Bioactive Properties and Metabolite Profiles of Endolichenic Fungi in Mangrove Ecosystem of Negombo Lagoon, Sri Lanka

Ramani H. Weerasinghe[1], Kasun Maduranga[1], Renuka N. Attanayake[2], Chaitrali Shevkar[3], Abhijeet S. Kate[3], Gothamie Weerakoon[4], Kiran Kalia[3] and Priyani A. Paranagama[1] (iD)

Abstract

Endolichenic fungi (ELF) serve as a novel source of natural products with distinctive bioactivities. A total of 58 ELF isolated from 29 lichens collected from mangrove and mangrove-associated plants of Negombo lagoon, Sri Lanka were identified using morphological and DNA barcoding techniques. Ethyl acetate extracts of 18 such identified ELF isolates were subjected to *in vitro* assays to determine antioxidant, anti-inflammatory, tyrosinase inhibitory and antibacterial potency. Liquid chromatography–mass spectrometry (LC–MS) dereplication was conducted on the crude extracts in order to detect the secondary metabolites present. The extracts of *Daldinia eschscholtzii* and *Hypoxylon lividipigmentum* had the highest radical scavenging activity with SC_{50} values 14.27 ± 0.24 µg/mL and 18.34 ± 1.37 µg/mL, respectively. *D. eschscholtzii* also exhibited remarkable anti-inflammatory activity (IC_{50} 7.97 ± 0.09 µg/mL). Tyrosinase inhibitory activity was highest in *Cytospora xylocarpi* (IC_{50} 68.50 ± 0.34 µg/mL), while the highest activity against aerobic bacterial species *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and the anaerobic bacterial strain *Streptococcus mutans* was observed in the extracts of *Xylaria feegenesis* and *Curvularia lunata*. After a thorough study of the LC–MS profiles, it was found that the chemical profiles of *Neofusicoccum occulatum, H. lividipigmentum* and *Myramaececium rubricosum* were previously poorly explored in the literature.

Introduction

The discovery of biologically active compounds has opened a diverse "chemical space" in natural product chemistry.[1] Microorganisms have played a major role in the discovery of novel bioactive small molecules, contributing to the identification of more than 22 500 bioactive metabolites.[2] Among them, lichens and endolichenic fungi (ELF) are emerging as a treasure trove for novel bioactive metabolites.

Lichens are a symbiosis between a fungal component, which makes up the bulk of the thalli while providing access to water and minerals, and a cyanobacterium and/or alga, which provides the photosynthate. Interestingly, this is a physiological combination of microorganisms belonging to 3 different Kingdoms. Moreover, these lichens provide grounds for the exploration of more microorganisms that live on them asymptomatically.[3,4] ELF are one such group, which live inside the thallus of a lichen, analogous to endophytic fungi residing in plant tissues.[5]

Since the first report of heptaketides from ELF published in 2007,[5] many molecules have been identified with potent bioactivities around the world.[6] Such potent metabolites have been discovered during previous studies conducted in Sri Lanka as well, which include polyketides from ELF *Penicillium citrinum*[7] and *Curvularia trifolli*[8] and more metabolites from *Daldinia eschscholtzii*[9] and *Xylaria psidii*.[10]

[1]Department of Chemistry, University of Kelaniya, Kelaniya, Sri Lanka
[2]Department of Plant and Molecular Biology, University of Kelaniya, Kelaniya, Sri Lanka
[3]National Institute of Pharmaceutical Education and Research, Ahmedabad, India
[4]The Natural History Museum, London, UK

Corresponding Author:
Priyani A. Paranagama, Department of Chemistry, University of Kelaniya, Kelaniya, Sri Lanka.
Email: priyani@kln.ac.lk

Our previous research was conducted using the lichens in the mangrove ecosystem of Puttalam lagoon in the dry zone of Sri Lanka [11] while the current study was undertaken in the mangroves of the Negombo lagoon in the wet zone of the country. Constant exposure to high salinity, extreme tides and temperature changes in this area make it a challenging environment not only for lichens and ELF but also for any organism to survive. In the current study, ELF from the mangrove ecosystem were identified using DNA barcoding of the internal transcribed spacer (ITS) region and their crude extracts were screened for antioxidant, anti-inflammatory, antibacterial and tyrosinase inhibitory potentials. Liquid chromatography–mass spectrometry (LC–MS) dereplication, an integrated approach of combining traditional fraction collection, sample preparation, and multicomponent analysis into a single step, was performed for the understanding of metabolite profiles of each representative isolate obtained in the study. This is the first such study focusing on metabolic profiles of the mangrove associated ELF in Sri Lanka.

Results and Discussion

Identification of Lichens

It is reported that nearly 1200 lichen species are known from Sri Lanka.[12] Host plant and lichen species of the 29 lichen samples collected are shown in Table 1. The majority of the samples belonged to the crustose type, which included *Pyrenula ochraceoflava* (4), *Bactrospora myriadea* (9), *Graphis librata* (4), *Graphis furcata* (2), *Arthonia parantillarum* (1), *Opegrapha medusulina* (4), *Graphis caesiella* (2), and *Graphis* indet. (1).The other lichens identified were foliose *Pyxine cocoes* (1) and fruticose *Roccella montagnei* (1) (Figure 1). Morphological and molecular identification of all the lichen samples revealed that all of them were previously described species.[13]

Isolation and Identification of ELF

A total of 58 fungal isolates were isolated into pure cultures from 29 lichen samples. Species confirmation was achieved by comparing sequences of ITS-rDNA region with the established (voucher specimens or published) records with the highest % similarity of the GenBank accessions and are shown in Table 1. Isolates belonged to 23 different species, out of which 18 belonged to phylum Ascomycota and only 5 to Basidiomycota. Out of the Ascomycetes, 9 genera belonged to the class Sordariomycetes, 5 to Dothideomycetes and only 1 to Eurotiomycetes. All genera found under the phylum Basidiomycota belonged to the class Agaricomycetes. The highest number of isolates (23) belonged to the species *D. eschscholtzii*.

Molecular phylogenetic analysis was performed using ITS sequences. Multiple sequence alignment (MSA) was made using ∼500 to 600 length of ITS sequences. The maximum likelihood tree with the highest log likelihood (−813.33) is shown in Figure 2. The tree was drawn to scale in combination with the already available sequences for each species in GenBank. The branch lengths were measured by the number of substitutions per site. In the analysis, isolates were clearly grouped according to families and genera. Fungal species clustered together regardless of their lichen host demonstrating that in most cases, ELF diversity is not determined by the lichen host.

Qualitative Analysis of Metabolites in Extracts of ELF

Qualitative analysis of crude extracts performed using thin-layer chromatography (TLC) indicated that the chemical constituents of the same fungal species isolated from different lichens were similar to each other. Based on their close phylogenetic relationships, only one isolate per selected species was used in bioassays.

Bioassays and LC–MS dereplication were conducted for 18 selected isolates due to the sterility of the remaining 5 strains which obstructed further culturing.

Screening of Radical Scavenging Activity (DPPH Assay)

The extreme reactivity of free radicals can cause damage to macromolecules inside our body, making antioxidants important in protecting tissues from many noncommunicable diseases.[14] 2, 2-diphenyl-1-picrylhydrazyl (DPPH) consists of a delocalized electron and produces a characteristic purple color in a solution of methanol, which changes to a residual pale yellow in the presence of an antioxidant. This absorbance can be measured at 517 nm.[15]

The lowest half-maximal scavenging concentration (SC_{50}) values were shown by the crude extracts of *D. eschscholtzi* ($14.27 \pm 0.24\ \mu g/mL$) and *Hypoxylon lividipigmentum* ($18.34 \pm 1.37\ \mu g/mL$), presumably containing antioxidants even more active than the commercially available antioxidant, butylated hydroxytoluene (BHT) ($26.22 \pm 2.28\ \mu g/mL$). In line with the current observation, several other studies also reported that *D. eschscholtzi* isolated as ELF had lower SC_{50} values.[9,11] In contrast, isolates of *H. lividipigmentum* from any source have not been reported to possess antioxidant activity so far in the literature.
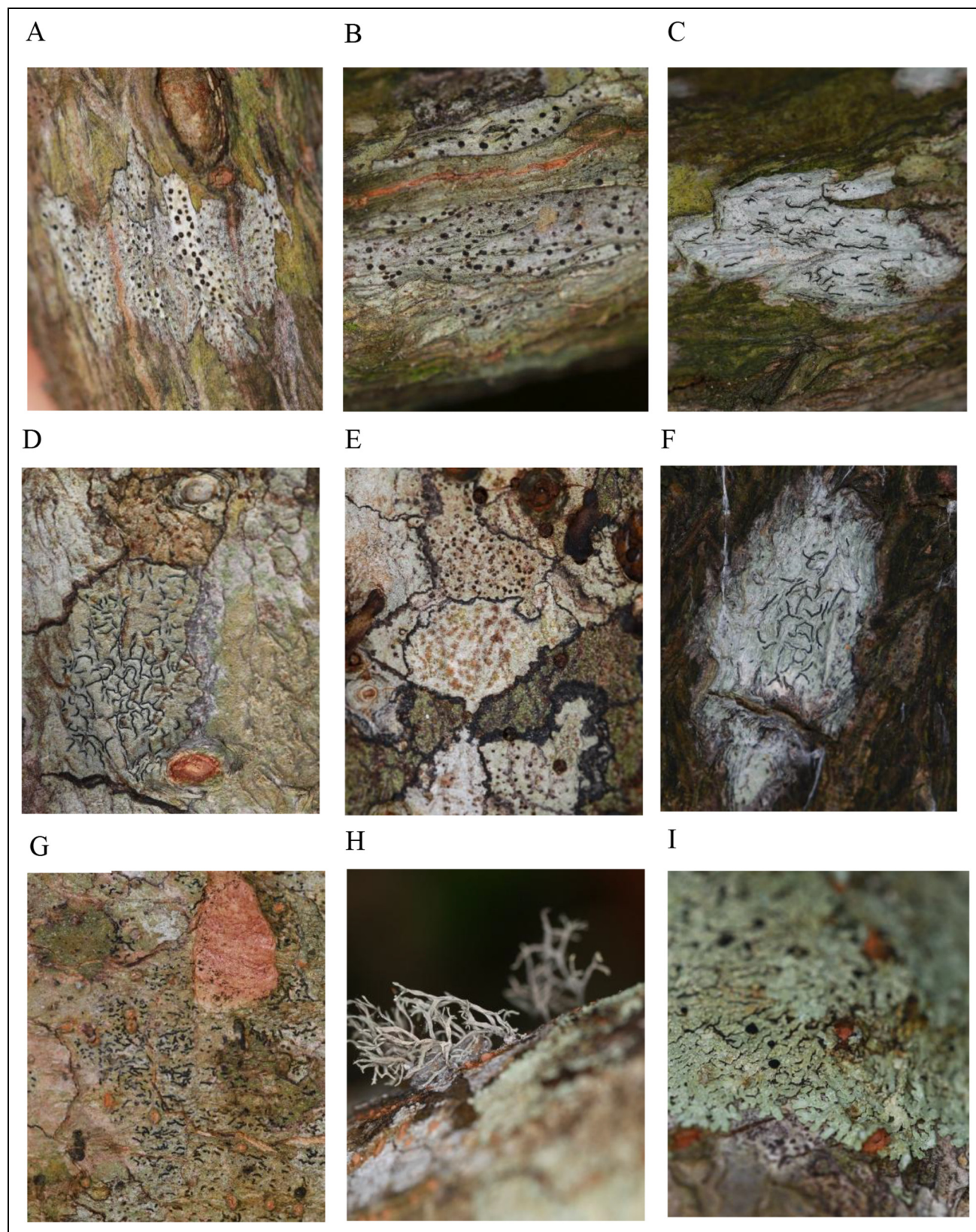
Moreover, a considerable amount of activity was observed for *Chaetomium globosum* (SC_{50} $58.55 \pm 1.65\ \mu g/mL$), *Cytospora xylocarpi* (SC_{50} $124.30 \pm 0.95\ \mu g/mL$), *Hypoxylon griseobrunneum* (SC_{50} $126.97 \pm 1.74\ \mu g/mL$), *Myramaececium rubricosum* (SC_{50} $288.73 \pm 2.45\ \mu g/mL$) and *Pseudothielavia terricola* (SC_{50} $620.33 \pm 2.79\ \mu g/mL$) in descending order. While this happens to be the first report of antioxidant activity of *C. globosum* as an ELF, this also becomes the first report for the other 4 species isolated from any source as per the species identified based on ITS-rDNA sequencing.

Activity displayed by the extracts of *Nodulisporium* sp. (SC_{50} $559.60 \pm 1.85\ \mu g/mL$), *Hypoxylon anthochroum* (SC_{50} $621.80 \pm 3.63\ \mu g/mL$) and *Schizophyllum commune* (SC_{50} $708.8 \pm 2.67\ \mu g/ml$) was observed to be similar to the reported values
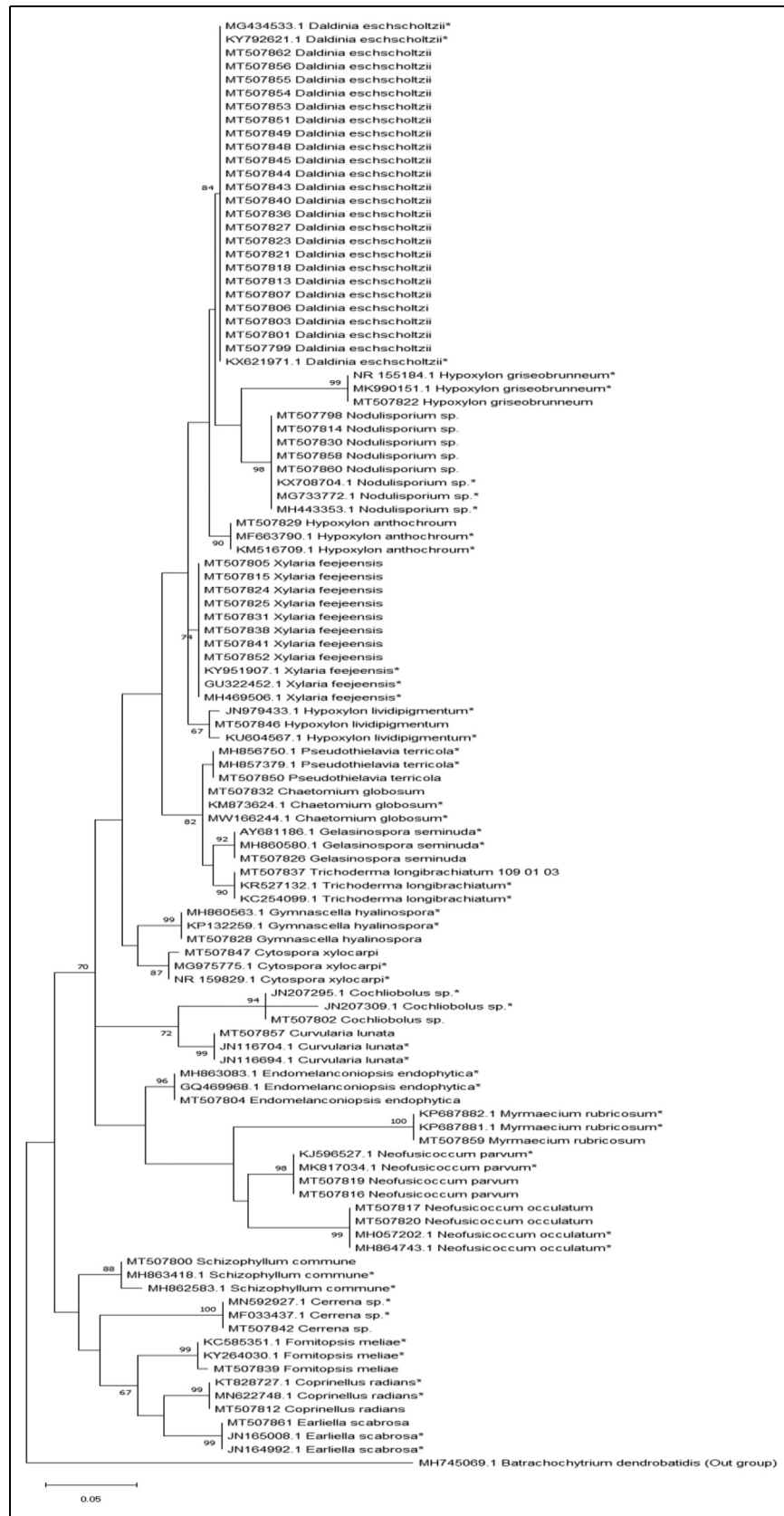
Table 1. Endolichenic Fungal Strains Isolated From the Lichens of Mangrove and Mangrove-Associated Plants in Negombo Lagoon, Sri Lanka.

Host plant	Sequence ID	Organism	Hit from GenBank	% Homology	GenBank Accession number
Graphis caesiella	100_01_02	*Nodulisporium* sp.	KX708704.1	100.00%	MT507798
	100_01_05	*Daldinia eschscholtzii*	MH368106.1	100.00%	MT507799
Pyrenula ochraceoflava	100_02_03	*Daldinia eschscholtzii*	MG434533.1	100.00%	MT507801
	100_02_01	*Schizophyllum commune*	MH863418.1	97.08%	MT507800
Bactrospora myriadea	103_01_02	*Daldinia eschscholtzii*	MG434533.1	100.00%	MT507803
	103_01_01	*Cochliobolus* sp.	JN207295.1	99.48%	MT507802
	103_01_03	*Endomelanconiopsis endophytica*	MH863083.1	99.83%	MT507804
Graphis librata	103_02_01b	*Daldinia eschscholtzii*	KY792621.1	99.83%	MT507806
	103_02_02a	*Xylaria feejeensis*	GU322452.1	99.45%	MT507805
Bactrospora myriadea	104_01_04	*Daldinia eschscholtzii*	KY792620.1	100.00%	MT507807
Pyrenula ochraceoflava	105_02_01	*Coprinellus radians*	KT828727.1	100.00%	MT507812
	105_02_03IALPHA	*Daldinia eschscholtzii*	MH748178.1	100.00%	MT507813
	105_02_03IBETA	*Nodulisporium* sp.	MG733772.1	100.00%	MT507814
	105_02_04	*Xylaria feejeensis*	MH469506.1	97.82%	MT507815
Graphis furcata	106_01_01	*Neofusicoccum parvum*	KJ596527.1	98.93%	MT507816
	106_01_02	*Neofusicoccum occulatum*	MH057202.1	99.11%	MT507817
	106_01_05	*Daldinia eschscholtzii*	MH748178.1	100.00%	MT507818
Graphis furcata	106_02_01	*Neofusicoccum parvum*	MK817034.1	98.91%	MT507819
	106_02_02	*Neofusicoccum occulatum*	MH057202.1	99.10%	MT507820
	106_02_03	*Daldinia eschscholtzii*	KX621971.1	99.46%	MT507821
	106_02_04	*Hypoxylon griseobrunneum*	NR_155184.1	99.21%	MT507822
Pyrenula ochraceoflava	106_03_02ALPHA	*Daldinia eschscholtzii*	MF380724.1	100.00%	MT507823
	106_03_06	*Xylaria feejeensis*	KY951907.1	99.82%	MT507824
Arthonia parantillarum	106_04_02	*Xylaria feejeensis*	KY951907.1	100.00%	MT507825
	106_04_03	*Gelasinospora seminuda*	AY681186.1	99.27%	MT507826
Bactrospora myriadea	107_01_03a	*Daldinia eschscholtzii*	MG434533.1	100.00%	MT507827
Graphis librata	107_02_01	*Gymnascella hyalinospora*	MH860563.1	99.50%	MT507828
Graphis caesiella	108_01_01	*Hypoxylon anthochroum*	MF663790.1	99.41%	MT507829
	108_01_02	*Nodulisporium* sp.	KX708704.1	100.00%	MT507830
Bactrospora myriadea	108_02_01	*Xylaria feejeensis*	KR025539.1	96.75%	MT507831
	108_02_02	*Chaetomium globosum*	KM873624.1	100.00%	MT507832
Opegrapha medusulina	109_01_02I	*Daldinia eschscholtzii*	KJ466979.1	99.81%	MT507836
	109_01_03	*Trichoderma longibrachiatum*	KR527132.1	100.00%	MT507837
Bactrospora myriadea	109_02_01a	*Xylaria feejeensis*	MH469506.1	100.00%	MT507838
	109_02_01bALPHA	*Fomitopsis meliae*	KC585351.1	95.59%	MT507839
	109_02_01bBETA	*Daldinia eschscholtzii*	KU304335.1	99.63%	MT507840
Bactrospora myriadea	110_01_02a	*Xylaria feejeensis*	AB285482.1	99.42%	MT507841
	110_01_02b	*Cerrena* sp.	MN592927.1	99.83%	MT507842
	110_01_03b	*Daldinia eschscholtzii*	KT151578.1	99.61%	MT507843
Opegrapha medusulina	111_01_01	*Daldinia eschscholtzii*	MH368106.1	100.00%	MT507844
Opegrapha medusulina	111_02_03	*Daldinia eschscholtzii*	KX621971.1	99.82%	MT507845
	111_02_04	*Hypoxylon lividipigmentum*	KU604567.1	99.81%	MT507846
Bactrospora myriadea	111_03_01	*Cytospora xylocarpi*	MG975775.1	99.65%	MT507847
	111_03_02	*Daldinia eschscholtzii*	KX621971.1	99.81%	MT507848
Bactrospora myriadea	112_01_02	*Daldinia eschscholtzii*	KX621971.1	99.64%	MT507849
Bactrospora myriadea	112_02_01	*Thielavia terricola*	MH856750.1	99.63%	MT507850
	112_02_03	*Daldinia eschscholtzii*	KX621971.1	99.82%	MT507851
Opegrapha medusulina	113_01_01b	*Xylaria feejeensis*	JX256824.1	99.43%	MT507852
	113_01_02b	*Daldinia eschscholtzii*	MF380766.1	99.61%	MT507853
Graphis librata	114_01_02	*Daldinia eschscholtzii*	MG434533.1	100.00%	MT507854
Graphis indet.	115_01_02	*Daldinia eschscholtzii*	MF380724.1	100.00%	MT507855
Pyrenula ochraceoflava	115_02_02	*Daldinia eschscholtzii*	KX621971.1	100.00%	MT507856
	115_02_04	*Curvularia lunata*	JN116704.1	100.00%	MT507857
Pyxine cocoes	116_01_02I	*Nodulisporium* sp.	EU363038.1	100.00%	MT507858
Graphis librata	116_02_01BETA	*Myrmaecium rubricosum*	KP687882.1	99.81%	MT507859
	116_02_03	*Nodulisporium* sp.	EU363038.1	100.00%	MT507860
Roccella montagnei	117_01_01	*Earliella scabrosa*	JN165008.1	100.00%	MT507861
	117_01_02	*Daldinia eschscholtzii*	KY792620.1	100.00%	MT507862

Figure 1. Selected lichens collected from mangrove and mangrove-associated plants in Negombo lagoon, Sri Lanka. (A) *Pyrenula ochraceoflava*, (B) *Bactrospora myriadea*, (C) *Graphis librata*, (D) *Graphis furcata*, (E) *Arthonia parantillarum*, (F) *Graphis caesiella*, (G) *Opegrapha medusulina*, (H) *Roccella montagnei*, (I) *Pyxine cocoes*.

Figure 2. Maximum likelihood tree with the highest log likelihood showing phylogenetic placement of endolichenic fungi isolated from lichens in the mangrove and mangrove-associated plants in Negombo lagoon, Sri Lanka. *Indicates the reference sequences from the GenBank.

of our previous ELF study conducted using samples from Puttalam lagoon, Sri Lanka.[11]

Anti-inflammatory Assay (HRBC Stabilization Method):

The regulation of an inflammatory response can be achieved by stabilizing the lysosomal membrane which otherwise would release the constituents to trigger inflammation. Since the lysosomal membrane is analogous to the erythrocytes' membrane in humans, this assay provides the technique to assess anti-inflammatory potency of the extracts using the heat-induced hemolysis principle.[16]

The extract of *Daldinia eschscholtzi* had the highest activity with an IC_{50} value of 7.97 ± 0.09 µg/mL, which was better than the positive control aspirin $(24.17 \pm 0.24$ µg/mL), while *Nodulisporium* sp. had the second-best activity with an IC_{50} value of 28.28 ± 0.79 µg/mL. According to the literature, no records of this activity can be found for *D. eschscholtzi*. However, in research conducted in China, *Nodulisporium* species, isolated from the lichen *Everniastrum* sp., are reported to contain compounds belonging to the viridin family, which are known to possess anti-inflammatory activity.[17]

The extracts of *H. anthochroum* and *H. lividipigmentum* showed substantial activity with IC_{50} values of 51.63 ± 1.78 and 81.08 ± 1.05 µg/mL, while *H. griseobrunneum, Neofusicoccum occulatum* and *Cerrena* sp. showed moderate activity with IC_{50} values of 131.4 ± 1.0, 166.8 ± 1.9, and 232.3 ± 2.7 µg/mL, respectively. It is to be noted that no records of anti-inflammatory activity are available for these 5 strains in previous literature.

The IC_{50} values shown by *Neofusicoccum parvum, C. globosum* and *Curvularia lunata* were 49.09 ± 0.82, 99.53 ± 1.34, and 162.7 ± 2.61 µg/mL, respectively. The available records show their potency against chronic inflammation,[18–20] but interestingly, none of them were reported from ELF.

Tyrosinase Inhibitory Assay

While giving protection from ultraviolet (UV) rays, overproduction of melanin can cause skin pigment disorders and therefore the cosmetic industry is also interested in the reduction of melanin production. This reduction can be achieved by inhibiting the enzyme tyrosinase, which will prevent a major step in the production of melanin inside melanocytes.[21]

In contrast to other bioassays, the tyrosinase inhibitory activity shown by extracts was slightly less. However, the extract of *C. xylocarpi* proved to possess a higher potency $(IC_{50}$ 68.50 ± 0.34 µg/mL) than the commercially available inhibitor Kojic acid $(IC_{50}$ 104.3 ± 2.30 µg/mL). According to the literature, this is the first time tyrosinase inhibition is reported for this strain. The second best IC_{50} value was exhibited by the extract of *H. lividipigmentum* $(121.2 \pm 2.55$ µg/mL). This is also the first report of this activity for this fungus.

The extract of *C. globosum* also showed a substantial inhibitory activity $(IC_{50}$ 308.4 ± 2.49 µg/mL). Interestingly, this fungus is known to be a low to moderate producer of Kojic

acid.[22,23] This genus is also reported to produce the metabolite "chaetominedione" with tyrosinase kinase inhibitory activity.[24] However, no records of activity are available from an ELF thus far.

Antibacterial Assay (Agar Well Diffusion Method)

Antibiotic resistance is of major global concern which has highlighted the importance of the discovery of clinically proven new antibiotics during the past few decades.[25] Hence this assay was carried out to assess activity against common aerobic bacterial species *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and the anaerobic bacterial strain *Streptococcus mutans*.

Considering the inhibition zone values, extract of *Xylaria feegenesis* showed very high activity against all 3 aerobic bacterial strains, *E. coli, S. aureus* and *B. subtilis* $(17 \pm 0.38$ mm, 20 ± 0.24 mm, 20 ± 0.28 mm, respectively) on par with the positive control Azithromycin $(21 \pm 0.15$, 22 ± 0.30, 24 ± 0.24 mm, respectively, for *E. coli, S. aureus* and *B. subtilis*). Apart from reported activity against *E. coli* and *S. aureus* from synthesized silver nanoparticles from the fungal filtrate and activity against different bacterial strains from the extract[26] and the presence of antimicrobial potent nonenolides,[27] there is no record of activity reported from an extract of ELF, *X. feegenesis* against the tested bacterial species.

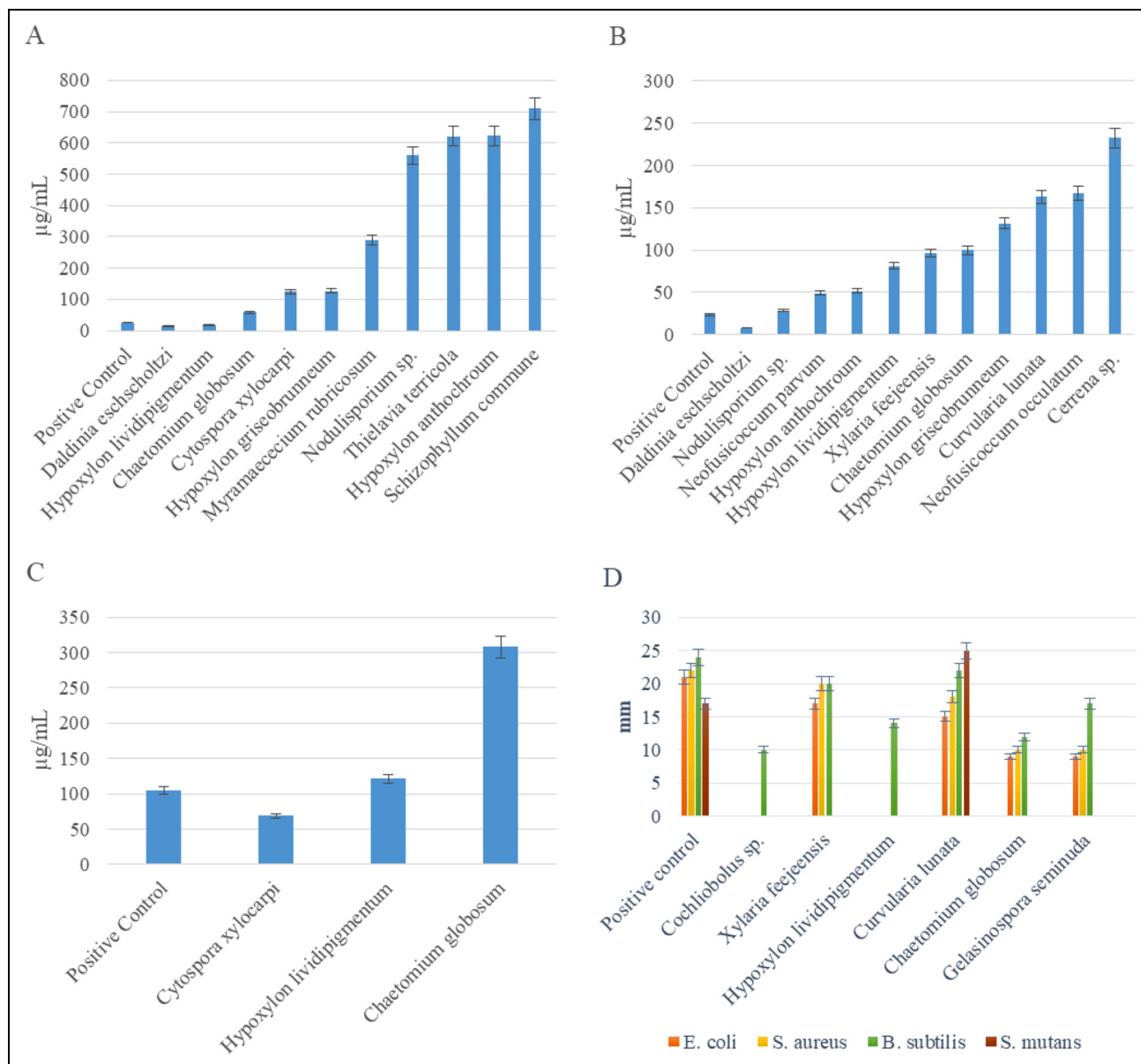The extract of *C. lunata* showed activity against all aerobic strains $(15 \pm 0.20$ mm, 18 ± 0.19 mm, 22 ± 0.15 mm, respectively for *E. coli, S. aureus* and *B. subtilis*), while showing a higher activity $(25 \pm 0.24$ mm) than the positive control, Chlorohexidine $(17 \pm 0.15$ mm) against the anaerobic species *S. mutans*. Although there is ample evidence for antibacterial activity of this fungus against *E. coli* and *S. aureus*[28] and silver nanoparticles of the fungal filtrate against *B. subtilis*,[29] none had been reported from an ELF. Furthermore, the antibacterial activity against *S. mutans* is not reported for the strain by any source.

The extracts of *C. globosum* and *Gelasinospora seminuda* exhibited substantial inhibition zones with diameters of 9 ± 0.28, 10 ± 0.15, 12 ± 0.31 mm and 9 ± 0.37, 10 ± 0.45, 17 ± 0.37 mm against *E. coli, S. aureus*, and *B. subtilis*, respectively. While many records verified the antibacterial activity of *C. globosum*,[30–32] even from an endophyte found in Sri Lanka,[33] none have been reported from ELF. Moreover, no reported records were found for *G. seminuda,* against the tested bacterial species.

Inhibition against *B. subtilis* was shown by the extracts of *H. lividipigmentum* and *Cochliobolus* sp. with zone diameters 14 ± 0.45 and 10 ± 0.20 mm, respectively. Even though previous activity is reported against *E. coli* and *S. aureus* from fungi in the genus *Cochliobolus*,[34,35] no records against *B. subtilis* or from an ELF could be found. For *H. lividipigmentum*, this becomes the first report of antibacterial activity reported from any source.

Only *Endomelanconiopsis endophytica* did not show a comparable activity in any of the conducted bioassays.

Activity comparisons in bioassays are presented graphically in Figure 3.

Figure 3. The activity comparison of bioassays. (A) SC_{50} comparison for DPPH (BHT as the positive control); (B) IC_{50} comparison for HRBC stabilization assay (aspirin as the positive control); (C) IC_{50} comparison for tyrosinase inhibitory assay (kojic acid as the positive control); (D) antibacterial assay inhibition zone comparison for highest dose (azithromycin for *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and Chlorohexidine for *Streptococcus mutans* as the positive control). Three replicates were used for each fungal extract and SC_{50}/IC_{50} values (µg/mL) were calculated by using GraphPad Prism (Version 7) software package. Abbreviations: DPPH, 2, 2-diphenyl-1-picrylhydrazyl; BHT, butylated hydroxytoluene; HRBC, human red blood cell; SC, scavenging concentration.

LC–MS Profile of Crude Extracts

LC–MS dereplication has been found to be an efficient method to scrutinize the chemical diversity of organisms in the field of natural product chemistry. It provides an opportunity to differentiate previously studied secondary metabolites from novel active compounds.[36] LC–HRMS analysis was performed and the accurate mass data were used along with the database Dictionary of Natural Products (DNP) to find the reported close hit for the particular peak. Multiple filters were applied in

this process such as the inclusion of genus, species and subsequently fungi as a broad category. All the suggested structures have shown similar UV profiles along with the accurate mass defect error below 10 PPM, which is well within the acceptable range. Accordingly, from the filter of species, 4 molecules were dereplicated from the fungus *C. globosum*, which are Chaetoglobosin Y, Chaetoglobosin J, 11-Epichaetomugilin A and Cochliodone A, while 8-Methoxy-1-naphthol was identified from the fungus *D. eschscholtzi*. The remaining peaks were analyzed

for their presence in the respective category of the genus. Longirostrerone B reported from *Chaetomium longirostre* showed the same mass as that observed in *C. globosum*. Moreover, its UV profile was the same, indicating that the above-mentioned molecule is produced by the latter as well. The structures identified using species and genus filters are presented in Table 2. The chromatographic data of the dereplicated compounds are available in Supplemental Material (File S1). Along with this, the remaining peaks present in the extracts from 15 different fungi were searched from the entire fungal category using accurate mass data and a total of 59 peaks were identified Supplemental Material (File S2). Although performing this study, it was observed that *N. occulatum* and *M. rubricosum* are yet to be studied for their secondary metabolites. The major peaks observed in the extracts of ELF *N. occulatum* (m/z: 542.3131, 319.1170, 552.4197, 1103.8159, 1132.8665, 767.4884), *H. lividipigmentum* (m/z: 919.4281, 746.4877), and *M. rubricosum* (m/z: 604.3099, 648.3360, 655.2750, 892.3669, 572.2901) have shown no hit in any of the applied filters of species, genus and fungal category, suggesting its further exploration for novel scaffolds.

Furthermore, TLC visualization conducted using visibility under UV at 254 and 366 nm proved the existence of aromatic and highly conjugated compounds in the extracts. The derivatization conducted using anisaldehyde–sulfuric acid reagent showed blue, violet, red, and yellow spots, which were indicative of phenols and terpenes being present.[44] The TLC profiles of the crude extracts in the respective optimized solvent systems are available in the supplemental material Supplemental Material (File S3).

Materials and Methods

Ethical Statement

A permit to collect lichen samples from the mangrove forest in Negombo lagoon was obtained from the Forest Department of Sri Lanka. The samples were collected from the trees in the area of "Kadolkele" under the observation of a representative of the department, without causing any disturbance to the surroundings.

Collection of Lichen Samples

Collection of 29 lichen specimens was carried out on March 31, 2018 at the mangrove forest of Negombo lagoon, Sri Lanka. The study site was "Kadolkele" area situated at 7.195528 N 79.84389 E GPS coordinates belonging to the lagoon area. The lichens were collected from mangrove and mangrove associated plants belonging to 6 different species positioned mainly at the water's edge and a few towards the land. Samples were collected from root, stem and bark with an approximated elevation of 1 m from the sea level. Collected specimens were placed in acid-free paper bags, labeled appropriately and transported to the Department of Chemistry, University of Kelaniya. Samples were stored at 4 °C to be processed for the isolation of ELF within 2 weeks of sampling.

Identification of Lichens

Portions of lichens of 2 × 2 cm were refrigerated for 2 weeks and air dried. The identification of these 29 specimens (Figure 2) was carried out using morphological and molecular techniques at the Natural History Museum, London and photographs were taken using Olympus stereomicroscopes and Olympus compound microscopes with interference contrast, connected to a Nikon Coolpix digital camera. Tap water was utilized for section mounting for all measurements. Duplicates of each specimen are deposited in the Department of Chemistry, University of Kelaniya.
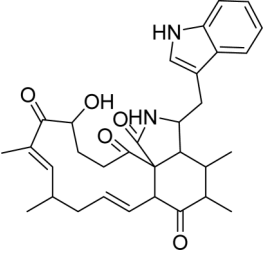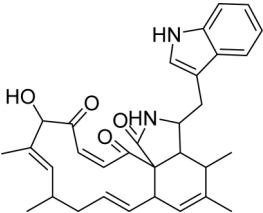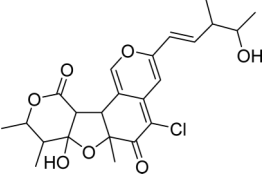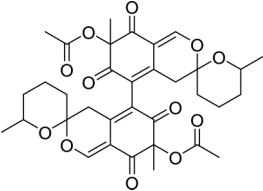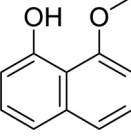
Isolation of Endolichenic Fungi

The lichen thalli were washed in running tap water to remove unwanted particles and then surface sterilized using consecutive immersion in 96% ethanol for 10 s, 0.5% sodium hypochlorite for 2 min, and 70% ethanol for 2 min and dried using sterile filter paper. Small segments of the lichen thallus (∼3 × 3 mm) were then cut and placed on 2% malt extract agar supplemented with 0.01% streptomycin. Sealed plates were then incubated under ambient conditions (room temperature 30 °C) for 14 days and observed regularly. Hyphal tips from each morphologically distinct colony were placed on new potato dextrose agar (PDA) plates and subjected to several sub-cultures to obtain a pure culture.

Extraction of Genomic DNA and Polymerase Chain Reaction

The isolates were inoculated in potato dextrose broth and incubated at room temperature (30 ± 32 °C) for 1-week. Mycelia were separated from the broth, washed with sterilized water, excess water was removed, and DNA extracted using a slightly modified method of the CTAB DNA extraction protocol.[45] The quality of extracted DNA samples was tested using 1% agarose gel electrophoresis and samples were stored at −20 °C for further utilization. The nuclear ribosomal DNA ITS region (rDNA-ITS) was amplified using polymerase chain reaction (PCR); the fungal specific ITS1/ITS5 forward and ITS4 reverse universal primers were used.[46] The PCR reaction mixture was comprised of 2 μL fungal DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 μM each forward and reverse primer, and 1.25 units of Taq DNA polymerase (Promega). PCR protocol consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension step of 72 °C for 10 min and was performed in a Veriti™ Thermal Cycler (Applied Biosystems). The amplification of the ITS region was confirmed by separating the amplified product using 1% agarose gel electrophoresis. The PCR products were subjected to Sanger dideoxy sequencing bi-directionally at Genentech Institute, Colombo, Sri Lanka. DNA sequences were manually edited using BioEdit sequence
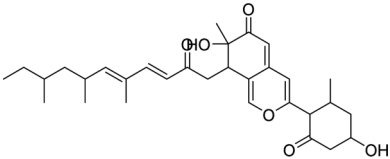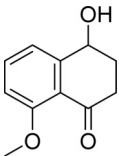
Table 2. Suggested structures in LC–MS dereplication in species and genus level search

Name	Observed *m/z* [M + H]⁺	Observed UV	Suggested structures with corresponding accurate mass observed from ELF	Mass Defect (ppm error value)	Reported UV	Reference
Suggested structures of molecules identified from *Chaetomium globusum* and *Daldinia eschscholtzi* at species level						
Chaetomium globosum	531.2833	310	Chaetoglobosin Y	4.9	238, 316	[37]
	451.1505	228, 285	Chaetoglobosin J	4.2	222, 273, 281	[38]
	433.1441	290, 375	11-Epichaetomugilin A	5.3	285, 373, 414	[39]
	639.2435	217	Cochliodone A	0.47	217, 312	[40]
Daldinia eschscholtzi	175.0743	230, 280, 330	8-Methoxy-1-naphthol	8.7	-	[41]
Suggested structures of molecules identified from *Chaetomium globosum* and *Nodulisporium* sp.						
Chaetomium globosum	511.2612	222, 290		5.3	225, 296	[42]

(Continued)

Table 2. Continued

Name	Observed m/z [M + H]$^+$	Observed UV	Suggested structures with corresponding accurate mass observed from ELF	Mass Defect (ppm error value)	Reported UV	Reference
			Longirostrerone B			
Nodulisporium sp.	193.0853	220,280,300	Nodulisporone 	5.2	219,289,300	[43]

Abbreviations: LC–MS, liquid chromatography–mass spectrometry; UV, ultraviolet; ELF, endolichenic fungi.

Alignment Editor (Version 7.2.5) and compared with the sequences available in the GenBank using Basic Local Alignment Search Tool (BLAST) to assess homology. DNA sequences of the identified ELF species were deposited at the National Center for Biotechnology Information (NCBI) database and accession numbers were obtained. ELFs identified in this study are shown in Table 1 with the percent similarity to the published/authenticated sequences at the GenBank and the accession numbers of the deposited sequences.

Phylogenetic Analysis of ELF

DNA sequences were aligned by using an MSA algorithm, Multiple Sequence Comparison by Log-Expectation (MUSCLE),[47] and poorly aligned positions and divergent regions of the alignment were removed/edited manually. All characters were equally weighted and the gaps were treated as missing data. A sequence of the ITS region of *Batrachochytrium dendrobatidis*, a fungus belonging to the Phylum *Chytridiomycota* (GenBank Accession: MH745069.1), was used as the out group. Molecular phylogenetic analysis was conducted using a Maximum Likelihood method using the bioinformatics software Molecular Evolutionary Genetics Analysis (MEGA) ver. X. One thousand bootstrap replications were used as statistical support for the nodes in the phylogenetic tree using Kimura-2 parameter model as the substitution model in MEGA.[48]

Extraction of Secondary Metabolites

Each endolichenic fungus was cultured on 5 PDA 150 × 25 mm plates and incubated at room temperature for 2 weeks. The mycelium of each fungus with the medium was cut into small pieces, extracted with ethyl acetate (6 × 500 mL) and the solvent evaporated under reduced pressure using a Rotary Evaporator (IKA RV 10B S96). The evaporated ethyl acetate extracts were then transferred to glass vials separately and N$_2$ was passed through the samples to remove any remaining solvent. The resulting semi-solid extracts were stored at 0 °C for further analysis.[7]

Radical Scavenging Ability by DPPH Method

The radical scavenging ability assay was carried out in a flat-bottom 96-well microtiter plate, according to the colorimetric method described by Chatatikunand Chiabchalard,[49] with slight modifications. A volume of 160 μL with a series of doses of each test sample and standard antioxidant, BHT (12.50, 25.00, 50.00, 100.00, 200.00, 400.00, 800.00 μg/mL) was added to 40 μL of 0.25 mM methanolic DPPH solution in a 96-well plate. All reagents were mixed and incubated for 15 min at room temperature protected from light. The absorbance of each well was measured at 517 nm with a Microplate Reader (Biotek). The percentage inhibition was calculated using Equation (1). The SC$_{50}$ values of all the crude extracts were calculated using GraphPad Prism 7 Statistical Software. The experiment was carried out in triplicates.

$$\% \text{ Activity } = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \tag{1}$$

Anti-inflammatory Assay (HRBC Stabilization Method)

The anti-inflammatory assay was conducted using the human red blood cells (HRBCs) stabilization method, which uses the heat-induced hemolysis principle.[50] Test samples and the positive control Aspirin (1 mg of each) were dissolved in 0.5 mL of dimethyl sulfoxide (DMSO) and diluted up to 5 mL by addition of normal saline. A half dilution series was prepared with a control for each sample and the reaction mixture was prepared with 2.5 mL of the solution and 0.25 mL of 10% v/v RBC suspension. All the centrifuge tubes containing reaction mixtures were incubated in a water bath at 56 °C for 30 min after which the tubes were cooled under running tap water. The reaction mixture was centrifuged at 3000 rpm for 10 min to obtain a clear liquid phase and the absorbance of the supernatants was measured at 560 nm using Microplate Reader (Biotek) by using aliquots of 200 µL from respective mixtures for each well in a 96 well-plate. The test was performed in triplicates and the percentage stability was calculated using Equation (1). IC_{50} values were calculated using the statistical software GraphPad Prism 7..

Tyrosinase Inhibitory Assay

Tyrosinase inhibitory assay was carried out in a flat-bottom 96-well microtiter plate according to a modified version of the method described by Souza et al[21] Crude extracts and positive control kojic acid were dissolved in 10% DMSO in phosphate buffer (pH 6.5) and a half dilution series of doses was prepared for each (0.0, 10.41, 20.83, 41.66, 83.33, 166.60, 333.30 µg/mL). An aliquot of 70 µL of each extract dilution was combined with 30 µL of tyrosinase (333 U/mL). Following incubation at room temperature for 5 min, 110 µL of substrate (2 mM L-3,4-dihydroxyphenylalanine [L−DOPA]) was added to each well. Incubation was continued for 30 min at room temperature under dark conditions and absorbance was measured at 490 nm with a Microplate Reader (Biotek). The percentage inhibition was calculated using the Equation (2).

$$\% \text{ Inhibition} = \frac{(A_{\text{control}} - A_{\text{blank}}) - (A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100 \qquad (2)$$

Antibacterial Assay (agar Well Diffusion Method)

Antibacterial assays against aerobic bacterial species *E. coli*, *B. subtilis*, and *S. aureus*, and the anaerobic bacterium *S. mutans* were conducted using the agar well diffusion method.[51] For the aerobic strains, an aliquot of overnight cultures in the nutrient broth of each bacterial strain was diluted 100 times and 100 µL was spread evenly on the surface of the nutrient agar plates. Wells with a diameter of 0.5 cm were made in the plates using a cork borer. Crude extracts and Azithromycin (positive control) were dissolved in DMSO to meet a 5 mg/mL concentration. The wells were filled with 100 µL of the test solutions while keeping a negative control of DMSO. After an incubation of 24 h at room temperature under aerobic conditions, the diameters of observable inhibition zones were measured in cm.

In the case of the anaerobic species, an aliquot of overnight cultures in trypticase soy broth (supplemented with blood with lysed RBC and grown under anaerobic conditions in a jar containing anaerobic sachets at 37 °C) was used in the same manner as previously described on trypticase soy agar (supplemented with lysed RBC) plates. Well and extract preparation and filling were also conducted in a similar manner with similar concentrations and volumes. Chlorhexidine (0.2%) was used as the positive control. After an incubation of 24 h at 37 °C under anaerobic conditions, the diameters of observable inhibition zones were measured in cm.

Analyzing LC–MS Profile of Crude Extracts

LC–MS dereplication was carried out for some extracts to discern interesting active ingredients and novel molecules present in them. Chromatographic separation of crude extracts was performed on an Agilent 1290 Infinity 2 utra high performance liquid chromatography (UHPLC) system (with photodiode-array (PDA) detector, electrospray ionization (ESI) source, quadrupole time-of-flight (Q-TOF) analyzer, 5 µm and 4.6 × 250 mm C_{18} column) using a 1 mL/min solvent gradient. Samples with a concentration of 5 mg/mL were prepared in LC–MS graded methanol. The solutions were filtered through a 0.22 µM durapore filter and transferred to clean and dry vials. In total, 5 µL was injected into the column from each extract. 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 DNP and SciFinder, using genus and species level specificity for rationalization of the obtained profiles. TLC visualization was also carried out to aid the validation of the presence of different metabolites in the extract. The visualizing was conducted under UV wavelengths 254 and 366 nm and derivatization was conducted using anisaldehyde–sulfuric acid reagent (0.5 mL anisaldehyde, 10 mL glacial acetic acid, 5 mL concentrated sulfuric acid and 85 mL methanol).

Conclusions

ELF has become a promising source for providing novel bioactive compounds and this study adds more evidence to that already proven statement. In the present study, 58 ELF strains, isolated from 29 lichens collected from mangrove and mangrove associated plants of Negombo lagoon, Sri Lanka, were identified using molecular and morphological techniques. In order to determine antioxidant, anti-inflammatory, tyrosinase inhibitory, and antibacterial potency, ethyl acetate extracts of ELF belonging to 18 species were

utilized. *D. eschscholtzi* was found to possess the highest antioxidant and anti-inflammatory activity in DPPH and HRBC stabilization assays, respectively. This is the first report of anti-inflammatory activity for this fungus thus far. The highest tyrosinase inhibitory activity was found for *C. xylocarpi* while making it the first report of tyrosinase inhibition reported for this species. The significant activity was shown against the aerobic bacteria *E. coli*, *B. subtilis*, and *S. aureus* by *X. feegenesis*, and anaerobic bacterium *S. mutans* by *C. lunata*, also becomes the first time report from an ELF. However, further studies are required to determine the bioactive compounds responsible for these activities of ELF extracts.

The LC–MS dereplication study conducted for the same 18 extracts revealed that they are comprised of interesting metabolites reported from the literature while containing some unknown major compounds as well. Many major peaks observed in the extracts of ELF *N. occulatum*, *H. lividipigmentum,* and *M. rubricosum* have shown no hit in any of the applied filters of species, genera and fungal category, suggesting further exploration for novel scaffolds.

Author Contributions

Experimentation: Collection, isolation, screening and data analysis: Ramani Weerasinghe; Collection assistance and phylogenetic analysis: Kasun Maduranga; LCMS analysis: Chaitrali Shevkar; Fungal molecular identification and analysis: Renuka Attanayake; Lichen identification: Gothamie Weerakoon; LCMS analysis and dereplication: Abhijeet Kate, Kiran Kalia. Writing-original draft: Ramani Weerasinghe, Kasun Maduranga, Chaitrali Shevkar. Writing-review and editing: Renuka Attanayake, Gothamie Weerakoon, Abhijeet Kate, Kiran Kalia, Priyani Paranagama. Funding acquisition: Priyani Paranagama. Overall supervision: Priyani Paranagama.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

ORCID iD

Priyani A. Paranagama https://orcid.org/0000-0001-5489-081X

Supplemental Material

Supplemental material for this article is available online.

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