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Phytochemical analysis, total flavonoid and phenolic content, antimicrobial properties of lichens extract of *Hypotrachyna cirrhata* (Fr.) Divakar

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Abstract
Despite the significant attention lichens have received for their unique secondary metabolites, research on edible varieties remains limited. The antioxidant activity of Hypotrachyna cirrhata *was evaluated using in vitro DPPH radical scavenging assays. The total phenolic content (TPC) and total flavonoid content (TFC) of* H. cirrhata *were measured as 42.165 ± 0.98 mg GAE/g and 11.789 ± 0.34 mg QE/g, respectively. In this assay, ethyl acetate demonstrated strong antioxidant potential with an IC_{50} value of 34.14 ± 0.17 µg/mL, whereas hexane showed a higher IC_{50} value of 74.3 ± 1.13 µg/mL, compared to the standard quercetin, which had an IC_{50} value of 62.87 ± 1.02 µg/mL. The methanolic extracts of the lichen demonstrated notable antimicrobial effects against pathogens, with minimum inhibitory concentration (MIC) values recorded at 195.312 g/mL and minimum bactericidal concentration (MBC) ranging from 195.312 to 390.625 g/mL. The study suggested that* H. cirrhata *may exhibit significant levels of total phenolic content (TPC) and total flavonoid content (TFC), which are closely associated with enhanced antibacterial and antioxidant activities.*

Keywords
Anti-oxidant, anti-bacterial, total phenolic content, total flavonoid

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1 Introduction

Lichens represent a form of symbiotic organism, composed of a mycobiont (fungus) in association with cyanobacteria or algae [1] . These organisms are capable of surviving prolonged desiccation without succumbing to starvation, thanks to their ability to sustain a slow metabolic rate. Additionally, lichens exhibit extraordinary adaptability to a wide range of habitats, thriving on almost any surface and in diverse environmental conditions, from sea level to high alpine regions. They also play a

role in forming biological soil crusts and are frequently found on exposed soil surfaces, walls, roofs, and rocks [2]. Lichens manifest in four fundamental growth morphologies: crustose, foliose, and fruticose [3]. Globally, approximately 20,000 lichen species have been documented, with Nepal's catalog identifying 1,129 species, including infraspecific taxa, distributed across 237 genera and 66 families [2].

Since prehistoric times, lichens have been utilized for various purposes including food, dyes, perfumes, medicinal treatments in folk traditions, and ornamental uses [4]. Lichen species produce a diverse array of secondary metabolites, which have been isolated and shown to exhibit a wide spectrum of biological activities [5]. Numerous lichen extracts have been employed in traditional remedies, and screening tests have frequently revealed metabolites with antibiotic [6], antimycobacterial [7], antiviral [8], antitumor [9], analgesic [10] and antipyretic [11] properties.Lichens are capable of enduring extended periods of desiccation without experiencing starvation, thanks to their slow metabolic processes. These efforts seek to explore the possible medicinal uses of compounds obtained from lichens. The metabolites found in any lichen species can be produced through one of three major metabolic pathways: the polymalonate pathway, the mevalonic acid pathway, or the shikimic acid pathway [12]. These pathways not only facilitate the storage of carbohydrates but also the production of secondary metabolites [13].

Lichens have garnered considerable interest from researchers due to their therapeutic potential, which is ascribed to their distinctive compounds. Despite this, comprehensive data on the edibility of lichens and detailed evaluations of their culinary and medicinal applications remain limited. To investigate the biological activities of the lichen *H. cirrhata* (Figure 1) studies have focused on their antioxidant and antimicrobial properties, alongside the identification of phytochemical constituents and the quantification of total phenolic and flavonoid content.

2 Materials and Methods

2.1 Collection and identification of lichens

Lichens *Hypotrachyna cirrhata* were collected from the Makwanpur District with geographical distribution (27° 60 81.83 N, 85° 09 22.81 E) around 2300 meter in altitude. The collected Lichens was identified from the National Herbarium and Plant Laboratories, Department of Plant Resources, Godawari, Lalitpur, Nepal.

Figure 1: Picture of *Hypotrachyna cirrhata*

2.2 Preparation lichen extract

After meticulously removing dust particles from the lichens, they were air-dried in the shade at room temperature for two weeks and then ground into a powder. The powdered lichens were stored in paper bags for future use. A 100 g portion of the dry powder was soaked in methanol at a 1:10 ratio for 24 hours. Following this, the mixture was filtered, and the filtrates were concentrated using a rotary evaporator before being dried in the air.

2.3 Phytochemicals screening

The solution containing extracts from the stem was tested, revealing the presence of phytochemicals such as alkaloids, steroids, tannins, saponins, glycosides, terpenoids, flavonoids, reducing sugars, and coumarins. These findings were obtained using the standardized procedure described by A. J. Harborne [14].

2.4 Determination of total phenolic content

The total phenolic content (TPC) in the extract was evaluated using the Folin-Ciocalteu reagent with some modifications to a previously established method [15]. In short, 20 L of the extract at a concentration of 0.5 mg/mL was added in triplicate to 96-well plates. Then, 100 L of Folin-Ciocalteu reagent and 80 L of Na_2CO_3 were added to each well. The absorbance was measured at 765 nm using a microplate reader (Epoch2, BioTek Instruments, Inc., USA) and expressed in mg of gallic acid equivalents (GAE) per gram of extract, based on a calibration curve created from standard gallic acid.

2.5 Determination of total flavonoid content

The total flavonoid content (TFC) in the extracts was determined using a method with slight modifications from a previously reported study [15]. Briefly, 20 L of plant samples at a concentration of 0.5 mg/mL was added to 96-well plates in triplicate. Next, 110 L of distilled water was added to each well containing the plant samples, followed by 60 L of ethanol, 5 L of $AlCl_3$, and 5 L of CH_3CO_2K. The absorbance was measured at 415 nm using a microplate reader and was expressed as milligrams of quercetin equivalent per gram of extract.

2.6 DPPH radical scavenging activity

The antioxidant capacity of the extracts was assessed using a modified 96-well plate method based on the colorimetric technique [16]. Quercetin at a concentration of 20 g/mL was used as the positive control, while 50% DMSO served as the negative control. Each well in the 96-well plate received 100 L of either the positive control (quercetin), negative control (DMSO), or plant samples, all in triplicate. Following this, 100 L of DPPH reagent was added to each well, and the plate was incubated in darkness for 30 minutes. After incubation, the absorbance was measured at 517 nm using a microplate reader. The DPPH radical scavenging activity was then calculated using the following formula:

$$\%\ Inhibition = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

where, A_{sample} = absorbance of the sample
A_{control} = absorbance of the control

2.7 Antibacterial activities

Lichen extracts were evaluated for antibacterial activity using the agar-well diffusion method. The test microorganisms were incubated for 12 hours at 37°C after being adjusted to a turbidity equivalent to 0.5 McFarland standards (1.5 x 10ˆ8 CFU/mL or 10ˆ8 bacteria/mL) in MHB. Using a sterile cotton swab, bacteria were evenly spread across MHA plates. Three wells, each approximately 4 mm deep and 6 mm in diameter, were made in the cultured MHA plates using a cork borer. Each well was then filled with 50 µL of lichen extract (50 mg/mL in 50% DMSO). Separate wells were used for the negative control, containing 50 µL of 50% DMSO, and the positive control, containing 50 µL of 1 mg/mL neomycin. The plates were left at ambient temperature for 15 minutes to allow diffusion, then incubated for 18 to 24 hours at 37°C. After incubation, the zone of inhibition (ZoI) for each extract was measured in millimeters

2.7.1 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the smallest concentration of a substance required to prevent any visible bacterial growth, determined using a slightly modified version of a previously established method [17]. It measures the effectiveness of a chemical against a specific type of bacteria and is typically expressed in mg/mL or µg. The MIC was assessed for methanolic extracts or other solvent fractions of the plant extract that showed significant antibacterial activity. To prepare solutions with varying concentrations from 12.5 mg/mL to 0.097 mg/mL, 100 L of stock solutions (50 mg/mL) were serially diluted twice in 96-well plates. Then, 5 L of bacteria (106 CFU/mL) was added to all wells except the negative control. The plates were covered and incubated at 37°C for 18 to 24 hours. After incubation, 0.003% resazurin solution was added to each well, followed by another incubation for three to four hours at 37°C. A blue color indicated no bacterial growth, while a pink color, resulting from the enzyme reductase, signaled bacterial presence. The well with a blue color represented the MIC, or the lowest concentration required to inhibit bacterial growth.

2.7.2 Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of a substance needed to kill specific bacteria. In MBC assay, resazurin serves as a dye that changes color upon its conversion to resorufin, signaling the presence of viable cells (Figure 2). Using serially diluted plant extracts, the microdilution method is used to determine this concentration following the CLSI protocol [18]. For the MIC, higher concentrations were applied to MHA plates and incubated for 18 to 24 hours at 37°C. Bacterial growth was then observed on the MHA plates, and the MBC was identified as the lowest concentration where no bacterial growth occurred.

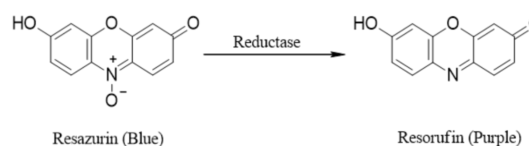

Figure 2: Reaction for the conversion of resazurin to resorufin during MIC determination.

3 Results and Discussions

3.1 Results

3.1.1 Phytochemicals Screening

The results from the phytochemical screening, obtained through chemical tests, are presented in Table 1 which also can indicate the different compound present in *H. cirrhata.*

Table 1: Phytochemical Screening

Serial No.	Group of Compounds	H. *cirrhata*
1	Alkaloids	++
2	Flavonoids	++
3	Terpenoids	++
4	Glycosides	++
5	Polyphenols	++
6	Quinones	++
7	Tannins	++
8	Steroids	–
9	Saponins	–
10	Fatty acids	–
11	Proteins (Xanthoprotein)	++
12	Coumarins	++
13	Anthraquinone	–
14	Reducing sugar	++

Note: (++) indicates presence; (–) indicates absence.

3.1.2 Estimation of Total Phenolic Content (TPC)

Gallic acid equivalent (mg GAE/g) was used to assess the total phenolic content in the crude extracts of *H. cirrhata.* The standard used to calculate TPC is gallic acid. The calibration curve is built using different final concentrations of gallic acid (10, 20, 40, 60, 80, and 100) µg/mL. Using the regression equation (Y = 0.017x+ 0.0552, R^2 = 0.9907) derived from the gallic acid calibration curve, the TPC of the extracts was determined. TPC value for *H. cirrhata* was found to be 42.165± 0.98mg GAE/g.The calibration curve of gallic acid is shown in Figure 2 and the TPC value of extracts is represented in Table 2.

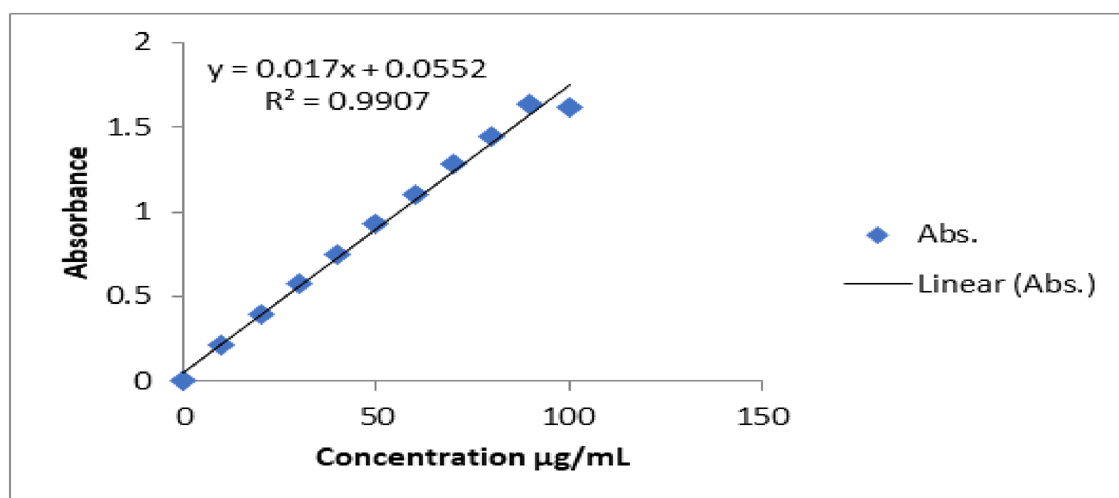

Figure 3: Calibration curve of gallic acid

3.1.3 Estimation of Total Flavonoid Content (TFC)

Using the usual technique and quercetin as a reference, the total flavonoid concentration of the crude lichen extract was estimated. The quercetin equivalent (mg QE/g of the extract's dry weight) was used to express the TFC value. The calibration curve is constructed using varying final concentrations of quercetin (100, 80, 60, 40, 20, 10) µg/mL. Using the regression equation (Y =0.0179x+ 0.0273, R^2 = 0.9997) derived from the quercetin calibration curve, the TFC of the extract was determined. *H. cirrhata* had a TFC value of 11.789± 0.34 mg QE/g. Figure 3 displays the quercetin calibration curve, and Table 2 displays the TFC value for the extracts of lichens *H. cirrhata.*

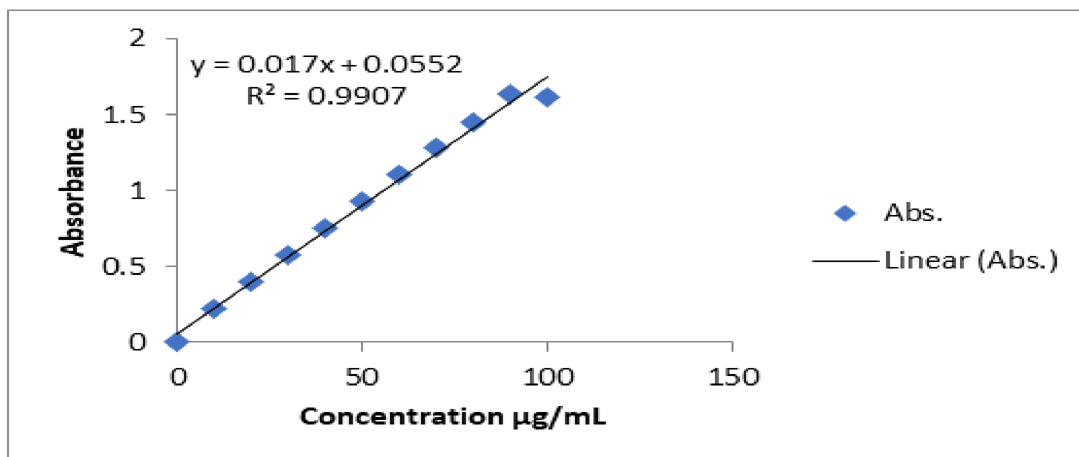

Figure 4: Calibration curve of Quercetin.

Table 2: Total Phenolic Content and Total Flavonoid Content with Standard Deviation of Crude Extract of *H. cirrhata*

Name of Lichen	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)
H. cirrhata	42.165 ± 0.98	11.789 ± 0.34

3.1.4 Anti-oxidant Inhibition Activities

The radical scavenging activity of methanolic extract of lichen and their fractions are detailed in Table 3. All extracts demonstrated concentration-dependent free radical scavenging activity in the DPPH assay. Among the different extracts, the ethyl acetate extracts of *H. cirrhata* exhibited the highest radical scavenging activity, with IC_{50} values of 34.14 ± 0.17 µg/mL. Conversely, the lowest activity was observed in the methanol extract of *H. cirrhata* with IC_{50} values of 74.3 ± 1.13 µg/mL.

3.1.5 Anti-bacterial Activities Along with MIC and MBC

The following zone of inhibition of lichens extract were evaluated against their antibacterial properties against the given bacteria. *Salmonella typhii* (ATCC 14028), *Shigella sonnei* (ATCC 25931), *Acinetobacter baumannii* (ATCC19606), *Escherichia coli* (ATCC 3292), *Staphylococcus aureus* (ATCC 25923), *Klebsiellapneumoniae* (ATCC 700603), and *Acinetobacter baumannii* (ATCC 19606) and zone of inhibition was observed which are presented in Table 4 and Figure 4. In addition, this lichen exhibited inhibition zones of 14 mm and 16 mm at identical concentrations against *Shigella sonnei* and *Staphylococcus aureus.*

Table 3: Antioxidant Activity of Different Fractions of *H. cirrhata* in IC_{50} Values

Lichen (Fractions)	**IC_{50} Values (µg/mL)**
Crude	74.3 ± 1.13
Hexane	44.35 ± 1.23
DCM	37.56 ± 0.49
Ethyl acetate	34.14 ± 0.17
Quercetin	62.87 ± 1.02

Note: Mean values ± SD (Standard Deviation) were measured.

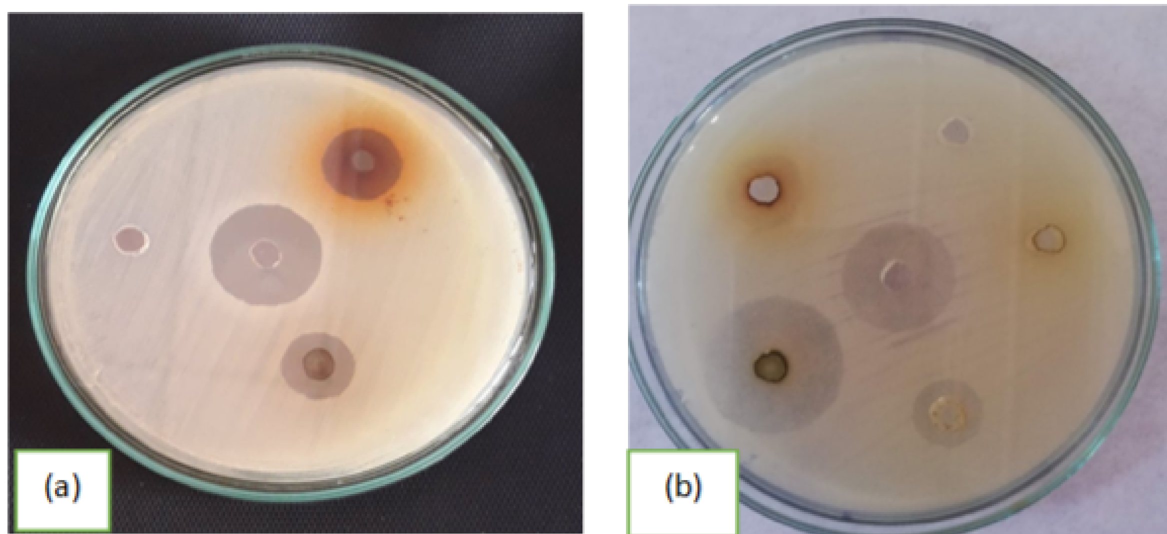

Figure 5: Showing inhibition zone of *H. cirrhata* in *Staphylococcus aureus b*) showing inhibition zone of *H. cirrhata* in *Shigella sonnei.*

Each methanolic lichen extract was evaluated against bacteria, showing the largest inhibition zones, along with corresponding MIC and MBC values (see Figure 5 and Table 5). The MIC for *Shigella sonnei* was determined to be 195.312 g/mL, which was also the observed MBC. For *Staphylococcus aureus*, the MIC was 390.625 g/mL, matching its MBC. Additional details of the MIC and MBC values are presented in Table 5, along with Figures 5 and 6.Each methanolic lichen extract was evaluated against bacteria, showing the largest inhibition zones, along with corresponding MIC and MBC values (see Figure 5 and Table 5). The MIC for Shigella sonnei was determined to be 195.312 g/mL, which was also the observed MBC. For Staphylococcus aureus, the MIC was 390.625 g/mL, matching its MBC. Additional details of the MIC and MBC values are presented in Table 5, along with Figures 5 and 6.

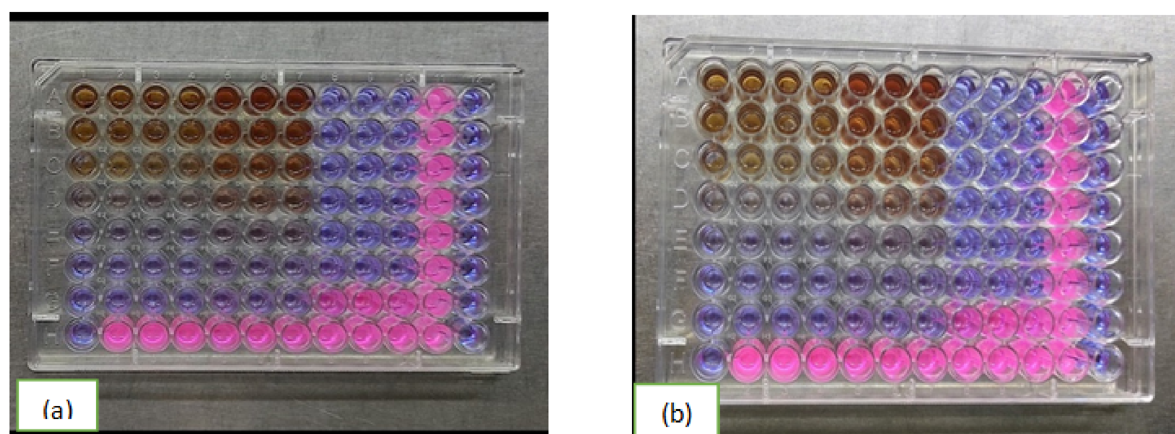

Figure 6: MIC of lichen extracts and antibiotics against *Shigella sonnei*: 5–7: (A–H: 12.5–0.098 mg/mL) for *H. cirrahata*, 8–10: Antibiotic (Neomycin A–H: 250–1.95 g/mL), 11: Positive control (AH: Media + bacteria), 12: Negative control (A–H: Media only), and b)MIC of lichen extracts and antibiotics against Staphylococcus aureus 5–7: (A–H: 12.5–0.098 mg/mL) for *H. cirrahata*, 8–10: Antibiotic (Neomycin A–H: 250–1.95 g/mL), 11: Positive control (AH: Media + bacteria), 12: Negative control (A–H: Media only).

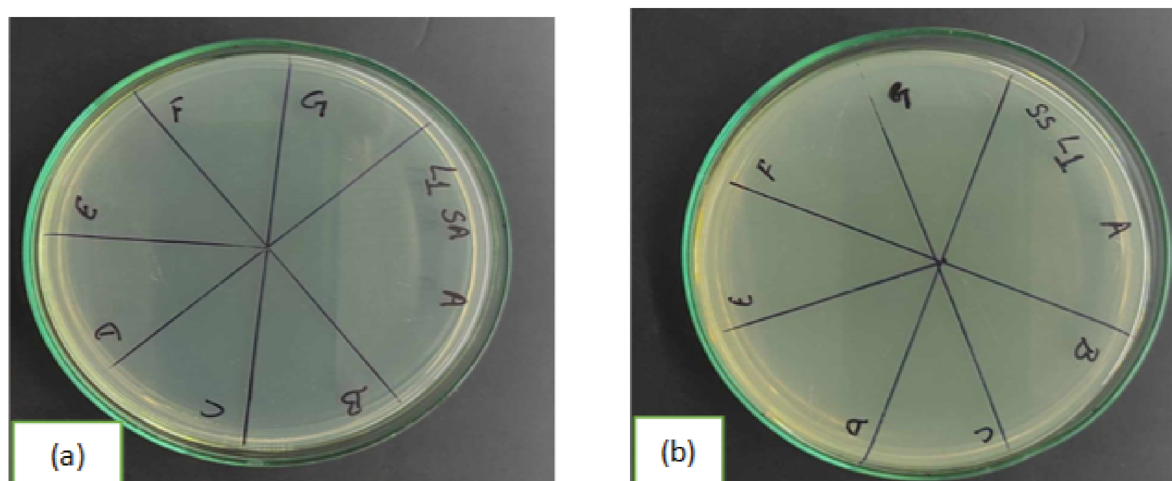

Figure 7: MBC of lichen extracts and antibiotics against (a) *Staphylococcus aureus* and (b)*Shigella sonnei*

Table 4: Zone of Inhibition of *H. cirrhata* Extract Against Bacterial Strains

Serial No.	Name of Bacteria	Positive Control (mm)	*H. cirrhata* Extract (mm)
1	*Escherichia coli*	18	–
2	*Shigella sonnei*	25	14
3	*Salmonella typhi*	20	–
4	*Acinetobacter baumannii*	24	–
5	*Klebsiella pneumoniae*	18	–
6	*Staphylococcus aureus*	22	16

Note: (–) indicates no zone of inhibition observed.

Table 5: MIC and MBC Values of *H. cirrhata*

Name of Bacteria	MIC (µg/mL)	MBC (µg/mL)
Shigella sonnei	195.3125	195.3125
Staphylococcus aureus	390.625	390.625

4 Discussions

Lichens have been traditionally employed in medicine worldwide, with much of this knowledge originating from cultural practices. The primary secondary metabolites in this species are phenolic compounds, notably depsides and depsidones [19] , [20–22]. In this study, the total phenolic content (TPC) of the methanolic extract of *H. cirrhata* was found to be 42.165 ± 0.98 mg GAE/g, surpassing the TPC values of acetone, water, and petroleum ether extracts of *R. taitensis*, which were 22.87 ± 0.12, 6.3 ± 1.06, and 12.58 ± 0.082 mg GAE/g, respectively [23]. This indicates higher phenolic content in *H. cirrhata*. Similarly, the total flavonoid content (TFC) in the crude extract was 11.789 ± 0.34 mg QE/g. In the methanolic extract of *Ramalina lacera*, the TFC was 3.97 ± 0.3 mg RE/g of dry weight (DW), while in the aqueous extract, it measured 0.01 ± 0.03 mg RE/g DW [24].The ethyl acetate extract of *H. cirrhata* demonstrated the highest radical scavenging activity, with an IC_{50} value of 34.14 ± 0.17 µg/mL, likely due to its abundance of phenolic compounds with hydroxyl groups that act through various antioxidant mechanisms, such as free radical scavenging [25]. *R. hossei* and *R. conduplicans* showed radical scavenging activity at 79.05% and 72.63%, respectively [26], whereas *C. furcata* exhibited lower antioxidant activity, with percentages of 44.83% for the acetone extract, 47.39% for the methanol extract, and 29.99% for the aqueous extract. In comparison, the methanol extract of *P. sulcata* showed 71% activity [27, 28].

The methanolic extract of *H. cirrhata* displayed inhibition zones of 14 mm and 16 mm against *Shigella sonnei* and *Staphylococcus aureus*, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 195.312 g/mL and 390.625 g/mL, respectively. Activity against Gram-negative bacteria (e.g., *Escherichia coli*, *Salmonella typhi*) was absent. The absence of activity against Gram-negative bacteria may be due to their unique outer membrane made of lipopolysaccharides, which acts as a barrier to many antimicrobial agents. Efflux pumps and selective porin channels further limit compound entry. Thus, the tested compound may either lack the ability to cross this barrier or be specific to Gram-positive bacteria.In contrast, the ethanolic and hexane extracts of *P. tinctorum* exhibited smaller inhibition zones of 9.4 mm and 7.7 ± 0.2 mm, respectively, at a concentration of 300 mg/mL against methicillin-susceptible *S. aureus* (MSSA, ATCC 25923) [29]. Additionally, studies show that methanol, ethanol, and acetone extracts from various lichens exhibit antibacterial activity against Gram-positive bacteria, such as *B. subtilis* and *S. aureus*, while showing limited inhibition against Gram-negative bacteria [29].

5 Conclusion

The study highlighted the biological potential of *H. cirrhata* in terms of antioxidant and antibacterial properties. Lichens have garnered significant interest recently due to their unique secondary metabolites. The biological activities of lichens are attributed to the secondary compounds produced by their mycobiont. Consequently, this study focused on isolating bioactive compounds that could enhance the commercial value of these lichens in the pharmaceutical industry.

Disclosure Statement

The authors do not have any conflict of interest to declare.

Data Availability Statement

Individual data sets that support the findings of this study will be available from corresponding authors upon reasonable request.

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