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Bioactive compounds from endophytic fungus *Penicillium thiomii* isolated from *Terminalia chebula* Retz

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ABSTRACT

*Chemical investigation of the ethyl acetate extract of the endophytic fungus Penicillium thiomii isolated from the medicinal plant Terminalia chebula Retz and cultured on potato dextrose agar led to the isolation of one new compound named terminatone (**2**) along with three known compounds namely, ergosterol (**1**), 4-hydroxy benzaldehyde (**3**) and 4-hydroxy-hexadec-6-enoic acid methyl ester (**4**). The structures of the isolated compounds were determined by spectroscopic techniques i.e. UV, IR, 1D & 2D NMR, and by comparison with the literature. The crude ethyl acetate extract and three column fractions were evaluated for antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria and fungi, general toxicity and antioxidant activity by disc diffusion method, brine shrimp lethality and free radical scavenging activities, respectively. Low activities were observed in all cases.*

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

INTRODUCTION

Fungal of the genus *Penicillium* is a rich source of novel natural products with diverse structural and different biological activities including antibacterial, antimalarial, anticancer, and antioxidant (**1,2,3** and **4**). Penicillin, the first natural antibiotic was discovered from *Penicillium notatum* by Fleming in 1929 and found to inhibit the growth of gram positive bacteria (**5**). There are approximately 300 *Penicillium* species that have been identified from various sources so far (**6 and 7**). An endophytic fungus *Penicillium thiomii*, belonging to the family Trichocomaceae, was isolated from the medicinal plants of *Terminalia chebula* Retz (**8**). Fungal endophytes are fungi that live within their host plants without causing any noticeable symptoms of disease to the plant (**9** and **10**). Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive (**11** and **12**). Our earlier investigation on endophytes of *T. chebula* yielded a new natural product 4,7-dimethyl-1,3-dioxa-cyclohepta-2-one from *P. thiomii* (**8**) and novel compound terminatol from an unidentified strain. In continuous of our search for novel bioactive compounds (**13** and **14**) from endophytic fungi we studied *P. thiomii* and report here the isolation of one new natural product named terminatone (**2**) along with known compounds namely ergosterol (**1**), 4-hydroxy benzaldehyde (**3**) and 4-hydroxy-hexadec-6-enoic acid methyl ester (**4**) from the ethyl acetate extract and bioactivity of the parent extract and pure compounds for antimicrobial, general toxicity and antioxidant activity.

MATERIALS AND METHODS

General Experimental Procedure

UV and IR spectra were recorded on Shimadzu UV 160A and Shimadzu IR-470 spectrometer, respectively. The ^{1}H and ^{13}C 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). Media was sterilized using HIRAYAMA autoclave (Hirayama MFG Corp.).

Fungal strains

Five endophytic fungal strains labeled as IR-1, IR-2, IR-3, IR-4, IR-6 and IR-7 were isolated from the medicinal plant *Terminalia chebula* Retz (**15**) The fungal strain IR-7 was identified as *Penicillium thiomii* (**8**).

Inoculation and extraction of fungal strain

The endophyte *P. thiomii* was subcultured on semisolid potato-dextrose agar media in large scale (350 petridishes) for chemical and biological studies. After 21 days, the fungi gave optimum growth and 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 (3 x 1 L, 24 h, room temperature) and was evaporated to dryness and 2.0 g of extract was obtained. A small part of the extract (~100 mg) was saved for anti-bacterial, antioxidant, antifungal 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 (1.9 g), solvents of increasing polarities from 100% hexane to ethyl acetate (EA) and finally 50% methanol (MeOH) with EA were used for elution. On the basis of their R_f values on TLC, seven fractions were obtained. Among them, two fractions eluted, respectively from 70% n-hexane in EA and 10% n-hexane in EA gave white needle shape crystals which were purified by recrystallization from hexane and dichloromethane mixtures and collected as pure compounds 1 (30.0 mg) and 2 (8.0 mg). Other two more fractions eluted from 50% n-hexane in EA and 30% n-hexane in EA were further purified by repeated silica gel column chromatography using solvent mixture of n-hexane and dichloromethane and compounds 3 (4.0 mg) and 4 (4.0 mg) were isolated, respectively.

Compound 1: White crystal. UV (in DCM): λ_{max} 262, 274 and 415 nm. IR (KBr pellet): ν_{max} 2900, 2850, 1720, 1450 and 1250 cm^{-1}. ^{1}H NMR ($CDCl_3$, TMS as standard): 5.57 (1H, d, *J*=7.1 Hz, H-6), 5.38 (1H, m, H-7), 5.20 (1H, m, H-22), 5.20 (1H, m, H-23), 3.63 (1H, br., H-3), 1.03 (3H, d, *J*=6.4 Hz, H-21), 0.94 (3H, s, H-19), 0.92 (3H, d, *J*=6.7 Hz, H-28), 0.84 (3H, d, *J*=7.0 Hz, H-27), 0.82 (3H, d, *J*=6.9 Hz, H-26), 0.63 (3H, s, H-18) ppm. ^{13}C-NMR ($CDCl_3$); 141.5 (C-8), 139.9 (C-5), 135.5 (C-23), 132.1 (C-22), 119.7 (C-6), 116.4 (C-7), 70.6 (C-3), 55.9 (C-17), 54.7 (C-14), 46.4 (C-9), 42.9 (C-13), 41.0 (C-4), 40.6 (C-20), 39.2 (C-12), 38.5 (C-1), 37.2 (C-10), 33.3 (C-25), 32.2 (C-2), 28.4 (C-16), 23.2 (C-15), 21.3 (C-11), 20.1 (C-21), 19.8 (C-26), 19.6 (C-27), 17.8 (C-28), 16.5 (C-19), 12.2 (C-18) ppm.

Compound 2: White solid. UV (DCM): λ_{max} 224, 254 and 260 nm, IR (KBr pellet): ν_{max} 3410, 2920, 1715, 1455 and 1265 cm^{-1}. ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) Table-1.

Compound 3: White solid. UV (DCM): λ_{max} 224, 254 and 260 nm, IR (KBr pellet): ν_{max} 3350, 2920, 1715, 1450 and 1260 cm^{-1}. ^{1}H NMR (400 MHz, CDC_{l3}): 7.60 (1H, d, *J*=8.0 Hz), 7.06 (1H, d, *J*=8.0 Hz), ^{13}C NMR (100 MHz, CDC_{l3}): 121.6, 119.4, 119.2 110.7, 110.7 ppm.

Compound 4: Yellow colored and gummy (6.8 mg), UV (DCM): λ_{max} 228 nm. IR (KBr pellet): ν_{max} 2900, 1730 and 1450 cm^{-1}. ^{1}H NMR ($CDCl_3$, TMS as standard): 5.33 (bs, 1H), 5.25 (bs, 1H), 4.28 (2H, dd, *J*=12.0, 3.6 Hz), 4.13 (1H, dd, *J*=5.6, 1.2 Hz), 2.60 (m, 4H), 2.29 (m, 2H) 1.24 (m, 22H), 1.99, 0.86 (t, *J*=7.2 Hz, 6H) ppm.

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 (**16**). 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 on methanol). Thirteen microbial strains, which included 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) 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 (**17**). Test samples was dissolved in DMSO and varying concentrations of samples such as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/mL were prepared and each sample solution (100 µL) was added to vial containing 5 mL of sea water and 10 *Artemia salina* shrimp nauplii. The median lethal concentration LC_{50} of the test samples after 24 hours was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

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) (**18**) 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 absorbance was determined at 517 nm against methanol as blank by UV spectrophotometer and from these values, the corresponding percentage of inhibitions was calculated. *Tert*- butyl-1-hydroxytoluene (BHT), a potential antioxidant, was used as positive control.

RESULTS AND DISCUSSION

The endophytic fungus *Penicillium thiomii* was isolated from the medicinal plant *Terminalia chebula* Retz. *P. thiomii* was sub-cultured on semisolid potato-dextrose agar media in large scale and extracted with ethyl acetate. Four compounds, 1, 2, 3, and 4 were isolated from the ethyl acetate extract by repeated silica gel column chromatography. The ^{1}H and ^{13}C NMR spectral data of 1 were compared with previously isolated secondary metabolite from endophytes and found identical with ergosterol (**14**).

The compound 2 was obtained as white needle shaped crystal. It appeared as a violet fluorescence spot on TLC plate under UV light at 254 nm and gave pink colour with vanillin-sulphuric acid reagent. The ^{1}H NMR spectrum gave four doublets at 7.45 (1H), 5.82 (1H), 5.28 (1H), 1.24 (3H) ppm, nine multiplets at 5.75 (1H), 4.23 (1H), 4.03 (1H), 2.38 (1H), 2.13 (1H), 2.01 (1H), 1.83 (2H), 1.59 (2H), 1.45 (2H) ppm and one singlet at 3.35 ppm for one methyl group. The signals at 7.45 and 5.82 ppm were due to the olefinic protons of H-2 & H-3 having large coupling constants of 15.6 Hz indicated that the olefinic protons were in *trans* configuration.[19] The signals at 5.75 and 5.28 ppm were also indicated olefinic protons of H-9 & H-10 protons. The signals at 3.35 and 1.24 ppm were due to the oxymethyl and methyl protons, respectively. The ^{13}C NMR spectrum displayed sixteen signals at 164.2, 150.9, 133.9, 127.2, 113.6, 72.5, 69.0, 68.8, 49.0, 41.3, 40.0, 37.8, 30.8, 28.8, 23.8 and 16.8 ppm. The DEPT indicated two methyl (49.0 and 16.8 ppm), five methylene (40.0, 37.8, 30.8, 28.8, 23.8 ppm) and eight methine groups (150.9, 133.9, 127.2, 113.6, 72.5, 69.0, 68.8, 41.3 ppm). The ^{1}H-^{1}H COSY spectrum showed strong correlation between 7.45 & 5.82, 5.75 & 5.28, 7.45 & 1.45, 4.03 & 2.1 and 4.23 & 1.83 ppm. The long range correlations were observed in the HMBC spectrum between δ_H 7.45 & δc 164.2 and δ_H 5.82 & δc 164.2. Combining all the spectral data of IR, ^{1}H- and ^{13}C NMR including DEPT, ^{1}H-^{1}H COSY, HMBC, the structure of 2 was elucidated and named as terminatone. To the best of our knowledge terminatone is a new natural product.

The ^{1}H and ^{13}C NMR spectral data of 3 indicated the presence of 1,4 di-substituted benzene ring system by signals at δ_H 7.60 (1H, d, *J*=8.0 Hz), 7.06 (1H, d, *J*=8.0 Hz) and δ_C 121.6, 119.4, 110.7 ppm, respectively. One singlet at δ_H 9.87 ppm could be assigned for an aldehydic proton. However, the carbonyl carbon signal could not assigned due to S/N ratio was very high, also sample was too small and accusion/scan was not very high. The presence of carbonyl group was also confirmed by absorbance in the IR spectrum (1745 cm^{-1}). Thus, the 3 was proposed as 4-hydroxy benzaldehyde (3). The ^{1}H NMR spectrum of 4 gave signals at 5.33, 5.25, 4.28, 4.13, 2.29, 1.99, 1.24, 0.86 ppm. Two signals at 5.33 and 5.25 ppm were due to the presence of two olefinic protons at C-6 and C-7. A distorted triplet at 0.86 ppm was assigned for two methyl groups. A broad signal at 1.24 ppm was due to methylene protons. One triplet at 2.29 (2H) ppm indicated methylene protons attached to carbonyl functional group (C-1) and one

doublet at 4.28 (2H) ppm assigned one oxygenated methylene group. The ^{1}H NMR spectral data confirmed 4 to be 4-hydroxy-octadec-6-enoic acid ethyl ester.

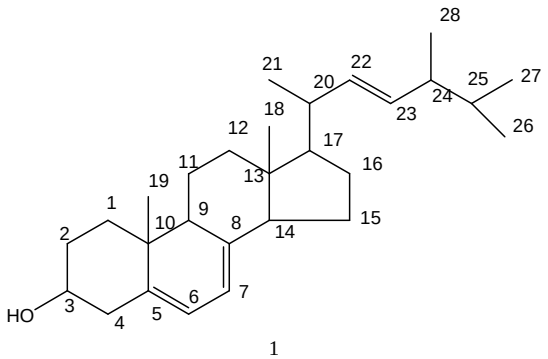
The antibacterial activities of the crude ethyl acetate extract, three column fractions, F-3 (eluted from 80% n-hexane in EA), F-7 (eluted from 50% n-hexane in EA) and F-9 (eluted from 100% EA) were carried out by the disc diffusion method (Table 2). The samples were screened at three different concentration (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 the fractions F-3, F-7, F-9 were ranged from 12-08, 12-08, 11-09 and 9-7 mm, respectively. The F-3 and F-5 exhibited mild activity against *P. aureus and S. paratyphi*, and *B. cereus*, *B. subtilis* and *S. paratyphi*, respectively. The parent extract showed mild activity against the bacteria of *Bacillus cereus*, *Bacillus subtilis*, *Sarcina lutea*, *Escherichia coli*, *Salmonella paratyphi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus*, and the fungi of *Candida albicans*, *Aspergillus niger*. The fraction F-3 showed mild activity against *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Salmonella paratyphi*, *Salmonella typhi* while the fraction F-9 showed mild activity against most of the cases.

The general toxicity of the crude ethyl acetate extract, three column fractions, F-3, F-7 and F-9 were screened by brine shrimp lethality assay. The LC_{50} values were found to be 2.39, 13.71, 8.47 and 24.29 µg/mL, respectively. It was evident that all the test samples were lethal to brine shrimp nauplii. However, crude extracts were comparatively more active than others.

The antioxidant activity of the crude ethyl acetate extract and three column fractions (F-3, F-5, F-7) were tested by DPPH scavenging assay. The IC_{50} value of crude ethyl acetate extract was found to be 40.73 µg/mL whereas, the inhibition of free radical scavenging (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 and could not determine.

Table 1 : ^{1}H NMR (400 MHz, CD_3OD), ^{13}C NMR (100 MHz, CD_3OD) and 2D NMR spectral data of 2

H/C	^{1}H (ppm)	^{13}C (ppm)	COSY	HMBC
1	--	164.2	--	--
OCH_3	3.35 (3H, s)	49.0	--	--
2	7.45 (1H, dd, *J*=15.6, 2.8 Hz)	150.9	H3	C1, H2
3	5.82 (1H, d, *J* =15.6, 2.0 Hz)	127.2	H2, H4	C1, H3
4	1.45 (2H, m)	40.0	H3	--
5	1.59 (2H, m)	23.8	--	--
6	2.13 (1H, m)	41.3	--	--
7	2.01 (1H, m)	68.8	H8	--
8	4.03 (1H, m)	72.5	H7	--
9	5.75 (1H, m)	113.6	H10	--
10	5.28 (1H, dd, *J*=15.2, 9.6 Hz)	133.9	H9	--
11	1.83 (2H, m)	37.8	H11	--
12	4.23 (1H, m)	69.0	H11	--
13	1.83 (2H, m)	30.8	H11	--
14	--	28.8	--	
15	--	41.3	--	--
16	2.38 (1H, m)	41.3	--	--
17	1.24 (3H,d, *J*=6.0 Hz)	16.8	--	--



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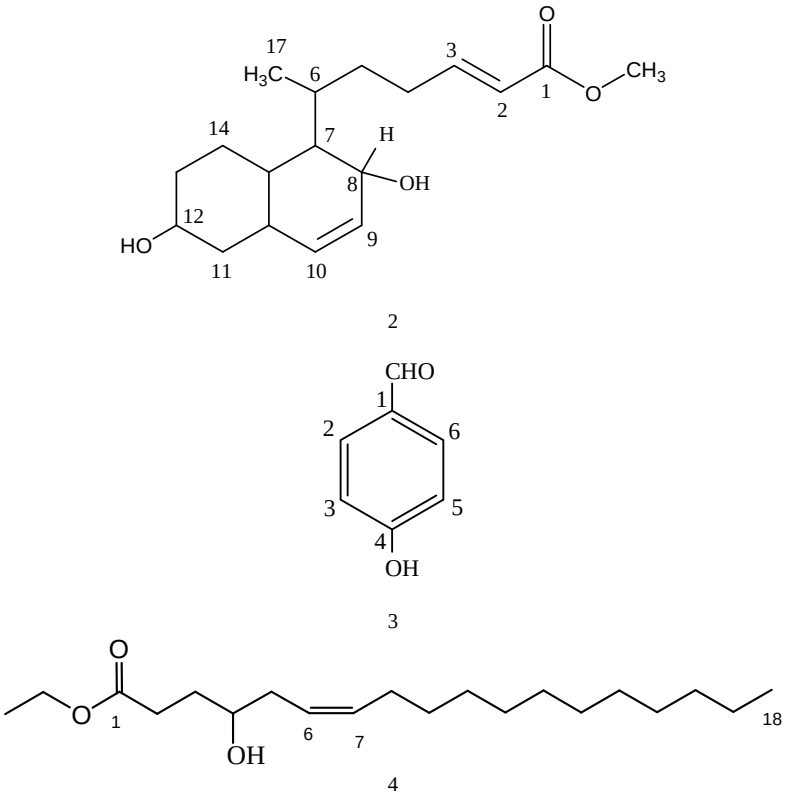

Figure 1: Structure of compounds 1-4

Table 2. Antimicrobial activity of EA extract, column fractions and Ciprofloxacin

Test bacteria and fungi	EA extract	F-3	F-7	F-9	Ciprofloxacin
Bacteria					
Bacillus cereus	10	10	--	10	44
B. megaterium	09	11	--	11	44
B. subtilis	10	10	--	10	44
Staphylococcus aureus	09	08	07	08	44
Sarcina lutea	10	09	--	09	43
Escherichia coli	10	08	07	08	43
Pseudomonas aeruginosa	09	09	--	09	42
Salmonella paratyphi	12	12	--	12	45
S. typhi	09	10	08	10	43
Shigella boydii	10	09	--	09	44
S. dysenteriae	11	09	--	09	44
Vibrio mimicus	10	08	07	08	44
V. parahemolyticus	08	09	--	09	44
Fungi					
Candida albicans	10	--	09	--	43
Aspergillus niger	11	--	10	--	44
Sacharomyces cerevacae	09	--	10	--	43

"--" indicates no activity

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