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Original Research Article

Antifungal and antibacterial activities of *Usnea* lichen extracts and associated Endolichenic fungi from the Western Ghats, India

Georgy Antony[1]*, Anusha Bhaskar[1], Sudheesh M.[2], Manikkumar R.[3]

[1]Department of Microbiology, Sarvepalli Radhakrishnan University, Bhopal, Madhya Pradesh, India
[2]Department of Biochemistry, Al Azhar Medical College, Thodupuzha, Kerala, India
[3]Department of Biochemistry, Takshashila Medical College, Ongur, Tamil Nadu, India

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***Correspondence:**
Dr. Georgy Antony,
Email: georgyantony@yahoo.com

ABSTRACT

Background: Lichens of the Usnea genus and their associated Endolichenic fungi from biodiversity hotspots like the Western Ghats, India, represent underexplored sources of secondary metabolites with antimicrobial potential. This study evaluates the antifungal activity of Usnea extracts and antibacterial efficacy of Endolichenic fungi to address the surge in antimicrobial resistance, demanding novel bioactive agents.
Methods: *Usnea* species (*U. subsordiata, U. perplexans, U. spinocula, U. undulata, U. maculata*) were collected from Kodayar, Tamil Nadu and extracted sequentially with methanol, acetone and diethyl ether. Antifungal minimum inhibitory concentrations were determined by performing broth macrodilution against *Aspergillus niger, A. flavus, Fusarium oxysporum, and F. solani*. ELF were isolated from *U. perplexans* thalli, cultured and their ethyl acetate extracts tested for antibacterial activity using agar well diffusion against *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae* and *Escherichia coli*.
Results: *U. subsordiata* extracts exhibited the strongest antifungal activity (MICs 93.72–109.34 µg/ml), while *U. maculata* showed the weakest (234.375–531.25 µg/ml). ELF isolates (*Acremonium lichenicola, Aspergillus* spp., *Fusarium* spp., *Penicillium* spp.) demonstrated broad-spectrum antibacterial effects, with *Aspergillus* spp. yielding the largest zones of inhibition, particularly against Gram-positive bacteria. Significant variations were confirmed by two-way ANOVA ($p<0.001$).
Conclusions: *Usnea* lichens and ELF harbor potent antimicrobial compounds, offering viable alternatives against antimicrobial resistant pathogens and emphasising the need for conservation and further metabolite characterization.

Keywords: Usnea lichens, Endolichenic fungi, Antimicrobial resistance, Antifungal activity, Antibacterial activity, Western Ghats

INTRODUCTION

The rising antimicrobial resistance poses a significant threat to public health, rendering many conventional treatments ineffective against bacterial and fungal infections. Natural products derived from secondary metabolic pathways in diverse organisms offer a promising avenue for addressing AMR, due to their structural diversity and bioactivity.[1] Lichens, the symbiotic associations between fungi and algae or cyanobacteria, represent a promising reservoir of bioactive secondary metabolites with pharmacological potential.[1-3] Lichens thrive in extreme environments, producing compounds such as depsides, depsidones, dibenzofurans, terpenes, and flavonoids to combat biotic and abiotic stresses.[4] *Usnea* genus comprises over 360 fruticose species worldwide, and is particularly noted for its ethnopharmacological applications.[3,5] *Usnea* spp. have been traditionally used across diverse cultures in medicine, cosmetics, and food preservation. They are involved in the production of

bioactive metabolites like usnic acid, exhibiting antimicrobial, antiviral, antiproliferative and anti-inflammatory properties.[6-13] *Usnea* spp. from the Himalayan regions of India have been documented in folklore medicine and subjected to bioactivity screening, revealing potent antimicrobial properties attributed to secondary metabolites such as usnic acid.[10,12,13] *U. articulata*, *U. longissima* and *U. blepharea* have demonstrated antibacterial as well as antifungal activities, along with anticancer and antimalarial properties, attributed to benzofurans, depsides and lactones.[14-21]

Endolichenic fungi (ELF), the non-mycobiont fungi residing asymptomatically within lichen thalli represent an underexplored reservoir of bioactive molecules in addition to lichen-derived compounds. ELF are analogous to plant endophytes and produce novel secondary metabolites with antimicrobial potential.[22,23] Commonly isolated ELF from *Usnea perplexans* such as *Aspergillus* spp. and *Penicillium* spp. are known as prolific "chemical factories" yielding alkaloids, quinones and terpenes, which differ from lichen metabolites.[24,25] The Western Ghats, a biodiversity hotspot in India, harbor diverse *Usnea* lichens and yet the antimicrobial properties of their extracts and ELF remain underexplored.

The present study evaluates the antifungal activity of methanol, acetone and diethyl ether extracts from *Usnea* spp. collected from Kodayar, Tamil Nadu, against pathogenic fungi and the antibacterial efficacy of ELF isolated from *U. perplexans* against clinically relevant bacteria. Our research aims to identify novel antimicrobial agents from lichens and their ELF, addressing the AMR crisis and highlighting the significance of conserving these ecologically vital organisms in the Western Ghats.

METHODS

The current study was conducted at the Department of Microbiology, Sarvepalli Radhakrishnan University, Bhopal, India, from March 2023 to January 2024. Lichen specimens were collected from Kodayar, part of Western Ghats in the Kanyakumari District, Tamil Nadu, India, during summer season and identified at the Plant Biodiversity and Conservation Biology Division, National Botanical Research Institute (NBRI-CSIR), Lucknow. The lichen specimens were identified as *Usnea undulata* Stirton (10025, 10026, 10029), *Usnea austroindica* G. Awasthi (10024), *Usnea maculata* Stirton (10030) and *Usnea cf. nilgirica* G. Awasthi (10027) and sample vouchers were deposited at NBRI, Lucknow.

Preparation of lichen extracts

The collected lichen specimens were meticulously cleaned to remove debris, air dried at room temperature and subsequently ground into a coarse powder. Sequential extraction was performed on 100 g portions of each powdered sample, using solvents of increasing polarity such as, diethyl ether (non-polar), acetone (intermediate polarity), and methanol (polar). Extractions were performed using a Soxhlet apparatus by the hot percolation method over 72 hours, maintaining temperatures below the boiling point of each solvent. The extracts were concentrated under reduced pressure using a rotary evaporator and were stored in glass vials inside a desiccator containing fused calcium chloride. For subsequent bioactivity assays, the extracts were reconstituted in their respective extraction solvents.

Antifungal susceptibility testing

Fungal strains and inoculum preparation

Fungal strains used in the study included *Aspergillus niger* MTCC 1344, *Aspergillus flavus* MTCC 277, *Fusarium oxysporum* MTCC 284, *Fusarium solani* MTCC 350. Strains were maintained on Sabouraud dextrose agar (SDA) slants at 4°C. Inocula were prepared by suspending conidia in sterile saline with 0.05% Tween 80, adjusted to 5 × 104 CFU/ml and confirmed by plating serial dilutions on SDA.

Broth macro-dilution assay and mic determination

In-vitro antifungal susceptibility testing for determination of MIC was performed on all fungal isolates by using the Broth Macrodilution method according to CLSI M38- Ed3 guidelines.[26] The extracts were serially diluted in dimethyl sulfoxide (DMSO) and added to Sabouraud dextrose broth, with the final volume adjusted to 2 ml. Each tube was inoculated with 20 µl of fungal suspension. Positive controls containing standard antifungal agent, Fluconazole and negative controls containing only DMSO were included. All tubes were incubated at 30°C and examined every two days for up to 14 days. MIC values were visually determined, compared with positive and negative controls and reported as mean MIC value from three replicates.

Antibacterial activity of endolichenic fungi

Isolation of Endolichenic fungi

Endolichenic fungi were isolated from lichen thalli of *Usnea perplexans* Stirton. The lichen thalli were surface sterilised by immersing in 30% hydrogen peroxide for 30 seconds, followed by 4% sodium hypochlorite for 30 seconds and finally in 75% ethanol for 30 seconds. The sterilised samples were aseptically cut into small fragments and placed onto PDA supplemented with 150 mg/L streptomycin to inhibit bacterial contamination. Plates were incubated at 25±2°C for 14 days. Emerging fungal colonies were subcultured onto fresh PDA plates to obtain pure isolates. The isolates were identified based on their morphological characteristics, microscopic appearances and nature of the spores. Pure cultures of endolichenic fungal isolate were grown in 250 ml Erlenmeyer flasks containing 50 ml of potato dextrose broth at 25±2°C with shaking at 170 rpm for 14 days.

Culture filtrates were extracted with ethyl acetate for subsequent antibacterial assays.

Agar well diffusion assay

Antibacterial activity of endolichenic fungi was determined against *Staphylococcus aureus* (MTCC 737), *Bacillus subtilis* (MTCC 441), *Klebsiella pneumoniae* (MTCC 3384) and *Escherichia coli* (MTCC 443), using the agar well diffusion method. Mueller-Hinton agar plates were prepared and inoculated with 100 µl of 0.5 McFarland standardised bacterial suspension, spread evenly to create a uniform lawn. Wells with 6 mm diameter were created in the agar using a sterile well-cutter. Each well was filled with 10 µl of the respective fungal suspension and incubated at 37°C for 24 hours. The zones of inhibition around each well were measured post incubation in mm. The assay was performed in triplicate and ZOI values were reported as mean±standard deviation.

Statistical analysis

The results of antifungal assays were expressed as the mean±standard error. Statistical significance among the three extract types and across four fungal pathogens was evaluated using two-way analysis of variance.

RESULTS

Antifungal activity

MIC of lichen extracts

The antifungal activity of acetone, methanol, and diethyl ether extracts from five lichen species including *U. subsordiata*, *U. perplexans*, *U. spinocula*, *U. undulata* and *U. maculata* was evaluated using broth macrodilution assay and the results are presented in Table 1 and visualised in Figure 1. *U. subsordiata* exhibited the highest antifungal activity across all extracts, with MIC values ranging from 93.72 to 109.34 µg/ml. The diethyl ether and methanol extracts showed MIC of 93.72 µg/mL against *A. flavus*, *A. niger*, and *F. oxysporum*, while *F. solani* required slightly higher concentrations (101.53 µg/ml- diethyl ether, 109.34 µg/ml- methanol and acetone). *U. perplexans* displayed moderate activity of 124.96 to 265.625 µg/ml MIC values. Diethyl ether extract was most effective against *A. niger* (124.96 µg/ml), while the acetone extract showed the highest MIC (265.625 µg/ml) against *A. flavus*. *U. spinocula* exhibited MICs between 124.96 and 268.75 µg/ml. The diethyl ether extract demonstrated strong activity against *A. niger* (124.96 µg/ml) and the acetone extract requires higher concentrations across all fungi (148.35–268.75 µg/ml). *U. undulata* and *U. maculata* showed the lowest antifungal activity, with MICs ranging from 187.5 to 468.75 µg/ml and 234.375 to 531.25 µg/ml, respectively. For *U. undulata*, the diethyl ether and methanol extracts were equally effective against *A. flavus* and *A. niger* (187.5 µg/ml, 375 µg/ml), while *F. oxysporum* and *F. solani* required higher concentrations. *U. maculata* demosntrated the highest MICs, particularly against *F. oxysporum* (531.25 µg/mL) and *F. solani* (500 µg/ml). All the MIC values obtained for the lichen extracts are found to be higher than the standard fluconazole MIC for the test fungi ranging from 12–16 µg/ml. Heat maps indicating the MIC variations across solvents and test fungi are presented in the Figure 2. Two-way ANOVA revealed significant differences among extract types and fungal species ($F(6, 48)=15.32$, $p<0.001$).

Table 1: MICs of methanol, acetone and diethyl ether extracts of *Usnea* spp. against *A.flavus*, *A.niger*, *F.oxysporum* and *F.solani*.

Lichens	Extract	MIC in microgram/mL			
		A.flavus	***A.niger***	***F.oxysporum***	***F.solanum***
Usnea subsordiata Stirton	D.E	93.72	93.72	93.72	101.53
	M	93.72	93.72	93.72	109.34
	A	101.53	101.53	101.53	109.34
Usnea perplexans Stirton	D.E	187.5	124.96	203.12	187.5
	M	250	140.58	218.75	203.125
	A	265.625	148.35	234.75	218.25
Usnea spinocula Stirton	D.E	234.375	124.96	187.5	187.5
	M	187.5	132.7	218.75	203.125
	A	268.75	148.35	250	234.375
Usnea undulata Stirton	D.E	375	187.5	437	406.25
	M	375	187.5	437	406.25
	A	406.25	203.125	468.75	437.5
Usnea maculata Stirton	D.E	406.25	234.375	500	468.75
	M	406.25	375	531.25	500
	A	468.75	375	531.25	500
Fluconazole		16	16	12	14

Table 2: Antibacterial activity ELF from *Usnea* lichen against *S. aureus, B. subtilis, K. pneumonia, E. Coli.*

Test pathogens	Zone diameter (mm)			
	Acremonium lichenicola	*Aspergillus* spp.	*Fusarium* spp.	*Penicillium* spp.
Staphylococcus aureus	13±0.89	18±0	12±0.56	12±1.12
Bacillus subtilis	14±0.9	19±0.84	17±0.2	15±1.15
Klebsiella pneumonia	10±0.82	14± 0.2	12±0.7	10±0.4
Escherichia coli	9±0.64	10±0.33	11±0.78	10±0.92

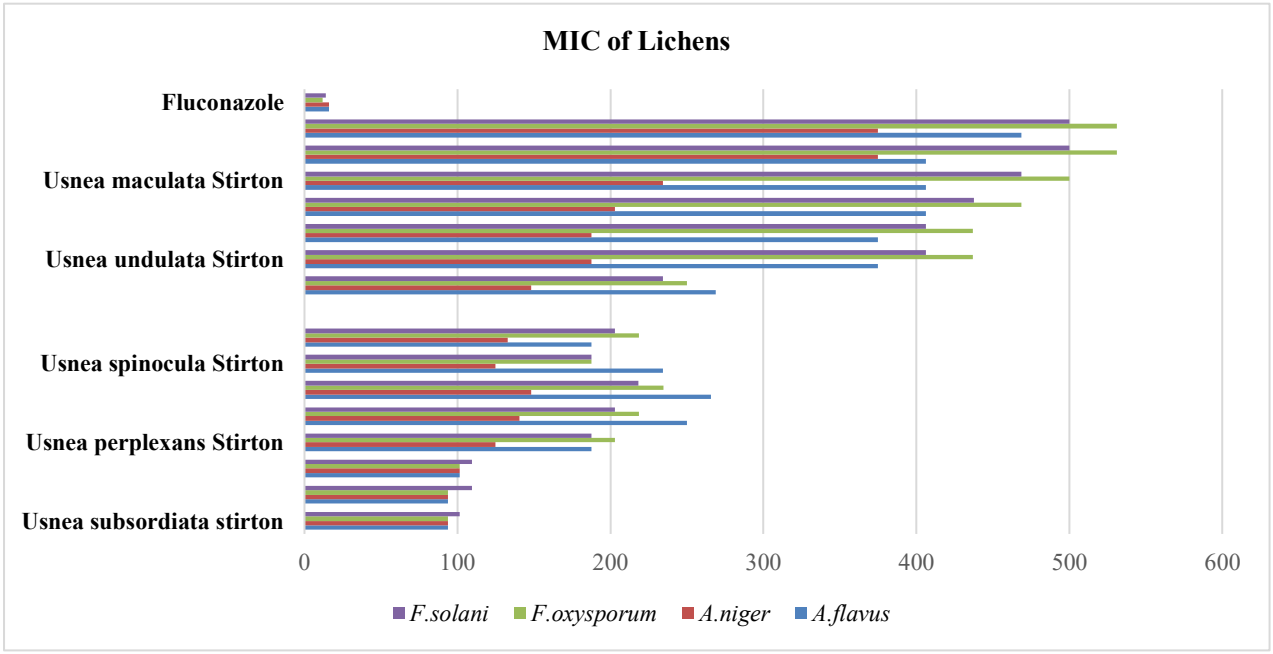

Figure 1: Antifungal activity of methanol, acetone and diethyl ether extracts of Usnea spp.

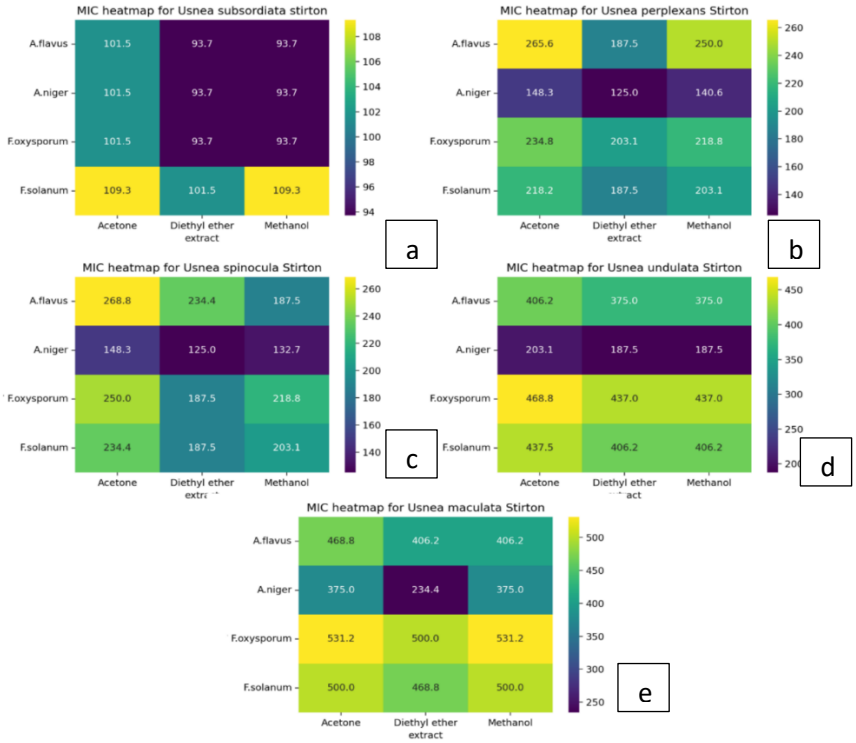

Figure 2 (a-e): MIC heatmaps of methanol, acetone and diethyl ether Usnea extracts against pathogenic fungi.

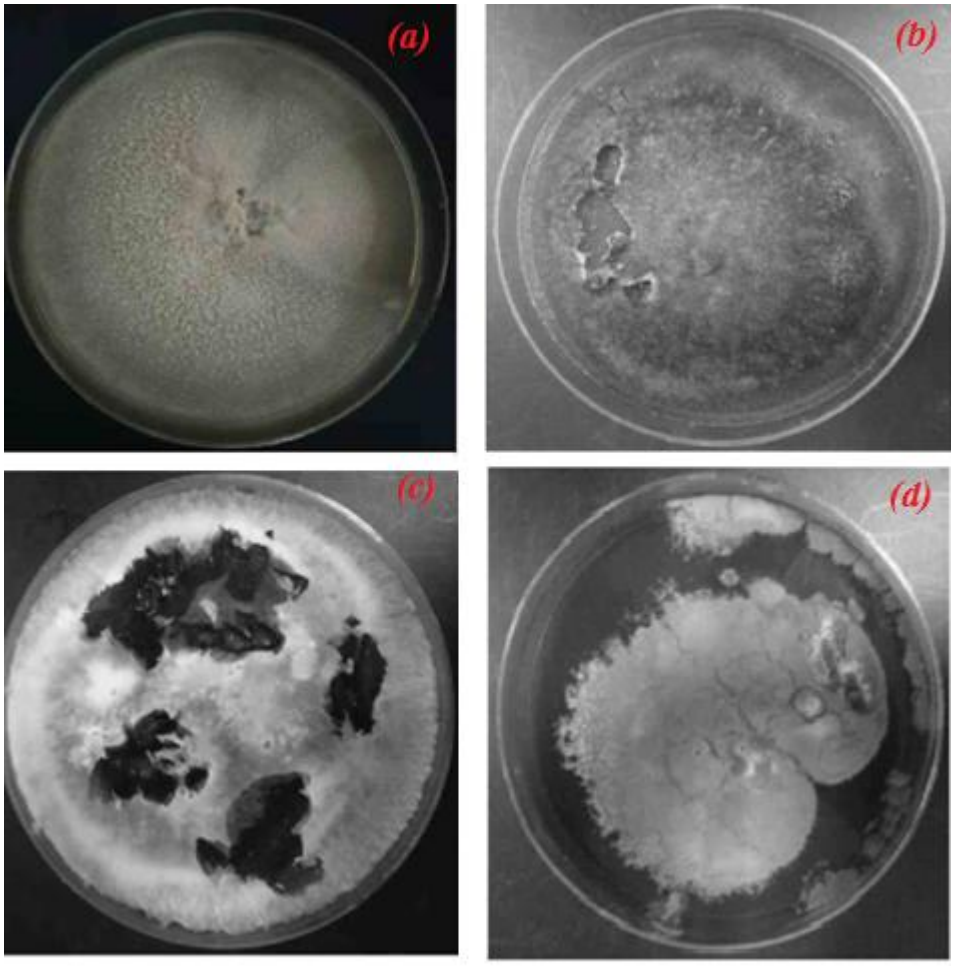

Figure 3: Fungal strains isolated from *Usnea perplexans Stirton*, *(a) A. lichenicola*, *(b) Aspergillus* spp., *(c) Fusarium* spp., *(d) Penicillium* spp.

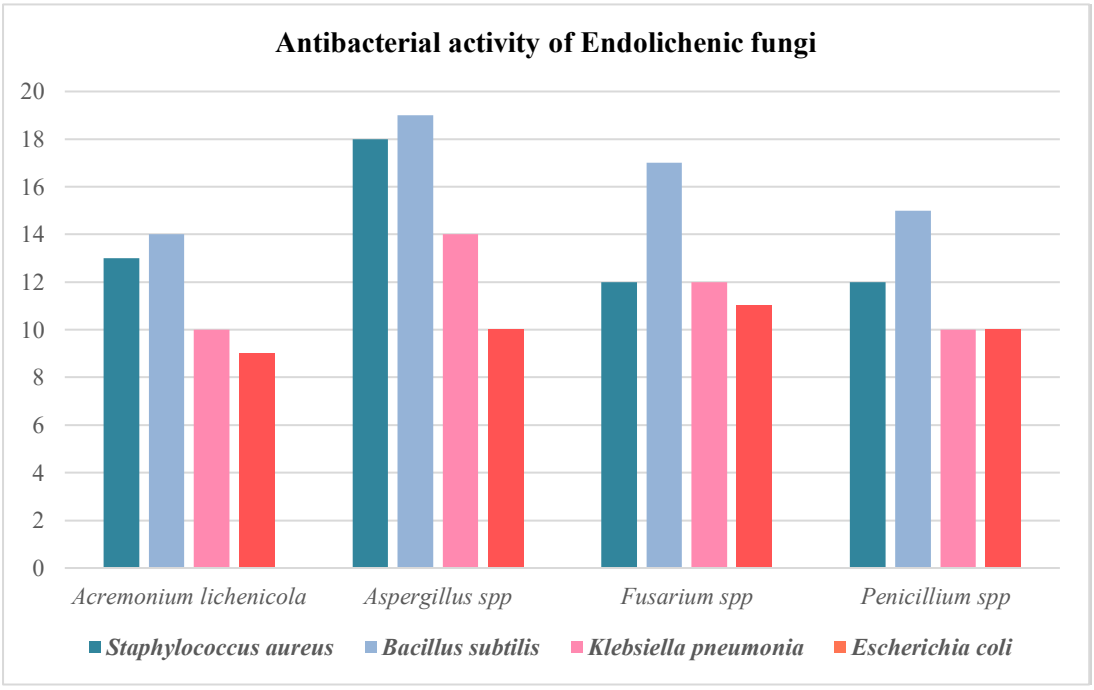

Figure 4: Antibacterial activity of *A. lichenicola*, *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp.

Antibacterial activity of Endolichenic fungi

A total of 9 endolichenic fungi were obtained from surface sterilised thalli of *Usnea perplexans* Stirton, including *Acremonium lichenicola, Alternaria alternata, Aspergillus* spp., *Fusarium* spp., *Nigrospora oryzae, Mucor racemosus, Papulospora* spp., *Penicillium* spp., *Xylaria hypoxylon* (Figure 3). The antimicrobial activity of four selected isolates, including *A. lichenicola, Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. was evaluated against *S. aureus*, *B. subtilis*, *K. pneumoniae*, and *E. coli* by performing the agar well diffusion assay (Figure. 4). *Aspergillus* spp. produced largest ZOI across all bacterial strains, with values of 18±0 mm (*S. aureus*), 19±0.84 mm (*B. subtilis*), 14±0.2 mm (*K. pneumoniae*), and 10±0.33 mm (*E. coli*). *Fusarium* spp. showed moderate activity, with ZOI values ranging from 11±0.78 mm to 17±0.2 mm. *Penicillium* spp. displayed comparable activity, with ZOI values from 10±0.4 mm to 15±1.15 mm. *A. lichenicola* was the least effective, with ZOI values ranging from 9±0.64 mm to 14±0.9 mm. The endolichenic fungi exhibited the strongest activity against *B. subtilis*, with ZOI values ranging from 14±0.9 mm (*A. lichenicola*) to 19±0.84 mm (*Aspergillus* spp.). *S. aureus* was also highly susceptible, with ZOI values ranging from 12±0.56 mm (*Fusarium* spp.) to 18±0 mm (*Aspergillus* spp.). Gram-negative bacteria showed reduced susceptibility. ZOI values ranged from 10±0.4 mm (*Penicillium* spp.) to 14±0.2 mm (*Aspergillus* spp.) for *K. pneumoniae*, while *E. coli* exhibited the smallest zones, ranging from 9±0.64 mm (*A. lichenicola*) to 11±0.78 mm (*Fusarium* spp.). Two-way ANOVA confirmed significant differences among fungal isolates and bacterial strains ($F(9, 32)=12.45$, $p<0.001$).

DISCUSSION

The present study demonstrates the bioactive potential of lichens and associated endolichenic fungi, particularly antifungal and antibacterial activities. Lichens harbour a rich array of secondary metabolites that confer ecological resilience and therapeutic potential. Antifungal activity is attributable to bioactive secondary metabolites including phenolics, terpenoids and alkaloids.[26] Minimum inhibitory concentration assays for *Usnea* spp. extracts revealed species and solvent specific variations, with *U. subsordiata* exhibiting the lowest MICs across all tested fungi. *U. maculata* showed the highest MIC, against *F. oxysporum*. These results indicate that *A. niger* is particularly susceptible to lichen extracts, while *F. oxysporum* and *F. solani* exhibit higher resistance, potentially due to differential interactions with lichen metabolites. The observed antifungal activity is found to be less than that of the standard antifungal fluconazole, but still positions the lichen extracts as viable natural antifungals, especially amid rising resistance to synthetic agents. The variation in MICs among lichen species and extracts highlights the lichen specific secondary metabolites and solvent properties on antifungal efficacy, warranting further investigation into their chemical composition and mechanisms of action.

Endolichenic fungi from *U. perplexans* represent an underexplored reservoir of bioactive diversity. *A. lichenicola*, *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. demonstrated broad-spectrum antibacterial activity in the agar well diffusion assay. *Aspergillus* spp. produced the largest inhibition zones. Gram positive bacteria were found to be more susceptible than Gram negative organisms, attributable to the differences in their cell walls facilitating metabolite penetration. Our findings align with the known chemical versatility of *Aspergillus* and *Penicillium* species as the prolific producers of alkaloids, quinones, terpenes and other compounds with antimicrobial properties.[27-30] The broad-spectrum efficacy against clinically relevant bacterial pathogens suggests therapeutic potential for secondary and opportunistic infections, reinforcing endolichenic fungi as "chemical factories". Despite the promising outcomes, challenges persist in harnessing endolichenic fungi such as, frequent rediscovery of known metabolites, purification hurdles, traditional screening limitations and unculturable strains.[31-33]

Our findings align with prior research highlighting the antioxidant capabilities of lichen phenolic compounds, which neutralise free radicals via hydroxyl mediated stabilisation, redox activity, electron donation and singlet oxygen quenching.[16,34,35] Flavonoids, a predominant subclass of these phenolics, further enhance this capacity by scavenging oxidising molecules, chelating trace elements, suppressing reactive oxygen species formation and bolstering endogenous antioxidant defenses. The current investigation affirms lichens and endolichenic fungi as potent sources of antimicrobials and antioxidants, with implications for drug discovery and ecological conservation. Future efforts should prioritise metabolite characterisation, mechanism of action studies and in vivo validations to address the global challenges in antimicrobial resistance and oxidative stress related diseases. Variability in MIC may arise from seasonal metabolite fluctuations and future HPLC-MS profiling could identify the active compounds. Necessitating advanced approaches like metabolomics and metagenomics is required in the future research studies related to ELF. Limitations of the present study include the lack of phytochemical profiling and in vivo validation, which are critical for drug development.

CONCLUSION

The current study underscores the significant bioactive potential of Usnea lichens as sources of antifungal compounds. Future studies should focus on detailed chemical characterisation of bioactive compounds, elucidation of their mechanisms of action and in vivo validation to enhance their applicability in drug discovery and ecological conservation.

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