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Original Research Article

Antioxidant Compound of Endophytic Fungi *Penecillium oxalicum* Isolated from *Swietenia mahagoni* L. Jacq

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ABSTRACT

Endophytic fungi are microorganisms that are symbionts in plant tissues. Endophytic fungi isolated from medicinal plants such as *Swietenia mahagoni* may produce a plethora of bioactive compounds. In this study, endophytic fungi was isolated from the leaves of *S. mahagoni* and cultivated in potato-dextrose broth (PDB) for six weeks at static condition. The antioxidant compound was isolated from the ethyl acetate extract of endophytic fungi by chromatographic method, and the chemical structure was determined by spectroscopic analysis. The antioxidant activity was tested by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, and the endophytic fungi was analyzed to identify its species by molecular method. Based on the spectroscopic analysis (^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC spectra), the antioxidant compound was identified as mono-*o*-methylcurvilinic acid with IC_{50} of 38.01 µg/mL. Phylogenetic analysis showed the endophytic fungi as *Penicillium oxalicum*.

Keywords: Antioxidant compound, Endophytic fungi, *Penicillium oxalicum*, *Swietenia mahagoni*

Introduction

Lifestyle factors, stress, and environmental influences can cause excessive free radical formation and trigger oxidative stress. Oxidative stress is an important risk factor in the pathogenesis of chronic diseases. Reactive oxygen species (ROS), such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), can cause oxidative damage to macromolecules such as DNA, proteins, lipids, and small molecules. ROS is recognized as an agent involved in the pathogenesis of diseases such as cardiovascular, asthma, arthropathy, inflammation, diabetes, cancer, anemia, ischemia, stroke, parkinson, alzheimer, atherosclerosis, neurodegenerative and premature aging.[1-4] Therefore, the balance between oxidation and antioxidants is vital to maintain the health of the biological system.

Antioxidants are substances that inhibit oxidative damage to target molecules.[3] The antioxidant activity will stop the chain reaction by eliminating free radical intermediates and inhibiting other oxidation reactions.[2] Antioxidant compounds have the ability to trap free radicals such as peroxides, hydroperoxides, or peroxyl lipids by inhibiting oxidative mechanisms that cause degenerative diseases.[3] Antioxidant compounds are needed to overcome cell damage. However, synthetic antioxidant compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) can cause various side effects.[5] Another alternative used to minimize synthetic antioxidants' side effects are to take advantage of medicinal plant extracts' benefits because medicinal plants have low toxicity and good therapeutic performance.[4] Antioxidant compounds of medicinal plants displays good protection against free radicals and an inhibitor of lipid peroxidation.[1] Medicinal plants also contain polyphenol and flavonoid compounds, useful as antioxidants.[2] Studies on plant activity are increasing rapidly due to interest in the potential used as a source of natural antioxidants.[4]

Endophytic fungi are type of fungi that can live in plant tissue at certain periods of their life without causing damage to the host.[5,6] Bioactive compounds from endophytic fungi are a potential source of new natural antioxidants for medicinal, agricultural, and industrial purposes.[7] The secondary metabolites of endophytic fungi are useful for plant defence, and some have potential use in modern medicine such as antibiotics, antidiabetic, anticancer and antioxidant.[8] Endophytic fungi isolated from different hosts showed significant differences in biological activity.[9] The same isolates inhabiting different hosts exhibit different and varied biological activities.[10] In any ecosystem, the production of secondary metabolites is based on the mutualistic relationship of endophytes and their hosts.[9]

Antioxidant compounds such as flavonoids and phenol derivatives have been obtained from *S. mahagoni.*[11-14] Endophytic fungi isolated from medicinal plants are a good source of bioactive compounds because they produce important and novel bioactive compounds in a short time and do not require large areas to grow.[15,16]

Materials and Methods

Plant material

Fresh leaves of mahoni (Swietenia mahagoni) were collected during last week of December 2018 from Ogan Ilir in the province of South Sumatra, Indonesia with identification number 24/UN9.1.7/4/PP/2018.

Isolation of endophytic fungi

The PDA medium was inserted in a petri dish using physical sterilization by heating. The medium was left to solidify. Leaves of mahoni were cleaned by running water, sterilized with 70% alcohol then rinsed with sterile distilled water. This sterilization was done three times. Then the samples were re-sterilized by 1% NaOCl (1

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min). Then, samples were cleaned again by sterile aquades. Leaf samples were cut to 0.5 cm in width and 2 cm in length. Then the leaf pieces were plated into a petri dish containing a solid field PDA medium containing chloramphenicol (150 mg/L).[15] The sample was incubated for 1–2 weeks at room temperature. Colonies were grown in a medium and observed after 3 to 14 days. Colonies that show differences characteristic was considered as different isolates, then separated and re-cultured in the PDA medium until a similar culture were obtained.[17]

Cultivation and extraction

Each fungal strain was cultivated into potato dextrose broth (PDB) medium (20 g dextrose monohydrates, 200 g potatoes, and 1,000 mL aquades) under static conditions at room temperature. The mycelia agar plugs of all strain (5 x 5 mm^2, 6 pieces) were cultivated into PDB medium (200 mL) for six weeks. After cultivation, The broth culture was extracted by partition technique with ethyl acetate (1 : 1) and concentrated under vacuum at 45^oC.[17,18]

Antioxidant activity test

The extract was weighed 0.002 g and dissolved with 2 mL of methanol. Then the mixture was diluted to 1000, 500, 250, 125, 62.5, 31.2 ppm. Each concentration was added by 3.8 mL DPPH 0.05 mM and left in a dark room for ± 30 minutes. Each sample was determined for its absorbance value using a UV spectrometer at a wavelength of 517 nm. Each sample was determined by % inhibition, using the following formula.[19]

$$\% \text{ Inhibition} = \frac{Standard\ Absorbance - Absorbance\ of\ sample}{Standard\ Absorbance} \times 100\%$$

Identification of endophytic fungus

Endophytic fungus was identified using the internal transcribed spacer (ITS) method conducted at LIPI Cibinong biological research center. Purified DNA extracts were analyzed by PCR method using ITS-4 and ITS-5 primers. Furthermore, nucleotide sequences were compared for homology with BLAST search (http://www.ncbi.nlm.nih.gov). That file was converted to txt, MSF, and PHYLIP files using the ClustalW program**.** The run file was renamed and entered into the SEQBOOT program then calculated by replication 1000 times. The resulting file was run with the DNAdist and Neighbor program with a lot of data processing 1000 times. The file was entered into the CONSENSE program. The phylogenetic tree was visualized by the Treeview and Mega4 program.[20]

Isolation and identification of pure Compound

Secondary metabolite of the active extract was separated using column chromatography with silica gel as the stationary phase and a gradient solvent as the mobile phase. Eluate was collected in vials and grouped into sub-fraction by thin layer chromatographic (TLC) analysis. The secondary metabolite of sub-fraction was separated and purified by column chromatography and recrystallization techniques to yield pure compound. The chemical structures were determined by NMR spectra, including NMR 1 and 2 Dimension (Agilent DD2 ^{1}H-500 MHz and ^{13}C-125 MHz).

Results and Discussion

A total of three fungal isolates (labeled: DMP1, DMQ2, and DMR3) were isolated from leaves *S. mahagoni* collected from Ogan Ilir in the province of South Sumatra, Indonesia in December 2018. Each fungi was harvested and the medium extracted with ethyl acetate until concentrated. Each extract was tested for antioxidant activity with a variety of sample concentrations. Antioxidant activity tests was carried out using the DPPH (2,2-diphenyl-1-picrylhidrazyl) method. The method was used because a sample's antioxidant reactivity can be easily detected when the DPPH solution changes colour (from violet to yellow) upon addition of an antioxidant compound.[21-24] DPPH is an active free radical that has very reactive free electrons. The presence of antioxidants can reduce the reactivity of free radicals by donating electrons. The 2,2-diphenyl-1-picrylhydrazine (violet) when reacted with antioxidant compounds produces 2,2-diphenyl-1-picrylhydrazine (yellow) which is non-reactive. The IC_{50} concentration was calculated based on the results of the percentage inhibition of each sample concentration. Based on these data, we find that the DMP1 extract had a higher activity compared other extracts (Table 1).

The DNA molecule of DMP1 was identified using the PCR method. The obtained sequences were subjected to BLAST program at the National Center for Biotechnology Information (NCBI). The ITS sequences of isolate DMP1 showed homology with 20 strain of fungus as *penicillium family*. The following is DNA of DMP1 counting:

TATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA
ACCTGGTTAAGATTGATGGTGTTCGCCGGCGGGCGCCGGCC
GGGCCTACAGAGCGGGTGACGAAGCCCCATACGCTCGAGG
ACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGCCCCCC
GGAAGCGGGGGGCGAGAGCCCAACACACAAGCCGTGCTTG
AGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAAT
ACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCAC
TGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTT
CTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTT
TTAACTGATTTAGTCAAGTACTCAGACTGCAATCTTCAGACA
AGAGTTCGTTTGTGTGTCTTCGGCGGCCGTGAGGCGGGCCC
GCCGAAGCAACAAGGTACGATAAACACGGGTGGG

The closest relationship of each sequence was further analyzed using the phylogenetic method by using the neighbor-joining method with 1000 matches. It was found that the DMP1 fungus had DNA similarity with the fungus *Penicillium oxalicum* isolate HC6 (MH558553)[25] with 100% similarity (figure 1).

Penicillium oxalicum is one of the fungi whose extracts are widely used as an anti-fungal, anti-microbial and antioxidant[26,27,28]. The ethyl acetate extract of the fungus *Penicillium oxalicum* inhibited the peroxidation of linoleic acid by 78% [29]. An antioxidant compound of *Penicillium oxalicum* chloroform extract had an IC_{50} value of 17.47 µg/ml for antioxidant test with the DPPH method.[30]

The crude extract of DMP1 (3 g) was separated using column chromatography (60 g silica gel 70-230 mesh) and eluted with a gradient solvent system, n-Hexane-ethyl acetate (10: 0 → 0: 10) and ethyl acetate-methanol (10: 0 → 8: 2) to give six fractions (F1-F6). Fraction 3 (F3, 402 mg) was further separated by column chromatography and eluted with n-Hexane-ethyl acetate (7:3→0:10) to give four subfractions. Fraction F3.3 (96 mg) was subjected to column chromatography and eluted with n-Hexane-ethyl acetate (4:6) to yield compound **1** (42 mg).

The chemical structure of compound 1 was identified by NMR spectra. The ^{1}H-NMR spectra (figure 2) identified the presence of five proton signals at δ_H 2.5 - 6.5 ppm, which is typical for aromatic, oxymethyl, methyl, and methylene protons. The spectrum showed the presence of two signals in the aromatic region at δ_H 6.36 (1H, d, J = 2.5 Hz) and 6.34 (1H, d, J = 2.5 Hz) ppm as doublet meta-coupled protons. The proton signal at δ_H 2.53 (3H, s) and 3.78 (3H, s) indicated the presence of one methyl and one methoxyl group. Other signals appeared at δ_H 3.70 (2H, s) as methylene protons of the acetate group.

The ^{13}C-NMR spectrum (figure 2) showed the presence of eleven signals carbon at δ_C 30-210 ppm which consists of oxymethyl (δ_C 55.8), ketone (δ_C 206.4), methyl ketone (δ_C 32.4), carboxylate (δ_C 137.8), methylene acetate (δ_C 40.8) carbon, and six aromatic carbons. The presence of two oxyaryl (= C-O) of aromatic carbons exhibited at δ_C 163.7 and 160.5 ppm.

Table 1: Antioxidant activity using DPPH method

Samples	IC_{50} (µg/mL)
DMP1	50.28
DMQ2	1963.02
DMR3	1036.15
Compound 1	38.01
Ascorbic acid	10.54

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The HMQC spectrum (Figure 2) showed the presence of five ^{1}H-^{13}C correlations through one bond consisting of two correlations in the aromatic ring, one correlation to the methoxyl, methyl ketone, and methylene acetate groups, respectively. The HMBC spectrum (Figure 2) showed a correlation between the methylene acetate proton signal at δ_H 3.70 ppm with the carboxylate carbon (δ_C 175.4), and aromatic carbons (δ_C 110.7; 121.9; and 137.8 ppm). The correlation indicates that the methylene acetate group is attach directly to the aromatic ring. The methyl ketone proton at δ_H 2.53 showed correlation with the carbonyl carbon of the ketone group at δ_C 206.4 ppm. The proton δ_H 3.78 is far from the carbon in the aromatic ring with a chemical shift value of δ_C 163.7. The relationship between HMQC and HMBC can be seen in table 2. Best on the spectroscopy data, compound 1 was identified as 2-(2-acetyl-3-hydroxy-5-methoxyphenyl)acetic acid or mono-O-methylcurvilinic acid (Figure 3) with good antioxidant activity IC_{50} 38.01 µg/mL (Table 1).

Mono-o-methylcurvilinic acid is not produced by the host *S. mahagoni*. However, the compound was also found in another endophytic fungi, namely *Drechslera indica* purchased from the Commonwealth Mycological Institute (CMI)[31] and *Penecillium mariae-crucis* was isolated from *Paeonia lactiflora Pall.*[32] The production of the same compound in some endophytic fungi is common. An example is (5-hydroxy-2-oxo-2H pyran-4-yl) methyl acetate produced by the endophytic fungus *Cladosporium tenuissimum* from *S. mahagoni*,[20] *Trichoderma* sp. from *Tinospora crispa*,[33] and *Aspergillus* sp from *Andrographis paniculata.*[34]

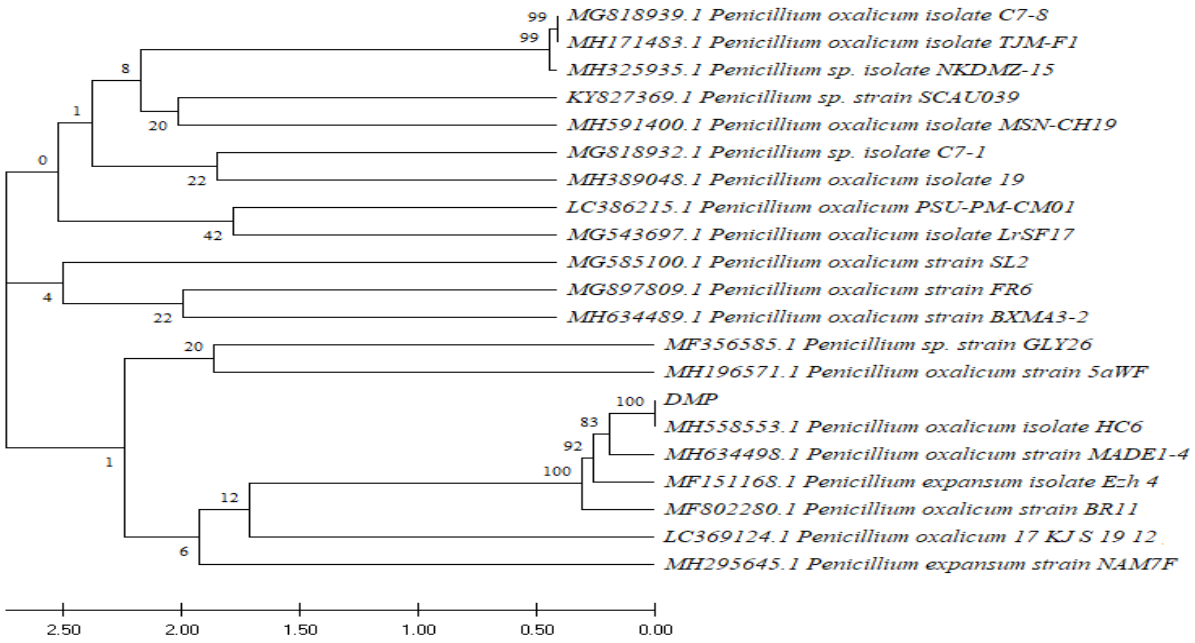

Figure 1: Phylogenetic tree of DMP1

Table 2: The data of NMR spectrum of compound 1 (^{1}H-500 MHz, ^{13}C-125 MHz, in CD_3OD) and mono-O-methylcurvilinic acid 1* (^{1}H-400 MHz in acetone)[31]

No. C	δ_C (ppm)	δ_H (ppm), ΣH, mult, *J* (Hz)	HMBC	δ_H (ppm), ΣH, mult, *J* (Hz)
	1	1	1	1*
1	137.8			
2	121.9			
3	160.5			
4	101.2	6.36 (1H, d,J = 2.5 Hz)	110.7; 160.5;163.7	6.45 (1H, d, J = 2.4 Hz)
5	163.7			
6	110.7	6.34 (1H, d,J = 2.5 Hz)	40.8; 101.2;121.9	6.55(1H, d, J = 2.4 Hz)
1'	40.8	3.70 (2H, s)	110.7; 121.9; 137.8; 175.4	3.81 (2H, s)
2'	175.4			
3'	206.4			
4'	32.4	2.53 (3H, s)	206.4	2.56 (3H, s)
5-OCH_3	55.8	3.78 (3H, s)	163.7	3.82 (3H, s)

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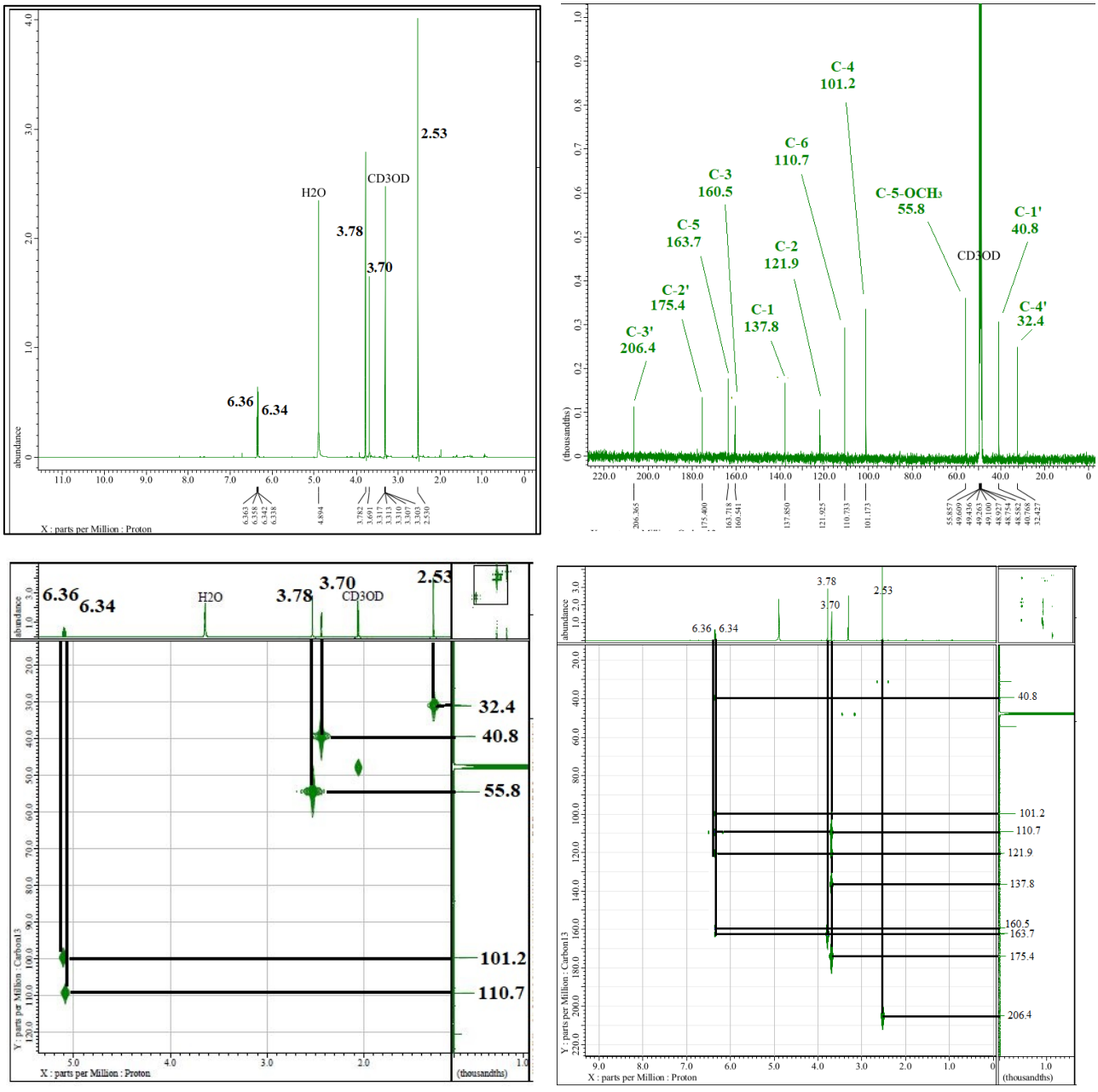

Figure 2: The NMR spectra of compound **1**

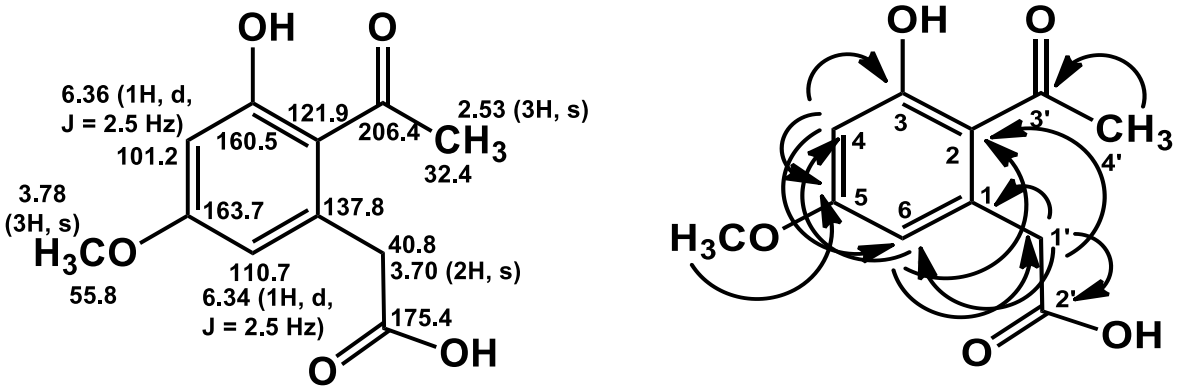

Figure 3: The δ-assignment and HMBC correlation of compound **1** (mono-*O*-methylcurvilinic acid)

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Conclusion

The endophytic fungus *Penicillium oxalicum* isolated from the leaves of *S. mahagoni* is able to produce mono-*o*-methylcurvilinic acid as an antioxidant compound. The compound apart from those produced by the host plant has been found in endophytic fungi.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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