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## Bioactive sesquiterpenes from an endophytic fungus *Bipolaris sorokiniana* isolated from a popular medicinal plant *Costus speciosus*

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### ABSTRACT

*Bipolaris sorokiniana*, a fungal endophyte, was isolated from the leaves of *Costus speciosus*, a popular medicinal plant used to control diabetes. Fermentation of the fungus in potato dextrose broth and chromatographic purification of the ethyl acetate (EtOAc) extracts of the culture broth and mycelium yielded two rare sesquiterpenes helminthosporal acid (**1**) and helminthosporol (**2**), together with ergosterol. Compounds **1** and **2** showed a strong phytotoxic effect on lettuce seed germination and toxicity against brine shrimps. Compound **1** also showed antifungal activity. Complete assignments of <sup>1</sup>H and <sup>13</sup>C NMR data of compound **1** are described for the first time.

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### KEYWORDS

Endophytic fungus; *Bipolaris sorokiniana*; *Costus speciosus*; Helminthosporal acid; helminthosporol

## 1. Introduction

Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed (Strobel and Long 1997). It is well documented that endophytic fungi are capable of producing bioactive secondary metabolites. The first discovery of endophytic fungi was recorded as far back as 1904 (Freemann 1904). However, endophytes did not receive much attention until the recent realisation of their ecological relevance and potential of yielding metabolites with diverse structures and biological functions (Gunatilaka 2006). In a continuation of our studies on the search for bioactive compounds from Sri Lankan flora, secondary metabolites produced by an endophytic fungus *Bipolaris sorokiniana* (Class: Ascomycetes & Order: Pleosporales) isolated from the leaves of *Costus speciosus* of the family Costaceae have been investigated. *C. speciosus* is a widely used medicinal plant in Sri Lanka and India especially due to its antidiabetic properties and for its ability to cure various diseases (Pawar and Pawar 2014).

## 2. Materials and methods

### 2.1. General

Extractions were taken using a sonicator (VWR Ultrasound cleaner, model-USC 1700 D). Thin layer chromatography (TLC) analysis was conducted on silica gel plates (Merck 1.05554, 60F<sub>254</sub>, 0.20 mm thickness). TLC spots were located using a UV lamp and by heating after spraying with acidic anisaldehyde. Silica gel (Merck Art. 7734 & 9385) and Sephadex LH-20 were used for column chromatography. <sup>1</sup>H NMR and <sup>13</sup>C NMR were recorded on a Bruker DRX-500 or JEOL JNM-ECP500 (both are 500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C) spectrometer in CDCl<sub>3</sub> solution. FABMS (+) were obtained on a JEOL JMS-700 spectrometer with NBA as matrix. UV absorptions were measured on a Thermo Scientific Multiskan GO Microplate spectrophotometer.

### 2.2. Isolation and identification of the endophytic fungus

Fresh leaves of *C. speciosus* were collected from the Central Province of Sri Lanka in January 2014. Endophytic fungus was isolated from the leaves as

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This paper is dedicated to the late Prof. G.P. Wannigama, Sri Lanka, an inspirational scientist and teacher, on the occasion of his second death anniversary.

Supplemental data for this article can be accessed [here](#).

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described in our previous publication (Qader et al. 2015; Thanabalasingam et al. 2015). The fungus was identified as *Bipolaris sorokiniana* by sequence analysis of the rDNA gene. DNA was extracted using Promega, Wizard Genomic DNA purification kit (A1120), and amplification of the ITS region was carried out using the universal eukaryotic primers of ITS1 and ITS4. These experiments were performed by the GeneTech Institute, Sri Lanka. BLAST search indicated that the sequence of the ITS region had 99% similarity to that of *Bipolaris sorokiniana* isolate A1S6-2 (Gene Bank Accession No. KJ767094.1). Photographic evidence of the leaves of *C. speciosus* and *B. sorokiniana* strain (IFS/MQ/TFP-2014) are deposited at the National Institute of Fundamental Studies.

### 2.3. Fermentation of fungus, extraction, bioassays and isolation of compounds

Large-scale culturing of the fungus was carried out by inoculating *B. sorokiniana* grown on potato dextrose agar (PDA) medium to Erlenmeyer flasks (1 L × 30), each containing 400 mL of PDB media, which were allowed to stand at room temperature for 10 days, while shaking every other day to complete a period of 4 weeks. The culture broth was filtered and the filtrate was extracted with EtOAc (×3) to give an EtOAc extract (0.32 g). The residual mycelium was extracted with EtOAc to give the other EtOAc extract (3.20 g). Both EtOAc extracts were found to show a similar TLC pattern. The combined EtOAc extract was screened for antifungal activity against *C. cladosporioides* using the TLC bioautography method (Homans and Fuchs 1970), antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (Alakolanga et al. 2015), brine shrimp lethality (Howlader et al. 2006), phytotoxicity against lettuce seed germination (Piyasena et al. 2015) and  $\alpha$ -amylase enzyme inhibitory bioassays (Nickavar et al. 2008). Repeated chromatographic separation of the EtOAc extract over silica gel (hexane-EtOAc), Sephadex LH-20 (30% CHCl<sub>3</sub>-MeOH) and final separation by PTLC (developed with hexane-EtOAc, 4:1 for **1** and 3:2 for **2**) furnished helminthosporal acid (**1**, 26 mg), helminthosporol (**2**, 30 mg) and ergosterol (115 mg).

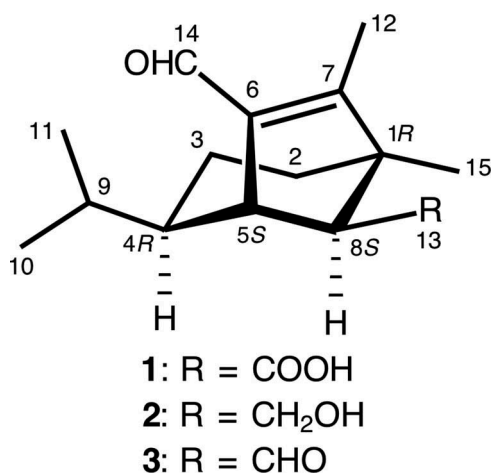
Helminthosporal acid (**1**): white solid, mp 120–122°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz):  $\delta$  1.39–1.50 (m, H<sub>2</sub>-2), 1.76 (m, Ha-3), 0.89–0.93 (m, Hb-3, H-4), 3.41 (brs, H-5), 2.36 (s, H-8), 1.07 (m, H-9), 0.76 (d,

$J = 5.6$  Hz, H<sub>3</sub>-10), 1.07 (s, H<sub>3</sub>-11), 2.04 (s, H<sub>3</sub>-12), 10.00 (s, H-14), 1.19 (s, H<sub>3</sub>-15); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz):  $\delta$  51.6 (C-1), 33.7 (C-2), 25.0 (C-3), 44.6 (C-4), 42.8 (C-5), 137.1 (C-6), 164.5 (C-7), 63.9 (C-8), 31.4 (C-9), 21.5 (C-10), 20.6 (C-11), 10.6 (C-12), 179.1 (C-13), 187.6 (C-14), 19.3 (C-15) (Supplemental data); FABMS:  $m/z$  251 [M + H]<sup>+</sup>.

Helminthosporol (**2**): colorless oil;  $[\alpha]_D^{25} -24.4^\circ$  (c, 1.1, CHCl<sub>3</sub>); lit.  $[\alpha]_D -28.7^\circ$  (Tamura et al. 1965); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz):  $\delta$  1.41 (m, H<sub>2</sub>-2), 1.75 (m, Ha-3), 0.88 (m, Hb-3), 1.04 (s, H-4), 3.19 (brs, H-5), 1.68 (dd,  $J = 8.9, 5.2$  Hz, H-8), 1.04 (m, H-9), 0.76 (d,  $J = 5.6$  Hz, H<sub>3</sub>-10), 1.08 (d,  $J = 5.4$  Hz, H<sub>3</sub>-11), 2.02 (s, H<sub>3</sub>-12), 3.64 (dd,  $J = 10.8, 5.2$  Hz, Ha-13), 3.32 (dd,  $J = 10.8, 8.9$  Hz, Hb-13), 10.00 (s, H-14), 1.04 (s, H<sub>3</sub>-15); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz):  $\delta$  50.8 (C-1), 34.1 (C-2), 25.2 (C-3), 44.8 (C-4), 41.2 (C-5), 137.3 (C-6), 166.3 (C-7), 61.2 (C-8), 31.7 (C-9), 21.7 (C-10), 20.7 (C-11), 10.6 (C-12), 62.2 (C-13), 188.3 (C-14), 18.3 (C-15) (Supplemental data); FABMS:  $m/z$  237 [M + H]<sup>+</sup>. <sup>1</sup>H NMR and <sup>13</sup>C NMR charts of compounds **1** & **2** are given as the supplemental data.

### 3. Results and discussion

An endophytic fungus isolated from the leaves of *C. speciosus* was identified as *B. sorokiniana* (syn. *Helminthosporium sativum* and *Cochliobolus sativus* (Duczek 1982) by molecular means. This is the first report of the isolation of endophytic fungus *B. sorokiniana* from a popular medicinal plant *C. speciosus*. Large-scale fermentation of *B. sorokiniana* in potato dextrose broth media for 4 weeks and extraction of the culture broth and mycelium with EtOAc yielded an extract that displayed antifungal activity against *C. cladosporioides* (minimum inhibitory concentration [MIC] <250 ppm), antioxidant activity against DPPH (IC<sub>50</sub> 346 ppm), lettuce (*Lactuca sativa*) seed germination assay (IC<sub>50</sub> 400 ppm), brine shrimp (*Artemia salina*) lethality (LD<sub>50</sub> 407 ppm) and  $\alpha$ -amylase inhibitory activity (IC<sub>50</sub> 627 ppm). Chromatographic separation of the EtOAc extract furnished compounds **1** and **2** and ergosterol. Compound **1** was characterised as helminthosporal acid by comparison of the <sup>1</sup>H NMR data with the reported values (Rodriguez et al. 1988). First complete assignments of <sup>1</sup>H and <sup>13</sup>C signals for **1**, which were established by 2D-NMR including COSY, HMQC and HMBC spectra, are reported. Compound **2** was identified by



**Figure 1.** Structures of Compounds 1, 2 and 3.

comparison of the <sup>1</sup>H and <sup>13</sup>C NMR data with literature values (Osterhage et al. 2002). The absolute configurations of **1** and **2**, shown in Figure 1, have been determined previously through the synthesis of helminthosporal (**3**) (Corey and Nozoe 1963) and chemical oxidative conversion of **2** and **3** to **1** (de Mayo et al. 1962; Tamura et al. 1965).

Compounds **1** and **2**, and related sesquiterpenes such as helminthosporal and helminthosporic acid, are rare sesquiterpenes, which were previously isolated from *B. sorokiniana* (Mayo et al. 1961; Tamura et al. 1965; Rodriguez et al. 1988). Isolation of **2** is also reported from the fungus *Drechslera dematoides*, which was isolated from the marine red alga *Liagora viscida* (Osterhage et al. 2002). *B. sorokiniana* is known to be a plant pathogenic fungus that produces root rot, leaf spot disease, seedling blight and head blight of barley and wheat, causing significant yield loss (Mayo et al. 1961). It was reported that helminthosporol (**2**) is a gibberellin-like plant growth regulator, which exhibited elongation effect to the shoots of rice seedlings at concentrations ranging from 10 to 300 ppm, whereas rice root growth was inhibited above 50 ppm of **2** and emergence of roots was suppressed at 300 ppm. Moreover, compound **2** also inhibits shoot growth of wheat seedlings above 30 ppm (Tamura et al. 1963).

In the present phytotoxic assay, both compounds **1** and **2** inhibited (100%) the germination of lettuce seeds at 62.5 ppm (the positive control (+)-abscisic acid inhibited the germination (100%) at 10 ppm). Compounds **1** and **2** inhibited hypocotyl growth with IC<sub>50</sub> values of 45.5 and 28.6 ppm,

respectively, and percentage radicle growth with IC<sub>50</sub> values of 31.6 and 20.6 ppm, respectively. Our results indicated that compounds **1** and **2** showed consistently deleterious effects on lettuce seed germination, hypocotyl and radicle growth at several 10 ppm, among which the effect on radicle growth of lettuce seeds was opposite to that reported for the shoots of rice seedlings. In addition, compounds **1** and **2** showed toxicity against brine shrimp (LD<sub>50</sub> 27.1 and 28.3 ppm, respectively). Furthermore, compound **1** displayed anti-fungal activity against *C. cladosporioides* (MIC 31.3 ppm).

In addition to these activities, compounds **1** and **2** were reported to have significant neuraminidase inhibitory activity (Zhang et al. 2011). Furthermore, compound **1** was shown to be an inhibitor of Acyl-CoA: cholesterolacyltransferase in rat liver microsome (Park et al. 1993).

It would be interesting to study about endophytic fungi diversity in other parts of *C. speciosus*, because the secondary metabolites produced by endophytic fungi of *C. speciosus* are less studied and the possibility of production of similar bioactive compounds in a large scale will be beneficial for agriculture, industry and medicine.

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## Disclosure statement

No potential conflict of interest was reported by the author.

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