PHYTOCHEMICAL SCREENING, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *PARMOTREMA PERLATUM* LICHEN EXTRACT

A Dissertation for

BOT-651 Discipline Specific Dissertation

16 Credits

Submitted in partial fulfilment of Master's Degree

in Botany

By

MAULI YESHWANT SHIRODKAR

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ABC ID -626-841-808-125

PRN- 201900209

Under the Supervision of

Dr. RUPALI BHANDARI

School of Biological Sciences and Biotechnology

Botany Discipline

Goa University

April 2024

Examined by: Seal of the School

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Phytochemical screening, antioxidant and antimicrobial activities of *Parmotrema perlatum* lichen extract" is based on the results of investigations carried out by me in Botany Discipline at School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Rupali Bhandari and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University will be not be responsible for the correctness of observations / experimental or other findings given the dissertation. I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Date: 8th April, 2024

Signature and Name of Student

Mauli Y. Shirodkar

Seat no: 22P0480011

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "Phytochemical screening, antioxidant and antimicrobial activities of *Parmotrema perlatum* lichen extract" is a bonafide work carried out by Ms Mauli Yeshwant Shirodkar under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University.

Signature and Name of Supervising Teacher: Dr. Rupali Bhandari

Date: 8th April, 2024

Signature of Dean of the School

School stamp

Date: 8-4-2024

Place: Goa University

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Ms Mauli Shirodkar

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ABBREVIATIONS

Entity	Abbreviation
2,2-diphenyl-1-picrylhydrazyl	DPPH
Ferric Reducing Antioxidant Power	FRAP
Fourier-transform infrared spectroscopy	FTIR
Gallic Acid Equivalent	GAE
Half-maximal inhibitory concentration	IC50
Hydrochloric acid	HCL
KOH -test	K-test
Muller- Hinton Agar	MHA
Parmelia perlata	*P. perlata*
Sodium hypochlorite test	C- test
Total phenolic content	TPC

ABSTRACT

Our study investigated the impact of maceration and cold extraction on Lichen *Parmelia perlata* using different solvents like acetone, chloroform, and water. The study covered preliminary identification, phytochemical screening, total phenolic content assessment, antioxidant activity, Usnic acid estimation using FTIR, and antimicrobial activity against *Bacillus subtilis*.

The results proved that cold extraction outperformed maceration, especially with water and chloroform solvents, in terms of extraction yield. Phytochemical screening unveiled a diverse range of secondary metabolites, with chloroform extracts exhibiting the most extensive variety. Total phenolic content assessment indicated higher concentrations in water and acetone extracts, with slightly elevated levels in acetone due to its intermediate polarity. Antioxidant activity assays revealed concentration-dependent scavenging abilities, with water and acetone extracts showing superior potential compared to chloroform. However, chloroform extract demonstrated moderate to high antioxidant activity. FTIR analysis offered insights into the presence of usnic acid in *P. perlata* extracts, suggesting its potential pharmacological importance. Antibacterial evaluation against *Bacillus subtilis* indicated concentration-dependent inhibitory effects, with acetone extract showing the highest potency. This work highlights the medicinal potential of *P. perlata* extracts, particularly in terms of antioxidant and antibacterial activities and underscore the value of *P. perlata* as a promising natural source for drug development, with implications for human health and well-being.

Chapter – 1
INTRODUCTION

1. INTRODUCTION

Lichens are symbiotic organisms consisting of a fungal partner (mycobiont) combined with one or more photosynthetic partners (photobiont). Photobionts can be green algae, cyanobacteria, or both. Lichens are ubiquitous and can be found throughout the world. They can colonize a variety of surfaces, such as tree bark, exposed rocks, and even contribute to biological soil crusts. Their adaptability enables them to survive in various environmental conditions. Lichen thallus is a well-balanced symbiotic system consisting of both heterotrophic and autotrophic components. It can be seen as a self-contained mini-ecosystem (Farrar 1976c; Seaward 1988). The parasitic fungi that invade lichens can be viewed as decomposers of the system. This highlights the ecological importance of lichens. Lichen fungi certainly benefit greatly from photobiont feeding, but the photobiont benefits from association are less clear. Photobionts are organisms that benefit from protection against high light, extreme temperatures, and to some extent drought. It is believed that an alliance between free-living algae or cyanobacteria and fungal partners is necessary for them to live together in harsh areas where they might not be able to survive independently.

Studies conducted recently have discovered that there are approximately 18,500 species of lichens (Boustié & Grube, 2005). Among these lichens, Ascomycota serves as the fungal partner for 98% of them, while Basidiomycota and anamorphic fungi serve as the other partners. Lichen formation involves about 40 genera, 25 species of algae, and 15 species of cyanobacteria, which act as photosynthetic partners. Interestingly, 10 species of lichens have the ability to colonize diverse substrates and can grow on or within rocks on the bark of woody

plants, on wood, soil, moss, leaves of vascular plants, on other lichens, as well as on artificial substrates such as concrete, glass, metal, and plastic (Joneson & Lutzoni, 2009).

Lichens contain approximately 53 to 79 % carbohydrates, 5 to 16% crude fiber, 6 to 16% proteins, 1.3 to 6.5 % lipids and 4 to 12 % ash (up to 17.2 g Ca / kg, 5.5 g K). /kg, 1 g P/kg, 0.56 to 0.88 g Mg/kg and lower amounts of Na, Fe, Cu and Zn) (Kumar and Pandey, 2010; Kambar *et al*., 2014; Meli *et al*., 2018; Zhao *et al*., 2020). Due to their nutrient content, lichens theoretically have high edible value and can be an important source of essential elements, which vary between varieties, especially depending on the region (Meli *et al*., 2018). A notable feature of lichens is that they are valuable and sensitive biological indicators of environmental and industrial pollution.

Lichens are type of organism that produce unique secondary metabolites and have significant biological activity. While many lichens can be eaten, some are toxic. Throughout history, lichens have been used for a variety of purposes, including dyes, perfumes, and folk medicine remedies. In the Indian spice market, a mixture of two or more species of *Parmelia, Usnea longissima, Ramalina subcomplanata* and *Heterodermia tremulans* is sold under the name 'Chharila'. Chharila is believed to have astringent, resolving, laxative, carminative, and even aphrodisiac properties. Additionally, Chharila smoke is said to relieve headaches. The powdered medicine made from lichen can be applied to wounds and has a very good head-steaming effect. The different types of lichen are also used to treat various diseases.

1.1 General Description

Parmotrema perlatum (Huds.) M. Choisy / *Parmelia perlata* lichens (*Parmelia perlata* (Huds.) Ach.) originates from Indian subcontinent and is known as “stone flower”. *Parmelia perlata* has a greyish-brown flattened thallus with broad, quite rounded, more closely attached and more distinctly wrinkled centrally, with narrower (3– 8 mm broad) and shorter, occasionally forked marginal cilia (1–2 mm long); soralia linear and marginal or sometimes submarginal, causing lobe margins to roll back and appear labriform; soredia fine, rarely spreading laminally as globose clusters. Apothecia are not commonly found. The thalline margin is partially sorediate and uneven.

Parmotrema reticulatum and *Parmotrema pseudoreticulatum* can be easily distinguished from *P. perlatum* by the fine mosaic of hair-line cracks present on the upper surface, the soredia at the tips of incised lobe margins. This lichen is mostly found in well-lit, neutral to somewhat acid-barked broad-leaved trees. It can also be found in siliceous rocks and walls, coastal rocks, short coastal turf, and shingle beaches.

1.2 Taxomomy

Kingdom- Fungi

Phylum- Ascomycota

Class- Lecanoromycetes

Order- Lecanorales

Family- Parmeliaceae

Genus- *Parmotrema*

Species- *Parmotrema perlatum* (Huds.) M. Choisy

Fig. 1: Lichen *Parmotrema perlatum* (Huds.) M. Choisy

1.3 Uses

The lichen *Parmotrema perlatum* is a species that is widely distributed in both the northern and southern hemispheres. It is a key ingredient in spice powders used in Chinese and Indian cuisine to enhance the flavour. It usually grows in hilly regions such as Kerala and the Himalayas, including Kashmir. This lichen is well-known for its carminative, diuretic, astringent, and antibacterial properties (Goyal *et al*., 2016). It is used in conventional medicine to treat stomach issues, inflammation, and bronchitis. It is also a significant component of Ayurveda remedies intended to cure various ailments.

The secondary substances produced by this lichen are most likely light filters that protect the photobiont from intense radiation. They also act as antibiotics (acids), help to transfer carbohydrates from the photobiont to the mycobiont (atranorin), or contribute to breaking down the mineral substrate.

1.4 Chemical spot test for lichen identification

A contemporary monograph of a lichen taxon illustrates the significance of chemistry in lichen taxonomy. One explanation for this is that, although lichens are hard to distinguish morphologically, they can be identified by their secondary metabolites, which are frequently found in large amounts. The use of chemical criteria in lichen taxonomy has been summarized by W.L. Culberson (1969), W.L. Culberson and C.F. Culberson (1970), Hawksworth (1976), and Brodo (1978).

In lichens, secondary metabolites can be tightly restricted to particular tissues or widely distributed throughout the fungal hyphae's surface. The majority of colourless substances, primarily depsides and depsidones, are typically found inside the medulla and algal layer of the thallus, whereas atranorin and pigments like usnic acid are frequently located in the thallus cortex. Throughout a specimen's life, the thallus's overall chemical complement doesn't change,

but apothecia, as well as occasionally soralia, pycnidia, pseudocyphcllae, and cephalodia, may have additional or different chemistry from the parent vegetative thallus. Spot tests for lichen tissue colour responses are widely employed as a quick, non-specific way to find specific, unidentified lichen substances. One salient characteristic of these tests is their general ease of use and simplicity, even in situations where material collection is not feasible.

1.5 Qualitative Phytochemical screening

Phytochemicals (Greek: phyton = plant) are chemical compounds naturally present in plants/ lichens attributed to positive or negative health effects. These biological materials are the richest bioreservoirs of different phytochemicals and are employed in the treatment of various illnesses. The medicinal qualities of a plant or lichen are determined by the phytochemical components present in them. Alkaloids, flavonoids, phenolics, tannins, saponins, steroids, glycosides, terpenes, etc. are some of the significant phytochemicals. Even though these compounds may seem non-essential to the plant that produces them, they are crucial to its survival. They play a vital role in mediating ecological interactions with competitors, protecting it from diseases, pollution, stress, and UV radiation, and giving the lichen its colour, scent, and flavour. The metabolites produced to shield plants from biotic and abiotic stresses have been developed into medications that are used to treat various illnesses.

1.6 Lichens secondary metabolites

A great variety of secondary metabolites are synthesized by lichens with distinct biological properties. These aliphatic and aromatic lichen metabolites have relatively low molecular weight. These metabolites are complex, but predominantly small molecules, which comprise up to 20% of lichen’s dry weight. Lichen secondary metabolites comprise many classes of compounds including amino acid derivatives, sugar alcohols, aliphatic acids, macrolytic

lactones, monocyclic aromatic compounds, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers. The mycobiont is responsible for producing secondary metabolites which are then stored in the cortex (like atranorin, parietin, usnic acid, and fungal melanins) or in the medullary layer (like physodic acid, physodalic acid, and protocetraric acid), forming small crystals on the outer surface of the hyphae. The photobiont also influences the secondary metabolism of the mycobiont. To produce specific secondary metabolites, suitable culture conditions need to be maintained, such as nutrient medium, added sugars or polyols, pH, temperature, light, and stress.

Lichen compounds are also known to show some biological activities against microorganisms. The list of the antifungal and antibacterial activities of lichen compounds and lichens against bacteria and fungi can be found (Huneck, 2001). Usnic acid is one of the most common and investigated lichen compounds. Its antimicrobial, antiprotozoal, antiviral, anti-proliferative, anti-inflammatory, analgesic, antipyretic, and anti-tumour activities as well as some other properties such as UV protection, allergen, and toxicity have been summarized in two recent reviews (Cocchietto *et al*., 2002; Ingo´lfsdo´ttir, 2002). Usnic acid is a natural lichen compound used in pharmaceutical preparations. It is commonly used in treating hyperproliferative skin diseases like psoriasis and parasitic infestations. It is a yellowish pigment and occurs in two enantiomeric forms, depending on the projection of the angular methyl group at the chiral 9b position. Usnic acid containing lichens and their extracts have been utilized in medical, pharmaceutical, cosmetic and agricultural applications.

1.7 Total phenolic content in lichens

Lichens are fascinating symbiotic organisms formed by the union of fungi and algae. They are rich in secondary metabolites, particularly phenols, making them remarkable sources of natural antioxidants. These phenolic compounds are known for their antioxidant properties, which are essential in combating the dangers posed by overproduced free radicals. Lichens play a crucial role in neutralizing these harmful molecules and safeguarding biological systems from oxidative stress. The constituents of lichens act as natural antioxidants, helping to maintain the delicate balance between ROS generation and elimination. Some common phenolic acids found in lichens are caffeic acid, gallic acid, and ferulic acid. Phenolic acids are widely dispersed in plant kingdom. They contain two distinguishing constitutive carbon frameworks, hxydroxycinnamic (C_6C_3) and hydroxybenzoic (C_6C_1) structure. Phenolic acids have antioxidant properties due to their high redox potential, which allows them to act as reducing agents and singlet oxygen quencher.

1.8 Antioxidant activity

Free radicals, which are reactive oxygen species like hydroxyl radical, superoxide anion, and hydrogen peroxide, as well as reactive nitrogen species like nitric oxide, play a crucial role in various chemical processes within cells. However, they can also cause undesirable side effects, leading to damage to the cellular structures. They target unsaturated fatty acids found in cell membranes as well as proteins, nucleic acids, and other substances. Enzymes such as glutathione reductase, superoxide dismutase, catalase, and glutathione peroxidase can help reduce the harmful effects of free radicals by scavenging them and terminating chain reactions. Synthetic antioxidants are carcinogenic, which is why natural antioxidants are a better option. Lichens produce a wide range of secondary metabolites that possess strong antioxidant activity. These metabolites can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals, and acting as oxygen scavengers.

1.9 Characterisation of compounds found in lichen using FTIR

Fourier Transform Infrared (FTIR) has been developed as a tool for the simultaneous determination of organic components, including chemical bond, as well as organic content (e.g., protein, carbohydrate, and lipid). IR spectroscopy is a reliable method for the identification of lichen substances. In the FTIR analysis procedure, samples are subjected to contact with infrared (IR) radiation. The IR radiations then have impacts on the atomic vibrations of a molecule in the sample, resulting the specific absorption and/or transmission of energy. This makes the FTIR useful for determining specific molecular vibrations contained in the sample (Kirk and Othmer, 1953). Solid compounds can be measured either in solution (CCl4 or CHCl3), suspension or preferably in KBr.

Research on lichens and their secondary metabolites dates back several decades, with early studies primarily focused on identifying and characterizing the chemical compounds produced by these symbiotic organisms. In the mid-20th century, researchers began isolating and identifying secondary metabolites from lichens using techniques such as chromatography and spectroscopy. This period saw the discovery of numerous compounds, including depsides, depsidones, dibenzofurans, pulvinic acid derivatives, and usnic acid. Usnic acid, one of the most well-known secondary metabolites found in lichens, was first isolated in 1844. It gained attention for its antibiotic properties and was used in traditional medicine for wound healing and topical infections. Early research focused on elucidating its chemical structure and biological activities. Lichen secondary metabolites were recognized as valuable chemotaxonomic markers for species identification and classification. Researchers investigated the chemical composition of lichens from various taxonomic groups to understand their evolutionary relationships and ecological adaptations. Early research revealed the diverse biological activities of lichen secondary metabolites. Usnic acid demonstrated antimicrobial properties against bacteria, fungi, and some viruses. Other compounds showed antioxidant,

anti-inflammatory, and cytotoxic effects, sparking interest in their potential pharmaceutical applications. Studies in the mid-20th century began exploring the ecological roles of lichen secondary metabolites. Researchers investigated how these compounds protect lichens from environmental stressors such as UV radiation, desiccation, and herbivory. Some compounds were found to inhibit the growth of competing organisms, contributing to lichen dominance in certain ecosystems. Early research also explored potential industrial applications of lichen secondary metabolites. Usnic acid was investigated for its preservative properties in food and cosmetics. Other compounds showed promise as natural dyes, perfumes, and bioactive agents in agriculture.

Technological advancements in analytical chemistry, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS), facilitated more precise identification and quantification of lichen secondary metabolites. These techniques allowed researchers to explore the chemical diversity of lichens in greater detail. Overall, early research on lichens and their secondary metabolites laid the foundation for subsequent studies investigating their ecological, pharmacological, and biotechnological significance. It provided valuable insights into the chemical diversity and biological activities of these symbiotic organisms, shaping our understanding of their roles in ecosystems and their potential applications in various fields.

Significance of the research:

Research on lichens and their secondary metabolites is a fascinating area of study that encompasses various fields such as ecology, chemistry, pharmacology, and medicine. Lichens are symbiotic organisms composed of a fungus (the mycobiont) and a photosynthetic partner (the photobiont), usually a green algae or cyanobacterium. They are known for their ability to produce a wide array of secondary metabolites, which serve various ecological roles including protection from predators, UV radiation, and desiccation, as well as aiding in nutrient uptake.

Lichens produce a remarkable diversity of secondary metabolites, including phenolics, terpenoids, alkaloids, and depsides. These compounds exhibit a wide range of biological activities and have attracted significant attention from researchers for their potential pharmaceutical and industrial applications. Many secondary metabolites isolated from lichens exhibit interesting pharmacological properties, including antimicrobial, antioxidant, anticancer, anti-inflammatory, and antiviral activities. Researchers are exploring the therapeutic potential of these compounds for drug development. Secondary metabolites play crucial roles in the ecological interactions of lichens with their environment. Some compounds act as allelochemicals, inhibiting the growth of competing organisms, while others deter herbivores or protect against environmental stressors. Lichens are sensitive to environmental changes and have been used as bioindicators of air quality. Monitoring the presence and abundance of certain lichen species, as well as their secondary metabolites, can provide valuable information about air pollution levels, particularly heavy metals and sulfur dioxide.

Secondary metabolites are often used as chemotaxonomic markers to classify lichen species and elucidate their phylogenetic relationships. Analyzing the chemical composition of lichens can provide insights into their evolutionary history and ecological adaptations. Lichen-derived secondary metabolites have potential applications in various biotechnological fields, including agriculture, cosmetics, and food preservation. For example, some compounds exhibit herbicidal or insecticidal properties, while others have been used in natural dyes and perfumes. Continued research on lichen secondary metabolites aims to discover novel bioactive compounds with pharmaceutical or industrial significance. Techniques such as bioassay-guided fractionation, metabolomics, and genome mining are employed to identify and characterize new compounds from lichen extracts. Overall, research on lichens and their

secondary metabolites contributes to our understanding of symbiotic relationships, biodiversity, and the potential for biotechnological and pharmacological applications.

Research gaps:

While research on lichens and their secondary metabolites has made significant strides, there are still several gaps and areas for further investigation. There is a good understanding of some ecological roles of lichen secondary metabolites, such as protection against herbivory and UV radiation, the full extent of their ecological interactions remains unclear. Further research is needed to explore how lichen secondary metabolites influence nutrient cycling, community dynamics, and symbiotic relationships within ecosystems.

There is a lack of comprehensive studies on the biogeography of lichens and how it is influenced by climate change and other environmental factors. Investigating the distribution patterns of lichen species and their secondary metabolites across different habitats and regions can provide insights into their responses to changing environmental conditions. While many lichen secondary metabolites have been identified, there is still much to learn about their biosynthesis pathways and the genetic mechanisms underlying their production. Understanding the biosynthetic pathways of lichen secondary metabolites could enable researchers to engineer lichen-associated fungi for the production of bioactive compounds. Advancements in genomics and metabolomics have the potential to revolutionize lichen research by allowing for a more comprehensive exploration of the genetic and chemical diversity of lichens.

Integrating omics approaches with ecological and evolutionary studies can provide a deeper understanding of lichen biology and the ecological significance of their secondary metabolites. While some lichen secondary metabolites have shown promising pharmacological

activities, such as antimicrobial and anticancer properties, their therapeutic potential remains largely untapped. Further research is needed to evaluate the safety, efficacy, and mechanisms of action of lichen-derived compounds for drug development.

Lichens are sensitive to environmental changes and are often used as indicators of ecosystem health. However, there is a lack of research on the conservation and restoration of lichen populations, particularly in the face of habitat loss, pollution, and climate change. More studies are needed to develop effective conservation strategies for lichen biodiversity. Indigenous communities around the world have long relied on lichens for food, medicine, and cultural practices. There is a need to document and integrate traditional knowledge of lichens into scientific research, ensuring that indigenous perspectives are valued and incorporated into conservation and management efforts. Addressing these gaps in lichen research will not only advance our understanding of these fascinating symbiotic organisms but also contribute to broader scientific knowledge, conservation efforts, and the development of sustainable biotechnological and pharmaceutical applications.

OBJECTIVES

The present study aims at understanding the effects of maceration and cold extraction on the extraction yield of Lichen *Parmelia perlata* using different solvents acetone, chloroform and water. The cold extraction extracts were further used to analyse different phytochemical components, pharmacological activities and characterisation of usnic acid in the lichen extracts obtained.

1. Preliminary identification of the lichen using chemical spot tests.
2. Preparation of lichen extracts using solvents, acetone chloroform and water by maceration and cold extraction method and compare the extraction yield obtained.
3. Preliminary phytochemical screening of the lichen extracts of both cold extraction and maceration technique.
4. Analysis of total phenolic content in lichen extracts using Folin–Ciocalteu method.
5. Study of antioxidant activity of lichen extracts using DPPH and FRAP method.
6. Estimation of usnic acid in the lichen extract by using FTIR.
7. Study antimicrobial properties of the lichen extract prepared against *Bacillus subtilis* using disc diffusion method.
8. Study the larvicidal activity of the lichen extract on the mosquito larvae

Chapter – 2

REVIEW OF LITRETURE

2. REVIEW OF LITERATURE

Leela & Devi, (2017) conducted the study on secondary metabolites of lichen, *Parmelia perlata*. These metabolites were isolated, purified, and tested. The extraction process was carried out using a cold method with methanol, which resulted in a 2.56% yield of lichen extract. From a 50g sample of dry lichen, the total yield was 0.27%. Several techniques were employed in this study, including TLC, column chromatography, and GC-MS. The findings suggest that *Parmelia perlata* has potential for the development of drugs with therapeutic significance. Specifically, the glycosides and alkaloids derived from lichens exhibited antibacterial and antifungal properties. This indicates a promising avenue for future research and drug development.

Rahman *et al*., (2014) showed that *Parmelia perlata*, is rich in phenolic compounds and exhibits strong antioxidant properties. The extraction process was conducted using methanol on 100g of the plant material. The study revealed that *P. perlata* demonstrated anti-cholesterol activity at a rate of 48 %, when compared to the drug Simvastatin. Furthermore, it showed a cytotoxic effect against HCT 116 cell lines, with an IC50 value of 202.1 µg mL^{-1}. This suggests that *P. perlata* could potentially be used in the treatment of cholesterol-related conditions and in cancer therapies.

In the study, Payal *et al*., (2016) identified 49 compounds in *P. perlata* extracts of pet ether, chloroform, and acetone. The pet ether extract was found to be active against *S. grieveces*, while the chloroform extract was effective against *B. subtilis*. The acetone extract demonstrated moderate activity against both *B. subtilis* and *E. coli*. Both the pet ether and acetone extracts showed potential activity against *P. funiculosam*. Additionally, the acetone

extract exhibited strong free radical scavenging activity. This suggests that these extracts could have potential therapeutic applications.

Momoh & Adikwu, (2008) investigated the interaction between lichen extract and colloidal silver against *Staphylococcus aureus*. It was found that the presence of colloidal silver enhanced the activity of the ethanol extract against this bacterium. The lichen was extracted using a cold maceration process in ethanol. Phytochemical tests revealed the presence of flavonoids, saponins, tannins, and glycosides in the lichen. The ethanol extract of the lichen demonstrated activity against *S. aureus*. Interestingly, the inhibitory effect increased when colloidal silver was present. This suggests that the combination of lichen extract and colloidal silver could potentially enhance antibacterial activity.

Vidyalakshmi *et al*., (2012) focused on the antibacterial activity of *Parmelia perlata* extracts against *Staphylococcus aureus*. The extracts demonstrated inhibitory effects, suggesting their potential use as antibacterial agents. Specifically, the methanol, ethyl acetate, and acetone extracts of *P. perlata* were found to inhibit *S. aureus*. The study also found that a concentration of 5 mg mL^{-1} of these extracts showed the greatest inhibitory effect against *S. aureus*. This suggests that *P. perlata* could be a promising source for developing new antibacterial treatments.

In the thallus of *Parmelia perlata*, Kyslychenko *et al*., (2018) identified 32 volatile compounds. Additionally, 5 terpene compounds were detected in the raw plant material. These findings suggest that the thalli of *Parmelia perlata* could potentially be a valuable source for the development of new medicinal products.

Sharma *et al*., (2012) studied lichens bioactive compounds that have applications in traditional medicine and cosmetic products. From 1.5 kg of plant material, an extraction yield of 7.0 gm was obtained. The solvents used for this process were petroleum ether and benzene, and the separation method employed was column chromatography. The extracts have analgesic properties. Furthermore, usnic acid, which is derived from the *Parmelia* species, has demonstrated antitumor and antimicrobial effects. In order to identify the functional groups present in these compounds, an IR spectrum analysis was conducted. This suggests that lichens, particularly the *Parmelia perlata*, could be a promising source for the development of new therapeutic agents and cosmetic products.

Aoussar *et al*., (2020) study suggest, in lichen extracts particularly those from *P. furfuracea*, have demonstrated antioxidant and antibacterial properties. These extracts have shown minimal cytotoxic effects on a variety of cell lines. The antibacterial effects are particularly noticeable against Gram-positive bacteria, including MRSA. Interestingly, no significant relationship was found between the content of flavonoids and the antioxidant effect of the extracts. Acetone was the solvent of choice for the extraction of lichen species. The extraction process itself was carried out using a maceration method. This suggests that lichen extracts could potentially be a valuable resource in the development of new therapeutic agents.

Madheshwar *et al*., (2017) in their study, *Parmotrema perlatum* found to possess anti-inflammatory, antibacterial, and thrombolytic properties. The extraction process was carried out using a Soxhlet apparatus at room temperature over a period of 72 hours. A phytochemical analysis of *P. perlatum* revealed the presence of flavonoids, saponins, and tannins. These findings suggest that lichens like *P. perlatum* could potentially be used in the preparation of drugs. The screening process also confirmed the presence of flavonoids, saponins, and tannins.

The lichens exhibited thrombolytic activity similar to that of streptokinase. Various chemical constituents, including atranorin, chloroatranarin, and usnic acid, were isolated from the lichens. This suggests that lichens could potentially be a valuable source of bioactive compounds for therapeutic applications.

Study by Thippeswamy *et al*., (2012) shows that compounds found in the lichen *Parmelia perlata* have demonstrated antimicrobial properties. The extracts and compounds derived from *Parmelia perlata* have shown both antibacterial and antifungal activities. A hot extraction method was used, employing solvents such as petroleum ether, chloroform, and methanol. Techniques like thin layer chromatography and column chromatography were utilized in the study. It was found that compound-II is particularly effective against and *Pseudomonas solanacearum*. The crude extracts from *P. perlata* was most effective against *Clavibacter michiganensis*, moderately effective against *Pseudomonas solanacearum*, and less effective against *Escherichia coli* when compared to the antibiotic streptomycin. In terms of antifungal activity, *Fusarium oxysporum* and *Rhizopus nigricans* were more susceptible to the crude extracts, while *Aspergillus niger* was less susceptible when compared to the antifungal agent bavistin. This suggests that *P. perlata* could potentially be a valuable source of antimicrobial agents.

Paul at el., (2014) conducted qualitative analysis was conducted to identify the presence of various compounds such as terpenoids, anthraquinones, flavonoids, saponins, tannins, and phenol. In addition, nutritional studies were carried out to examine the content of amino acids and proteins. Among the samples studied, *G. lucidum* was found to have a higher content of vitamin A. This suggests that mushrooms and lichens could potentially be a valuable source of nutrients and medicinal compounds.

Esimone *et al*., (2007) dound that the crude polysaccharide fraction derived from *Parmelia perlata* demonstrate antiviral activity against the yellow fever virus. However, the water and acetone extracts did not show any antiviral activity against the viruses tested. The lichen extracts, which contain anthraquinones and plant polysaccharides, have shown antiviral properties. This suggests that lichens like *Parmelia perlata* could potentially be a valuable source of antiviral agents.

Shiromi *et al*., (2021) have demonstrated, lichens *P. rampoddense* and *P. tinctorum* as a potential sources of antimicrobial agents against both Gram-negative and Gram-positive bacteria. This includes twenty clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). The extraction process was carried out using a cold method with hexane and ethanol. The solvents used in this process were hexane, acetonitrile, and chloroform. Among these, the ethanol extract of *P. rampoddense* exhibited the highest activity against MSSA. This suggests that these lichens could potentially be a valuable source of antimicrobial agents.

Fernández-Moriano *et al*., (2016) the research focused on the antioxidant capacities and cytotoxic effects of various species of Parmeliaceae lichens. There were significant correlations found between the results of the antioxidant assays and the total phenolic content. Additionally, several lichen species exhibited cytotoxic effects on HepG2 and MCF-7 cells. This suggests that these lichens could potentially be a valuable source of therapeutic agents.

Ratna *et al*., (2021) focused on the phytoconstituents and antioxidant capacity of P*armotrema perlatum*, assessed through molecular docking. The methanolic and n-hexane extracts of *Parmotrema perlatum* demonstrated significant antioxidant potential. The primary constituents identified were terpenoids, coumarins, flavonoids, and alkaloids. Molecular

docking studies revealed a good binding score with the target enzymes, indicating a strong interaction. In vitro antioxidant studies further confirmed the remarkable antioxidant potential of both extracts. The methanol extract had a total phenolic content of 24.6 mg GAEg, while the n-hexane extract had a total flavonoid content of 6.7 mg RUEg. This suggests that *Parmotrema perlatum* could potentially be a valuable source of antioxidants and other bioactive compounds.

S & Garampalli, (2014) conducted phytochemical analysis on lichen extracts to identify potential medicinal compounds. The solvents used for this process included petroleum ether, chloroform, ethyl acetate, acetone, and methanol. The extraction of these lichen extracts was performed using a cold maceration method. The study mentioned secondary compounds such as barbatic acid, atranorin, and lecanoric acids. A spot test was also carried out to detect the presence of Atranorin in nine lichen species. This suggests that lichens could potentially be a valuable source of medicinal compounds.

EngiN *et al*., (2023) studied bioactive compounds found in lichens and demonstrated antimicrobial, antioxidant, and antitumor properties. Extracts from *Usnea longissima*, as well as usnic acid, have shown cytotoxic activity on cells, particularly exhibiting high cytotoxic activity on cancer cells. The methanol extract has proven to be effective on AGS and NIH/3T3 cells. The extraction process was carried out using a Soxhlet apparatus with methanol and water as the solvents. This suggests that lichens, particularly *Usnea longissima,* could potentially be a valuable source of therapeutic agents.

Candan *et al*., (2007) study focused on the antimicrobial activity of extracts from the lichen *Parmelia sulcata*. Salazinic acid and atranorin were identified in different lichen extracts. The extraction process involved the use of solvents such as methanol, acetone, diethyl ether, petroleum ether, and chloroform. The extraction method included sonication for one hour, leaving it at room temperature overnight, and then filtration. All of the extracts, except for the petroleum ether extract, demonstrated antimicrobial activity against a range of organisms including *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Proteus vulgaris*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Candida albicans*, *Candida glabrata*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Penicillium notatum*. This suggests that *Parmelia sulcata* could potentially be a valuable source of antimicrobial agents.

Kosanić *et al*., (2012) Lichen samples were processed using a Soxhlet extractor with acetone as the solvent. The extracts from these lichens demonstrated potential in antioxidant, antimicrobial, and anticancer applications. The acetone extracts, in particular, displayed a high degree of DPPH radical-scavenging activity, indicating strong antioxidant potential. Furthermore, species of the *Parmelia* genus exhibited a growth-inhibitory effect on FemX and LS174 cell lines, suggesting potential anticancer properties. This suggests that lichens could potentially be a valuable source of therapeutic agents.

Mayilsamy and Geetharamanan (2016) studied the larvicidal activity of *Parmelia perlata* against Artemia salina, showing the highest cytotoxic potential in the hexane extract with 100% brine shrimp mortality at 100ppm. Hexane extract of *P. perlata* showed highest cytotoxic potential while methanol extract of P. perlata had least activity.

Chapter - 3

MATERIALS AND METHOS

3. MATERIALS AND METHODS

3.1 Material Used

Dried samples of Lichen *Parmelia perlata* were obtained from a local spice shop in Vasco, Goa.

3.2 Sample Preparation

The lichen sample was cleaned, dried, and powdered using a mixer. The powder was then stored in a clean bottle for further analysis.

3.3 Chemical spot test

Some colour and other reactions give useful hints for the presence of certain functional groups or elements in the lichens. The colour is also known as spot test. The presence of certain substances in tissues of lichen thallus produces a change in their colour when certain chemicals are applied on the surface of thallus. A positive change is denoted by a positive (+) symbol, followed by the colour produced, and no change in colour is denoted by a negative (-) symbol (S & Garampalli, 2014). The chemicals are routinely used as follows:

K- test: 10-25% aqueous solution of KOH, applied to the cortex, medulla, parts of apothecium.

C-test: 4% Sodium hypochlorite, applied to the cortex, medulla, parts of apothecium.

3.4 Extraction of Samples:

The crude extract from the lichen samples were obtained by means of maceration and cold extraction method using acetone, chloroform and water as solvent. For maceration, about 10 g of the powdered lichen sample was added to 200 ml of acetone, chloroform and water respectively in conical flasks, covered with aluminium foil and kept for 3 days with in between mixing on a rotary shaker for 3 days at room temperature. The solution was filtered with the

help of Whatman No.1 filter paper and the filtrate obtained was evaporated using rotary evaporator. The dried extracts were then stored for further experiments.

For cold extraction, about 10 g of the powdered lichen sample was added to 200 ml of acetone, chloroform and water respectively in conical flasks, covered with aluminium foil and kept for 3 days on a rotary shaker for 3 days at room temperature. The solution was filtered with the help of Whatman No.1 filter paper and the filtrate obtained was evaporated using rotary evaporator. The dried extracts were then stored for further experiments (Rashmi S. and Rajkumar H.G, 2014). The yield of respective crude extract was calculated as:

Percentage yield (%) = (dry weight of extract/dry weight of samples) × 100.

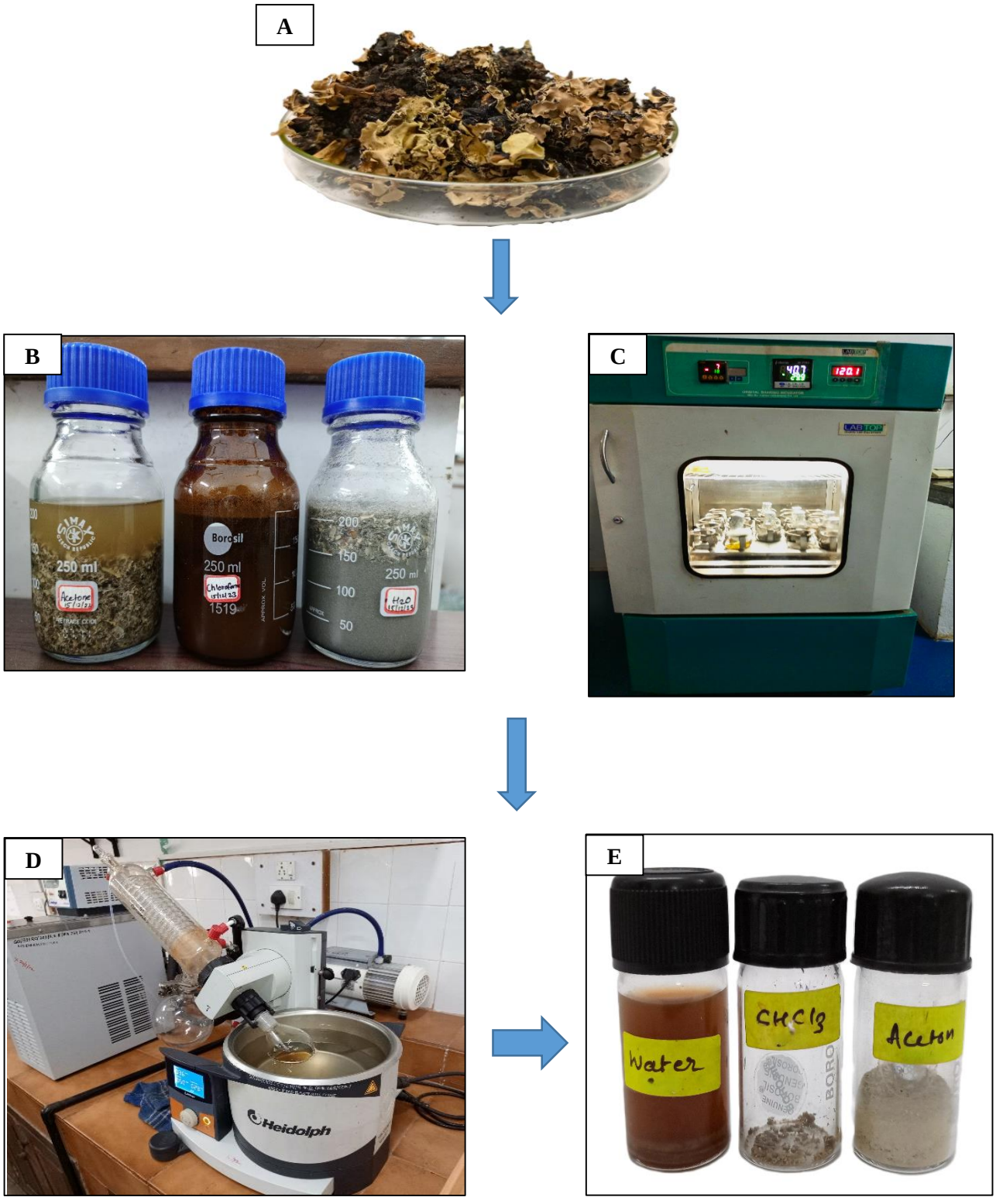


Fig. 2 : Flow chart depicting the extract preparation; A- lichen *Parmelia perlata*; B, C – extraction methods maceration and cold extraction respectively; D- extract concentration using rotary evaporator; E- water, chloroform and acetone extracts.

3.5 Preliminary phytochemical screening

The different extracts thus obtained were qualitatively tested for the presence of various phytochemical constituents (S & Garampalli, 2014).

A. **Test for Tannins (Ferric chloride Test):** 2 ml crude extract was mixed with a few drops of 5% ferric chloride solution. The formation of blue colour indicated the presence of hydrolysable tannins.

B. **Test for Alkaloids (Dragondroff's test):** 2 ml of crude extract is added to 1% HCl, steam for 10 minutes. To this added 6 drops of Dragondroff's reagent. The reddish brown precipitate indicates the presence of alkaloids.

C. **Test for Saponins (Frothing test):** 2 ml of crude extract was mixed with 5 ml of distilled water in a test tube, which was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

D. **Test for glycosides (Keller-kilani test):** 2 ml of Crude extract was mixed with 2 ml of glacial acetic acid containing 1-2 drops of 2% solution of FeCl3. The mixture was then poured into another test tube containing 2 ml of concentrated sulphuric acid. A brown ring at the interphase indicated the presence of cardiac glycosides.

E. **Test for Flavonoids (NaOH solution test):** 2 ml of crude extract is added to 2 ml of 10% NaOH solution. A yellow to orange colour indicates the presence of flavanoids.

F. **Test for Proteins (Xanthoproteic test):** 2 ml of crude extract is added to 2 ml of HNO3, boiled in a water bath. A orange colour indicates the presence of proteins.

G. **Test for Triterpenoids (Salkowski Test):** 2 ml of crude extract is shaken with 1 ml of chloroform and a few drops of concentrated sulphuric acid were added along the side of the test tube. A red brown colour formed at the interface indicated the test as positive for triterpenoids.

H. **Test for carbohydrates (Benedict's test):** 2 ml of Crude extract when mixed with 2 ml of Benedict's reagent and boiled, a reddish brown precipitate formed which indicated the presence of the carbohydrates.

I. **Test for Steroids (Liebermann-Burchard reaction):** 2 ml of crude extract is added to 2 ml acetic anhydride and a few drops of concentrated sulphuric acid is added. The blue-green ring indicates the presence of steroids.

3.6 Determination of Total Phenolic Content

Total phenolic content was estimated by Folin Ciocalteu's method. Standard Gallic acid solution was prepared by dissolving 10 mg in 10 mL of distilled water (1 mg ml^{-1}.). The total phenolics were expressed as mg of Gallic acid equivalents (GAE) /g of extract. Various concentrations of gallic acid solutions in distilled water (1, 5, 10, 15, 20 and 25 µg ml^{-1}) were prepared from the standard solution. Concentration of 1mg ml^{-1} of the acetone, chloroform and water extracts made using only cold extraction were prepared in respective solvents and 200 µl of each sample were introduced into test tubes and final volume was made to 500 µl using distilled water. Then 150 pl. of 10 fold dilute Folin- Ciocalteu reagent was mixed and shaken. After 5 minutes, 2 ml of 7.5% sodium carbonate was added. The test tubes were covered with aluminium foil and incubated for 1 hour at room temperature. After incubation, absorbance was read at 760 nm spectrophotometrically. All determinations were performed in triplicate. The Folin- Ciocalteu reagent being sensitive to reducing compounds including polyphenols

produced a blue colour upon reaction which is measured spectrophotometrically. The calibration curve was plotted using standard gallic acid (Savitree *et al*., 2004).

3.7 Antioxidant Activity

3.7.1 Reducing power assay

The reducing power assay method is based on the principle that substances with reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric-a ferrous complex that has an absorption maximum at 700 nm. The extract concentration of 1 mg ml^{-1} was prepared of the all three solvents. Further the concentrations of 2, 4, 6, 8, 10, µg ml^{-1} and was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml. of 1% potassium ferricyanide. The mixtures were incubated at 50^{o}C for 20 min in the water bath, followed by adding 2.5 ml of 10% trichloroacetic acid. An aliquot of 1 ml of the reaction mixture was added with 1 ml of distilled water and 200 µl of 0.1% $FeCl_3$ and incubated at room temperature for 10 min. The absorbance of the resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicates the increased reducing power. Ascorbic acid was used as the positive control (Leela & Devi, 2017).

3.7.2 DPPH Assay

Free radical scavenging activity of acetone, chloroform and water extracts of lichen *Permelia* perlata were determined according to the DPPH methods Ahmed *et al*., (2013) and Brand-Williams *et al*., (1995). The hydrogen atom donating ability of the plant extracts was determined by the decolourization of ethanol solution of 2. 2-diphenyl- 1-picrylhydrazyl (DPPH). DPPH produces violet/ purple colour in ethanol solution and fades to shades of yellow colour in the presence of antioxidants. The stock solution of DPPH radical (24 mg/100 mL in

ethanol) was prepared and stored in -20°C until further use. By diluting the stock solution of DPPH with ethanol (10 ml/45 ml ethanol), a working solution was prepared. Stock solutions of lichen extracts were prepared in respective solvents with concentration 1 mg mL^{-1}. From this, different dilutions (12.5-200 µg ml^{-1}) were made. The test mixture contained 3 ml DPPH working solution and 1 ml of a sample. The mixture was incubated at room temperature for 30 min in dark. The absorbance of each sample was recorded at 517 nm. For a negative control, 1ml of acetone, chloroform and water was added in place of lichen sample, respectively. Ascorbic acid (10 mg/10 ml distilled water) was used as a positive control. Each extract was analyzed in triplicate. Percent inhibition curves were made and IC_{50} values were calculated for all samples. The percentage of inhibition was calculated by using the formula:

Scavenging of DPPH (%) = [(Absorbance of blank − Absorbance of the sample)/Absorbance of blank] × 100

3.8 Estimation of usnic acid in the lichen extract by using FTIR

The water, acetone and chloroform extracts of lichen were further lyophilized to yield ultra-dry powder in lyophilizer. The dried lichen extracts were prepared for analysis using KBr: Extract (100:1 mg) ratio. The determination of functional groups within the organic compound structure and bonds present in the dried lichen powders was accomplished using Shimadzu IR Spirit instrument. The software used is LabSolutions to get the IR spectrum. The range of spectrum ranges from 400-4000 cm^{-1}.

3.9 Antibacterial Activity

The antibacterial activity of acetone, chloroform and water extract of Lichen (*Parmelia perlata*) were screened against *Bacillus subtilis*, a gram-positive bacteria. *P. perlata* extracts

against *Bacillus subtilis* was determined by Kirby and Bauer disc diffusion method (National Committee for Clinical Laboratory Standards, 1993). Mueller-Hinton agar plates inoculated with 1 ml of bacterial suspension was used for the antibacterial assay. The sterile filter paper discs of 6 mm diameter were impregnated with 1 ml of crude extracts of lichen of varying concentrations such as 1 mg mL^{-1}, 5 mg mL^{-1} and 10 mg mL^{-1} and after complete evaporation were placed on the surface of the inoculated agar plates. These plates were incubated at 37°C for 48 h. At the end of the incubation period, the antimicrobial activities were evaluated by measuring the zone of inhibition. In this assay, negative control was the respective pure solvent and the positive control was Ampicillin (10 mg mL^{-1}). All the tests were performed in triplicates.

3.10 MOSQUITO LARVICIDAL ACTIVITY

3.10.1 Source of *Culex quinquefasciatus* larvae

Culex quinquefasciatus larvae were obtained from the insectary of National Institute of Malaria Research, Field Station, Panaji, Goa. The cyclic colony of this mosquito species is maintained under laboratory conditions at temperature 27 ± 2°C, relative humidity 70 ± 5% with a photoperiod of 12h of day and night cycle. From this cyclic colony, 3 instar larvae were used for conduction the bioassays.

3.10.2 Larval Bioassays

The dried crude extract was weighed and different doses were prepared with the solvent acetone, chloroform and water. Doses such as 50ppm, 75ppm, 100ppm and 150ppm were prepared and tested against 3 instar larvae of *Culex quinquefasciatus*. For each of the doses, 3 replicates were maintained. 20 healthy 3 instar larvae of *Culex quinquefasciatus* were introduced in 250 ml plastic bowl containing 100 ml sterile RO (reverse osmosis) water and

the dose was administered with the help of a micropipette. Concurrent controls were maintained under similar conditions using the same doses of the solvent acetone, chloroform and water. The mortality was recorded by counting the number of dead larvae after 24 and 48 hours of exposure. Percentage mortality was determined for each time interval using the following formula (Kalaivani *et al*., 2012).

$$\text{Percentage Mortality} = \text{Number of Dead larvae Number of Larvae taken} \times 100$$

Chapter -4
RESULTS

4. RESULTS

4.1 Preliminary identification of the lichen using chemical spot tests

The preliminary identification of the lichen *Parmelia perlata* was done using two chemical test: K-test and C-test. The presence of certain substances in tissues of lichen thallus produces a change in their colour when certain chemicals are applied on the surface of thallus. On application of KOH to lichen the tissue turned yellow and when Sodium hypochlorite was applied, the thallus turned orange red in colour indicating presence of possible lichen compounds found in *P. Perlata* (**Table 1 & Fig. 2)**.

Table 1: Spot Test of Lichen *Parmelia perlata*

Spot test	Colour obtained (+ / -)	Possible secondary compounds
K-test	Yellow (+)	Salazinic acid, Thamnolic acid
C-test	Orange - Red (+)	Atranorin

Fig. 3: Chemical spot test on lichen *Parmelia perlata;* A- hydrated lichen sample; B- positive K- test; C- Positive C- test.

4.2 Determination of extraction yield

The already dried Lichen sample was powdered and was kept for maceration and cold extraction in three solvents: Acetone, Chloroform and Water and their extraction yield was calculated **(Fig 2)**. The percentage yield obtained in water cold extraction yields more than Maceration, in acetone both methods yield nearly the same percentage, with cold extraction slightly higher and Chloroform cold extraction again yields more than Maceration. Cold extraction tends to yield a higher percentage of extractive values compared to Maceration, except for Acetone, where the results are almost identical. This suggests that Cold extraction might be a more efficient method for extracting compounds using Water and Chloroform as solvents. **(Table 2 & Fig. 4)**.

Table 2: Extractive value (% yield) of *P. Perlata* by Maceration and Cold extraction method. The data represents mean values ± Standard deviation (n=3).

Weight of powdered Lichen (g)	Colour of Extract	Solvent Extract	Extractive Value % (g/dw)	
			Maceration	Cold Extraction
10	Orange	Water	3.187 ± 0.06	3.106 ± 0.01
10	Yellow	Acetone	16.48 ± 0.11	17.38 ± 0.06
10	Yellow	Chloroform	2.68 ± 0.10	1.391 ± 0.10

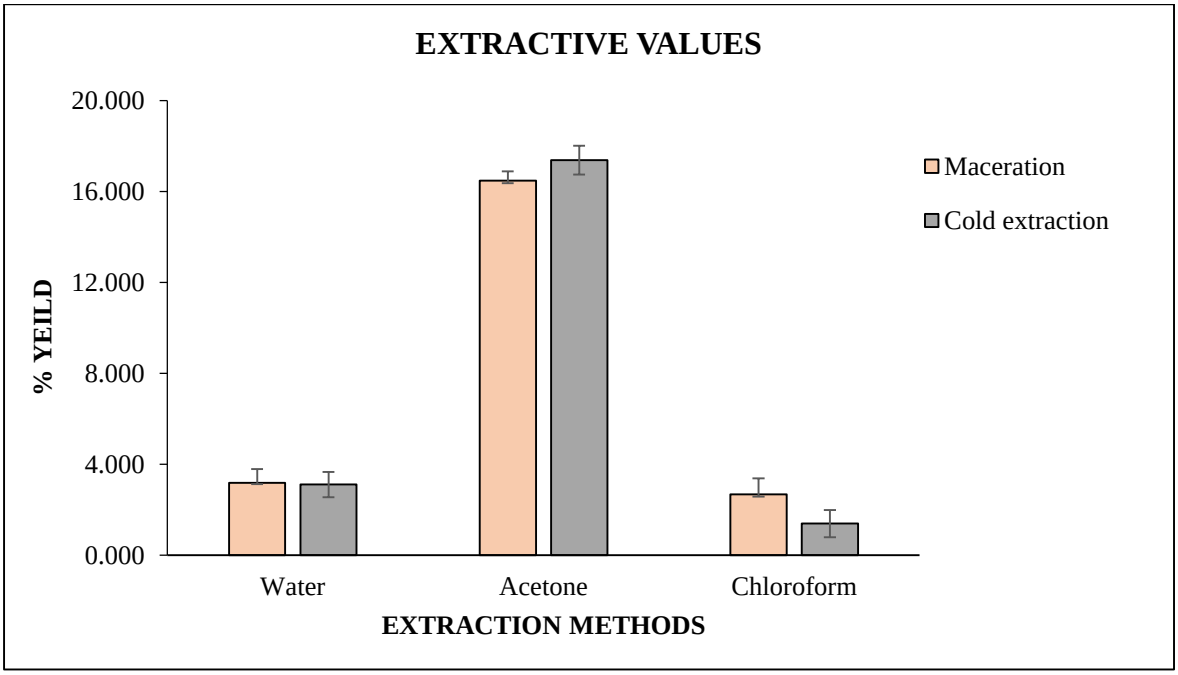


Fig. 4: Comparison of % yield of *Parmelia perlata* obtained through Maceration and Cold extraction methods with three different solvents. The data represents mean values ± Standard deviation (n=3).

3.3 Preliminary phytochemical screening

The qualitative analysis of phytochemical constituents present in water, acetone and chloroform extracts of lichen *Parmelia perlata,* extracted using maceration and cold extraction method are shown in **Table no 3, 4** and **Figures 5, 6**.

The phytochemicals present in water extract of both extraction methods showed presence of glycosides, proteins and triterpenoids. Tannins are only detected in cold water extraction while flavonoids, carbohydrates and saponnins were detected in maceration. In both extraction types, alkaloids and steroids were absent.

The phytochemicals present in Acetone extract of both extraction methods showed presence of glycosides, proteins and triterpenoids. Saponnins are only detected in cold water extraction while flavonoids and carbohydrates were detected in maceration. In both extraction types, tannins, alkaloids and steroids were absent.

The phytochemicals present in chloroform extract of both extraction methods showed presence of glycosides, proteins, carbohydrates, saponins, flavonoids and triterpenoids. Tannins and steroids are only detected in cold water extraction while in both extraction types, alkaloids were absent.

Overall, more phytochemicals are extracted and detected in chloroform extract of both maceration and cold extraction. The cold extraction method showed more phytochemicals extracted for lichen *P. perlata* when compared with maceration.

Table 3: Phytochemical screening of Lichen extracts using Maceration extraction method.

Sr. No.	Secondary metabolites	Phytochemical test	Lichen extracts		
			Water	Acetone	Chloroform
1	Tannins	Ferric chloride Test	-	-	-
2	Alkaloids	Dragondroff's test	-	-	-
3	Saponnins	Frothing test	+	-	+
4	Glycosides	Keller-kilani test	+	+	+
5	Flavonoids	NaOH solution test	+	+	+
6	Proteins	Xanthoproteic test	+	+	+
7	Triterpenoids	Salkowski Test	+	+	+
8	Carbohydrates	Benedict's test	+	+	+
9	Steroids	Liebermann-Burchard reaction	-	-	-

(-) Negative, (+) Positive

Table 4: Phytochemical screening of Lichen extracts using Cold extraction method.

Sr. No.	Secondary metabolites	Phytochemical test	Lichen extracts		
			Water	Acetone	Chloroform
1	Tannins	Ferric chloride Test	+	-	+
2	Alkaloids	Dragondroff's test	-	-	-
3	Saponnins	Frothing test	-	+	+
4	Glycosides	Keller-kilani test	+	+	+
5	Flavonoids	NaOH solution test	-	-	+
6	Proteins	Xanthoproteic test	+	+	+
7	Triterpenoids	Salkowski Test	+	+	+
8	Carbohydrates	Benedict's test	-	-	+
9	Steroids	Liebermann-Burchard reaction	-	-	+

(-) Negative, (+) Positive

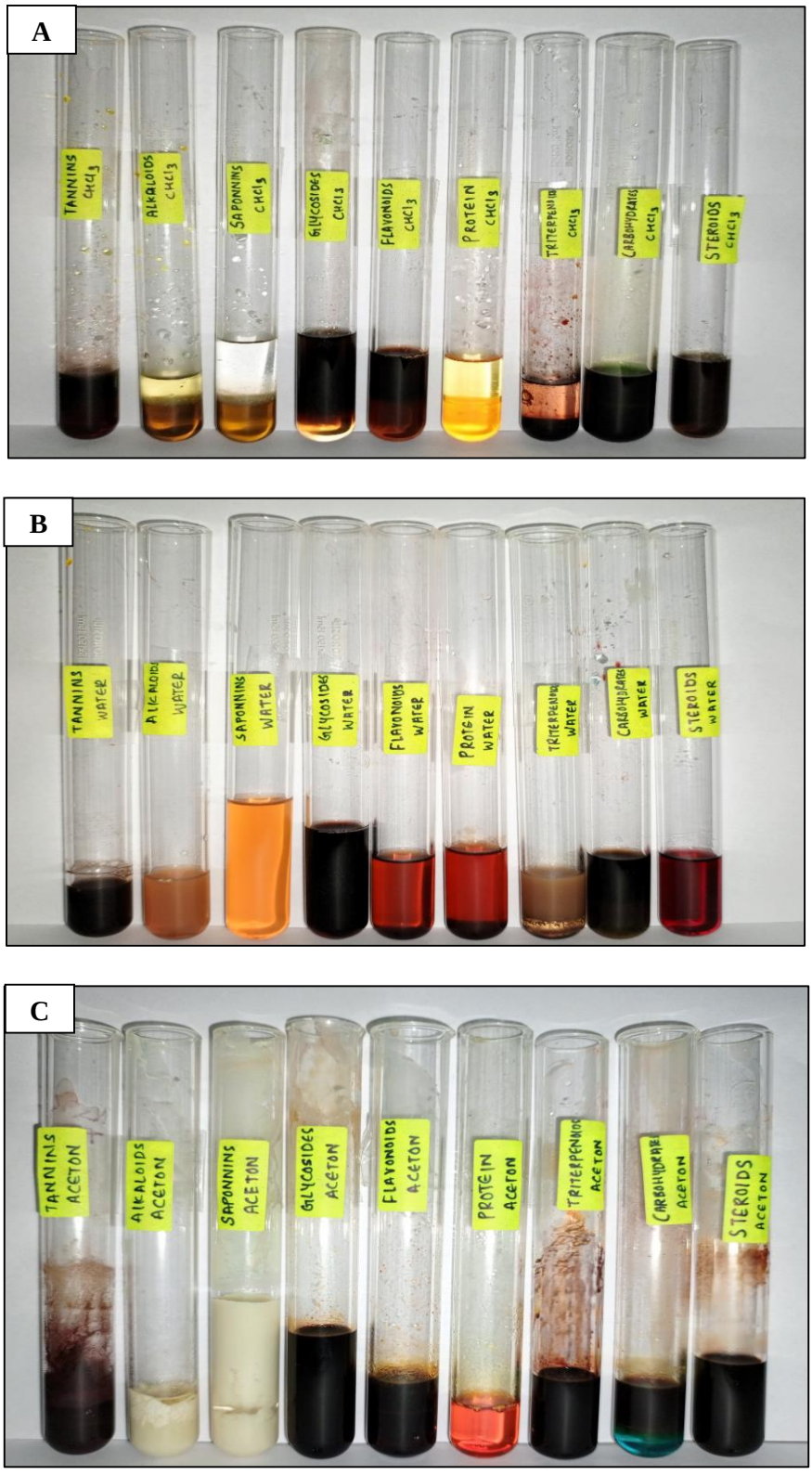


Fig. 5: Preliminary Phytochemical analysis of A- Chloroform; B- Water and C- Acetone extract of *P. Parleta* using Maceration.

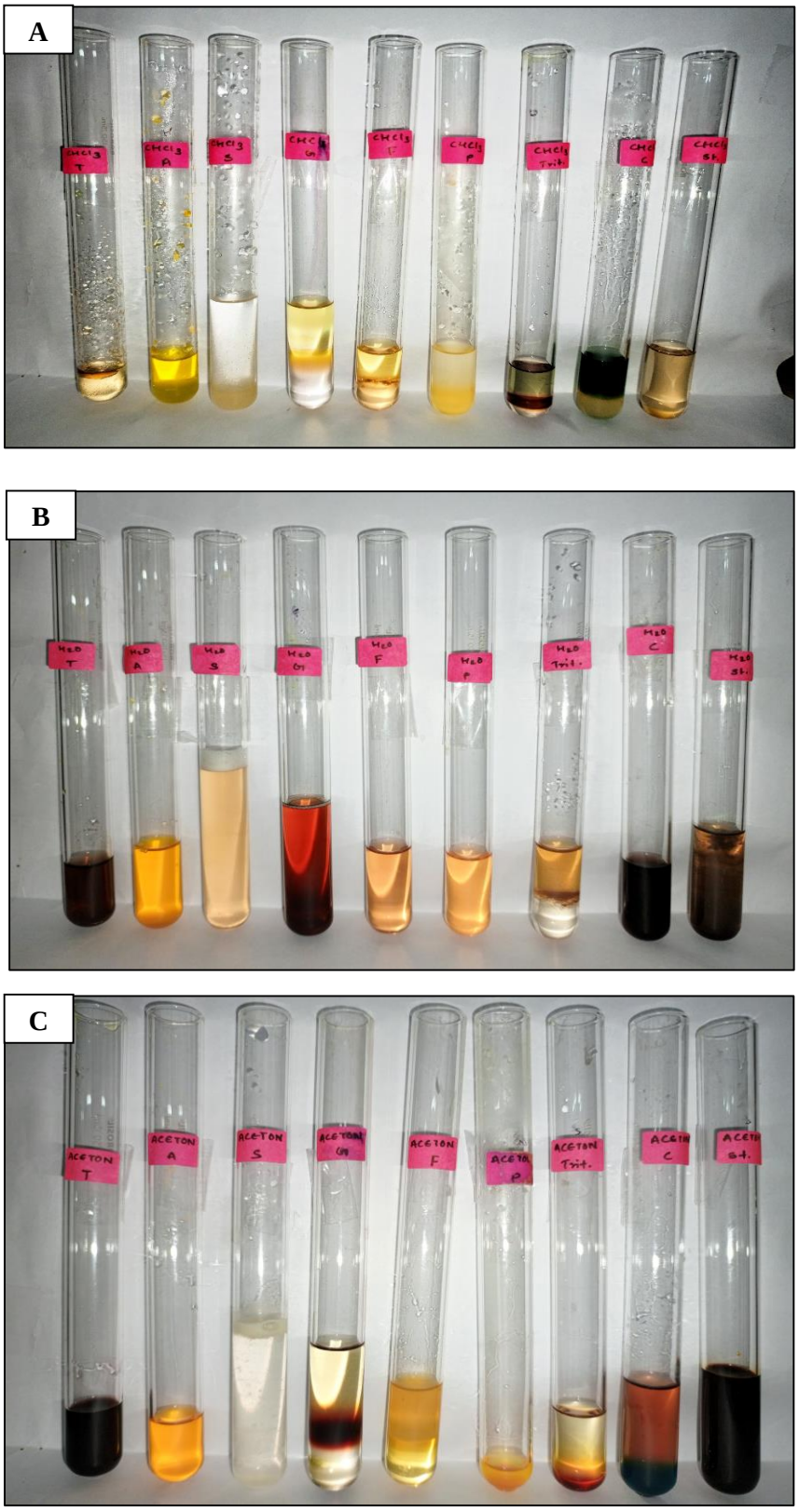


Fig. 6: Preliminary phytochemical analysis of A- Chloroform; B- Water and C- Acetone extract of *P. Perlata* using Cold extraction.

3.4 Determination of Total Phenolic Content

The total phenolic content with water, acetone and chloroform extract of the Lichen *Parmelia perlata,* which was extracted using cold extraction method was measured using Folin-Ciocalteu reagent and external calibration with Gallic acid as standard are represented in **Table 5** and **Figure 7**. The water extract, Acetone extract and chloroform extract showed the total phenolic content of 28.53%, 28.17 % and 23.74 % respectively.

Table 5: The Total phenolic content (TPC) in water, acetone and chloroform extract of lichen *Parmelia Perlata.*

Lichen Extract	**Total Phenolic Content (mg of GAE/ g of extract)**
Water	28.53 ± 0.502
Acetone	28.17 ± 0.608
Chloroform	23.74 ± 0.616

GAE: Gallic Acid Equivalent. Data represent mean values ± Standard Deviation (n=3)

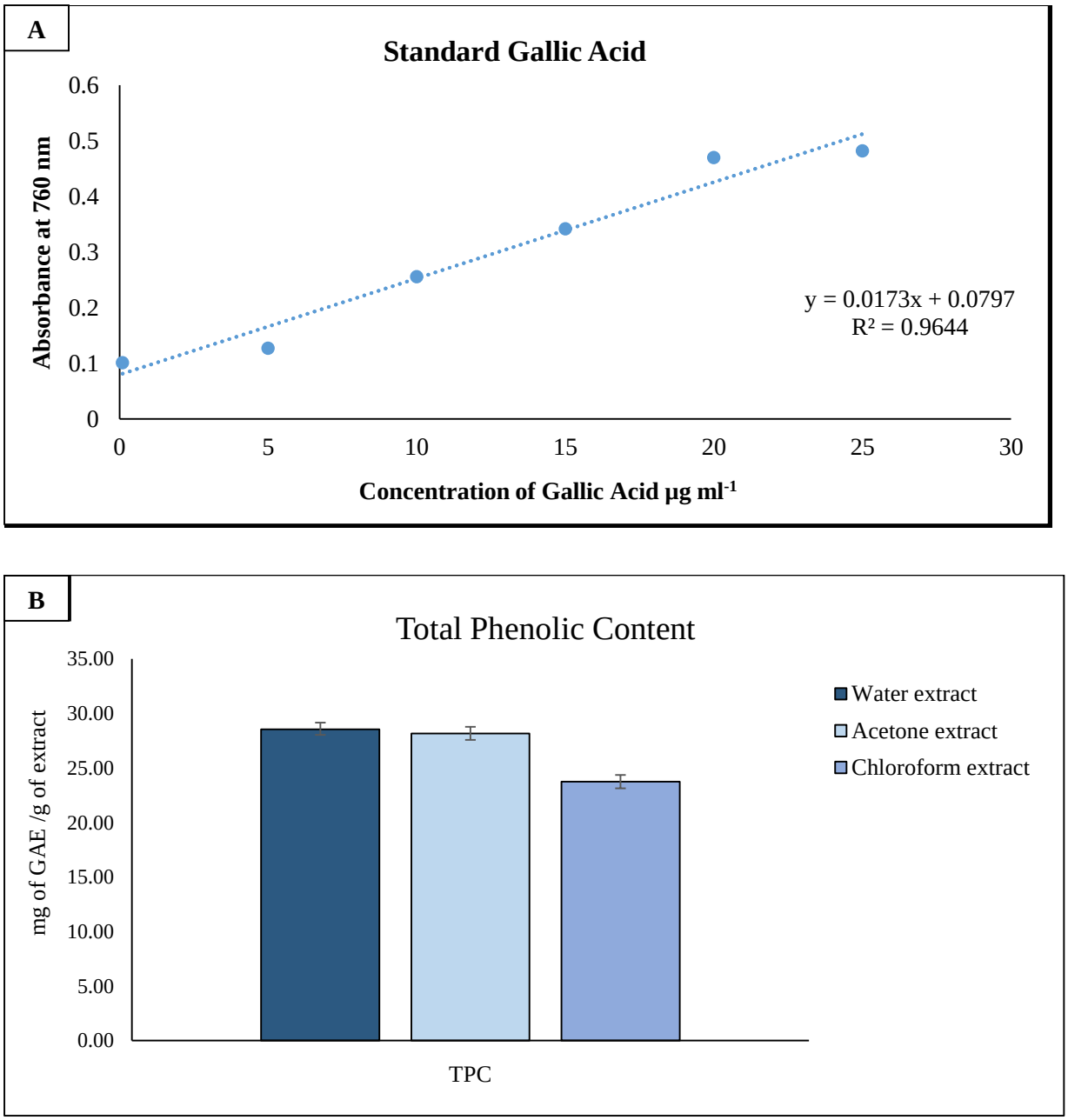


Fig. 7: Determination of Total Phenolic Content (TPC) of different solvent extracts of lichen *Parmelia perlata*. A- Curve for Gallic acid; B- Total phenolic content (mg of Gallic acid equivalent (GAE)/ g of extract) in different solvents. Bars represent mean values ± SD (n=3).

3.5 Antioxidant Activity

In present study, water, acetone and chloroform extracts of lichen *Parmelia perlata* were tested for two antioxidant assays: DPPH (2, 2- diphenyl-1- picrlhydrazyl) free radical scavenging activity and reducing power assay.

3.5.1 Reducing Power Assay

The FRAP assay is useful in these studies as it provides a measure of the reducing power of the lichen extracts, which is an indicator of their ability to act as antioxidants. The higher the absorbance, the greater the antioxidant capacity. Here, *Parmelia perlata* water extract showed significantly higher absorbance (A_{700nm} 0.374 - 0.395) than the acetone extract of lichen (A_{700nm} 0.293 - 0.301). The chloroform extract showed the lowest absorbance (A_{700nm} 0.068 - 0.067) indicating lowest reduction of Fe^{3+} (ferric ion) to Fe^{2+} (ferrous ion) activity **(Fig. 8)**.

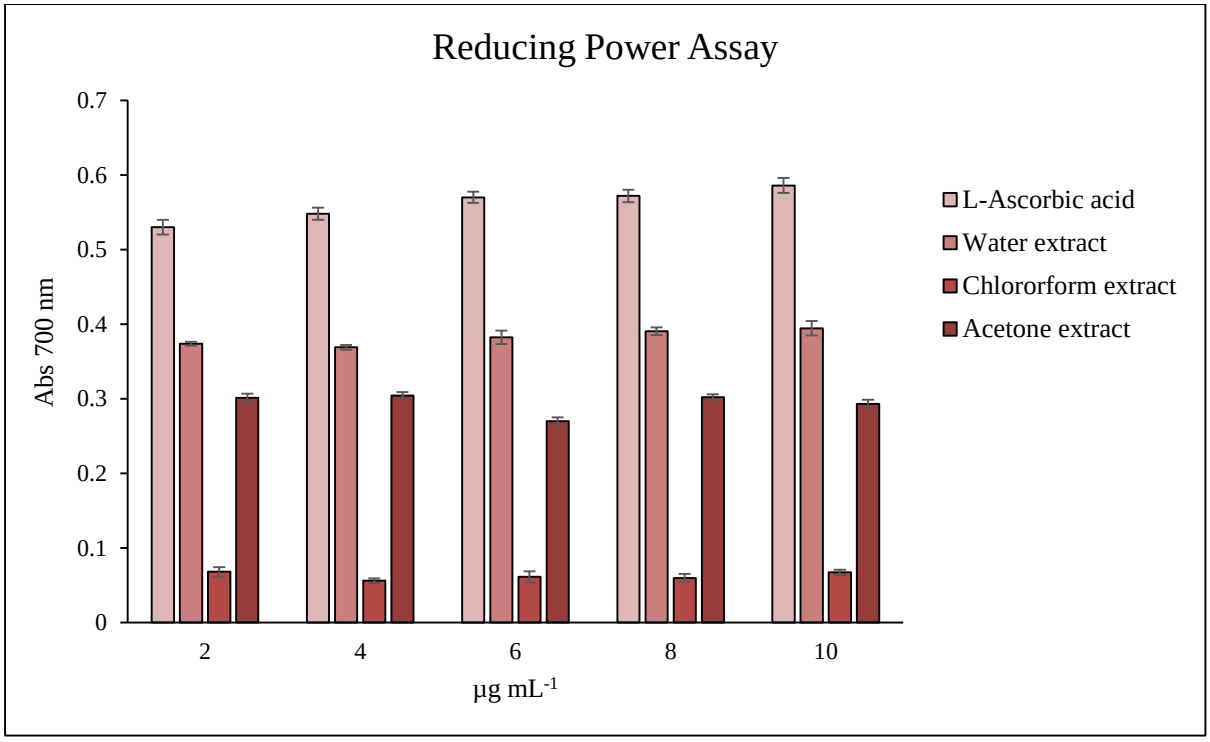


Fig. 8: Antioxidant activity using reducing power assay of water, acetone and chloroform extracts of lichen *Parmelia perlata.* Bars represent mean values ± SD (n=3).

3.5.2 DPPH (2, 2- diphenyl-1- picrylhydrazyl) free radical scavenging

The DPPH assay is a widely used method for evaluating the antioxidant capacity of compounds. It is a stable free radical with an unpaired electron. Antioxidants react with DPPH by donating electrons, leading to the reduction of DPPH. The reduction results in a colour change from purple to yellow. The DPPH scavenging activity of the three extracts of lichen *Parmelia perlata* are shown in **Table 6** and **Fig. 9**.

The percent (%) scavenging activity of water, acetone and chloroform extracts of lichen *P. perlata* is compared with standard L-ascorbic acid. In this work, % scavenging activity of the lichen extracts is seen to increase as the concentration increases. Ascorbic acid is used as a positive control because it is a well-known antioxidant and at all concentrations, it exhibits the highest percentage inhibition. Chloroform extract shows moderate antioxidant activity and its % scavenging activity increases with higher concentrations but remains lower than that of ascorbic acid.

Water extract exhibits lower antioxidant activity compared to chloroform extract and its % scavenging activity is less pronounced across all concentrations. The Acetone extract demonstrates the least antioxidant activity and its % scavenging activity is consistently lower than that of the other extracts.

Lower IC50 values indicate stronger antioxidant activity. Ascorbic acid serves as a positive control and has 78.665 µg mL^{-1} IC50 value. Among the lichen extracts, the chloroform extract of IC50 value 152.01 µg mL^{-1} shows the highest antioxidant activity, followed by water extracts with IC50 value of 482.41 µg mL^{-1} and acetone with IC50 value of 1842.6 µg mL^{-1} **(Table 7).**

Table 6: Antioxidant activity using DPPH free radical scavenging by ascorbic acid and water, acetone and chloroform extracts of *P. perlata*. Data represent mean values ± Standard deviation (n=3).

Sr. No.	Concentration (µg/mL)	L-Ascorbic acid	*P. perlata* extract		
			Water	Acetone	Chloroform
1	12.5	13.30 ± 0.66	2.822 ± 0.66	6.947 ± 0.62	18.78 ± 0.65
2	25	26.99 ± 0.54	4.715 ± 0.57	4.207 ± 0.72	22.02 ± 0.87
3	50	45.66 ± 0.65	5.832 ± 0.73	2.414 ± 0.77	32.88 ± 0.66
4	100	64.49 ± 0.83	17.32 ± 0.60	8.350 ± 0.69	45.61 ± 0.46
5	200	97.08 ± 0.60	20.31 ± 0.72	9.198 ± 0.61	56.31 ± 0.56

Table 7: Antioxidant activity: IC 50 value of Water, Acetone and Chloroform extracts of lichen *P. perlata* with L- Ascorbic acid as Standard.

Sr. No.	Sample / Standard	IC 50 Values (µg/mL)		
		Water	Acetone	Chloroform
1.	*Parmelia perlata*	482.41	1842.6	152.01
2.	L- Ascorbic acid	78.665		

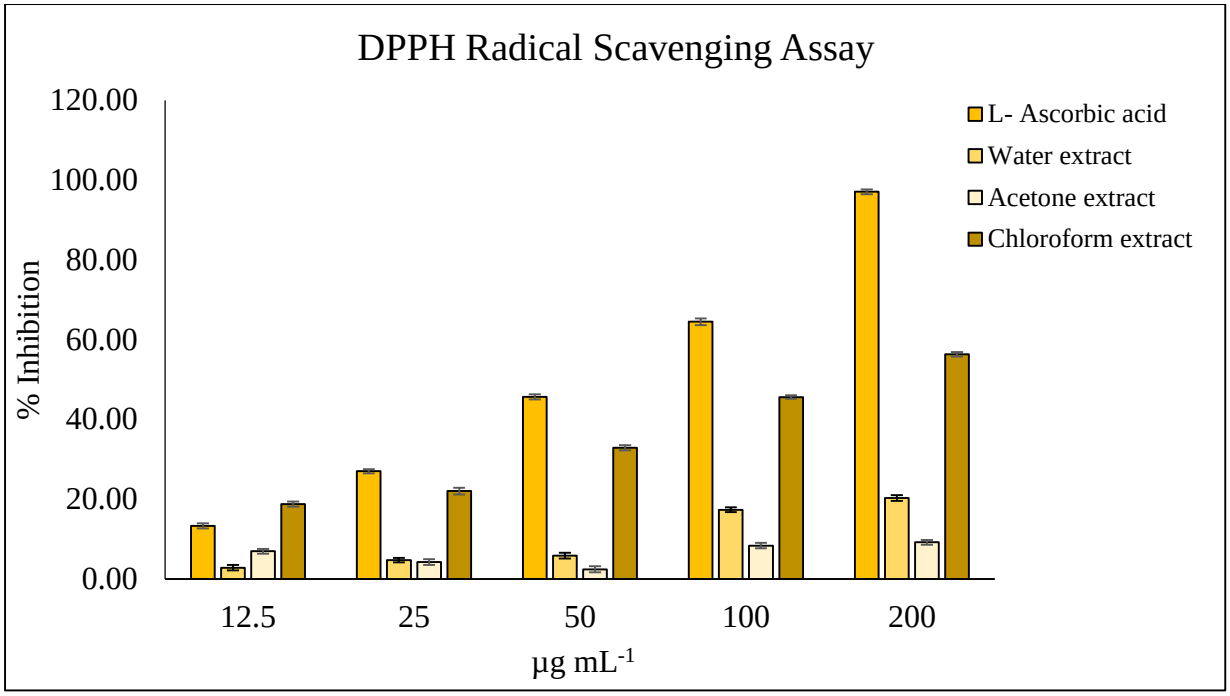


Fig. 9: Antioxidant activity: DPPH free radical scavenging activity of water, acetone and chloroform extracts of *P. perlata*. Bars represent mean values ± SD (n=3).

3.6 Estimation of usnic acid concentrations in the lichen extract by using FTIR

FTIR spectra of water extract of lichen showed adsorption bands at 3852.10, 2362.49, 2342.46, 1650, 1335.07, 1210.58, 974.47, 835.67, 686.85, 595.27, 523.73, 510.85, 457.90, 452.18, 423.56, 417.84, 403.53 cm^{-1} **(Fig.10) (Table 8)**.

The strongest peak at 3852.10 cm-1, is usually a sign of O-H stretching vibrations, indicating the presence of hydroxyl groups or water in the sample. An additional noteworthy peak, measuring 2342.46 cm-1, may be linked to C=O stretching vibrations, suggesting the existence of carbonyl groups.

Asymmetric stretching or C=O stretching may also be connected to the peak at 2362.49 cm-1 with an intensity of 60.46, indicating the presence of carbonates or carbonyl compounds. The fingerprint region is typically defined as peaks in the 400–1500 cm-1 range, which can provide intricate details about the molecular structure.

O-H stretching may be the cause of the peak at 3852.10 cm-1; nevertheless, it is displaced to a higher wavenumber than is typical for usnic acid. The peak, at 1650 cm^{-1}, might be a match for the C=O stretching vibrations which indicate carbonyl group. By comparing with standard usnic acid spectra **(Fig 13)** our results also showed stretch at 1650 which indicate presence of usnic acid 1700 cm^{-1} must have shifted to 1650 cm^{1}

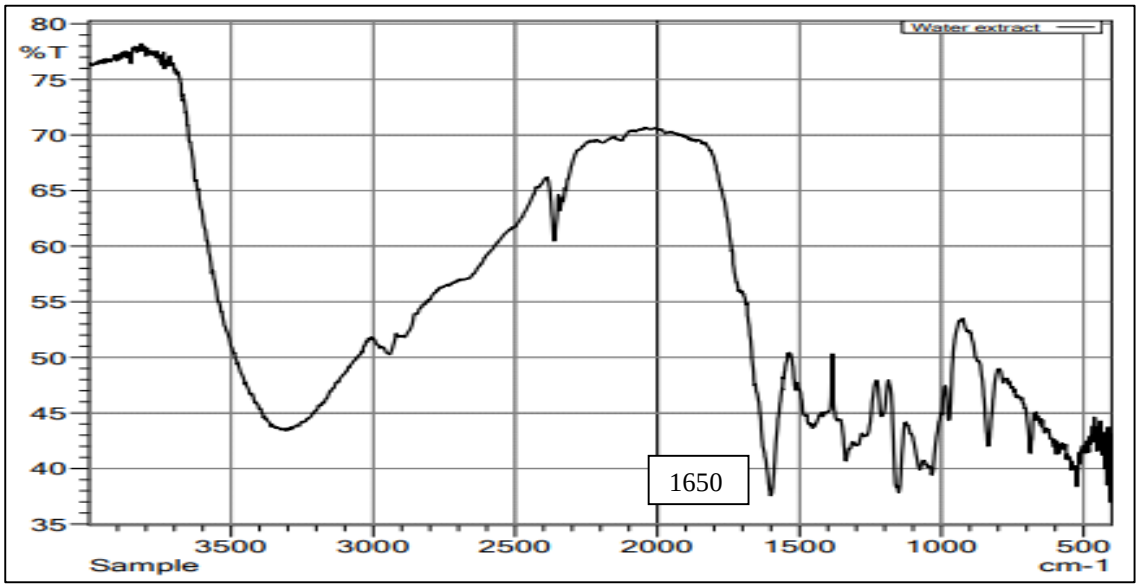
The C-O stretching vibrations that correlate to the peaks at 1210.58 cm-1 and 1335.07 cm-1 may be the cause of the observed range for usnic acid. Peaks at 3436.16 (OH), 2916.61 (CH), 1692.31 (C=O), 1632.10 (C=C aromatic), and 1144.21 cm^{-1} (C-O-C) were found in the

usnic acid nanocrystals. These locations are a little closer to those found in the lichen water extract.

Table 8: FTIR profile of *P. perlata* water extract.

Sr. no.	Peak (cm^{-1})	Functional group	Class
1	3852.10	O-H stretching	water
2	2342.46	C=O stretching	carbonyl groups
3	2362.49	O=C=O stretching	carbon dioxide
4	1650	C=O stretching	carbonyl groups

Fig. 10: FTIR spectra of *P. perlata* water extract.



Acetone extract

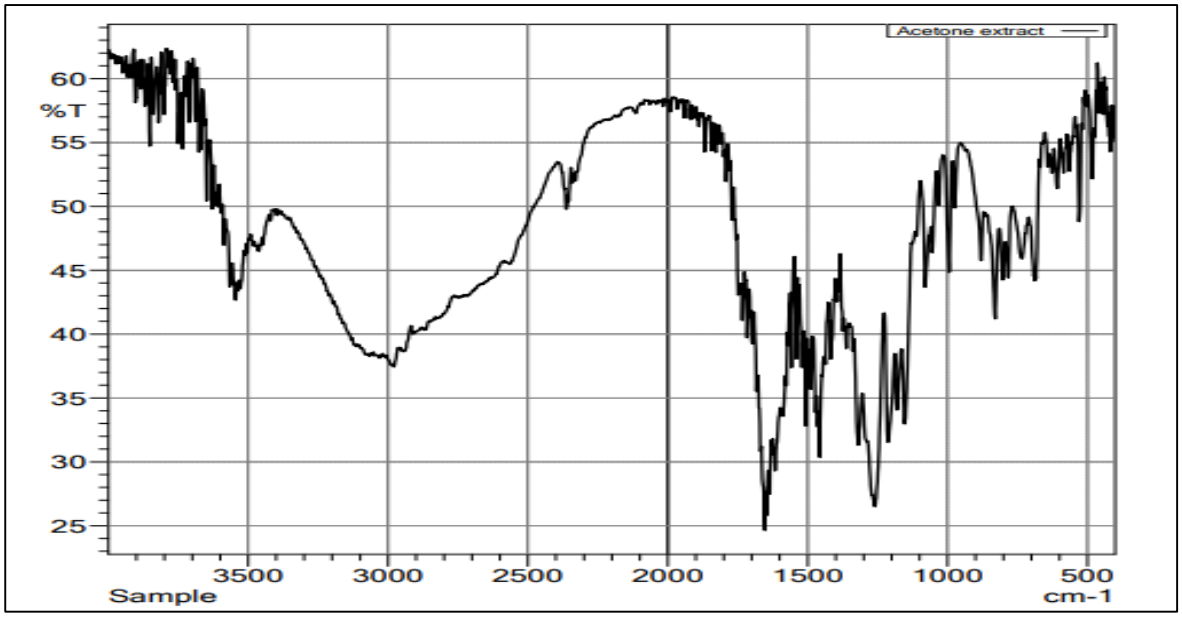
FTIR spectra of acetone extract of lichen showed adsorption bands at cm^{-1} which indicate 529.45 cm^{-1} - C-Br stretching (halo compound), 628.18 cm^{-1} - C-Cl stretching (halo compound), 880.03 cm^{-1} - C-H bending (1,2,4-trisubstituted), 1031.71 cm^{-1} - C-N stretching (amine), 1373.71 cm^{-1} - O-H bending (phenol), 1388.02 cm^{-1} - C-H bending (aldehyde), 1418.07 cm^{-1} - O-H bending (alcohol), 1521.09 cm^{-1} - N-O stretching (nitro compound), 1576.90 cm^{-1} - C=C stretching cyclic (alkene), 2361.06 cm^{-1} - O=C=O stretching (carbon dioxide), 1991.87 cm^{-1} - C=C=C stretching (allene), 3502.95 cm^{-1} - O-H stretching (alcohol). **(Fig.11) (Table 9)**.

It is seen that the acetone extract of lichen has some similar peaks to usnic acid, such as the O-H stretching and C=O stretching. By comparing with standard usnic acid spectra **(Fig 13)**, our results also showed stretch at 1650 which indicate presence of usnic acid. 1700 cm^{-1} must have shifted to 1650 cm^{-1}.

Table 9: FTIR profile of *P. perlata* acetone extract.

Sr. no.	Peak (cm^{-1})	Functional group	Class
1	529.45	C-Br	Halo compound
2	628.18	C-Cl	Halo compound
3	880.03	O-H bending	Phenol
4.	1388.02	C-H bending	Aldehyde
5	1418.07	O-H bending	Alcohol
6	1521.09	N-O stretching	Nitro compound
7	1991.87	C=C=C stretching	Allene
9.	3502.95	O-H stretching	Alcohol

Fig. 11: FTIR spectra of *P. perlata* acetone extract.



Chloroform extract

FTIR spectra of chloroform extract of lichen showed adsorption bands at which indicate 666.82 cm^{-1} - C=C bending (aklene), 1435.24 cm^{-1} - O-H bending (carboxylic acid), 1521 cm^{-1} - N-O stretching (nitro compound), 1568.32 cm^{-1}- C=C stretching (cyclic alkene), 1652.74 cm^{-1} - C-H bending (aromatic compound), 2850.44 cm^{-1}- C-H stretching (alkane), 2923.42 cm^{-1}- O-H stretching (carboxylic acid) **(Fig.12) (Table 10)**.

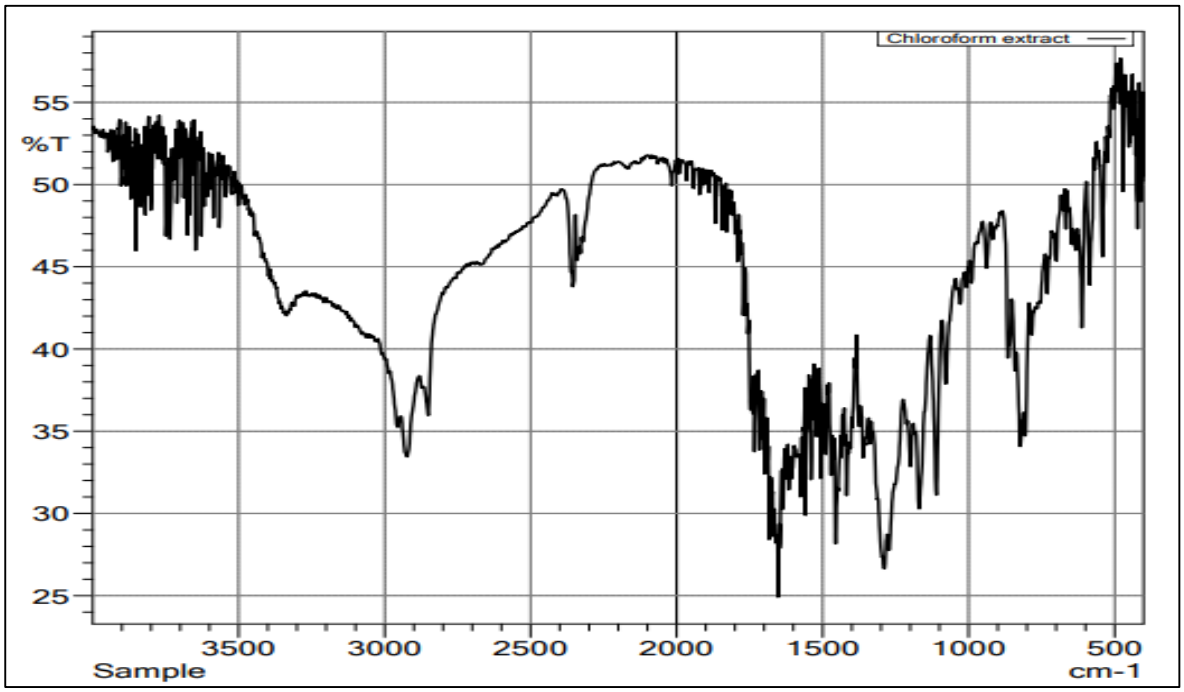
It is seen that the chloroform extract of lichen has some similar peaks to usnic acid, such as the C=C stretching and O-H bending. By comparing with standard usnic acid spectra **(Fig 13)** our results also showed stretch at 1650 which indicate presence of usnic acid. 1700 cm^{-1} must have shifted to 1650 cm^{-1}.

Table 10: FTIR profile of *P. perlata* chloroform extract.

Sr. no.	Peak (cm^{-1})	Functional group	Class
1	666.82	C=C bending	aklene
2	1435.24	O-H bending	carboxylic acid
3	1521	N-O stretching	nitro compound
4	1568.32	C=C stretching	cyclic alkene
5	1652.74	C-H bending	aromatic compound
6	2850.44	C-H stretching	alkane
7	2923.42	O-H stretching	carboxylic acid

.

Fig. 12: FTIR spectra of *P. perlata* chloroform extract.



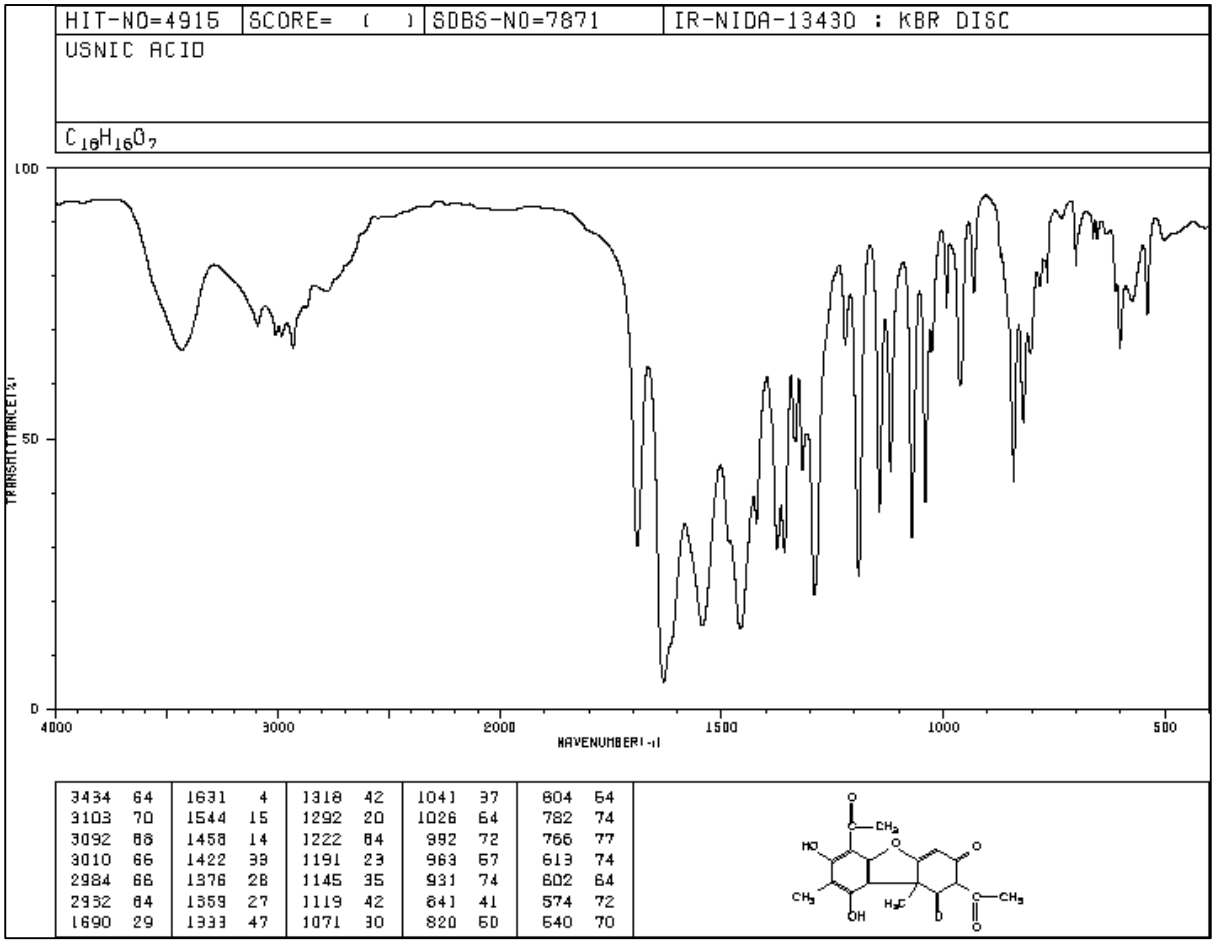


Fig. 13: FTIR spectra of standard usnic acid.

3.7 Antibacterial activity

The antibacterial activity of water, acetone and chloroform extract of lichen *Parmelia perlata* on the gram-positive bacteria, *Bacillus subtilis* are shown in **Table 11** and **Fig. 14**.

The lichen extract of water, acetone and chloroform showed increased zone of inhibition as the concentration of extract increases from 1 mg mL^{-1}, 5 mg mL^{-1} to 10 mg mL^{1} gradually. Acetone extract at highest concentration showed the largest zone of inhibition of 14 mm against the bacteria while chloroform extract also showed similar inhibition zone of 13 mm in diameter. On the other hand, water extract showed the least diameter for zone of inhibition of 11 mm. The positive control with antibiotic Ampicillin showed 12 mm zone of inhibition against *Bacillus subtilis* while there was no activity seen for solvents used as negative control.

Table 11: Antibacterial assay with zone of inhibition of *Bacillus subtilis* in the presence of various concentrations of lichen extracts.

Sample/ Control	**Solvent**	**Zone of Inhibition**		
		1 mg mL^{-1}	**5** mg mL^{-1}	**10** mg mL^{-1}
Parmelia perlata (Extract)	Water	8	9.5	11
	Acetone	6	9	14
	Chloroform	11.5	8	13
Positive control (Ampicillin)		12		
Negative control (solvent)	Water	No Activity		
	Acetone	No Activity		
	Chloroform	No Activity		

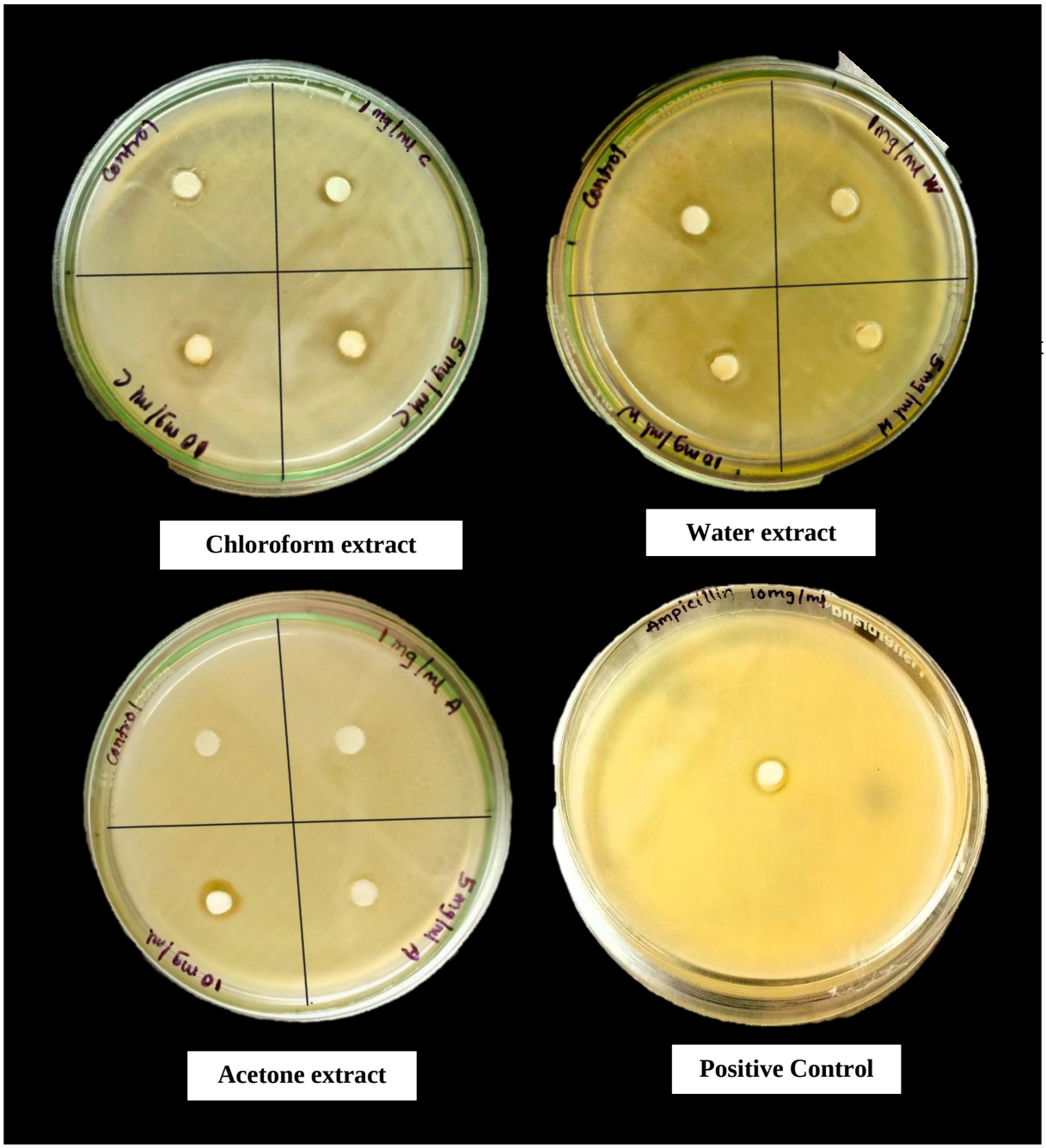


Fig. 14: Antibacterial activity of Chloroform, Water and Acetone extracts of *P. perlata* against *Bacillus subtilis*

3.8 Mosquito larvicidal activity

This study embarked on an exploration of the larvicidal potential of *P. perlata* lichen extracts against the larvae of the mosquito species *Culex quinquefasciatus*. Three different solvents were used in the extraction process: acetone, chloroform, and water. To determine how effective these extracts were at killing larvae, a preliminary mortality test was conducted **(Fig. 15).**

Nevertheless, the results of this exploratory study did not show any appreciable larvicidal action. It is hypothesised that the extract concentrations used in these experiments may not have been high enough to produce any discernible action.

Additional trials within the scope of this study were not possible due to time constraints and other unanticipated reasons. These would have sought to test the extracts at higher concentrations and optimize the extraction procedure. Future research endeavors could aim to overcome these limitations, thereby enabling a more comprehensive investigation into the potential larvicidal properties of these botanical specimens.

Fig. 15: Preliminary bioassay for larvicidal potential of *P. perlata* lichen extracts against *Culex quinquefasciatus.* A- Experimental setup; B- Larvae along with water, acetone and chloroform extracts and solvent as control.

Chapter-5
DISCUSSION

5. DISCUSSION

Lichens, symbiotic organisms composed of fungi and algae or cyanobacteria, exhibit remarkable chemical diversity. Among their secondary metabolites, phenolics, terpenoids, alkaloids, and depsides have drawn significant attention due to their potential pharmaceutical and industrial applications. In this study, the effects of maceration and cold extraction on Lichen *Parmelia perlata* were investigated using acetone, chloroform, and water as solvents. We have worked on the preliminary identification, phytochemical screening, total phenolic content assessment, antioxidant activity evaluation, Usnic acid estimation using FTIR, and antimicrobial testing against *Bacillus subtilis* to understand the activities of lichen extracts. The K-Test and C-Tests for preliminary identification of the lichen *Parmelia perlata* showed yellow colour change with KOH and orange-red colour with Sodium hypochlorite **(Fig. 3 & Table 1)** aligning with the presence of specific lichen compounds in *Parmelia perlata* (Leela & Anchana Devi, 2017).

In this study, the extractive value (% yield) of *P. Perlata* by maceration and cold extraction method indicates that cold extraction is generally more effective than maceration in extracting substances, particularly using water and chloroform as solvents **(Table 2 & Fig. 4)**. Consistent with prior research, cold extraction generally outperforms maceration in extracting substances from *P. perlata*, especially with water and chloroform solvents (Thippeswamy, 2013). The exception is with acetone, where both extraction methods are almost equally efficient, suggesting acetone as a versatile solvent for optimal yield (Vidyalakshmi & Kruthika, 2012).

The presence of glycosides, proteins, and triterpenoids in acetone extracts aligns with previous studies on lichen compounds (Leela & Anchana Devi, 2017). The broader spectrum of phytochemicals in chloroform extracts, including saponins, carbohydrates, and flavonoids, diverges from some earlier findings, suggesting a more diverse profile for *P. perlata*. Methanol and acetone extracts are optimal for the extraction of total phenolics, flavonoids, tannins, and proanthocyanidin with antioxidant properties (Mendili *et al*., 2019). Furthermore, cold extraction proved to be superior for the lichen *P. perlata*, yielding a higher quantity of phytochemicals compared to maceration (Leela & Anchana Devi, 2017) **(Table 3, 4 & Fig. 5, 6)**.

The quantification of phenolic compounds in *Parmelia perlata* extracts was obtained via cold extraction and assessed using the Folin-Ciocalteu method with Gallic acid calibration **(Fig. 7 & Table 5)**. Comparatively, the water and acetone extracts exhibited a marginally higher phenolic concentration than the chloroform extract, suggesting solvent-specific extraction efficiencies for phenolic compounds in this lichen species. Acetone's intermediate polarity enhances the extraction of phenolic compounds due to its ability to dissolve a wide range of polar and nonpolar compounds, facilitating the extraction of diverse phenols (Díaz-Reinoso *et al*., 2021).

The Ferric Reducing Antioxidant Power (FRAP) assay serves as a valuable indicator of the antioxidant potential in lichen extracts, with the higher absorbance equates to a stronger antioxidant effect. In this study, the water extract of *Parmelia perlata* demonstrated a higher absorbance, suggesting a robust capacity for reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). On the other hand, the acetone extract exhibited a significantly lower absorbance, indicating moderate antioxidant activity **(Fig. 8)**. The chloroform extract presented the minimal

absorbance value which implies the least effectiveness in the reduction of Fe^{3+} to Fe^{2+}. These findings underscore the superior antioxidant properties of the water and acetone extract compared to chloroform. The data is supported by previous work done by Leela & Anchana Devi (2017) which shows higher FRAP values for acetone extract but was yielded using hot extraction.

In the comparative analysis of antioxidant activities, the DPPH radical scavenging efficiency of lichen *P. perlata* extracts in water, acetone, and chloroform is evaluated against the benchmark antioxidant, L-ascorbic acid (IC50 of 78.665 µg mL^{-1}). The study reveals a concentration-dependent increase in scavenging activity for all extracts, with L-ascorbic acid consistently demonstrating superior inhibition across all tested concentrations **(Fig 9 & Table 6, 7)**. Among the extracts, the chloroform variant exhibits moderate antioxidant capabilities (IC50 of 152.01 µg mL^{-1}), surpassing the water extract (IC50 of 482.41 µg mL^{-1}), which shows a subdued response, and significantly outperforming the acetone extract, which presents the lowest activity (IC50 of 1842.6 µg mL^{-1}). The chloroform extract emerges as the most effective among the extracts, followed by the water extract and the acetone extract highlighting a hierarchy in antioxidant potential within the lichen extracts. The hierarchy of antioxidant potential observed in lichen extracts aligns with previous findings where chloroform extracts exhibited moderate to high antioxidant capabilities compared to water and acetone extracts but the exceptionally low antioxidant activity of the acetone extract contradicts the general expectation that acetone extracts often demonstrated as significant antioxidant potential (Pratibha & Mahesh, 2016).

The water, acetone, and chloroform extracts of the lichen *Parmelia perlata* were tested for antibacterial activity against *Bacillus subtilis* **(Fig. 14 & Table 11)**. The results showed that the antibacterial effect increased with the concentration of the extracts. The acetone extract was the most effective, with a 14 mm inhibition zone at the highest concentration, closely followed by the chloroform extract at 13 mm. The water extract had the least effect with an 11 mm zone. The standard antibiotic Ampicillin showed a 12 mm zone, indicating that the acetone and chloroform extracts were more effective at the highest concentration, while the solvents alone had no antibacterial activity. The smaller zone of inhibition showed by the antibiotic ampicillin is possible is the density of bacterial inoculum is too dense, the bacterial resistance towards the antibiotic is also possible. The antibacterial activity of lichen extracts against *Bacillus subtilis* increased with concentration, consistent with findings in other studies on antibacterial effects of lichen extract. In the study, the acetone extract of *Parmelia perlata* was the most effective against *Bacillus subtilis*, while the water extract had the least effect, differing from results where water extracts showed superior antibacterial activity (Vidyalakshmi & Kruthika, 2012). Chloroform extract demonstrated significant antibacterial activity against *Bacillus subtilis*, contrasting with studies where chloroform extracts exhibited moderate to high antioxidant capabilities but not specifically against *Bacillus subtilis* (Ranković *et al*., 2007).

Chapter -6
CONCLUSION

6. CONCLUSION

The present study comprehensively investigated the effects of maceration and cold extraction techniques on Lichen *Parmelia perlata*, focusing on the extraction yield, phytochemical composition, total phenolic content, antioxidant activity, and estimation of usnic acid and antibacterial properties

The extraction yield varied depending on the solvent and extraction method utilized. The cold extraction consistently outperformed maceration, particularly with water and chloroform solvents, indicating its superior efficiency in extracting bioactive compounds from *P. perlata*. Phytochemical screening revealed a diverse array of secondary metabolites, including glycosides, proteins, triterpenoids, saponins, carbohydrates, flavonoids, and phenolic compounds. Notably, chloroform extracts exhibited the broadest spectrum of phytochemicals, underscoring the solvent-dependent extraction efficiency.

Quantification of total phenolic content revealed substantial concentrations in water and acetone extracts, with acetone exhibiting slightly higher levels. The antioxidant activity, evaluated through FRAP and DPPH assays, demonstrated concentration-dependent scavenging capabilities, with water and acetone extracts exhibiting superior antioxidant potential compared to chloroform extract. Notably, chloroform extract displayed moderate to high antioxidant activity, suggesting its utility as a potential source of natural antioxidants.

FTIR analysis provided insights into the presence of usnic acid in *P. perlata* extracts. While the spectral data indicated characteristic vibrations associated with usnic acid, further confirmation is required through additional analytical techniques. Usnic acid, known for its pharmacological properties, could contribute significantly to the biological activities exhibited by *P. perlata* extracts. The antibacterial evaluation against *Bacillus subtilis* revealed

concentration-dependent inhibitory effects, with acetone extract exhibiting the highest potency followed by chloroform and water extracts. These findings underscore the potential of *P. perlata* extracts as promising sources of antibacterial agents, particularly against gram-positive bacteria.

The findings of this study contribute to the growing body of knowledge regarding the pharmacological potential of lichen species, particularly *P. perlata*. The observed bioactivities, including antioxidant and antibacterial properties, highlight the therapeutic significance of *P. perlata* extracts in pharmaceutical and nutraceutical applications. Future research endeavors could focus on elucidating the specific bioactive compounds responsible for the observed activities through advanced analytical techniques such as chromatography and spectroscopy.

Additionally, in vivo studies are warranted to validate the efficacy and safety of *P. perlata* extracts for therapeutic use. Moreover, exploring novel extraction techniques and solvent systems could enhance the extraction efficiency and broaden the spectrum of bioactive compounds obtained from *P. perlata*. Overall, the findings of this study underscore the potential of *P. perlata* as a valuable natural resource for drug discovery and development, with implications for human health and well-being.

Chapter -7
REFERENCES

7. REFERENCES

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