e-ISSN: 2582-5208
International Research Journal of Modernization in Engineering Technology and Science
(Peer-Reviewed, Open Access, Fully Refereed International Journal)
Volume:05/Issue:10/October-2023 Impact Factor- 7.868 www.irjmets.com

IDENTIFICATION OF LICHEN AND ITS SECONDARY METABOLITES FOR PRODUCTION OF EFFECTIVE ANTIMICROBIAL AGENT

Afrin Fathima. A*1, Ms. Abirami. S*2

*1M.Sc Microbiology, Department Of Microbiology, Hindusthan College Of Arts & Science, Coimbatore, Tamil Nadu, India.

*2Assistant Professor, Department Of Microbiology, Hindusthan College Of Arts &Science, Coimbatore. Tamil Nadu, India.

DOI : https://www.doi.org/10.56726/IRJMETS45675

ABSTRACT

Lichens Are Symbiotic Organism Composed Of Fungus And Analgae. They Produce Chacterstic Secondary Metabolites "Lichen Substance" That Seldom Occur In Other Organisms. Lichen Mycobionts As Other Fungi Could Therefore Be A Potential Source In The Search For Pharmaceutical Useful Chemicals. Lichen Produces Unique Metabolites That Has Various Effect As Antimicrobial And Antifungal Agents. Lichens And Their Metabolites Have Many Biological Activities Such As Antimicrobial, Antiviral, Anti-Oxidant, Anti-Inflammatory And Analgesic Activity. The Spot Test Is Done To Identify The Lichens And The Extractions Are Analyzed For The Phytochemical Screenings Their Antibacterial And Antifungal Properties Are Described By Plating Techniques.

Keywords: Lichen Isolation-Spot Test-Secondary Metabolites Biopotential Activity Antimicrobial Agent.

I. INTRODUCTION

Lichens are symbiotic organisms composed of fungus and an algae. They produce characteristic secondary metabolites 'lichen substances' that seldom occur in other organisms. Lichens are composed of fungal partner my cobiont and one or more photosynthetic partner photobiont. The photosynthetic partner is generally algae or cyanobacteria. There are about 13,500 species of lichen on the earth. In lichen, the mycobiont produces a thallus, which houses the photobiont .There are three major morphological types of thalli Foliose, Crustose and fruticose. Lichens can survive severe conditions because they can withstand drying. The lack of water interrupts photosynthesis. In this suspended state, some lichens are able to great extremes in temperature.

Lichens produce an unique variety of extracellular secondary metabolites known as lichen substances. These compounds exist within the thalli either in an amorphous form or as crystals. A lichen absorbs most of its mineral nutrients from the air and rainfall. Pollution in the atmosphere can be especially dangerous to lichens because they retain, and can accumulate, deadly amounts of heavy metals, sulfur, radioactive elements, NO_2, and ozone. Sulfur dioxide (SO_2) is especially lethal to lichens because it lowers pH and deteriorates chlorophyll, which causes photosynthesis to cease. Anti-sulfur dioxide legislation in the last 25 years is allowing lichens to return to formerly polluted areas. Lichens have been used to monitor the amount of pollutants in an environment. This is done by observing the condition of lichens as well as their chemical composition.

Lichenometry is a technique used in dating rock surfaces on which certain lichens grow by means of their rate of growth. Lichens produce many secondary compounds which play an important role in distinguishing species in the laboratory. A practical use for these same compounds is seen in medicine as well as natural dyes and as a component in perfume. In nature these compounds may serve as a defense against herbivores and may also help break down rock substrates. Lichens colonize places that have not had any previous growth, such as rocks. Lichens having cyanobacteria as a photobiont provide fixed nitrogen to their environment.

Ecologically, they are also important in providing food and shelter to wildlife including deer, moose, and elk, as well as certain species of squirrels, mice, and bats. Lichens contribute to the background diversity of the natural landscape and can be appreciated by all, even if only subliminally to the untrained eye. Lichen mycobionts as the fungi, could therefore be a potential source in the search for pharmaceutical useful chemical .Lichens and their metabolites have many biological activities such as antimicrobial ,antiviral, anti-oxidant, anti-inflammatory and analgesic activity.

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In spite of the wide spectrum of biological activities shown by the lichens, they have long been neglected by mycologists and overlooked by pharmaceutical industry because if its slow growth in nature and difficulties in the artificial cultivation of the organisms. Hence the large-scale industrial production of the lichen metabolites have never been accomplished.

Lichen extracts and their metabolites have been widely studied for their antimicrobial properties. But their anti bio film potential is still poorly explore. Some of these studies ,particularly on cultured mycobionts, tired to improve the culture to trigger and enhance the synthesis of secondary metabolites, to reveal the factors involved in this process, to understand the role of each of the partners in the synthesis of the lichens substances, or to find sources of therapeutic an gents.

In some cases the asymbiotic fungal strains synthesized the same secondary metabolites as the natural lichen. All or only some of them .Other examples showed that some mycobionts produced secondary metabolites different from those present as major compounds in the symbiotic state, including novel molecules such as the graphislactones, graphenone and xanthones. The primary intracellular metabolites include proteins , amino acids, cartenoids, polysaccharides and vitamins are generally soluble in water and can be easily isolated from the lichens by boiling water.

Some of the primary metabolites are produced by fungi and some by algae. Most of these metabolites are non specific and also may occur in free living fungi and some algae. In general, the amount pf nitrogen compounds is between 1.6% and 11.4% dry weight of the lichen thallus among vitamins. Lichen contains ascorbic acid, biotin , α-tocophenol, nicotinic acid, pantothenic acid. Riboflavin, thiamine and folic acid. Vitamins were identified as metabolic products which biosynthesis algae. While more than 800 secondary metabolites are known from lichens. All of the secondary substances in in lichens are of fungal origin. These substances are the crystals deposited on the surface if the hyphae, which are poorly soluble in water, and usually can be isolated from the lichens by organic solvent.

Morphology: Father of lichenology is Erick Acharius, who have started the study of lichen morphology. Study on lichen structures began over a century ago when the light microscope became readily available. The accurate account of the internal structure of several lichens is presented by Schwendener in 1860. Different parameters are needed to lichen identification by morphology

Vegetative part: The characterization of lichens by variety of vegetative structures rhizines, tomentum, cilia are also known as in fungi. But soredia to lichenized fungi. Pycnidia and conidia are non-symbiotic reproduction parts.

Growth form: Lichen morphology is usually determined by the organization of fungal filaments. Their vegetative part is known as Thallus. The most visually prominent part of the lichen was thallus, They are grouped by thallus type as crustose (crusts that are strongly attached to substrate), squamulose (having scale like lobes), Foliose(leafy structure), Umbilicate(attached t single point), Fruticose (shrubby), Gelatnous (its mucusy-gelly type and its photobiont is cyanobacterium) and Leprose (powdery). Macro and micro lichens are differ from their growth form will be the despite of wide diversity ,but they all have similar outer surface , where it comes in contact with the environment, are packed tightly together to form the intensity of light ,which may damage the algal cells. The algal cells are distributed just below the cortex in a layer. Medulla is below algal layer

Sexual reproduction It depends upon sexual life cycle of Ascomycota and according to different projections on the surface of thallus for sexual reproduction , lichens can be identified. These non vegetative bodies includes Mazaedia and Apothecia Lecanorine, Lecideine, Biatorine, Zeorin and perithecia, Hysterothecia, pseudothecia.

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II. METHODOLOGY

SAMPLE COLLECTION:

Fig 1: TN Map

Fig 2: Nilgiris Map

Lichen samples were collected from nilgiri mountain (Doddabetta), ooty, Nilgiris. Foliose and Fruticose types were found commonly, hence foliose and fruticose types of lichens were collected. The collected samples were packed in the acid free packets and stored at 4º C for the furthur experiments and studies.

SPOT TEST FOR THE IDENTIFICATION OF LICHENS

Spot test is chemical method, the chemicals were applied on the lichen fragments and their secondary metabolites were help to the identification of the lichen species.

K test: 10% aqueous solution of potassium hydroxide (KOH) is prepared by adding 20 grams of potassium hydroxide pellets in to the 100 ml of distilled water.The reagent is then sprayed on the lichen fragments .

C test: 5-25% of calcium hypochlorite were prepared by adding 50 grams of common bleach in to 100 ml of distilled water and mixed well, this mixture allowed to settle down and the supernatant is used as reagent . The prepared reagent is sprayed on the lichen fragments.

I test: The iodine reagent prepared by dissolving 0.5 grams of iodine and 1.5 g of potassium iodide in 100ml of distilled water, the mixed solution reacts with the certain polysaccharides in lichen.

MICROCRYSTALLOGRAPHY:

A small lichen fragment is placed over the slide. Few drops of acetone added and allowed to evaporate and the thallus is removed from the fragment. Then, few drops of crystallization agents like Glycerol, Ethanol, Water in 1:1:1 ratio to the slide. The slide has kept in warm place. Then the cover slip is placed and observed under microscope.

PREPARATION OF EXTRACTS:

The extracts were prepared in six different solvents like Ethanol, Methanol, Chloroform, Acetone, Ethyl acetate and Water.

ETHANOL The sample is made in to powder and one gram of powdered sample is packed in a filter paper and immersed in 100ml Ethanol and kept at room temperature for 24 hours.

METHANOL The powdered sample is packed in a filter paper and immersed in 100ml methanol and kept at room temperature for 24 hours.

CHLOROFORM The Powdered sample is packed in a filter paper and immersed in 100ml chloroform and kept at room temperature for 24 hours.

ACETONE The powdered sample is packed in a filter paper and immersed in 100ml acetone and kept at room temperature for 24 hours.

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ETHYL ACETATE The powdered sample is packed in a filter paper and immersed in 100ml ethyl acetate and kept in room temperature for 24 hours.

WATER The powdered sample is packed in a filter paper and immersed in 100ml distilled water and kept at room temperature for 24 hours.

COLLECTION OF EXTRACTS

The extracts from each solvent is collected by vaporizing the each solvent using water bath. 2ml of extraction taken in a petriplates and kept undisturbed at room temperature for 48 hours later the vaporized extracts forms like a paste and those extracts are collected for further process.

PHYTOCHEMICAL ANALYSIS OF EXTRACTS

Phytochemical analysis is performed to identify the compounds that are present in the particular extracts. The test for some major compounds like alkaloids, protein and aminoacids, carbohydrates, cholesterol, lignins, tannins etc…are confirmed by performing different analysis

IDETECTION OF PROTEIN AND AMINO ACIDS

In a sterile test tube 2ml of lichen extract is taken, few drops of Millons reagent is added the positive result forms white participate.

DETECTION OF CARBOHYDRATES

To the 1ml of lichen extract 1ml of Barfoeds reagent is added, red precipitate shows positive result.

DETECTION OF LIGNINS

To the 2ml of lichen extract few drops of gallic acid, prepared by dissolving 10 mg of gallic acid in 10ml of methanol and added to the lichen extract and observed for a olive green colour.

DETECTION OF ALKALOIDS

To detect alkaloids in a extract iodine test is performed, to the 3ml of lichen extract few drops of iodine solution is added and boiled in water bath for few minutes a blue colour forms which disappears on boiling and reappears while cooling.

DETECTION OF TANNINS

To the 0.4ml of lichen extract 4ml of 10% NaoH is added and shaken well and observed for emulsification which shows positive result.

DETECTION OF CHOLESTROL

To the 2ml of lichen extract 2ml of chloroform is added and 10 drops of acetic anhydride is added then 2-3 drops of concentrated sulphuric acid also added and observed for red rose colour change.

DETECTION OF REDUSING SUGARS:

Benedict's test:

To the 0.5 ml of lichen extract 0.5 ml of Benedicts reagent is added and boiled for 2 minutes. Then it is observed for green or yellowish colour change for the positive result.

DETECTION OF CARBOXILIC ACID

Effervescence test:

To the 1 ml of the lichen extract 1 ml of sodium bicarbonate solution is added and observed for effervescence. Appearance of effervescence shows positive result.

DETECTION OF FLAVANOIDS

To the 5 ml of lichen extract few drops of concentrated sulphuric acid is added. Orange colour change indicates the positive result.

DETECTION OF PHLOBATANNINS:

HCL test:

To the 2 ml of lichen extract 2 ml of 1% of HCL was added and then boiled for few minutes. Formation of red precipitate indicates the positive result.

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DETECTION OF ANTHOCYANINS:

HCL test.:

To the 2 ml of lichen extract ,2ml of 2N HCL and few drops of ammonia is added. A pink red solution which turns blue – violet after addition of ammonia indicates positive result.

DETECTION OF TITERPINOIDS

Salkowski's test:

To the 2ml of lichen extract few drops of concentrated sulphuric acid is added, shaken well and allowed to rest for few minutes. A golden colour layer at the bottom indicates positive result.

DETECTION OF SAPONINNS

In a test tube 3ml of extract is taken and then 10 ml of distilled water is added, shaken vigorously and kept undisturbed for 30 minutes. Formation honey comb layer indicates positive result.

THIN LAYER CHROMATOGRAPHY TLC

Thin layer chromatography was performed to determine the number of components in a mixture, the identity of compounds and the purity of a compounds.

Pre coated aluminium silica gel plates are cut in to size of 20*15 cm. A pencil mark is made at a distance of 2cm from bottom end and points made at appropriate distance. Small fragments of thallus are kept in small test tubes and lichen substances are extracted with acetone, with the help of capillaries extracts are spotted on the silica gel plates. Spotted plates are kept in a beaker containing buffer solution. Then the plates are removed and dried, then the plates are sprayed using ninhydrin and spotters are observed. Then the Rf value of a compound is calculated. The Rf value of a compound is equal to distance traveled by the compound divided by the distance traveled by the solvent front.

ANTIMICROBIAL PROPERTY OF LICHEN EXTRACT

The antimicrobial property of lichen extract was analysed by well diffusion method for common pathogens like bacillus, E,coli, and proteus. The MHA (Mueller Hinton Agar) was prepared, the plates were kept for sterilization and MHA was poured into the plates and plates were allowed to solidify and kept under UV. The each culture was swabbed on the MHA plates. The well is cutted on the MHA plates and 100µl extracts of each solvent was dropped into the well and the plates are incubated at 37ºC for 24 hrs. And zone of inhibition was observed .The 5 common pathogens like proteus mirabilis, staphylococcus aureus, E.coli, klebsiella pneumoniae, bacillus subtilis.

III. RESULT AND DISCUSSION

COLLECTION OF LICHEN SAMPLE

Foliose and Fruticose lichens were collected from the nilgiris mountain, Tamilnadu on 2nd February 2023 and those sample were stored in 4ºC.

Fig 3: Fruticose

Fig 4: Foliose

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SPOT TESTS

K test:

The aqueos solution of KOH was sprayed on the lichen thallus, quinonoid lichen pigments reacts to the solution and forms dark red colour.

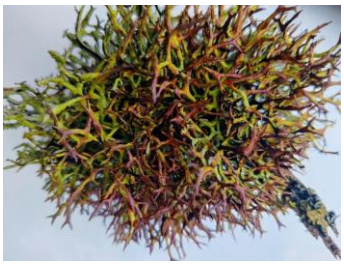

Fig 5: Colour change on fruticose lichen

C TEST:

The solution of calcium hypochlorite was sprayed and the free -OH- Meta group reacts to this solution by forming red colour.

Fig 6: Foliose fragments color change

I TEST:

The potassium iodide solution were sprayed in to the lichen fragment and forms lite yellow and black colour.

Fig 7: Foliose fragments colour change

MICROCRYSTALLOGRAPHY

On the addition of crystallization agent to the fragment of lichens sample various types of crystal formation was observed under microscope.

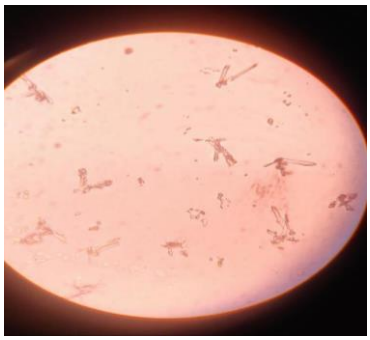

Fig 8: Lichen thallus under microscope

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THIN LAYER CHROMATOGRAPHY:

The flavonoids sovent system shows yellow spots on the TLC plates

PHYTOCHEMICAL ANALYSIS

The screening and identification of bioactive compounds were done using the extract of lichens.

TESTS	ACETONE	ETHANOL	ETHYL ACETATE	CHOLOROFORM	METHANOL	H2O
Protein	+ve	+ve	-ve	+ve	-ve	-ve
Tannins	-ve	+ve	-ve	+ve	-ve	-ve
Lignins	+ve	-ve	-ve	+ve	-ve	-ve
Reducing sugar	+ve	+ve	+ve	+ve	+ve	+ve
Carboxylic acid	+ve	-ve	-ve	-ve	-ve	-ve
Flavonoids	+ve	-ve	+ve	+ve	-ve	-ve
Phablotaninns	-ve	-ve	-ve	-ve	-ve	-ve
Anthocyaninns	-ve	-ve	-ve	-ve	-ve	-ve
Alkaloids	-ve	-ve	-ve	-ve	-ve	-ve
Triterpenoids	-ve	-ve	+ve	+ve	-ve	-ve
Saponins	-ve	-ve	+ve	-ve	-ve	-ve
Carbohydrates	-ve	-ve	-ve	-ve	-ve	-ve
Cholestrol	-ve	-ve	-ve	-ve	-ve	-ve
Quinones	-ve	-ve	+ve	+ve	-ve	-ve

GCMS

The ethyl acetate extract were used for GCMS analysis and compounds were identified.

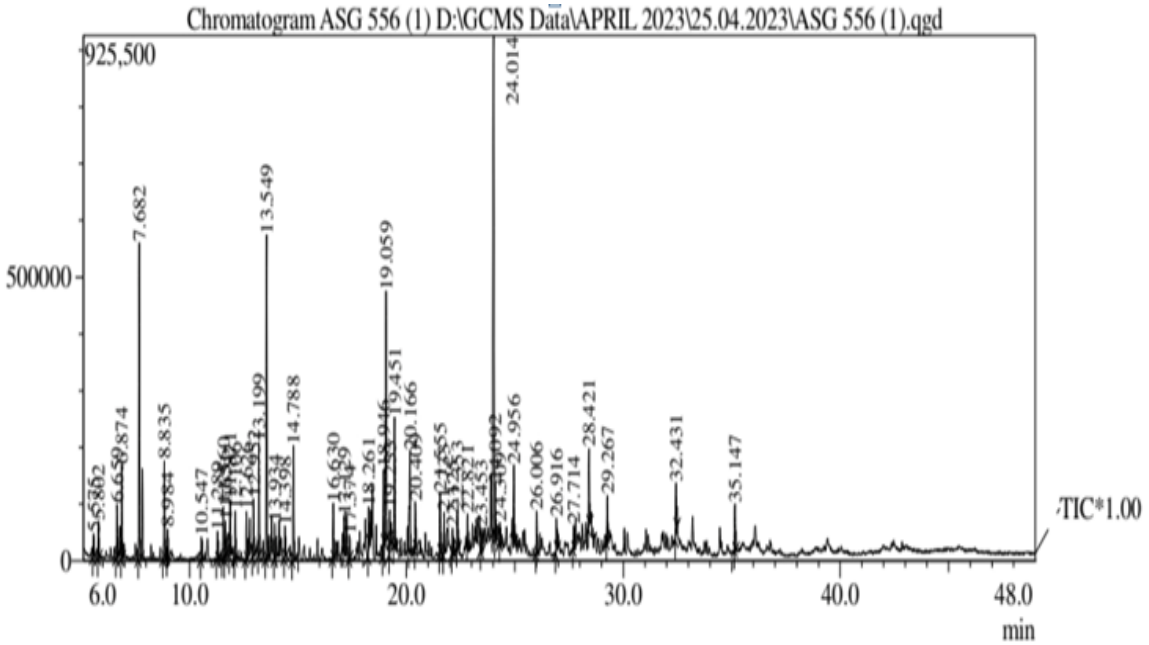

Fig 9:

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Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	5.575	78601	0.51	40789	0.64	NONANE, 2-METHYL-	57.05
2	5.802	149007	0.96	64415	1.01	Nonane, 2,5-dimethyl-	57.10
3	6.659	207337	1.34	94261	1.48	Decane, 5-methyl-	57.05
4	6.874	318497	2.06	165587	2.60	Nonane, 2,6-dimethyl-	57.05
5	7.682	1177481	7.61	557902	8.77	OCTANE, 5-ETHYL-2-METHYL-	57.05
6	8.835	387241	2.50	173369	2.73	Decane, 3,7-dimethyl-	57.05
7	8.984	98960	0.64	50009	0.79	NONANE, 4,5-DIMETHYL-	57.10
8	10.547	85922	0.56	40631	0.64	UNDECANE, 2-METHYL-	57.05
9	11.289	111809	0.72	50300	0.79	1-DODECENE	55.95
10	11.560	228922	1.48	91741	1.44	UNDECANE, 4,7-DIMETHYL-	57.05
11	11.651	155185	1.00	64807	1.02	2-PENTADECYL-4,4,5,5-TETRADEUTERO-1,3-DIOXOLANE	77.00
12	11.871	225150	1.45	95543	1.50	DODECANE, 6-METHYL-	57.10
13	12.106	184254	1.19	83641	1.32	UNDECANE, 4,8-DIMETHYL-	57.05
14	12.626	200199	1.29	84805	1.33	Dodecane	57.05
15	12.952	255329	1.65	101263	1.59	DODECANE, 4,6-DIMETHYL-	57.05
16	13.199	417480	2.70	197741	3.11	UNDECANE, 2,4-DIMETHYL-	57.05
17	13.549	1297998	8.39	571488	8.99	TETRADECANE	57.05
18	13.934	132171	0.85	64123	1.01	UNDECANE, 3,8-DIMETHYL-	57.05
19	14.398	119221	0.77	55728	0.88	Decane, 2,3,7-trimethyl-	57.05
20	14.788	480687	3.11	202487	3.18	HEPTADECANE	57.05
21	16.630	228182	1.47	97890	1.54	1-Tridecene	55.05
22	17.129	145938	0.94	62245	0.98	PENTADECANE	57.05
23	17.374	91269	0.59	42184	0.66	UNDECANE, 2,8-DIMETHYL-	57.05
24	18.261	174755	1.13	74904	1.18	Hexadecane	57.05
25	18.946	335328	2.17	138047	2.17	OCTADECANE	57.05
26	19.059	1190377	7.69	438834	6.90	Eicosane	57.05
27	19.233	151564	0.98	69417	1.09	NONADECANE	57.00
28	19.451	508461	3.29	229012	3.60	2,4-DITERT-BUTYLPHENOL	191.10
29	20.166	545923	3.53	178042	2.80	HEPTADECANE, 8-METHYL-	57.05
30	20.409	197458	1.28	83270	1.31	-Methano-2H-cyclopenta[b]furan-2,4(5H)-dione, 6-bromo-3,3a,6,6a-tetrahydro-, (3r,3a-trans,5-cis,6-trans,6a-trar	151.10
31	21.555	254340	1.64	106638	1.68	E-14-Hexadecenal	55.05
32	21.733	153617	0.99	72654	1.14	PENTADECANE	57.00
33	22.125	101972	0.66	47045	0.74	1,3-BENZODIOXOLE, 4,5-DIMETHOXY-6-(2-PROPENYL)-	222.05
34	22.353	152647	0.99	66590	1.05	TRICOSANE	57.05
35	22.821	129896	0.84	56152	0.88	Pentadecane, 2,6,10-trimethyl-	57.05
36	23.453	93207	0.60	41327	0.65	2-Bromotetradecane	57.05
37	24.014	2749830	17.77	876265	13.78	BENZOIC ACID, 2,4-DIHYDROXY-3,6-DIMETHYL-, METHYL ESTER	136.10
38	24.092	225268	1.46	96700	1.52	2,6,10,14-TETRAMETHYLHEXADECANE	57.05
39	24.309	88266	0.57	40297	0.63	OCTADECANE	57.05
40	24.956	283233	1.83	125124	1.97	HEXADECANE, 2,6,10,14-TETRAMETHYL-	57.05
41	26.006	176541	1.14	75283	1.18	E-15-Heptadecenal	55.00
42	26.916	128159	0.83	58711	0.92	HEXACOSANE	57.00
43	27.714	101359	0.65	41560	0.65	TRICOSANE	57.00
44	28.421	311904	2.02	136315	2.14	DOTRIACONTANE	57.05
45	29.267	188721	1.22	83109	1.31	Hexadecane, 2,6,11,15-tetramethyl-	57.10
46	32.431	247342	1.60	89396	1.41	OCTADECANE	57.05

Fig 10:

ANTIMICROBIAL PROPERTY TESTING

The zone of inhibition was observed against proteussp., and bacillus sp in chloroform extraction, and no zone formation observed against staphylococcus sp., E.coli sp., klebsiella sp.,

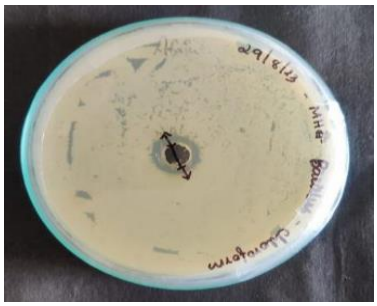

Fig 11: Bacillus sp.,

Fig 12: Proteus.,sp

DISCUSSION

The lichens are recognized and gathered by different spot test like K test, C test, I test. Lichen fragments change color because of their pigments, most commonly foliose and fruticose, which react with the reagents and cause color changes. The lichens extractions are from different solvents gives different outcome gracious phytochemical examination. For major analysis, the extraction of ethyl acetate yields a linear result. The lichen sort of fruticose and foliose extraction were taken from solvents like CH3)2CO, Ethanol, Methanol, Chloroform, Ethyl acetic acid derivation and Water. The phytochemical screening and TLC results show that the water extraction does not move. In ethyl acetate extraction, the various compounds are displayed by the GCMS. The

lichen extraction of chloroform structures zone of restraint for bacillus sp., furthermore, proteus sp.,. The lichens are identified and collected using a variety of spot tests, such as the K test, the C test, and the I test. Lichen extraction therefore contains various compounds against particular microbes. Lichen fragments change color because of their pigments, most commonly foliose and fruticose, which react with the reagents and cause color changes. The lichens are extracted using a variety of solvents, which results in different phytochemical analysis results. For major analysis, the extraction of ethyl acetate yields a linear result. The lichen kind of fruticose and foliose extraction were taken from solvents like CH3)2CO, Ethanol, Methanol, Chloroform, Ethyl acetic acid derivation and Water. The phytochemical screening and TLC results show that the water extraction does not move. In ethyl acetate extraction, the various compounds are displayed by the GCMS. The chloroform extraction of lichen creates a barrier for bacillus sp., and the genus Proteus Therefore, lichen extraction contains a variety of antimicrobial compounds.

IV. CONCLUSION

Microbial symbiont groups like lichens are understudied and understudied. According to studies, they produce potent bioactive metabolites with numerous therapeutic applications. My research indicates that lichens can be utilized in the creation of antimicrobial specialists, and the ongoing review is a step in this direction. This investigation may lead to the discovery of a potent antimicrobial. The kinds of lichen species shows different mixtures that are separated and disengaged for additional investigation. The lichen species can be used as any antimicrobial by the phytopharmaceutical. The lichens species can be finished the restorative field particularly Usnea species. My research demonstrates that lichens are an extremely predictable natural resource that can be utilized in the phytopharmaceutical industry.

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