Isolation and Identification of Three Bioactive Compounds from Endophytic Fungus *Trichoderma sp.*

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Abstract

The endophytic fungus *Trichoderma* sp. was isolated from *Ocimum basilicum* L. plant and cultivated in the laboratory on potato dextrose agar PDA medium. Three bioactive chemical compounds were isolated and purified from culture of *Trichoderma sp*. using potato dextrose agar and potato dextrose broth. An investigation of the secondary metabolites by using Thin layer chromatography, column chromatography and Gas chromatography technique was carried out ,The molecular weight of purified compounds 1, 2 and 3 were 202 KD, 137 KD and 197 KD respectively and chemical name of compound 1 is1,10-Decanedioic acid, chemical name of compound 2 is Phenol, 4-(ethylamino)-4-Ethylaminophenol and chemical name of compound3 is Pyrimido [5,4-E]-1,2,4-triazine-5,7(4ah,6H)-dione,8,8a-dihydo-4a-hydroxy-6-methyl-. The antibacterial activity of the purified compounds against two bacterial species *E.coli and S. aureus* were tested by using a disc diffusion agar method reaching to 32,20mm for compound 1and 24,19 mm for compound 2 and 27, 26mm for compound 3. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by the standard serial dilution assay.
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Keywords: Bioactive compounds, Identification, Endophytic fungi, Antibacterial.

Introduction

Endophytes are microbes that colonize in internal tissues of plants without causing any immediate, negative effects [1]. Almost all vascular plant species appear to be inhibited by endophytic bacteria or fungi, these represent important components of microbial diversity. The relationship between the host plant and its endophyte shows symbiotic characteristics as the endophytic occupant usually obtains nutrients and protection from the host plant and in return profoundly enhances the fitness of the host by producing certain functional metabolites [2]. Fungal endophytes are a polyphyletic group of primarily ascomycetes fungi, whereas basidiomycetes, deuteromycetes and Oomycetes are rarely found [3]. Medicinal application of natural products can be traced back several millennia in human history. Natural products have been an integral part, in one form or another, of several indigenous therapeutic systems including traditional Chinese medicine [4]. Although they do not show host specifity, certain fungal lineages appear with greater frequency in plants representing particular families and thus denote host preference [5]. Between 1987 and 2000 approximately 140 new natural products were isolated from endophytic fungi [2]A similar number was subsequently characterized in half of this time span, i.e. between 2000 and 2006 [6]. Many of these exhibit interesting activity profiles. Cryptocin for example, is an tetramic acid isolated from the endophytic fungus *Cryptosporiopsis quercina* [7]. Endophyte *Phomopsis* sp. was isolated, which produces the anti-inflammatory as well as antifungally and antibacterially active polyketide lactone and phenol [8].

Materials and Methods

Isolation of endophytic fungus

Healthy roots of *Ocimum basilicum* collected from Misan city south of Iraq and processed separately within 48hrs were of collection. The Root samples were surface sterilized [9]. Surface sterilized roots segments were equally spaced in Petri dishes on a potato dextrose agar medium (PDA) (medium was amended with chloramphenicol 150 mg-l). The Petri dishes were closed by Para film and incubated at 27°C in a light space for 12hrs followed by 12hrs of dark cycles. The cultures were observed every day to check the growth of endophytic fungal in the roots *O.basilicum*.

Identification of endophytic fungi

The identification of endophytic fungi was performed at the Microbiology Laboratory in the College of Science University of Misan., by means of analysis of macroscopic and microscopic characteristics of colonies.

The test microorganisms

The test bacteria used in this study were *Esherichia coli* as gram negative bacterium and *Staphylococcus aureus* as gram positive bacterium. All bacterial strains were obtained from the Microbiology Laboratory in the College of Science University of Misan.

Preliminary screening of antibacterial assay

The endophytic fungi were subjected to an antibacterial assay using a solid medium [10], which permits a rapid and qualitative selection of the bioactive microorganisms. Each endophytic strain was cultivated on the surface of PDA in Petri dishes, at 27◦C, for 7days.Then disks were cut from the PDA plate (6mm diameter) and transferred to the surface of Petri dishes previously spread with bacteria (Muller–Hinton agar, MHA).The Petri dishes were incubated at 37◦C for 24hrs Antibacterial activity was assayed by the measurement of inhibition diameter zones (IDZ).

Extraction and purification of compounds

The cultures of broth medium fungi were filtered on filter paper type Watmann No. 1, the filtrate was extracted 3 times using ethyl acetate (1:1 v/v) using separating funnel [11]. Organic layer was collected then dehydrated with Na2SO4 then placed in a Glass vial and dehydrated at 25c^{o}. Thin layer chromatography (TLC) was applied for the isolation of extracted metabolites using silica gel(c-60) of 2×10 cm and rate flow value (Rf) was measured. Purification of extracted compounds was made on silica gel (mesh 60) column chromatography 1.5×50 cm using eluent methanol- ethyl acetate (1:1). A further purification of fractions was made by using another column 1.5×50 cm and using eluent cyclohexane and ethyl acetate (1:1). The identification of the purified compounds was made using Gas chromatography (GC-Mass) technique.

Antibacterial bioactivity assay

Filter paper discs (0.6 mm) after being sterilized by autoclave were, socked in secondary metabolites solution for 5 min., filter paper discs with extract were placed on the surface of Muller-Hinton agar medium in Petri-dishes streaked with 0.2 ml of bacterial suspensions of bacteria strains. Plates were incubated at 37ċ for 24 hrs, an appearance of inhibition zones around the filter paper disc indicating the bioactivity of secondary metabolites against tested bacteria [12]. The diameters of the clear zones were measured and compared with control agar plates containing discs with solvent only (control), triplicates were made.

Minimum inhibitory concentration and minimum bactericidal concentration test

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were determined by the standard serial dilution assay [13]. The inhibitory test was carried out on Muller-Hinton agar medium.

Toxicity test

Cytotoxicity of the fungal secondary metabolites was examined by using human RBC following a previously described method [14].

Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA) between any pair of variables.

Results and Discussion

A total of 7 endophytic strains were isolated from roots of *O.basilicum* and subsequently submitted to a preliminary antimicrobial screening on solid medium. Isolation of endophytic fungi from medicinal plants produce bioactive compounds which have greater activity against various pathogenic microbes [15]. One endophytic isolate showed an activity against tested bacteria which was *Trichoderma sp*. Endophytic fungi are a good source for antimicrobial products [16]. Endophytes are reported as novel source of antimicrobial compounds [17]. Endophytic microorganisms are excellent sources of

bioactive natural products that can be used to satisfy demand of pharmaceutical industry, since a single endophyte may be able to produce a variety of bioactive metabolites [18]. The production of Hypericin (C30H16O8), a naphthodianthrone derivative and Emodin (C15H10O5) believed to be the main precursor of hypericin [19].Isolation and purification of three compounds from *Trichoderma sp*. Mycelium were reported. Based on Gas chromatography (GC-mass) apparently the molecular formula of compound (1) is C10H18O4 with molecular weight 202Kd and its chemical name is1,10-Decanedioic acid as show in Fig.(1),While the molecular formula of compound (2) is C8H11NO and its name is Phenol, 4-(ethylamino)-4-Ethylaminophenol with molecular weight 137Kd Fig.(2), and the molecular formula of compound (3) is C6H7N5O3 and its name is Pyrimido[5,4-E]-1,2,4-triazine-5,7(4ah,6H)-dione,8,8a-dihydo-4a-hydroxy-6-methyl-with molecular weight 197Kd Fig.(3).

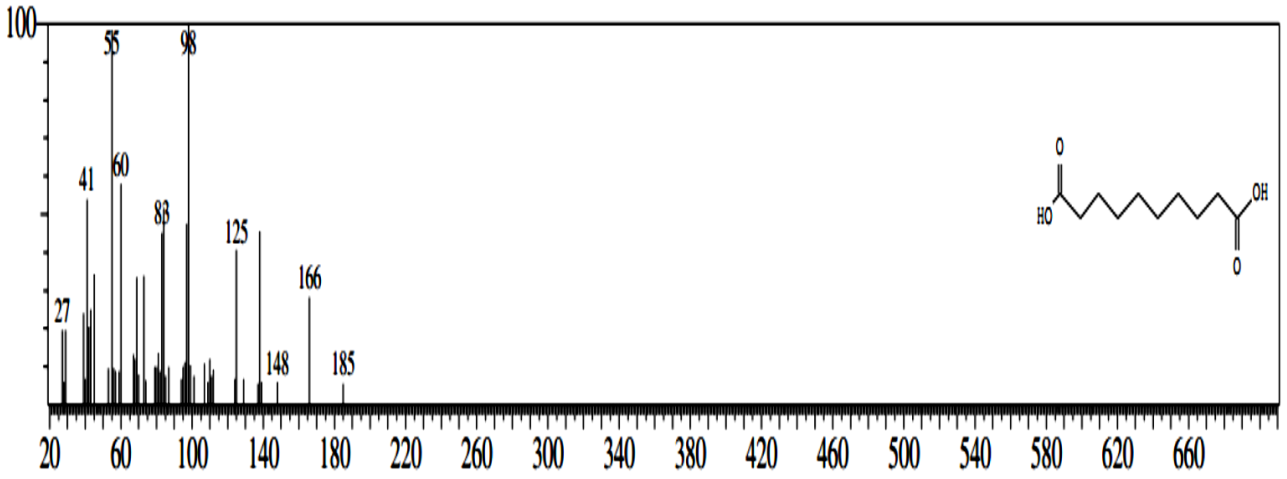


Fig.(1): Chemical structure and GC –Mass spectroscopy of compound 1,10-Decanedioic acid.

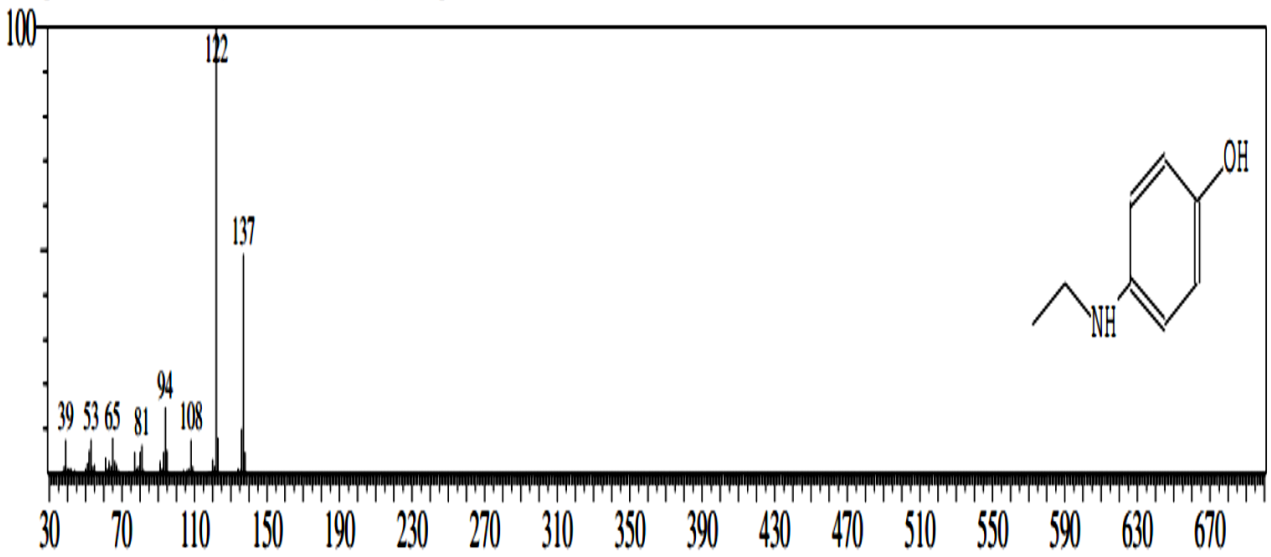


Fig.(2): Chemical structure and GC –Mass spectroscopy of compound Phenol, 4-(ethylamino)- 4-Ethylaminophenol.

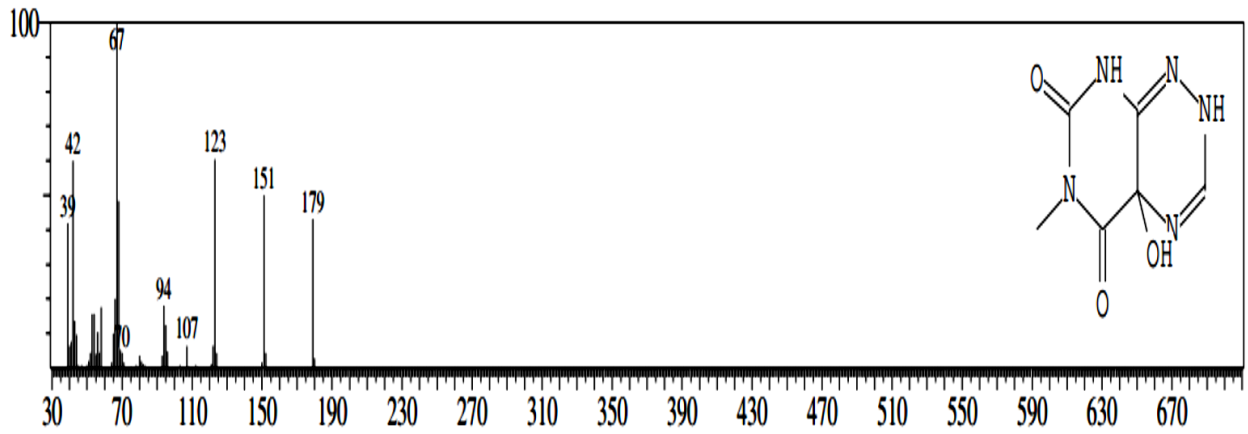


Fig.(3): Chemical structure and GC –Mass spectroscopy of compound Pyrimido[5,4-E]-1,2,4-triazine-5,7(4ah,6H)-dione, 8,8a-dihydo-4a-hydroxy-6-methyl.

All purified compounds exhibited antibacterial activity against bacterial species. [15] Studied the antimicrobial potential of endophytic fungi *Alternaria sp, Colletotrichum* and *Nigrospora* and sterile mycelia isolated from the leaf tissues of *Tectona grandis sp* and *Samanea saman.*The inhibition zone diameter of compound (1) reached to32 and 20 mm for *E. coli* and *S. aureus* respectively and 24 and 19 mm of compound (2) for bacterial species respectively and 27 and 26 mm of compound (3) Fig.(4). Purified compounds in the present study contained the active chemical groups. Other studies also indicated that 1,10 Decanedioic acid compound has antibacterial activity against bacteria[19]. Compounds1and 2 contained phenol, amino, hydroxyl and other groups identified as antimicrobial molecule. These biological activities are associated with the molecules structures, via their hydroxyl groups. The antimicrobial inhibitory impact of secondary metabolites can be related to the bioactivity of these compounds. It has been reported that several phenolic compounds including tannin are potent inhibitors of microbial enzymes [20]. Because phenolic compounds conjugate with proteins and bacterial membrane [21, 22]. The MIC and MBC are shown in Table (1). Since our study was the primary screening for the antibacterial activity of these extracts, assaying MIC is suggested in order to strengthen the findings of the current study[23].Varied values of MIC and MBC were obtained, the reason for this is due to the difference in the cell wall of gram negative bacteria and gram positive bacteria as it is characterized by a wall of negative bacteria lack the permeability of the outer shell of antibiotics because of the presence of the outer layer, which prevents the arrival of antibiotics to the target zone. The wall of gram negative bacteria contains on lipopolysaccharides and lipoprotein and the walls of gram positive bacteria are characterized by permeability for effective compounds more than gram negative bacteria [24].

Table (1)
The minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of purified three compounds (mg/mL) Compounds.

	E .coli		*S .aureus*	
Compounds	**MIC**	**MBC**	**MIC**	**MBC**
Compound 1	6.25	12.5	3.12	6.25
Compound 2	3.12	6.25	6.25	12.5
Compound 3	6.25	12.25	6.25	12.5

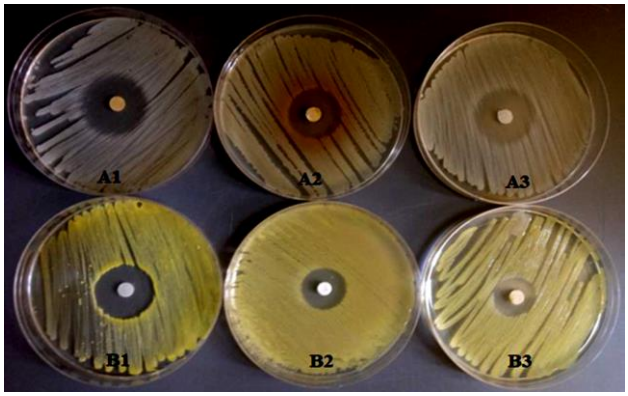


Fig.(4): Inhibition zone diameter (mm): 1: compound NO. (1), 2: compound NO.(2) and 3: compound NO.(3).

Conclusion

Endophytic fungi have proven to be rich sources of novel natural compounds with a wide spectrum of biological activities. This study revealed that this endophytic fungus showed a significant antibacterial activity.

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