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Volatile metabolites profiling of a Chinese mangrove endophytic *Pestalotiopsis* sp. strain

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***Pestalotiopsis* JCM2A4, an endophytic fungus originally isolated from leaves of the Chinese mangrove plant *Rhizophora mucronata*, produces a mixture of volatile metabolites. As determined by gas chromatography and gas chromatography/mass spectrometry (GC/GC-MS), 18 compounds representing all of the hexane extract were identified. Higher amounts of oil-based straight-chained alkyl (mono- and di-methyl) esters and fatty acids were found to compose major volatile chemotype which accounted for 78.65 and 14.52% of this organism, respectively. The main components was demonstrated to be pentadecanoic acid, 14-methyl-, methyl ester (35.92%); octadecanoic acid, methyl ester (13.10%); nonanedioic acid, dimethyl ester (11.21%); and *n*-hexadecanoic acid (10.54%). Two of these components were isolated and determined to be *n*-hexadecanoic acid and elaidic acid by ^{1}H NMR and ^{1}H-^{1}H COSY spectroscopy. Antioxidant activity of the hexane extract and isolated compounds were screened using 2,2'-diphenyl-b-picrylhydrazyl (DPPH) free radical scavenging method. This is the first report to describe the volatile metabolites of mangrove endophytic *Pestalotiopsis* sp. strain; its specific fatty acid methyl esters (FAME) profile can be used as a tool for microbial source tracking.**

Key words: Mangrove endophytic fungus, *Pestalotiopsis* sp., volatile metabolites, fatty acid methyl esters (FAME) profile.

INTRODUCTION

Mangrove endophytic fungi are microbes that colonize in the inter- and/or intra-cellular spaces of the healthy tissues of the mangrove host and do so in a variety of relationships, ranging from symbiotic to pathogenic. These microbes can be said to hold special ecological status, which for more than 20 years produced and excreted effective biomolecules that may be useful to humans as novel physiological agents (Xu, 2011a; Zhang et al., 2006).

Fungus species *Pestalotiopsis* JCM2A4 isolated from the Chinese Mangrove plant *Rhizophora mucronata* proved to be particularly productive with regard to the accumulation of diverse natural products and yielded so far over 40 unvolatile compounds with 34 of them being new natural products with chromones, cytosporones, coumarins, pyrones and alkaloids predominating (Xu et

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al., 2009a, 2009b, 2011b).

Morphological characters used to differentiate species of *Pestalotiopsis* and similar genera are limited currently; classification and identification of *Pestalotiopsis* was previously based on morphology with conidial characters (Hu et al., 2007). Comparative taxonomic analyses have been hindered by inadequately defined characters and ambiguous distinctions between morphologically similar species (Maharachchikumbura et al., 2011). Thus, molecular and biochemical characters are an important supplement to morphological characters used to define closely related genera in the order *Pestalotiopsis*. Since the introduction of gas chromatographic analysis of cellular fatty acid methyl ester (FAME), this technique has been used frequently in various taxonomic studies. FAME analysis has become established in many laboratories involved in fungal taxonomy and diagnostics (Yousef et al., 2012).

To the best of our knowledge, however, no attempt has been made previously to explore the volatile metabolites from hexane extract of *Pestalotiopsis*. In the current paper, we report for the first time the results of a study aimed to define the volatile metabolites of the mangrove endophytic fungus *Pestalotiopsis* JCM2A4 and construct its specific FAME profiles.

MATERIALS AND METHODS

Isolation and identification of the fungus

Pestalotiopsis sp. was isolated from fresh healthy leaf material of *R. mucronata* (Rhizophoraceae) collected in October 2005 in Dong Zhai Gang-Mangrove Garden on Hainan Island, China. The fungus (strain no. JCM2A4) was isolated under sterile conditions from the inner tissue of the leaf following an isolation protocol, cultured on solid rice medium as described previously (Aly et al., 2008) and identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region (GenBank accession no. FJ465172). A voucher strain was deposited at one of the authors' laboratory (P.P.).

Cultivation of the fungus

Mass growth of the fungus for the isolation and identification of new metabolites was carried out in Erlenmeyer flasks (1 L each). The fungus was grown on rice solid medium (to 100 g commercially available rice was added 110 mL of distilled water and kept overnight prior to autoclaving, two flasks) at room temperature under static conditions and daylight for 40 days.

Extraction and Isolation

The mycelia and solid rice medium were extracted with ethyl-acetate. The extract was further extracted with *n*-hexane to get the volatile metabolites and yield of 1.2 g residue. This residue was subjected to vacuum liquid chromatography (VLC) on a silica gel column employing a step gradient of dichloromethane-methanol. Each fraction containing 50 ml was dried and examined by thin layer chromatography (TLC) on premade silica gel plates (Merck, Germany) using a dichloromethane-methanol based solvent system. Promising fractions were subjected to further chromategraphic separation using Sephadex LH-20 with methanol as solvent. Final purification was achieved by repeatedly chromategraphed on silica gel to yield one (9 mg) and two (4 mg). The obtained oil and substances were dried and stored in an amber vial at +4°C until tested and analyzed.

Gas chromatography (GC) analysis

Hexane extract obtained from *Pestalotiopsis* JCM2A4 was analyzed using Hewlett Packard 6890 GC equipped with a flame ionization detector (FID) and HP-FFAP MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). GC oven temperature was kept at 60°C for 3 min initially, and then raised at the rate of 3°C/min to 250 °C. Helium was the carrier gas, at a flow rate of 1 ml/min. Diluted samples 1/1000 in n-pentane, v/v) of 1.0 µL were injected manually and in the splitless mode. Peaks area percents were used for obtaining quantitative data.

Gas chromatography/mass spectrometry (GC/MS) analysis

The analysis of the hexane extract was performed under the same conditions with GC, using a Hewlett Packard 6890 gas chromategraph equipped with a Hewlett Packard 5973 mass selective detector in the electron impact mode (70 eV). Identification of the components was based on comparisons of their relative retention times and mass spectra with those obtained from standards and/or the NIST98 and Wiley275 library data.

Identification of the volatile compounds

^{1}H NMR and ^{1}H-^{1}H COSY (chemical shifts in ppm) spectra were recorded on Bruker ARX 500 NMR spectrometers in $CDCl_3$.

Free radical-scavenging activity

The radical scavenging ability (RSA) of the hexane extract of *Pestalotiopsis* JCM2A4 was estimated by using 2,2'-diphenyl-b-picrylhydrazyl (DPPH) method described previously (Li et al., 2012). Thus, an aliquot of EO solution 1 mL was added to 3 mL of ethanolic DPPH (60 µM). The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark and absorbance was measured at 517 nm. The free radical scavenging activity was calculated as follows:

$$\%RSA = [(A_{blank} - A_{sample} / A_{blank}] \times 100\%$$

Where, A_{blank} was the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} was the absorbance of the test compound.

RESULTS AND DISCUSSION

GC-MS results

The analysis of the hexane extract from mangrove *R. mucronata* endophytic *Pestalotiopsis* JCM2A4 was

Table 1. Volatile organic compounds identified from *Pestalotiopsis* sp. by GC-MS.

Number	R.I.[a]	MF[b]	Component	Composition (%)
1	3.79	$C_{15}H_{30}O_2$	Tetradecanoic acid,methyl ester	0.45
2	4.20	$C_{10}H_{18}O_3$	Nonanoic acid, 9-oxo-, methyl ester	1.01
3	4.84	$C_{10}H_{18}O_4$	Nonanedioic acid monomethyl ester	1.91
4	4.97	$C_9H_{18}O_2$	Nonanoic acid	0.49
5	6.07	$C_{17}H_{34}O_2$	Pentadecanoic acid, 14-methyl-, methyl ester	35.92
6	8.71	$C_{19}H_{38}O_2$	Octadecanoic acid, methyl ester	13.10
7	8.87	$C_{19}H_{36}O_2$	9-Octadecenoic acid (*Z*)-, methyl ester	5.45
8	13.04	$C_9H_{16}O_4$	Suberic acid monomethyl ester	2.38
9	14.79	$C_{11}H_{20}O_4$	Nonanedioic acid, dimethyl ester	11.21
10	15.87	$C_{16}H_{32}O_2$	*n*-Hexadecanoic acid	10.54
11	17.82	$C_{13}H_{24}O$	Tetrahydroedulan	1.06
12	18.46	$C_{25}H_{50}O_2$	Tetracosanoic acid, methyl ester	5.67
13	18.56	$C_{12}H_{18}O_2$	4,5-Epoxy-5,7,7-trimethylspiro[3.5]nonan-1-one	2.69
14	19.13	$C_{18}H_{36}O_2$	Octadecanoic acid	2.29
15	19.52	$C_{18}H_{34}O_2$	Elaidic acid	1.20
16	22.55	$C_8H_{13}NOS$	3H,6H-Thieno[3,4-c]isoxazole,3a,4-dihydro-6-(1-methylethyl)-	0.81
17	24.32	$C_{12}H_{11}NO$	4-[4-methoxyphenyl]Pyridine	2.26
18	25.36	$C_{10}H_{20}O_3$	8-Methoxy octanoic acid, methyl ester	1.55

simultaneously performed using gas chromatography-mass spectrometry (GC-MS). The detected volatile components of the fungus and its relative percentages according to their relative retention indices (RI) are given in Table 1. 18 components were identified representing all of the extract. Pentadecanoic acid, 14-methyl-, methyl ester (35.92%); octadecanoic acid, methyl ester (13.10%); nonanedioic acid, dimethyl ester (11.21%); and *n*-hexadecanoic acid (10.54%) were the main constituents and totally comprising 70.77% of the extract. In addition, high amounts of straight-chained alkyl (mono- and di-methyl) esters and fatty acids were found to compose a major chemotype of the extract, such as tetradecanoic acid,methyl ester; nonanoic acid, 9-oxo-, methyl ester; nonanedioic acid, dimethyl ester; pentadecanoic acid, 14-methyl-, methyl ester; octadecanoic acid, methyl ester; 9-octadecenoic acid (*Z*)-, methyl ester; suberic acid monomethyl ester; nonanedioic acid, dimethyl ester; tetracosanoic acid, methyl ester; and 8-methoxy octanoic acid, methyl ester, accounted for 78.65% of this organism; nonanoic acid; *n*-hexadecanoic acid; octadecanoic acid; and elaidic acid accounted for 14.52% of this organism, respectively. Since every microorganism has specific microbial fingerprinting such as FAME profile, its stability and heritability was justified previously (Chowdhury and Dick, 2012). On the basis of the above results, the specific FAME profile of *Pestalotiopsis* can be used as a taxonomic tool for microbial source tracking in the future. In addition, some pharmaceutical components were discovered (Figure 1). Research has indicated that elaidic acid increased low density lipoprotein cholesterol and decreased high density lipoprotein cholesterol in human *via* increase plasma cholesteryl ester transfer protein activity (Brouwer et al., 2010).

Identification of the volatile components of hexane extract

Mycelia and culture medium of the endophytic fungus *Pestalotiopsis* sp. were extracted with ethylacetate and following by hexane. This extract was concentrated and then repeatedly chromatographed on silica gel and followed by Sephadex LH-20 to yield two known compounds, *n*-hexadecanoic acid and elaidic acid. Their structures were unambiguously elucidated on the basis of ^{1}H NMR and ^{1}H-^{1}H COSY spectroscopic data analysis as shown in Figure 2. Although these two compounds has been isolated previously from a wide range of organisms (Kandhro et al., 2008; Silici et al., 2005), this is the first report of its presence in the *Pestalotiopsis* species.

n-Hexadecanoic acid (10)

Colorless amorphous residue (MeOH); ^{1}H NMR(500 MHz, $CDCl_3$) δ 2.35(2H, d, J = 7.3 Hz, H-2), 1.63(2H, m, H-3), 1.30(20H, m, H4-15), 0.89(3H, t, J = 6.9 Hz, H-16); EI-MS $[M]^+$ *m/z* 256.24 ($C_{16}H_{32}O_2$).

Elaidic acid (15)

Colorless amorphous residue (MeOH); ^{1}H NMR (500

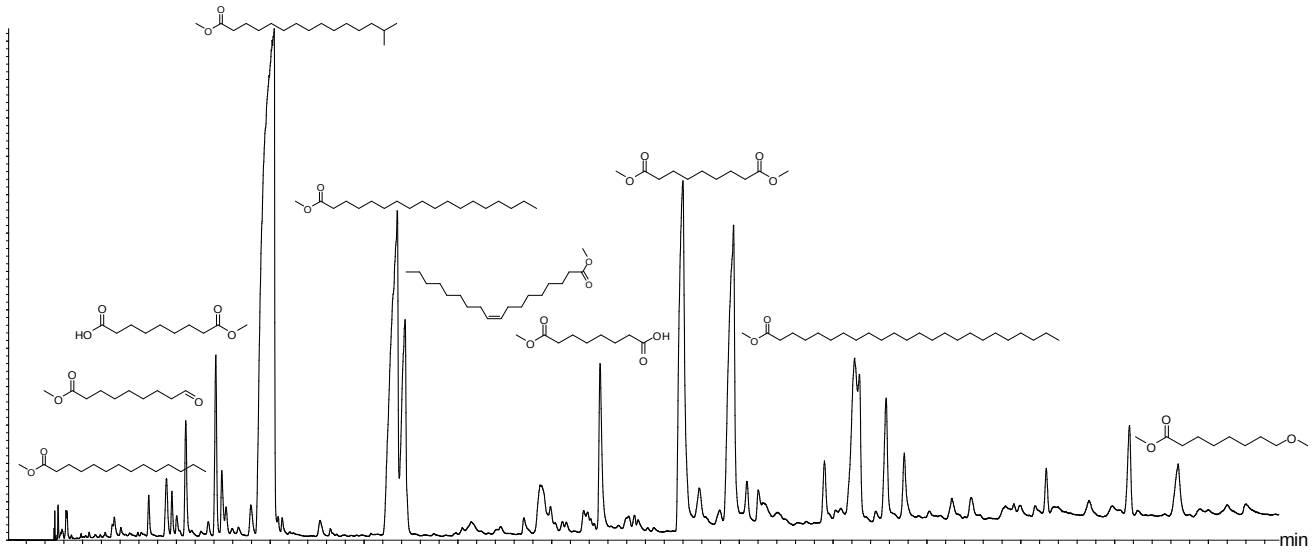

Figure 1. Fatty acid methyl esters (FAME) profile of *Pestalotiopsis* sp.

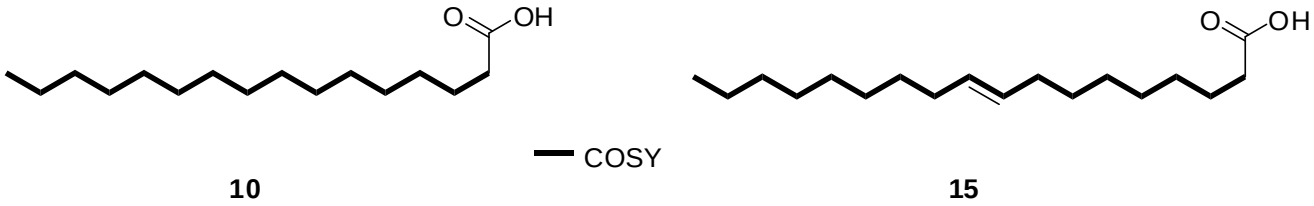

Figure 2. Key ^{1}H-^{1}H COSY correlations of n-Hexadecanoic acid (10) and Elaidic acid (15).

MHz, $CDCl_3$) δ 5.35 (2H, m, H-9, H-10), 2.34(2H, d, J = 7.5 Hz, H-2), 2.02(4H, m, H-8, H-11), 1.62(2H, m, H-3), 1.30(20H, m, H4-7, H11-17), 0.87(3H, t, J = 6.9 Hz, H-18); EI-MS $[M]^+$ *m/z* 282.26 ($C_{18}H_{34}O_2$).

Antioxidant activity

The volatile extract and their constituents generally displayed strong antioxidant property, which are useful in daily life in foods and pharmaceutical agents against various diseases. In the case of *Pestalotiopsis* sp., the hexane extract and all isolated compounds were devoid of significant activity at 500 µg/mL in the DPPH radical-scanvenging bioassays used.

Conclusions

In this study, 18 components were identified by GC-MS representing all of the hexane extract of Chinese mangrove plant *R. mucronata* endophytic *Pestalotiopsis* JCM2A4. Oil-based straight-chained alkyl (mono- and di-methyl) esters and fatty acids were found to have major chemotypes of the chemical composition. The specific FAME profile of *Pestalotiopsis* JCM2A4 was constructed. Meanwhile, two of these components were isolated and determined to be *n*-hexadecanoic acid and elaidic acid by ^{1}H NMR and ^{1}H-^{1}H COSY spectroscopy. Antioxidant capacity was evaluated by measuring the scavenging effect on 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical. However, none of hexane extract and two isolated compounds investigated proved to be devoid of significant activity when tested at an initial concentration of 500 µg/mL in the bioassay used. These results encourage complementary and more in-depth studies on the other biological activities or for the non-volatile fractions as a natural source of functional ingredients.

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