Phytochemistry Letters 66 (2025) 24–30

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Phytotoxicity of secondary metabolites from a fungal endophyte *Muyocopron laterale* isolated from leaves of *Centella asiatica*

Kavindya Samarakoon [a], Dilhara Dissanayake [a], Nilupa R. Amarasinghe [b], Savitri Kumar [a], Deepthi Yakandawala [c], Nimal Adikaram [a], Lalith Jayasinghe [a,*], Hiroshi Araya [d], Yoshinori Fujimoto [a,d]

[a] National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka
[b] Department of Pharmacy, University of Peradeniya, Sri Lanka
[c] Department of Botany, University of Peradeniya, Sri Lanka
[d] School of Agriculture, Meiji University, Kawasaki, Kanagawa 214-8571, Japan

ARTICLE INFO

Keywords:
Endophytic fungus
Centella asiatica
Muyocopron laterale
Polyketides

ABSTRACT

An endophytic fungus isolated from the leaves of a common medicinal plant, *Centella asiatica*, was identified as *Muyocopron laterale* through molecular means. The fungus was cultured in potato dextrose broth medium. After five weeks, the broth and mycelium were extracted with EtOAc. Chromatographic separation of the combined EtOAc extract yielded two new polyketides, named muyokopyrone (**3**) and muyokoenone (**5**), along with four known compounds, austdiol (**1**), 4-(hydroxymethyl)-3-methoxy-5-methylcyclopent-2-enone (**2**), eugenitin (**4**) and 6-methoxymethyl eugenin (**6**). The fermentation broth showed necrotic and wilting symptoms when sprayed on the leaves of cucumber (*Cucumis sativus*) plants. The EtOAc extracts of the broth and mycelium showed necrotic symptoms in the leaf puncture assay using cucumber leaves. The EtOAc extract of the broth inhibited root and shoot elongation with IC_{50} values of 23.7 μg/mL and 55.8 μg/mL, respectively in the lettuce seed germination assay. Compound **1** inhibited root elongation of lettuce seeds with an IC_{50} value of 5.38 μg/mL while compounds **1** and **2** showed necrotic symptoms in the leaf puncture assay.

1. Introduction

Secondary metabolites are a highly diverse group of natural products and act as an inexhaustible source of leads for many uses such as new antimicrobials, anti-tumour drugs, many other pharmacological and agricultural uses (Dias et al., 2012; Vicente et al., 2003). Fungal endophytes show a great biological diversity and each of the wide range of host plants harbours one or more endophytic fungal species (Jia et al., 2016; Strobel and Daisy, 2003). Therefore, endophytic fungi are a rich source of compounds with various applications including agriculture. We have previously reported some phytotoxic metabolites from endophytic fungi and plants (Piyasena et al., 2015; Gunawardena et al., 2015). In a continuation of our research work towards the search for bioactive compounds from Sri Lankan flora, the chemistry and bioactivity of secondary metabolites produced by endophytic fungi from *Centella asiatica* were studied. *C. asiatica* (L.) Urb. commonly known as Gotukola in Sri Lanka, belongs to the family Apiaceae. It has been used for various medicinal and cosmetic purposes, including for the treatment of various skin conditions, diarrhoea, fever, and diseases in the female genito-unirnary tract since prehistoric times (Brinkhaus et al., 2000). Furthermore, the traditional uses of *C. asiatica* have been supported by several in-vitro, pre-clinical and clinical studies which included wound healing, anticancer, antimicrobial, neuroprotective, antidiabetic, hepatoprotective, antioxidant, anxiolytic, antidepressant, antiepileptic and anti-inflammatory activities (Gohil et al., 2010; Prakash et al., 2017). Synthetic weedicides and herbicides have been a threat to the environment and health for decades and weed resistance to herbicides has become a major concern. The discovery of efficient and less hazardous herbicides has become important and bioactive secondary metabolites from endophytic fungi have shown to be promising sources of novel compounds that can be used in agriculture, including weed control (Macías-Rubalcava and Garrido-Santos, 2022). In this study, we report the isolation of two novel compounds together with four known compounds from an endophytic fungus *Muyocopron laterale* isolated from the

* Correspondence to: National Institute of Fundamental Studies, Kandy, Sri Lanka.
E-mail address: lalith.ja@nifs.ac.lk (L. Jayasinghe).

https://doi.org/10.1016/j.phytol.2025.01.004
Received 31 October 2024; Received in revised form 26 December 2024; Accepted 8 January 2025
Available online 11 February 2025

leaves of *C. asiatica* and their phytotoxic activity.

2. Methods

2.1. General methods

Silica gel coated aluminum sheets (Merck 1.05554.0007, 60 F_{254}, Darmstadt, Germany) were used for TLC and silica gel coated glass plates (Merck 1.05715.0001, 60 F_{254}, Darmstadt, Germany) were used for preparative thin layer chromatography (PTLC). Silica gel (Merck Art. 7734 and 9385, Darmstdt, Germany) or Sephadex® LH-20 (Fluka, Switzerland) were used for chromatography. High performance liquid chromatography (HPLC) was performed with Shimadzu, SPD-20A UV–VIS detector, LC-6A Liquid Chromatograph, SCL-6A System controller and C-R6A Chromatopac equipped with an ODS column. Inertsil Ph-3 HPLC column (5 µm, 4.6 x 250 mm, GL Sciences, Japan) was used,when stated. Nuclear Magnetic Resonance (NMR) spectra were recorded on a JNM-ECP500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) or JMN-AL-300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer (JEOL, Japan) in $CDCl_3$ or CD_3OD. IR spectra were recorded on an FT-IR 4200 spectrometer (JASCO, Japan). UV spectra were obtained on a MD-4017 photodiode array detector (JASCO, Japan). Optical rotations were measured on a P-2200 polarimeter (JASCO, Japan). HR-ESI-MS was obtained with an Impact II instrument (Bruker, USA). Spectrophotometric readings were obtained from a Synergy HTX multimode plate reader (Biotek Instruments, USA).

2.2. Isolation of endophytic fungi

Healthy, fresh leaves of *C. asiatica* were collected from a home garden in Kandy, Sri Lanka. Leaves were washed gently with running water. Then leaves were blot-dried on sterile tissue papers and the leaf surfaces were sterilized in the laminar flow cabinet with 96 % EtOH for 1 minute, 2.5 % NaOCl for 1 minute, 96 % EtOH for 30 seconds, and finally with sterile distilled water and this procedure was repeated three times to achieve triple sterilization. Segments (5 mm×5 mm) of triple sterilized leaf samples were then placed aseptically on the surface of Petri dishes (90 mm) poured with potato dextrose agar (PDA) medium and incubated at room temperature (25°C) for 5–7 days. Plates were examined for the emergence of fungi from leaf segments and repeated subculturing was done to obtain a pure fungal strain of the emerged fungus.

2.3. Identification of the endophytic fungus

2.3.1. DNA extraction

A pure culture of the emerged fungus grown on PDA for about 14 days was used for the DNA extraction. DNA extraction was performed using Promega Wizard® Genomic DNA Purification Kit A1120 (Promega Corporation, USA). Mycelia were scraped from the culture using a sterile blade and was performed according to the manufacturer's protocol with modifications where 20 µL of Proteinase K (20 mg/mL) (Promega Corporation, USA) was added after cooling to room temperature, following the addition of cell lysis solution (20 µL), nuclei lysis solution (20 µL) and incubating for 1 hour at 65°C. After the addition of Proteinase K, incubation was carried out for 1 hour at 65°C and allowed to cool to room temperature. Protein precipitation solution (20 µL) was added next followed by vortexing and centrifuging at 14000 rpm for 5 minutes. The supernatant was transferred to a new Eppendorf tube, and it was centrifuged again at 14000 rpm for 5 minutes. The supernatant was transferred again to a new Eppendorf tube and 600 µL of ice-cold isopropanol was added followed by incubation in ice for 5 minutes. Then it was centrifuged at 14000 rpm for 15 minutes and the supernatant was discarded. Then, 600 µL of 70 % EtOH was added and centrifuged at 14000 rpm for 1 minute. The supernatant was discarded, and the tube was allowed to air dry to remove any traces of EtOH, after which 100 µL of DNA rehydration solution was added. Finally, the DNA sample was stored at –20°C freezer (Kurera et al., 2023).

2.3.2. PCR amplification and identification

Internal Transcribed Spacer (ITS) region of the rDNA was amplified using the primer pair ITS 1-F- 5' CTT GGT CAT TTA GAG GAA GTA A 3' (Gardes and Bruns, 1993) and ITS 4 – 5' TCC TCC GCT TAT TGA TAT GC 3' (White et al., 1990). Forward and reverse primers were purchased from Integrated DNA Technologies Inc., USA. All polymerase chain reaction (PCR) amplifications were carried out using the Promega - 20 µL GoTaq® Green Master Mix, 0.8 µL (10 µM) of each forward and reverse primers, and 5 µL of unquantified DNA template, 1.6 µL $MgCl_2$ (25 mM), 2 µL dimethylsulfoxide (DMSO), 0.8 µL Taq Polymerase (5 units/µL) and topped up to a total volume of 40 µL with nuclease-free water. The PCR reaction was performed using a thermal cycler (Applied Biosystems Veriti). The thermal cycler was programmed to perform the PCR reactions using an initial denaturation at 95°C for 4 minutes, 35 cycles of denaturation at 95°C for 30 seconds and annealing at 54°C, for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes and then at 4°C (Weir et al., 2012; Wickramasinghe et al., 2020). The amplified PCR products were separated by electrophoresis in 1 % agarose gel (Sigma-Aldrich, Type I-B) in 1x TAE (Tris-Acetate-EDTA) buffer. Gel was run at 120 V for 30 minutes loaded with 3 µL of PCR product and stained with ethidium bromide and visualized with a UV transilluminator (UviTec – BTS-20.MS) at 254 nm. The PCR products were sequenced (Applied Biosystems, 3500 genetic analyzer) at the Department of Molecular Biology, University of Peradeniya. Sequenced data of the gene regions of the fungus were edited by manually editing ambiguous bases using BioEdit Sequence Alignment Editor (Version 7.2.5). Edited and aligned forward and reverse sequences were subjected to a similarity-based search in the National Centre for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST) for nucleotides (Blastn) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find similar sequences.

2.4. Large scale culturing of endophytic fungus in liquid media

Large-scale culturing of the fungus was carried out in potato dextrose broth (PDB) media. Erlenmeyer flasks (1 L x 90 flasks), each containing 400 mL of PDB were sealed with cotton wool plugs covered in aluminium foil and were sterilized by autoclaving for 20 minutes at 121°C and 15 psi. Each flask with the media was then inoculated with the fungus and were allowed for undisturbed incubation for 10 days and then incubated while shaking on a laboratory shaker (Stuart® SSL1 and SSL2) (90 rpm) till 5 weeks until extraction at room temperature. The resulting culture broth was filtered, and the filtrate was extracted with EtOAc. Mycelium was extracted to EtOAc by sonicating for 30 minutes three times. Removal of the solvent in vacuo afforded an oily residue (4.5 g) from the broth and an oily residue (3.0 g) from the mycelium.

2.5. Determination of phytotoxicity

2.5.1. Broth spraying assay

Cucumber (*Cucumis sativus)* seeds were planted on a potting mixture of sand: coir dust: compost (1:1:1) and were watered regularly to obtain plantlets for three weeks. The assay was conducted as stated in Brun et al. (2016) with modifications. Freshly filtered broth (5 mL) of the fungus was sprayed onto the whole plant including either side of all the leaves. The control experiment was done in the same manner with an un-inoculated PDB medium. Both test and control pots were watered and were kept in a greenhouse. Observations on wilting properties of plants and leaves were taken for 7 days daily.

2.5.2. Leaf puncture assay

The assay was conducted as stated by Brun et al. (2016) with some modifications. The leaf puncture assay was conducted using 2-week-old well-expanded Cucumber (*Cucumis sativus*) leaves. Leaves were removed

from the plant with a sterile blade and the stem end was cut slightly. Petri dishes (90 mm) with filter papers (Whatman®) were poured with 2 mL of distilled water. One leaf per plate was placed on the filter paper making sure the cut-end of stalk was in contact with the wet filter paper. Each leaf was punctured slightly with a sterile fine pointed needle on the upper surface on either side of the midvein to make small wounds/punctures. Samples were dissolved in distilled water to obtain a serial dilution and 10 µL of the test solution was placed on each puncture. Control was done with distilled water. Both test and control were done in triplicates. Petri dishes were then placed in a humidity chamber. Observations were taken at 24, 48 and 72 hours with photographic evidence. The diameter of leaf injury if present was measured after 72 hours.

2.5.3. Lettuce seed germination inhibition assay

The assay was conducted as stated in Piyasena et al. (2015) with some modifications. *Lactuca sativa* (Lettuce) seeds (Rapido 344, East-West Seed International Ltd., Nonthaburi, Thailand) were washed with distilled water and floating seeds were discarded. The remaining seeds were blot dried. Test samples were dissolved in distilled water or 2 % DMSO. Samples were prepared in a concentration series with triplicates for each concentration. For crude extracts, Petri dishes with filter papers (Whatman®) were poured with 2 mL of sample. For compounds, 6-well plates with filter papers were poured with 400 µL of test solution of compounds. Ten lettuce seeds were placed on each filter paper. Plates were sealed with parafilm and kept for 5 days at room temperature under dark conditions. After five days, root length and shoot length were measured. Distilled water or 2 % DMSO was used as the negative control. Abscisic acid was used as a positive control. The percentage inhibition of root and shoot elongation were calculated against control as given below.

$$\%\ \text{Inhibition of root elongation} = \frac{\text{Control root length} - \text{Test sample root length}}{\text{Control root length}} \times 100$$

2.6. Isolation and identification of compounds

The TLC patterns of EtOAc extracts of culture broth and mycelium of the fungus were found to be similar. Therefore, the EtOAc extracts of broth and mycelium were combined. The combined EtOAc extract (7.5 g) was chromatographed over silica gel with increasing polarity of hexane, EtOAc and MeOH to give five fractions (Fr-1 to Fr-5). Fr-1 was further chromatographed over silica gel with increasing polarity of hexane, CH_2Cl_2 and MeOH furnishing five fractions (Fr-1–1 to Fr-1–5). Fr-1–1 was subjected to HPLC separation using an ODS column (MeOH-water, 85:15) to give eugenitin (**4**) (6.7 mg) (Andrioli et al., 2014). Fr-1–2 was similarly separated by HPLC (MeOH-water, 80:20) to give three major fractions. Compound (16.7 mg) eluted in the first major fraction was identified as 6-methoxymethyleugenin (**6**) (Feng et al., 2002). The second major fraction was further purified by HPLC using Inertsil Ph-3 column eluting with MeOH-water 2.5:1 to give muyokoenone (**5**) (2.0 mg). The third major fraction afforded additional amounts of compound **4** (2.5 mg). Fr-3 (770 mg) was further chromatographed over silica gel with increasing polarity of hexane and CH_2Cl_2. The fraction eluted with hexane-CH_2Cl_2, 1:2 was chromatographed over Sephadex LH-20 eluting with MeOH to obtain two fractions (Fr-3–1 and Fr-3–2). Fr-3–1 was subjected to HPLC separation using an ODS column eluting with MeOH-water, 70:30 to give two major UV-absorbing fractions, more mobile fraction containing compound **2** and less mobile containing compound **3**. Purification of the more mobile fraction by PTLC (developed with $CHCl_3$-ethanol, 20:1) afforded 4-(hydroxymethyl)-3-methoxy-5-methylcyclopent-2-enone (**2**) (2.5 mg) (Nong et al., 2013). Further purification of the less mobile fraction by HPLC using Inertsil Ph-3 column with eluting MeOH-water, 2.5:1 yielded muyokopyrone (**3**) (2.0 mg). By rinsing Fr-3–2 with $CHCl_3$ austdiol (**1**) (35.5 mg) (Andrioli et al., 2014; Vleggaar et al., 1974) was obtained. The known compounds were identified by comparison with published spectral data.

Muyokopyrone (3) Yellowish gum; $[\alpha]_D^{22}$ + 127.4 (c 0.42, MeOH); UV λ_{max} 252 and 356 nm (MeOH); IR (KBr) ν_{max} 3425, 2960, 2925, 1660, 1615, 1535 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 2; negative-ion HR-ESI-MS *m/z* 371.1500 [M - H]$^-$ (calcd for $C_{21}H_{23}O_6$, 371.1495).

Muyokoenone (5) Yellowish gum; $[\alpha]_D^{22}$ + 173.0 (c 0.15, MeOH); UV λ_{max} 232 nm (MeOH); IR (KBr) ν_{max} 3440, 2960, 2930, 2880, 1720, 1682, 1600, 1455 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 3; negative-ion HR-ESI-MS *m/z* 447.2388 [M - H]$^-$ (calcd for $C_{25}H_{35}O_7$, 447.2383).

3. Results and discussion

The endophytic fungus isolated from the leaves of *C. asiatica* was identified as *Muyocopron laterale* since the sequence of ITS regions of the fungal rDNA gene matched 98.79 % percentage identity with those of *M. laterale* (GenBank accession No. NR_164055.1). The mycelium of the fungus was off-white/pale yellow and was seen with black colour spots/pigmentation on the reverse surface of the fungus (Fig. 1).

The fungal broth was subjected to broth spraying assay. Necrotic leaf symptoms were observed on cucumber leaves sprayed with the fungal broth after 24 h. Further, the leaves showed wilting symptoms between 24 h and 72 h after spraying. Small plants were completely wilted while others showed increased necrotic symptoms on leaves. Plants sprayed with un-inoculated broth did not show any injury symptoms. The broth and mycelium EtOAc extracts were subjected to the leaf puncture assay. Leaf necrosis with surrounding chlorosis was shown for both EtOAc extracts at 1000, 500, and 250 µg/mL after 24 h with increasing necrosis by 72 h. The broth EtOAc extract and mycelium EtOAc extract showed the highest leaf injury at 1000 µg/mL with 1.5 ± 0.2 cm and 0.7 ± 0.10 cm respectively after 72 h (Table 1). Leaves treated with distilled water (control) did not show any necrotic or chlorosis symptoms (Fig. 2).

The broth EtOAc extract showed a potent phytotoxic effect on root and shoot elongation inhibition with IC_{50} values of 23.7 µg/mL and 55.8

Table 1
Diameter of necrotic symptoms of leaves treated with EtOAc extracts and compounds **1** and **2**.

Concentration (µg/mL)	Average diameter of leaf injury (cm) at 72 h (n = 6)			
	Broth EtOAc extract	Mycelium EtOAc extract	Compound **1**	Compound **2**
1000	1.5 ± 0.2	0.7 ± 0.10	1.4 ± 0.05	0.8 ± 0.05
500	1.2 ± 0.5	0.4 ± 0.00	1.4 ± 0.00	0.3 ± 0.00
250	0.5 ± 0.1	0.3 ± 0.10	1.3 ± 0.07	0.2 ± 0.00
125	ND	ND	1.3 ± 0.27	NA
62.5	ND	ND	0.7 ± 0.26	NA
31.25	ND	ND	0.4 ± 0.50	NA

ND, not determined; NA, not active.

Table 2
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **3** (in $CDCl_3$).

No	δ_C	δ_H (mult., J in Hz)
1	144.5, 144.6	7.35 (s), 7.34 (s)
3	159.5, 159.6	———
4	103.6, 103.4	6.00 (s), 5.94 (s)
4a	143.1	———
5	111.3, 111.0	———
6	198.0	———
7	76.2, 76.1	———
8	72.4	4.46 (s), 4.43 (s)
8a	119.8, 120.9	———
9	29.4, 29.6	2.54 (m), 2.66 (m)
10	19.7, 19.6	2.17 (s), 2.16 (s)
11	18.7	1.07 (s), 1.06 (s)
1′	34.1, 34.5	3.27 (m)
2′	166.8, 166.5	———
3′	119.6, 119.4	———
4′	180.2, 180.1	———
5′	112.2, 112.1	6.07 (s), 6.04 (s)
6′	164.3	———
7′	9.6	1.82 (s), 1.80 (s)
8′	19.9	1.25 (d, 10.0), 1.24 (d, 10.1)
9′	18.1, 17.8	2.28 (s), 2.27 (s)

Table 3
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **5** (in $CDCl_3$).

No	δ_C	δ_H (mult., J in Hz)
1	200.5	———
2	130.7	———
3	145.1	———
4	35.7	2.58 (m), 2.93 (brd, 9.8)
5	74.6	5.35 (q, 1.8)
6	74.4	———
7	49.9	3.53 (d, 17.8), 3.60 (d, 17.8)
8	202.9	———
9	66.6	3.30 (d, 11.5)
10	195.8	———
11	128.9	6.06 (d, 10.1)
12	150.8	7.04 (ddd, 10.1, 6.0, 2.6)[a])
13	33.0	2.16 (m), 2.59 (m)
14	31.7	2.65 (m)
15	11.8	1.84 (brs)
16	23.6	1.43 (s)
17	19.8	1.04 (d, 6.4)
1′	175.2	———
2′	43.9	2.58 (dq, 8.2, 7.3)[a])
3′	75.6	3.55 (dd, 8.2, 3.2)[a])
4′	36.4	1.42 (m)[a])
5′	26.8	1.27 (m)
6′	11.7	0.89 (t, 7.3)
7′	12.4	0.83 (d, 6.9)
8′	14.4	1.08 (d, 7.3)

[a])The *J* values were determined by HOMO decoupling studies.

μg/mL, respectively, in lettuce seed germination inhibition assay. The mycelium EtOAc extract showed phytotoxicity with IC_{50} values of 470.4 μg/mL and 842.2 μg/mL for root and shoot elongation inhibition, respectively.

Due to the presence of phytotoxic properties in the crude extracts, the combined EtOAc extract was separated by chromatography to isolate two new polyketides, named muyokopyrone (**3**) and muyokoenone (**5**), and four known compounds, austdiol (**1**) (Andrioli et al., 2014; Vleggaar et al., 1974), 4-(hydroxymethyl)-3-methoxy-5-methylcyclopent-2-en one (**2**) (Nong et al., 2013), eugenitin (**4**) (Andrioli et al., 2014) and 6-methoxymethyleugenin (**6**) (Feng et al., 2002) (Fig. 4).

The phytotoxicity of the isolated compounds was determined using the lettuce seed germination assay and the leaf puncture assay. In the leaf puncture assay, the leaves treated with compound **1** started showing larger leaf necrosis and chlorosis symptoms by 48 h in 1000 μg/mL to 125 μg/mL and necrotic patches and chlorosis were seen as low as 31.25 μg/mL by 72 h. The compound **1** showed the highest leaf injury at 1000 μg/mL with 1.4 ± 0.05 cm and the lowest at 31.25 μg/mL with 0.4 ± 0.05 cm. Leaves treated with compound **2** showed smaller leaf necrotic patches by 48 h in 1000 μg/mL and 500 μg/mL and the necrotic patches were increased by 72 h, but leaves treated with concentrations below 250 μg/mL only showed slight chlorosis with no measurable necrotic symptoms. For the compound **2**, the highest leaf injury was at 1000 μg/mL with 0.8 ± 0.05 cm and the lowest at 250 μg/mL with 0.2 ± 0.00 cm (Table 1). Control leaves did not show any necrotic or chlorosis symptoms (Fig. 3).

Compound **1** showed root inhibition with 88.9 % and shoot inhibition of 36.4 % at 100 μg/mL, and IC_{50} values were determined as 5.38 μg/mL and 805 μg/mL, respectively. At the same concentration, compound **2** showed lower root inhibition (12.5 %) and shoot inhibition (20.0 %) than compound **1**. Abscisic acid used as the positive control showed IC_{50} of 0.99 μg/mL for root inhibition and 1.11 μg/mL for shoot inhibition. Compounds **3**-**6** did not show any phytotoxicity in the lettuce seed germination assay and the leaf puncture assay.

M. laterale is an Ascomycetes fungi belonging to the order Muyocopronales with *Mycoleptodiscus lateralis* as a basionym (Sutton and Alcorn, 1990). *M. laterale* has been isolated as an endophytic fungus from several plant families. Compounds **4** and **6** were previously isolated from *M. laterale* (Nakashima et al., 2020). Compound **2** is a rare secondary metabolite that has only been reported from a marine-derived fungus *Xylariaceae* sp. (Nong et al., 2013). The relative structure for compound **2** is depicted in Fig. 4 (the $J_{H\text{-}4,H\text{-}5}$ value was 3.0 Hz in agreement with the literature data), since absolute stereochemistry has not been established.

Recently *M. laterale* was found to cause brown blight disease of tea plants (Jiang et al., 2022). Metabolites from the *Mycoleptodiscus indicus* UFSM54 isolated from the leaves of *Conyza* sp. with disease symptoms were reported to show phytotoxic effects on germination and seedling growth and lesion development of some commercial plants and weeds (Portela et al., 2022). A fungal pathogen *Mycoleptodiscus terrestris* was shown to produce necrotic shoot lesions and disintegration of an aquatic weed, Eurasian watermilfoil under experimental conditions (Verma and Charudattan, 1993). It was reported that austdiol (**1**) did not show herbicidal activity at 100 μg/mL against a fast-germinating grass *Eragrostis tef* (Valter, 2022). In the present study, austdiol (**1**) showed high root inhibition against lettuce seeds at 100 μg/mL and leaf necrosis of cucumber at 1000 μg/mL, which shows the potential of this compound for the development of new herbicides. 6-Methoxymethyleugenin (**6**) has shown phytotoxic effects on both aseptic *Pinus armandi* seedlings and field *P. armandi* seedlings (Li et al., 2012). However, compound **6** did not show any phytotoxicity in our assay.

Structures of new compounds **3** and **5** were determined as follows. Compound **3** was isolated as 4:3 epimeric mixture at the C-1' position. The compound had the molecular formula, $C_{21}H_{24}O_6$ (by HR-ESI-MS). The NMR data of **3** was closely similar to those of the known compounds dothideomynones E and F, which were isolated from the endophytic fungus *Dothideomycete* sp. (Wijesekera et al., 2017). The ^{1}H NMR spectrum of **3** showed signals of five methyl groups at δ_H 1.07/1.06, 1.25/1.24, 1.82/1.80, 2.17/2.16 and 2.28/2.278, one methylene group at δ_H 2.54 and 2.66 (H_2-9), two sp^3 methine protons at δ_H 3.27 (H-1′) and 4.46/4.43 (H-8), and three sp^2 methine protons at δ_H 6.07/6.04 (H-5'), 6.00/5.94 (H-4) and 7.35/7.34 (H-1) (Table 2). The ^{13}C NMR spectrum had 21 sets of signals (seven sets were identical shifts) attributable to five methine, one methylene, five methyl, and ten non-hydrogen-bearing carbons. The two downfield carbons at δ_C 198.0 (C-6) and 180.2/180.1 (C-4′) were of ketones, while the carbons at δ_C 76.2/76.1 (C-7) and 72.4 (C-8) were of oxygenated sp^3 carbons. The COSY spectrum of **3** displayed coupling of the methine proton at δ_H 3.27 (H-1′) and the methylene proton at δ_H 2.54 and 2.66 (H_2-9) as well as H_3-8′ at δ_H 1.25/1.24, and allylic coupling of H-4/H_3-10 and H-1/H-8. The HMBC spectrum of **3** showed the correlations from H-1 to C-3, C-4a, C-8 and C-8a; H-4 to C-3, C-8a and C-10; H-8 to C-1, C-4a, C-7, C-8a and

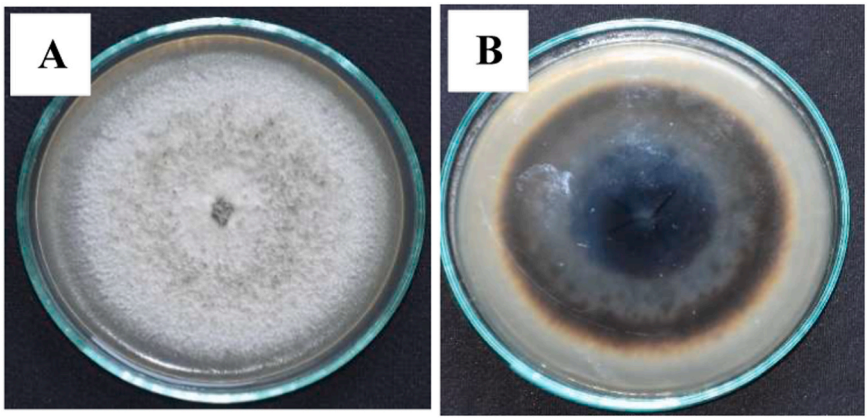

Fig. 1. Culture of *M. laterale* grown on PDA medium. A: Upper surface, B: Reverse surface.

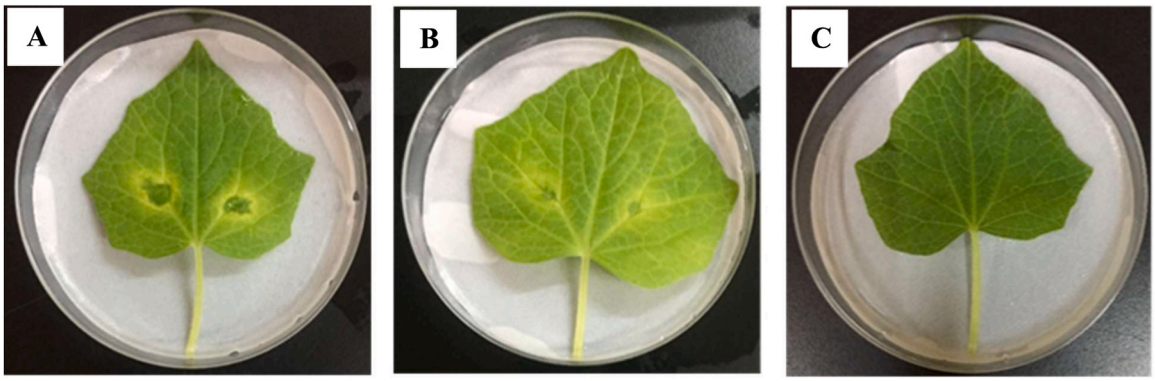

Fig. 2. Leaf injury symptoms of the leaf puncture assay at 1000 μg/mL. (A) Broth EtOAc extract, (B) Mycelium EtOAc extract, and (C) Distilled water.

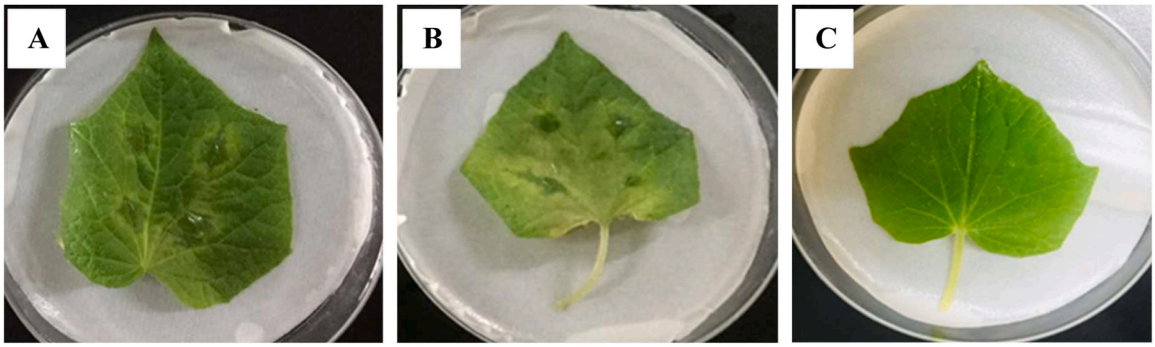

Fig. 3. Leaf injury symptoms of the leaf puncture assay at 1000 μg/mL. (A) Compound **1**, (B) Compound **2**, and (C) Distilled water.

C-11; H_3-10 to C-3 and C-4; H-9 to C-4a, C-5 and C-1′; H_3-11 to C-7, C-6 and C-8; H-5′ to C-3′ and C-6′; H_3-9′ to C-5′ and C-6′; H_3-7′ to C-2′, C-3′ and C-4′; H_3-8′ to C-9, C-1′ and C-2′. The HMBC correlations mentioned above established the partial structure from C-1 to C-11, which was identical to that of austdiol (**1**) carbon skeleton except that the aldehyde of **1** was changed to a CH_2 group. The aforementioned HMBC correlations of **3** also established the structure linked to the C-9 position of the core azaphilone as 1-(3,6-dimethyl-4-oxo-4H-pyran-2-yl) ethyl moiety. Therefore, the gross structure of compound **3** was determined as shown in Fig. 4. The relative and absolute (7 *R*,8*S*-configuration) stereochemistry of the core azaphilone from C-1 to C-11 of **3** can be considered to be the same as that of austdiol (**1**), which was also isolated in the present work and is thought to share the similar biosynthetic origin. Therefore, the epimeric stereo center was assigned at C-1′ as found in dothideomynone E and dothideomynone F, and muyokopyrone (**3**) was determined to be a C-1′ epimeric mixture of the structure shown in Fig. 4. Careful comparison of the NMR of data of dothideomynone E and dothideomynone F supported the structure of **3**.

Compound **5**, named muyokoenone, had a molecular formula $C_{25}H_{36}O_7$ (by HR-ESI-MS). The IR spectrum of **5** showed absorption bands for carbonyl peaks at 1720, 1682 and 1600 cm^{-1}, while ^{13}C NMR demonstrated signals for three ketones (δ_C 202.9, 200.5 and 195.8) and a carbonyl ester (δ_C 175.2). ^{13}C NMR and DEPT experiments revealed 25 signals including six methyl, four methylene, six sp^3 methine, two sp^2 methine and three non-protonated carbons. ^1H–^1H COSY spectrum of **5** showed the connectivity of H-5 and H_2-4; from H-9 to H-11 passing through H-14, H-13, H-12 including H_3-17. The ^1H–^1H COSY spectrum also established a structure of 3-hydroxy-2,4-dimethylhexanoic acid unit in **5**, showing correlations from H-2′ along the chain to H_3-6′, and correlations of H-4′/H_3-8′, and H-2′/H_3-7′. In addition, homoallylic

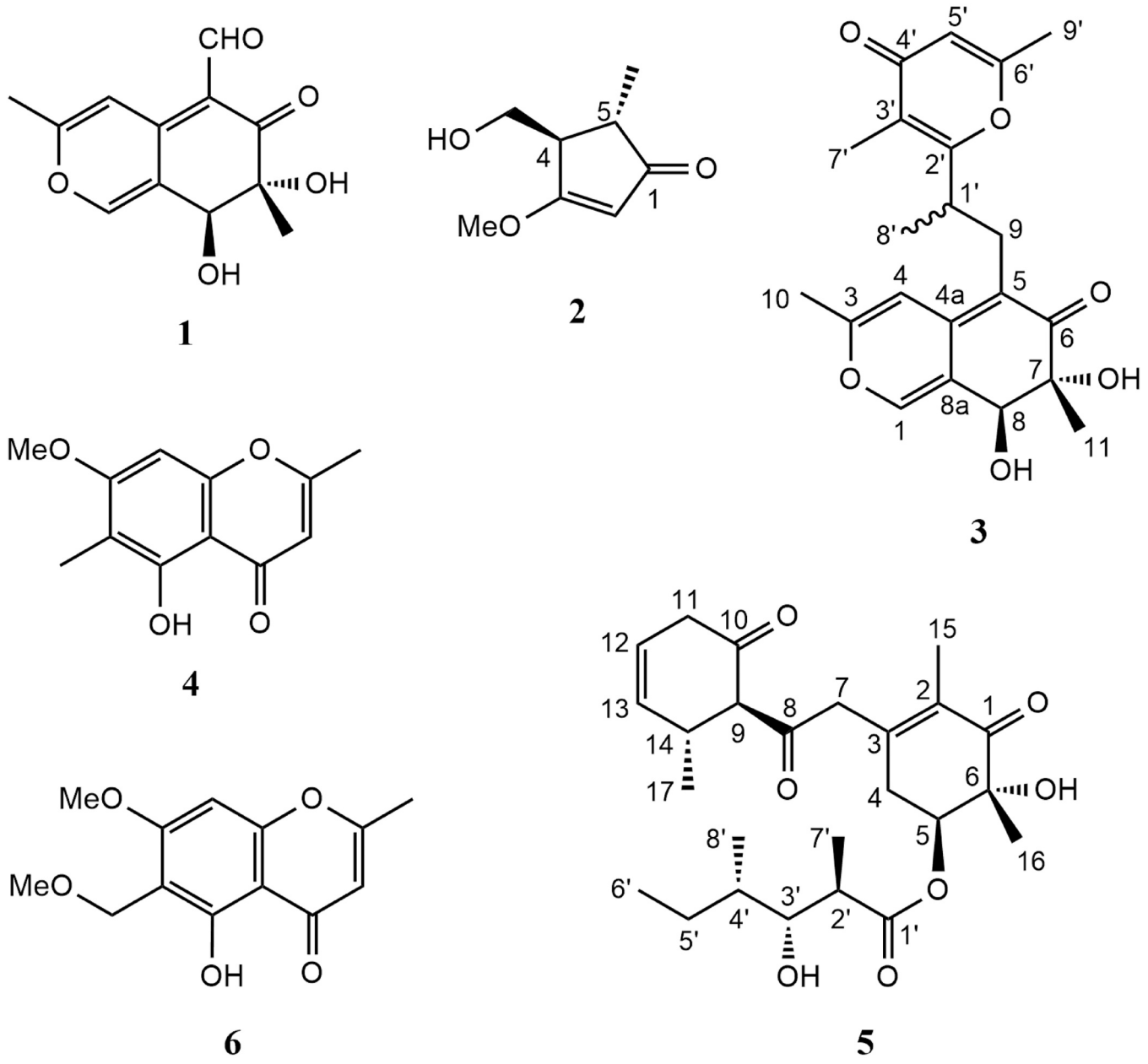

Fig. 4. Chemical structure of compounds **1**-**6**.

coupling between H_3-15 and H_2-4 was observed in the ^1H–^1H COSY spectrum. The HMBC spectrum of **5** showed the following correlations: H-5 to C-1, C-3, C-6 and C-1′; H_2-4 to C-2 and C-3; H_3-16 to C-1, C-5 and C-6; H_3-15 to C-1, C-2, C-3; H_2-7 to C-2, C-3, C-4 and C-8; H-9 to C-8, C-10, C-13 and C-17; H-11 to C-13; H-13 to C-11 and C-12; H_3-17 to C-9, C-13 and C-14. Upon these spectroscopic data, a gross structure of **5** was unambiguously established. Assignments of ^1H and ^{13}C resonances for **5** are shown in Table 3. The assigned structure was found to be similar to that of dothideomycetone A, which was isolated from the endophytic fungus *Dothiodemycete* sp. (Senadeera et al., 2012). The NMR data of **5** was in good agreement with those of dothideomycetone A except for the cyclohexenone moiety containing C-9. Thus, the relative and absolute stereochemistry for the core C-1 to C-6 cyclohexenone moiety was assumed to be (5*S*,6 *R*) as shown in Fig. 4, which is the same as in the biosynthetically correlated compounds **1** and **3**. A large coupling constant of $J_{H\text{-}9,H\text{-}14} = 11.5$ Hz clearly indicated trans orientation of H-9 and H-14, although the absolute configuration at C-9 and C-14 has not been elucidated. In Fig. 4, the same (9*S*,14*R*) configuration as that of dothideomycetone A is tentatively shown. As for 3-hydroxy-2,4-dimethylhexanoyl moiety, it is known that $J_{H\text{-}2,H\text{-}3}$ vicinal coupling constant for anti diastereoisomer (7.0–9.0 Hz) are larger than those for the syn isomer (5.9–6.3 Hz) (Senadeera et al., 2012). Also, the $J_{H\text{-}3,H\text{-}4}$ for 3-hydroxy-2,4-dimethylhexanoic acid derivatives of anti diastereoisomer (8.5 Hz) is larger than those of syn isomer (5.7 Hz) (Senadeera et al., 2012). The relative stereochemistry of the 3-hydroxy-2,4-dimethylhexanoyl moiety of **5** was determined as anti-orientation of H-2′ and H-3′ and syn relationship between H-3′ and H-4′ on the basis of $J_{H\text{-}2',H\text{-}3'}$ of 8.2 Hz and $J_{H\text{-}3',H\text{-}4'}$ of 3.2 Hz. The absolute configuration of the hexanoyl moiety was not determined due to the limited amount of sample, but the same stereochemistry as dothideomycetone A was tentatively depicted in Fig. 4. A biosynthetic pathway of dothideomycetone A from an austditol-like compound has been proposed (Senadeera et al., 2012).

4. Conclusion

The endophytic fungus *Muyocopron laterale* was isolated from the leaves of the medicinal plant *Centella asiatica* for the first time. Large-scale cultivation of the fungus in PDB medium led to the isolation of six compounds, including two new polyketides, muyokopyrone (**3**) and muyokoenone (**5**). Phytotoxicity of the broth and the isolated secondary metabolites were assessed using broth spraying and leaf puncture assays on cucumber and lettuce seed germination assay. Austdiol (**1**) exhibited necrotic activity in the leaf puncture assay and inhibited lettuce root elongation with an IC_{50} of 5.38 μg/mL. The phytotoxicity demonstrated by the fungal broth as well as austdiol (**1**) and 4-(hydroxymethyl)-3-methoxy-5-methylcyclopent-2-enone (**2**) suggests the potential use of the fungal metabolites as herbicides or weedicides.

CRediT authorship contribution statement

Jayasinghe Lalith: Writing – review & editing, Supervision, Project administration, Conceptualization. **Adikaram Nimal:** Writing – review & editing, Supervision. **Yakandawala Deepthi:** Resources, Methodology. **Kumar Savitri:** Funding acquisition. **Fujimoto Yoshinori:** Writing – review & editing, Data curation. **Araya Hiroshi:** Writing – review & editing, Data curation. **Amarasinghe Nilupa R:** Writing – review & editing, Methodology. **Dissanayake Dilhara:** Methodology, Investigation. **Samarakoon Kavindya:** Writing – original draft, Methodology, Investigation.

Declaration of Competing Interest

Authors have no conflict of interest to declare.

Acknowledgements

Financial support from the National Research Council Sri Lanka (Grant number NRC 18-03) is gratefully acknowledged.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phytol.2025.01.004.

Data availability

No data was used for the research described in the article.

References

Andrioli, W.J., Conti, R., Araújo, M.J., Zanasi, R., Cavalcanti, B.C., Manfrim, V., Toledo, J.S., Tedesco, D., De Moraes, M.O., Pessoa, C., Cruz, A.K., Bertucci, C., Sabino, J., Nanayakkara, D.N.P., Pupo, M.T., Bastos, J.K., 2014. Mycoleptones A-C and polyketides from the endophyte *Mycoleptodiscus indicus*. J. Nat. Prod. 77 (1), 70–78. https://doi.org/10.1021/np4006822.

Brinkhaus, B., Lindner, M., Schuppan, D., Hahn, E.G., 2000. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomedicine 7 (5), 427–448. https://doi.org/10.1016/S0944-7113(00)80065-3.

Brun, T., Rabuske, J.E., Todero, I., Almeida, T.C., Junior, J.J.D., Ariotti, G., Confortin, T., Arnemann, J.A., Kuhn, R.C., Guedes, J.V.C., Mazutti, M.A., 2016. Production of bioherbicide by *Phoma sp*. In a stirred-tank bioreactor. 3 Biotech 6 (2). https://doi.org/10.1007/s13205-016-0557-9.

Dias, D.A., Urban, S., Roessner, U., 2012. A historical overview of natural products in drug discovery. Metabolites 2 (2), 303–336. https://doi.org/10.3390/metabo2020303.

Feng, Y., Blunt, J.W., Cole, A.L.J., Munro, M.H.G., 2002. The isolation of two new chromone derivatives from the New Zealand fungus *Tolypocladium extinguens*. J. Nat. Prod. 65 (11), 1681–1682. https://doi.org/10.1021/np020160j.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2 (2), 113–118. https://doi.org/10.1111/j.1365-294x.1993.tb00005.x.

Gohil, K.J., Patel, J.A., Gajjar, A.K., 2010. Pharmacological review on *Centella asiatica*: a potential herbal cure-all. Indian J. Pharm. Sci. 72 (5), 546–556. https://doi.org/10.4103/0250-474X.78519.

Gunawardena, D.C., Jayasinghe, L., Fujimoto, Y., 2015. Phytotoxic constituents of the fruits of Averrhoa carambola. Chem. Nat. Compd. 51, 532–533. https://doi.org/10.1007/s10600-015-1332-6.

Jia, M., Chen, L., Xin, H.L., Zheng, C.J., Rahman, K., Han, T., Qin, L.P., 2016. A friendly relationship between endophytic fungi and medicinal plants: a systematic review. Front. Microbiol. 7, 1–14. https://doi.org/10.3389/fmicb.2016.00906.

Jiang, H., Zhang, M., Zhou, Y., Li, X., Li, J., Song, C., 2022. First report of *Muyocopron laterale* causing a new leaf disease of *Camellia sinensis* in China. Beverage Plant Res. 2 (1), 1–4. https://doi.org/10.48130/BPR-2022-0010.

Kurera, W.M.S., Adikaram, N.K.B., Yakandawala, D.M.D., Maharachchikumbura, S.S., Jayasinghe, L., Samarakoon, K., 2023. Molecular and phenotypic characterization of *Colletotrichum plurivorum* and *Colletotrichum musae* causing banana anthracnose disease in the Central Province of Sri Lanka. J. Natl. Sci. Found. Sri Lanka 51 (2), 311–326. https://doi.org/10.4038/jnsfsr.v51i2.11217.

Li, X.J., Gao, J.M., Chen, H., Zhang, A.L., Tang, M., 2012. Toxins from a symbiotic fungus, *Leptographium qinlingensis* associated with *Dendroctonus armandi* and their in vitro toxicities to *Pinus armandi* seedlings. Eur. J. Plant Pathol. 134 (2), 239–247. https://doi.org/10.1007/s10658-012-9981-9.

Macías-Rubalcava, M.L., Garrido-Santos, M.Y., 2022. Phytotoxic compounds from endophytic fungi. Appl. Microbiol. Biotechnol. 106 (3), 931–950. https://doi.org/10.1007/s00253-022-11773-w.

Nakashima, K.I., Tomida, J., Tsuboi, T., Kawamura, Y., Inoue, M., 2020. Muyocopronones A and B: azaphilones from the endophytic fungus Muyocopron laterale. Beilstein J. Org. Chem. 16 (1), 2100–2107. https://doi.org/10.3762/bjoc.16.177.

Nong, X.H., Zheng, Z.H., Zhang, X.Y., Lu, X.H., Qi, S.H., 2013. Polyketides from a marine-derived fungus *Xylariaceae sp*. Mar. Drugs 11 (5), 1718–1727. https://doi.org/10.3390/md11051718.

Piyasena, K.G.N.P., Wickramarachchi, W.A.R.T., Kumar, N.S., Jayasinghe, L., Fujimoto, Y., 2015. Two phytotoxic azaphilone derivatives from *Chaetomium globosum*, a fungal endophyte isolated from *Amaranthus viridis* leaves. Mycology 6 (3–4), 158–160. https://doi.org/10.1080/21501203.2015.1089332.

Portela, V.O., Moro, A., Santana, N.A., Baldoni, D.B., de Castro, I.A., Antoniolli, Z.I., Dalcol, I.I., Jacques, R.J.S., 2022. First report on the production of phytotoxic metabolites by Mycoleptodiscus indicus under optimized conditions of submerged fermentation. Environ. Technol. 43 (10), 1458–1470. https://doi.org/10.1080/09593330.2020.1836030.

Prakash, V., Jaiswal, N., Srivastava, M., 2017. A review on medicinal properties of *Centella asiatica*. Asian J. Pharm. Clin. Res. 10 (10), 69. https://doi.org/10.22159/ajpcr.2017.v10i10.20760.

Senadeera, S.P.D., Wiyakrutta, S., Mahidol, C., Ruchirawat, S., Kittakoop, P., 2012. A novel tricyclic polyketide and its biosynthetic precursor azaphilone derivatives from the endophytic fungus *Dothideomycete* sp. Org. Biomol. Chem. 10 (35), 7220–7226. https://doi.org/10.1039/C2OB25959A.

Strobel, G., Daisy, B., 2003. Bioprospecting for microbial endophytes and their natural product. Microbiol. Mol. Biol. Rev. 67 (4), 491–502. https://doi.org/10.1128/MMBR.67.4.491.

Sutton, B.C., Alcorn, J.L., 1990. New species of *Mycoleptodiscus* (Hyphomycetes). Mycol. Res. 94 (4), 564–566. https://doi.org/10.1016/S0953-7562(10)80025-5.

Valter, S., 2022. Exploring the Chemical Space around Austdiol by Semi-synthesis. Macquarie University. https://doi.org/10.25949/21951032.v1.

Verma, U., Charudattan, R., 1993. Host range of *Mycoleptodiscus terrestris*, a microbial herbicide candidate for *Eurasian Watermilfoil*, *Myriophyllum spicatum*. Biol. Control 3 (4), 271–280. https://doi.org/10.1006/bcon.1993.1036.

Vicente, M.F., Basilio, A., Cabello, A., Peláez, F., 2003. Microbial natural products as a source of antifungals. Clin. Microbiol. Infect. 9 (1), 15–32. https://doi.org/10.1046/j.1469-0691.2003.00489.x.

Vleggaar, R., Steyn, P.S., Nagel, D.W., 1974. Constitution and absolute configuration of austdiol, the main toxic metabolite from *Aspergillus utus*. J. Chem. Soc. 1, 45–49. https://doi.org/10.1039/P19740000045.

Weir, B.S., Johnston, P.R., Damm, U., 2012. The *Colletotrichum gloeosporioides* species complex. Stud. Mycol. 73, 115–180. https://doi.org/10.3114/sim0011.

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, pp. 315–322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1.

Wickramasinghe, P., Adikaram, N., Yakandawala, D., 2020. Molecular characterization of *Colletotrichum* species causing Begonia anthracnose in Sri Lanka. Ceylon J. Sci. 49 (5), 363–371. https://doi.org/10.4038/cjs.v49i5.7803.

Wijesekera, K., Mahidol, C., Ruchirawat, S., Kittakoop, P., 2017. Metabolite diversification by cultivation of the endophytic fungus Dothideomycete sp. in halogen containing media: cultivation of terrestrial fungus in seawater. Bioorg. Med. Chem. 25 (11), 2868–2877. https://doi.org/10.1016/j.bmc.2017.03.040.