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Phytotoxic and Cytotoxic Polyketides Produced by Fungal Endophytes Isolated from *Psidium guajava*

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ABSTRACT

Endophytic fungi reside within their host plants with no obvious symptoms. They have been proven as abundant sources of new bioactive compounds with diverse structures. In our continuous search of the new compound from endophytic fungi, a new compound, 5,7-dihydroxy-3(R)-methylphthalide (1), was isolated from *Xylaria brevipes* PGR1, and three known compounds, (–)-altenuene (2), alternariol (3), and altertoxin I (4), were isolated from *Alternaria alternata* D-8. Both endophytic fungi were isolated from the branch of an Indonesian medicinal plant, *Psidium guajava*. Their structures were elucidated using extensive spectroscopy analyses, including 1D/2D NMR, HRESITOFMS, and data comparisons with the literature. Compounds 1–3 exhibited phytotoxicity at IC_{50} (µg/ml) values of 4.6, 23.07, and 77.28, respectively. Additionally, 1–4 showed significant cytotoxicity in the brine shrimp lethality test (BSLT), with mortality rates at 75%, 50%, 90%, and 80% at the concentration of 0.19 µg/ml, respectively.

1. Introduction

Endophytic fungi are an essential part of the plant micro-ecosystem, which significantly influences the formation of metabolic products in plants by establishing a sophisticated relationship (Jia *et al.* 2016). Alongside the plant host, endophytic fungi have been proven to produce compounds with high-potential therapeutic activities (Gouda *et al.* 2016). Additionally, endophytic fungi have developed the ability to produce similar or identical metabolites as their plant hosts, as reported for paclitaxel which was initially isolated from *Taxus brevifoli* (Wani *et al.* 1971) and podophyllotoxin from *Podophyllum emodi* (Leiter 1950), which at the later time, both compounds or their analogs were also reported to be produced by endophytic fungi (Stierle and Stierle 2015). Our recent investigation of an endophytic fungus also resulted in the isolation of bioactive compounds with interesting structures and exhibited potent osteoclastic differentiation activity in RAW264.7 cells derived from murine macrophages (Ariefta *et al.* 2019).

In part of our continuing effort to explore bioactive metabolites from endophytic fungi, we investigated a medicinal plant, *Psidium guajava*, as a source of endophytic fungi. *P. guajava* is a well-known tropical tree that is grown for fruit. It has also been used to treat pain, diabetes, hypertension, diarrhea, dysentery, and gastroenteritis. (Gutiérrez *et al.* 2008; Naseer *et al.* 2018). Some studies also reported that *P. guajava*-associated fungi were found to be potential sources of biologically active compounds, including cytochalasins (cytotoxic agent) (Okoye *et al.* 2015), fusaric acid derivatives (antifungal agent) (Yang *et al.* 2019), and depsides (antimicrobial agent) (Dea Medeiros *et al.* 2011). In this study, we isolated two strains, PGR1 and D-8, from *P. guajava* collected from Yogyakarta, Indonesia; both fungi were subsequently identified as *Xylaria brevipes* and *Alternaria alternata*, respectively. Herein, we reported the characterization of the new compound and the bioactivities of all isolated compounds.

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2. Materials and Methods

2.1. General Experimental Procedures

In the separation processes, either octadecyl silica (ODS, Fuji Silysia, Japan) or silica gel 60 (Kanto Chemical Co., Inc., Japan) was used for column chromatography. The packed column Biotage® SNAP Ultra (10 g, 25 m) and Büchi Flash Chromatography C-601 (Büchi, Switzerland) were used for flash chromatography. Shimadzu LC-20AT and UV-vis detector SPD-20A (Shimadzu, Japan) were used for HPLC separations on Mightysil (Kanto Chemical Co., Inc., Japan) packed column for reverse phase-high performance liquid chromatography (RP-HPLC). Precoated silica gel 60 F254 plates from Merck, Germany, were used for thin-layer chromatography (TLC). Spots were observed by heating or UV irradiation after being sprayed with 10% vanillin in H_2SO_4. A Horiba SEPA-300 polarimeter (Horiba, Japan) was used to measure the optical rotations. IR and UV-vis spectra were independently recorded with Horiba FT710 (Horiba, Japan) and Shimadzu UV-1,800 spectrometer (Shimadzu, Japan). Using a JEOL HX110 mass spectrometer (JEOL, Japan) and a Synapt G2 (Water Corporation, USA), mass spectra were obtained. Chemical shifts are presented in ppm with TMS serving as an internal standard on a JEOL ECZ-600 spectrometer (JEOL, Japan) at 600 MHz for 1H and 150 MHz for ^{13}C. Standard JEOL pulse sequences were used to record the ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC spectra.

2.2. Fungal Materials and Fermentation

A plant specimen of *Psidium guajava* was collected in Sleman, Yogyakarta, Indonesia (southern latitude 7°77'31", east longitude 110°37'35"). The plant specimens were cut about 1 × 1 cm and then sterilized with 70% ethanol for 1 minute, followed by 5% NaClO solution for 5 minutes and then 70% ethanol for 1 minute (Schulz *et al.* 1993). After the sterilization process, plant specimens were dried on a clean bench and dissected to expose the inner part. Next, the samples were inoculated on potato dextrose agar (PDA) media at a 25°C incubator. Within 3-7 days, the developed mycelia on the plate were picked up and transferred to PDA as stock culture. Two fungal strains, PGR1 and D-8, were selected based on morphological features and thin-layer chromatography (TLC) profile screening. These strains were identified as *X. brevipes* and *A. alternata* using ITS DNA analysis of the ITS rDNA regions by the Research Center for Biology, Indonesian Institute of Sciences (Report Number: B-2836/IPH.1/IF.07/X/2019). These fungi were deposited in our laboratory. Each fungus was axenically cultivated at 25 °C for four weeks on brown rice (120 g in ten 1 L flasks) for the compound isolation. Before use, the brown rice was previously sterilized by autoclaving at 120°C, 100 kPa, and 20 minutes.

2.3. Extractions and Purifications

2.3.1. *Xylaria brevipes* PGR1

The molded media was extracted three times using methanol, a total of 3 L, and the methanol extract was dried using a vacuum evaporator. The dried methanol extract was then suspended in distilled water and partitioned with EtOAc (three times using the same volume as distilled water); a vacuum evaporator was used to collect and dry the organic layer. First, the crude EtOAc extract (45.52 g) was subjected to silica gel column chromatography (*n*-hexane: EtOAc = 10:0-0:10; EtOAc: MeOH = 1: 1; and MeOH) to obtain 13 fractions (1.1-1.13). Next, fraction 1.9 (80% EtOAc) was further subjected to silica gel column chromatography (CHCl3: EtOAc = 10:0-0:10; and methanol) to obtain 12 fractions (1.9.1-1.9.12). Next, fraction 1.9.11 (100% EtOAc) was subjected to an ODS column to obtain compound **1** (9.4 mg).

2.3.2. *Alternaria alternata* D-8

Similarly, as described for PGR1, the molded media was extracted using methanol, 3 L, three times; then, the methanol extract was dried using a vacuum evaporator. The dried methanol extract was then suspended in distilled water and partitioned with EtOAc (three times using the same volume as distilled water); a vacuum evaporator was used to collect and dry the organic layer. The crude EtOAc extract (17.00 g) was subjected to silica gel column chromatography with (*n*-hexane: EtOAc = 100:0–0:100) to give 11 fractions (2.1-2.11). Fraction 2.11 was further separated on a silica gel column with a stepwise elution of $CHCl_3$: EtOAc (100:0–0:100) to obtain 11 fractions (2.11.1-2.11.11). Fractions 2.11.8 and 2.11.9

(70% and 80% EtOAc) were further separated using an ODS column chromatography (H_2O: MeOH = 100:0–0:100) which yielded 11 fractions (2.11.8.1-2.11.8.11). Compound **4** was obtained from fraction 2.11.8.4 (30% MeOH). Fraction 2.11.8.8 (70% MeOH) was subjected to a silica gel column with ($CHCl_3$/EtOAc = 100:0-0:100) to provide 11 fractions (2.11.8.8.1-2.11.8.8.11). Fraction 2.11.8.8.7 (60% EtOAc) was further separated using ODS column chromatography (H_2O: MeOH = 100:0–0:100) to obtain 11 fractions (2.11.8.8.7.1-2.11.8.8.7.11). Fraction 2.11.8.8.7.7 (60% MeOH) was further purified on RP-HPLC with an isocratic system (H_2O: MeOH = 3:7, 1 ml/min) to obtain compound **2** (8 mg). Fraction 2.11.8.8.7.5 and 2.11.8.8.7.6 were further separated using a silica gel column ($CHCl_3$/EtOAc = 100:0–0:100) to provide 11 fractions (2.11.8.8.7.5.1-2.11.8.8.7.5.11). Compound **3** (10 mg) was obtained from fraction 2.11.8.8.7.5.8 (70% EtOAc).

2.4. Bioassay

2.4.1. Phytotoxic Assay of 1–4

Bok choy seeds (*Brassica chinensis* var. parachinensis) were used for the phytotoxic assay. In a Petri dish (8 cm id.), ten seeds were sown on filter paper at specific concentrations of the test compound (100, 50, 25, 12.5, and 6.25 µg/ml). The Petri dish was filled with distilled water (2 ml), and for seven days, it was incubated at 25°C under constant light. The roots' and shoots' elongations were compared to those of the negative control. The positive control utilized 2,4-dichlorophenoxyacetic acid, whereas the negative control utilized only distilled water. The IC_{50} was calculated using GraphPad Prism 8.

2.4.2 Brine Shrimp Lethality Test of 1–4

Each compound (200 µg) was dissolved in DMSO and diluted in varying final concentrations (100, 50, 25, 12.5, 6.25, 3,13, 1.56, 0.78, 0.39, and 0.19 µg/ml) in artificial seawater. Artificial seawater was set up with 35 g/L salinity, temperatures between 23-25°C, and aeration under a continuous light lamp. Brine shrimp eggs (2 g) were reared in artificial seawater, and the nauplii were used after 24 hours. Ten live brine shrimps were added to 2 ml simulated seawater containing isolated compounds or DMSO. The number of surviving nauplii in each tube was counted after 24 hours. Then the percent of the lethality of the brackish water shrimp nauplii for every fixation and control was determined.

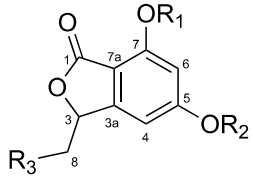
3. Results

3.1. New compound 1

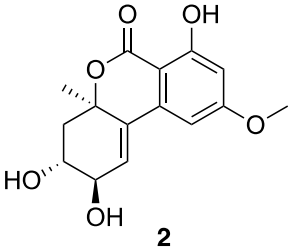
Compound **1** (Figure 1) was obtained as a yellow oil with specific rotation of $[\alpha]_D^{26}$ +165.0 (c 0.1, MeOH). UV-vis analysis showed (MeOH) λ_{max} (Log e) 255 (2.1) and 292 (1.7), and FTIR analysis showed (KBr) ν_{max} 3275, 2993, 1670, 1473, 1338, and 1230 cm^{-1}. From ^1H NMR (600 MHz, Methanol-d_4, Figure 2) spectrum showed resonances of δ 6.33 (dt, *J* = 1.9, 1.0 Hz, 1H, H-4), 6.20 (d, *J* = 1.9 Hz, 1H, H-6), 4.46 (q, *J* = 6.8 Hz, 1H, H-3), 1.32 (dd, *J* = 6.8, 1.0 Hz, 3H, H-8), and from ^{13}C NMR (125 MHz, Methanol-d_4) spectrum showed resonances of δ 173.3 (C-1), 164.8 (C-7), 158.3 (C-5), 154.3 (C-3a), 109.6 (C-7a), 102.6 (C-6), 102.4 (C-4), 54.3 (C-3), 20.7 (C-8). HRESITOFMS showed *m/z* 181.0690 $[M + H]^+$ (calcd. for $C_9H_9O_4$, 181.0501, Figure 2).

3.2. Biological Activities of 1–4

Compounds **1–4** exhibited phytotoxicity against Brassica chinensis var. parachinensis at IC_{50} (µg/ml) values of 4.6, 23.07, 77.28, and >100, respectively



1 3(*R*); R_1,R_2,R_3 = H
5 3(*S*); R_1,R_2 = CH_3, R_3 = H
6 3(*R*); R_1,R_2 = H, R_3 = CH_3



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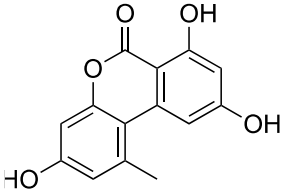

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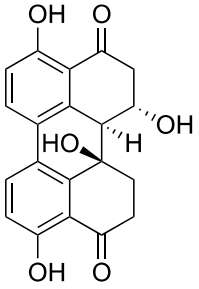

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Figure 1. The structures of **1–6**

(Figure 3A and 3B). Additionally, **1–4** also showed significant cytotoxicity in the brine shrimp, Artemia salina, with mortality rates at 75%, 50%, 90%, and 80% at the concentration of 0.19 µg/ml, respectively (Figure 3C).

4. Discussion

The molecular formula of **1** was determined as $C_9H_8O_4$ (m/z 181.0690 $[M + H]^+$, Figure 2A) through a combination of HRESITOFMS and NMR, indicating

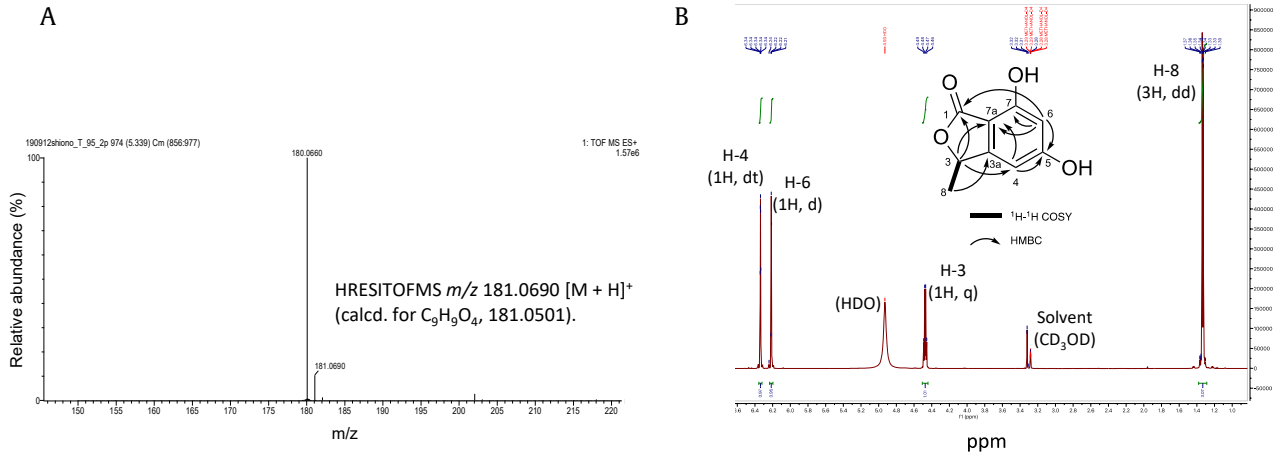

Figure 2. (A) The HRESITOFMS spectrum of **1**, (B) the ^{1}H NMR Spectrum, ^{1}H–1H COSY, and key HMBC correlations observed for **1**

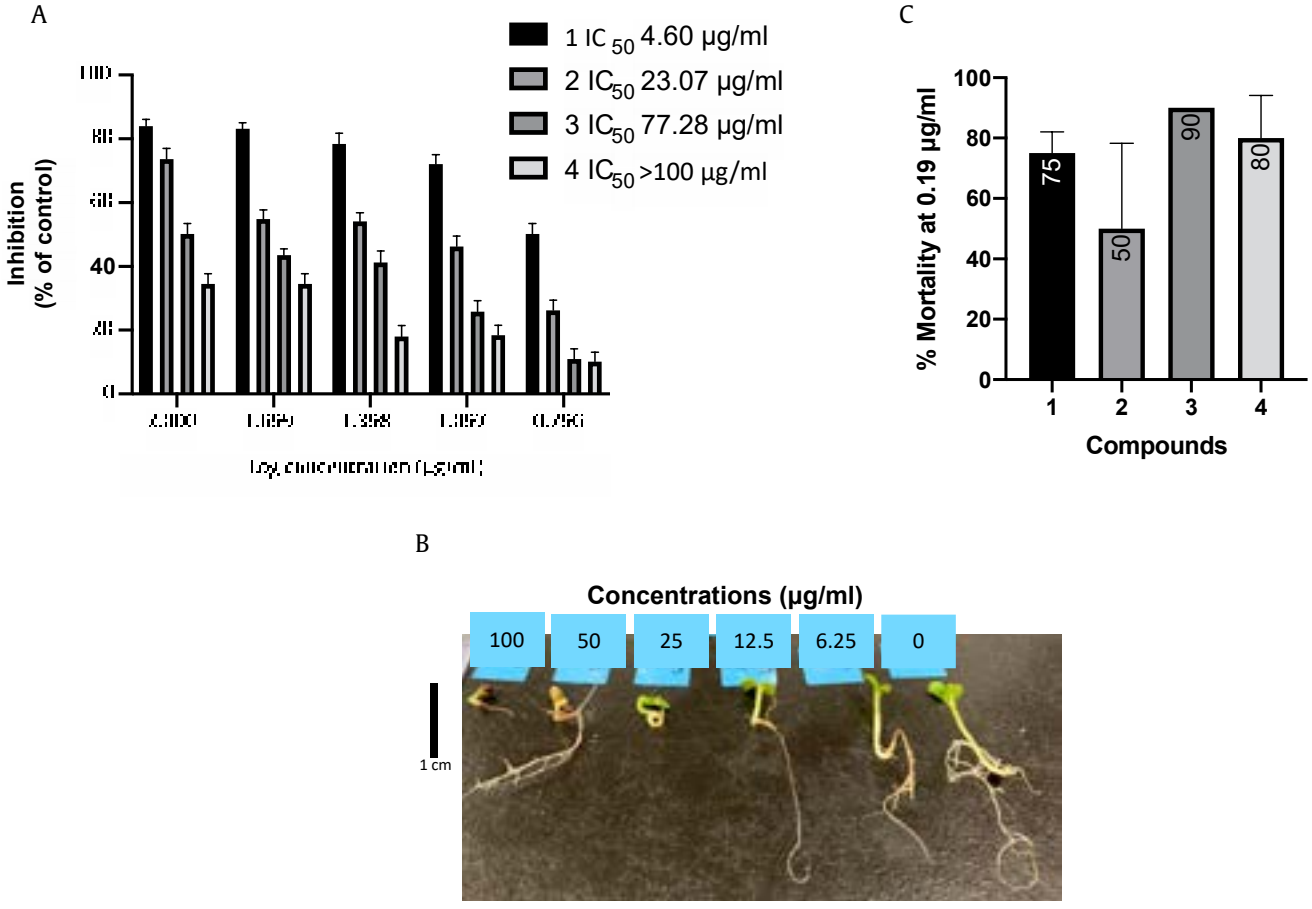

Figure 3. (A) The phytotoxic activity of **1–4** under various concentrations against *Brassica chinensis*, (B) representative comparison of the growth inhibition assay of **1** against *B. chinensis*, (C) % mortality values of **1–4** against *Artemia salina* at 0.19 µg/ml

six degrees of unsaturation. The UV-vis spectrum of **1** showed two absorptions maxima at 255 and 292 nm, indicating the presence of a conjugated chromophore. The FTIR spectrum showed major absorption bands at 3275 and 1670 cm^{-1} corresponding to a hydroxyl and an α,β-unsaturated carbonyl groups, respectively. The ^{13}C NMR spectral data of **1** revealed the presence of nine carbon resonances, including one methyl [δC 20.7 (C-8)], one sp^3 methine [δC 54.3 (C-3)], two sp^2 methines [δC 102.4 (C-4) and 102.6 (C-6)], three sp^2 quaternary carbons [δC 109.6 (C-7a), 158.3 (C-5), and 164.8 (C-7)], and one carbonyl [δC 173.3 (C-1)]. The ^1H NMR spectral data of **1** showed signals for one methyl at δH 1.32 (dd, J = 6.8, 1.0 Hz, 3H, H-8), one methine at δH 4.46 (q, J = 6.8 Hz, 1H, H-3), and two meta-coupled protons at δH 6.33 (dt, J = 1.9, 1.0 Hz, 1H, H-4) and 6.20 (d, J = 1.9 Hz, 1H, H-6). The cross peak was found between H-3/H-9 in the ^1H–^1H COSY spectrum of **1** (Figure 2B). The HMBC correlations from H-3 to C-1 and C-7a, from H-6 to C-5, C-7, C-7a, and C-1, and from H-4 to C-5 and C-7a, allowed to construct a 5,7-dihydroxyphthalide core on the structure of **1**. Furthermore, the HMBC correlations from H-8 to C-3a confirmed the attachment of the methyl group, and the planar structure of **1** was then determined as 5,7-dihydroxy-3-methylphthalide. Compound 1 is structurally related to 5,7-dimethoxy-3(S)-methylphthalide (5) and 3(R)-ethyl-5,7-dihydroxyphthalide (6), previously isolated from an endophytic fungus *Tubakia* sp. ECN-111(Nakashima *et al.* 2017) and synthesized in the preparation of spiromastilactone A (Chaumont-Olive *et al.* 2019), respectively. There was a positive value for the specific optical rotation of **1**, $[\alpha]_D^{26}$ +126 (c 0.1, MeOH), which is opposite to the previously reported **5**, $[\alpha]_D^{26}$ -36 (c 0.1, $CHCl_3$), and similar to **6**, $[\alpha]_D^{20}$ +47 (c 0.85, MeOH). The smaller specific optical rotation values of reference compounds possibly arise from a mixture of R/S configurations. Thus, the structure of **1** was tentatively determined as 5,7-dihydroxy-3(*R*)-methylphthalide (Figure 1).

The structures of **2–4** were identified as (–)-altenuene, alternariol, and altertoxin I, respectively, by comparing our spectroscopic and physical data with the reported literature (Aly *et al.* 2008; Pero *et al.* 1971; Stinson *et al.* 1982).

All isolated compounds were tested for phytotoxicity against *Brassica chinensis* var. parachinensis and cytotoxicity against brine shrimp, *Artemia salina*. Compared to the commonly used faunal tests, toxicity research utilizing higher plants has expanded in recent years. Root elongation inhibition is a valid and sensitive biomarker of environmental toxicity; these bioassays are simple, affordable, and only require a small sample (Priac *et al.* 2017). Brassica chinensis was among the most frequent plant species recommended by the Organization for Economic Cooperation and Development (OECD 2003). Meanwhile, brine shrimp is one of the useful test organisms for toxicity assessment and has numerous uses in toxicology and ecotoxicology assessments (Nunes *et al.* 2006). The *A. salina* testing of cytotoxicity of various classes of NPs was used because of the speed, convenience, and low cost (Rajabi *et al.* 2015). Compounds **1–4** exhibited phytotoxicity at IC_{50} (μg/ml) values of 4.6, 23.07, 77.28, and >100, respectively (Figures 3A and 3B). Additionally, **1–4** also showed significant cytotoxicity in the brine shrimp lethality test, with rates of mortality at 75%, 50%, 90%, and 80% at the concentration of 0.19 μg/ml, respectively (Figure 3C). Phthalide derivatives are a group of structurally very diverse secondary metabolites; they also exhibit a large of bioactivity (Lin *et al.* 2005), including antioxidant (Gao *et al.* 2019), cytotoxicity (Lan *et al.* 2014), antimicrobial, anti-acetyl cholinesterase, and anti-α-glucosidase (Liu *et al.* 2019). Phthalide pharmacophore is essential in the exhibition of these broad spectrums of biological activities (Karmakar *et al.* 2014). Despite its simple structure, **1** in this study exhibited significant phytotoxic and cytotoxic activities and demonstrated the importance of phthalide pharmacophore. (–)-altenuene (2), alternariol (3), and altertoxin I (4) belong to *Alternaria* mycotoxins and are reported to be not acutely toxic (Ostry 2008). Altenuene has been reported to express antioxidant, insecticidal, and acetylcholinesterase inhibitor properties (Bhagat *et al.* 2016). Alternariol can frame receptive oxygen species and connect with DNA topoisomerase, resulting in single and twofold strand DNA breaks and further triggering various DNA damage responses pathway (Solhaug *et al.* 2016), and altertoxin I act as a significant mutagenic species and could pose a potent carcinogenic health hazard (Schrader *et al.* 2001). One or more mechanisms, as mentioned earlier, might be possible to explain the phytotoxicity and cytotoxicity of **2** and 3. These results confirmed

that endophytic fungi could act as symbionts and opportunistic pathogens by producing phytotoxic components. Additional research is required to unrevealed other biological activities of the new compound.

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