Studies on Chemical Constituents and Biological Activities of an Endophytic Fungi from *Magnifera indica* L.

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ABSTRACT: Two compounds, ergosterol (**1**) and 4-hydroxy-hexadec-6-enoic acid methyl ester (**2**) were isolated from the ethyl acetate extract of the endophytic fungal strain labeled as MI-3, isolated from the leave of *Magnifera indica* L. The structures of the isolated compounds were elucidated by ^{1}H NMR studies and comparing with published data. The crude ethyl acetate extract, three column fractions and ergosterol were tested for antimicrobial activity against five Gram-positive and eight Gram-negative bacteria and three fungi by disc diffusion method. The general toxicity and antioxidant activity of the parent extract, column fractions and ergosterol were also evaluated by using brine shrimp lethality assay and free radical scavenging assay, respectively. Low activities were observed in all cases.

Key words: Endophytic fungi, ergosterol, antimicrobial activity, general toxicity, antioxidant activity.

INTRODUCTION

Endophytic fungi are the fungi that live within their host plants without causing any noticeable symptoms of disease to the plant.[1,2] Endophytic fungi are found in all divisions. Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive agents.[3,4] There are approximately 300,000 higher plant species on this earth which can be host for one or more endophytes and subsequently produce many interesting secondary metabolites. However, endophytic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with different biological activities. As part of our ongoing research on endophytic fungi for the isolation of novel bioactive compounds,[5,6] we studied leaves of *Magnifera indica* L and report here the isolation of two compounds, ergosterol (**1**) and 4-hydroxy-hexadec-6-enoic acid methyl ester (**2**) from the ethyl acetate extract of an endophytic fungi (MI-3) and bioactivity of the parent extract and purified compounds for antimicrobial activity, general toxicity and antioxidant screening.

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MATERIALS AND METHODS

General experimental procedures. UV and IR spectra were recorded on Shimadzu UV 160A and Shimadzu IR-470 spectrometer, respectively. The ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer using tetramethylsilane (TMS) as the internal reference. Media was prepared under Laminar flow (Thermo Forma. Class 11 A1; Biological Safety Cabinet) and was sterilized using HIRAYAMA autoclave (Hirayama Mfg. Corp.).

Plant collection and sterilization. The leaves of plant, *Magnifera indica* L were collected from a local nursery of Dhaka city. Different parts of leaves were cleaned and washed with water. Each part of leaves was surface sterilized with 70% ethanol, 3% sodium hypochlorite and sterile water. The cleaned leaves were kept successively into each of the solution for 3 minutes.

Inoculation and extraction of fungal strain. The surface sterilized plant materials were inoculated on autoclaved potato-dextrose agar medium on a sterilized petridish (90 mm in diameter). Five pieces of leaves were inoculated on each petridish. After 21 days of inoculation, seven fungal strains were isolated for identification. Out of them, one fungal strain labeled as MI-3 was found to have optimum growth and was subcultured on semisolid potato-dextrose agar medium in large scale for chemical and

biological studies. After 21 days, the fungi were collected in a round bottom flask and ground by Ultra-Turrax followed by freeze-drying. The dried powdered material was extracted with ethyl acetate (1500 ml x 3 for 24 h, at room temperature) and was evaporated to dryness to provide 1.50 g. A small portion of the extract (~30 mg) was reserved for anti-bacterial, antioxidant and brine shrimp lethality assays.

Chemical studies of the extract. A column was packed with silica gel using hexane as column equilibrating solvent. After application of the sample (1.2 g), solvents of increasing polarities from 100% hexane to ethyl acetate (EA) and finally 50% methanol (MeOH) in EA were used for elution. On the basis of their R_f values on TLC, eight fractions were obtained. Among them, one fraction eluted with 70% *n*-hexane in EA gave white needle shaped crystals which was purified by re-crystallization from hexane and dichloromethane mixture (8:2) and collected as pure compound **1** (28.0 mg). Another fraction collected from 80% n-hexane in EA also gave a single spot on TLC and was collected as pure compound **2** (36.0 mg) as yellowish gum.

Compound **1**: White crystal. UV (in DCM): λ_{max} 260 and 272 nm. IR (KBr pellet): ν_{max} 2900, 2840, 1630, 1440 and 1240 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.63 (3H, s, H-18), 0.81 (3H, d, *J*=6.0 Hz, H-26), 0.82 (3H, d, *J*=6.4 Hz, H-27), 0.91 (3H, d, *J*=6.8 Hz, H-28), 0.93 (3H, s, H-19), 1.04 (3H, d, *J*=6.4 Hz, H-21), 3.62 (1H, m, H-3), 5.18 (1H, d, *J*=7.2 Hz, H-22/23), 5.38 (1H, s, H-7) and 5.56 (1H, s, H-6).

Compound **2**: Yellowish gum. ^1H NMR (400 MHz, $CDCl_3$): δ 0.87 (3H, t, *J*=7.2 Hz, H-16), 1.24 (2H, br. s, H-15), 1.53 (2H, br. s), 1.59 (2H, br. s), 2.05 (2H, t, *J*=7.2 Hz), 3.18 (3H, s, O-CH_3) 3.98 (1H, d, *J*=4.8 Hz, H-4), 5.09 (1H, br. s, H-6) and 5.10 (1H, br. s, H-7)

Biological studies

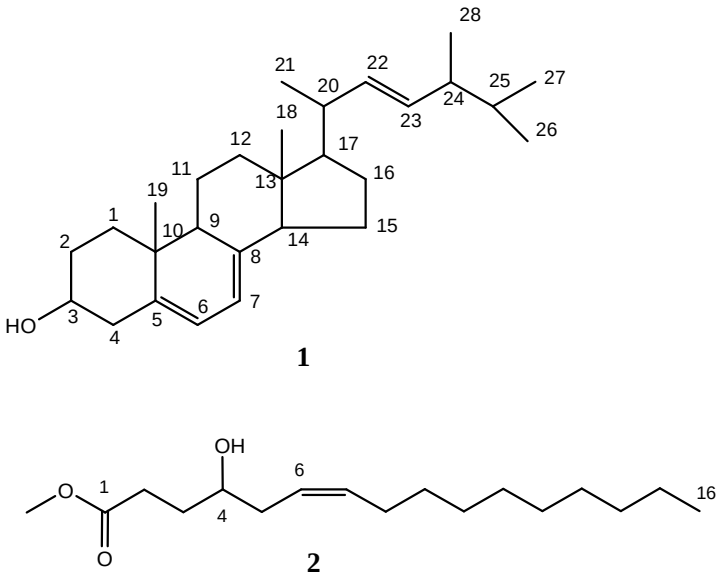
Antimicrobial assay. The *in vitro* antibacterial and antifungal activities of the crude extracts as well as the isolated purified compound were determined by the disc diffusion technique.[7] Solutions of known concentration (µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents (chloroform or methanol). Five Gram positive and eight Gram negative antibacterial and three fungal strains, were collected from the Institute of Nutrition and Food Sciences, University of Dhaka. Nutrient agar media was used for the culture of bacteria and potato dextrose agar media was used for the culture of fungi. Dried and sterilized filter paper discs (7 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Ciprofloxacin) discs and blank discs (impregnated with solvents followed by drying) were used as positive and negative control, respectively. The plates were incubated at 37°C for 24 hours to allow maximum growth of the organisms. The antimicrobial activities were measured from the zone of inhibition expressed in mm. All experiments were carried out in triplicate.

General toxicity: Brine shrimp lethality assay was carried out to evaluate the general toxicity following the experimental details in published paper.[8]

Antioxidant activity. The antioxidant activity (free radical scavenging activity) of the test samples on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the modified method of Takao (Takao, 1994).[9] Samples (2.0 mg) was dissolved in methanol and solution of varying concentrations such as 500, 250, 62.50, 32, 16, 8, 4 and 2 µg/mL were obtained by serial dilution technique. Methanol solution of samples (2 mL) were mixed with 3 mL of a DPPH methanol solution (20 µg/ mL) and allowed to stand in dark place for 30 min for the reaction to occur. Then the absorbances were determined at 517 nm against methanol as blank and the corresponding percentage of inhibitions was calculated. *Tert*-butyl-1-hydroxytoluene (BHT), a potential antioxidant, was used as positive control.

RESULTS AND DISCUSSION

Two compounds, **1** and **2** were isolated from the ethyl acetate extract of the fungal strain MI-3 derived from *M. indica* by silica gel column chromatography. The compound **1** was obtained as white needle shaped crystal. It gave purple colour with vanillin-sulphuric acid reagent. The ^{1}H NMR spectrum of **1** showed two broad singlets at δ 5.56 and 5.38 ppm typical for H-6 and H-7 of a steroidal nucleus.[10] Moreover, a down field overlapping signals of two proton intensity at δ 5.18 ppm, is also characteristic for the position of 22 and 23 protons of the steroidal moiety. A multiplet at δ 3.62 was assigned to C-3 proton of the steroidal nucleus.[10,11] The ^{1}H NMR spectral data also indicated the presence of six methyl groups at δ 0.63, 0.81, 0.82, 0.91, 0.93 and 1.04 ppm. These ^{1}H NMR data were compared with that reported for ergosterol and found to be identical.[11] Thus, the structure of compound **1** was confirmed as ergosterol (Fig 1).



1

2

Fig. 1. Structure of compounds **1** and **2**

Compound **2** was obtained as yellowish gum. The ^{1}H NMR spectrum of **2** displayed signals at δ 5.10 and 5.09 ppm which accounted for the presence of two olefinic protons. The signals at δ_H 3.98 and 3.18 ppm could be attributed were due to the oxygenated methine and methyoxyl group, respectively. A broad signal at δ 1.24 and a singlet at 0.87 ppm were due to the presence of methylene and methyl protons, respectively. On this basis compound 2 was identified as 4-hydroxy-hexadec-6-enoic acid methyl ester (Fig. 1) and confirmed from published data.

The antibacterial activity of the crude ethyl acetate extract, three column fractions, F-3 (eluted from 80% n-hexane in EA), F-5 (eluted from 50% n-hexane in EA), F-7 (eluted from 100% EA) and compound **1** were carried out by the disc diffusion method (Table 1). The samples were screened at three different concentration (500, 300, 200 µg/disc) against five gram positive, eight gram negative bacteria and three fungi, and zone of inhibition were measured. The zone of inhibition produced by the crude ethyl acetate extract and column fractions, F-3, F-5, F-7 were ranged from 8-10, 8-11 and 7-9 mm, respectively. However, all the test organisms were found to be resistant to the crude ethyl acetate extract and compound **1** (data not shown). The F-3 and F-5 exhibited mild activity against *P. aureus, S. paratyphi,* and *B. cereus, B. subtilis* and *S. paratyphi*, respectively.

The general toxicity of the crude ethyl acetate extract, three column fractions, F-3, F-5, F-6 and compound **1** were screened by brine shrimp lethality assay. The LC_{50} values were found to be 2.98, 13.30, 12.02, 22.32 and 27.13 µg/mL, respectively. It was evident that all the test samples were lethal to brine shrimp nauplii. However, crude extract was comparatively more active than the others.

Table 1. Antimicrobial activity of EA extract, column fractions F-3, F-5, F-7 and Ciprofloxacin

Test bacteria and fungi	EA extract (500 µg/disc)	F-3 (300 µg/disc)	F-5 (300 µg/disc)	F-7 (300 µg/disc)	Ciprofloxacin (30 µg/disc)
Bacteria					
Bacillus cereus	08	08	11	08	44
B. megaterium	08	08	10	08	44
B. subtilis	09	10	11	09	44
Staphylococcus aureus	07	09	09	07	44
Sarcina lutea	08	09	10	08	43
Escherichia coli	09	10	09	08	43
Pseudomonas aeruginosa	10	11	09	07	42
Salmonella Paratyphi	08	12	11	09	45
S.Typhi	08	10	10	08	43
Shigella boydii	07	09	10	08	44
S. dysenteriae	07	10	10	07	44
Vibrio mimicus	09	10	09	08	44
V. parahemolyticus	08	09	10	07	44
Fungi					
Candida albicans	09	09	10	08	43
Aspergillus niger	08	09	10	08	44
Saccharomyces cerevisiae	08	10	10	07	43

Mild activity: 08-11 mm

The antioxidant activity of the crude ethyl acetate extract, three column fractions (F-3, F-5, F-7) and compound **1** were tested by DPPH scavenging assay. The inhibition of free radical scavenging (IC_{50}) value of crude ethyl acetate extract was found to be 32.35 µg/mL whereas, the IC_{50} value of *tert*-butyl-1-hydroxytoluene (standard) was found to be 21.37 µg/mL. The IC_{50} for other samples was very low. To the best of our knowledge the study of endophytic fungi from the *M. indica* is reported here for the first time in Bangladesh.

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