MATERIALS AND METHODS

1. Collection, identification and extraction of phytochemicals from lichens:

1.1) Collection of lichen samples:
Lichen species were collected during March 2013 from Ooty, Tamil Nadu mainly at coordinates of 11.4064°N to 11.436160°N and 76.6032° E to 76.696408° E with average altitude of 2,240 meters. No crustose lichen from bark of tree was collected so most of lichens were collected as they were fallen from the tree since they were of foliose and fruticose types. Collected lichen sample were kept in lichen wrap bags and were transferred to laboratory. They were soaked in water and washed to remove their debris and insects, then were dried and some part of it was measured and powdered by grinder for the extraction process. Remaining parts were preserved in acid-free packets for further use.

1.2) Identification:

A) Lichen samples were identified on the base of their morphology and chemical characteristics. Lichens were screened for their morphology based on their growth type, presence or absence of vegetative parts (Rhizines and cilia), and sexual reproductive parts (Their types of Apothecia and Perithecia, if any) and color of thallus \(^{[5, 245]}\). Spot tests, TLC and micro-crystallography were chemical methods applied to lichen fragment and their extract to help the identification of species.

B) Spot test: For Calcium or ‘C’ test, a drop of Ca(OCl)\(_2\)solution, which was prepared freshly applied on lichen fragment. Aromatic compounds with two free-OH- groups react to this solution and form a red color on thallus of lichen \(^{[194]}\). For potassium or ‘K’ test, 10-25% aqueous solution of potassium hydroxide is used as the reagent. Quinonoid lichen compounds react to this solution and form a dark red color \(^{[194]}\). For phenyl-enediamine or ‘Pd’ test, 1-5% ethanolic solution of \(p\)-phenyl-enediamine is used; it reacts with aromatic aldehydes and gives yellow to red color on tested fragment. For KC test, first K solution applied followed by immediate use of C. Some depsides and depsidpnes give red color after applying this procedure \(^{[194]}\). Iodine or ‘I’ test also was used by a 0.5% potassium iodide solution which reacts with certain polysaccharides in lichen.

C) Micro-crystallography: A small piece of each lichen thallus was placed on a slide and lichen substances extracted by drop wise adding crystallizing solution which is glycerol:
ethanol: water (1:1:1) \[194\]. After light heating of slide, they were observed under microscope and photos were compared with reference photograph available in literature like; Culberson, 1969 \[246\] and 1970 \[247,248\], Huneck and Yoshimura (1996) \[249\] and Hale (1974) \[195\].

D) Thin Layer Chromatography: Lichen extracts were spotted on heat-activated silica coated TLC Aluminum sheets (Silica gel 60 F254, Merck, Germany) and were run separately by four standard solvents. Four different organic mobile phase used for TLC were as follows: EA (Diethyl ether: Acetic acid; 200 ml: 2 ml); HEF (Hexane: Diethyl ether: Formic acid; 140 ml: 100 ml: 20 ml); TDA (Toluene: 1-4 Dioxane: Acetic acid; 180 ml: 45 ml: 5 ml); TEF (Toluene: Ethyl acetate: Formic acid; 65.5 ml: 41.5 ml: 4 ml). TEF solvent showed better separation of bands so in further study this mobile phase was used as standard solvent. Plates were dried for evaporation of solvents \[164\]. Then developed bands were visualized under UV chamber and by spraying 10% Sulphuric acid solution. Lichen materials were also sent for authentication Lichenology Lab at CSIR-National Botanical Research Institute (NBRI), Lucknow. A part of each lichen species is deposited as dried herbarium specimen in National Lichen Herbarium, NBRI, Lucknow, India.

1.3) Extraction: 5 grams of each powdered lichen material was kept in handmade thimble and placed in Soxhlet apparatus (Fig-8). Extraction was carried out in various organic solvents because lichen substances are poorly soluble in water \[250\]. In order to obtain maximum extraction of lichen substances, solvents were used according to increasing polarity \[164\] as follows: hexane, ethyl acetate, methanol and ethanol (Fig-7). Extraction time for each solvent was about 8 hrs. Concentration of each lichen crude extract was recorded and percentage of yield was calculated. Final obtained yield is a measure of solvent’s efficiency to extract specific components from sample \[251\]. Percentage of yield was calculated by below formula.

\[
\text{Yield (\%) = (dry weight of extract/dry weight of sample) x 100}
\]
Fig-7: Extraction process in this study.

Fig-8: Powdered *C. subradiata* getting fractionated in hexane.

2. Qualitative and quantitative phytochemical analysis of lichen extracts
2.1) Qualitative analysis: Different tests were carried out on all extracts by standard procedure to identify the constituents present in lichen extracts as described by Sofowora (1993) [252], Harborne (1998) [253, 254], Uma, et al., 2014 [255], Vijay et al., 2015 [256], Ajay et al., 2011 [257] and Zohra et al., 2012 [266].

1) Test for alkaloids (Dragendorff's test): In 2ml of each extract 5ml of aqueous HCl 1% was added and transferred to a water bath for few minutes. After few minutes, 1ml of the filtrated extract was treated with 2-4 drops of Dragendorff's reagent. The presence of alkaloids is indicated by the appearance of an orange reddish precipitation.

2) Test for saponins (Frothing test): A small amount of extract was shaken with distilled water and observed for the formation of persistent foam.

3) Test for Tannins (Braymer’s test): 1ml of extract was treated with few drops of ferric chloride solution and observed for formation of blue or greenish colour solution.

4) Test for anthraquinones (Borntrager's test): 1ml of the extract was boiled with 1ml of sulphuric acid (H₂SO₄) and filtered while hot. The filtrate was shaken with 2ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of diluted ammonia was added. The resulting solution was observed for colour changes.

5) Test for cardiac glycosides (Keller-Killiani test): 1ml of extract was added to 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

6) Test for flavonoids: 5ml of diluted ammonia was added to a portion of an aqueous filtrate of the extract, then 1ml of Concentrated sulphuric acid was added. Yellow color that disappears on standing indicates the presence of flavonoids.

7) Test for terpenoids (Salkowski test): To 1ml of each of the extract 1ml of chloroform was added. Concentrated H₂SO₄ (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.
8) Test for carbohydrates (Molisch’s test): Few drops of Molisch's reagent was added to each extract, followed by addition of 1 ml of conc. H₂SO₄ by the side of the test tube. The mixture was kept for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet color at the interphase of the two layers was a positive test.

9) Test for Steroid (Libermann-Burchard test): 1ml of extract was added to 1ml of acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added to it. Formation of green or bluish color indicates the presence of steroids.

10) Test for Reducing sugar (Fehling’s test): In a test tube 2 ml of the filtrate were treated with 2 ml Fehling's solutions (A & B) and heated; the appearance of a red precipitate indicates the presence of reducing sugars.

11) Test for Anthocyanin: To 1ml of extract, 1ml of 2M NaOH was added; development of blue or green color indicates presence of anthocyanin.

12) Test for Xantoproteins: This test gives a positive result in those proteins with aminoacids carrying aromatic groups. 1 ml of strong HNO₃ was added to 2ml of each lichen extract in test tube. An intensely yellow color was recorded as positive result.

13) Test for Carotenoids: 1 g of each specimen sample was mixed with 10 ml of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85 % sulphuric acid was added. A blue color at the interface showed the presence of carotenoids.

14) Test for Phenols (Ferric chloride test): to each lichen extract, few drops of 5% ferric chloride solution. A dark green color indicates positive reaction.

15) Test for Volatile Oils: 1ml of the extract was mixed with dilute hydrochloric acid. A white precipitated was formed which indicated the presences of volatile oils.

### 2.2) Qualitative analysis

#### 2.2.1) Total Phenolic Content (TPC): The concentration of phenolic compounds in lichen extracts was determined using spectrophotometric method [258]. The Folin-Ciocalteu method is widely used to determine phenol content in extracts. It is accepted that the FC reagent contains phosphomolybdic / phosphotungstic acid complexes and the chemistry behind the FC method counts on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue
complexes that can be monitored spectrophotometrically at 750–765 nm. The phenolic compounds react with FC reagent only under basic conditions. The total phenol assay by FC method is convenient, simple, and reproducible \cite{259,260}. To investigate phenolic content of lichen extracts, each solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of extract, 2.5 ml of 10% Folin-Ciocalteu’s reagent dissolved in water and 2.5 ml 7.5% NaHCO$_3$ (sodium bicarbonate). Blank also was prepared concomitantly using respected solvent instead of extract. Samples were incubated at 45°C for 45 min. The absorbance was determined using spectrophotometer at $\lambda_{\text{max}} = 765$ nm. The same procedure was repeated for the standard solution of Gallic acid (GA) and Tannic acid (TA) at different concentrations and calibration line of each standard was constructed. The phenolic content of each extract is expressed in terms of Gallic acid equivalent as $\mu$g of GA/g of extract and mg of TA/g of extract.

2.2.2) Determination of flavonoid concentrations (TFC): The content of flavonoids in the tested lichen extracts was determined using spectrophotometric method \cite{261}. 1 ml of each lichen extract in concentration of 1 mg/ml was added to 1 ml of 2% of methanolic AlCl$_3$ solution. The reaction complex was incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at $\lambda_{\text{max}} = 415$ nm. The same procedure was repeated for the standard solution of Quercetin and calibration line was construed. Content of flavonoids in each lichen extract was expressed in terms of quercetin equivalent (mg of QU/g of extract).

2.2.3) Total Carotenoid content (TCA): Total carotenoids concentration was quantified according to Rodriguez-Amaya and Kimura (2014) \cite{262}. Briefly, 1 g of fresh lichen was homogenized in 20 mL acetone and supernatant decanted. This process was repeated until a colorless solution was obtained. After filtration, each solution was washed with 30 mL acetone, after evaporation, dissolved in 60 mL petroleum ether. This solution was filtered and made up to 100 mL by petroleum ether. 2 mL of this solution was mixed with 8 mL petroleum ether and absorbance measured at 475 nm. Concentration of carotenoid content of each sample was calculated with a $\beta$-carotene calibration curve which is constructed by different concentration.

2.2.4) Determination of Total Antioxidant Capacity (TAC): Total antioxidant capacity of lichen extracts was evaluated by the method of Prieto \textit{et al.} (1999) \cite{263}. The assay is
based on the reduction of Mo (VI) to Mo (V) by antioxidant agents and subsequent formation of a green phosphate/Mo (V) complex \[264\] at acidic pH.

0.1ml of each lichen extract (100 μg) was combined with 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes got capped and incubated in boiling water bath at 95° C for 90 min. Samples were kept at room temperature to get cooled and their absorbance were measured at 695 nm against blank using spectrophotometer. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent was used and incubated under same conditions as rest of the samples. Ascorbic acid (AA) was used as standard and TAC was expressed as milligrams of ascorbic acid per gram of the dry extract \[265, 267\].

### 2.2.5) Correlation study among all quantified phytochemical classes:

To understand correlation between the content of phenolic, Flavonoid and carotenoid with antioxidant activity, each of these groups was investigated to calculate their correlation coefficient (r), and coefficient determination (r\(^2\)), p-value and t-test \[238, 268, 269\]. Also data were subjected to normality test to confirm whether each two sets of compared population are normally distributed or not. Correlation was also examined between phenol and Flavonoid content as well as carotenoid and phenol content. Based on all computed values, each two set data were interpreted to know their strength of correlation.

### 2.2.6) TLC bio-autography for screening antioxidant activity:

In this technique, the antioxidant constituents in each lichen metanolic extract was analyzed using thin layer chromatography (TLC) by application of DPPH (2, 2- Diphenyl-1-picrylhydrazyl). A 100μg of each lichen extract was loaded on TLC plates. The plates then were developed in solvent system of chloroform: methanol (97:3) to separate the various constitutes of the extracts. The developed plates were air dried and observed under visible and UV light \[219\]. Various separated spots and their Rf values were observed. Then, 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plates and incubated for 10 min at room temperature. The active antioxidant lichen constituents were detected as yellowish white spots against a purple background of TLC plates.
3. Biological activities of lichen extracts and characterization of potential metabolites

Bacterial strains *Salmonella typhimurium* (NCIM 2501), *Pseudomonas aeruginosa* (NCIM 2200), *Bacillus subtilis* (NCIM 2063), *Escherichia coli* (NCIM 2065), *Proteus vulgaris* (NCIM 2027) and *Staphylococcus aureus* (NCIM 2654), were used in this study. All the microorganisms had been obtained from National Collection of Industrial Microorganisms (NCIM) at National Chemical Laboratory, Pune, India and were maintained on recommended Muller-Hinton medium.

3.1) Anti-Bacterial activity of lichen extracts:

3.1.1) Determination of microbial concentration and agar disc diffusion: In order to determine the suitable concentration of microbial culture for antimicrobial activity of lichen extract, microorganisms were inoculated in Muller-Hinton broth and incubated at 34°C for 24 hrs. After completion of incubation period microorganisms were serially diluted in 0.86% sodium chloride saline in the concentration of $10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-8}$. From each concentration 100μl and 10μl of microbial suspension were inoculated in separate petri plates containing Muller Hinton agar media and colony-forming units (CFU/mL) were calculated. Preliminary antimicrobial activity was done by disc diffusion method of Bauer et al., (1966) \([271,272]\). In this method two sets of microbial suspension; 6 Log$_{10}$ CFU/mL (10 μl) and 9 Log$_{10}$ CFU/mL (100 μl) with $10^{-2}$ concentration (as the most suitable one) were used to examine efficacy of lichen extracts on different concentration of bacteria. Muller Hinton agar plates were inoculated by microbial suspension and spread completely by glass spreader, then kept for 30 min for adsorption of microorganism on the surface of the media. Whatman filter paper No. 1 were used to prepare 0.6 cm diameter discs and soaked in different lichen extract (200μg/ml) and implanted on surface of media \([21]\). All the petri plates were incubated at 34°C for 24 hrs. After completion of incubation period, zone of inhibition were measured. Tetracycline (30μg/ml), Streptomycin (10μg/ml), Ofloxacin (5μg/ml) and Cefixime (5μg/ml) were used as positive controls which are most potent standard antibiotics available against tested bacteria to compare lichen extracts with highly effective antibiotics \([270]\).
3.1.2) Determination of MIC, NIC, MBC

A) The minimum inhibitory concentration (MIC): MIC of extracts was determined by micro dilution method in 96 wells microtitre plates according to NCCLS guidelines. A twofold serial dilution of extracts in μg/ml values; (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.7, 0.35, 0.175, 0.087) were added to Muller-Hinton broth in separate test tubes. Bacterial suspensions of 6 Log\textsubscript{10} CFU/mL were inoculated with the medium and all the tubes were incubated at 34°C for 24hrs. The O.D. was recorded at 600 nm by a multimode plate reader (Thermo Fischer, Varioskan™). MIC values were determined by Lambert and Pearson (2000) method. Data were fitted to a modified Gompertz function in GraphPad prism software, from which highly accurate values can be obtained for the MIC. This method provides a mathematical approach to determine the exact value of the MIC by the standard microdilution method and allows more accurate adjustment of the PK/PD parameters for tested drugs.

B) Non-Inhibitory concentration (NIC): NIC value also can be described by the modified Gompertz function in GraphPad prism software. This value can be determined by the below equation and can be seen in the Gompertz graph as a final point where growth is not affected by tested samples and after that point antimicrobial effect begins.

C) Analysis of MIC and NIC by Gompertz model: Lambert and Pearson described a non-linear regression model for the MIC and NIC determination of a tested drug using values obtained by the micro-dilution method. These factors, in this model, are defined mathematically based on a Gompertz function modified to fit the data of the reference method as (MIC=10\textsuperscript{(M+1/B)}) and (NIC=10\textsuperscript{(M+1.718/B)}), where B is a slope parameter and M is the log concentration of the inflection point. In general, plotting the inhibitor concentration on a logarithmic scale gives a characteristic sigmoid-shaped curve. The curve can be split into three principle regions: a region where tested extracts have no effect on the of the organism relative to the control growth (as measured by O.D.), a region where there is increasing inhibition of growth, and a region where there is no measurable growth relative to the control. By this curve, two terms had assigned to two specific concentrations; the non-inhibitory concentration (NIC), the concentration above which the inhibitor begins to have a negative effect on growth, and the minimum
inhibitory concentration (MIC) that marks the concentration above which no growth is observed relative to the control \[277\].

**D) Minimal Bactericidal Concentration (MBC):** The MBC was determined by sub culturing the test dilutions (1 to 4 fold above MIC) on Mueller Hinton Agar and further incubated for 24h \[278\]. The MBCs were recorded as the lowest dilution that produced a 99.9% reduction in growth observed in comparison to the growth of the control \[279\].

**3.1.3) Kinetic-time study and Determination of area under curves and fractional areas**

**A) Kinetic-time study:** In order to study the efficacy of different lichen extracts against human pathogens from bacterial growth curve changes after treatment, Kinetic time assay was performed which analyzed bacterial life cycle behavior under constant condition at different time intervals \[281\].

In the first model to study microbial kinetics, O.D./ time values were used to make the graph. From this study graphs were made based on measured O.D. against each time interval. In the preliminary study by disc diffusion method, methanolic extract was found as the most effective solvent so it was used in this study. Basically the test was carried out by inoculating 6 log\textsubscript{10} CFU/ml of bacteria into Muller-Hinton broth (PH: 7.3) supplemented with lichen extracts (100 μg/ml) and tubes were incubated at 30°C. At each interval (0, 3, 6, 24 and 48 h), 1 ml of aliquot was taken under sterile condition and OD was measured at 600 nm by UV-visible spectrophotometer (Beckman-Coulter) to observe bacterial growth. The same was performed for negative control (methanol instead of lichen extract) and tetracycline was used as positive control since it was effective on all tested microorganisms and showed maximum inhibition zone compared to other standard antibiotics.

**B) Determination of area under curves and fractional areas:** From the kinetic based curves, Area Under Curve (AUC) was calculated using GraphPad Prism\textsuperscript® V 6.1. This value is directly related to bacterial density; higher turbidity (O.D) means more reproduced bacteria, hence higher AUC. In other words, efficacy of tested extract is manifested by amount of reduction in the area under O.D/time curve \[78\]. By comparing AUC of control and test (fractional area) percent inhibition caused by extract can be calculated by the following formula \[282\].

\[
\text{Inhibition} \% = \frac{(\text{AUC control} - \text{AUC test})}{\text{AUC control}} \times 100.
\]
C) Development of growth curves by exponential (Mathusian) growth model: 

In order to get more precise growth curves based on cell number as microbial population against time, it is necessary to predict the cell number in broth culture of each sample. Hence, to predict cell numbers in broth media according to the concept of equation 1, it is presumed that the growth curves calculated by equation 3 is a function of time, then cell number \( N \) at each time interval could be predicted by multiplying the growth rate percentage of bacteria by observed O.D. (as a values of cells density) multiplied by time duration \( (d_t) \). At zero time since no time was elapsed, just growth rate is multiplied by O.D. of zero time (Eq. 5). To get specific growth rate, O.D. of final point of lag phase and final point of log phase were considered. The calculated method for predicting cell numbers at each time interval gives reliable result with less effort and time. To achieve this, growth rate \( \mu \) of each treated and untreated culture was calculated as per equation 3. As growth rate is a function of time \( t \) and is related to O.D. at each specific time, cell population in broth medium was predicted by the following formula:

\[
\text{Cell number (N) at each time interval} = \% \mu \times t \times \text{O.D.}_t
\]

This formula found to be more accurate and is an easier way to compare the cell number in broth media instead of laborious traditional methods like colony counting which are time and effort consuming.

A direct colony counting method was performed to test the reliability of calculated cell number. Same procedure under constant condition was followed: Muller-Hinton broth \( (pH: 7.3) \) supplemented with 100 \( \mu \)g/ml of lichen extracts was inoculated with 6 log10 CFU/mL of bacteria and incubated at 30\(^{\circ}\)C. All environmental and nutritional conditions were same as followed for kinetic study. After 24h of incubation, the inoculated media was poured on Muller-Hinton agar \( (pH: 7.3) \) plates and further incubated for 24h and colonies were counted. F-test and t-test were carried out to compare mean value and variance of two data sets, after 24 of incubation of treated and non-treated bacteria, seeking for significance difference. The predicted cell number was used to make growth curve based on exponential growth equation model against time.

Equation of exponential growth curve for each bacterial cultures treated with extracts was made as per the reference equation (5) from Graphpad Prism\(^\circ\), where \( Y_0 \) is starting point of graph on y axis which is the number of bacteria at time 0 and \( K \) is growth rate. Respective equation of each sample is shown in table 10.
D) Development of growth curves by Gomertz model: Biologists often utilize growth experiments to analyze basic properties of an organism or effect of a specific substrate on their growth \(^{70}\). To develop growth curves for analytical studies, usually different models use based on diverse criteria. These models differ in their number of parameters and also in the biological significance of their parameters \(^{71}\). In 1825 Benjamin Gompertz \(^{285}\) showed relationship of proportional population through time in a mathematical way \(^{284}\). Gompertz curve was used by actuaries till some biologists started to use this model to make growth curve for biological phenomena \(^{284}\). Winsor (1932) \(^{284}\) described research results of some scientists who used this equation for their biological model, which worked well on its parameters and made it easier to use. This model is now well-accepted and according to different citation like; Zwietering et al., 1990 \(^{69}\); Buzrul, 2009 \(^{287}\); Kahm et al., 2010 \(^{70}\); Xiangbo et al., 2014 \(^{58}\); Palacios et al., 2014 \(^{288}\); Baty et al., 2003 \(^{71}\), it most closely approximates bacterial growth behavior.

Another advantage of this model is its easiness to find lag phase duration. The lag phase of microbial growth was defined by Penfold (1914) \(^{289}\) as the interval between the inoculation of a bacterial culture and the time of commencement of its growth rate. Lag is inherently more difficult to predict than growth rate because it depends on the physiological state of the inoculum as well as growth conditions \(^{68}\). Lag phase helps to better understanding the effect of a substrate or condition on organisms and to know how much it can stringent the environment for the organism to grow. According to these backgrounds, Data were fitted to Gompertz model in Graphpad Prism\(^{6}\) and lag phase duration was calculated from the point that number of cells was not increasing exponentially.

3.1.4 Correlation study between LPD and growth rate, growth rate and fractional area

It is presumed that the duration of lag depends on 1) the quantum of biological processes that a cell needs to do to adapt to its environment and prepare for division and 2) the rate at which it is able to do the work \(^{68}\). In order to seek the relation between lag phase and growth rate, correlation study was performed using Pearson’s r. It was also carried out for fractional area and growth rate. In AUC, it was shown that less growth rate causes less fractional area and less AUC \(^{68}\). This study was performed to calculate correlation
coefficient (r) and coefficient of determination (r²) between growth rate and lag phase as well as growth rate and FA to analyze the significance level of correlation among them.

3.1.5 TLC-bio-autography overlay assay

TLC bio-autography overlay technique of Gibbons & Gray (1998) was performed for detection of antimicrobial active components in lichen’s crude extracts. Briefly, lichen extracts in concentration of 200μg/ml were spotted on a pre-coated silica gel plates (60F254, Merck Co, USA) and were developed using chloroform: methanol solvents (97:3) in a closed TLC chamber. The developed TLC plates were sterilized under UV lamp for 30 min before encasing in nutrient agar and then covered with another layer of molten nutrient agar (45 °C) containing 6log10 CFU/mL of bacterial suspension. After 4 h of diffusion at 4 °C, the plates were incubated for 24h at 34 °C and then sprayed with 2.5 mg/ml of methyl thiazolyl tetrazolium bromide (MTT) (yellow color), which converts to a formazan dye (purple color) by live bacteria. The zone of inhibition observed as a clear spot against purple background.

3.2) Anti-Diabetic activity of lichen extract

3.2.1) α-Amylase inhibitory activity of lichen extract: In order to investigate extracted lichen species for their anti-amylase activity, three different concentrations (5, 10, 15 mg/ml) were tested for their amylase inhibitory against fungal Diastase (from Aspergillus oryzae) by the method of Karthik et al. (2011) and Narkhede et al. (2011). A 500 μl of each lichen extract in three different concentrations were added to 500 μl of 0.1 M phosphate buffer (pH 6.8) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After incubation period, 500 μl of a 1% starch solution in 0.1M phosphate buffer (pH 6.8) was added to each tube. Each test tube was then incubated at 25°C for 10min to allow the reaction to take place. The enzymatic reaction was stopped with 1.0 ml of 3, 5-dinitrosalicylic acid (DNSA) color reagent. Samples were then incubated in boiling water bath for 5 min and cooled at room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control test performed by replacing solvent with extracts and assumed to have 0% enzyme activity. To compare tested extracts with standard drug, commercially available acarbose was used as positive control in 0.2, 0.4 and 0.6 mg/ml concentration. From this standard drug, a calibration line was constructed and extracts where evaluated
for their content of acarbose, so it shows if activity of tested samples are related to acarbose or it is because of lichen unique secondary metabolites\textsuperscript{[294]}. The percentage (\%) inhibition of each sample was calculated using the formula\textsuperscript{[114]}:

\[
\% \text{ Inhibition} = \left( \frac{A_{540\text{Control}} - A_{540\text{ Extract}}}{A_{540\text{Control}}} \right) \times 100
\]

IC\textsubscript{50} (half maximal inhibitory concentration), which is measure for effectiveness of substances in inhibiting biochemical function\textsuperscript{[292]}, was estimated by nonlinear regression graph using Log (inhibitor) Vs. Normalized response model\textsuperscript{[293]} in Graphpad Prism with the following equation:

\[
Y=\frac{100}{1+10^{(X-\log IC50)}},
\]

Where Y is response and X is log of inhibitor concentration.

3.2.2) \(\beta\)-glucosidase inhibitory activity of lichen extract: In order to evaluate extracted lichen species for anti \(\beta\)-glucosidase property, a spectrophotometry method was followed according to Verma \textit{et al.}, 2012\textsuperscript{[237]}. In this method \(p\)-nitro phenyl-\(\beta\)-D- glucopyranoside (PNGP) was used as substrate and enzyme inhibitory was measured by % inhibition based on turbidity of each sample. The concept behind O.D measurement here is, more inhibition cause less conversion of substrate to product, hence sample contains more substrate and more turbidity. A 2 mM \(p\)-nitro phenyl-\(\beta\)-D- glucopyranoside (0.5 mL), 0.2 mL lichen extract (20μg/mL) and 50 mM potassium phosphate buffer (0.3 mL) pH 5 were placed in a test tube and incubated at 37\(\degree\) for 10 min in a water bath. A 20 mU of enzyme \(\beta\)-glucosidase (Enzyme activity: 3500U/mg) was added and incubated at 37\(\degree\) for 30 min. After completion of incubation period, the enzymatic reaction was terminated by addition of 2.6 mL of potassium phosphate buffer pH 10. The same was done with acarbose, a commercially available enzyme inhibitory drug to control Diabetes mellitus and final results were compared with this standard drug. For negative control, phosphate buffer of pH 10 was added at the beginning of the reaction to block enzyme activity. Absorbance was measured at 410 nm for each sample\textsuperscript{[295]}. The percentage inhibition calculate using this formula:

\[
\% \text{ Inhibition} = \left( \frac{\text{Abs 410 Control} - \text{Abs 410 Extract}}{\text{Abs 410Control}} \right) \times 100
\]

Reports had been shown that high content of phenolic components could inhibit glucosidase activity\textsuperscript{[363,364,365]}. In order to investigate the effect of phenolic content on
glucosidase inhibitory a correlation study was performed by comparing % of inhibition if each extract with its quantified phenolic content.

3.2.3) TLC Bio-autography for screening anti β- Glucosidase activity: The lichen extracts were examined for β-glucosidase inhibitors by the cleavage of 2- naphthyl-β-D-glucopyranoside to form 2-naphthol on TLC plates, which in turn reacts with Fast Blue B salt to give a purple-coloured diazonium dye [119]. β-D-glucosidase (1000 U) get dissolved in 100mL of buffer solution (10.25 g of sodium acetate in 250 mL with addition of acetic acid 0.1 M to pH 7.5) and the stock solution was stored at 4°C. After migration of the samples with an appropriate solvent, The TLC plate was dried under a stream of cold air for complete removal of solvent. The plate was then sprayed with enzyme solution. For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing little water, avoiding direct contact of water with the plate. The tank was covered in order to keep the atmosphere humid and incubated at 37°C for 20 min. For detection of the active compounds in lichen extract, the solution naphthyl-β-D-glucopyranoside (for β-D-glucosidase) at 2 mg/mL in ethanol and Fast Blue Salt at 2.5 mg/mL in distilled water was prepared. After incubation of the TLC plate, the naphthyl-glucopyranoside solution and the Fast Blue B salt solution was mixed at a ratio of 1:4 and was sprayed onto the plate to give a purple coloration after 2–5 min [108, 296].

3.3) Anti-Cancer activity of lichen extract

3.3.1) Passaging and counting cells to get desired concentration: Transferring some or all cells from a previous culture to fresh growth medium is passaging [297,298]. Cells were checked for any contamination under inverted microscope (Invi, Magnus), and then, in case of adherent cells, media was removed and flask was washed with PBS to remove all traces of serum. To dissociate the cells, Trypsin/EDTA solution was added till it covered monolayer of cells and flask was placed in 37 °C incubator for 2 minutes. Then complete growth medium was added by twice the volume of trypsin solution, and pipetted vigorously. Cells were centrifuged at 200 × g for 5 to 10 minutes and then were re-suspended in a complete growth medium in flask [301].

Sub culturing suspension cells is less complicated than passaging adherent cells as the cells are already suspended in growth medium. Centrifugation is required when suspension cells medium is acidic which indicates the cells have overgrown or if cells are not in single cell form. Then cells were reseeded at a slightly higher cell density and 10-
20% of complete medium to the fresh medium was added to them. A sample (100-200μl) from each flask was removed for counting cells and concentration of present cells was calculated according to following formula:

Concentration (Cells/ml) = (Total cell counts / number of squares) *10^4

The desired cell numbers was seeded into flasks containing media.

3.3.2) Viability test by trypan blue dye exclusion method: Trypan Blue is a blue acid dye that has two chromophores group. It is an essential dye, use in estimating the number of viable cells present in a population. 50uL of cell suspension was mixed with an equal volume of trypan blue. After vigorously pipetting it got transferred to a hemocytometer and observed under inverted microscope. Live cells were appeared as clear form and dead cell as blue cells. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue unlike dead cells. Following formula was used to calculate percent viability:

% Viability = (live cell count/total cell count)*100

3.3.3) Microculture tetrazolium assay (MTT): This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the intensity of purple formazan dye is a measure of the viability of the cells. Desired concentration of each cell lines were plated in 98-well plates. After 24 hours incubation, they got incubated in the presence of various concentrations of lichen extracts in 0.1% DMSO for 24h at 37°C. Further, 200μl/well (5mg/ml) of 0.5% MTT phosphate-buffered saline solution was added to each well. After 4h incubation, 0.04M HCl/ isopropanol was added as solubilization solution. Then absorbance was measured at 570nm by a bench-top microplate photometer. Percent inhibition of cells by lichen extracts was calculated by following formula:

% Inhibition = ([Abs_{control} – Abs_{test}]/Abs_{control}) ×100

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test compound) and Abs test is the absorbance of the test compound.
IC\textsubscript{50} of extracts was determined by a non-linear regression model to fit the obtained data to the log (inhibitor) vs. response (variable slope) curve \cite{393}.

### 3.3.4) DNA isolation and estimation of most affected cell by lichen extracts:

**A) DNA isolation:** DNA of most affected cell by lichen extract was subjected to isolation and estimation to run it on the gel electrophoresis for DNA laddering. DNA isolation was done with DNA-Xpress reagent, which is designed for rapid purification of DNA from different samples \cite{310}. Sample was homogenized with 1 ml of DNA-XPress reagent, then DNA was precipitated by 1 ml of absolute ethanol and was mixed repeatedly. After 3 minutes incubation at room temperature, DNA became visible. Then DNA pellet was removed and washed twice with 1ml of 95% ethanol. Ethanol was decanted and sample was kept to dry. Isolated DNA was dissolved in sufficient amount of freshly prepared 8mM NaOH to reach the desired concentration \cite{310}.

**B) Estimation of most affected cell:** Estimation of isolated DNA: Isolated DNA was quantified by Qubit® 2.0 Fluorometer (Invitrogen), a highly sensitive and accurate fluorescence-based machine used for the quantitation of DNA, RNA, and protein by Qubit quantitation assay \cite{311}.

### 3.3.5) Mechanism of Cell Death:

#### 3.3.5.1) DNA laddering technique

This method (also called DNA fragmentation assay) is used to visualize the endonuclease cleavage products of apoptosis \cite{313}. In apoptosis mechanism of cell death, DNA degrades by Caspase-activated DNase (CAD), which cause fragmentation of DNA. This fragmentation may be seen when isolated DNA is run in an agarose gel electrophoresis \cite{312,314}. DNA loading buffer and Novel juice (fluorescent reagent, GeneDirex) was added to isolated DNA sample and this solution along with 1kb DNA ladder as marker was loaded into dry wells of 1.5% agarose gel in TAE buffer. Gel was run at low voltage to improve resolution of DNA fragments for about 4 hours. Developed gel was visualized by UV-LED transilluminator (BLook, GeneDirex). Apoptotic cells form a distinct DNA ladder, whereas necrotic samples may generate a smear pattern. DNA from viable cells will stay on the top as a high-molecular-weight band.

#### 3.3.5.2) Morphological studies on biological hallmarks of cell death mechanisms:

In order to understand the efficacy of lichen extract on cell lines, morphological changes in the cell structures after treatment were compared with cells shape without treatment. Cells
were fixed on the slide by cytology cytopspin centrifuge (Cyto-Tek®Centrifuge). Giemsa and May Grunwald solutions, which are used for In Vitro diagnostic purposes, were applied to stain fixed cells. Briefly, Fixed slides were dipped in May-Grunwald Stain for 5 minutes. Then washed with PBS pH 7.2 for 1.5 minutes. Slides were placed in diluted Giemsa solution for 15 minutes and then rinsed with deionized buffer \[316,317\], slides were dried and observed at 10X and 40X under digital research stereo zoom microscope (SteREO Discovery.V20, ZEISS, Germany).

3.3.5.3) Cell Cycle Arrest: Cell cycle arrest analysis uses the fluorescent nucleic acid dye: propidium iodide (PI) to identify the proportion of cells that are in each of the three-interphase stages (G1, S, G2) of the cell cycle. PI dye bind in proportion to the amount of DNA present in the cell \[318,319\]. So as the amount of DNA in S phase is more, more fluorescent emission is scattered from PI-DNA complex, intensity of scattered light is analyzed by a flowcytometer to distinguish treated cell stages \[320\]. So distribution of cells among different phases of their growth cycles was achieved by following method: Briefly, most affected cell line was chosen and treated with the highest cytotoxic lichen extract for 24 hrs. Then sample cells were harvested and washed twice with PBS and then got fixed in cold 70% ethanol and vortex repeatedly. Again cells were washed in PBS and centrifuged, supernatant was discarded. Then 50 µl of a 100µg/ml stock of RNase was added to sample to remove traces of RNA. And finally 200 µl PI (from 50 µg/ml stock solution) was added and incubated at room temperature for 30 min \[321,322\]. The stained cells were analyzed by a BD LSRFortessa™ cell analyzer.

3.3.5.4) Detection of early apoptosis by Annexin V- FITC and propodium iodide (PI): Sometimes an early apoptosis event causes cell death, which usually occurs within 3-4hrs of treatment of cells with drug \[312,323\]. One of the earlier events of apoptosis includes translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface \[324\]. Annexin V, a phospholipid-binding protein, has high affinity for PS (early apoptosis marker), and fluorochrome-labeled Annexin V (Annexin V-FITC) can be used for the detection of early apoptosis. The BD Pharmingen™ Annexin V-FITC apoptosis detection kit was used to perform this study. So to analyze early apoptosis, sample cells were treated with the drug for 4 hrs. After harvesting, the cells were washed twice with PBS and re-suspended in 100µl 1X binding buffer (10 mM HEPES/NaOH, of pH 7.4, 140 mM NaCl and 2.5 mM CaCl2) \[324\]. Then 5 µl of FITC-Annexin V was added to the cells followed by the addition of 5 µl of PI.
Sample was incubated for 10 min in the dark at 4°C and then analyzed by BD FACS Flow cytometer™.

3.4) Isolation and Characterization of potential metabolites

The most effective lichen extract among all studied samples was chosen and subjected to isolation of its effective compounds, which were previously detected by TLC-bioautography.

A) Thin Layer Chromatography was performed to detect and separate the active bands. Most suitable solvent in order to get a good separation was standardized as Chloroform: Ethanol (97:3). From different TLC-Bioautography assays, which were done during this study, three active bands were marked as biologically active metabolites and were subjected to isolation and purification by column chromatography.

B) A column was set and packed by silica gel (60-120 mesh) and chloroform (as non-polar solvent) using slurry method (Fig-50). In order to find suitable solvent system and its ratio for obtaining better separation in column chromatography, various solvents with different ratio were used to run the spotted TLC plates. Finally, Chloroform: methanol was used for successive elution of metabolites in the ratio of 100:0 to 75:25 by gradually increasing the polarity. Non-polar compounds were eluted first and polar metabolites were eluted last. All obtained fractions were tested for their purity by TLC and finally three different pure metabolites were isolated. Isolated and purified samples were concentrated using rotary evaporator. Dried samples were analyzed further for an accurate characterization.

C) LC-MS was done using solvent system of acetonitrile–formic acid in a “Waters Acquity” UPLC BEH C18 1.7um 1.0 x 50mm column run at a flow rate of 0.25 μL/min.

D) GC-MS analysis was performed where 100μl of each sample was dried completely using Nitrogen drying technique. Samples were suspended in pyridine and derivatized using methylamine hydrochloride followed by BSTFA: TMCS. Then they were subjected to GCMS after dilution (1:20) with methanol. GC was of Agilent 7890A and MS was of 5975C MSD series, Single Quadrupole mass analyzer with a DB5 column (30m L x 0.25mm ID x 0.25um film thickness). With an autoinjector (7683B) and Electron Impact Ionization system where Helium used as carrier gas at a flow rate of 1 ml/min with split ration of 10:1 and injection volume of 1μl. Range of scanning mass was 30m/z – 600m/z.
E) $^1$H NMR and $^{13}$C NMR
Purified samples (in DMSO-$d_6$) were analyzed by $^1$H NMR and $^{13}$C NMR to elucidate their chemical structures, using a Agilent NMR spectrometer (USA; 400 MHz for $^1$H and 100 MHz for $^{13}$C) with tetramethylsilane as an internal standard. Chemical shifts ($\delta$) are expressed in ppm, with the coupling constants ($J$) reported in Hertz (Hz).

F) 2D NMR (COSY, HMBC, HMQC)
To adequately solve the structure of isolated metabolites, three different types of 2D NMR spectroscopy were performed; COSY (COrrelated SpectroscopY) gave information about concerning coupled (homonuclear) systems and helped to determine the configuration of molecule. HMBC (Heteronuclear Multiple Bond Correlation) and HSQC (Heteronuclear Single Quantum Correlation) showed connectivity between protons and carbons.

3.5) Determination of MIC and NIC of isolated compounds
In order to investigate that whether effect of chosen extract is due to synergetic action of its metabolites or it’s the effect of individual compound, a micro-dilution method was used to determine their MIC and NIC values. Data were fitted to a modified Gompertz model and results were compared with the crude extract data to conclude the efficacy of isolated compounds.

Statistical Analysis:
All analyses were carried out in triplicates and the mean value of absorbance ± SD was taken for analysis. Calibration lines and correlation graphs in this study were constructed using Microsoft Excel and GraphPad Prism 6® Software. In GraphPad data were presented at 95% confidence. To find out correlation coefficients ($r$) between individual groups, Pearson’s bivariate correlation test was done. $r$ near to 1 indicates strong positive relationship and near to -1 shows reverse (negative) relation. P-value < 0.05 considered as significant value to reject null hypothesis. For this level of significance ($\alpha<0.05$) in a directional test, $t \leq 1.70$ considered to reject null hypothesis and indicates the correlation is not by chance coincidence.