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Alternariol 9-methyl ether from the endophytic fungus *Alternaria* sp. Samif01 and its bioactivities

Jingfeng Lou[a], *Ruiting Yu*[a], *Xiaohan Wang*[a], *Ziling Mao*[a], *Linyun Fu*[a], *Yang Liu*[b], *Ligang Zhou*[a,*]

[a] *MOA Key Laboratory of Plant Pathology, Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China*

[b] *Institute of Agro-products Processing Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China*

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ABSTRACT

One bioactive compound, identified as alternariol 9-methyl ether, was isolated from the crude extract of the endophytic fungus *Alternaria* sp. Samif01 residing in the roots of *Salvia miltiorrhiza* Bunge. Alternariol 9-methyl ether was active against bacteria with minimum inhibitory concentration values ranging from 25 to 75 μg/mL and median inhibitory concentration (IC_{50}) values ranging from 16.00 to 38.27 μg/mL. The IC_{50} value of alternariol 9-methyl ether against spore germination of *Magnaporthe oryzae* was 87.18 μg/mL. Alternariol 9-methyl ether also showed antinematodal activity against *Bursaphelenchus xylophilus* and *Caenorhabditis elegans* with IC_{50} values of 98.17 μg/mL and 74.62 μg/mL, respectively. This work is the first report on alternariol 9-methyl ether and its biological activities from the endophytic fungus *Alternaria* sp. Samif01 derived from *S. miltiorrhiza* Bunge. The results indicate the potential of *Alternaria* sp. Samif01 as a source of alternariol 9-methyl ether and also support that alternariol 9-methyl ether is a natural compound with high potential bioactivity against microorganisms.

Introduction

Plant endophytic fungi are defined as fungi that live asymptomatically within plant tissues.[1] They are rich potential resources for producing bioactive metabolites such as antimicrobial, insecticidal, anti-viral, anti-tumor and antioxidant compounds.[2,3] Some endophytic fungi have the ability to produce the same or similar bioactive compounds as the ones that originate from their host plants.[4] Isolation of the endophytic fungi that produce bioactive substances has also become an efficient method to screen broad-spectrum, stable agents with low phytotoxicity.[5,6]

Salvia miltiorrhiza Bunge (Lamiaceae) has been widely used in traditional oriental medicine for improving body functions, treatment of cardiovascular diseases, and liver diseases.[7,8] Some endophytic fungi isolated from *S. miltiorrhiza* were

* *Corresponding author at*: Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China.
E-mail: lgzhou@cau.edu.cn (L. Zhou).
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determined to contain tanshinones by TLC, HPLC, and LC-MS analyses,[9,10] though these tanshinone-producing endophytic fungi should be further verified.

In this work, we report the endophytic AME-producing fungus *Alternaria* sp. Samif01 derived from *S. miltiorrhiza*. Both the fungus and the AME structure were identified. The antimicrobial and antinematodal activities of AME were also evaluated to provide data supporting the development and utilization of *Alternaria* sp. Samif01.

Materials and methods

General

The melting point (m.p.) of the compound was determined on an XT4-100B microscopic melting-point apparatus (Tianjin Tianguang Optical Instruments Company, China) and uncorrected. NMR spectra were recorded on a Bruker Avance DRX-400 (^{1}H at 400 MHz and ^{13}C at 100 MHz) NMR spectrometer using tetramethylsilane (TMS) as an internal standard, and chemical shifts were recorded as δ values. A high-resolution electrospray mass spectrometry (HR-ESI-MS) spectrum was measured using a Bruker Apex IV FTMS mass spectrometer. A microplate spectrophotometer (PowerWave HT, BioTek Instruments, USA) was applied to measure light absorption values of the samples. The silica gel (200–300 mesh) for column chromatography (CC) and silica gel GF_{254} for thin layer chromatography (TLC) were purchased from Qingdao Marine Chemical Company, China. Sephadex LH-20 and silica gel RP-18 were obtained from Pharmacia Biotech, Sweden. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Amresco (USA). Streptomycin sulfate and carbendazim were purchased from Sigma–Aldrich (USA). Avemectin with a purity of 97.2% was kindly provided by Dr. Shankui Yuan at the Institute for the Control of Agrochemicals, Chinese Ministry of Agriculture. All other chemicals used in the study were of analytical grade.

Plant materials and isolation of endophytic fungi

The three-year old healthy roots of *S. miltiorrhiza* Bunge were collected from the Institute of Medicinal Plant Development (116°16′27″ E, 40°1′59″ N), Chinese Academy of Medical Sciences, Beijing, China, in July 2011. The plant was identified according to its morphological features by Prof. Yuhai Guo, a botanist from the College of Agronomy and Biotechnology at China Agricultural University. The voucher specimen (BSMPMI-201107001) of this plant was deposited in the Herbarium of the Institute of Chinese Medicinal Materials, China Agricultural University. The plant samples were stored in sealed plastic bags at 4 °C for processing within 24 h of collection. The isolation of endophytic fungi was performed according to previous reports[11,12] with some modifications.

The root samples were rinsed thoroughly with tap water to remove soil residues and dust, sterilized with 75% ethanol for 2 min and immersed in 0.2% mercuric chloride for 20 min, then rinsed 4 times with sterile distilled water. After surface sterilization, both root epidermis and remnant tissues were cut into small pieces of 0.5 cm × 0.5 cm and placed under aseptic conditions on potato dextrose agar (PDA) plates containing 500 μg/mL of streptomycin sulfate and incubated at 25 °C until the mycelia were apparent on PDA plates. Pure cultures were finally isolated by hyphal tip isolation on PDA plates without antibiotics and stored at 4 °C.

Morphological characterization

The morphological characteristics of the fungal isolate Samif01 were observed and described according to the methods of Ainsworth et al.,[13] Photita et al.[14] and Li et al.,[11] including colony morphology and microscopic observation of mycelia and asexual/sexual spores.

DNA extraction, ITS-rDNA amplification and sequence analysis

Total genomic DNA of the fungal isolate Samif01 was prepared according to the protocols described by Wang et al.[15] and Jasalavich et al.[16] The ITS region was amplified by polymerase chain reaction (PCR) with the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described previously.[11,12,17] For identification, the PCR product was purified using the QIA quick Gel Purification Kit (Qiagen, Hilden, Germany) as described by the manufacturer's protocol and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer (Applied Biosystem, USA). Then, the sequence was run by the BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov) and submitted to GenBank, where the accession number was obtained.

Mycelia suspension culture and crude extract preparation

After growing on a PDA plate at 25 °C for 5 days, two or three mycelia plugs from the actively growing colony edge of the endophytic fungus Samif01 were inoculated into 1000 mL Erlenmeyer flasks containing 300 mL potato dextrose broth (PDB). All flasks were incubated in a rotary shaker at 150 rpm and 25 °C for 15 days. Afterwards, a total of 30 L fermentation broth was harvested. The fermented broth was filtered under vacuum to separate the mycelia from the filtrate. The mycelia were dried and powdered (40.2 g), then extracted in methanol with ultrasound. The methanol solution was concentrated in vacuum at 50 °C to obtain a crude methanol extract (12.6 g), which was further thoroughly mixed with water and fractionated with ethyl acetate to obtain a concentrated ethyl acetate fraction (4.6 g). The filtrate was fractionated with ethyl acetate to afford a concentrated ethyl acetate fraction (3.0 g). As the TLC profiles of the mycelia and filtrate fractions were similar, they were combined to obtain a crude total ethyl acetate extract (7.6 g).

Separation and identification of alternariol 9-methyl ether

The crude ethyl acetate extract (7.0 g) was first subjected to chromatography over a silica gel column (200–300 mesh) eluted with petroleum ether-ethyl acetate (3:2, v/v) to obtain eight fractions (fractions A–H) using TLC detection. According