Isolation of Bioactive Secondary Metabolites from the Endophytic Fungus of *0cimum basilicum*.

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ABSTRACT: Twenty three endophytic fungi from leaf, stem and root of *0cimum basilicum* (Tulshi) were isolated and purified. One of the fungi (2L-5) isolated from the leaves was fermented in a large scale and extracted with ethyl acetate. The fungal extract was found to be active against two bacteria, *Bacillus cereus* and *Staphylococcus aureus*. From the ethyl acetate extract two steroids, ergosterol and cerevisterol were isolated. Structures of the compounds were elucidated by high-resolution 1- and 2-D NMR spectroscopy.

Key words: *0cimum basilicum*, Secondary Metabolites, Endophytic fungi, Ergosterol, Cerevesterol

INTRODUCTION

Fungi are plant-like organisms that lack chlorophyll. An endophytic fungi is a fungal microorganism, which spends the whole or part of its life cycle colonizing inter and /or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of diseases.[1] In recent years, there has been evidence that the production of secondary metabolites by an endophyte is not random, but seems to be correlated with his ecological niche.[2] Thus, the microorganisms from ecological habitats have not been thoroughly investigated. The metabolic interactions of endophytes with its host may favor the synthesis of biologically active secondary metabolites.[3] Therefore, in the present study endophytic fungus of *Ocimum basilicum* has been selected for chemical and biological studies due to its medicinal importance.

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MATERIALS AND METHODS

Experimental. For column chromatographic separation silica gel G-60 (230-400 mesh, particle size 0.04-0.063 mm, Ar.7734, ASTM, Merck) was used as stationary phase. IR spectra were recorded by making KBr pellets using Shimadzu IR-470 spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer using tetramethylsilane (TMS) as the internal reference. Two-dimensional NMR (H-H COSY, HSQC & HMBC) spectra were obtained using standard pulse sequences.

Media was prepared under Laminar flow (Thermo Forma. Class 11 A1, Biological safety cabinet). Media was sterilized using HIRAYAMA autoclave (Hirayama MFG. Corp.). For semisolid media all petri dishes were sterilized (at 180°C) using a Sterilizer (EYELA natural sterilizer NDS- 600D). For liquid media shaking were carried out at 28^{0}C using an orbital shaker (Thermo Forma, Refrigerated). Shaking rate was done at 150 rpm/min. Cultured fungi of semisolid media were kept inside incubator (Sanyo cool incubator).

Collection of Plant Materials. O*cimum basilicum* seedling (Tulsi), was collected from a local nursery of Dhaka city, Bangladesh.

Surface Sterilization of Plant Material. Optimization of surface sterilization of leaves, stems and root of *Ocimum basilicum* was done with 70% ethanol, 3% sodium hypochlorite and sterile water. Different parts of plants were cleaned and washed with water. The cleaned plant parts were kept successively into each of the solution for 3 minutes. The effectiveness of surface sterilization was checked by making an imprint of the treated portion on agar media.[4]

Preparation of Media. Potato-carrot agar media was used for fungal growth. Grated potato 200 g and grated carrot 200 g in 1L of water were boiled for 30 minutes. The mixture was cooled, mashed and the extract was collected by squeezing through a pre-cleaned cloth filter. Volume of the extract was adjusted up to 1L by adding water. 100 mL of this extract was used to prepare 1L media. For solidification of media 12 g/L agar was used. For liquid media no agar was used.

Isolation of Fungi. The surface sterilized plant materials were inoculated on autoclaved potato-carrot agar media on sterile petri dish (90 mm diameter). After 32 days of inoculation it was found that leaves, stems and roots gave seven, nine and seven fungi, respectively. The seven fungi from leaves were sub-cultured on semisolid potato-carrot agar media and on liquid media in a small scale (10 Petri dishes for each fungus in semisolid media and two flasks each for liquid media) for 21 days. Each of the cultured fungi was extracted with ethyl acetate and TLC pattern of both the extract were found to be same and were combined. The fungus 2L-5 was cultivated on large scale on semisolid as well as in liquid media for chemical and biological investigation.

Extraction of Fungi. The fermented fungus in 300 petri dishes (90 mm in diameter) was made homogeneous using an Ultra-Turrax and resultant mixture was extracted with ethyl acetate (3 × 200 ml) and total of 150 mg extract was obtained. The fermented fungus in liquid media in 75 conical flasks (500 ml) and in 25 conical flasks (250 ml) was filtered by using suction pump and then the residue was made homogeneous using an Ultra-Turrax. The extracts were dried by using rotary vacuum evaporator followed by a freeze dryer. The amount of total extract was 450 mg. A part of the combined extract was used for antibacterial assay.

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Figure 1. Picture of fungi 2L-5 in liquid media

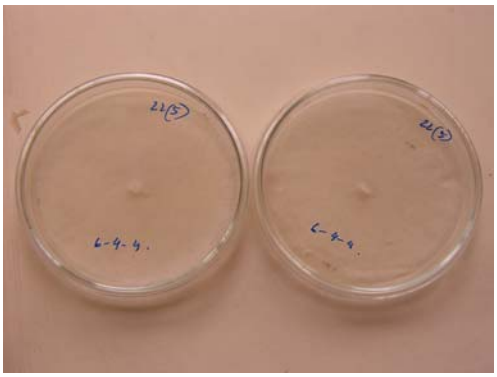


Figure 2. Picture of fungi 2L-5 in semisolid media

Isolation of Compound. The extract (400 mg) was fractionated by silica gel column chromatography to isolate compounds. By fractionation with different proportion of mobile phase (ethyl acetate, hexane, methanol) two white solid compounds were isolated AH-1 (2.4 mg) and AH-2 (2.2 mg).

Purification of Compounds. Compounds AH-1 and AH-2 was purified by recrystalisation from n-hexane.

Spectroscopic data of Compound AH-1

IR (KBr pellets) ν_{max}3450, 1650, 1450, cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.62 (3H, s, CH_3-18), 0.82 (3H, d, J=6.9 Hz, CH_3-26), 0.84 (3H, d, J=7.0 Hz, CH_3-27), 0.90 (3H, d, J=6.8 Hz, CH_3-28), 0.94 (3H, s, CH_3-19), 1.04(3H, d, J= 6.6 Hz, CH_3-21), 3.63 (1H, bm, H-3α), 5.17 (1H, m, H-22), 5.21(1H, m, H-23), 5.38 (1H, m, H-7), 5.56 (1H, dd, J=5.8, 2.6 Hz, H-6); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ : 38.4 (C-1), 32.0 (C-2), 70.5 (C-3), 40.8 (C-4), 139.8 (C- 5), 119.6 (C-6), 116.3 (C-7), 141.4 (C-8), 46.3 (C-9), 37.1 (C-10), 21.2 (C-11), 39.1 (C-12), 42.9 (C-13), 23.0 (C-15), 28.3 (C-16), 55.8 (C-17), 12.1(C-18), 16.3 (C-19), 40.4 (C-20), 21.1 (C-21), 135.6 (C-22), 132.0 (C-23), 42.9 (C-24), 33.1 (C-25), 20.0 (C-26), 19.7 (C-27), 17.6 (C-28).

DEPT-135 (100 MHz, $CDCl_3$) δ: 12.07, 16.30, 17.62, 19.66, 19.96, 21.13, 21.57, 23.02, 28.30, 32.02, 33.11, 38.40, 39.11, 40.43, 40.82, 42.85, 46.28, 54.58, 55.77, 70.50, 116.31, 119.61, 132.01, 135.59.

DEPT-90 (100 MHz, $CDCl_3$) δ: 33.11, 40.43, 42.85, 46.28, 54.58, 55.77, 70.50, 116.31, 119.62, 132.01, 135.59.

Spectroscopic data of Compound AH-2

IR (KBr pellets) ν_{max}3400,1650, 1450 cm^{-1}; **^{1}H-NMR** ($CDCl_3$+CD_3OD, 400 MHz) : δ: 5.14 (1H, m, H-7), 5.10 - 5.02 (2H, m, H-22,H-23), 3.87 (1H, m, H-3), 3.62 (1H, m, H-6), 0.90 (3H, s, H-19), 0.87 (3H, d, J=6.7 Hz, H-21), 0.77 (3H, d, J=6.6 Hz, H-28), 0.69 (3H, d, J=6.3 Hz, H-26), 0.65 (3H, d, J=6.9 Hz, H-27), 0.45 (3H, s, H-18);. ^{13}C-NMR($CDCl_3$, 100 MHz) δ: 32.5 (C-1), 30.2 (C-2), 67.0 (C-3), 39.1 (C-4), 75.7 (C-5), 72.9 (C-6), 117.3 (C-7), 143.1 (C-8), 42.9 (C-9), 36.8 (C-10), 22.4 (C-11), 38.7 (C-12), 44.0 (C-13), 54.5 (C-14), 22.7 (C-15), 27.7 (C16), 55.8 (C-17), 11.9 (C-18), 18.0 (C-19), 40.2 (C-20), 19.3 (C-21), 131.8 (C-22), 135.3 (C-23), 42.6 (C-24), 32.8 (C-25), 19.6 (C-26), 20.8 (C-27), 17.2 (C-28).

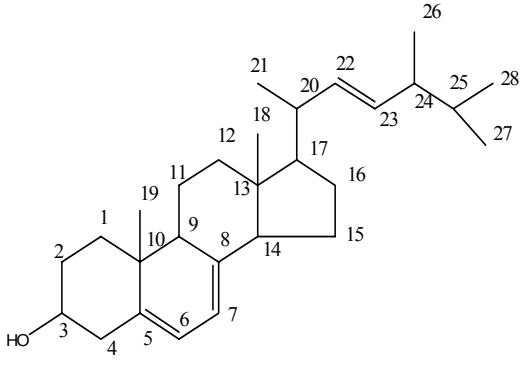
DEPT -135 (400 MHz, $CDCl_3$) δ: 11.91, 17.24, 17.95, 19.26, 19.58, 20.76, 22.39, 22.65, 27.70, 30.16, 32.52, 32.82, 38.67, 39.06, 40.17, 42.59, 42.87, 54.47, 55.75, 67.00, 72.87, 117.25, 131.82, 135.27 .

Biological Studies

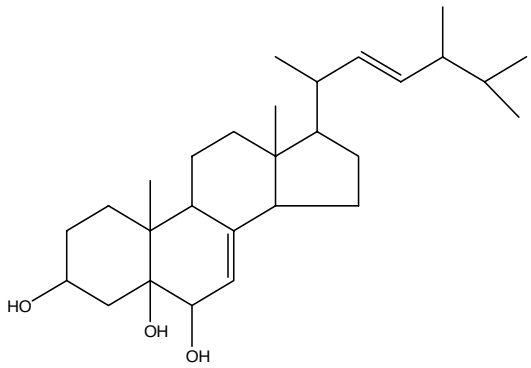
Antibacterial Activity. The ethyl acetate extract of the fungal strain 2L-5 was studied for antibacterial against four bacteria - *Bacillus cereus, Bacillus megaterium, Sarcina lutea* and *Staphylococcus aureus*. The agar disc diffusion protocol was used for antibacterial assay.[5] Ethyl acetate extract (8 mg) was used to prepare solutions of specific concentration (250μg/μL) in ethanol. Sterile filter paper disc of 6 mm in diameter were loaded with 250 μg/disc and 150 μg/disc using micropipette and were dried under laminar air flow hood. Standard antibiotic, *Streptomycin* was used as a positive control. The loaded discs were placed in petri dish (90 mm in diameter) containing sterile nutrient agar medium seeded with test microorganisms.

RESULTS AND DISCUSSION

Twenty three fungi were isolated from different parts of the plant. The fungus 2L-5 was cultivated on large scale in semisolid as well as in liquid media for chemical and biological investigation since it had minimum growth time and the extract showed well-separated spots on TLC. The extract was fractionated by silica gel column chromatography to isolate pure compounds. This provided two white solid compounds, AH-1 (2.4 mg) and AH-2 (2.2 mg). Compounds AH-1 and AH-2 were further purified using recrystalisation from hexane.



Ergosterol



Cerevesterol

Comparing the spectral date of AH-1 and AH-2 with those of known steroids it was found that AH-1 matches the data of ergosterol and AH-2 matches those of cerevesterol.[6] Therefore, the structure of AH-1 was assigned as ergosterol and that of AH-2 as cerevesterol

The ethyl acetate extract of the fungal strain 2L-5 was studied for antibacterial activity against four Bacteria - *Bacillus cereus*, *Bacillus megaterium*, *Sarcina Lutea*, *Staphylococcus aureus* and was found active against *Bacillus cereus* and *Staphylococcus aureus* with zone of inhibition of 14 mm and 15 mm respectively**.**

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