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New naphthoquinone derivatives from *Fusarium napiforme* of a mangrove plant

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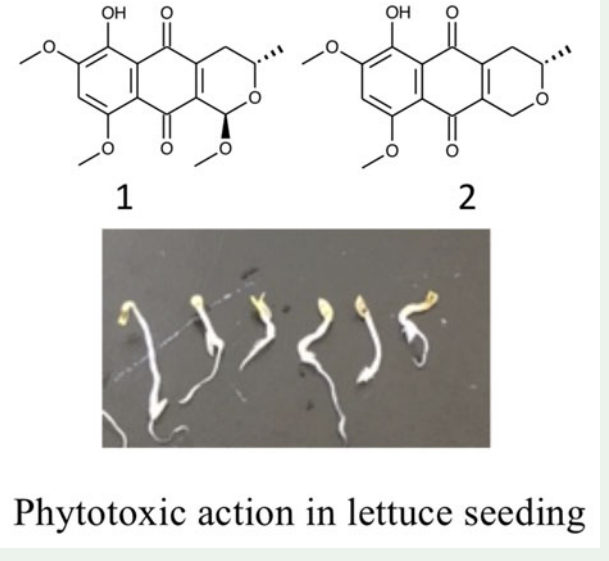
ABSTRACT

Two new naphthoquinone derivatives, 6-hydroxy-astropaquinone B (**1**) and astropaquinone D (**2**) as well as the known compound 3-*O*-methyl-9-*O*-methylfusarubin (**3**) were isolated from *Fusarium napiforme*, an endophytic fungus isolated from the mangrove plant, *Rhizophora mucronata*. The structures of **1** and **2** were determined by 1D and 2D NMR spectroscopic analyses. Compounds **1**, **2** and **3** exhibited moderate antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Furthermore, **1**, **2** and **3** were phytotoxic action in lettuce seeding at a concentration of 30 $\mu g \cdot mL^{-1}$.



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1. Introduction

Endophytic fungi, which asymptomatically inhabit plant tissues, may produce specific biologically active secondary metabolites in some cases. Actually, endophytes have also been a prominent source of new secondary metabolites with unique structures over past two decades. In addition, endophyte can evolve to produce the same or similar bioactive compounds as their host plants (Venugopalan and Srivastava 2015). Further, to date, several novel endophytic fungal-derived natural products have been isolated and described (Li et al. 2018). A previous investigation by our team has demonstrated that several secondary metabolites secreted by endophytic fungi isolated from different habitats were biologically active and had interesting structures (Shiono et al. 2016).

In our continuing search for fungal metabolites, a batch of approximately 50 fungal strains isolated from mangrove plants collected from Makassar, South Sulawesi, Indonesia, were fermented on a small scale. Subsequently, the methanolic fermentation extracts were subjected to TLC chemical screening. The fungal strain *F. napiforme* IP-28 isolated from the stem of *Rhizophora mucronata* was selected for further chemical investigation. The genus *Fusarium* is known as one of the mycotoxin producing fungi with diverse structures (Zakaria 2017). *F. napiforme* has been firstly isolated from millet and sorghum from Southern Africa, and soil debris from one grassland in Australia (Marasas et al. 1987) and ability to produce fumonisin B1 has been reported (Zakaria 2017). Fungal culture of IP-28 strain was extracted with solvents and fractionated using different chromatographic techniques, which yielded two new compounds and one previously identified compound. Based on different spectroscopic data including HRESITOFMS, as well as ^{1}H, ^{13}C, and 2D NMR, the structures of the new compounds were found to be naphthoquinone derivatives, which were named 6-hydroxyastropaquinone B (**1**) and astropaquinone D (**2**). We found that **1** and **2** exhibited antimicrobial activities against *S. aureus* and *P. aeruginosa*. Additionally, all three compounds regulated the growth of lettuce seedlings.

2. Result and discussion

Compound **1** was obtained as a red amorphous powder. The molecular formula of **1** was determined as $C_{17}H_{18}O_7$ by HRESITOFMS, in combination with ^{13}C NMR spectroscopy. The IR spectrum showed major absorption bands at 3397 and 1650 cm^{-1} corresponding to a hydroxyl and a carbonyl group, respectively. The UV absorption bands at 226, 283 and 491 nm together with IR absorption bands indicated the presence of a 1,4-naphthoquinone moiety in **1**. The ^{13}C NMR spectrum of **1**, when analysed in combination with the HSQC data, revealed the presence of two quinone carbonyls (δ_C: 190.3 and 179.5), eight aromatic carbons, of which three were oxygenated (δ_C: 148.4, 156.0 and 155.5) and one was protonated (δ_C: 102.8), two oxygenated methine carbons, of which one was dioxygenated (δ_C: 94.1), three OCH_3 carbons (δ_C: 56.4, 56.5 and 56.8), one methylene carbon (δ_C: 28.6), and a methyl carbon (δ_C: 21.1). The ^{1}H NMR spectrum of **1** showed signals for one aromatic proton [δ_H: 6.74 (1 H, s, H-8)], two methine protons [δ_H: 4.21 (1 H, m, H-3), 5.52 (1 H, s, H-1)], three methoxy groups [δ_C: 56.4 (q, 1-OMe), 56.5 (q, 7-OMe), 56.8 (q, 7-OMe)], one doublet methyl group [δ_H: 1.38

(3 H, d, $J = 6.7$ Hz, H-11)], and one methylene group [δ_H: 2.22 (1 H, dd, $J = 18.0$, 10.0 Hz, H-4), 2.66 (1 H, dd, $J = 18.0$, 3.5 Hz, H-4)]. The cross peaks between H-4/H-3/H-11 were found in the ^{1}H-^{1}H COSY spectrum of **1** (Supplementary information Figure S1). The HMBC correlations from H-8 to C-6, C-9a, and C-10, from H-1 to C-10, from H-4 to C-5 and from H-11 to C-3 and C-4 established a methyl-pyranonaphtoquinone nucleus (Supplementary information Figure S1). In addition, NOE correlations between H-8 and two methoxys at C-7 and C-9 were found, which established the positions of the methoxyl groups at C-7 and C-9 in the aromatic ring (Supplementary information Figure S1). The location of a methoxyl group at C-1 of the pyran ring was indicated by HMBC correlations between these protons and the corresponding carbon (Supplementary information Figure S1). Comparison of the C-6 ^{13}C NMR chemical shifts (δ_C: 148.4) of **1** with that for the corresponding C-atom (δ_C: 148.4) of **3** (Kornsakulkarn et al., 2011) revealed the position of the hydroxyl group at C-6. Thus, the planar structure of **1** was assigned as depicted in Figure 1. Compound **1** is structurally related to astropaquinones B (**4**) and C (**5**) previously isolated from *Astrosphaeriella papuana* (Geng et al. 2012; Wang et al. 2009). The relative configuration of **1** was deduced on the basis of NOE experiments (Supplementary information Figure S2). The NOE correlation of OMe-1/H-3 suggested that its relative configuration was identical to that reported for **4**. In addition, the specific optical rotation of **1** exhibited a positive specific rotation value [[α] $_D$ +45.1° (*c* 0.12, $CHCl_3$)] which was similar to the previously described **4**. Thus, the structure of **1** was identified as (1*R*, 3*S*)-6-hydroxy-astropaquinone B.

Compound **2** was obtained as an orange amorphous powder. The molecular formula of **2** was determined as $C_{16}H_{16}O_6$ by a combination of its HRESITOFMS, ^{13}C NMR, and HSQC data, which also indicated that the molecule has nine degrees of unsaturation. Its UV spectrum exhibited absorption maxima at 280 and 490 nm, which is characteristic of a pyranonaphthoquinone (Masi et al. 2018). Furthermore, its IR spectrum showed absorption bands at 3502 and 1631 cm^{-1}, indicating the presence of hydroxy and quinone ketone functions. The ^{1}H NMR data of **2** indicated that it was structurally related to **1**. The essential difference between the ^{1}H NMR spectra of **1** and **2** was the absence of a C-1 methoxy group in **1**, which instead contained a methylene group [δ_H: 4.50 (1H, dt, $J = 19.0$, 2.0 Hz, H-1), 4.86 (1H, dd, $J = 19.0$, 2.4 Hz, H-1)]. Correlations from H-1 to C-3 and C-4a in the HMBC spectrum of **2** confirmed that a methylene moiety was located at C-1. In addition, the specific optical rotation of **2** exhibited a positive specific rotation value [[α]$_D$ +100° (*c* 0.3, MeOH)] similar to that of the

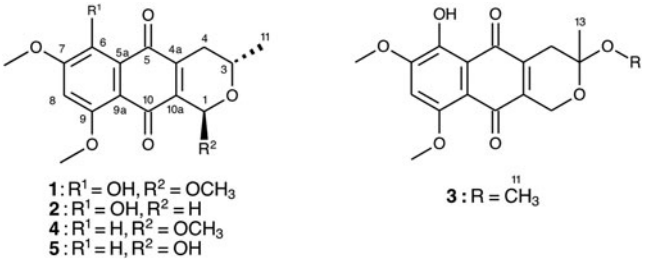

Figure 1. Chemical structures of **1**–**5**.

previously described synthetic compound, (+)-(3*S*)-7,9-dimethoxy-3-methyl-3,4-dihydro-1H-benzo[g]isochromene-5,10-dione [$[\alpha]_D$ +112.5° (*c* 1.3, CH_2Cl_2)] (Bulbule et al. 2007). Thus, the structure of **2** was established and given the trivial name astropaquinone D.

The structure of **3** was identified as 3-*O*-methyl-9-*O*-methylfusarubin by comparing our physical and spectroscopic data with the reported data (Tatum et al. 1985).

Naphthoquinones isolated from fungi have been reported to possess antimicrobial activity against bacteria (Wang et al. 2009). The antimicrobial mechanism involves the generation of superoxide anion and hydrogen peroxide in bacterial membranes. Consequently, superoxide anion and hydrogen peroxide can induce damage to bacterial membranes, proteins and DNA, which induces apoptosis (Ravichandiran et al. 2019). Compounds **1**–**3** were evaluated for antimicrobial activity against Gram-positive and Gram-negative bacteria, yeast, and other fungal strains. Compounds **1**, **2** and **3** showed moderate antibacterial activity against *S. aureus* NBRC 13276 and *P. aeruginosa* ATCC 15442 [MIC values ($\mu g \cdot mL^{-1}$): **1**: 6.3, **2**: 12.5, **3**: 6.3 for *S. aureus* NBRC 13276, and **1**: 6.3, **2**: 6.3, **3**: 6.3, for *P. aeruginosa* ATCC 15442]. In contrast, neither *Aspergillus clavatus* F 318a, nor *Candida albicans* ATCC 2019 were inhibited by **1**, **2** or **3** (at 25 $\mu g \cdot mL^{-1}$). It has been reported that **4** and **5** exhibited antimicrobial activity against *S. aureus* YMF 3.17 with MIC values ($\mu g \cdot mL^{-1}$) of 20 and 10, respectively. A comparison of the published MIC values for *S. aureus* to those of the present study suggested that **1** and **2** might be more potent inhibitors than **4** and **5**. Higher activity was observed when the structure had the naphthoquinone unit with a hydroxy at C-6. Since some endophytes produce biological active myctoxins in the plant tissues, and the accumulation of these mycotoxins often enhance the phytotoxicity or symptoms of plant diseases (Ismaiel and Papenbrock 2015), the phytotoxic activities of **1**, **2** and **3** were investigated by seed germination test on lettuce (*Lactuca sativa* L.) with 2,4-dichlorophenoxyacetic acid (0.3 $\mu g \cdot mL^{-1}$) as the positive control (Supplementary Information Figure S16). Compounds **1**, **2** and **3** each inhibited the growth of both roots and hypocotyls at 30 $\mu g \cdot mL^{-1}$. Furthermore, **1** suppressed seed germination at 100 $\mu g \cdot mL^{-1}$.

3. Experimental

3.1. General experimental procedures

See detailed experimental section in supplementary material.

3.2 Fungal material and fermentation

See detailed experimental section in supplementary material.

3.3. Extraction and purification

The extract was separated by silica gel column chromatography with a stepwise elution of *n*-hexane/EtOAc (100:0–0:100) to give 11 fractions (Frs.1-1 to 1-11). Frs. 1-4 and 1-5 (2.1 g) were further separated on a silica gel column with $CHCl_3$/EtOAc to provide

11 fractions (Frs. 2-1 to 2-11). Fr. 2-4 was chromatographed on silica gel CC using a stepwise gradient of $CHCl_3$–EtOAc to give fractions 1 – 12 (Frs. 2-4-1 to 2-4-12). Fr. 2-4-5 (150 mg) was subjected to ODS CC by eluting with H_2O-MeOH (20:80) to afford 6-hydroxy-astropaquinone B (**1**, 11.0 mg), and astropaquinone D (**2**, 13.5 mg). Fr. 1-6 (1.2 g) was combined and chromatographed on a silica gel column chromatography using a stepwise gradient of $CHCl_3$/EtOAc (100:0–0:100) to give 11 fractions (Frs. 1-6-1 to 1-6-11). Fr. 1-6-4 (62.0 mg) was subjected to ODS column chromatography by eluting stepwise with 100% H_2O to 100% MeOH (100:0–0:100), which yielded 11 fractions (Frs. 1-6-4-1 to 1-6-4-11). Fr. 1-6-4-5 (30.5 mg) was subjected to flash silica gel CC ($CHCl_3$/MeOH, 50:1, *v*/*v*) to afford 3-*O*-methyl-9-*O*-methylfusarubin (**3**, 3.2 mg).

6-Hydroxy-astropaquinone B (**1**): an orange amorphous powder; $[\alpha]_D^{20}$ +55° (*c* 0.3, MeOH); UV (MeOH); λ_{max} (log ε) 226 (4.3), 283 (3.7), 491 (3.2) nm; HRESITOFMS *m/z* 357.0931 $[M+Na]^+$, (calcd for $C_{17}H_{18}O_7Na$, 357.0950); IR (KBr): 3397, 2927, 2923, 1650, 1616 1457, 1376, 1256, 1051 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm, *J* Hz): 1.38 (3 H, d, *J* = 6.7 Hz, H-11), 2.22 (1 H, dd, *J* = 18.0, 10.0, H-4), 2.66 (1 H, dd, *J* = 18.0, 3.5, H-4), 3.57 (3 H, s, 1-OMe), 4.00 (3 H, s, 7-OMe), 4.03 (3 H, s, 9-OMe), 4.21 (1 H, m, H-3), 5.52 (1 H, s, H-1), 6.74 (1 H, s, H-8); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 21.1 (C-11), 28.6 (C-4), 56.4 (1-OMe), 56.5 (7-OMe), 56.8 (7-OMe), 62.0 (C-3), 93.8 (C-1), 102.8 (C-8), 110.0 (C-9a), 114.5 (C-5a) 140.8 (C-4a), 142.7 (C-10a), 148.4 (C-6), 155.5 (C-9) 156.0 (C-7), 179.5 (C-10), 190.3 (C-5).

Astropaquinone D. (**2**): an orange amorphous powder; $[\alpha]_D^{20}$ +100° (*c* 0.3, MeOH); UV (MeOH): λ_{max} (log ε): 227 (4.3), 284 (3.6), 490 (3.2); HRESITOFMS *m/z* 327.0823 $[M+Na]^+$, (calcd for $C_{16}H_{16}O_6Na$, 327.0844); IR (KBr): 3502, 2923, 1631,1616, 1434, 1469, 1265, 1051 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H (ppm, *J* Hz): 1.37 (3 H, d, *J* = 6.7, Me-11), 2.28 (1 H, m, H-4) 2.69 (1 H, dt, *J* = 18.3, 3.6 Hz, H-4), 3.65 (1 H, m, H-3), 3.99 (3 H, s, 7-OMe), 4.01 (3 H, s, 9-OMe) 4.50 (1 H, dt, *J* = 19.0, 2.0 Hz, H-1), 4.86 (1 H, dd, *J* = 19.0, 2.4, H-1), 6.71 (1 H, s, H-8); ^{13}C-NMR (150 MHz, $CDCl_3$) δ_C (ppm): 21.3 (C-11), 28.8 (C-4), 56.5 (7-OMe), 56.9 (9-OMe), 64.1 (C-1), 70.0 (C-3), 102.3 (C-8), 111.3 (C-9a), 114.3 (C-5a), 139.2 (C-4a), 146.4 (C-10a), 148.5 (C-6), 155.7 (C-9), 156.0 (C-7), 180.5 (C-10), 189.5 (C-5).

3.4. Bioassays

3.4.1. Antimicrobial activity

The minimal inhibition concentration (MIC) was determined by the agar dilution method, using nutrient agar for bacteria (1% beef extract, 1% peptone and 0.5% NaCl at pH 7.2). This test was performed in Petri dishes (4 cm. id.) in duplicate. Each test compound was dissolved at 1 $mg \cdot mL^{-1}$ in 10% aqueous DMSO. A suitably quantified volume of the test solution was mixed with the appropriate agar medium (2 mL) to prepare a plate with a given concentration (0–100 $\mu g \cdot mL^{-1}$) of a test compound. Each plate was subsequently inoculated with a test microorganism (100 µL of approximately 10^6 CFU $\cdot mL^{-1}$) and incubated at 30 °C for 18–24 h for bacteria. MIC is defined as the lowest concentration resulting in no visible growth after incubation. Chloramphenicol and kanamycin were used for positive control against *S. aureus* and *P. aeruginosa* (each 1 $\mu g \cdot mL^{-1}$), respectively.

3.4.2. Phytotoxic assay

Lettuce seeds (*Lactuca sativa* L.) were used for the bioassay. Fifteen seeds were sown on filter paper containing a defined concentration of the test compound in a Petri dish (4 cm id.). Distilled water (1 mL) was added to the Petri dish, and incubation was carried out at 25 °C under continuous light for 7 days. The elongation of the roots and shoots was measured to compare with those of the control. The negative control was using only distilled water and the positive control was using 2,4-dichlorophenoxyacetic acid.

4. Conclusion

As a result, the chemical investigation of an endophytic fungus *F. napiforme* IP-28 led to the discovery of three compounds **1–3**. Their structures were established on the basis of NMR spectroscopic data. Compounds **1–3** showed the moderate antibacterial activity and phytotoxicity on lettuce seeding at a concentration of 30 $\mu g \cdot mL^{-1}$. The results in this article implied that endophytes living special environment such as mangrove area might be able to have survival strategy including production of the metabolites with unique skeletons displaying a wide range of biological effects.

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showed moderate antibacterial activity against S. aureus NBRC 13276 and P. aeru...

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Phytotoxic assayLettuce seeds (Lactuca sativa L.) were used for the bioassay

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and discussionCompound 1 was obtained as a

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4. ConclusionAs a result, the chemical investigation of an endophytic fungus

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