MATERIALS AND METHODS

3.1. Media used *(g/l unless otherwise stated)*

(pH of the media was adjusted before autoclaving unless otherwise stated).

3.1.1 Nutrient Broth

Beef Extract	3.0
Peptone	5.0
NaCl	5.0
pH	6.8±2

3.1.2 Nutrient Agar

Beef Extract	3.0
Peptone	5.0
NaCl	5.0
Agar	20.0
pH	7.0±2

3.1.3 King's medium B

K_2PO_4	3.0
Na_2HPO_4	1.0
NH_4Cl	1.0
$MgSO_4.7H_2O$	0.4
Glycerol	15ml
Agar	15.0

3.2. Reagents used

3.2.1 Protein estimation

a. *Biuret reagent*

0.2N NaoH Solution:

Dissolved 8gm of Sodium hydroxide in 1000ml of dH_2O. Dissolved 3gm $CuSo_4.5H_2O$ and 9 gm of sodium potassium tartarate in 500ml of 0.2N of NaOH solution. Potassium iodide 5gm was added and volume was made to 1 litre by adding 0.2N NaOH solution.

b. BSA stock solution

Powdered 1200mg of BSA was weighed out into 100ml volumetric flask and was dissolved gradually in 10ml distilled water with careful shaking; a few drops of 0.2 (N) NaOH solution was added to facilitate the solution and volume was made up to 100ml.

3.2.2. Determination of DPPH Radical scavenging activity

a. 0.3mM of DPPH

Dissolved 1mg of DPPH in 25ml ethanol (100%)

3.2.3 Determination of total antioxidant activity

a 0.6M H_2SO_4

5.8ml of H_2SO_4 was added to dH_2O and volume was made up to 100ml.

b. 28 mM Sodium phosphate

Dissolved 1.064gm NaH_2PO_4 in 100ml dH_2O.

c. 4 mM Ammonium molybdate

Dissolved 494 mg of $NH_4.MoO_4$

in 100ml dH_2O.

3.2.4 Determination of Reducing power ability

a. Phosphate buffer (pH 6.6)

Dissolved 2M Monobasic sodium phosphate 276g/l (stock solution A) and 2M Dibasic sodium phosphate 284g/l (stock solution B).62.5 (A) +37.5(B), and volume was made up to 200ml by adding water.

b. Potassium ferricyanide

Dissolved $K_3Fe(CN)_6$ 1.64 gm in

100ml of dH_2O

c. Trichloroacetic acid (10%)

Dissolved 1.63gm of Trichloroacetic acid in dH_20 and volume made up to 100ml.

d. Ferric chloride (1%)

One gm of $FeCl_3.6H_2O$ in dH_2O
and volume made up to 100ml

3.2.5 Determination of Total phenolic content

*a. Folin ciocalteu reagent (*1:10 v/v with water)

To 10 ml of reagent 100 ml of dH_2O was added

b. Sodium carbonate (75g/lit)

Dissolved 75 gm of sodium carbonate and volume was made up to 1000 ml by adding of dH_2O

3.2.6 *Determination of Total Flavonoid content*

a. Aluminium nitrate (10%) in 80% ethanol

Dissolved 2 gm of aluminum nitrate in 20 ml ethanol (80%)

b. Potassium acetate (1M)

Dissolved 1.96gm of *Potassium acetate* in 20ml ethanol (80%).

3.2.7 *Determination of Catalase activity*

a. 0.05M phosphate buffer pH 6.5

Dissolved 1.56gm of $NaH_2PO_4.2H_2O$ in 50 ml of d H_2O (A) + 1.78gm of $Na_2HPO_4.2H_2O$ in 50 ml of dH_2O (B)

34.25 ml of reagent (A) + 15.75 ml of reagent (B), pH was adjusted to 6.5 and volume was made up to 100 ml.

b. *0.0025M H_2O_2*

H_2O_2 (0.028 ml) 30% mixed to 100 ml of d. H_2O

c.*0.1% Titanium sulphate*

$TiSO_4$ (15%) 0.1ml added to 14.9 ml of 25%

H_2SO_4.

c.*25% H_2SO_4*

(Conc. H_2SO_4) 25.5ml was added to 74.5 ml of dH_2O

3.2.8 *Determination of Peroxidase activity*

*a.300µM phosphate buffer (*pH -6.8*)*

Dissolved 2.34gm of $NaH_2PO_4.2H_2O$ in 50 ml dH_2O(A).Dissolved 2.66gm of $Na_2HPO_4.2H_2O$ in 50 ml dH_2O(B)

25.5 ml of (A) + 24.5 ml (B) was mixed and volume was made up to 50 ml. By adding d.H_2O

b. *Pyragallol*

Dissolved 189mg of Pyragallol in 100ml of dH_2O.

c. 5% H_2SO_4

Added 2ml of 25% H_2SO_4 to 8ml of distilled water.

3.3. Instruments used

1. Laminar air flow cabinet (MSW-161, Macro Scientific Works, New Delhi, India).

2.Orbital shaking incubator (MSW-232, Macro Scientific Works, New Delhi).

3. Digital Balance (Sartorius, Germany)

4. Hot air oven (MSW-211, Macro Scientific Works, New Delhi)

5. Spectrophotometer (Shimadzu UV-1700, Japan)

6. Sonicator (Labsonic M, 100 W, Sartorius, Japan)

7. Cooling centrifuge (REMI, C-30 BL, India)

8. Rotary evaporator (Ricon, India)

9. UV-Transilluminator (Bangalore Genei)

10. Vortex mixer (REMI, India)

11.Digital camera (SONY, Japan)

3.4. Methodology

3.4.1. Area of Study

Darjeeling District (27° 13" N to 26° 27" N and 88° 53" E to 87° 59" E), is the northernmost District of West Bengal. The area under Darjeeling District is flanked by North Dinajpur District from South, Bangladesh from South-East, Bihar from South-West, Nepal from West, Sikkim from North and Bhutan from North-East.

The District has four Administrative Sub-divisions namely, Darjeeling, Kalimpong, Kurseong and Siliguri. The area of Darjeeling District falls under the Northern Hill Zone and Teesta is the most important river of this zone. It's principle tributaries are the Rangphu and Relli on the left bank; Rangeet, Reang and Sevoke on the right bank. In the west of Teesta, the Mahanadi, Balasan and Mechi merge into Ganges.

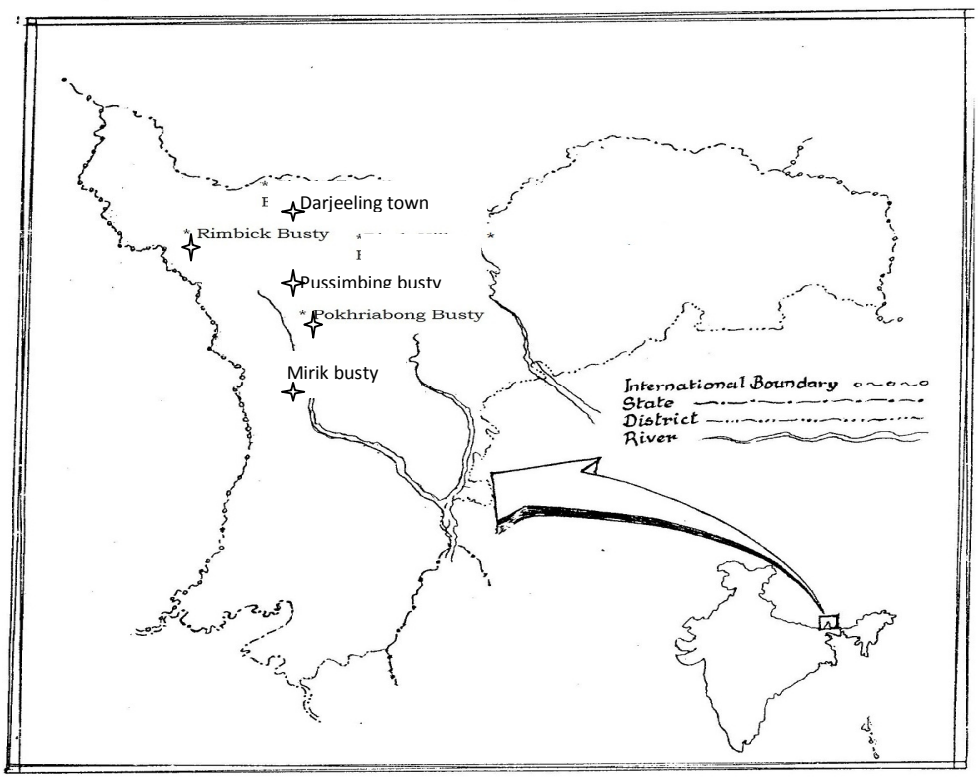

Figure 3.1 Area of study showing sampling sites

The geographical area of hill areas of Darjeeling District is 247800 hectare. About 18% of the soil is highly acidic (pH below 4.9), 60% is

moderately acidic (pH 5.0 to 5.9) and the rest 22% is normal (above 6.0). The rainfall varies from 2941mm to 1391mm of which 80% is received during June to September. The average maximum and minimum temperature round the year records are 20˚C and 2˚C respectively. Details of climatic conditions are given in Table 3.1 and 3.2 respectively.

Table 3.1 Climatic conditions of Darjeeling District- Average monthly rainfall for last five years

Rainfall (mm)					
Month	2011	2012	2013	2014	2015
January	0	0	0	0	0
February	39	0	28.2	3.4	0
March	14	0	19.2	33.6	0
April	48	204	54	47	98.7
May	123	81	294.9	159	87
June	629	339	261.6	331.8	244
July	961	529	310.5	452.6	356.6
August	667	284	319.2	543.5	278.1
September	438	505	199.7	305.8	326.6
October	14	18	159.7	4.8	0
November	8	0	0	0	0
December	0	0	0	0	0
Total	2941	1960	1647	1881.5	1391

Source: Deputy Director of Agriculture (Administrative), Darjeeling)

Table 3.2.Climatic conditions of Darjeeling District- Average monthly temperature for last five years

Month	Temperature (°C)									
	2011		2012		2013		2014		2015	
	Mean maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini	Mean Maxi	Mean Mini
January	9.8	1.3	9.5	2.0	8.1	1.5	9.5	1.7	10.2	1.7
February	15.1	4.3	10.5	4.0	9.1	1.9	12.4	3.3	12.6	3.8
March	19.1	7.6	10.5	6.0	13.5	5.6	20.2	8.1	18.4	5.7
April	19.7	8.0	22.3	9.0	16.9	9.2	19.5	9.1	20.5	8.3
May	20.0	12.3	21.5	8.8	17.8	11.2	19.8	11.3	22.2	9.8
June	19.4	14.5	20.0	16.0	18.6	13.4	20.3	13.5	24.1	15.1
July	18.5	15.0	20.0	15.5	19.1	14.3	20.8	14.0	21.5	15.0
August	19.9	14.8	22.0	15.6	18.8	14.1	21.5	15.8	22.2	16.0
September	20.2	14.2	21.5	15.0	18.3	13.2	24.2	13.5	23.4	15.2
October	18.8	12.7	20.1	12.0	16.3	9.9	19.5	11.1	18.6	13.0
November	12.0	6.8	17.0	6.5	12.8	5.9	15.8	6.2	14.8	6.5
December	12.5	4.5	14.0	5.5	9.6	2.4	14.6	4.4	12.9	6.2

Source: Deputy Director of Agriculture (Administrative), Darjeeling

3.4.2. Sampling sites: The sampling sites were selected on the basis of elevation, vegetation and population status (Table 3.3.). Two sampling sites located at sub-tropical region are Mirik, Pokhriabong Busty and three other are located at temperate region [Darjeeling town, Pussimbing Busty and Rimbick busty].

Table 3.3 Elevation and habitat of sampling sites

Sl No.	Sampling sites	Altitude (msl)	Habitat
1.	Darjeeling town	2130	*Alnus* trees
2.	Pussimbing Busty	2000	*Erythrina* trees, rock
3.	Mirik Busty	1300	*Citrus* trees
4.	Pokhriabong Busty	1550	*Macaranga*, *Alnus* trees
5.	Rimbick Busty	2600	*Prunus*, *Betula*, *Alnus* trees

3.4.3. a. Collection of lichen samples:

Lichen samples were collected in paper polypacks from different sampling sites described in Table 3.3. Samples were collected from the barks of trees like *Alnus*, *Erythrina, Macaranga, Citrus*, *Betula*, *Prunus* as well as rocks and brought to the laboratory. Each specimen was preliminarily identified with the help of available literature, Key to Macrolichens (Awasthi, 1988). The taxonomic identity of lichen samples was confirmed from the Lichenology Laboratory, National Botanical Research Institute, Lucknow, Uttar Pradesh, India and the voucher specimens were deposited in the Herbarium of the Postgraduate Department of Botany, Darjeeling Government College, Darjeeling, India.

3.4.3. b. Collection of medicinal plant samples:

The medicinal plants under study were collected from profusely grown places of Darjeeling and surrounding areas. The lichen and medicinal plant samples are deposited in P.G Department of Botany, Darjeeling Government College, Darjeeling, West Bengal, India

3.4.4. Extraction of samples

Each lichen and medicinal plant sample was washed to remove debris, dried and ground to powder and was stored in sterile glass bottle in the refrigerator. The 10g portions of sieved powder were added to 100 ml of solvents (ethanol and methanol), sonicated for 30 min and left overnight at room temperature. The crude extract was prepared by decanting, followed by filtration through muslin cloth and further filtered with Whatman No. 1 filter paper to obtain a clear filtrate. Fifty ml of the filtrate was evaporated to obtain 10 ml of concentrated extract and sterilized by membrane filtration using 450 nm bacteriological filters. Such sterilized filtrate was stored in screw capped airtight containers in the refrigerator and used for antimicrobial screening. The remaining (50 ml) filtrates were concentrated to paste in reduced pressure at 40°C using a rotary evaporator and were used for the determination of minimal inhibitory concentration (MIC) value, protein content and growth of the test microorganisms and antioxidant assays.

Table 3.4 List of lichens and medicinal plant samples

Sl No.	Name of lichens and medicinal plants	Extraction solvent	Extract code
1	*Everniastrum* sp	ethanol	EVRE
		methanol	EVRM
2	*Parmotrema reticulatum*	ethanol	PARE
		methanol	PARM
3	*Ramalina hossei*	ethanol	RARE
		methanol	RARM/CLRM
4	*Sterecaulon pomiferum*	ethanol	STRE
		methanol	STRM
5	*Usnea baileyi*	ethanol	USRE
		methanol	USRM
6	*Berginia ciliata*	ethanol	BERE
		methanol	BERM
7	*Panax pseudoginseng*	ethanol	PNXE
		methanol	PNXM
8	*Sapindus mukrossi*	ethanol	SAPE
		ethanol	SAPM
9	*Urtica dioica*	methanol	URRE
		methanol	URRM

3.4.5. Extract yield (%) of extracts

Extract yields of dried extracts were calculated the following equation: % Extract yield = (W_1x100) / W_2. W_1 shows the remaining solid lichen extract weight after evaporation of the solvent used in extraction; W_2 shows the weight of lichen powder form used in extraction (Aydin and Kinadiglu, 2013).

3.4.6. Test microorganisms

Test microorganisms (seven bacteria and one fungus) were obtained from Institute of Microbial Technology, Chandigarh, India (table.3.5).the bacterial culture was preserved in N.A medium and fungal culture in King's medium B.

Table 3.5. List of test microorganism

Sl No.	Test Microorganisms	Gram nature	MTCC Code
1	*Alcaligenes faecalis*	Gram negative	MTCC9780
2	*Bacillus megaterium*	Gram positive	MTCC 7192
3	*Bacillus subtilis*	Gram positive	MTCC 3972
4	*Candida albicans*	-	MTCC 4748
5	*Escherichia coli*	Gram negative	MTCC 6365
6	*Enterobacter aerogenes*	Gram negative	MTCC 111
7	*Staphylococcus aureus*	Gram positive	MTCC 7443
8	*Pseudomonas aeruginosa*	Gram negative	MTCC 424

3.4.7. Screening of antimicrobial activity

This procedure is based on disc diffusion method of Bauer *et. al.,* (1966). Overnight grown bacterial cultures of approximately (0.1ml) were spread plated on nutrient agar plates to achieve semi confluent growth. Sterile filter paper discs were soaked in concentrated extracts, allowed to dry between the applications and placed on plates which were then incubated at 37°C for 24 hrs. Streptomycin (10µg/ml) and sterile distilled water were taken as positive control and negative control respectively. Growth was evaluated and inhibition zone were measured. All the experiments were repeated thrice and data presented are average of three independent readings.

3.4.8. Determination of minimal inhibitory concentration of extracts against test microorganisms

The filtrate was evaporated to dryness under a rotary evaporator and the residues were dissolved in Dimethlyl sulfoxide (DMSO) and further diluted with sterile distilled water to obtain the concentrations (250 µg/ml, 500 µg/ml, 1000 µg/ml, 5000 µg/ml and 10000 µg/ml), (Javeria *et. al.*, 2013).

All of the test microorganisms in this study were used and the bioactivity was based on agar-diffusion assay (Dubey and Maheshwari, 2002). Overnight grown cultures (0.1 ml) were spread plated on nutrient agar plates. Cylindrical holes were made with the help of sterile cork borers on the petriplates containing test microorganisms. Different extract concentrations were filled to the holes and after overnight incubation at 37°C; the plates were screened for the production of inhibition zone. Minimum extract concentration which yielded inhibition zone was considered as their respective MIC value. The MIC values of lichen sample were compared to antibiotic, Streptomycin.

Plate 3.1 Lichen samples under study

Plate 3.2 Medicinal plants under study

3.4.9. DPPH radical scavenging assay

The free radical scavenging activity of the extracts was measured *in vitro* by 1, 1-diphenyl-2- Picryl-hydrazyl (DPPH) assay (Nagarajan *et. al.*, 2008). Solution having strength of 0.3 mM DPPH in ethanol and methanol was prepared and 1 ml of this solution was added to 3 ml of the extract residue dissolved in ethanol/ methanol at different concentrations (25-200 µg/ml). This mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm. Ascorbic acid was taken as reference. The ability to scavenge DPPH radical was calculated by the following equation (Adedapo *et. al.*, 2009):

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(Abs_{control} - Abs_{sample})]}{(Abs_{control})]} \times 100$$

Where $Abs_{control}$ is the absorbance of DPPH radical + solvent; Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

For the study of combined activities respective solvents extracts from different samples were mixed in equal proportion and used.

3.4.10. Reducing power ability

The reducing power was investigated by the Fe^{3+}-Fe^{2+} transformation in the presence of the extracts as described (Nagarajan *et. al.*, 2008). The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. One ml of the extract (25-200 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. Supernatant (2.5 ml) was diluted with 2.5 ml of water and was shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm using spectrophotometer. Butylated hydroxy toluene, BHT (25-200 µg/ml) was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations. For the study of combined activities respective solvents extracts from different samples were mixed in proportion and used. The more change in colour of extracts to blue indicates the increased reduction of Fe^{3+} to Fe^{2+}.

3.4.11. Determination of total antioxidant capacity

The total antioxidant capacity of the extracts was determined with phosphomolybdate method using α-tocopherol as the standard (Nagarajan *et. al.*, 2008). An aliquot of 0.5 ml of the extracts solution was combined with 5 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. The extracts concentration (25-200 µg/ml) was prepared by dissolving the extract residue in respective solvents (ethanol and methanol). After the samples had cooled to room temperature, the absorbance was measured at 695 nm against the blank using an UV spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. All tests were performed in triplicate, the total antioxidant capacity was expressed as µg equivalents of α-tocopherol by using the standard α-tocopherol graph ($Y=0.141x-0.039$; $R^2 = 0.941$).

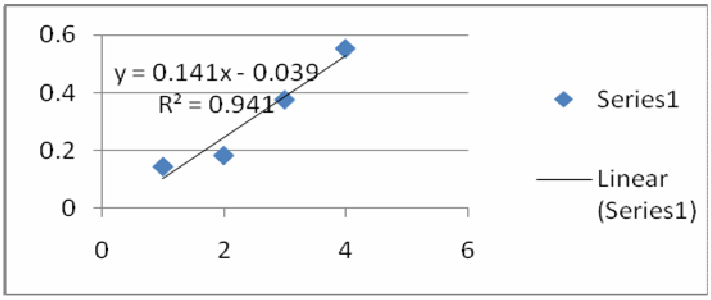

Fig 3.2 Standard α-tocopherol graph

3.4.12. Estimation of total flavonoid content

Total soluble flavonoid content of the extracts was determined with aluminium nitrate using quercetin as the standard (Nagarajan *et. al.*, 2008). One mg of the extract was added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was measured at 415 nm in UV spectrophotometer after incubation at room temperature for 40 min. The lichen extracts concentrations (25-200 µg/ml) were made by dissolving the residue extract in respective solvents (ethanol/methanol). The total flavonoid content in the

extracts was determined as µg quercetin equivalent by using the standard quercetin graph using following equation based on calibration curve:- y=356x -0.461; R^2 = 0.697.

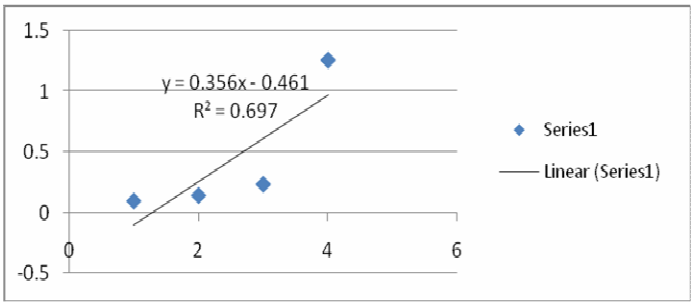

Fig.3.3 Standard quercetin graph

3.4.13 Determination of total phenolics

Total soluble phenol contents in the extracts were determined using Folin-Ciocalteu reagent using tannic acid as standard with some modifications (Adedapo *et. al.*, 2009). Five ml Folin-Ciocalteu reagent (previously diluted with water at 1:10 v/v) was added to 4 ml (75 g/l) of sodium carbonate and 0.1mg/ml extract. The mixtures were vortexed for 15 sec and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm using the UV spectrophotometer. The lichen extract (25-200µg/ml) were made by dissolving the residue extract in respective solvents i.e., ethanol and methanol separately. Total phenolic content were expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: - y=0.007x-0.186; R^2= 0.938.

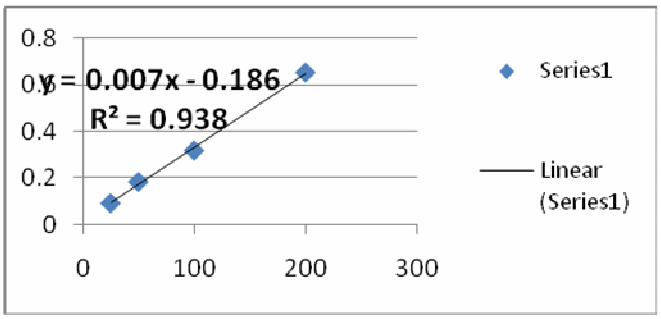

Fig. 3.4 Standard tannic acid curve

3.4.14. Catalase activity

One gm of freshly collected lichen thallus was homogenized with 10 ml of 0.05 M cold phosphate buffer (pH-6.5). The homogenate was then centrifuged at 5000 rpm for 10 minutes and supernatant was taken as enzyme extracts. One ml of 0.0025M H_2O_2 added to 1ml of crude enzyme extract, the mixture was then incubated at 37°C for 5 minutes. The reaction was stopped by adding 2 ml of 0.5 % $TiSO_4$. The content was further centrifuged for further 10 minutes and O.D of the golden yellow supernatant was measured using the UV spectrophotometer at 420nm. For control set, $TiSO_4$ added to the enzyme extract before the addition of H_2O_2 was taken and same procedure as for reaction mixture was followed. The enzyme activity was calculated by the formula of Luck (1974).

$$\text{Enzyme activity} = \frac{\Delta A \times TV}{t \times V}$$

[Where Δ A= O.D. difference; TV = total volume of enzyme extract; t = time of incubation taken; V= volume of enzyme extract taken for the reaction].

Peroxidase activity

One gram of freshly collected lichen thallus was homogenized with 10 ml of 300µM cold phosphate buffer (pH-6.8). The homogenate was then centrifuged at 5000 rpm for 10 min and supernatant was taken as enzyme extract. For reaction mixture 1ml of phosphate buffer and 1ml of pyragallol added to 1ml of enzyme extract which was incubated at 25° C for 5 minutes. The reaction was stopped by adding 5% H_2SO_4 and mixture was further centrifuged at 5000 rpm for 10 minutes. The O.D of the yellow coloured supernatant was measured using the UV spectrophotometer at 430 nm. For contol set 1ml of 5% H_2SO_4 added to the mixture of enzyme extract, pyragallol and H_2O_2 before the addition of buffer. The enzyme activity was calculated by the formula of Luck (1974):

$$\text{Enzyme activity} = \frac{\Delta A \times TV}{t \times V}$$

(Where Δ A=O.D. difference; TV = total volume of enzyme extract; t = time of incubation; V= volume of enzyme extract taken for the reaction).

3.4.16. Effect of lichen extracts on total protein content of the test microorganisms

Effect of lichen extracts on total protein content of the microorganisms grown culture filtrate was performed following the method of Ray *et. al.*, (2003) with some modification. Different concentration of extracts (100-500µg/ml) was prepared filtered and sterilized. Sterile nutrient broth each of 5 ml was inoculated with 24 hours old broth cultures of different microorganisms (0.1ml) each, to which was added 0.5ml of extract of different concentration. The whole content was incubated at 37ºC for 24 hours. The content was sonicated and filtered through Whatman No.1filter paper and was centrifuged at 10,000 rpm for 5 minutes.

The clear supernatant was taken and Biuret method was employed for protein estimation. Three ml of Biuret reagent was added to 3 ml of the supernatant incubated at 37°C for 10 minutes and was allowed to stand at room temperature for 30 minutes. Then absorbance of the mixture was measured at 540nm. The OD_{540} values were recorded and total protein content of the supernatant was estimated using BSA as standard.

3.4.17. Effect of lichen extracts on the growth of the test microorganisms

Effect of lichen extracts on growth of microorganisms was performed following the method of Ray *et. al.*, (2003) with some modifications. Nutrient broth (50 ml) were inoculated with different volume (0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml) of lichen extracts (ethanol and methanol) from the stock (1mg/ml) mixed well, inoculated with 0.5ml of overnight grown different test microorganisms and incubated in shaking incubator (150 rpm) at 37ºC. Broth without any lichen extract was taken as control. OD_{620} value of the content was taken at initial i.e., 0 hour and at intervals of one hour repeatedly until stationary phase of the test cultures was obtained. Incubation time taken by the microorganisms to reach stationary growth

phase was determined using a plot with O. D. values on Y axis and time interval on the X axis.

3.4.18. Preliminary separation of lichen compounds by TLC

Extraction and preliminary identification of lichen compounds was done following the method of Santiago *et. al.*, (2010). One gram, air-dried thalli of lichen specimens were initially ground to powder and soaked overnight in 10 ml acetone. Acetone was used for the microscale extraction of the lichen acid, as most lichen substances are soluble in this solvent (Huneck and Yoshimura, 1996). After 24 hours, the extracts were filtered, concentrated by air-drying for 4 – 5 days or until the extracts crystallized, and the weight/yield of the crude extracts were determined. To identify the lichen acids present, crude extracts were dissolved in acetone to a final concentration of 10 mg/ml. The crude extracts were then spotted on silica gel thin layer chromatography (TLC) plates (silica gel 60 F_{254} aluminum plates, Merck) and run in three different solvent systems: (1) Solvent System A- 36:9:1 toluene/dioxane/glacial acetic acid, (2) Solvent System B- 24:18:4 hexane/diethyl ether/formic acid, and (3) Solvent C- 20:3 toluene/glacial acetic acid (Culberson *et. al.*, 1972). Each TLC plate was then sprayed with 0.5 ml glacial acetic acid and 1 ml 97 % sulfuric acid and heated at 105°C for 5 minutes to visualize the lichen acids (Santos and Mondragon, 1969). The RF values for each spot were determined using the formula:

$$RF = \frac{\text{distance travelled by the solute}}{\text{distance travelled by the solvent}}$$

3.4.19. Determination of the bioactive lichen compounds using TLC bioautography

To determine the bioactive lichen acids (Santiago *et. al.*, 2010), lichen extracts from representative lichen species were initially spotted on TLC plates. The TLC plates were then run in Solvent System A (36:9:1 toluene/dioxane/glacial acetic acid) 2) Solvent System B: 24:18:4 hexane/diethyl ether/formic acid, and (3) Solvent C: 20:3 toluene/glacial acetic acid (Culberson *et. a*l., 1972) and the spots were visualized under ultraviolet (UV) light (254 nm). Prior to the TLC bioautography, the TLC plates were allowed to air-dry for at least 24 hours to remove any traces of

the solvent system. A bacterial suspension was prepared from a 24-hour old *S. aureus* and *E. coli* culture. About 100 µl of the bacterial suspension were mixed with 100 ml cooled, melted NA. The seeded NA was poured on top of the base medium (approximately 15 ml solidified NA) and allowed to solidify.

The TLC plates were placed on top of the seeded layer and stored for two hours inside a refrigerator. This was to allow the metabolites to diffuse directly into the seeded layer without allowing the growth of the test organisms. After two hours, the TLC plates were carefully removed and the culture plates were then incubated at 37°C for 18-24 hours. After incubation, the spots having zones of inhibition were noted.

3.4.20. Identification of active principle in lichen extract

Four lichen samples was air dried at room temperature (26°C) for until complete drying and then it was ground to powder. Powdered lichen material (10g) was added to 100ml methanol, sonicated and shaken for 7 days in shaking incubator at room temperature. The extract was filtered through whatman filter paper no 42 and was concentrated using a rotary evaporator the obtained extracts were sent to SAIF, CDRI, Lucknow for LCMS analysis.

The mass spectrum as LCMS chromatogram of EVRM, USRM, STRM and CLRM obtained from SAIF was studied following the literature - A catalogue of standardized chromatographic data of synthetic relationship for lichen substances (Elix, 2014) and lichen substances were determined.

3.4.21Statistical analysis:

Statistical analysis were calculated using Excel software (Microsoft 2007) and SPSS version 21.0 for Windows 2007. Statistical significance was determined by One way ANOVA using Duncan's post hoc test. Correlation coefficients (r) were determined by using Pearson's bivariate correlation test. All the results are shown as mean ± standard deviation (SD) of three parallel measurements.