Conference Paper No: BF-06

Bioactive properties and metabolite profile of an endolichenic fungus, *Hypoxylon lividipigmentum*

W.R.H. Weerasinghe[1], C.D. Shevkar[2], R.S. De Silva[3], R.N. Attanayake[4], G. Weerakoon[5], A.S. Kate[2], K. Kalia[2] and P.A. Paranagama[1*]

[1]Department of Chemistry, University of Kelaniya, Sri Lanka
[2]National Institute of Pharmaceutical Education and Research, India
[3]College of Chemical Sciences, Institute of Chemistry Ceylon, Sri Lanka
[4]Department of Plant and Molecular Biology, University of Kelaniya, Sri Lanka
[5]Department of Life Sciences, The Natural History Museum, United Kingdom
priyani@kln.ac.lk*

Abstract

Endolichenic fungi (ELF) serve as a novel source of secondary metabolites. *Hypoxylon lividipigmentum* is an ELF isolated from the lichen *Opegrapha medusulina*, collected from mangrove plant *Xylocarpus granatum* from Negombo lagoon, Sri Lanka. The fungus was identified to the species level using morphological and DNA barcoding techniques. Ethyl acetate extract of the fungus was subjected to in vitro assays to determine antioxidant, anti-inflammatory, tyrosinase inhibitory and antibacterial potency. Liquid Chromatography-Mass Spectrometry (LCMS) dereplication was conducted on the crude extract in order to detect the secondary metabolites present. The extract reported a IC_{50} value of 18.34±1.37 µg/ml on par with the positive control BHT, in DPPH radical scavenging assay. It also exhibited moderate anti-inflammatory activity with an IC_{50} value of 81.08±1.05 µg/ml. Tyrosinase inhibitory activity was fairly comparable with an IC_{50} value of 121.20±2.55 µg/ml. Agar well diffusion assay was conducted to determine antibacterial activity against aerobic bacterial species *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and the anaerobic bacterial species *Streptococcus mutans*. Suppression of growth was shown only against *B. subtilis*. Five major mass peaks were observed during the study of LCMS profile of the extract. After a thorough dereplication process, two masses could be presumed to be from novel scaffolds. Since none of the mass peaks could be dereplicated within the species or genus level, it could be speculated that the chemical profile of *Hypoxylon lividipigmentum* was previously poorly explored in literature thus making it an interesting organism to study further for novel metabolites.

Keywords

Bioactivity, Endolichenic Fungi, *Hypoxylon lividipigmentum*, LCMS dereplication, Lichens

Introduction

Among diverse microorganisms, endolichenic fungi (ELF) play an important role as secondary metabolite producers. Since the first research on isolating secondary metabolites from ELF (Paranagama *et al*., 2007), so far more than 172 compounds have been isolated with various activities such as anticancer, antifungal, antibacterial, antioxidant, anti-inflammatory, UV protectant, antiviral and anti-Alzheimer's disease (Agrawal *et al*., 2020).

ELF associated with the mangrove ecosystem might be adapted to face diverse array of challenges such as high salinity, extreme tides and temperature fluctuations. These fungi should have novel metabolites with significant bioactivities, as evident by the recent study

conducted in the mangrove ecosystem of Puttalam lagoon, Sri Lanka (Maduranga *et al*., 2018).

Although there have been numerous studies conducted on the phylogenetic and ecological aspects of the fungus *Hypoxylon lividipigmentum* isolated from various sources (Sir *et al*., 2016), its bioactivity and metabolic profile is scarcely explored in the literature. This study attempts to explore the bioactivities such as antioxidant, anti-inflammatory, tyrosinase inhibitory and anti-bacterial potency of this particular ELF and its metabolite profile using LCMS dereplication. LCMS dereplication method used in this study is a novel and convenient approach as opposed to the conventional bioactivity-guided fractionation, to understand the metabolic profile of the extract before proceeding with further analyses and separation phases (Wolfender *et al*., 2019).

Methodology

Ethical statement

Permit to collect lichen samples from the mangrove forest in Negombo lagoon was obtained from the Forest Department of Sri Lanka.

Collection of lichen sample and lichen identification

Lichen host was collected on 31st of March, 2018 at the mangrove forest of Negombo lagoon, Sri Lanka from the mangrove plant *Xylocarpus granatum*. The study site was "Kadolkele" area situated at 7.195528 N 79.84389 E GPS coordinates belonging to the lagoon area. The identification of this lichen was carried out using morphological techniques (Maduranga *et al*., 2018) at Natural History Museum, London.

Isolation and identification of endolichenic fungi

The lichen thallus was surface sterilized, plated on 2% malt extract agar (MEA) supplemented with 0.01% streptomycin and ELF was isolated using the method described in (Maduranga *et al*., 2018). DNA extraction of the isolated pure culture was carried out using a slightly modified method of CTAB DNA extraction protocol. The nuclear ribosomal internal transcribed spacer region (rDNA- ITS) was amplified, sequenced and identified as described in (Maduranga *et al*., 2018).

Extraction of secondary metabolites

The ELF was cultured on 5 PDA plates of the size 150×25 mm and incubated at room temperature for a duration of 2 weeks. Extraction was carried out according to the method described in (Maduranga *et al*., 2018).

Radical scavenging ability by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method

The radical scavenging ability assay was carried according to the colorimetric method described by (Maduranga *et al*., 2018), with slight modifications. BHT was used as the positive control and activity was calculated using Equation 1.

$$\%\, Activity = \frac{A_{control} - A_{sample}}{A_{control}}\, x\, 100\% \qquad \text{Equation 1}$$

Anti-inflammatory assay (HRBC Stabilization method)

The anti-inflammatory assay was conducted using human red blood cells stabilization method which uses the heat induced hemolysis principle (Sakat *et al*., 2010). Aspirin was used as the positive control and the test was performed in triplicates and the percentage stability was calculated using Equation 1.

Tyrosinase inhibitory assay

Tyrosinase inhibitory assay was carried out in a flat bottom 96-well microtiter plate according to a modified version of (Souza et al., 2012). Kojic acid was used as the positive control and the percentage inhibition was calculated using the Equation 2.

$$\%\ Inhibition\ = \frac{(A_{control} - A_{blank}) - (A_{sample} - A_{blank})}{(A_{control} - A_{blank})} \text{ x } 100\%$$

Anti-bacterial assay (Agar well diffusion method)

Anti-bacterial assays against aerobic bacterial species *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 25923) and the anaerobic bacterial strain *Streptococcus mutans* (ATCC 700610) were conducted using agar well diffusion method (Holder and Boyce 1994). Azithromycin and chlorohexidine were used as positive controls against aerobic and anaerobic strains respectively.

Statistical analysis

IC_{50} values for the bioassays were calculated using the statistical software GraphPad Prism 7.0.4. To statistically determine the significant difference between the extract of *H. lividipigmentum* and positive control, paired t-test was conducted using Wilcoxon matched-pairs signed rank test using 95% confidence level with the same statistical software.

LC-MS dereplication

Chromatographic separation of crude extract was performed on the Agilent (5µm, 4.6×250 mm) C_{18} column and the mobile phase system consisted of 0.1 % Formic acid (A) and Methanol (B) and the gradient was set up as: 0 to 2 min 30% A/70% B, a linear gradient from 2 to 8 min to 5% A/95% B and a linear gradient from 12 to 15 min to again 30% A/70% B. The obtained masses were thoroughly searched in Dictionary of Natural Products (DNP) and SciFinder, using species, genus and fungi level specificity for rationalization of the obtained profiles.

Results and Discussion

Isolation and identification of endolichenic fungi:

The lichen was collected from the mangrove plant *Xylocarpus granatum* and the specimen was identified as *Bactrospora myriadea* using morphological techniques. The obtained ITS sequence matched with *Hypoxylon lividipigmentum* sequence (published record) of KU604567.1 in GenBank with 99.81% homology. The sequence was deposited under the

accession number MT507846 in the GenBank. The isolate showed moderate growth on PDA, covering a 150 mm diameter Petri-dish within two weeks.

Screening for biological activities:

The radical scavenging activity was assessed using the DPPH assay. As shown in the Figure 1(a), the IC_{50} value shown by the extract against DPPH (IC_{50} 18.34±1.37 µg/ml) is less than the value shown by the positive control BHT (IC_{50} 26.22±2.28 µg/ml). Furthermore, paired t-test revealed that the difference is significant with $P < 0.05$ (0.0156). This interestingly displays that the extract contains metabolites which lead to a better antioxidant activity than BHT.

The anti-inflammatory activity shown by the extract (IC_{50} 81.08±1.05 µg/ml) in the HRBC stabilization assay is fairly comparable to the positive control aspirin (IC_{50} 24.17±0.24 µg/ml) with a significant difference of P value (0.0156) as well, confirming its moderate potency.

Inhibition of tyrosinase enzyme is the most convenient way of controlling melanogenesis. Hence, the demand for tyrosinase inhibitors has increased immensely over the past few years. This extract seems to possess quite a similar activity (IC_{50} 121.20±2.55 µg/ml) compared to the commercially available inhibitor Kojic acid (IC_{50} 104.35±2.30 µg/ml) with no significant difference according to statistical analysis (P value 0.0938).

In an era where the antibiotic resistance has become a major issue, emergence of compounds with promising antibacterial activity is vital. The agar well diffusion method carried out to assess the antibacterial activity of the extract with a maximum dose of 5 mg/ml, revealed that the ethyl acetate extract of *H. lividipigmentum* possess antibacterial activity against *B. subtilis* bacterial strain only. This activity statistically has no significant difference (P value 0.2500) (zone diameter 14±0.45 mm) compared to the positive control azithromycin (zone diameter 24±0.24 mm).

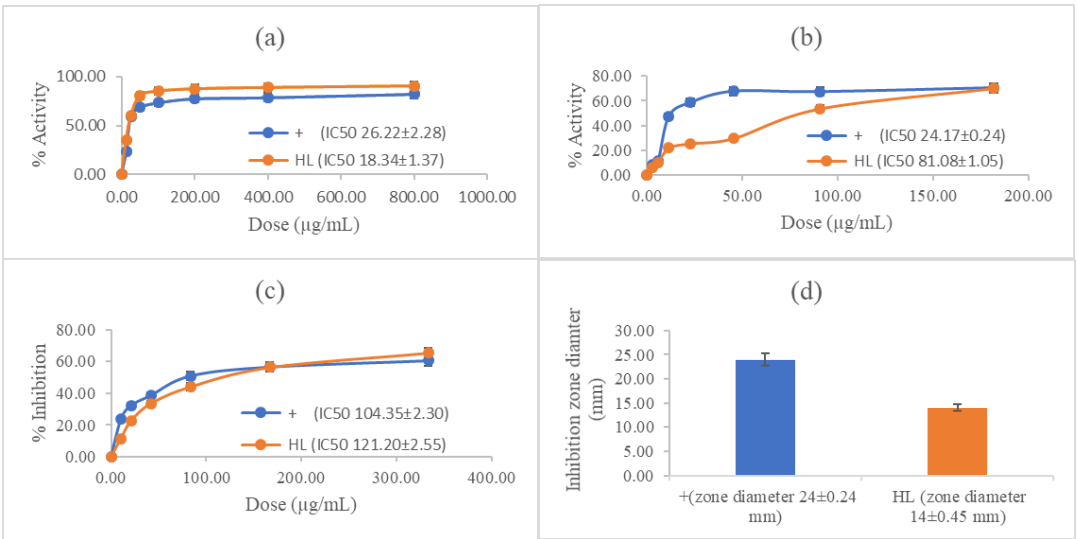
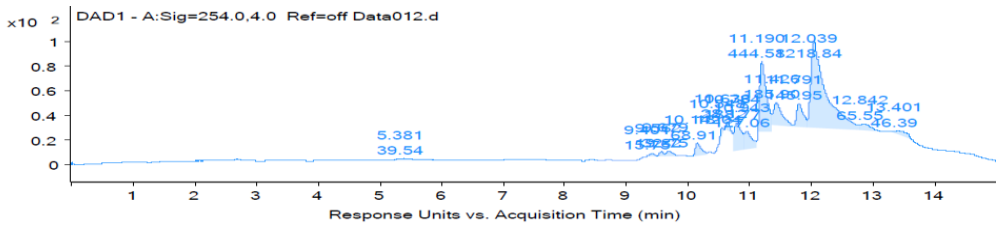


Figure 1. *Activity comparison of in vitro assays; (a) DPPH assay (b) HRBC stabilization assay (c) Tyrosinase inhibitory assay (d) Antibacterial assay against B. subtilis (+: positive control, HL: extract of H. lividipigmentum). Three replicates were used for each assay and error bars represent percentage error.*

It is to be stated that this is the first report of the above mentioned activities for the fungus *H. lividipigmentum* thus far in literature.

LCMS Dereplication

The chromatogram with the cluster of peaks observed at 254 nm is presented in Figure 2. All the proposed structures were confirmed with relevant UV profiles along with the accurate mass defect error below 10 PPM, which is well within the acceptable range.



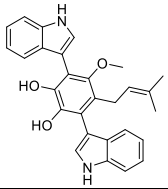
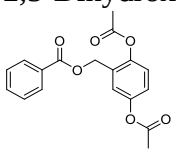
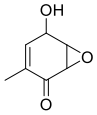
Integration Peak List

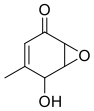
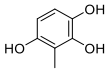
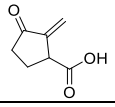
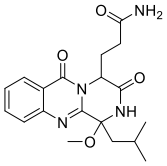
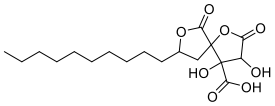
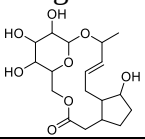
Peak	Start	RT	End	Height	Area	Area %
1	10.074	10.142	10.302	10.45	68.91	5.65
2	10.497	10.548	10.575	5.69	18.04	1.48
3	10.593	10.636	10.705	7.32	28.12	2.31
4	10.722	10.784	10.897	22.87	188.77	15.49
5	10.897	10.943	11.1	14.96	127.06	10.42
6	11.13	11.19	11.352	58.61	444.58	36.48
7	11.352	11.426	11.69	18.85	185.9	15.25
8	11.69	11.791	11.927	19.17	145.95	11.97
9	11.927	12.039	12.767	71.8	1218.84	100
10	12.767	12.842	13.126	5.43	65.55	5.38

Figure 2. *The chromatogram at 254 nm showing cluster of peaks*

Major observed mass peaks for ethyl acetate extract of the fungus were *m/z*: 141.0561, 373.1871, 493.2016, 746.4877 and 919.4281. It is to be stated that none of the mass peaks could be dereplicated at species and genus level specificity. The mass peaks 141.0561, 373.1871, 493.2016 could be dereplicated under the broad filter of fungi, which are presented in the Table 1. No hit in any of the applied filters of species, genus and fungi category could be found for the peaks 919.4281 and 746.4877, suggesting further exploration could be pursued for *H. lividipigmentum* in search of novel scaffolds.

Table 1. *Proposed structures in LC-MS dereplication in fungi category search*

Observed *m/z* $[M+H]^+$	Proposed structures with corresponding accurate mass	Reference
493.2016	Ochrindole C 	(De Guzman *et al.*, 1994)
141.0561	2,5-Dihydroxybenzenemethanol,  Epoformin, 	(Cala *et al.*, 2018)

	Isoepiepoformin,  2,3,6-Trihydroxytoluene,  5-Methylpyrogallol,  2-Methylene-3-oxocyclopentanecarboxylic acid 	
373.1871	Aurantiomide A,  Cinatrin B,  Jiangolide 	(Tohme *et al.*, 2011)

Conclusion

In the present study, *H. lividipimentum* ELF strain, was investigated for its biological activities and metabolic profile. Noteworthy antioxidant, anti-inflammatory, tyrosinase inhibitory and antibacterial activities could be observed from the *in vitro* screenings carried out for the extract. This also becomes the first report of such activities from the fungal strain of *H. lividipigmentum*. However, further studies are required to determine the exact bioactive compounds responsible for these activities. The LCMS dereplication study conducted for the same revealed that it comprises of interesting metabolites reported from literature under the broad fungal category while containing two unknown major compounds. Overall, this fungus seems to be an interesting strain to explore further, in order to uncover novel scaffolds with useful bioactivities.

Acknowledgment

Authors thank Prof. Mala Amarasinghe, Department of Plant and Molecular Biology, University of Kelaniya for helping in identification of the mangrove host plants. Financial support is provided by Ministry of Science, Technology and Research, Sri Lanka (Grant No: MSTR/TRD/AGR/03/02/07).

References

Agrawal, S., Deshmukh, S. K., Reddy, M. S., Prasad, R., & Goel, M. (2020). Endolichenic fungi: A hidden source of bioactive metabolites. *South African Journal of Botany*, *134*(2019), 163–186. https://doi.org/10.1016/j.sajb.2019.12.008

Cala, A., Masi, M., Cimmino, A., Molinillo, J. M. G., Macias, F. A., & Evidente, A. (2018). (+)-epi-Epoformin, a Phytotoxic Fungal Cyclohexenepoxide: Structure Activity Relationships. *Molecules*, *23*(7), 1–12. https://doi.org/10.3390/molecules23071529

De Guzman, F. S., Bruss, D. R., Rippentrop, J. M., Gloer, K. B., Gloer, J. B., Wicklow, D. T., & Dowd, P. F. (1994). Ochrindoles A-D: New bis-indolyl benzenoids from the sclerotia of *Aspergillus ochraceus* NRRL 3519. *Journal of Natural Products*, *57*(5), 634–639. https://doi.org/10.1021/np50107a011

Holder, I. A., & Boyce, S. T. (1994). Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture. *Burns*, *20*(5), 426–429. https://doi.org/10.1016/0305-4179(94)90035-3

Maduranga, K., Attanayake, R. N., Santhirasegaram, S., Weerakoon, G., & Paranagama, P. A. (2018). Molecular phylogeny and bioprospecting of Endolichenic Fungi (ELF) inhabiting in the lichens collected from a mangrove ecosystem in Sri Lanka. *PLoS ONE*, *13*(8), 1–22. https://doi.org/https://doi.org/ 10.1371/journal.pone.0200711

Paranagama, P. A., Wijeratne, E. M. K., Burns, A. M., Marron, M. T., Gunatilaka, M. K., Arnold, A. E., & Gunatilaka, A. A. L. (2007). Heptaketides from Corynespora sp. inhabiting the Cavern Beard Lichen, *Usnea cavernosa*: First Report of Metabolites of an Endolichenic Fungus. *Journal of Natural Products*, *70*(11), 1700–1705. https://doi.org/10.1021/np070466w

Sakat, S. S., Juvekar, A. R., & Gambhire, M. N. (2010). In-vitro antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* linn. *International Journal of Pharmacy and Pharmaceutical Sciences*, *2*(1), 146–155.

Sir, E. B., Kuhnert, E., Lambert, C., Hladki, A. I., Romero, A. I., & Stadler, M. (2016). New species and reports of Hypoxylon from Argentina recognized by a polyphasic approach. *Mycological Progress*, *15*(4). https://doi.org/10.1007/s11557-016-1182-z

Souza, P. M., Elias, S. T., Simeoni, L. A., de Paula, J. E., Gomes, S. M., Guerra, E. N. S., Fonseca, Y. M., Silva, E. C., Silveira, D., & Magalhães, P. O. (2012). Plants from Brazilian Cerrado with Potent Tyrosinase Inhibitory Activity. *PLoS ONE*, *7*(11), 1–7. https://doi.org/10.1371/journal.pone.0048589

Tohme, R., Darwiche, N., & Gali-Muhtasib, H. (2011). A journey under the sea: The quest for marine anti-cancer alkaloids. *Molecules*, *16*(11), 9665–9696. https://doi.org/10.3390/molecules16119665

Wolfender, J. L., Nuzillard, J. M., Van Der Hooft, J. J. J., Renault, J. H., & Bertrand, S. (2019). Accelerating Metabolite Identification in Natural Product Research: Toward an Ideal Combination of Liquid Chromatography-High-Resolution Tandem Mass Spectrometry and NMR Profiling, in Silico Databases, and Chemometrics. *Analytical Chemistry*, *91*(1), 704–742. https://doi.org/10.1021/acs.analchem.8b05112