

# Exploring the Bioactive Potential of Himalayan Lichens: Phytochemical and Antimicrobial Profiling of *Xanthoparmelia tinctina* and *Pyxine subcinerea* Extracts

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## Abstract

The symbiotic association that develops between cyanobacteria, algae, and fungi results in the composite organism known as lichens, which has traits similar to those of plants but differs in its separate parent organisms. Lichens can have a variety of morphological characteristics, including foliose structures that resemble flat leaves, crustose structures that resemble flake crystals, fruticose branches that have short, leafless branches, and leprose-powder-like appearances. Due to their therapeutic potential, lichens have long been used in traditional medicine. They are commonly utilized in traditional medicine as an external disinfectant as well as an antihemorrhagic agent in dressing wounds. After careful phytochemical screening, the extracts revealed a complex mixture of several bioactive substances, including cardiac glycosides, alkaloids, steroids, and saponins. The present investigation explores the potential antioxidant and antibacterial properties of ethyl acetate and aqueous extracts obtained from two different species of Himalayan lichen, *Xanthoparmelia tinctina*, and *Pyxine subcinerea*. The evaluation of antioxidant capacity, carried out using the ferric ion-reducing antioxidant power (FRAP) and (DPPH) 2,2-diphenyl-1-picrylhydrazyl assays, demonstrated significant antioxidant capabilities by scavenging free radicals. Moreover, ethyl acetate extracts have shown strong inhibitory effects against a variety of harmful bacteria, such as *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, and *Pseudomonas fluorescens*, according to antimicrobial testing. These thorough results validate the dormant potential of lichen extracts as sources of bioactive substances possessing antibacterial and antioxidant properties. In conclusion, this study highlights the potential benefits of lichen species that are native to the Himalayas for improving human health and well-being, in addition to highlighting their extraordinary biological capabilities.

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## INTRODUCTION

Oxidative stress is a major factor in many degenerative and chronic diseases, including cancer and diabetes [1, 2]. Irrespective of their origin, antioxidants have proven to be efficient in either preventing the formation or release of free ions or removing existing free ions [3–6]. The toxicity of many synthetic and commercially available antioxidants such as propyl gallate, tert-butylhydroquinone, and butylated hydroxyanisole is a major cause of concern [7]. Therefore, in the past few decades, attention has been focused on the

identification of natural sources of antioxidants that are safe for human consumption. Many researchers have demonstrated a keen interest in the identification of antioxidant constituents from natural sources because of their potential to maintain the good health of human health.

Algae, cyanobacteria, and fungi symbiotically associate to give rise to a composite organism referred to as lichens. Significant therapeutic effects of lichens have been observed for traditional medicinal uses in China and India. Recently, lichens have been given attention as a potential source of good antioxidants [8–10]. The secondary metabolites produced by lichens have demonstrated antitumor, antibacterial, and antiviral activities [10]. Humans have used *Pseudocyphellaria aurata*, *Usnea bismolliuscula*, *U. longissimi*, *Xanthoparmelia conspersa*, *Sulcaria sulcata*, and *Solorina crocea* in traditional medicinal systems since ancient times. Lichens are relatively unexplored medicinal plants, yet they have significant potential. According to recent studies, lichens have the potential to be a significant source of bioactive chemicals with antibacterial, anticancer, and other bioactivities. The antimicrobial potential of lichens has also been extensively explored owing to the increasing resistance of microorganisms to existing antimicrobial drugs [11–13]. Therefore, the aim of this in vitro study was to assess the antioxidant and antimicrobial activities of ethyl acetate and aqueous extracts of *Xanthoparmelia tinctoria* and *Pyxine subcinerea*.

## MATERIAL AND METHODS

### Sample Collection

Lichen material (*Xanthoparmelia tinctoria*) was collected from the tree bark using a sharp blade and chisel from Chadwick Falls, Shimla (H.P.). We ensured that lichens were attached to the substrate while isolating it from its natural habitat. The scraps were stored in sealed paper bags. Later, the bags were labeled according to their geographical location, date, and time of collection. A similar process was followed for *Pyxine subcinerea*, except that the sample was isolated from rocks using a hammer and chisel.

### Extract Preparation

The lichen thalli were dried in the air for 5 days to eliminate moisture. Subsequently, 5 g of the thallus was crushed using a mortar and pestle. Solvents, ethyl acetate (EA), and distilled water (AQ), were added to the crushed thallus at a ratio of 1:15. The extract was kept untouched for 24 hours and filtered. This process was repeated for the remaining thallus, and the resulting extract was filtered again. The obtained extract was then subjected to evaporation in a water bath set at 40°C. The dried extract was subsequently dissolved in the respective solvents and stored at -20°C for future analysis.

### Phytochemical Screening

#### Saponin

Distilled water was added to the extract at a ratio of 1:1. The mixture was exposed to rigorous shaking, which led to the formation of stable frothing, determining the presence of saponins in the extract [14].

#### Tannin

A ferric chloride solution (1%) was added to the extract at a ratio of 2:1. The appearance of a greenish-black color indicates the presence of tannins [14].

#### Alkaloids

Sulfuric acid (2%) was added to the extract, which was subsequently warmed. After warming, a few drops of the Dragendorff reagent were added. The presence of alkaloids was confirmed by the formation of an orangish-red precipitate [14].

#### Phlobatannins

A 2 ml extract was reacted with a few drops of hydrochloric acid, and the formation of a red precipitate determined the presence of phlobatannins in the extract [14].

### **Steroids**

After adding 10 ml of chloroform to the extract (0.5 g), the solution was filtered and concentrated sulfuric acid was added to the filtrate. The reddish-brown ring at the interface indicated that steroids were present in the extract [14].

### **Cardiac Glycosides**

Glacial acetic acid (2 ml) was placed in a test tube and drops of 2% ferric chloride were added to the test tube. Subsequently, 2 ml of the extract and 1 ml of concentrated hydrochloric acid were added to the test tube in sequence. The presence of cardiac glycosides was determined using the brown ring formed at the interface [14].

### **DPPH ASSAY**

The free radical scavenging activity of the extracts was assessed using a DPPH assay. A solution of the extract at a concentration of 1 mg/ml was prepared using the appropriate solvent. After adding 160  $\mu$ l of 0.039% DPPH to 50  $\mu$ l of the extract, the mixture was incubated in the dark at room temperature for 30 min. Following incubation, absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used as the standard [15].

### **Ferric Ion Reducing Antioxidant Power**

In the ferric ion-reducing antioxidant power (FRAP) assay, a standard protocol involves preparing a fresh FRAP reagent mixture comprising 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, and 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . Following the preparation of standard solutions with known antioxidant capacity and dilution of test samples, the FRAP reagent was warmed to 37°C, and 180  $\mu$ L was added to each well of a microplate. Subsequently, 10  $\mu$ L of each standard and sample were added to separate wells, mixed thoroughly, and incubated at 37°C. Absorbance readings were recorded at various time points using a microplate reader at a wavelength of approximately 593 nm. The antioxidant capacity of the samples was calculated by generating a standard curve with known standards, expressing the results as ascorbic acid equivalents or another chosen antioxidant standard, and reporting the antioxidant capacity in micromoles of ascorbic acid equivalents per unit volume of the sample. It is crucial to include controls, such as a blank with only the FRAP reagent, and run replicates to ensure the accuracy and reproducibility of the assay [16].

### **Antimicrobial Assay**

The antimicrobial activity of the *Xanthoparmelia tinctoria* lichen was initially assessed using agar culture plugs, following the method described by Bauer et al. in 1966. Gram-positive bacteria, including *Bacillus subtilis* (MTCC-121) and *Micrococcus luteus* (MTCC-106), and gram-negative bacteria, such as *Escherichia coli* (MTCC-1679) and *Pseudomonas fluorescens* (MTCC-2421), were selected as test strains. The agar plates were then incubated at 37 °C for 24–48 hours, and subsequently, the zones of inhibition were measured [17].

## **RESULTS AND DISCUSSION**

### **Phytochemical Analysis**

The collection, identification, extraction, and phytochemical analysis of a variety of extracts from frequently occurring lichens growing in Shimla district were conducted as part of the current study. Two extracts were prepared from the two lichen species used in this study using solvents such as ethyl acetate and water. The extracts were subjected to phytochemical screening to detect the presence of tannins, alkaloids, saponins, glycosides, and steroids. Most of the lichen extracts analyzed contained significant phytochemicals such as saponin, carbohydrates, alkaloids, and steroids. The existence of components that are known to have physiological and therapeutic properties was discovered by phytochemical examination of lichen extracts. Both the EA and AQ extracts of *Xanthoparmelia tinctoria* produce saponins, alkaloids, steroids, and cardiac glycosides. The EA extract of *Pyxine subcinerea* contained steroids, phlobatannins, saponins, and alkaloids. The variety of secondary chemicals that lichens make makes them useful. Interest in the pharmacological properties of chemicals produced from

lichens has increased because of the wide range of biological actions that lichen substances exhibit, including antibacterial, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects [18]. Lichen depsides, barbatic acid, atranorin, and lecanoric acid, are frequently found in numerous lichens and have been claimed to have analgesic, antipyretic, and fungitoxic properties. The phytochemical profiles of both species obtained by initial phytochemical analysis have been demonstrated in Table 1.

**Table 1.** Phytochemical analysis of lichen extracts.

Species	Extract	Saponin	Tannin	Alkaloid	Phlobatannins	Steroid	Cardiac glycoside
<i>Xanthoparmelia tinctoria</i>	EA	+	+	+	-	+	+
	AQ	+	-	+	-	+	+
<i>Pyxine subcinerea</i>	EA	+	+	+	+	+	-
	AQ	+	-	+	-	+	-

### Antimicrobial Assay

The EA extract demonstrated antimicrobial properties against all selected non-pathogenic isolates. The most pronounced antimicrobial activity was observed against *Mycobacterium luteus*. The aqueous extract also displayed antimicrobial activity against all non-pathogenic isolates, except for *B. subtilis* and *M. luteus*. The AQ extract of *Pyxine subcinerea* failed to exhibit inhibitory effects on most of the isolates, whereas the EA extract did not demonstrate similar activity against any of the isolates (Table 2). Notably, the largest inhibition zone was observed for *M. luteus*. The results of the antimicrobial activity of the EA extracts showed relatively strong inhibitory activity, depending on the type of lichen related to the tested bacteria. Depending on the lichen species, the extracting solvent utilized, and the concentration of the lichen extract, there are either parallels or variations between our results and those of many other studies on the antibacterial activity of lichen extracts. The physical differences between the organisms, namely in the variations in cell wall porosity, may be the cause of the varying sensitivities between Gram-positive and Gram-negative bacteria [19]. The aqueous lichen extracts examined lacked any discernible antibacterial action. This is most likely due to the fact that the active ingredients that lichens generate are either water-soluble or very water-soluble [20]. Similar lichens have also been found to possess important bioactive properties in earlier studies. According to Gulluce et al. (2006), similar polar lichens have been found to have important bioactive properties in earlier studies. The polar extract of *P. saxatilis* was found to have a high antimicrobial impact [21]. Candan et al. (2007) observed similar results using extracts obtained from the lichen *P. sulcata* [22].

According to our data, the studied extracts exhibited significant yet distinct antibacterial action. This implies isolating the active ingredients from the extracts of lichen species and studying their antibacterial properties, as well as their potential applications in food preservation and the pharmaceutical sector.

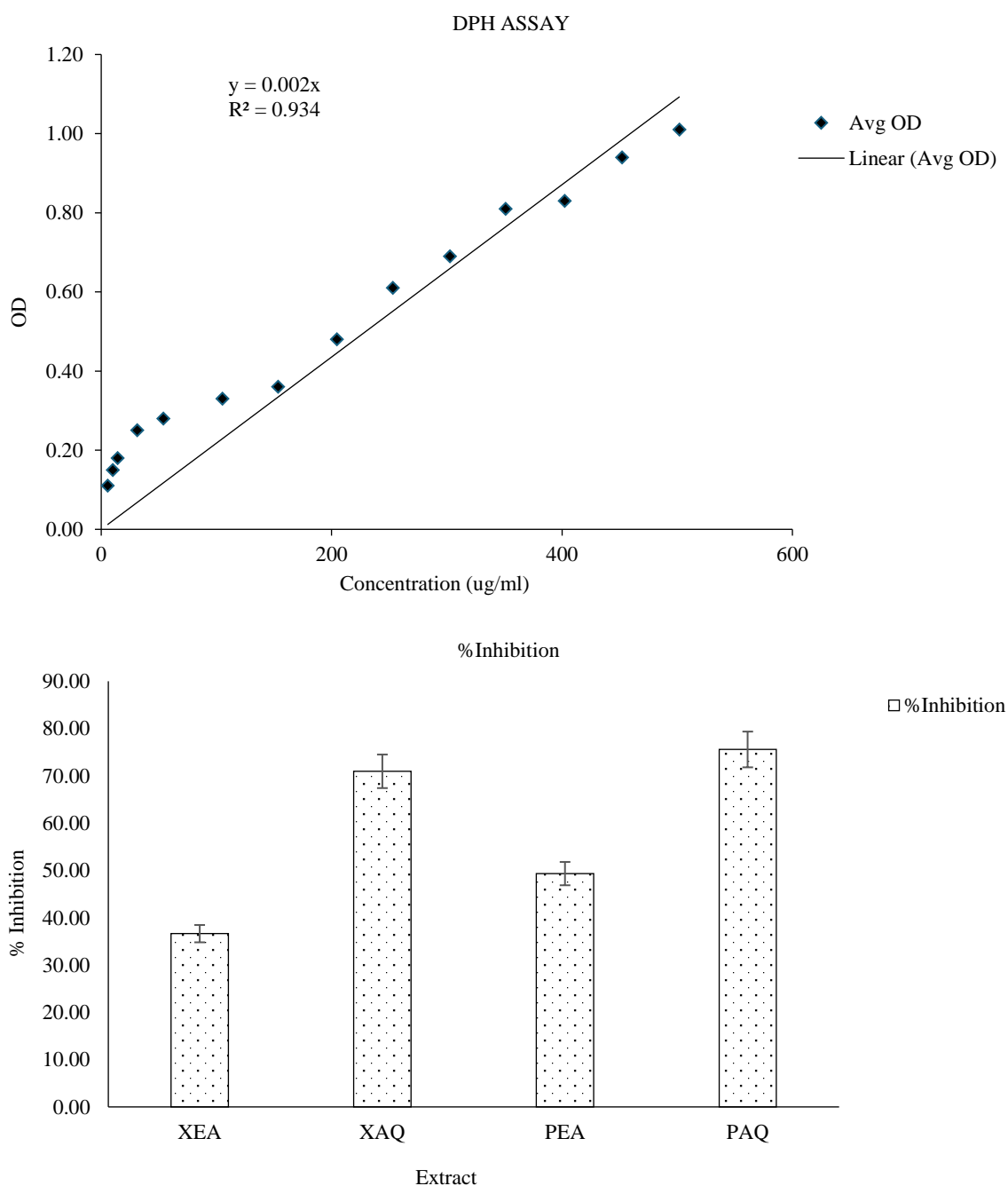
**Table 2.** Antimicrobial activity of lichen extracts.

Species	Extract	<i>Bacillus subtilis</i>	<i>Micrococcus luteus</i>	<i>Escherichia coli</i>	<i>Pseudomonas fluorescens</i>
<i>Xanthoparmelia tinctoria</i>	EA	2.17 ± 0.12	3.53 ± 0.05	1.08 ± 0.15	2.83 ± 0.12
	AQ	0 ± 0.00	0 ± 0.00	1.05 ± 0.09	0.9 ± 17
<i>Pyxine subcinerea</i>	EA	0.9 ± 0.12	2.7 ± 0.05	1.6 ± 0.2	1.8 ± 0.09
	AQ	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.92 ± 02

### DPPH ASSAY

One of the many ways that antioxidation works is by scavenging free radicals. The antiradical activity of the lichen extracts was investigated by testing whether they could neutralize the stable DPPH radical. According to Anandjiwala et al. (2008), this technique is based on the synthesis of the non-radical form DPPH-H in the presence of an alcoholic DPPH solution and a hydrogen-donating antioxidant (AH) via

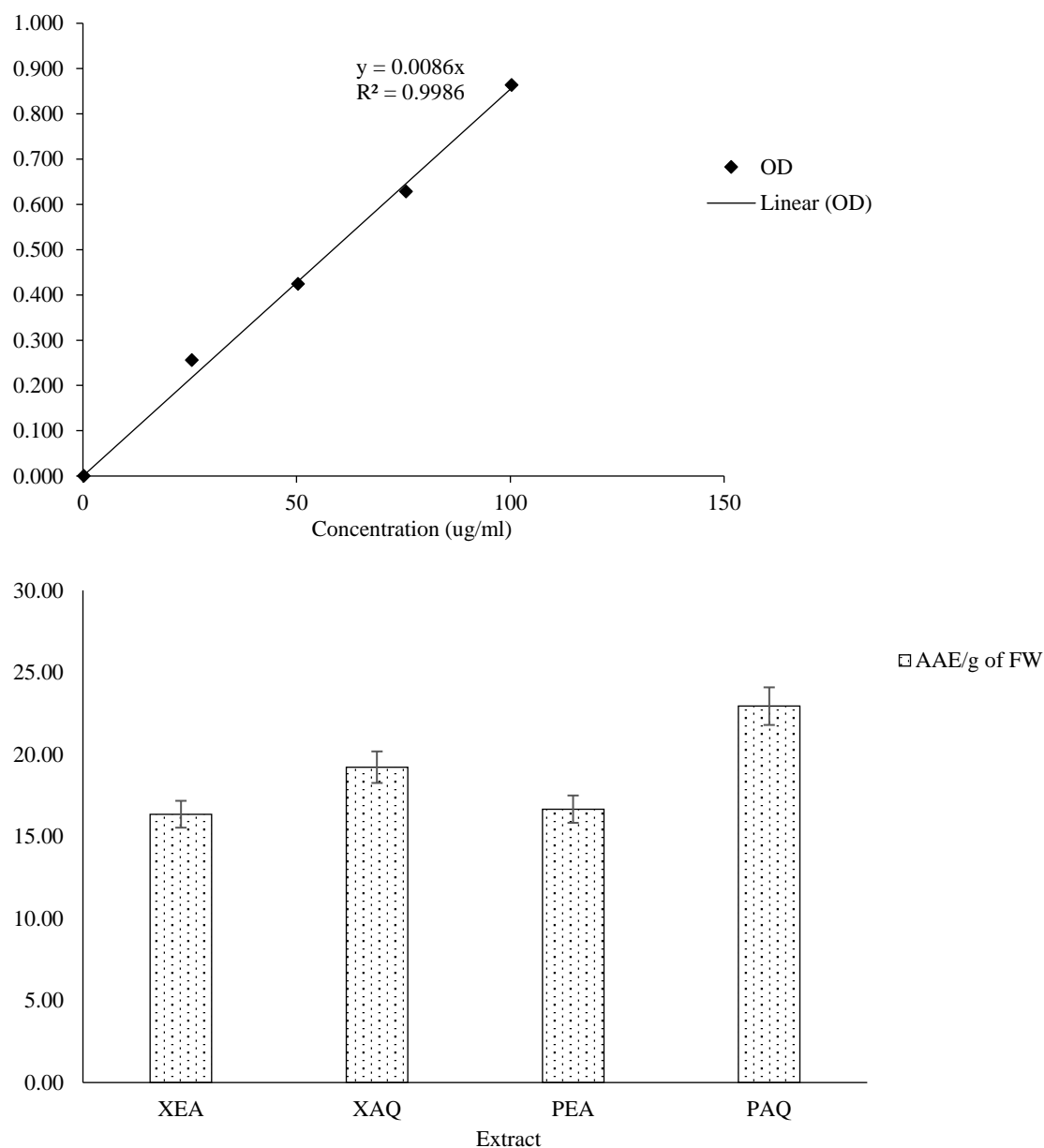
the reaction  $\text{DPPH} + \text{AH} \rightarrow \text{DPPH-H} + \text{A}$  [23]. The aqueous and ethyl acetate extracts demonstrated strong DPPH radical scavenging properties. All lichen extracts had scavenging activities ranging from to 36.26–75.21%. Compared with the other samples, the lichen *Pyxine subcinerea* extracts exhibited the highest DPPH radical scavenging capabilities. The ethyl acetate and aqueous extracts of lichen showed percentage inhibition on the DPPH radical of 49.48 and 75.21%, respectively. The lichen *Xanthoparmelia tinctoria* exhibited high scavenging activity (36.26% for ethyl acetate and 70.43% for aqueous extracts). It is clear from the data that the examined lichen extracts exhibit potent antioxidant activity in vitro. It is clear from the data that the examined lichen extracts exhibit potent antioxidant activity in vitro. The radical scavenging activities of both the species are shown in (Figure 1). The studied lichen species and extraction solvents had an impact on the level of antioxidant activity. The ability of various solvents to extract bioactive compounds determines their antioxidant activities [24].



**Figure 1.** DPPH assay of aqueous and ethyl acetate extracts of *X. tinctoria* and *P. subcinerea*.

### FRAP Assay

The reducing power of several chemical components found in lichen extracts was investigated using a ferric-reducing power assay. The ability of the extracts to transfer a single hydrogen atom from ferric ions was also assessed using this method [25]. Ferrocyanide ( $\text{Fe}^{2+}$ ) is produced by reducing ferric ions from potassium hexaferricyanide ( $\text{Fe}^{3+}$ ). Subsequently, a colored complex is formed by its reaction with ferric chloride. The absorbance intensity was measured at 700 nm. Extracts with the highest absorbance exhibited the strongest reducing power. Aqueous extracts of *Pyxine subcinerea* had the maximum reducing power at 1000  $\mu\text{g}$  of concentration, measuring 22.87; they were followed by ethyl acetate extracts (16.49), and *Xanthoparmelia tinctoria* extracts (16.2) showed the least amount of activity. Ascorbic acid equivalents per gram of lichen extract were used to compute the reduction potential, allowing for a direct comparison of the potential with the benchmark antioxidant molecule (Figure 2). According to these results, a few different lichen species have modest levels of ferric ion reduction, which can serve as a pro-oxidant for the food chain. For the human system, a moderate degree of reduction is advantageous. The ferric ion reducing activities of both species are shown in (Figure 2).



**Figure 2.** FRAP assay aqueous and ethyl acetate extracts of *X. tinctoria* and *P. subcinerea*.

## CONCLUSION

In conclusion, comparative phytochemical, and bioactive analyses of two lichen species, *Xanthoparmelia tinctoria* and *Pyxine subcinerea*, from the Himalayas revealed promising antioxidant and antimicrobial properties. This study aimed to explore the potential of lichens as natural sources of antioxidants and antimicrobial agents because of the increasing interest in finding safe alternatives to synthetic compounds. Phytochemical screening of lichen extracts revealed the presence of various bioactive compounds, such as saponins, alkaloids, steroids, and cardiac glycosides. These compounds are known for their physiological and therapeutic properties, making them valuable resources for traditional medicine and pharmacological research. The FRAP and DPPH assays were used to evaluate the antioxidant activities of both extracts.

Both *Xanthoparmelia tinctoria* and *Pyxine subcinerea* extracts exhibited significant scavenging activity against free radicals, indicating their potential as natural antioxidants. The observed variations in antioxidant activity among the extracts could be attributed to the differences in lichen species and extraction solvents. Additionally, antimicrobial assays revealed significant inhibitory effects of the ethyl acetate extracts on pathogenic bacteria, including *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, and *Pseudomonas fluorescens*. These findings suggest that lichen extracts possess promising antimicrobial properties, which could be explored further for various applications including food preservation and pharmaceuticals. Overall, these results highlight the potential of lichen species from the Himalayas as sources of bioactive compounds with antioxidant and antimicrobial activities. Further research is warranted to elucidate the specific bioactive components responsible for these effects and explore their potential applications in various fields of medicine and industry.

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