

RESEARCH ARTICLE

A secondary metabolite with *in vitro* radical scavenging activity from endolichenic fungus *Daldinia eschscholzii* found in lichen, *Parmotrema* sp. in Sri Lanka

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Abstract: Endolichenic fungi, an unexplored group of microorganisms, are a promising source of bioactive compounds. Secondary metabolites were isolated from the chloroform fraction of crude ethyl acetate extract of endolichenic fungus *Daldinia eschscholzii* inhabiting the lichen, *Parmotrema* sp. in Sri Lanka. Two pure compounds, 1 and 2 were isolated and the structures were identified using ¹H-, ¹³C-, 2D- nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) data. Compound 1 did not show any radical scavenging activity in DPPH assay. Compound 2, identified as 8-methoxynaphthalen-1-ol showed strong radical scavenging ability in the DPPH assay with a half maximal inhibitory concentration (IC₅₀) of 10.2 ± 5.8 µg/ mL. The activity of compound 2 was higher than that of the standard, butylated hydroxy toluene (BHT).

Keywords: *Daldinia eschscholzii*, endolichenic fungus, radical scavenging activity, secondary metabolites.

INTRODUCTION

Natural products with biological activities are promising leads for novel therapeutic agents (Koehn & Carter, 2005). Isolation and characterisation of bioactive natural compounds as pharmacophores have been the focus of ongoing research. Fungi are well known producers of naturally occurring bioactive compounds (Samanthi *et al.*, 2014). Endolichenic fungi (ELF), analogous

to plant endophytes (Wang *et al.*, 2010) are one of the ecological groups of fungi that occur asymptotically within the lichen thalli (Kannangara *et al.*, 2009). Although they have shown a great potential to be a rich source of structurally diverse bioactive compounds, there are only a few studies reported (Kulasekera *et al.*, 2013; Pary *et al.*, 2013; Samanthi *et al.*, 2013; 2015a; 2015b) on isolation and identification of secondary metabolites from ELF (Paranagama *et al.*, 2007). ELF in Sri Lanka still remains almost untapped as a source of bioactive compounds (Samanthi *et al.*, 2015a).

The objective of this study was to isolate secondary metabolites with radical scavenging activity from the ELF *Daldinia eschscholzii* inhabiting the lichen *Parmotrema* sp. in the Hakgala Botanical Garden, Sri Lanka and screen them for biological activity. Two major compounds were isolated. The isolation procedures and the results of bioactivity screening are reported here.

METHODOLOGY

¹H-, ¹³C- and 2D- NMR spectra for the isolated compounds were recorded in CDCl₃ with Bruker Ascend TM 400 Spectrometer at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR. LCMS data were recorded on

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Waters e 2695 separations module using 2998 PDA detector and Zspray™ SQ mass detector 2. Thin layer chromatography (TLC) (analytical and preparative) was performed on 0.2 mm pre-coated silica (60 Å pore size, Fluka, Germany) plates with fluorescent indicator 254 nm.

Isolation of the endolichenic fungal species

The lichen *Parmotrema* sp., was collected from Hakgala Botanical Garden located at an altitude of about 1745 m. Samples were transported in sterile polythene bags to the laboratory at the Department of Chemistry, University of Kelaniya and were processed within 24 h.

Surface sterilisation method (Kannangara *et al.*, 2009) was used to isolate fungal species from the lichen thalli. Healthy lichen thalli cleaned up with tap water were surface-sterilised by sequential immersion in 95 % ethanol, 0.5 % sodium hypochloride and 75 % ethanol for 10 s, 3 min and 30 s, respectively. The surface of the thalli was dried with sterile filter papers. Sterilised lichen thalli were aseptically cut into small pieces of 1×1 cm and plated on 2 % malt extract agar containing 0.01 % streptomycin (five replicate thalli samples of the selected lichen species, 20 small segments from each). Plates were incubated at room temperature (28 ± 2 °C) for 14 d. Pure cultures of different types of fungi growing from each lichen segment were isolated by sub-culturing from actively growing edge.

The growing fungi sub-cultured on 2 % MEA were photographed and vegetative mycelia of each fungus in sterile water were deposited at room temperature in the Department of Chemistry, University of Kelaniya.

Screening of endolichenic fungi for antioxidant activity

A small-scale analysis was carried out for the crude ethyl acetate extracts of 6 endolichenic fungi sp. selected based on the absence of previous studies for isolation and identification of antioxidant active compounds. Each fungus was grown on 5 PDA plates (150 × 25 mm) and secondary metabolites were extracted after 1 week of incubation at room temperature (28 ± 2 °C). The crude ethyl acetate extracts of the selected endolichenic fungi were evaluated for their radical scavenging activity using DPPH assay to identify promising candidates for further studies.

Extraction and isolation of bioactive compounds from selected fungus

Daldinia eschscholzii, which showed a higher *in vitro* radical scavenging activity in DPPH assay during small-scale analysis was cultivated on 48 PDA plates (150 × 25 mm) and incubated at room temperature (28 ± 2 °C). After one week of incubation, mycelia were cut into small pieces along with the PDA medium and secondary metabolites were extracted into ethyl acetate. The extract was dried with anhydrous Na₂SO₄ and the solvent was evaporated under reduced pressure. The dark brown semi-solid crude extract (1.5 g) was subjected to antioxidant assay using DPPH method. The extract was found to be an active radical scavenger. A portion (1.4 g) of the bioactive crude extract was dissolved in 100mL of aqueous 80 % methanol and extracted with hexane (2 × 100 mL). The methanol fraction was diluted with distilled water to a 60 % aqueous methanolic solution and extracted with chloroform (2 × 100 mL). The hexane, chloroform and aqueous methanol fractions were separately dried with anhydrous Na₂SO₄ and solvents were evaporated under reduced pressure to yield 222 mg, 660 mg and 37 mg of the fractions, respectively. The insoluble sticky material from the crude extract that could be particles from fungal cultures was removed during partitioning. All three fractions were subjected to DPPH assay. The chloroform fraction which had the highest antioxidant activity was then subjected to bioassay guided fractionation using silica gel column chromatography.

Silicagel column (70–230 mesh, 19.2 g, 2.5 cm × 53 cm) was packed with dichloro methane (CH₂Cl₂). A slurry of sample (510 mg) was packed on top of the column and was eluted with 100 % CH₂Cl₂ followed by CH₂Cl₂ containing increasing amounts of methanol and finally with 100 % methanol. Fractions were analysed by TLC (developed in 4% methanol in CH₂Cl₂ and observed for UV absorption at 254 nm). The solvent was evaporated and eight fractions, F1 to F8 were obtained. Fraction F2 (25 mg) which had radical scavenging activity was then subjected to gel-filtration column chromatography on Sephadex LH-20 made up in hexane (1.0 g, 1 cm × 30 cm). The sample was eluted with 100% hexane, hexane containing increasing amounts of CH₂Cl₂, 100 % CH₂Cl₂, followed by CH₂Cl₂ containing increasing amounts of methanol and finally with 100 % methanol. Column fractions were combined after running the TLC (developed in 50% hexane in CH₂Cl₂ and observing under UV absorption at 254) to obtain three combined

fractions F2-1, F2-2 and F2-3. Fraction F2-1 (16 mg), which showed the presence of a compound with radical scavenging ability upon qualitative analysis by spraying DPPH on TLC plate, was further purified using normal phase preparative thin layer chromatography (PTLC) (40 % CH_2Cl_2 in hexane) to isolate pure compound 1 (6 mg) and the fraction F2-1-2 (5 mg) with slight impurity. The fraction F2-1-2 was subjected to PTLC (30 % CH_2Cl_2 in hexane) to remove the impurity and isolate pure compound 2 (4 mg).

Structure elucidation of the isolated pure compounds was done with the aid of 1D-, 2D- NMR and mass spectroscopic data.

2, 2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability

Assay was carried out in a 96-well plate according to the method described by Chatatikun and Chiabchalard (2013) with slight modifications. Butylated hydroxy toluene (BHT) and methanol were used as the positive and negative controls, respectively. A volume of 160 μL of each dilution of test sample was mixed with 0.25 mM methanolic DPPH solution (40 μL). Absorbance measurements were taken at 517 nm with the micro plate reader (Thermo Scientific Multiskan GO, Finland) after 20 min of incubation at room temperature in the dark. The percentage inhibition was calculated and plotted against the concentration to determine the IC_{50} .

Morphological and molecular identification of the endolichenic fungus

Identification keys (Barron, 1968) were used in morphological identification of pure fungus culture prepared on slides using sticky tape method (Felgel, 1980).

Genomic DNA extraction and amplification followed by sequencing was used in molecular identification of the pure strain of endolichenic fungus used for the study (Samanthi *et al.*, 2015a). Fungal DNA extraction was carried out using Promega Wizard® Genomic DNA Purification Kit. Ribosomal internal transcribed spacer (ITS) region analysis of extracted DNA, and selective amplification by polymerase chain reaction (PCR) with the use of ITS 1 and 4 primers (Arnold & Lutzoni, 2007) were used to identify the fungus. The excess nucleotides, remaining primers and enzymes etc., in the PCR product (500-600 bp) were removed using a gel purification

protocol before sequencing. First, the amplified DNA bands that are of interest were separated from undesired bands using agarose gel electrophoresis. Promega Wizard® SV Gel and PCR Clean-Up System were used (Promega, 2002) to isolate DNA from the gel. Nucleotide sequence of the amplified, purified DNA was submitted to GenBank as a nucleotide query. The nucleotide sequence was compared with existing nucleotide sequences using NCBI BLAST® (<https://www.ncbi.nlm.nih.gov/nucore/> MN862623) to identify the fungal strain.

RESULTS AND DISCUSSION

Out of a total of 11 different fungi species isolated and identified from the lichen *Parmotrema* sp. based on the morphological and molecular studies, the fungus BM/ F7, identified as *D. eschscholzii* revealed a higher *in vitro* radical scavenging activity in small-scale analysis and was selected for further analysis. The lichen identification was done as described in Kannangara *et al.* (2009) and this paper presents results of further study. The fungus has a white colour hyphae that grows from

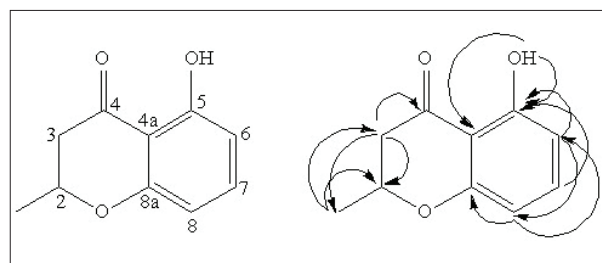


Figure 1: Structure and selected HMBC correlations of compound 1 isolated from the chloroform fraction of *D. eschscholzii*

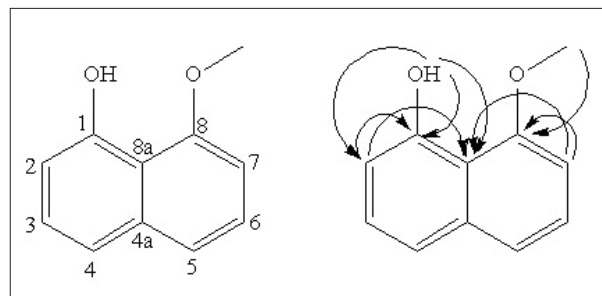


Figure 2: Structure and selected HMBC correlations of compound 2 isolated from the chloroform fraction of *D. eschscholzii*

the site of inoculation and during sporulation it turns to smoky gray colour. The reverse side of the PDA plate with the fungus appeared blackish-gray colouration. The nucleotide sequences matched 99 % with the existing *D. eschscholzii* sequences at the GenBank (Accession No. MN862623).

The *in vitro* radical scavenging activity of the crude extract of *D. eschscholzii* was determined using DPPH assay and the activity was compared with that of the standard antioxidant, BHT. The standard showed higher activity (~ 67 % inhibition at 50 µg/ mL) than the crude extract (~ 36 % inhibition at 50 µg/ mL). Figure 3 shows the DPPH radical scavenging activity for hexane, chloroform and methanol fractions separated from ethyl acetate extract of *D. eschscholzii* and pure compound 2.

According to results, the chloroform fraction showed the highest activity ($IC_{50} = 63.8 \pm 4.8$ µg/ mL) out of all three fractions. However, activity of chloroform fraction was higher than that of ethyl acetate extract ($IC_{50} = 77.9 \pm 5.1$ µg/ mL) and less than that of BHT ($IC_{50} = 38.2 \pm 4.0$ µg/ mL). The hexane fraction showed the lowest activity.

Bioassay guided fractionation of the chloroform fraction using column chromatography and PTLC yielded two pure compounds; 1 and 2 and only compound 2 showed radical scavenging activity.

The spectral data analysis confirmed the structure for compound 1 as 5-hydroxy-2-methylchroman-4-one. ¹H- NMR (400 MHz, chloroform-*d*) and ¹³C NMR

Table 1: ¹³C (100 MHz, CDCl₃) and ¹H (400 MHz, CDCl₃) NMR data for compound 1

Position	δ ¹³ C	δ ¹ H (multiplicity, nH, J/Hz)	HMBC
2	73.9	4.63 - 4.55 (m, 1H)	
3	43.8	2.69 (dd, 1H, 17.2, 3.7, H-a) 2.77 (dd, 1H, 17.2, 11.9, H-b)	2-CH ₃ , 2, 4
4	198.5		
4a	108.0		
5	162.2		
6	109.3	6.52 (dd, 1H, 8.3, 0.8)	8, 5
7	138.2	7.37 (t, 1H, 8.3)	5
8	107.2	6.45 (dd, 1H, 8.3, 0.8)	6, 4, 8a
8a	161.0		
2-CH ₃	20.9	1.53 (d, 3H, 6.3)	3, 2
5-OH		11.73 (s, 1H)	5, 4a

Table 2: ¹³C (100 MHz, CDCl₃) and ¹H (400 MHz, CDCl₃) NMR data for compound 2

Position	δ ¹³ C	δ ¹ H (multiplicity, nH, J/Hz)	HMBC
1	154.5		
2	110.4	6.90 (dd, 1H, 7.4, 0.9)	1, 8a
3	118.9 - 127.7	7.29 - 7.44 (m, 1H)	
4	118.9 - 127.7	7.29 - 7.44 (m, 1H)	
4a	136.7		
5	118.9 - 127.7	7.29 - 7.44 (m, 1H)	
6	118.9 - 127.7	7.29 - 7.44 (m, 1H)	
7	103.8	6.81 (bd, 1H, 7.7)	8, 8a
8	156.1		
8a	115.1		
1-OH		9.36 (s, 1H)	1, 2, 8a
8-OMe	56.1	4.09 (s, 3H)	8

(100 MHz, chloroform-*d*) data of compound 1 (Figure 1) are given in Table 1. This compound has previously been reported from a soil fungus *Daldinia loculata* (Nadeau & Sorensen, 2011) and during a biosynthetic study of Aspyrone (Copeland *et al.*, 1984).

Molecular formula for compound 2 was determined as C₁₁H₁₀O₂ with six degrees of unsaturation on the basis of pseudomolecular ion at m/z 175.05 (calculated for C₁₁H₁₀O₂). NMR data are given in Table 2. The compound (Figure 2) is a slightly yellow coloured solid.

The ¹³C NMR along with DEPT 90, DEPT 135 and HMQC data indicated the presence of ten aromatic/olefinic carbons of which six were protonated (δ_c 103.8, 110.4, 118.9, 121.9, 125.6 and 127.7), two were oxygenated (δ_c 156.1, δ_c 154.5) and two were quaternary carbons (δ_c 115.1 and 136.7). In addition ¹³C NMR data also revealed the presence of one methoxy carbon (δ_c 56.1).

HMBC correlation analysis (Figure 2) allowed the assignment of signals and substitutions on the carbon skeleton. The -OH group was placed at C-1 with the help of HMBC correlations of the proton (δ_H 9.36) with C-1 (δ_c 154.5), C-2 (δ_c 110.4) and C-8a (δ_c 115.1). HMBC correlations of protons at δ_H 4.09 with the C-8 (δ_c 156.1) suggested the attachment of the methoxy group to the C-8. HMQC data analysis showed clear correlations of protons at δ_H 4.09 with carbon at δ_c 56.1. Aromatic methine protons at δ_H 6.81 showed HMBC correlations with C-8 (δ_c 156.1), C-8a (δ_c 115.1), and δ_H 6.90 with C-1 (δ_c 154.5), C-8a (δ_c 115.1) which helped the assigning of the protons as H-7 and H-2 directly attached to C-7 (δ_c 103.8) and C-2 (δ_c 110.4), respectively. The

four remaining methine carbons C-3–C-6 were shown at δ_c 118.9, 121.9, 125.6 and 127.7, and the corresponding protons in the range δ_H 7.29 - 7.44. The spectroscopic data analysis and comparison with the values reported in literature (Nadeau & Sorensen, 2011) confirmed the identification of compound 2 as 8-methoxynaphthalen-1-ol. The compound has been reported earlier from the fungus *Daldinia loculata* in soil (Nadeau & Sorensen, 2011), from endophytic fungus, *Coniothyrium* sp. (Krohn *et al.*, 2008) and from roots of *Engelhardia roxburghiana* (Wu *et al.*, 2007).

The pure compound 2 showed strong radical scavenging activity with an IC₅₀ value of 10.2 ± 5.8 µg/ mL, compared to BHT (Figure 3).

CONCLUSION

In this study, two major compounds were isolated from the ethyl acetate extract of *D. eschscholzii* using bioassay guided fractionation. The structures of compounds 1 and 2 were identified using spectroscopic data, of which only one compound (8-methoxynaphthalen-1-ol) showed high radical scavenging activity in DPPH assay. The activity was even higher than that of the standard BHT.

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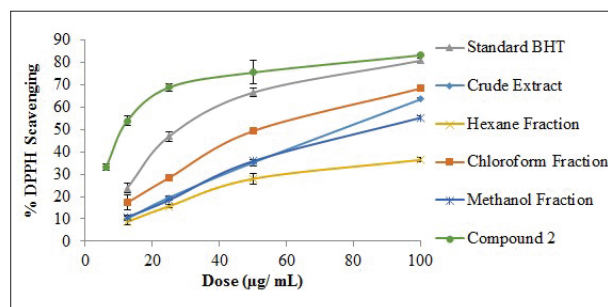


Figure 3: *In vitro* DPPH radical scavenging activity of crude extract, hexane, chloroform, methanol fractions of *D. eschscholzii* and compound 2 isolated from the chloroform fraction compared with the positive control BHT. Each value is expressed as mean ± SD (n=3).

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