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Lipid profiling, phenolic content and antioxidant activity of the endophytic fungus *Alternaria* sp. isolated from Dracaena plant

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Abstract

The aim of this study is to present the lipid profile along with the total phenolic contents and antioxidant activities of the ethyl acetate (EtOAc) and methanol (MeOH) extracts of the endophytic fungus *Alternaria* sp. isolated from *Dracaena* sp. leaves (Family: Asparagaceae). GC/MS analysis of the petroleum ether (pet. ether) fraction demonstrated high contents of oleic (23.15%), palmitic (23.33%), and linoleic (43.92%) acids. The EtOAc and MeOH extracts displayed a high total phenolic content, which was established by the Folin-Ciocaltue method. Moreover, the EtOAc extract showed considerable free radical scavenging activity (IC_{50}= 0.52 mg/ml) using the DPPH assay and also exhibited substantial reducing power (13.322 mg Ascorbic equiv./g dried extract) by means of the phosphomolybdate complex assay.

Key words
Endophytic fungi, Total phenolic content, Antioxidant activity, Lipid profile, Alternaria sp.

1. Introduction

Terrestrial fungi are considered a rich source for bioactive secondary metabolites with both medicinal and pharmaceutical applications. Fungal endophytes have received the attention of natural product researchers as they look for different sources and ecological niches to avoid frequent rediscovery of previously isolated metabolites of fungi from traditionally investigated habitats [1 - 4]. Endophytic fungi spend a part or all of their life cycle inside tissues of the host plant. The relationship between the endophytic fungus and its host ranges from symbiotic to pathogenic. The fungus obtains nutrition and protection from the host, while its metabolites may enhance the host's growth and resistance [2, 3].

Alternaria is a cosmopolitan fungal genus, and several species are known as plant pathogens, which cause both pre- and post-harvest decay [5]. Although several endophytic Alternaria strains have been investigated before [4, 6, 7], the isolates from particular habitats frequently yield novel natural products [3]. This provoked us to investigate *Alternaria* sp., isolated from the leaves of *Dracaena* sp.,(Family: Asparagaceae) for its fatty acids, phenolic content, and antioxidant potential.

2. Materials and Methods

2.1. Chemicals

Ascorbic acid, gallic acid, Folin-Ciocalteu reagent, and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Co. (St. Louis, MO, USA). Sulphuric acid, sodium carbonate, potassium hydroxide, ammonium molybdate, andsodium dihydrogen phosphate were obtained from Wako Co. (Osaka, Japan).The organic solvents MeOH (99.8%), EtOAc (99.8%), pet. ether (99.8%), and dichloromethane (DCM) (99.8%) were of analytical grade and purchased from Merck Co. (Darmstadt, Germany).

2.2. Isolation of endophytic fungi

The endophyte was isolated from the leaves of *Dracaena* sp.plant, collected from Governorate of Alexandria, Egypt. The isolated endophyte was cultured in Petri dishes with Potato Dextrose Agar medium (fresh potato tubers (200 g); dextrose (10 g); agar (15 g), and distilled water up to 1 L) and incubated at 28 °C for 21 days. The fungus *Alternaria* sp. was identified on the basis of conidial morphology by Dr. Ahmad Moharram (Mycology Center, Faculty of Science, Assiut University, Egypt). Subculture of this fungal strain was deposited in the culture collection of Assiut University Mycology Centre (AUMC) with a voucher number (6873).

2.2.1. Preparation of fungal extracts

The fresh fungal culture was transferred to 15 Erlenmeyer flasks (1 L each) containing rice solid cultures (100 ml of distilled water were added to 100 g commercially available rice and kept overnight prior to autoclaving). The cultures were then incubated at 28 °C for 30 days under aseptic conditions. The culture was successively extracted using EtOAc then MeOH and concentrated under vacuum.

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2.2.2. Preparation of fatty acids

The fungal EtOAc extract was defatted using pet.ether. The fat fraction (3g) was then subjected to alkaline hydrolysis for saponification by refluxing with 50 ml of N/2 alcoholic potassium hydroxide for eight hours. The alcohol was distilled off and the liquid left was diluted with twice its volume of water then extracted with several portions of DCM to eliminate the unsabonifiable matter. The remained alkaline aqueous solution (soap) was acidified with sulphuric acid (10%) and the liberated fatty acids were then extracted with successive portions of DCM. The combined DCM extracts were washed with distilled water and DCM was distilled off to obtain the free fatty acids residue [8, 9].

2.2.3. Preparation of fatty acid methyl esters

The fatty acids were converted to their methyl esters by refluxing with 50 ml methanol and 1.5 ml conc. sulphuric acid for two hours. The alcohol was distilled off and the obtained residue was diluted with twice its volume of distilled water and then extracted with several portions of DCM. The combined DCM extracts were washed with distilled water. The DCM extract was concentrated and the residue of fatty acids methyl esters was finally dried over calcium chloride overnight [8, 9].

2.3. GC/MS analysis of fatty acid methyl esters

Analysis of fatty acids methyl esters was carried out using a Shimadzu GC-MS Model (QP-2010 Ultra) equipped with a Head Space AOC-5000 auto injector. The Rtx-5 MS fused-silica column (30 m length, 0.25 mm ID; 0.25 µm film thickness) and a flame ionization detector (FID), which was operated in EI mode at 70 eV were used under the following conditions: The injector temperature was 210 ºC and the oven temperature was held at 100 °C for 2 min, then programmed to increase to 270 °C at 10 °C /min and held for 20 min. The FID detector temperature was 270 °C. The air flow was 400ml/min and helium was used as the carrier gas at a flow rate of 60ml/min. Mass range was 35–500 m/z, scan speed was 1.666 and the run time was 39 min. Fatty acids methyl esters were identified by comparison of their Rt and MS spectra with the customized database and confirmed through comparison with data reported in the literature [10].

2.4. Estimation of total phenolic content

The total phenolic contents of the fungal EtOAc and MeOH extracts was determined by the Folin–Ciocalteu method [11]. The analysis was achieved by mixing 3.5 ml of deionized water, 50µl of sample extract (10 mg/ml) , 50 µl of Folin-Ciocalteu reagent (2N) and 300µl of Na_2CO_3 (10 %). The reaction was kept for 30 min and then the absorbance was measured at 730nm in triplicate. A standard curve was prepared using gallic acid and the total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram dried extract.

2.5. Antioxidant assays

2.5.1. DPPH free radical scavenging activity assay

Both the EtOAc and MeOH extracts were separately dissolved in 95% MeOH to make a concentration of 10 mg/ml and next diluted to prepare the series concentrations for the free radical scavenging activity using DPPH [12]. Briefly, 200µl of each extract at various concentrations were mixed with 2 ml of DPPH solution (0.1 mM).The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature, then the absorbance was measured at 517 nm. All samples were tested in triplicate. The potential to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = $[(A_0 - A_1/A_0) \times 100]$,

where A_0 is the absorbance of the control reaction and A1 is equivalent to the absorbance in the presence of the extract. The extract concentration providing a50% inhibition (IC_{50}) was calculated from the graph of DPPH scavenging activity versus extract concentration.

2.5.2. Phosphomolybdate assay

The total reducing power of the EtOAc and MeOH extracts was determined by the phosphomolybdate method using ascorbic acid as a standard [13]. An aliquot of 0.3 ml of sample solution (10 mg/ml) was added to 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95 °C for 90 min. After cooling the samples to the room temperature, the absorbance of the mixture was measured at 695 nm against a blank in triplicate. Ascorbic acid was used for preparing the standard curve; consequently, the antioxidant activity was expressed relative to that of ascorbic acid.

3. Statistical analysis

The records are expressed as mean ± SEM, and analyzed by the one-way analysis of variancse (ANOVA) test using the Graph Pad Prism 6 software (Version 6.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

4. Results and Discussion

GC/MS analysis of fatty acid methyl esters established a high content of polyunsaturated fatty acids (PUFA) 43.92% in addition to 23.83% monounsaturated fatty acids (MUFA), and 28.66% saturated fatty acids (SFA). The major identified fatty acid was linoleic acid (43.92%). The extract also showed considerable amounts of oleic acid (MUFA) and palmitic acid (SFA) (23.15% and 23.33%, respectively)as demonstrated in (**Table 1**) and (**Figure 1**). PUFAs have been proved to have several valuable impacts on cardiovascular diseases as improving blood lipid profile [14], causing a decline in plasma lipoproteins, lipids and incidence of atherosclerosis, and body fat accumulation [15], in addition to anti-arrhythmic effects [16]. They have also been found to improve insulin resistance [17], reduce the incidence of type 2 diabetes [18], and have an antimalarial activity [19].

Table 1: Fatty acids identified as methyl esters in *Alternaria* sp.

Peak No.	Compounds	Molecular Formula (methyl ester)	Molecular Weight	R_t (min)	Relative Area (%)
1	Myristic acid methyl ester	$C_{15}H_{30}O_2$	242	11.937	1.04
2	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	13.041	0.20
4	Palmitoleic acid, methyl ester	$C_{17}H_{32}O_2$ ω 6	268	13.895	0.38
5	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	270	14.096	23.33
7	6-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$ ω 12	296	14.882	0.30
8	Margaric acid, methyl ester	$C_{18}H_{36}O_2$	284	15.093	0.64
9	Linoleic acid methyl ester	$C_{19}H_{34}O_2$ ω 6,9	294	15.792	43.92
10	Oleic acid methyl ester	$C_{19}H_{36}O_2$ ω 9	296	15.834	23.15
11	Stearic acid methyl ester	$C_{19}H_{38}O_2$	298	16.050	2.44
17	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326	17.843	0.17
20	Behenic acid methyl ester	$C_{23}H_{46}O_2$	354	19.528	0.33
22	Lignoceric acid methyl ester	$C_{25}H_{50}O_2$	382	21.565	0.51
Monounsaturated fatty acids (MUFA)					23.83
Polyunsaturated fatty acids (PUFA)					43.92
Saturated fatty acids (SFA)					28.66
Unidentified					3.59

This study was also concerned with the estimation of the phenolic contents and the in vitro antioxidant activities of the EtOAc and MeOH extracts of *Alternaria* sp. The EtOAc extract had a higher phenolic contents compared to the MeOH extract (4.09 and 3.64 mg GAE/ g, respectively) (**Figure 2**). The capacity of *Alternaria* sp. to biosynthesize a variety of phenolic compounds has been previously reported [4, 7, 20, 21]. Likewise, the DPPH free radical scavenging activity of the EtOAc extract was also found to be higher than that of the MeOH extract (IC_{50}=0.52 and 2.162 mg/ml, respectively). Moreover, the EtOAc extract was found to display lightly higher reducing power (13.32 mg ascorbic acid equivalent/ g) than the MeOH one (12.88 mg ascorbic acid equivalent/ g) using the phosphomolybdate complex assay (**Figure 4**). The high phenolic components found in the EtOAc extract explained its generous antioxidant properties that have the aptitude to track down and neutralize oxygen reactive free radicals. Consequently, polyphenols are proficient to inhibit LDL oxidation and minimize the hazards of atherosclerosis and are chemoprotective agents to fight against cancer [22, 23].

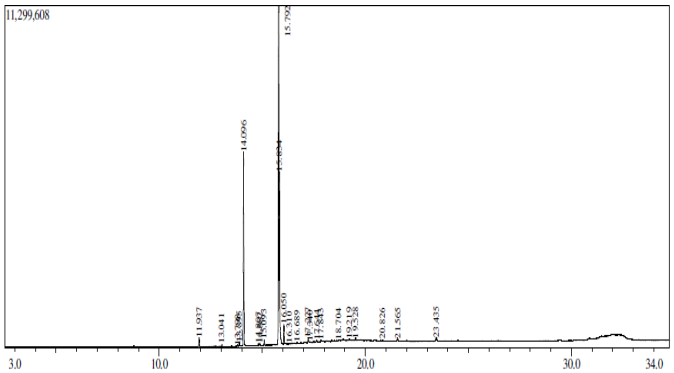

Figure 1: Total GLC chromatogram of fatty acids methyl esters of *Alternaria* sp.

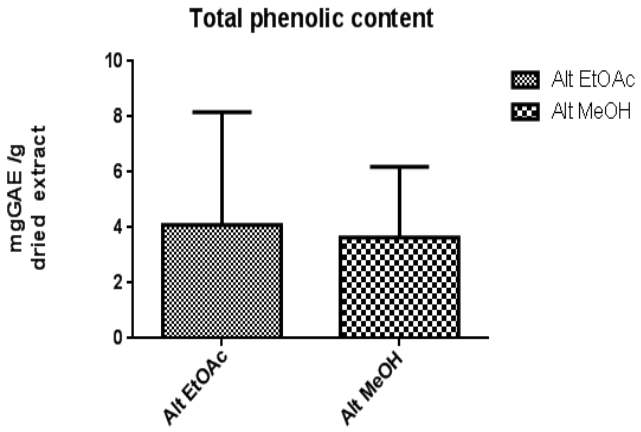

Figure 2: Total phenolic contents of the EtOAc and MeOH extracts of *Alternaria* sp.

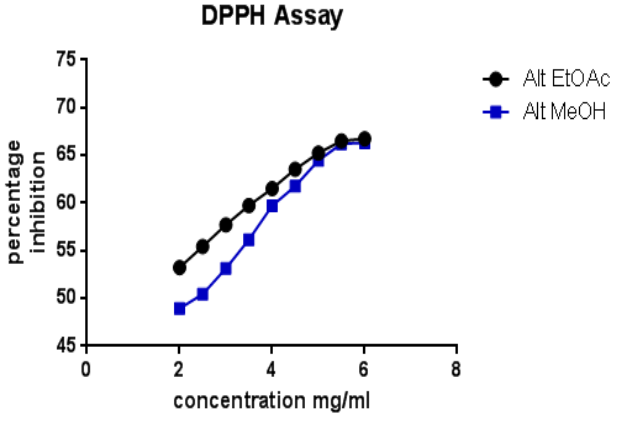

Figure 3: DPPH free radical scavenging activity of the EtOAc and MeOH extracts of *Alternaria* sp.

Figure 4: phosphomolybdate total reducing power of the EtOAc and MeOH extracts of *Alternaria* sp.

Conclusion

Evaluation of the fatty acids present in the petroleum ether extract of the endophytic fungus *Alternaria* sp showed the presence of a high content of PUFA, mainly linoleic acid. PUFA are of great medical importance. Additionally, the high phenolic content and consequently the antioxidant activity of the EtOAc extract indicate that this endophytic fungus has great prospects as a source of natural health products, especially for cardiovascular system and for fighting cancer.

Declarations of interest: none

References

[1] Bugni TS, Ireland CM. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Natural Product Reports*. 2004;21(1):143-63.

[2] Kjer J, Debbab A, Aly AH, Proksch P. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. *Nature Protocols*. 2010;5(3):479.

[3] Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. *Natural Product Reports*. 2001;18(4):448-59.

[4] Kjer J, Wray V, Edrada-Ebel R, Ebel R, Pretsch A, Lin W, et al. Xanalteric Acids I and II and Related Phenolic Compounds from an Endophytic *Alternaria* sp. Isolated from the Mangrove Plant *Sonneratia alba*. *Journal of Natural Products*. 2009;72(11):2053-7.

[5] Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Diversity*. 2010;41(1):1-16.

[6] Aly AH, Edrada-Ebel R, Indriani ID, Wray V, Müller WE, Totzke F, et al. Cytotoxic metabolites from the fungal endophyte *Alternaria* sp. and their subsequent detection in its host plant Polygonum senegalense. *Journal of Natural Products*. 2008;71(6):972-80.

[7] Zheng C-J, Shao C-L, Guo Z-Y, Chen J-F, Deng D-S, Yang K-L, et al. Bioactive hydroanthraquinones and anthraquinone dimers from a soft coral-derived Alternaria sp. fungus. *Journal of Natural Products*. 2012;75(2):189-97.

[8] Johnson AR, Davenport JB. *Biochemistry and Methodology of Lipids*. New York: John Wiley and Sons, Inc. ; 1971.

[9] Christie WW. *Gas chromatography and lipids*: The Oily Press Ltd.; 1989.

[10] Adams RP. Identification of essential oils by ion trap mass spectroscopy. New York: *Adams Academic Press*; 1989.

[11] V. L. Singleton JRR. Colorimetry of total phenolics with Phosphomolybdic–phosphotungstic acid Reagents. *American Journal of Enology and Viticulture*. 1965;16(3):144–58.

[12] Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature* 1958;26:1199–200.

[13] P. Prieto MP, & M. Aguilar. Spectrophotometric quantitation of antioxidant capacity through the formation of a Phosphomolybdenum Complex: Specific application to the determination of vitamin E. *Analytical Biochemistry*. 1999;269(2):337-41.

[14] Keys A, Parlin RW. Serum-cholesterol response to changes in dietary lipids. *American Journal of Clinical Nutrition*. 1966;19: 175–81.

[15] Kennedy A, Martinez K, Chuang C-C, LaPoint K, McIntosh M. Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. *The Journal of Nutrition*. 2008;139(1):1-4.

[16] Abeywardena MY, McLeannan PL, Charnock JS. Differential effects of dietary fish oil on myocardial prostaglandin 12 and thromboxane A2 production. *American Journal of Physiology*. 1991;260:379–85.

[17] Lovejoy JC. Dietary fatty acids and insulin resistance. *Current Atherosclerosis Reports*. 1999;1(3):215-20.

[18] Salmeron J, Hu FB, Manson JE, Stampfer MJ, Colditz GA, Rimm EB, et al. Dietary fat intake and risk of type 2 diabetes in women–. *The American Journal of Clinical Nutrition*. 2001;73(6):1019-26.

[19] Melariri P, Campbell W, Etusim P, Smith P. In vitro and in vivo antimalarial activity of linolenic and linoleic acids and their methyl esters. *Advanced Studies in Biology*. 2012;4(7):333-49.

[20] Okamura N, Mimura K, Haraguchi H, Shingu K, Miyahara K, Yagi A. Altersolanol-related compounds from the culture liquid of Alternaria solani. *Phytochemistry*. 1996;42(1):77-80.

[21] Coombe R, Jacobs J, Watson T. Metabolites of some Alternaria species. The structures of altenusin and dehydroaltenusin. *Australian Journal of Chemistry*. 1970;23(11):2343-51.

[22] Meiyanto E, Hermawan A, Anindyajati A. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pacific *Journal of Cancer Prevention*. 2012;13(2):427-36.

[23] Reddy L, Odhav B, Bhoola K. Natural products for cancer prevention: a global perspective. *Pharmacology and Therapeutics*. 2003;99(1):1-13.