

Bioactive Compounds from an Endophytic Fungi *Nigrospora Aurantiaca*

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Abstract

Background:

Many groups of fungi live as an endophyte in plants. Endophytic fungi could produce the bioactive compounds which was new or known compounds. Various biological activities of bioactive compounds from endophytic fungi had been reported, including anti-inflammatory and anticancer.

Chemical investigation and biological activities of bioactive compounds from endophytic fungi from *Melaleuca leucadendra* Linn. have not yet been reported

Results: One new compound, namely nigauidiol (**1**), together with five known compounds, xyloketal K (**2**), bostrycin (**3**), deoxybostrycin (**4**), xylanthraquinone (**5**), and ergosterol (**6**), were isolated from the *Melaleuca leucadendra* Linn. associated fungal strain *Nigrospora aurantiaca* #TMU062. Their chemical structures were elucidated by spectroscopic data and compared with literatures. All isolated compounds were evaluated for NO production inhibitory activity in LPS-activated microglial BV-2 cells.

Conclusions: Compound **6** exhibited markedly inhibitory activities on NO production with IC₅₀ values of $7.2 \pm 1.4 \mu\text{M}$ and the survival of the cells was $90.8 \pm 6.7\%$ at the concentration of $10 \mu\text{M}$.

Background

Endophytes are defined as the microorganisms that inhabit at least parts of their life cycle in its host plants without causing apparent harm to the host (Hardoim et al. 2015). Endophytic fungi are one of the potential resources for obtaining bioactive compounds because of the complex interaction between endophytic fungi and their host plants or with other microorganisms in the host plants. Previous studies showed that many bioactive compounds produced by endophytic fungi exhibiting antioxidant, anticancer, anti-inflammatory, antimicrobial, or other biological activities (Kumari et al. 2018; Ukwatta et al. 2020). Some of the medicinal plants have been found to exist a number of highly diversified endophytic fungi, which could even produce the same compounds as in their host plants, such as ginkgolide B produced by both *Fusarium oxysporum* and its host plant *Ginkgo biloba* (Cui et al. 2012). Thus, many of the folk medicinal plants were chosen to screen the associated fungal strains with significant biological activities in the recent past.

Melaleuca leucadendra Linn. belongs to the family Myrtaceae, which is distributed in Australia to Southeast Asia including Indonesia (Pujiarti et al. 2011). The leaves in this family are known containing a high concentration of terpenes in qualitative and quantitative variation (Keszei et al. 2008). As a folk medicine, *M. leucadendra* Linn. was reported to exhibit antioxidant, antiproliferative, and anticancer activities (Rini et al. 2012; Monzote et al. 2020). However, the activity and chemical investigation of the endophytic fungi from *M. leucadendra* Linn. have not yet been reported. This study focused on the chemical investigation and the bioactivity of *Nigrospora aurantiaca* isolated from *M. leucadendra* Linn.

Methods

General Experimental Procedures

^1H , ^{13}C , DEPT, and 2D NMR were acquired on Agilent DD2 600 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). Optical rotation was measured on a JASCO-P-2000 polarimeter (Tokyo, Japan). IR spectra were recorded on a JASCO FT/IR 4100 spectrometer (Tokyo, Japan). Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) was used for open column chromatography. High-resolution mass spectrometry data were acquired using Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to the Dionex UltiMate™ 3000 RSLCnano UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA). Semi-preparative HPLC experiments for compound purification were performed using HPLC pump L-7100 (Hitachi, Japan) with refractive index (Bischoff, Leonberg, Germany) for detector.

Fungal Material

The fungal strain *Nigrospora aurantiaca* was isolated from a healthy leaf of *Melaleuca leucadendra* linn collected in National Taiwan University Campus yard and was identified by sequencing of the internal transcribed spacer regions of the rDNA (ITS). A BLAST search of the sequence led to the best match as *Nigrospora aurantiaca*. Mycelium *Nigrospora aurantiaca* #TMU062 was inoculated into two different media, liquid medium and solid medium. Inoculated on liquid media using 5 L serum bottles, each containing 50 gram malt extract (Becton, Dickinson and Company, Sparks, USA) add 3.5 L deionized water. The fermentation was conducted with aeration at 25–30°C for 14 days. Solid medium using 250 mL flasks, each containing 20 g of barley and 0.2 g of potato dextrose agar (Becton, Dickinson and Company, Sparks, USA), add 15 mL of deionized water, and then fermentation for 30 days at 27–30°C.

Extraction and Isolation

The fermented broth (9.5 L) was filtered and partitioned five times with equal volumes of EtOAc and then concentrated under vacuum to obtain crude extract (5.8 g). The crude extract was re-dissolved in 50 mL MeOH to obtain MeOH layer and sediment (2.3 g), then the sediment was dissolved in 10 mL DMSO and purified by HPLC semipreparative reversed-phase column (Phenomenex Luna PFP, 5 μm , 10 $\times$ 250 mm, Torrance, CA, USA) eluted by 65% MeOH, 2 mL/min, to obtain **3** (t_R : 12 min; 50.0 mg) and three fractions (Fr.S2–Fr.S4). Further purification of Fr.S3 on HPLC on a semipreparative reversed-phase column (Thermo Hypersil HS C18, 5 μm , 10 $\times$ 250 mm, Bellefonte, PA, USA) eluted by 50% MeOH, 2 mL/min, to obtain **4** (t_R : 9 min; 4.9 mg). The MeOH layer was concentrated under vacuum until around 15 mL, then applied into a Sephadex LH-20 column (2.5 i.d. $\times$ 68.5 cm) eluted by MeOH with a flow rate of 2.5 mL/min to give forty-five fractions (25 mL) and then combined into seven fractions as Fr.A – Fr.G based on similar compositions of TLC analysis. The Fr.B (1.3 g) and Fr.C (1.05 g) were purified by HPLC on a semipreparative reversed-phase column (Phenomenex Luna PFP, 5 μm , 10 i.d. $\times$ 250 mm, Torrance, CA, USA) eluted by MeOH (respectively, 60% and 65%) to obtain four subfractions (Fr.B1–Fr.B4) and eight subfractions (Fr.C1–Fr.C8) from Fr.B and Fr.C, respectively. Further purification of Fr.B1 and Fr.C7 by HPLC semipreparative reversed-

phase column (Thermo Hypersil HS C18, 5 μ m, 10 i.d. $\times$ 250 mm, Bellefonte, PA, USA) eluted by MeOH_{aq} (respectively, 30% and 50%) to give **1** (t_R : 21 min; 3.2 mg), **2** (t_R : 25 min; 3.5 mg) and **5** (t_R : 13 min, 7.0 mg). The solid fermented products were into a powder after cryodesiccation and than extracted four times with MeOH (equal volumes). The crude extracts were suspended with H₂O and partitioned three times with EA, *n*-hexane, and *n*-butanol, respectively (equal volumes). The dried *n*-hexane extract (3.4 g) was subjected gravity column chromatography (5.0 i.d. $\times$ 17 cm) with silica, eluted with *n*-hexane, EA, and MeOH by gradient system to give 52 fractions, and than combined into 12 fractions (fr.A-Fr.L) based on similar compositions of TLC analysis. Compound **6** (10.0 mg) was obtained from the recrystallization of fraction Fr.B at -4°C for 12 hours.

Nigaurdiol (1)

colorless oil; $[\alpha]_D^{25} = -1.2$ (c 0.3, MeOH); IR (ν_{max} , KBr): at 3334 and 1646 cm^{-1} ; HR-ESI-MS: $[M + H]^+$ m/z 183.1381 (calcd 183.1380 for C₁₁H₁₉O₂); ¹H and ¹³C NMR see Table 1.

Microglial Culture

The murine BV-2 microglial cell line cultured was followed the procedure of our previous reports (Hsiao et al. 2020). In summary, BV-2 cells were cultured with DMEM containing Fetal Bovine Serum (FBS), streptomycin/penicillin, Lglutamine and HEPES at 37°C, humidified 5% CO₂. Prior to the study, BV-2 cells were cultured in FBS media (5%), then pretreated with carrier media or various concentrations of compounds for 15 minutes, and collected after 24 h of stimulation with LPS (150 ng/mL).

Cell Viability Assays

The cellular viability assays procedure was followed our previous report using BV-2 cells with 24 h treatment of all compounds and assessed using MTT test (Hsiao et al. 2020).

Detection of Nitric Oxide Production

The level of nitric oxide (NO) metabolites from productions of activated BV-2 cells was measuring for detection of cellular NO production followed the Griess method (Wang et al. 2018).

Results And Discussion

Chemical investigation on the liquid and solid fermented products of *N. aurantiaca*, an endophytic fungi from *M. leucadendra*, afforded one new compound together with five known compounds. By comparison with literature data, the known compounds were identified as xyloketal K (**2**) (Sun et al. 2016), bostrycin (**3**) (Stevens et al. 1979; Chen et al. 2012), deoxybostrycin (**4**) (Chen et al. 2012; Wang et al. 2013), xylanthraquinone (**5**) (Sommart et al. 2008), and ergosterol (**6**) (Kawai et al. 2018).

Compound **1**, a colorless oil, was determined to have a molecular formula of C₁₁H₁₈O₂, ($[M + H]^+$ m/z 183.1381, calcd 183.1380) by HRESIMS analysis and evidenced by ¹³C NMR spectrum. The IR spectrum

confirmed the presence of a hydroxy and an olefinic functionalities at 3334 and 1646 cm^{-1} , respectively. The ^1H NMR data ($\text{MeOH-}d_4$, 600 MHz) spectrum shows two methyl groups at δ_{H} 1.67 (3H, d, J = 6.2 Hz, H_3 -1) and δ_{H} 1.77 (3H, dd, J = 6.7, 1.2 Hz, H_3 -9); six methine signals at δ_{H} 3.00 (1H, q, J = 7.0 Hz, H-4), δ_{H} 5.43 (1H, dd, J = 16.4, 7.0 Hz, H-3), δ_{H} 5.52 (1H, dq, J = 16.4, 6.2 Hz, H-2), δ_{H} 5.70 (1H, dq, J = 14.8, 6.7 Hz, H-8), δ_{H} 5.94 (1H, d, J = 11.0 Hz, H-6), and δ_{H} 6.43 (1H, ddq, J = 14.8, 11.0, 1.2 Hz, H-7); and two oxygenated methylene signals at δ_{H} 3.55 and 3.63 (each 1H, dd, J = 10.7, 7.0 Hz, H_2 -11) and δ_{H} 4.14 and 4.18 (each 1H, d, J = 12.0 Hz, H_2 -10). The DEPT ^{13}C NMR in combination with the ^{13}C NMR ($\text{MeOH-}d_4$) and HSQC spectrum of **1** contained 11 carbon signals corresponding to two methyls at δ_{C} 16.8 (C-1) and 17.00 (C-9); six methines at δ_{C} 50.7 (C-4), 126.2 (C-2), 126.9 (C-7), 129.2 (C-6), 129.6 (C-8), and 130.7 (C-3); and two methylenes at δ_{C} 58.1 (C-10) and 64.4 (C-11). The COSY spectrum (Fig. 2) revealed contiguous protons of H-9/H-8/H-7/H-6 and H-1/H-2/H-3 /H-4 /H-11. Key cross-peaks of HMBC spectrum (Fig. 2) including δ_{H} 4.18 (H_2 -10)/ δ_{C} 137.6 (C-5), 50.4 (C-4), and 129.2 (C-6); δ_{H} 3.63 (H_2 -11)/ δ_{C} 137.6 (C-5), 50.4 (C-4), and 130.7 (C-3); δ_{H} 3.00 (H-4)/ δ_{C} 130.7 (C-3), 137.6 (C-5), 129.2 (C-6), and 126.2 (C-2) were observed. The structure of **1** was thus determined to be as shown in Fig. 1, and it was named nigaordioli. The chemical skeleton of **1** has not been reported previously, and it could be a recemate since the optical rotational value of **1** was close to zero.

All six isolates were evaluated for nitric oxide (NO) production inhibitory activity assays in LPS-activated microglial BV-2 cell and also its cytotoxic activity to these cells. For positive control, curcumin was used with an IC_{50} value of $6.0 \pm 0.3 \mu\text{M}$. Compounds **3**, **4**, and **6** showed potently inhibitory activities with IC_{50} value of 2.3 ± 0.3 , 2.5 ± 0.5 , and 7.2 ± 1.4 , respectively; however, compounds **3** and **4** exhibited significant cytotoxicity against microglial BV-2 cell with viability of 10.7 ± 0.8 and $11.3 \pm 1.3\%$ ($10 \mu\text{M}$), respectively. Furthermore, compounds **6** showed no cytotoxic activity with the survival of cells at concentration $10 \mu\text{M}$ of $90.8 \pm 6.7\%$. Compounds **1**, **2** and **5** showed weak inhibitory activities and no cytotoxic activity (see Table 2). Ergosterol (**6**) is the major sterol endogenous produced by fungi and protozoa with diverse bioactivity including anti-inflammatory, anti-cancer, and immune-modulatory (Lee et al. 2017; Papoutsis et al. 2020).

Table 1 NMR data of compound **1** in CD_3OD .

Position	δ_C	δ_H (J, in Hz)
1	16.8	1.67 (d, 3H, J = 6.2 Hz)
2	126.2	5.52 (dq, 1H, J = 16.4, 6.2 Hz)
3	130.7	5.43 (dd, 1H, J = 16.4, 7.0 Hz)
4	50.4	3.00 (q, 1H, J = 7.0 Hz)
5	137.6	
6	129.2	5.94 (d, 1H, J = 11.0 Hz)
7	126.9	6.43 (ddq, 1H, J = 14.8, 11.0, 1.2 Hz)
8	129.6	5.70 (dq, 1H, J = 14.8, 6.7 Hz)
9	17.0	1.77 (dd, 3H, J = 6.7, 1.2 Hz)
10a	58.1	4.18 (d, 1H, J = 12.0 Hz)
10b		4.14 (d, 1H, J = 12.0 Hz)
11a	64.4	3.63 (dd, 1H, J = 10.7, 7.0 Hz)
11b		3.55 (dd, 1H, J = 10.7, 7.0 Hz)

Table 2 IC₅₀ and cell viability values of compounds in BV-2 microglial cells. Data are as the mean $\pm$ SD (n = 3). * p < 0.05, ** p < 0.01, and *** p < 0.001 compared with the stimulation (V); ### p < 0.001 compared with the resting (R)

Compounds	IC ₅₀ (μ M)	Cell viability (%)
1	32.2 $\pm$ 3.3	102.6 $\pm$ 8.8
2	30.1 $\pm$ 3.0*	98.3 $\pm$ 7.6
3	2.3 $\pm$ 0.3***	10.7 $\pm$ 0.8***
4	2.5 $\pm$ 0.5***	11.3 $\pm$ 1.3***
5	32.1 $\pm$ 6.7	102.8 $\pm$ 6.9
6	7.2 $\pm$ 1.4***	90.8 $\pm$ 6.7
R	1.4 $\pm$ 0.8	100.0 $\pm$ 0
V	38.2 $\pm$ 4.7###	-
Curcumin	6.0 $\pm$ 0.3	-

Conclusions

In this report, we have identified one new compound, nigaardiol (1), along with five known compounds (2–6) from an endophytic fungus associated with *Melaleuca leucadendra* Linn., than that identified as *Nigrospora aurantiaca* #TMU062. Of the compounds identified, the chemical skeleton of nigaardiol (1), we have shown for the first time. All compounds were evaluated by *in-vitro* NO inhibitory assay in the LPS-stimulated murine BV-2 microglial cell line. The results showed, bostrycin (3), deoxybostrycin (4), and ergosterol (6) potently inhibitory activities, than that nigaardiol (1), xyloketal K (2) and xylanthraquinone (5) weak inhibitory activities. Bostrycin (3) and deoxybostrycin (4) exhibited significant cytotoxicity against microglial BV-2 cell.

Declarations

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

S. S. performed the experiments and wrote the manuscript. G. H. performed the biological assays. T-H L. performed the data curation, supervision, and methodology. C -K. L. performed the conceptualization, funding acquisition.

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Figures

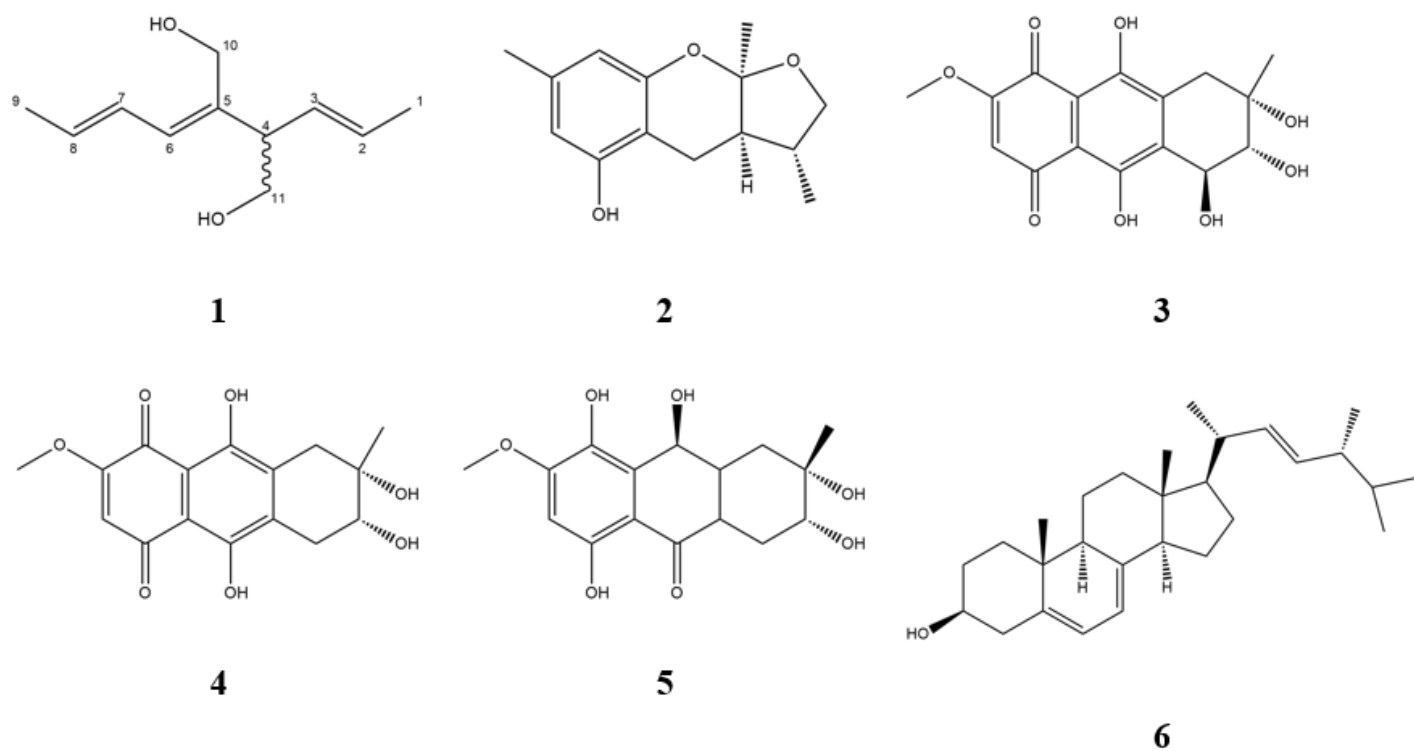


Figure 1

Chemical structures of compounds 1–6.

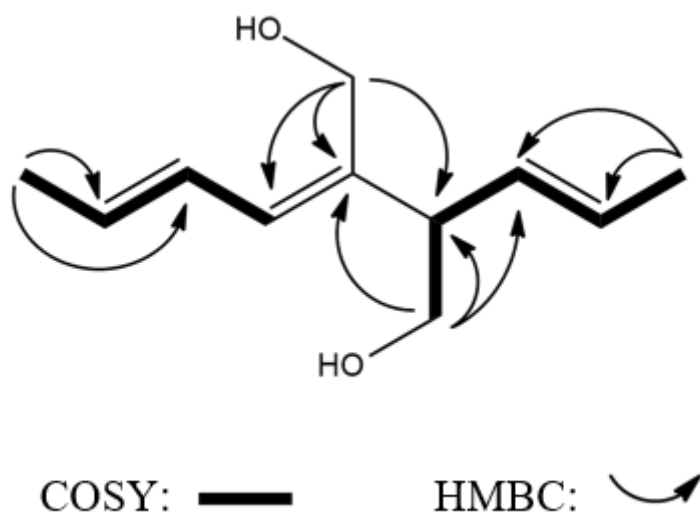


Figure 2

Key HMBC and COSY correlations for compound 1.