CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

The present study was carried out in Dhubri district of Assam, situated in the extreme western part of Assam and is a gateway to North-East India. The district is bounded by both inter-state and international border i.e. West Bengal and Bangladesh in the west, Goalpara and Bogaigoan district of Assam in the east, Kokrajhar district in the north, South Salmara–Mankachar district and state of Meghalaya in the south.

The district is located between 89.42° to 90.12° E longitude and 26.22° to 25.28° N latitude. It covers an area of 2,176 sq. km. The district is situated 30 meters above the mean sea level on average. The region has humid, subtropical, dry winter climate with annual temperature ranging from 23°C to 31°C, 83.15 mm of precipitation and humidity 64.21%. Generally the topography of Dhubri district is plain with several patches of small hillocks like Tokorabandha, Dudhnath, Chandardinga, Boukumari and Boropahar which are located in the north eastern part of the district. The enormous River Brahmaputra flows through the district from east to west with its tributaries Champabati, Gadadhar and Gangadhar.

Soil is alluvial type and dominated by deciduous type of vegetation. Commonly available flora are viz. *Acacia* sp., *Albizia* sp., *Aquilaria agallocha, Areca catechu, Artocarpus heterophyllus, Azadirachta indica, Bombax ceiba, Cassia fistula, Cedrus deodara, Citrus* sp., *Coccos nucifera, Dalbergia sissoo, Delonix regia, Dillenia indica, Emblica* sp., *Eucalyptus* sp., *Ficus benghalensis, F. hispida, Gmelina arborea, Lannea coromandelica, Litchi chinensis, Mallotus tetraphylla, Michelia champaca, Mangifera indica, Mesua ferrea, Neolamarckia kadamba, Oroxylum indicum, Pongamia pinnata, Shorea robusta, Syzygium cuminii, Tectona grandis*.

Total population of the district is 19, 49,258 (approx.) according to 2011 census. The economy of Dhubri district is primarily dependent on agriculture, and the main source of income is paddy production followed by jute and mustard cultivation occupies the major share of the cash crops.

The district acquires an appreciable grade quality in the domain of handicraft, such as Terracotta (national recognition), bamboo crafts, and jute crafts. The district is enriched culturally due to various art, religion, races and caste of local and migrated people.

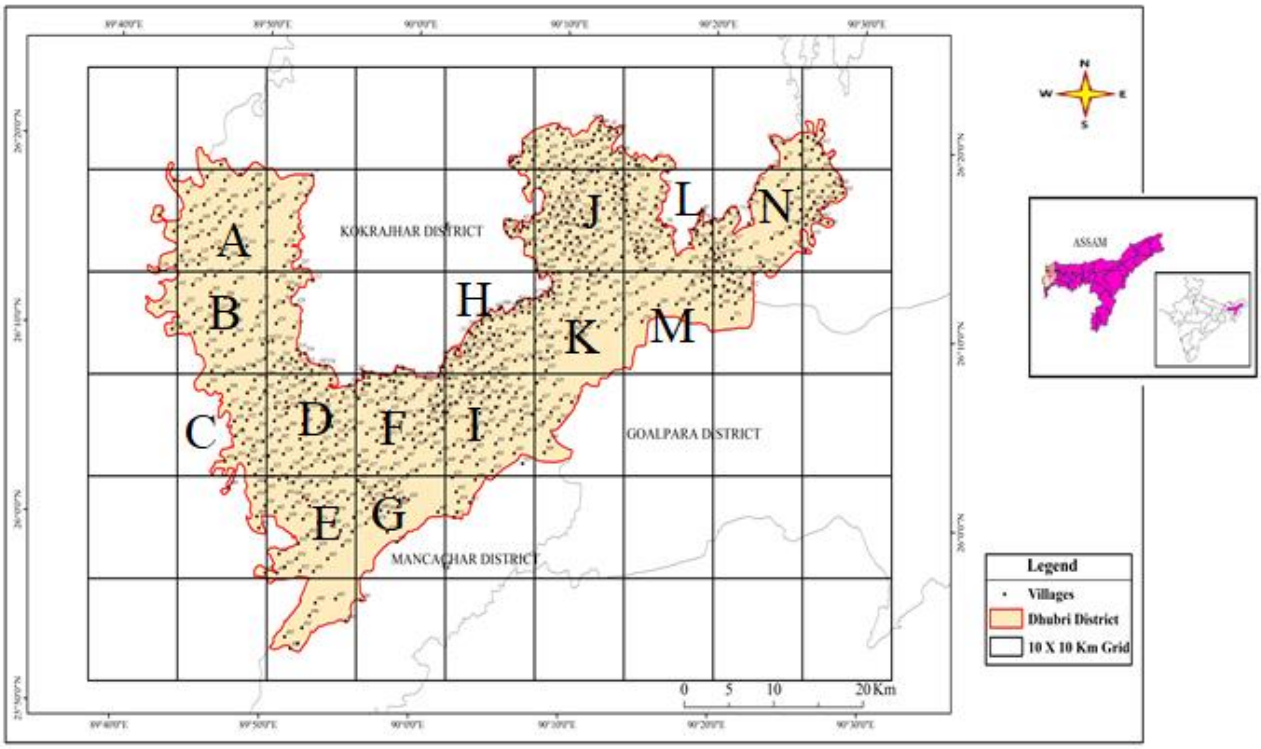

Fig 3.1: Grid map of the study area (10×10 Km. sq.)

3.2 Sampling sites

The entire map of Dhubri district was divided into grids (quadrat) of 10×10 km^2 using ArcGIS 9.3 software using the Hawaath tool for exploring lichen diversity in individual grid. The location of the villages were collected using the GPS points and Google earth maps and overlaid on the grid map to identify the locations available in the particular grid. Final map was generated using the ArcGIS software 9.3. Parts of the map covering 50-100 % of the grid were considered for sampling. In total, 14 grids (A-N) represented the map (**Fig 3.1**) of which seven grids viz. A, D, G, H, J, L, N were considered for the present study. Selection of the grids were done such that it gives the right representation of the district. From each grid covering 100 % of the map, five villages were considered and three villages for those grids covering >50<100 % of the map. In this way a total of 31 sampling sites (equivalent to villages) were considered (**Table 3.1)**. Selection of villages within the grids were made such that it represents the grid at its best. Further, ten trees were selected from each sampling site throughout the entire village which leads to 310 trees. Only the corticolous lichens were considered for the present study.

Besides these, additional lichen samples were collected from seven sites viz. Alokjhari, Bilashipara Florican Garden, Chandor Dinga Pahar, Gauripur Matiabag Hawakhana, Panbari tea garden, Satrasal and South Tokrerchara pt-IV (**Table 3.2**). Alokjhari and Chandor Dinga Pahar are small hillocks of historical importance. Gauripur Matiabag Hawakhana, once upon a time was the palace of Jamindar of Gauripur and is situated on the bank of the river Godadhar; Panbari tea garden is one of the two tea gardens of the district; Satrasal, situated at Indo-Bangladesh border is one of the Satras of Mahapurush Sankardeva viz. Ramrai kuti and South tokrerchara pt-IV is my residential area. From each of these seven sites, 10 trees were selected accounting to 380 trees in total for lichen collection.

Table 3.1: Details of the sampling sites for collection of lichen samples

Grid no.	Sl. no.	Sampling sites	Latitude	Longitude
A	1	Chagolia pt-I	26°29′50″ N	89°78′33″ E
	2	Kherbari pt-II	26°25′23″N	89°74′16″E
	3	Bidyadabri pt -V	26°25′82″N	89°77′87″E
	4	kaldoba pt- III	26°21′54″N	89°79′17″E
	5	Barobaluchar (Uttar maragadadhar)	26°23′79″N	89°83′07″E
D	6	Uchita	26°11′12″N	89°85′83″E
	7	Pub- gaikhowa pt-I	26°08′41″N	89°84′46″E
	8	Debotar hasdaha pt-IV	26°07′93″N	89°89′43″E
	9	Dumardaha pt-II	26°08′18″N	89°92′42″E
	10	Kismat hasdaha pt-II	26°04′59″N	89°88′73″E
G	11	Chagolchara pt-III	26°02′77″N	89°94′59″E
	12	Dhubri town	26°01′78″N	89°99′61″E
	13	Khalilpur	26°03′10″N	89°97′09″E
H	14	Rangamati pt-III	26°15′69″N	90°05′74″E
	15	Gauripur town	26°08′10″N	89°96′51″E
	16	Alomganj pt-IX	26°13′73″N	90°04′08″E
J	17	Bhalukmari	26°30′57″N	90°13′48″E
	18	Pataner kuti	26°28′41″N	89°87′45″E
	19	Khajurbari pt-I	26°27′05″N	90°18′49″E
	20	Fakiranir jhar pt-I	26°22′20″N	90°14′58″E
	21	Ananda nagar	26°22′60″N	90°21′91″E
L	22	Bhasani goan	26°30′55″N	90°22′91″E
	23	Hatipota pt-II	26°22′38″N	90°24′73″E
	24	Jamduar pt-I	26°24′13″N	90°26′95″E
	25	Gopigoan pt-III	26°26′63″N	90°23′66″E
	26	Gourangtari pt-II	26°24′95″N	90°31′71″E
N	27	Falimari	26°30′31″N	90°40′36″E

	28	Sreegram pt-III	26°26′04″N	90°33′85″E
	29	Bhelupara pt-II	26°22′92″N	90°34′34″E
	30	Brahmin para	26°24′26″N	90°24′44″E
	31	Arear jhar pt-II	26°25′64″N	90°39′72″E

Table 3.2: Details of the additional sampling sites

Grid no.	Sampling sites	Latitude	Longitude
L	Alokjhari	26°25′09″N	89°86′30″E
G	Bilashipara Florican Garden	26°15′30″N	90°16′31″E
N	Chandor Dinga Pahar	26°19′75″N	90°35′83″E
F	Gauripur Matiabag Hawakhana	26°50′00″N	89°58′00″E
G	Panbari tea garden	26°15′42″N	90°05′06″E
B	Satrasal	26°16′30″N	89°73′50″E
D	South Tokrerchara pt-IV	26°11′06″N	89°83′32″E

3.3 Collection of lichen samples:

The field survey was carried out throughout the year from 2019 December to March 2021, except Covid-19 lockdown periods. Lichen samples were collected along with the bark of any tree species on which they grew as per the standard criteria proposed by Pinho *et al*. (2004) and Nayaka (2005). About 2000 epiphytic lichen samples were collected. The following criteria of tree species were considered for collection of lichens:-

1. Trunk ≥35 cm in diameter,
2. Healthy and
3. Reachable height of 1.5–2.0 m above the ground

Hammer and chisel were used for collection of the samples. Geographical details of the sampling sites such as latitude, longitude and elevation were recorded in the field using Geographical Positioning System (Garmin, etrex 10). Samples collected were stored in paper envelopes and other informations such as tree species, sampling site, date of collection were noted on each envelope.

3.4 Preservation of the samples:

As the humidity of the region is high, the samples were dried properly using blotting papers. Dried samples were then stored in herbarium packets made of acid free paper of dimension 13.5×11.5 inches and contain details of collection site, lichen species, family, date of collection, notes on substratum, interesting characters if any, collectors name and accession number. Naphthalene balls are used for preservation of samples. All the specimens are deposited in the Bodoland University Botanical Herbarium (BUBH), Department of Botany, Bodoland University, Kokrajhar, Assam. A set of voucher specimens of some of the species are deposited in the 'National Repository' of CSIR-National Botanical Research Institute (LWG), Lucknow, India.

3.5 Identification of lichen samples:

Identification of lichen samples involved morphological, anatomical observations and identification of chemical substances present in the lichen thallus (Awasthi 1991, 2007).

3.5.1 Morphological observations:

The morphological characters were studied under stereozoom microscope (Leica EZ4W). External features such as colour of thallus and medulla, presence or absence of rhizines, cilia, pseudocyphellae, soredia, isidia, pruina, pycnidia; perithecia, apothecia and their types are some of the important characters required to be noted.

3.5.2 Anatomical observations:

For anatomical observations, a thin hand cut sections of the thallus and fruiting bodies were mounted on water, taken on a slide and observed under the compound microscope (Leica DM 750). Features like colour of epithecium, hymenium, hypothecium; excipulum and its carbonization, presence or absence of crystals and oil globules, shape of ascus, number of spores per ascus, colour and type of septation, spore shape and size were observed.

3.5.3 Chemical identification: Spot colour tests and Thin Layer Chromatography were performed to know the chemical constituents present in lichens.

3.5.3.1 Spot tests: It was performed by applying some chemical reagents (K, C, KC, P) on the thallus and medulla. The reagents used are as follows:

K test (Potassium hydroxide): It was prepared by dissolving 10 g potassium hydroxide pellets in 100 ml of distilled water.

C test (Calcium hypochlorite): It is consists of calcium hypochlorite in distilled water in the ratio 1:2.

KC test: It implies application of potassium hydroxide, immediately followed by calcium hypochlorite on the thallus or medulla.

P test (Para-phenylenediamine): It was prepared by dissolving 1 g of para-phenylenediamine and 10 g of sodium sulphite in 100 ml of distilled water with 1 ml of a liquid detergent.

I test (Iodine): Composed of 2 g of iodine dissolved in 100 ml of distilled water with 0.5 g of potassium iodide. It is generally used on sections of fruiting body.

These reagents were applied either on the thallus or the medulla and the change in colour was noted. Chemistry behind this is that reagents reacts with the secondary metabolites present in lichen thallus.

3.5.3.2 Thin layer chromatography (TLC):

Lichen substances extracted in acetone were loaded on TLC plate and placed in TLC jar containing solvent system comprising Toluene: 1, 4-dioxane: glacial acetic acid in the ratio 180:45:5. *Parmelinella wallichiana* was used as the control. Dried TLC plates were then sprayed with 10 % sulphuric acid solution and then placed in pre-heated oven at 110°C for about 15 minute. The spots developed on the plates were noted and the Rf (Retention factor) value was calculated. Lichen substances were identified based on the colour of spots and Rf value following Orange *et al*. (2001).

Rf value of the lichen substances were calculated as follows:

$$\text{Rf value} = \frac{Distance\ travelled\ by\ the\ lichen\ substance}{Distance\ travelled\ by\ the\ solvent}$$

3.5.4 Observations of fluorescence*:* Some lichen species due to the presence of certain compounds such as lichexanthone or secondary metabolites like depsides and depsidones emits fluorescence (yellowish, bluish) when exposed to UV light at 254 nm.

Above all these, relevant literatures (**Table 3.3**) were referred for identification of the lichen specimens upto species.

Table 3.3: Literatures referred for the identification of lichens upto the rank species

Sl. no.	Genera	Literature cited
1	*Allographa*	Lücking *et al*. (2009); Sharma *et al*. (2012)
2	*Arthonia*	Awasthi (1991)
3	*Arthopyrenia*	
4	*Arthothelium*	
5	*Astrothelium*	
6	*Bacidia*	
7	*Bacidina*	
8	*Chrysothrix*	
9	*Clathroporina*	Upreti (1994)
10	*Coenogonium*	Plata *et al.* (2006)
11	*Coniocarpon*	Awasthi (1991)
12	*Cratiria*	Elix (2000)
13	*Cryptothecia*	Awasthi (1991)
14	*Diorygma*	Kalb *et al*. (2004)
15	*Dirinaria*	Awasthi (2007)
16	*Enterographa*	Sparrius *et al*. (2006); Jagadeesh Ram *et al.* (2008)
17	*Glyphis*	Awasthi (1991)
18	*Graphis*	Lücking *et al*. (2009)
19	*Herpothallon*	Frisch *et al.* (2014); Jagadesh Ram and Sinha (2009)
20	*Lecanora*	Nayaka (2004)
21	*Lepraria*	Bajpai *et al.* (2018)
22	*Letrouitia*	Awasthi (1991)
23	*Nigrovothelium*	Aptroot and Lücking (2016)
24	*Oxneriopsis*	Kärnefelt (1990)
25	*Pallidogramme*	Awasthi (1991)
26	*Parmotrema*	Awasthi (2007)
27	*Phaeographis*	Awasthi (1991)
28	*Physcia*	Awasthi (2007)
29	*Porina*	Liu *et al.* (2020); Orange (2013); Upreti (1994)
30	*Pyrenula*	Aptroot (2012); Upreti (1998b)
31	*Pyxine*	Awasthi (2007)
32	*Sarcographa*	Awasthi (1991)
33	*Stirtonia*	Aptroot *et al.* (2014)
34	*Synarthonia*	Awasthi (1991); Van den *et al.* (2018)

35	*Trypethelium*	Awasthi (1991); Aptroot and Lücking (2016)
36	*Viridothelium*	Aptroot and Lücking (2016)

The classification of lichen species were assigned as per the literature of Wijayawardene *et al.* (2020). The names of the specimens were updated following Index Fungorum for lichen taxonomy. Singh and Sinha (2010); Sinha *et al.* (2018); Joseph *et al*. (2020) were referred for the distribution of the identified lichen species in India and as per Global Biodiversity Information Facility (GBIF) database for worldwide distribution of the recorded species.

3.6 Ecological parameters:

Quantitative ecological parameters viz. Alpha diversity, Beta diversity, Abundance and Frequency for identified lichen species were used for describing the community structure.

3.6.1 Alpha diversity (α)

According to Whittaker (1972) richness of species in a particular ecosystem or an area is determined by the alpha diversity. More the value of alpha diversity higher the number of species of the area.

3.6.2 Beta diversity (β)

According to Whittaker (1972), the beta diversity compares the diversity of species in different ecosystems. Beta diversity determines the similarity or differences of a range of habitats in terms of the variety of species found. Beta diversity increases with the increase in dissimilarity between the ecosystems. The formula for calculating the beta diversity is mentioned below:

$$\beta \text{ diversity} = \frac{(No.\ of\ species\ in\ quadrat\ 1\ only) + (No.\ of\ species\ in\ quadrat\ 2\ only)}{N}$$

Where N is the number of species in both the sites.

3.6.3 Abundance

Abundance is the distribution pattern of species within a particular area. Rareness or commonness of a species in an area can be determined from this. The formula for calculating abundance is mentioned below:

$$\text{Abundance} = \frac{(Total\ no.\ of\ species\ in\ all\ the\ quadrates)}{Total\ no.\ of\ quadrats\ in\ which\ the\ species\ occur}$$

3.6.4 Frequency

Frequency is the distribution and degree of dispersion of individual species in a particular area. It is expressed in terms of percentage occurrence and is calculated as follows:

$$\text{Frequency} = \frac{(No.\ of\ quadrates\ in\ which\ the\ species\ occur)}{Total\ no.\ of\ quadrats\ studied}$$

3.7 Phytochemical screening of the lichen species selected for antimicrobial activity:

3.7.1 Selection of the species

Foliose and fruticose lichens are mostly explored for evaluation of antimicrobial activities because of the considerable amount of biomass compared to crustose lichens. Further as crustose lichens have to be scraped off from the substratum there is greater chance of adulteration of the lichen compounds with that of the substratum. So, based on the availability of the lichen species in the study area, their chemical constituents and availability of data against the tested organisms following macrolichens were considered for phytochemical and antimicrobial studies (**Table 3.4**).

Table 3.4: List of lichen species considered for phytochemical and antimicrobial test

Sl. no.	Lichen species	Growth Form (GF)	Habitat	Family
1	*Dirinaria appalanata* (Fèe) D.D. Awasthi	Foliose	Corticolous	Caliciaceae
2	*Dirinaria consimilis* (Stir t.) D.D. Awasthi	Foliose	Corticolous	Caliciaceae
3	*Dirinaria papillulifera* (Nyl.) D.D. Awasthi	Foliose	Corticolous	Caliciaceae
4	*Dirinaria picta* (Sw.) Clem. & Shear	Foliose	Corticolous	Caliciaceae
5	*Parmotrema saccatilobum* (Taylor) Hale	Foliose	Corticolous	Parmeliaceae
6	*Pyxine cocoes* (Sw.) Nyl.	Foliose	Corticolous	Caliciaceae
7	*Pyxine reticulata* (Vain.) Vain.	Foliose	Corticolous	Caliciaceae

3.7.2 Preparation of sample extracts: The lichen thalli were cleaned and removed from the substratum with the help of blade and forcep under stereozoom microscope, washed with distilled water and dried at room temperature for 24 hours. Then it was powdered using mortar

and pestle. For extraction of the lichen substances, 10 g of the powdered samples were extracted in soxhlet apparatus using methanol and hexane.

The extracts obtained were concentrated in vacuo at 40°C using a rotary evaporator. The residues obtained were preserved in deep freezer for future use.

3.7.3 Qualitative phytochemical analysis of the lichen species: Methanolic and hexane extracts were used for screening of phytochemicals viz. Alkaloids, Flavonoids, Phenols, Tannin, Saponin, Triterpenoids using standard procedures (Harbone 1973; Yadav and Agarwala 2011; Roopalatha and Nair 2013; Devi *et al.* 2017).

Table 3.5: Details of the solvents used for extraction of the lichen compounds

Sl. no.	Solvent	Boiling point	Melting point	Density (g/ml)	Relative polarity
1	Methanol (CH_3OH)	64.4°C	- 98°C	0.791	0.762
2	Hexane (C_6H_{14})	69°C	-96°C to -94°C	0.655	0.009

Test for Alkaloids

a. **Dragondroff's test**: To 2 ml of crude extract, 1% HCl was added, steamed for 10 minutes and to these, 6 drops of Dragondroff's reagent was added. Formation of reddish–brown precipitate indicates the presence of alkaloids.
b. **Mayer's reagent test**: To 2 ml of crude extract, 2% (V/V) H_2SO_4 was added and warmed for 2 minutes. To this, few drops of Mayer's reagent was added and creamy white precipitation indicates the presence of alkaloids.
c. **Wagner's reagent test**: To 2 ml of crude extract 2% (V/V) H_2SO_4 was added and warmed for 2 minutes. To this, few drops of Wagner's reagent was added and reddish brown precipitation indicates the presence of alkaloids.

Test for Flavonoids

a. **NaOH solution test**: To 2 ml of crude extract, 2 ml of 10% NaOH solution was added and a yellow to orange precipitation indicates the presence of flavonoids.

b. Ammonium test: Crude extract of 1 ml was heated in water bath for 3 minutes and to this, 1 ml of 1% NH_4OH was added and shaken. Yellow coloration indicates the presence of flavonoids.

c. Aluminium Chloride test: About 1 ml of crude extract was heated in water bath for 3 minutes. To this, 1 ml of 1% $AlCl_3$ solution was added and shaken. Light yellow coloration indicates the presence of flavonoids and on addition of dilute NaOH and HCl the solution turns colourless.

Test for Phenols

a. Nitrous Acid test: To 1 ml of crude extract, few drops of 5% (V/V) acetic acid solution and 5% (W/V) $NaNO_2$ solution were added. Muddy or Nigar brown colour indicates the presence of Phenol.

b. Ferric Chloride test: About 1 ml of crude extract was treated with 2-3 drops of 5% (W/V) $FeCl_3$ solution and appearance of black colour indicates the presence of Phenol.

Test for Tannin

Ferric Chloride test: To 1 ml of crude extract, 2-3 drops of 5% (W/V) $FeCl_3$ solution was added and appearance of transient green or black colour indicates the presence of Tannin.

Test for Saponin

Foam test: About 1 ml of crude extract was mixed with 5 ml of distilled water and shaken. Appearance of foamy layer on the top indicates the presence of saponin.

Test for Triterpenoids

Salkowski test: About 2 ml of crude extract was treated with 2 ml of chloroform and to it 3 ml of concentrated H_2SO_4 was added carefully by the side wall of the test tube. Reddish brown coloration in the interface indicates the presence of triterpenoids.

3.8 Quantitative estimation of total phenol and flavonoids contents

3.8.1 Total Phenol Content (TPC)

The total phenol content in lichen extracts was determined using Folin-Ciocalteu reagent following methods recommended by Iloki-Assanga *et al.* (2015). The Methanolic and hexane extracts were prepared at a concentration of 1mg/ml then 1 ml of extracts were added with 1.5 ml Folin-Ciocalteu reagent (10%) and 0.5 ml Na_2CO_3 (7% W/V). The mixture was then vortexed for 15second and incubated for 30 minutes at room temperature. The absorbance was measured at

765 nm using UV–visible Spectrophotometer (Systronics PC Based Double Bean Spectrophotometer 2206). The total phenolic content (mg/ml) was calculated using Gallic acid as the standard. The experiment was carried out in triplicate.

3.8.2 Total Flavonoid Content (TFC): Total flavonoid content was determined using Quercetin equivalent assay recommended by Ordonez *et al.* (2006). The Methanolic and hexane extracts were prepared at the concentration of 1 mg/ml, then 1 ml of the extract is mixed with 1 ml (w/v) 2% Aluminium chloride ($AlCl_3$). The mixture was then incubated for 10 minutes at room temperature. The absorbance was measured at 420 nm using UV–visible Spectrophotometer (Systronics PC Based Double Bean Spectrophotometer 2206). The total flavonoid content (mg/ml) was calculated using Quercetin equivalent (QE) curve as the standard. The experiment was carried out in triplicate.

3.9 Determination of Antimicrobial activity of the selected lichen species:

For antimicrobial activity, a phytopathogenic bacterial and three fungal strains known to cause various diseases in crop plants viz. rice, jute and mustard (**Table 3.6**) were considered and were obtained from CSIR- Institute of Microbial Technology Sector 39- A, Chandigarh 160036 India, Microbial Type Culture and Gene Bank (MTCC). Strains used in this study were *Pyricularia oryzae* (MTCC 1477), *Xanthomonas oryzae* (MTCC 11102), *Colletotrichum gloeosporioides* (MTCC 10529) and *Sclerotinia sclerotiorum* (MTCC 8785). Bacterial strain was maintained on Nutrient Agar (NA) and fungal strains on Potato Dextrose Agar (PDA).

Table 3.6: Detailed informations about the phytopathogens used for antimicrobial screening

Sl no.	Crop plant	Disease	Symptoms	Causal organism	Pathogen type
1	*Oryza sativa* (Rice)	Blight of rice	Leaves with yellowish white or golden yellow marginal necrosis, drying of leaves from tip and curling	*Xanthomonas oryzae*	Bacteria
2	*Oryza sativa* (Rice)	Rice blast	On leaves the lesions start as small water soaked bluish green specks, soon enlarge and form boat shaped spots with grey centre and dark brown margin	*Pyricularia oryzae*	Fungus
3	*Corchorus* sp. (Jute)	Anthracnose	On mature plants, initially light yellowish patches are	*Colletotrichum gloeosporioides*	Fungus

			seen on stem which turns to brown/black depressed spots		
4	*Brassica nigra* (Mustard)	*Sclerotinia* stem rot	Stems develop water soaked spots near crown region which later may be covered with cottony white mycelium.	*Sclerotinia sclerotiorum*	Fungus

3.9.1 Minimum Inhibitory Concentration (MIC) by Broth Dilution Method

The evaluation of MIC was done as per National Committee for Clinical Laboratory Standards (NCCLS 2001). Approximately 50 µl of each microbial strain containing 1×10^6 CFU/ ml were added to 5 ml of lichen crude methanolic and hexane extracts ranging from (300-3000 µg/ml) prepared in nutrient broth for bacteria and potato dextrose broth for fungal pathogenic strains as a diluent. Tubes were then incubated at 30°C for bacteria and 25°C for fungal strain for 24 hrs and 48 hrs respectively. Amoxicillin and Nystatin were used as positive control. Tubes were examined for visible signs of bacterial and fungal growth and absorbance for each sample was read at 600 nm in UV spectrophotometer (Systronics PC Based Double Bean Spectrophotometer 2206). Least concentration of the lichen extracts resulting in complete inhibition of the growth of the pathogens was considered as the MIC values. IC_{50} values for the extracts were calculated using AAT Bioquest calculator.

3.9.2 Disc Diffusion Method: Antimicrobial activity of the lichen extracts against the phytopathogenic microbes were further done by disc diffusion method (Clinical and Laboratory Standard Institute 2009). Methanolic and hexane lichen extracts were dissolved in dimethyl sulphoxide (5% DMSO) to prepare desired concentrations. Inoculum of the microbial strains (50 µl) were plated using sterile glass spreader on petridishes with nutrient agar for bacteria and PDA for fungal strains. Then sterile discs of Whatman paper no. 1 of 5 mm in diameter were soaked in 50 µl of lichen extracts (3 mg/ml) and placed onto inoculated petri dishes. Standard antibiotic Amoxicillin (3 mg/ml) and antifungal Nystatin (3 mg/ml) were used as a positive control and DMSO as a negative control. The petri dishes were pre-incubated for 1 hr at room temperature, allowing complete diffusion of the lichen extracts and then incubated bacterial strains for 24 hrs at 30°C and fungal strains for 48 hrs at 25°C (Das *et al.* 2010). The zones of inhibition were measured.

3.10 Statistical analysis: The obtained experimental data of **t**otal phenolic, flavonoid content and antimicrobial assays were expressed as mean ± standard deviation.