ISSN 0974 – 5211

Journal of Natural Products
Volume 4 (2011)
www.JournalofNaturalProducts.com

Research Paper

Utilization of Agro-industrial by-products for production of bioactive natural products from endophytic fungi

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(Received 04 March 2011; Revised 06 March -06 April 2011; Accepted 06 April 2011)

ABSTRACT

The utilization of four different agro-industrial by-products, Sugarcane Bagasse (SB), Corn-Bran (CB), Wheat-Bran (WB), Fenugreek Straw(FS), in addition to the well known solid medium, Cooked Rice (CR) without any supplemental addition as productive media for fungal natural products were described. Endophytic fungal strain, *Alternaria alternate* was isolated from the Egyptian land-weed *Euphorbia helioscopia*, and grown in the fine grounded and autoclaved media by the system steady state fermentation for about 30 days at 30ºC. The growing fungi and its products were homogenized in the presence of methyl alcohol (MeOH). The main products of this fungus extracted were: Alternariol (**1**), alternariol methyl ether (**2**) and tenuazonic acid (**3**), purified and identified using different spectroscopic analysis. Cytotoxic and antimicrobial activities of the isolated compounds were tested.

Keywards: Endophytic fungi; *Euphorbia helioscopia*; Alternariol; Alternariol methyl ether; Tenuzonic acid.

INTRODUCTION

Endophytes have proven to be rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity. The use of endophytes as biocatalysts in the biotransformation process of natural products assumes greater importance (Pimentel, et al., 2011) and recently several novel bioactive substances have been isolated from these microorganisms (Nuclear, et al., 2011). There is growing evidence that bioactive substances produced by microbial endophytes may not only be involved in the host-endophyte relationship, but may also ultimately have applicability in medicine, agriculture and industry (Strobel, 2002). Additionally, it is of great relevance in this context that the

number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganism class (Zhang, et al., 2006).

One of the factors having great impact on growth and production of secondary metabolites from microorganisms is the medium composition (Bills, 1995). Thus it may be necessary to use several media and growth conditions for the strains to be investigated their full metabolic potential in order to generate a broad range of secondary metabolites as possible, (Larsen, et al., 2005). Some authors strongly argue in favor of using solid substrate fermentations in studies of fungal metabolites since fungi, unlike other microorganisms; typically grow in nature on solid substrates such as wood, roots and leaves of plants (Nielsen, et al., 2004).

Large amounts of solid by-products are generated during the processing of agro-industrial products, such as SB, CB, WB, and FS. These solid wastes, especially the SB have a limited use in Egypt due to low nutritional value and cannot be used as animal feed without previous biological treatment. It can produce alcohol due to uncontrolled fermentation in animal rumen and stimulate environmental problems through violent enhancement of microbial and insect spreading. SB is produced not only as an agro-industrial by-products but also as a waste of sugarcane pressing that made inside the sugarcane-drink shops which are spread all over the country.

The main goal of the present work was to verify the possibility of producing bioactive endophytic natural products through the utilization of accumulated agro-industrial by-products to save our environment, as well as minimizes the cost of commercial production of endophytic secondary metabolites. In this study, an alternative economical cultivation of endophytic strain of *Alternaria alternate* which was reported to has cytotoxic activity against HeLa cells in vitro (Fernandes, et al., 2009) on different agro-industrial by-products mentioned above was used for the production of medicinally-interesting natural products.. The endophytic fungus *Alternaria alternate* was isolated from the land weed *Euphorbia helioscopia*, purified and re-cultivated using solid state fermentation on different wastes without any supplemental addition to the media. The study includes also the isolation, structural elucidation, antimicrobial activity and cytotoxicity studies of the main phytoconstituents from the solid productive medium composed of sugarcane bagasse.

MATERIALS AND METHODS

***General experimental procedure*:** For HPLC analysis, samples were injected into an HPLC system with a photodiode-array detector (Dionex, Munich, Germany). Routine detection was at 254 nm in aqueous MeOH. The separation column (125 x 4 mm, i.d.) was prefilled with C18 (Knauer, Berlin, Germany). TLC was performed on TLC plates precoated with Si 60 F254 (Merck, Darmstadt, Germany). Column chromatography (CC) was carried out on silica gel (Merck) type 100 (70-230 Mesh ASTM). The compounds were detected from their UV absorbance. 1D and 2D NMR spectra (chemical shifts in ppm) were recorded on Bruker Unity 500, Bruker ARX 400, and/or DMX 600 NMR spectrometers using standard Bruker software, and DMSO-d_6 or MeOH-$d_4$was used as a solvent. ESI mass spectra were obtained on a Thermofinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector.

***Plant material and fungal strain*:** The plant was collected in April 2009 from Giza, Egypt and identified as *Euphorbia helioscopia* L. by Prof. Dr. Batanouny, K.H., Department of Botany, Faculty of Science; Cairo University, Egypt. The endophytic fungus *Alternaria alternate* was identified at "The Regional Center for Mycology & Biotechnology", Al-Azhar University, Cairo, Egypt.

***Purification of fungal strains*:** Plant materials were cut into small pieces, washed with sterilized demineralized water, then thoroughly surface treated with 70% ethanol for 1-2 min, rinsed in sterilized demineralized water and ultimately air dried under a laminar flow hood (according to Hassan, 2007). This is done in order to eliminate surface contaminating microorganisms. With a sterile scalpel, outer tissues were removed from the plant samples and the inner tissues were carefully dissected under sterile conditions and placed onto malt agar plates containing antibiotics (chloramphenicol/streptomycin). After 3 weeks of incubation at room temperature, hyphal tips of the fungi were transferred to fresh malt agar medium. Plates are prepared in duplicates to eliminate the possibility of contamination. Pure strains were isolated by repeated inoculation.

***Malt agar (MA) medium*:** MA medium was used for isolation and purification of the fungal strain and composed of: malt extract (15g), agar-agar (15g), distilled water (to 1000ml). pH was adjusted with 0.1 N NaOH to 7.4-7.8). For the isolation of endophytic fungi from plant tissues, 0.1g of chloramphenicol /streptomycin (1:1w/w) were added to the medium to suppress bacterial growth.

***Productive medium for solid state fermentations*:** It was consisted of 100g of each (SB, CB, WB, FS and CR), 200ml distilled water was added then autoclaved at 121ºC for 15 min. Fungi were separately grown on the solid state media at room temperature for 30 days in 1L Erlenmeyer flasks.

***Extraction procedure*:** MeOH (500 Ml) were added to the cultures and left overnight. Culture media were then cut into pieces to allow complete extraction and left for 3-5 days. Then filtration was done followed by repeated extraction with MeOH till exhaustion.

Cytotoxicity Assay: Antiproliferative activity was examined against mouse lymphoma cell, L1578Y and was determined by an MTT assay as described earlier (Mossman, 1983; Ashour, et al., 2009).

Antimicrobial Activity: Agar diffusion assay was performed according to the Bauer-Kirby-Test (Bauer, et al., 1966). Agar diffusion method was used to examine the antimicrobial activity of the isolated fungal metabolites against *Pseudomonas aeruginosa, Bacillus subtilis, Geotrichum candidum* and *Escherichia coli.*

RESULTS

The total mycotoxins which are produced by cultivation on different media, of SB, CB, WB, FS and CR were 9.6, 8.12, 7.3, 3.4 and 3.0 g/100g media respectively.

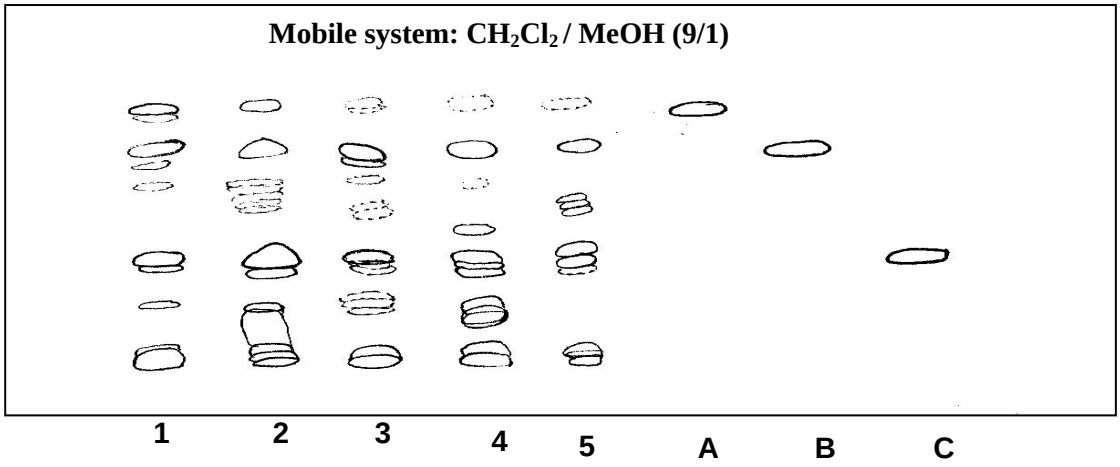


Figure- 1: TLC chromatogram of total extracts of *Alternaria alternate* grown on five different solid media, together with pure isolated fungal metabolites, 1- CR, 2- SB, 3- WB, 4- CB, 5- FS. A- alternariol methyl ether, B- alternariol, C- tenuazonic acid.

***Compounds isolated from the endophytic fungus Alternaria alternate*:** Three main fungal metabolites were isolated and purified after incubation on the SB, their structures were identified and verified after spectroscopic analysis. These compounds are alternariol (**1**) (Bradburn, et al., 1994)., alternariol methyl ether (**2**) (Onocha, et al., 1995) and tenuazonic acid (**3**) (Nolte, et al., 1980). Fig. 1 showed the results of TLC of the total alcohol extracts of the fungal metabolites after inoculation on five solid media, chemical screening studies indicated a clear difference in the relative concentrations of the main compounds from all extracts under the same condition, as well as a great differences in number and occurrence of minor components which may be attributed to the enzymatic manipulation of the nutrients in different solid media.

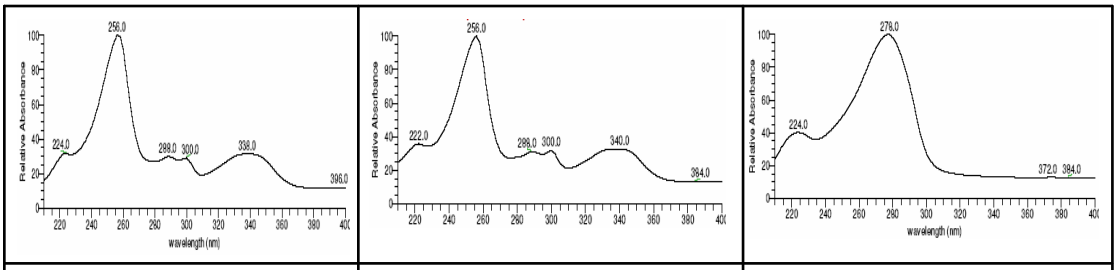


Figure- 2: Comparison of UV spectra of the isolated fungal metabolites from HPLC connected with diode array detector.

***Isolation and Purification*:** The total fungal extract (2.0g) of obtained from the sugarcane bagasse media were chromatographed using VLC with ten subsequent mobile phases, pet. ether 100%, C_6H_6 100%, C_6H_6/MeOH 95/5, C_6H_6/MeOH 9/1, C_6H_6/MeOH 8/2, $CHCl_3$/MeOH 9/1, $CHCl_3$/MeOH 8/2, $CHCl_3$/MeOH 7/3, $CHCl_3$/MeOH 1/1 and finally methanol 100%, to give ten fractions , Fr1 ---→ Fr10 respectively. Four fractions (Fr4 ---→Fr7) were combined (850 mg), and chromatographed using silica gel column. The sample was eluted first with n-hexane/EtOAc 75/25 to give compound **2** as colourless needle (5 mg, after purification), then, compound **1** was eluted with n-hexane/EtOAc 70/30 as colourless needles (22mg). the rest of the sample was eluted with n-hexane/EtOAc/MeOH 45/45/10 to give the third compound which was eluted again with $CHCl_3$/MeOH 85/15 to obtain compound **3** (101.2mg as yellowish-brown amorphous solid).

***Alternariol (1)*:** (R_f 0.4, using N-Hexane/EtOAc/MeOH, 7/3/0.3 and 0.73, using CH_2Cl_2/MeOH, 9/1) was isolated from the EtOAc extracts in the form of white needles (22mg). It exhibited UV absorbances at λ_{max} (MeOH) 224.0, 256.0, 288.0, 300.0 and 338.0 nm, having the typical pattern of alternariol derivatives (Fig 2). Positive and negative ESI-MS showed molecular ion peaks at m/z 259.4 [M+H]+ (base peak) and m/z 257.3 [M-H]- (base peak), indicating a molecular weight of 258 g/mol. The ^1H, ^{13}C NMR spectra, HMBC correlations (table 1) and mass spectral data were found to be identical to published data for alternariol, previously reported from several *Alternaria* species (Bradburn, et al., 1994).

***Alternariol-5-O-methyl ether (2)*:** (R_f 0.35, using N-Hexane: Ethylacetate, 7:3, and 0.85, using CH_2Cl_2//MeOH, 9/1) was isolated from the EtOAc extracts in the form of white needles (5.0mg). It exhibited UV absorbances at λ_{max} (MeOH) 222.0, 256.0, 287.0, 300.0 and 340.0 nm, having the typical pattern of alternariol derivatives (Figure 2)**.** Positive and negative ESI-MS showed molecular ion peaks at *m/z* 273.4 $[M+H]^+$ (base peak) and *m/z* 271.1 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 272 g/mol with an increase of 14 mass units compared to alternariol (**1**) and thus supporting a molecular formula of $C_{15}H_{12}O_5$. The

structure was confirmed by comparison of UV, 1H, NMR (table 1) and mass spectral data with published data for alternariol-5-O-methyl ether (Onocha, et al., 1995), also known as djalonensone, previously reported from several Alternaria species (Bradburn, et al., 1994) as well as from Anthocleista djalonensis (Onocha, et al., 1995).

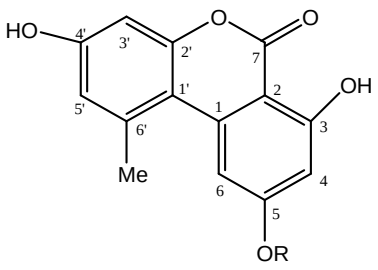
Table -1: ^{1}H and ^{13}C NMR data of 1 and 2.

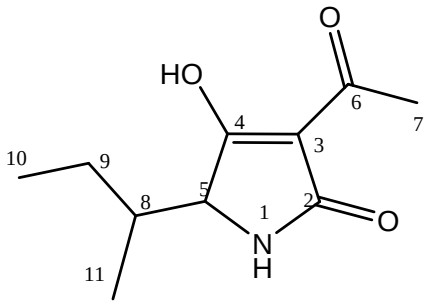
	1 (DMSO)			2 (MeOD)
Carbon No.	**^{13}C, Multi.**	**^{1}H, multi., (Hz)**	**HMBC**	**^{1}H, multi., (Hz)**
1	138.1, *s*			
2	97.4, *s*			
3	164.1, *s*			
4	100.7, *d*	6.34, *d*, 1.89 Hz	2, 3, 5, 6	6.52, *d*, 2.2 Hz
5	165.5, *s*			
6	104.4, *d*	7.20, *d*, 1.89 Hz	1', 2, 7	7.28, *d*, 2.2 Hz
7	164.7, *s*			
1'	109.0, *s*			
2'	152.6, *s*			
3'	101.6, *d*	6.6, *d*, 2.35 Hz	1', 2', 4', 5'	6.58, *d*, 2.5 Hz
4'	158.4, *s*			
5'	117.6, *d*	6.68, *d*, 2.35 Hz	1',3', 4'	6.68, *d*, 2.5 Hz
6'	138.3, s			
6'-CH_3	25.6, *q*	2.67, *s*	1', 5', 6'	2.74, *s*
5-OCH_3				3.88, *s*
3-OH		11.75, *s*	2, 3, 4	
5-OH		10.3, *br s*		
4'-OH		10.8, *br s*		

Tenuazonic acid (3): It was obtained as yellowish brown amorphous solid (101.2 mg). (R_f 0.37, using CH_2Cl_2/MeOH, 9/1). It displayed UV absorbances at λ_{max} (MeOH) 224.0 and 278.0 nm (Figure 2). Positive and negative ESIMS showed molecular ion peaks at *m/z* 198.1 $[M+H]^+$ (base peak) and *m/z* 196.2 $[M-H]^-$ (base peak), respectively, indicating a molecular weight of 197 g/mol and thus the presence of an odd number of N-atoms in the structure. The ^{13}C NMR spectrum revealing 10 carbon atoms, together with ^{1}H NMR and mass spectral data supported a molecular formula of $C_{10}H_{15}O_3N$. The comparison of UV, ^{1}H, ^{13}C NMR (table 2), and mass spectral data with those published for tenuazonic acid (Nolte, et al., 1980) confirmed unambiguously compound **3** as tenuazonic acid.

Table- 2: ^{1}H and ^{13}C NMR data of 3.

Carbon No.	**^{13}C, Multi.**	**^{1}H, multi., (Hz)**	**HMBC**
1		7.61, *s*	3, 4
2	177, *s*		
3	101.6, *s*		
4	194.9, *s*		
5	64.7, *d*	3.35, *br s*	2, 4, 5
6	190.3, *s*		
7	26.2, *q*	2.18, *s*	3, 6
8	36.3, *d*	1.72, *br s*	
9a	22.8, *t*	1.03, *m*	
9b		1.24, *m*	10
10	11.9, *q*	0.78, *t*, 7.25 Hz	9
11	15.9, *q*	0.88, *d*, 6.95 Hz	8





3

1 ---------- R = H
2 ---------- R = Me

***Cytotoxic activity*:** Compounds **1**, **2** and **3** showed cytotoxicity against mouse lymphoma cancer cell line L5178Y (11.8%, 72.0% and – 13.0 % growth rates at conc. of 10µg/ml respectively),

Antimicrobial Activity: Compounds **1**, and **3** showed mild antibacterial activity (7 mm inhibition zone) against *Bacillus subtilis*, compound 1, showed moderate antibacterial activity against *Pseudomonas aeruginosa* (8 mm)., while compound **3** showed strong antibacterial activities (9mm) against *Pseudomonas aeruginosa*. Both compounds **1** and **3** have no activities against, *Geotrichum candidum* and *E. coli*. Compound **2** showed no activity against the above mentioned microorganisms**.**

DISCUSSION

Although endophytes have proven to be rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity, the cost factor should be kept in consideration. New and non classical culture media provide nutrition stress to force the fungal strain to produce novel chemical entities and improve the quantities of their secondary metabolites. These costless solid wastes, especially the SB have a limited use in Egypt due to low nutritional value and cannot be used as animal feed without previous biological treatment, therefore, the utilization of SB for production of bioactive secondary metabolites is useful also for saving the environment. Some biological activities and medicinal benefits of the isolated compounds were published previously (Lehmann, et al., 2006; Griffin and Chu, 1983; Davis and Stack, 1994; Miller, et al., 1963; Royles, 1995).

CONCLUSIONS

This work confirms utilization of approximately costless agro-industrial by-products especially SB as nutritive media for endophytes to minimize the cost of production of bioactive natural products from endophytic fungi.

Acknowledgements: We thank Prof. Dr. Werner E. G. Mueller *Institute of Physiological Chemistry und Pathobiochemistry, Johannes-Gutenberg-university, Duesbergweg 6, 55128 Mainz, Germany* for cytotoxicity studies, and Prof. Dr. Batanouny, K.H., Department of Botany, Faculty of Science; Cairo University, Egypt for identification of the plant.

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