ORIGINAL ARTICLE

Investigating the Efficacy of *Parmotrema nilgherrense* in Inhibiting *Acinetobacter baumannii* and Methicillin-Resistant *Staphylococcus aureus*: Assessing Antimicrobial and Anti-Biofilm Properties

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ABSTRACT

Introduction: Microbial biofilm communities display a high degree of resistance to antimicrobial agents, resulting in biofilm-mediated infections leading to significant economic losses within public healthcare systems. *Acinetobacter baumannii* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) are major biofilm-producing bacteria known for their ability to adhere to biological interfaces. Consequently, contributing to elevated morbidity and mortality rate in hospitals. This research, explored the antibacterial and antibiofilm abilities of the ethanol extract of *Parmotrema nilgherrense* - a type of lichen (EEPn) - against *A. baumannii* and MRSA. **Methods:** Bioactive compounds found in EEPn were identified using Gas Chromatography-Mass Spectrometry (GC-MS); antimicrobial activity was determined through well diffusion, microdilution, and time-kill kinetic assays. Anti motility assays were also performed with *A. baumannii*. The biofilm inhibition and anti adhesion potential of EEPn were evaluated using Modified Congo red agar (MCRA) method, Bacterial Adherence to Hydrocarbons (BATH) Assay and Anti-adhesive assay. Finally, the ability of EEPn to eradicate biofilm on biomaterials was also assessed using EEPn. **Results:** The GC-MS results of EEPn detected various lichenochemicals with potential pharmacological properties. In this study, EEPn from *P. nilgherrense* exhibited significant activity against *A. baumannii* and MRSA (zone of inhibition: 9-15mm and 8-12mm, respectively). EEPn, at a concentration of 600µg/mL, was found to demonstrate potential inhibitory activity on both isolates. **Conclusion:** The outcome from this study emphasises the strong antimicrobial and antibiofilm potency of EEPn against both test strains which provides leads for elucidating active antibiotic agents from *Parmotrema nilgherrense*.
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Keywords: Biofilm, Antimicrobials, *Acinetobacter baumannii*, Methicillin Resistant *Staphylococcus aureus*, *Parmotrema nilgherrense*

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INTRODUCTION

Biofilm is a collective assemblage of microbes wherein the organisms cohere to diverse surfaces, including tissues and bio-implants such as cardiac pacemakers, prosthetic heart valves, cerebrospinal fluid shunts, orthopedic implants, intravascular catheters, urinary catheters, and more (1). Microorganisms produce and release extracellular polymeric substances (EPS) that aid in this adhesion by forming a structural framework (2). Biofilms are known to increase the ability of microorganisms to resist a wide range of antimicrobial agents and enhance their resilience against various host elements. This is primarily due to the limited penetration of antimicrobials into the biofilm matrix (3). According to estimates from the National Institute of Health (NIH), biofilms are the cause of 60% of microbial and 80% of total chronic infections (4).

The Emergence and spread of biofilm-producing *A. baumannii* is a serious medical concern as it is turning resilient to frequently used antibiotics such as quinolones, broad-spectrum β-lactams and

aminoglycosides. Methicillin-Resistant *Staphylococcus aureus* (MRSA) is among the primary pathogen for bacteremia, endocarditis, softness in tissues, skin, pleuropulmonary and implant infections (5). The biofilm-forming abilities and specific phenotype render host immune system defenseless against biofilm-associated bacteria thus making infections associated with them difficult to treat (6). Researchers are currently focused on the advancement of non-toxic anti-biofilm agents to address infections associated with biofilms because of which alternative ways to eradicate biofilms using bioactive phytoconstituents are gaining more popularity. Secondary metabolites present in plants are now being developed as potential therapeutic agents, which have proven efficacy in inhibiting and managing several infections (7). The present study has explored the antagonistic activity of lichens - the symbiotic partnership of a fungal and algal species or a cyanobacterium against biofilm forming bacterial pathogens- *A. baumannii* and MRSA.

According to Molnar and Farkas, more than 1000 different low molecular weight bioactive compounds have been reported from lichens, and many are yet to be explored (8). These lichen metabolites possess various biological properties, such as bactericidal, fungicidal, virucidal, anti-inflammatory, antipyretic, pain releaving, anticancer and cytotoxic effects (9-12). The antimicrobial efficacy of lichenochemicals has been proven against a restricted group of drug-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci (13-15). *Parmotrema nilgherrense*, the lichen used in this study, belongs to the Parmeliaceae family. Previous studies have reported the presence of biologically active components in Parmotrema species. Furthermore, organic solvent-based extracts of the species *P. grayana, P. praesorediosum, P. pseudotinctorum, P. stuppeum*, and *P. tinctorum* have been shown to offer multiple useful biological properties (16).

There exists few studies regarding the antimicrobial efficacy of lichen extracts and their bioactive compounds, but their anti-biofilm activities remain largely unexplored. Mitrovic et al. unveiled the anti-biofilm property of *Platismatia glauca* and *Pseudevernia furfuracea* against S.aureus consortia in a 2011 study (17). Consequently, lichens emerge as a significant source of antibacterial and antibiofilm compounds. Given the demand for new therapeutic agents, the present study assesses the *in-vitro* antibacterial activity and antibiofilm efficacy of the Ethanolic Extract of *P. nilgherrense* lichen (EEPn) against biofilm-forming strains of *A. baumannii* and MRSA.

MATERIALS AND METHODS

Lichen material

Lichen samples used for the present study was collected from Kodaikanal (Tamil Nadu); and have been identified and authenticated successfully by Dr.Vinayaka K.S. (Plant Biology Lab, Department of Botany, Sri Venkataramana Swamy College, Vidyagiri, Bantwal, Dakshina Kannada, Karnataka) as *Parmotrema nilgherrense* (Parmeliaceae).

Preparation of Lichen Extract

The lichen sample (*P. nilgherrense*) was thoroughly washed to eliminate all debris, air-dried, powdered and stored in airtight containers for extraction purposes (18). Exactly 25g of lichen powder was packed into a thimble and placed in a Soxhlet extractor. Subsequently, 250mL of ethanol was taken in a round-bottom flask as a solvent, and its temperature was raised to its boiling point (55-65˚C), where it was extracted for about 6-12hrs. Following this, the ethanol used as a solvent was removed by evaporation at room temperature, leaving a small amount of lichen extract. These extracts were refrigerated in airtight containers until their next use. The ethanol extract (EEPn), dissolved in dimethyl sulfoxide (DMSO) at different concentrations, was used for experimental studies.

Chemical Profiling of EEPn

Gas Chromatography-Mass Spectrometry (GC-MS) conducted at the South India Textile Research Association (SITRA), Coimbatore (19), revealed the presence of several compounds of biological importance in EEPn. The sample was analyzed using Thermo GC – Trace Ultra: 5.0 and Thermo MSDSQII. A sample volume of 1µL was utilized; the temperature of oven was programmed from 70°C to 260°C at a rate of 6°C/min, and helium was used as the carrier gas (flow rate – 1mL/min). Specific biomolecules were detected by examining their mass spectra and retention indices with the standards.

Microorganisms

The bacterial strains used for the study, *A. baumannii* MTCC 1425 and MRSA MTCC 1430, were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) in Chandigarh. A loopful of bacteria was inoculated into Mueller-Hinton (MH) broth (Hi-Media) and incubated for 18 hours at 37°C to obtain active cultures for further experiments.

Antibacterial Efficacy of EEPn

The antibacterial potential of EEPn was assessed using the well diffusion method (20). In brief, sterile cotton swabs were used to spread 100 µL of fresh *A. baumannii* and MRSA culture onto Mueller Hinton agar (MHA) plates. After keeping it undisturbed for a while, 6mm punctures were made within the agar. EEPn dissolved in dimethyl sulfoxide (DMSO) at various concentrations (300, 400, 500, 600, 700 µg/mL) were pipetted onto the wells and incubated for 24hrs at 37°C. Clear zones around each well without bacterial growth revealed the antimicrobial activity of EEPn. The diameter of inhibition zones was measured and noted for further evaluation.

Minimum Inhibitory Concentration

Minimal Inhibitory Concentration (MIC) represants the minimum amount of extract that inhibits the growth of tested microorganisms visibly in *in-vitro* settings. The CLSI-recommended microdilution technique was employed to measure the MIC of EEPn against *A. baumannii* and MRSA. Different concentrations of EEPn were dispensed in a 96-well microtiter plate along with 100µL of MH Broth (Hi-Media) and bacterial suspension. The plates were incubated at 37 °C for 24hrs. After incubation, absorbance was measured at OD 560 nm. Bacterial culture without EEPn and Mueller-Hinton Broth devoid of bacteria served as positive and negative controls, respectively.

Minimum Bactericidal Concentration

Minimum Bactericidal Concentration (MBC) is the minimum concentration of the extract that can destroy 99.9% of microbes in the original inoculum. An aliquot of 100µL from each well in the microtiter plate without any visible growth was re-inoculated onto Mueller Hinton agar (MHA) plates and incubated for 24 hours at 37 °C. The MBC was evaluated by counting the bacterial growth using the plate count technique as mentioned by Eduardo et al. (21).

Time-Kill Kinetics Assay

The time-kill kinetics of EEPn was determined using the method described by Appiah et al. (22). The *A. baumannii* and MRSA cultures were inoculated in MH Broth (Hi-Media) with 600µg/mL of EEPn and incubated at 37 °C for 24 hours. At time intervals of 0, 1, 2, 3, 4, 5, 24, and 48hours, an aliquot of 1mL of the culture medium was taken, and the absorbance was measured at OD580 nm. A control was also included for both organisms without adding EEPn. Finally, the values obtained were plotted against time to get the final results.

Inhibition of slime development

The addition of 600µg/mL of EEPn inhibited slime development of *A. baumannii* and MRSA on Congo red agar (CRA). The CRA plates were prepared using Brain Heart Infusion (BHI) medium with 3.6% sucrose, 1.5% NaCl, 0.8g/L Congo red dye and 1% agar. The plates inoculated with bacteria were infused with EEPn and incubated for 24hrs at 37 °C. CRA plates devoid of EEPn were used as controls (23)

Motility Assay

The effect of EEPn on the motility of *A. baumannii* was explored using EEPn (600µg/mL) infused Tryptone medium (1% tryptone, 0.5% NaCl) solidified with 0.3% agar (24). Bacteria from an overnight culture were introduced onto the center of the semi-solid agar and incubated at 37 °C for 24hrs to observe the effect of EEPn on motility. Plates inoculated with *A. baumannii* devoid of EEPn served as controls for comparison.

Cell Surface Hydrophobicity of EEPn

The Bacterial Adherence to Hydrocarbons (BATH) assay employed to assess changes in surface hydrophobicity of *A. baumannii* and MRSA cells (25). Both strains were grown to the stationary phase in 5mL of nutrient broth, after which the cells were separated by centrifugation at 3000g for 15 min. The cells were washed thrice in phosphate buffer (PBS) before being re-suspended in PBS to achieve an OD500nm of 0.5. Then, 1mL of each bacterial suspension and 100µL of toluene, along with different concentrations of EEPn (400, 500, 600 µg/mL), were taken in glass tubes and agitated intensely. About one hour later, the liquid phase was separated with a micropipette to measure absorbance at 500nm using a UV-Visible spectrophotometer. The following formula was used to calculate the bacteria's affinity for the solvent:

$$\% \text{ CSH} = (1\text{-}A/A_0) * 100$$

Here, A_0 denotes OD500 of the bacterial suspension before mixing and A denotes OD after mixing with the solvent.

Anti-adhesive Effect of EEPn

Overnight cultures of *A. baumannii* and MRSA were utilized to assess the impact of EEPn on bacterial adhesion. Both strains were cultured in 10mL of BHI broth infused with 5% sucrose and different concentrations of EEPn (500, 600µg/mL). After 24hrs, the non-adherent cells along with the growth medium were discarded, and adhered cells were rinsed with 0.5M NaOH, which was then clarified and re-suspended in saline after vortexing. A spectrophotometer set to OD630nm was used to measure the anti-adhesive activity. BHI tubes with bacteria devoid of EEPn were taken as a control.

Biofilm Eradication on Biomaterial

Individual catheter pieces were inoculated with test strains (*A. baumannii* and MRSA) for 24hrs at normal room temperature along with 600µg/mL of EEPn. After incubation, the catheter pieces were washed with Phosphate Buffer Saline (PBS) and stained with crystal violet dye. EEPn-free catheters were used as controls.

RESULTS

Chemical Profile of EEPn

The major bioactive compounds present in EEPn is disclosed in Table I. Most of these bioactive compounds are known to exhibit promising biological activity.

Antibacterial activity of EEPn

EEPn was screened to assess its antimicrobial potency against the test bacteria at varying concentrations (300, 400, 500, 600, 700µg/mL). EEPn inhibited the growth of *A. baumannii* and MRSA with zones without visible growth from 9 to 15mm and 8 to 12 mm, respectively. The largest inhibition area was given by 700µg/mL

Table I. Bioactive Compounds Identified in EEPn

Sl.No.	Compound name	Molecular formula	Molecular weight	Probability	Bioactivity*
1.	1-Tetradecanol (CAS)	C14H30O	214	13.09	Anti-microbial
2.	1-Adamantanamine, N, N-Dimethyl	C12H21N	179	6.23	Anti-viral
3.	2-Ethylquinolin-8-ol	C11H11NO	173	0.76	Anti-fungal
4.	Lucenin 2	C27H30O16	610	4.87	Anti-bacterial
5.	Iron iodide complex I	C26H26FeIN4O4	641	3.05	Anti-bacterial
6.	2,2-dideutero heptadecanal	C17H32D2O	254	2.33	Anti-microbial
7.	Ethyl 2,4-dihydroxy-6-Methylbenzoate	C10H12O4	196	75.36	Anti-fungal
8.	1H-2,8a-Methanocyclopenta[a] cyclopropa[e]cyclo-decen-11- one,1a,2,5,5a,6,9,10,10a-octahydro-5,5a,6-trihydroxy-1,4-bis (hydroxymethyl)-1,7,9-trimethyl-[1S-(1a,1aa,2a,56, 5a6,66,8aa,9a,10aa)]	C20H28O6	364	25.71	Anti- microbial
9.	5aH-3a,12-Methano-1H cyclopropa [5′,6′] cyclodeca [1′,2′:1,5] cyclopenta[1,2-d][1,3]dioxol-13 one,1a,2,3, 9,12,12a-hexahydro-9-hydroxy- 10-(hydroxymethyl)-1,1,3,5,7,7- hexamethyl-,[1aR (1aa,3a,3aa, 5aa,8aR*,96,12a,12aa)]-	C23H32O5	388	51.52	Anti- microbial
10	13-Docosenamide	C22H43NO	337	46.74	Antibacterial and Anti-adhesive

**Activity source: Dr. Duke's Phytochemical and Ethnobotanical Database

concentration for both A. baumannii MRSA as given in (Table II).

Determination of MIC

The MIC values were in line with the results in well diffusion method, with the lowest MIC values obtained using EEPn demonstrating the best antimicrobial activity. The MIC value for EEPn against *A. baumannii* and MRSA was 600µg/mL. The positive control showed bacterial growth, while bacterial growth was absent in the negative control.

Determination of MBC

MBC values demonstrated the robust antimicrobial effects of EEPn on both *A. baumannii* and MRSA. A concentration of 600µg/mL of EEPn effectively inhibited the growth of both microorganisms. Control plates without EEPn showed bacterial growth.

Time -Kill Kinetics

The time-kill profile of *A. baumannii* and MRSA were tested at 600µg/mL of EEPn, and resulted in a gradual decrease in the viable cells count over time (Fig. 1). The antimicrobial action of EEPn was found to be concentration-dependent and bacteriostatic. The area under the curve for EEPn against *A. baumannii* and MRSA at different concentrations showed a significant reduction viable cell count in comparison with the control.

Table II. Inhibition zone of EEPn against *A. baumannii* and MRSA

Bacterial strains	Zone of inhibition (mm) according to different concentrations of EEPn (µg/ml)				
	300	400	500	600	700
A. baumannii	9	9	10	12	15
MRSA	8	8	9	10	12

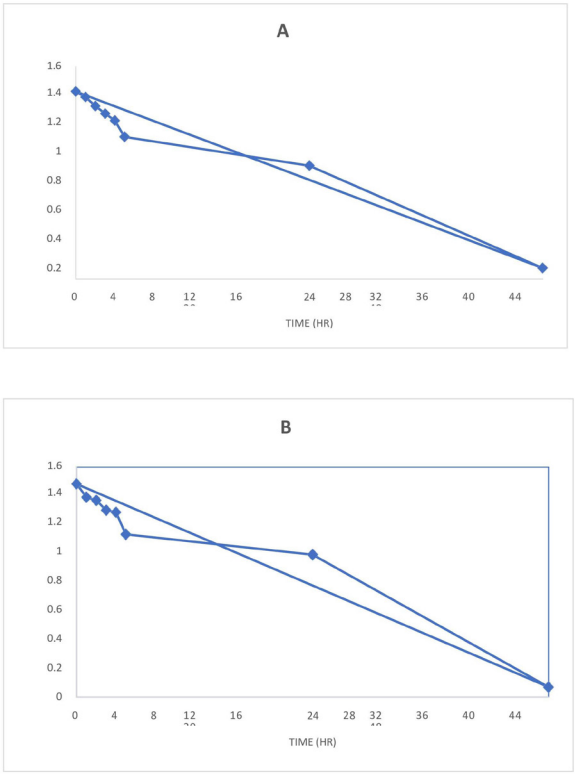

Figure 1. Time-kill kinetics of EEPn at 600µg/mL at time intervals of 0, 1, 2, 3, 4, 5, 24, 48hrs against (A) *A. baumannii* (B) MRSA

Screening of Biofilm Formation and Biofilm Inhibition

Modified Congo Red Agar Method (MCRA)

The results clearly indicate that the method can be effectively employed to asses' biofilm production in both *A. baumannii* and MRSA, as depicted in Fig. 2. Successful inhibition of biofilm production by both test strains was achieved at a concentration of 600µg/mL of EEPn.

Motility Assay

An inhibitory activity against the motility of *A. baumannii* was observed with EEPn at a concentration of 600µg/mL. The area under the motility zone decreased on the addition of EEPn than the control, as illustrated in Fig. 3.

Bacterial Adherence to Hydrocarbons (BATH) Assay

Cell surface hydrophobicity (CSH) has a crucial role in attachment of biofilms to surfaces. The results of the BATH assay demonstrate that the CSH of both *A. baumannii* and MRSA increase as the concentration of EEPn increases, as indicated in Table III. Upon treatment with 600µg/mL of EEPn, the % hydrophobicity of *A. baumannii* and MRSA increased from 34.4% to 69.95% and from 37.73% to 71.11%, respectively. Consequently, EEPn was successfully found to influence the CSH of the tested microorganisms thus reducing the chances of attachment to host/ biomedical surfaces .

Anti-Adhesive activity

The adhesiveness of *A. baumannii* and MRSA were tested at 500 and 600µg/mL of EEPn. As the concentration of EEPn increased, the adherence of the tested microorganisms decreased. The adherence of *A. baumannii* decreased from 0.529 to 0.015%, while that of MRSA decreased from 0.401 to 0.05% (Table III). EEPn exhibited a greater effectiveness against MRSA in comparison to *A. baumannii*. This reduction in adhesive nature can be attributed to the presence of the bioactive compound 13-Docosenamide in EEPn.

Surface Colonization and Biofilm Eradication on Biomaterial

Suction catheter segments were chosen for this study because they are highly susceptible to persistent biofilm colonization. When treated with 600µg/mL of EEPn, the biofilms of *A. baumannii* and MRSA on the surface of suction catheters were visibly eradicated (Fig. 4). This indicates that EEPn effectively reduces biofilms of *A. baumannii* and MRSA on biomaterials.

DISCUSSION

The emergence of multidrug-resistant bacterial strains like *A. baumannii* and MRSA is a growing global concern in both hospital and community settings. The reduction in the effectiveness of available medications and the subsequent rise in multidrug-resistant strains have prompted researchers worldwide to search for new antibacterial agents. Furthermore, these drug-resistant microbes can exhibit variations in their virulence nature, both temporally and geographically (26).

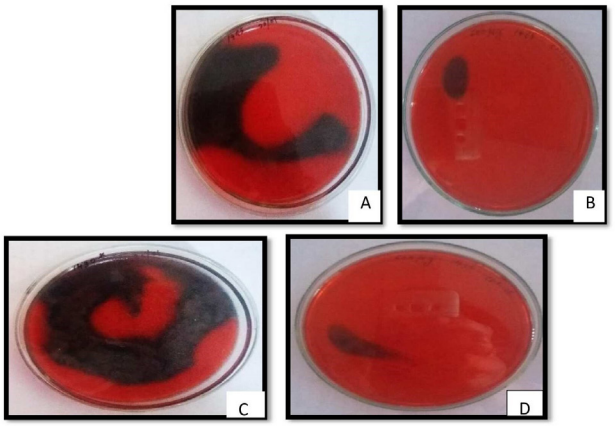

Figure 2. Bacterial colonies of *A. baumannii* and MRSA exhibiting formation and inhibition of biofilm in the absence and presence of EEPn respectively. (A) Contol *A. baumannii* plate showing black colonies indicating bioilm formation. (B) *A. baumannii* treated with 600µg/mL of EEPn showing red colonies indicating biofilm inhibition. (C) Contol MRSA plate showing black colonies indicating bioilm formation. (D) *A. baumannii* treated with 600µg/mL of EEPn showing red colonies indicating biofilm inhibition.

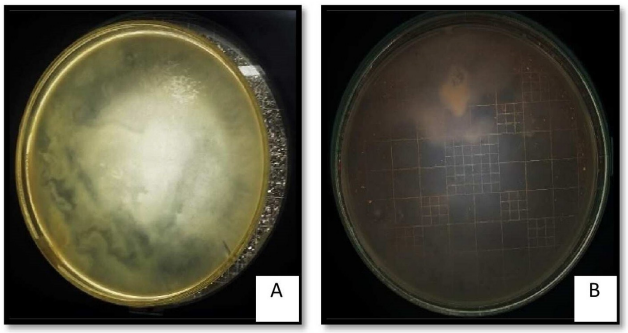

Figure 3. Effect of EEPn on bacterial motility was examined on semi-solid agar plates in the presence of 600µg/mL of EEPn. (A) Diameter of twitching motility zone of untreated *A. baumannii*. (B) Inhibition of motility zone in treated *A. baumannii*

Table III. Effect of EEPn on Cell surface hydrophobicity and adherence of *A .baumannii* and MRSA

Concentration of EEPn (µg/ml)	CSH % of Microorganisms	
	A. baumannii	MRSA
400	64.28	62.64
500	64.68	69.11
600	69.95	71.11
Control	34.42	37.73
	OD at 630 nm	
	A. baumannii	MRSA
500	0.033	0.158
600	0.015	0.05
Control	0.529	0.401

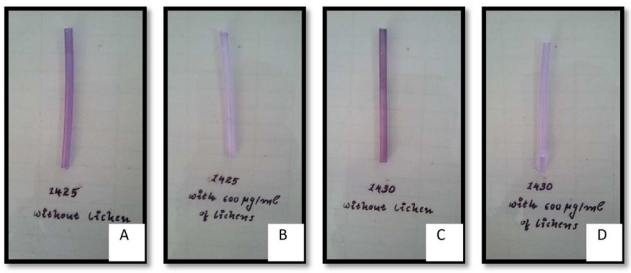

Figure 4. Surface colonization on biomaterial. (A) Untreated *A. baumannii*. (B) Treated *A. baumannii* with 600µg/mL of EEPn. (C) Untreated MRSA. (D) Treated MRSA with 600µg/mL of EEPn.

The pursuit of non-toxic antibiotics from biogenic sources is gaining momentum in the pharmaceutical industry to enhance and acknowledge traditional knowledge. Lichen compounds are no exception to this trend. The increasing popularity of lichen secondary metabolites is driven by their effectiveness and the ineffectiveness of previously considered reliable drugs (27).

While previous studies (28-30) mainly reported the inhibition of gram-positive bacteria by lichens, our study demonstrated that EEPn inhibited both gram-positive and gram-negative strains. The results indicate that EEPn extract effectively restricts the growth of MRSA at all tested concentrations, which is consistent with many other published studies (15, 31-36).

Interestingly, while some studies suggested that lichen extracts had no negative impact on Gram-negative strains like *E. coli* and *P. aeruginosa* (33, 37, 38), our study found that the crude extract EEPn restricted the growth of Multi-Drug Resistant (MDR) *A. baumannii*. Notably, no existing literature provides data on the antibiotic effects of EEPn crude extracts on MRSA and *A. baumannii*. Furthermore, the ethanolic extract was found to be more effective against *A. baumannii*. This difference is attributed to the distinct cell membrane composition, with gram-positive bacteria having peptidoglycan and teichoic acids, while gram-negative bacteria possess lipopolysaccharides and lipoproteins (39, 40).

Gas Chromatography-Mass Spectrometry revealed the bioactive compounds in *P. nilgherrense* responsible for antibacterial, antifungal, antiviral, and anti-adhesive activities. The enhanced antimicrobial activity of *P. nilgherrense* can be attributed to major components such as 1-Tetradecanol, 2,2-dideutero heptadecanal, 1H-2,8a-Methanocyclopenta, 1-Adamantanamine N, N-Dimethyl, 2-Ethylquinolin-8-ol, Ethyl 2,4-dihydroxy-6-Methylbenzoate, Lucenin 2, Iron iodide complex I, and 13-Docosenamide. The antagonistic or synergistic interactions between these components may contribute to the exhibited antimicrobial and antibiofilm activities, leading to the disruption of bacterial cell wall integrity.

This study also unveiled the anti-biofilm activity of the lichen *P. nilgherrense*, which was previously underexplored in scholarly information. Javeria et al. previously described the antimicrobial properties of *P. nilgherrense* against drug-resistant bacterial strains (41). In the current study, EEPn demonstrated antibacterial activity against multidrug-resistant *A. baumannii* (MTCC 1425) and MRSA (MTCC 1430), which can be attributed to the presence of secondary metabolites with antibacterial activity, such as 1,1-Cyclobutanedicarboxamide, Lucenin 2, and Iron iodide complex I. Prior researches also highlighted the antibacterial activity of different extracts of Parmotrema species like *P. reticulatum* against bacteria like *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* etc (42). The MIC value of EEPn against *A. baumannii* and MRSA was observed to be 600µg/mL in this study which also points the strong antimicrobial effects of Parmotrema species against multi drug resistant strains.

Biofilm-producing microorganisms can heavily contaminate implants, including catheters, contributing to hospital-acquired infections. Infections involving biofilm-forming *A. baumannii* and MRSA on infected catheters can complicate diagnosis and treatment. New research strategies, such as small molecules, matrix-targeting enzymes, bactericidal and anti-adhesion coatings, are being developed to manage infections associated with biofilms. In our study, the use of suction catheter pieces, known for their susceptibility to persistent biofilm colonization, demonstrated the visible reduction of biofilms formed by *A. baumannii* and MRSA at a concentration of 600µg/mL of EEPn. These findings suggest that the ethanolic extract of *P. nilgherrense* (EEPn) could be used as a coating agent to prevent cross-contamination and biofilm-mediated infections in medical devices.

CONCLUSION

The antimicrobial and antibiofilm efficacy of ethanolic extracts of *P. nilgherrense* (EEPn) warrant further investigation against significant number of both gram-positive and negative clinical strains to ascertain the independence of these activities from specific strains. This study highlights the potential of EEPn in reducing biofilm formation in MRSA and *A. baumannii*, which could serve as a potent strategy for mitigating infectious reservoirs in hospital and community settings. Additionally, the antibacterial activity of the studied lichen could be further elucidated through cutting-edge research approaches, involving various solvents and extraction techniques. It's important to note that this study exclusively focused on the antibacterial and antibiofilm potential of *P. nilgherrense* ethanolic extract and thus other biological effects of EEPn are also yet to be explored.

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