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Original Research Article

Preliminary evaluation of the anti-*Candida*, antibiofilm and phytochemical potential of *Graphis ceylanica* Zahlbr

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ABSTRACT

Background: *Graphis ceylanica* Zahlbr., a script lichen, was screened for its antioxidant, TLC profile and antibiofilm potentials.
Methods: The methanol fraction (MF) and hexane fraction (HF) from an acetone extract were screened for DPPH scavenging, phosphomolybdate total antioxidant capacity (TAC), total phenolic content (TPC), and total flavonoid content (TFC), along with antibiofilm assays against *Candida albicans* and *C. tropicalis*.
Results: MF displayed higher antioxidant activity than HF, with TAC (A695=1.382±0.02), TPC (=87.81±1.5 mg GAE/g), and TFC (=5.63±0.29 mg QE/g), thus confirming phenolic enrichment of the MFs reported in other *Graphis* species. TLC profiling revealed multiple UV-active bands consistent with depsides, depsidones, and aromatic metabolites characteristic of *Graphidaceae*. Both fractions exhibited promising DPPH scavenging activity, with 46.51% for MF and 40.47% for HF. Most importantly, strong antibiofilm effects against both *Candida* strains were evident, with HF at 3-5 mg/ml and MF at 50-100 mg/ml, inhibiting >75-95% of biofilm formation.
Conclusions: Therefore, the current study highlights *G. ceylanica* as a chemically diverse and bioactive lichen, presenting the first baseline evidence of its antioxidant and antibiofilm properties.

Keywords: TLC profile, Antibiofilm, *Candida* species, *Graphis,* Lichens

INTRODUCTION

Lichens have been recognized for their ethnopharmacological use in treating infections, respiratory and digestive conditions, wounds, and inflammatory disorders.[1] Systems such as Ayurveda, Siddha, and European folk medicine mention the use of several well-known species of lichen. *Graphis ceylanica* Zahlbr. is a relatively understudied lichen from the family *Graphidaceae*, *Ascomycota*, and has not been reported for its medicinal or homeopathic properties. There are no validated uses or preparations for this species, and scientific research is equally scarce. Pharmacological, antimicrobial, antioxidant, or metabolite studies have not been published to date, and its chemical profile remains entirely uncharacterized.

Belonging to the Genus *Graphis*, commonly grouped as 'script' lichen, *G. ceylanica* may contain bioactives that have been reported for this genus.[2] Congeners such as *G. nakanishiana* and *G. schizograpta* contain moderate levels of phenolic bioactives, typically classified as depsides and depsidones, with reported antimicrobial, antioxidant, and enzyme-inhibitory activities. In addition, *Graphis* depsidones, such as norsticitic, stictic, and protocetraric acids, have shown notable antifungal activities.[3] Similar research gaps exist for the antioxidant and radical-scavenging activities of *G. ceylanica,* while other members of the family display significant activity. To the best of our knowledge, this study is the first to report the biological activity of *Graphis ceylanica*, including its anti-*Candida* biofilm and antioxidant potential. It is a compelling candidate for initial screening as one of the

least studied lichens. The present study provides essential baseline data for future pharmacological investigations and offers insights into the underexplored tropical lichen diversity.

METHODS

Extract preparation

The lichen sample was collected from the bark of tea plants in Tapesia, Guwahati, Assam, India. This 'script' lichen was taxonomically identified as *Graphis ceylanica* Zahlbr. by the ICFRE-Bamboo and Rattan Center, Aizawl. The samples were then washed with distilled water and dried in a hot air oven. The dried samples were ground using a mortar and pestle, mixed with acetone at a w/v ratio of 1:15, and macerated for 16 h. After mixing, the acetone extract was filtered through a Buchner funnel, mixed with an equal volume of hexane, and stirred with a magnetic stirrer for an hour. The mixture was allowed to stand until a clear separation between the solutions occurred. The HF was pooled into a sterilized pre-weighed beaker and evaporated; the remaining solution was mixed with an equal volume of 1:2 methanol:water and processed in the same manner to obtain the MF. The yield of the extracts was measured using the following formula: [W_a is the weight of the empty beaker and W_b is the weight of the beaker and dried extracts]. The dried samples were stored at 4°C until further analysis.

Antioxidant activity

Antioxidant activity was measured by mixing the solvent fractions with freshly prepared 0.12 mM DPPH and recording the decrease in absorbance at 570 nm after 30 min of incubation in the dark.[4] Concentrations of fractions tested were HF: 5 mg ml^{-1} and MF: 100 mg ml^{-1}. The percentage of DPPH radical scavenging was calculated as follows:

$$DPPH\ scavenging\ activtity\ (\%) = \frac{\left(Absorbance\ of\ control - Absorbance\ of\ sample\right)}{Absorvance\ of\ control} \times 100$$

TAC was measured by reacting 0.3 mL of each extract with the phosphomolybdate reagent and incubating the mixture at 90 °C for 90 min. Absorbance was recorded at 695 nm against a methanol blank.

Determination of total phenolic and flavonoid contents

The TPC of the extracts was determined using the Folin-Ciocalteu method with minor modifications. For the assay, 200 µl of each solvent fraction was diluted with distilled water to a final volume of 800 µl, followed by the addition of 300 µl of Folin-Ciocalteu reagent. After 10 min at room temperature, 1 ml of 20% sodium carbonate solution was added and incubated in the dark for 60 min before measuring the absorbance at 650 nm. A modified method was used to quantify the TFC. Briefly, 200 µl of the solvent fraction was mixed with 800 µl of methanol, followed by the addition of 200 µl of 10% aluminium chloride and 200 µl of 1 M sodium acetate. The reaction mixture was incubated in the dark for 45 min, and the absorbance was recorded at 415 nm against a methanol blank without aluminium chloride. Gallic acid and quercetin were used as standards for TPC and TFC, respectively, and all measurements were performed in triplicate.[4]

Candida isolates and antibiofilm assay

Two clinical isolates each of *Candida tropicalis* and *C. albicans,* showing variable resistance to fluconazole, were collected from the Microbiology Laboratory, Ayursundra Superspeciality Hospital, Guwahati, Assam, India, in September 2022, and maintained on Sabouraud dextrose agar plates. Both *Candida* isolates formed *in vitro* biofilms in test tube assays. To assess the ability of the fractions, HF and MF, to inhibit biofilm formation, a crystal violet inference assay was conducted in microtiter plates.

The tests were performed as per published literature, and the concentrations of the fractions tested were 5 and 3 mg/ml for HF and 100 and 50 mg/ml for MF.[5] The absorbance values at 570 nm were compared to those of the blank control, in which autoclaved distilled water was used instead of the test fractions. The percentage (%) attachment was calculated as follows:

$$Attachment\ (\%) = \left(\frac{Absorbance\ of\ the\ Blank - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control}\right) \times 100$$

Thin-layer chromatography

HF and MF were dissolved in their respective solvents and spotted onto silica-G TLC plates for chromatographic separation. The solvent systems used were hexane-acetone (9:1 v/v) for HF and methanol for MF.

The developed plates were examined under short- and long-wave UV light and subsequently exposed to iodine vapor. The R_f values of all the visible spots were recorded.

Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean±standard deviation. The antibiofilm activities of the hexane and MFs against each *Candida* isolate were compared using a one-way analysis of variance (one-factor ANOVA).

Statistical calculations were performed using Microsoft excel (Office 2021), and differences were considered statistically significant at $p<0.05$.

RESULTS

Antioxidant activity

Both assays showed that the MF and HF produced detectable antioxidant effects. The DPPH assay results showed that MF could eliminate 46.51±3.75% of free radicals while HF could eliminate 40.47±8.28%of free radicals. The phosphomolybdate assay showed that TAC was greater in MF (A_{695}=1.382±0.02) than in HF (1.015±0.02). The control group results showed significant differences from all the measured values ($p<0.05$) (Table 1).

Determination of total phenolic and flavonoid contents

The MF showed a TPC of 87.81±1.50 mg GAE/g extract. The HF system did not show any detectable phenolic compounds. The TFC measured 5.63±0.29 mg QE/g for MF and 3.57±0.26 mg QE/g for HF. The measured values reached statistical significance with $p<0.05$ (Table 1).

Candida isolates and antibiofilm assay

The two fractions showed strong ability to fight against *Candida albicans* and *C. tropicalis* through their antibiofilm properties with significant reductions in biofilm at all tested concentrations (Figure 1). HF treatment at 3 and 5 mg/ml^{-1} decreased *C. albicans* biofilm biomass to 0.23±0.15 and 0.16±0.15 OD_{570} which resulted in 87.17% and 87.84% biofilm reduction respectively. *C. tropicalis* showed OD_{570} values of 0.15±0.04 and 0.10±0.05 which produced 88.59% and 92.21% biofilm reduction. The treatment of MF at 50 and 100 mg mL^{-1} decreased *C. albicans* biofilm biomass to 0.25±0.17 and 0.22±0.14 OD_{570} which resulted in 85.80% and 87.11% biofilm reduction respectively. For *C. tropicalis*, OD_{570} values showed 0.25±0.10 and 0.16±0.15 which produced 76.04% and 80.64% biofilm reduction. The study found that all reductions reached a level of statistical significance ($p<0.05$).

Thin-layer chromatography

The fractionated acetone extract was analyzed by TLC profiling which showed multiple UV-active bands in the sample. HF displayed four bands which had Rf values measuring 0.16 0.28 0.59 and 0.85. MF showed three bands with the Rf values of the 0.78 0.82 and 0.97. The bands were detected through the UV light which showed multiple secondary metabolites existed in the sample (Figure 2).

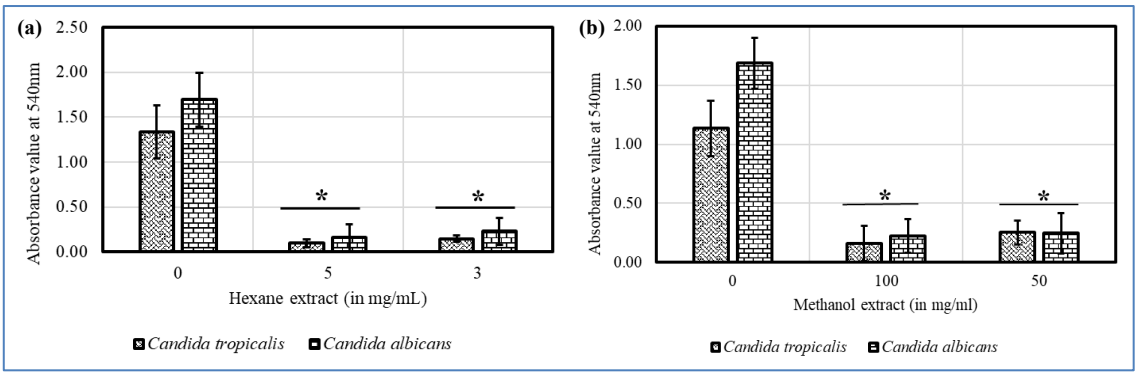

Figure 1 (A and B): Effects of hexane (A) and methanol (B) extracts on *Candida tropicalis* and *Candida albicans* biofilm formation.

*Indicate significant reduction vs. control.

Table 1: Summary of antioxidant, phytochemical, and antibiofilm activities of *Graphis ceylanica* hexane and MFs against *Candida albicans* and *C. tropicalis*.

***Graphis ceylanica* Zahlbr.**								
Experiment	**HF**				**MF**			
Antioxidant activity								
RSA% (DPPH assay)	46.51±3.75%*				40.47±8.28%*			
Total antioxidant activity (OD_{695})	1.015±0.02*				1.382±0.02*			
Phytochemical assay								
TPC mg GAE/g)	n.d.				87.81±1.50*			
TFC (mg QE/g)	3.57±0.26*				5.63±0.29*			
R_f values for TLC	0.16, 0.28, 0.59, 0.85 (Acetone:Hexane, 9:1)				0.78, 0.82, 0.97 (Methanol)			
Antibiofilm assay	**OD_{570}**		**% inhibition**		**OD_{570}**		**% inhibition**	
Concentration (mg ml^{-1})	3	5	3	5	50	100	50	100
Candida albicans	0.23±0.15*	0.16±0.15*	87.17±7.23	87.84±7.52	0.25±0.17*	0.22±0.14*	85.8±8.42	87.11±7.37
Candida tropicalis	0.15±0.04*	0.10±0.05*	88.59±4.45	92.21±4.7	0.25±0.10*	0.16±0.15*	76.04±14.42	80.64±14.52

*Indicates values significantly different from the untreated control (one-way ANOVA, $p<0.05$).

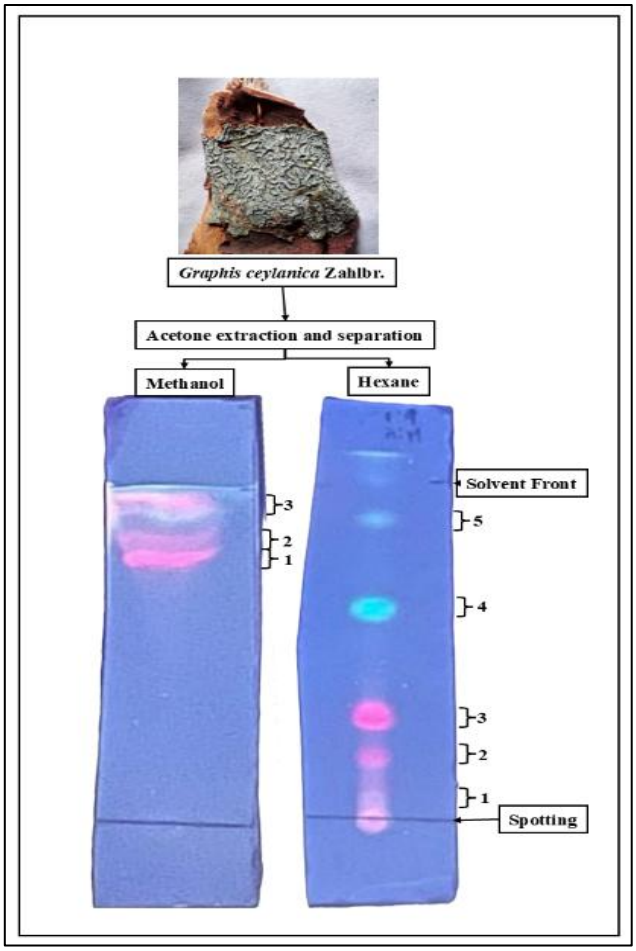

Figure 2: TLC plates for the methanol and HFs of the acetone extract of *Graphis ceylanica* Zahlbr. under UV light.

DISCUSSION

Graphis ceylanica Zahlbr. functions as a script lichen which belongs to the *Graphis* lichen genus. The genus contains lichens which show both antimicrobial and anti-*Candida* properties while displaying substantial antioxidant capabilities. The study investigates *G. ceylanica*'s biofilm inhibition and antioxidant properties through its extraction from two solvent fractions. *G. ceylanica* research so far has focused on its ecological and the bioindicator functions while scientists have not yet studied its secondary metabolite compounds which accumulate arsenic and the heavy metals from the contaminated sites.[9]

The TPC and TFC results show the same pattern as previous findings which show that *G. ajarekarii* produces more phenolic and flavonoid compounds through its polar fractions than through its non-polar extracts.[10] The chemical diversity studies of *Graphis* spp. report that phenolic depsidones which include norstictic and stictic and protocetraric acids provide antioxidant effects.[3]

The MF showed better DPPH radical scavenging results than the HF because it contained more phenolic and flavonoid compounds. The methanolic extracts of *G. scripta* show similar activity because its phenolic compounds provide antioxidant effects. The HF exhibits lower RSA because its main components are non-polar lipophilic substances. The MF showed higher TAC than HF based on the results of the phosphomolybdate assay. The antioxidant profile shows a relationship with other *Graphis* species which display phenolic depsidones that exhibit redox activity.[11]

The antibiofilm assays demonstrated substantial inhibitory effects of both fractions against *C. albicans* and *C. tropicalis*. The non-polar elements in HF contribute to its antibiofilm activity because it displays powerful effects at minimal concentration. The two fractions both exhibited similar inhibitory patterns against the two species which demonstrates their ability to fight multiple *Candida* strains. The results confirm earlier findings which showed that *Graphis* species possess antifungal and antibiofilm properties.[12]

The TLC analysis showed several UV-active bands which demonstrated that the sample contained various secondary metabolites. HF showed four separate bands which displayed R_f values of 0.16, 0.28, 0.59, and 0.85 that extended from the polar section to the nonpolar section of the plate. The R_f values match the documented range for

depsidone and depside metabolites which occur in Graphis species.[3] The R_f value of 0.16 for the test showed the range which exists for polar acidic depsidones that include constictic derivatives. The R_f value of 0.28 represents mid-polarity compounds that resemble stictic acid. The band at R_f 0.59 is compatible with alkylated depsides such as sekikaic or lobaric acids.[6] The R_f spot 0.85 matches nonpolar esters such as barbatic acid. The MF consisted of three spots that displayed R_f values of 0.78, 0.82 and 0.97 (Figure 2 and Table 1). Atranorin and chloroatranorin-type β-orcinol depsidones appear at R_f 0.78 and 0.82 while R_f value of 0.97 likely represents a highly lipophilic phenolic or terpenoid compound.[7,8,13] The profiles indicate that the *Graphidaceae* family contains depsides, depsidones, and aromatic metabolites which show the specific characteristics of their family. The chemical profile obtained from TLC analysis shows consistency with the antibiofilm activity which was observed. The TLC-derived chemical profile is consistent with the observed antibiofilm activity. The tested concentrations of both fractions resulted in a major decrease of *Candida albicans* and *C. tropicalis* biofilms. The antibiofilm activity of MF reached its highest effectiveness when used at elevated concentrations which contained aromatic compounds. The agreement between TLC-inferred metabolites and bioactivity demonstrates that multiple compound classes produce their combined effects. The chemical diversity of *G. ceylanica* includes multiple active components which scientists should continue to research through their complete extraction process and structural analysis.

The use of two solvent fractions (hexane and methanol) derived from an acetone extract, would be acknowledging one of the limitations of the present study. A broader solvent series including ethyl acetate and chloroform fractions may reveal additional bioactive components. This further extends to TLC-based compound identification being presumptive. Beyond the preliminary studies presented here, a detailed analysis of the chemical profile and further evaluation of the antibiofilm activity against *Candida* isolates with a variable range of antifungal resistance would add value to the properties of the lichen, and its eventual scalability into isolating and characterizing promising drug candidates. The current work provides a valuable first characterization of the biological activities of *G. ceylanica* and lays the groundwork for more comprehensive investigations in pharmacology and chemical research.

CONCLUSION

This study presents the first baseline of the antioxidant, phytochemical and anti-*Candida* antibiofilm properties of *Graphis ceylanica* Zahlbr. It is an underexplored script lichen from the *Graphidaceae* family. Both the HF and MF derived from the acetone extract demonstrated significant DPPH radical scavenging activity and TAC. Here, MF showed higher phenolic and flavonoid contents consistent with its polar composition. TLC profiling indicated the presence of chemically diverse secondary metabolites, and aromatic compounds characteristic of the genus *Graphis*. Most notably, both fractions exhibited potent inhibition (75-95%) of biofilm formation in the tested clinical isolates of *C. albicans* and *C. tropicalis* at the tested concentrations. These findings help advance knowledge and in the understanding that *G. ceylanica* worthy of further pharmacological investigation.

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Ethical approval: The study was approved by the Institutional Ethics Committee of Assam State Technology and Environment Council (ASTEC), Government of Assam (ASTEC/206/2021-22/1114-1114).

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