American Journal of Essential Oils and Natural Products

Available online at www.essencejournal.com

AJEONP American Journal of Essential Oils and Natural Products

AkiNik

ISSN: 2321-9114
AJEONP 2020; 8(1): 01-05

Received: 01-11-2019
Accepted: 02-12-2019

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Fatty acid profile of an oleaginous endophytic *Pseudofusicoccum* sp. Isolated from *Annona muricata*

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Abstract
This study was carried out to determine the fatty acid composition of secondary metabolites produced by a lipid-producing endophytic fungus *Pseudofusicoccum* sp. The fungus was isolated from the leaves of *Annona muricata* growing in Ifite Dunu, Anambra State, South-East Nigeria. Solid state fermentation was carried out using rice medium and the metabolites were extracted with ethyl acetate. The crude ethyl acetate extract was subjected to vacuum liquid chromatography (VLC), and the fatty acid profiles of the resulting oleaginous fractions were analyzed using gas chromatography-mass spectroscopy (GC-MS). GC-MS analysis of the fractions revealed the abundance of both saturated and unsaturated fatty acids. Ethyl palmitate (17.31%) was the most abundant compound in F1; while bisabolol (11.45%), ethyl oleate (12.73%), and palmitic acid (22.22%) were predominant in F2. These compounds have been reported to show beneficial biological properties with potentials for pharmaceutical and industrial applications.

Keywords: *Annona muricata*, endophytic fungus, *Pseudofusicoccum* sp., fatty acids, oleaginous fungi

1. Introduction
Anonna muricata, a species of the Annonaceae family, is widely distributed throughout tropical and subtropical parts of the world, including Nigeria and is known for its edible fruit commonly called soursop. The plant has been used in traditional medicine for the treatment of diverse ailments like bacterial infections, skin diseases, fever, neuralgia, arthritis, diabetes, headaches, insomnia, abscesses, tumors and cancer [1-4].
The phytochemical composition of the plant includes alkaloids, flavonol triglycosides, phenolics, cyclopeptides and essential oils [5-9]. Gas chromatography-mass spectroscopy (GC-MS) analysis of *A. muricata* leaf oil showed the presence of volatile oil constituents such as β-pinene, germacrene D, ρ-mentha-2,4(8)-diene, α-pinene and β-elemene, δ-cadinene, epi-α-cadinol and α-cadinol [10, 11].
There are reports on the endophytic fungal populations of *A. muricata* [12-15, 16-21]. Secondary metabolites of endophytic fungi associated with this plant have been revealed to show anticancer, anti-HIV and antioxidant activities [12, 17, 20, 21]. Since endophytes are known to possess potentials in the discovery of metabolites for pharmaceutical, industrial, or agricultural applications, our study was aimed at analyzing the fatty acid content of the oleaginous secondary metabolites of an endophytic fungus associated with the leaves of *A. muricata*.

2. Materials and Methods
2.1 Isolation and identification of Endophytic Fungus
Fresh and healthy leaves of *A. muricata* were collected from Ifite Dunu in Anambra State, Nigeria. An endophytic fungus was isolated from the plant leaves and taxonomic identification of the fungus was achieved by DNA amplification and sequencing of the ITS region [22].

2.2 Fermentation and Extraction of Metabolites
The fungus was grown on sterile solid rice medium (100 g rice and 110 mL distilled water) in 1L Erlenmeyer flasks at 27 ºC under static conditions for 30 days. At the end of fermentation, the fungal secondary metabolites were extracted with EtOAc, and the extract was concentrated under reduced pressure.

2.3 Fractionation of Extract
After extraction a weight of 6.3 g of the fungal crude extract was obtained. Vacuum liquid chromatography on Silica gel 60 (70–230 mesh, Merck, Germany) as stationary phase was

employed as fractionation medium. Stepwise gradient elution was done using non-polar and moderately polar solvent system (*n*-hex: EtOAc) in the ratio of 90:10, 80:20, 60:40, 50:50, and 40:60. Increasing the polarity with a polar solvent system DCM: MeOH in the ratio of 100:0, 95:5, 90:10, 80:20 and 0:100, successive non-oleaginous fractions were obtained from the fungal crude extract. Two oily fractions (F1 and F2) were obtained following VLC of the crude extract of *Pseudofusicoccum* sp. F1 was obtained with *n*-hex: EtOAc at the ratio of 90:10; and F2 was obtained at an increased polarity of *n*-hex: EtOAc (80:20).

Using other experimental procedures such as HPLC, MS and NMR spectroscopy, three phenolic compounds tyrosol, protocatechuic acid and *p*-hydroxyphenyl acetic acid were isolated from the other non-oily fractions [23].

2.4 Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS analysis of the oleaginous fractions (F1 and F2) fractions was carried out using a GC-MS-QP-2010 PLUS (Shimadzu, Japan). For GC, separation was achieved using a VF-5ms fused silica capillary column (30 m x 0.25 mm, and 0.25 µm film thickness). Injection was in the split mode, and helium was used as a carrier gas at a constant column flow of 1.58 mL/min at inlet pressure of 108.0 kPa. The temperature programme was maintained from 80 °C to 280 °C, with injection temperature set at 250 ºC. For the mass spectroscopy, ACQ mode scanner with scan range of 40-600 m/z at the speed of 1250 was used. Ion source and interface temperatures were set at 230 °C and 250 °C respectively. The constituents of the fractions were identified by their GC retention time (RT) and comparison of their mass spectra with those of the National Institute for Standard Technology (NIST) mass spectral library.

3. Results

The endophytic fungus isolated from *A. muricata* was identified as *Pseudofusicoccum* sp. The DNA sequence data of the fungus was deposited in the NCBI database with Gen Bank accession number-MH022721. The crude EtOAc extract produced by *Pseudofusicoccum* sp. was found to contain large quantity of lipids. After VLC, the fungal extract yielded several fractions, including two oily fractions (Fractions F1 and F2).

Fraction F1 was obtained with *n*-hex: EtOAc at the ratio of 90:10. At room temperature, the fraction was a clear yellowish flowing free flowing oil. The fraction was obtained as 38.35% of the total fungal crude extract. Through GC-MS analysis, F1 was found to contain mostly short, saturated and straight chain fatty acids. Table 1 presents some of the compounds identified in the fraction. Ethyl palmitate (17.31%) was the most abundant compound in F1 (Table 1, Figure 1).

Fraction F2 was obtained at an increased polarity of *n*-hex: EtOAc (80:20). The fraction appeared cloudy and semi-solid at room temperature, and was about 18% of the total fungal crude extract. GC-MS analysis revealed mostly compounds with unsaturated, branched chain and cyclic alkenes with some –OH groups in their structures. They were more of building blocks (like the squalene and farnesene) for other molecules. Table 2 shows some of the compounds identified in the fraction. Bisabolol (11.45%), ethyl oleate (12.73%), and Palmitic acid (22.22%) were predominant in F2 (Table 2, Figure 2).

Table 1: Chemical composition of F1

Names	Molecular formula	MW	RT	PA%
2-Heptenal	$C_7H_{12}O$	112	3.725	0.22
Ethyl caprylate	$C_{10}H_{20}O_2$	172	6.808	0.42
2-Decenal	$C_{10}H_{18}O$	154	7.783	0.19
2,4-Decadienal	$C_{10}H_{16}O$	152	8.583	1.27
Neodene	$C_{14}H_{28}$	196	12.000	0.25
Hexadecane	$C_{16}H_{34}$	226	12.083	0.31
Ethyl myristate	$C_{16}H_{32}O_2$	256	14.467	3.60
Methyl palmitate	$C_{17}H_{34}O_2$	270	16.817	5.54
Ethyl palmitate	$C_{18}H_{36}O_2$	284	18.642	17.31
Methyl linoleate	$C_{19}H_{34}O_2$	294	19.883	3.23
Ethyl (9E)-9-Octadecenoate	$C_{20}H_{38}O_2$	310	21.242	4.21
Ethyl stearate	$C_{20}H_{40}O_2$	312	21.358	5.96
Isopentyl laurate	$C_{17}H_{34}O_2$	270	21.892	7.62
Ethyl icosanoate	$C_{22}H_{44}O_2$	340	23.433	2.09
Heptyl 2-phenylethyl oxalate	$C_{17}H_{24}O_4$	292	26.158	2.55
Squalene (Spinacene; Supraene)	$C_{30}H_{50}$	410	27.792	4.44

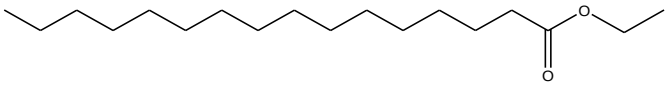
RT= retention Time in minutes, PA=Peak Area; MW=Molecular weight

Table 2: Chemical composition of F2

Name	Molecular formula	MW	RT	PA%
Limonene	$C_{10}H_{16}$	136	4.675	0.18
2-(1,1-dimethylethyl)-Cyclohexanol	$C_{10}H_{20}O$	156	5.417	0.31
Methyl caprylate	$C_9H_{18}O_2$	158	5.842	0.27
Ethyl caprylate	$C_{10}H_{20}O_2$	172	6.842	0.39
Woody acetate	$C_{12}H_{22}O_2$	198	7.667	0.14
2-Decenal	$C_{10}H_{18}O$	154	7.842	0.32
2,4 Decadienal	$C_{10}H_{16}O$	152	8.642	1.52
α-Copaene	$C_{15}H_{24}$	204	9.525	0.68
2,4-Diisopropenyl-1-methyl-1-vinylcyclohexane	$C_{15}H_{24}$	204	9.708	2.08
Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-,	$C_{15}H_{24}$	204	10.158	2.31
α-Humulene (α-Caryophyllene)	$C_{15}H_{24}$	204	10.600	1.90

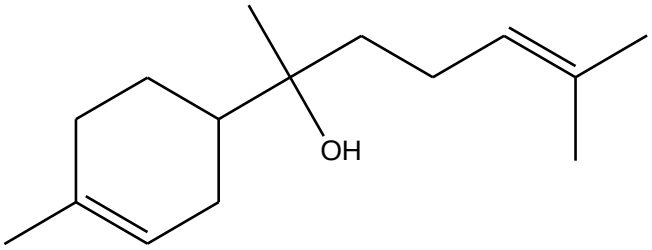
β-Farnesene	$C_{15}H_{24}$	204	11.167	4.53
β-Sesquiphellandrene	$C_{15}H_{24}$	204	11.350	1.40
(*S*,*Z*)-Nerolidol (Peruviol; Penetrol)	$C_{15}H_{26}O$	222	11.817	2.05
α-Bisabolol oxide B	$C_{15}H_{26}O_2$	238	13.050	2.78
Bisabolol (Levomenol)	$C_{15}H_{26}O$	222	13.425	11.45
Ethyl myristate	$C_{16}H_{32}O_2$	256	14.533	2.16
Methyl palmitate	$C_{17}H_{34}O_2$	270	16.992	4.04
Palmitic acid	$C_{16}H_{32}O_2$	256	17.892	22.22
Ethyl palmitate	$C_{18}H_{36}O_2$	284	18.525	4.87
Methyl (14E)-14-octadecenoate	$C_{19}H_{36}O_2$	296	20.092	2.95
Ethyl linoleate	$C_{20}H_{36}O_2$	308	20.833	6.25
Ethyl oleate (Ethyl-9-octadecanoate)	$C_{20}H_{38}O_2$	310	21.275	12.73
Isopentyl laurate (Isoamyl laurate)	$C_{17}H_{34}O_2$	270	22.092	3.33
2-Phenylethyl nonanoate	$C_{17}H_{26}O_2$	262	26.317	2.66
Ethyl tetracosanoate	$C_{26}H_{52}O_2$	396	27.458	1.58
Squalene (Spinacene; Supraene)	$C_{30}H_{50}$	410	27.892	1.34

RT= retention Time in minutes, PA=Peak Area; MW=Molecular weight

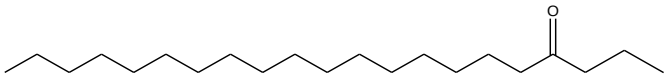


Ethyl palmitate- 17.31%

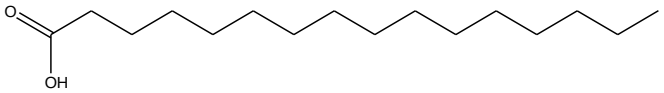
Fig 1: Major constituent(s) of Fraction F1



Bisabolol (Levomenol) - 11.45%



Ethyl oleate (Ethyl-9-octadecanoate) - 12.73%



Palmitic acid (*n*-hexadecanoic acid) - 22.22%

Fig 2: Major constituent(s) of Fraction F2

4. Discussion

This study reveals the ability of the fungus *Pseudofusicoccum* sp. to produce detectable amount of important fatty acids. *Pseudofusicoccum* sp. (family Botryosphaeriaceae) can exist as either an endophyte or a plant pathogen and has been reported to also cause canker disease in some plants [24-26]. Endophytic *Pseudofusicoccum* sp. has been isolated from several plants including *Adansonia gibbosa*, *Acacia synchronica*, *Eucalyptus* sp., *Ficus opposite*, *Mangifera indica*, *Pterocarpus angolensis*, and *Jatropha podagrica* [24-25, 27-28].

There are several reports of the production of important fatty acids by oleaginous endophytes [29-35]. Oil-rich microorganisms have been demonstrated to represent a promising alternative source of lipids for biodiesel production [36-37]. Oleaginous fungi are of special interest as they can be grown on a variety of starting materials (substrates), especially waste lignocellulosic materials; and biomass production can be scaled up in fermentation process to produce more total lipid [37-39].

GC-MS analysis of the VLC fractions (F1 and F2) of *Pseudofusicoccum* sp. crude extract showed the presence of fatty acids and their esters, as well as terpenes. F1 contained more of saturated fatty acids, while F2 had mostly unsaturated alkenes and fatty acids. The constituents of the oleaginous secondary metabolites of *Pseudofusicoccum* sp. are presented in Tables 1 and 2.

The major fatty acid detected in Fraction 1 is ethyl palmitate (17.31%). This compound ethyl palmitate, the ethyl ester of palmitic acid, can be used as a biomarker for assessing ethanol exposure [40]. In Fraction 2, the most abundant fatty acids include palmitic acid, also known as *n*-hexadecanoic acid (22.22%), ethy-9-octadecanoate (12.73%), and bisabolol (11.45%).

Palmitic acid, compared to other fatty acids, has been expressed in more significant concentrations and is a major component of lipids expressed by endophytes [32-34, 41]. Palmitic acid has been reported to show antimicrobial, antioxidant, ant androgenic, hypocholesterolemic, hemolytic 5-alpha reductase inhibition, and nematicidal activities. It has also been used as a pesticide, lubricant, and flavoring agent [42, 43]. Palmitic acid is equally valuable in the production soaps, cosmetics, and release agents [44]. Ethyl oleate (ethyl-9-octadecanoate) is useful as biological additive, PVC plasticizer, water-resisting agent, and for hydraulic fluid [45]. Ethyl oleate can also be used as a biomarker for assessing ethanol exposure [40]. Bisabolol (levomenol), a well-known monocyclic unsaturated sesquiterpene alcohol, is widely used in pharmaceutical and cosmetic preparations due to its anti-inflammatory, antibacterial, antiseptic, skin-soothing and moisturizing properties, as well as its low toxicity [46-49]. Bisabolol has also been reported to be a promising inducer of apoptosis in highly malignant Glioma cells [49, 50].

5. Conclusion

It has also shown that the fungus *Pseudofusicoccum* sp. can produce large quantities of good unsaturated fatty acids. These fatty acids show beneficial biological properties with potentials for pharmaceutical and industrial applications.

6. Acknowledgement

The authors are grateful to Prof. Dr. Peter Proksch of the Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Dusseldorf, Germany for the molecular identification of the fungus.

7. Conflicts of Interest

The authors declare no conflicts of interest.

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