sample were evaluated. For standard also the same procedure was followed. In infrared region the samples were run between 400 nm and 4000 nm.

Scanning Electron Microscope (SEM)

The morphological analysis of the invented AgNP's was done by Scanning Electron Microscope. The SEM images were recorded at (500 - 10,000) X magnification operating at 20.00ksv. The analysis was performed at DRDO, Bharathiar University, Coimbatore.

Statistical Analysis

All experiments were carried out in triplicates and results are expressed as mean ± SD (n=3). To analyse the differences among mean values of different assays oneway analysis of variance (ANOVA) was conducted. The data were analyzed using SPSS (2.0) software.