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Isolation and Identification of *Emericella Nidulans* Secondary Metabolites

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Abstract Within the scope of our program aiming to the search for bioactive secondary metabolites from *Emericella nidulans* sp. Emericellin and sterigmatocystine were isolated from ethanolic crude extract. The chemical structure of the isolated compounds was identified by the comparison of 1D, 2D NMR and HRESIMS data with authentic spectra. Sterigmatocystine was known to have an *in vitro* cytotoxicity against liver cancer HEP-G2. Interestingly, the metabolic profile of n-hexane lipophilic compounds were elucidated by (GC-MS) analysis that led to the identification of phytol and farnesol (terpenoid compounds), oliec acid, parafines and fatty acid esters.

Keywords *Emericella nidulans*, Emericellin, sterigmatocystine, terpenoids, fatty acid esters.

Introduction:

Emericella nidulans is a filamentous fungus which belongs to phylum Ascomycota, it was considered as a rich source of secondary metabolites with potential bioactivities. Polyketides with a benzophenone nucleus were previously isolated from the total extract of *E. nidulans* [1]. Emericellin was tested for its inhibitory activity against HCV protease using HCV NS3 protease inhibitor 2 as a positive control [2]. It showed a mild inhibitory effect with IC50 values of 50.0, µg/ml. Generally Xanthonic compounds showed interesting biological activities associated with their tricyclic scaffold depending on the nature and/or position of the different substituents [3]. The relationship between activity and the presence of prenyl groups in key-positions on the xanthone nucleus was associated with some biological activities, such as inhibition of human lymphocyte proliferation [4], PKC modulation [5], antitumor [6], and anti-inflammatory [7].

This study was conducted to explore the chemical profile of the total extracts that were produced from the marine endophyte, this was accomplished by Ethanol and n-hexane extraction, the isolated compounds were identified by means of NMR and (GC-MS) analysis.

Materials and Methods:

The NMR spectra were musured on Varian Inova 600 (150 MHz). Coupling constants (*J*) and Varian Unity 400 (400 MHz). Chemical shifts were measured relative to tetramethylsilane as internal standard. Mass spectra: EI MS at 70 eV with Varian MAT 731, Varian 311A, AMD-402, high resolution with perflurokerosine as standard. DCI-MS: Finnigan MAT 95 A, 200 eV, Reactant gas NH_3. ESI MS was recorded on a Finnigan LCQ. Column

chromatography (CC): MN silica gel 60: 0.05-0.2 mm, 70-270 mesh (Macherey-Nagel & Co); silica gel (230-400 mesh) for flash chromatography: 30-60 μm (J. T. Baker); size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany).

Biological Material:

The fungus *Emericella nidulans* was isolated as an endophyte from the brown alga *Turbinaria elatensis*. The fungus was identified by a morphological method and 18S rDNA sequence comparison [8]. The brown algae *T. elatensis* was collected from the Egyptian Red Sea site at a depth of 3–6m from the coast of Rass Mohamed (South Sina, Egypt). The sample was selected solely on the basis of a clean and healthy exterior and brought to the laboratory in ice. In the laboratory, the specimens were washed with sterile water and processed immediately. The sample was identified by the Coral Reef Ecology and Biology group, National Institute of Oceanography and Fisheries, Suez, Egypt.

Isolation of the Marine Endophyte:

E. nidulans was isolated as an endophyte using biomalt agar medium containing the following ingredients (g/L): bio malt (20), peptone (5) and agar (20). The previous nutrients were dissolved in 50% sea water supplemented with penicillin benzyl sodium salt (0.02) to avoid any bacterial growth; the cultured medium was adjusted at pH 7.5 and incubated statically at 28^{o}C. After 6-7 days sand brown, velvety colonies were observed. The strain was identified as *E. nidulans* from the morphological features of conidiophores. Stock cultures of the fungus, was used to inoculate 500 mL of liquid medium in an Erlenmeyer flask (3 L) containing bio malt broth in 50% seawater. It was then incubated at 28^{o}C in a rotary shaker for 72 h at 200 rpm and used as first stage inoculums. The same medium (10 L) was made in 75 Erlenmeyer flasks (1 L) and inoculated with 5% of first stage inoculums. The flasks were incubated statically for 15 days at 28^{o}C.

Extraction of the Total Metabolites:

At the end of the incubation period, the biomalt cultured broth media were filtered to separate the biomass away from the filtrate media, both were extracted with ethyl acetate three times and acetone for only one time. The total extract was evaporated and concentrated by a rotatory evaporator and weighed.

***In-vitro* Bioassays of Isolated Compounds:**

Antimicrobial activity

Antimicrobial activity of the ethyl acetate extract and isolated compounds was evaluated against different bacteria and fungi. The extract showed only moderate activity against *Bacillus-megaterium* (Gram-positive bacteria), whereas the isolated compounds were inactive against all tested pathogens.

Anticancer activity:

The extract of the culture broth of the fungus *E. nidulans* was screened for the in-*vitro* cytotoxicity against two type of leukaemia (murine L1210 and human CCRF-CEM), four solid tumours (murine colon 38, human colon HCT-116, human lung H-125, human liver HEP-G2), as well as human normal cells (CFU-GM) using the disc diffusion assay. The samples were initially prepared in DMSO and then applied to the filter disc. After 7 days of incubation with the examined cells, the cells that had survived had grown into colonies, and the zones of inhibition of colony formation were assessed.

Results and Discussion:

Emericellin (1):

Emericellin (1) was isolated from fraction II as yellow crystals and showed an absorbing band at 254 nm. Emericellin (1) was isolated with a molecular weight (*m/z* 408) corresponding to the molecular formula $C_{25}H_{28}O_5$. ^{1}H-NMR spectrum of emericellin (1) revealed signal at δ 12.63 (1H, s) assigned to the chelating hydroxyl group and

the signals at δ 6.72 (1H, d) and δ 7.42 (1H, d) were assigned to the protons of aromatic ring. The signals at δ 3.47 (2H, m) and δ 5.31 (1H, m) were assigned to the methylene and olefinic protons of cis prenyl group and the signals at δ 4.61, δ 4.72 (2H, m) were assigned to the methylene protons attached to the oxygen-bearing carbon. The singlet methyl signal at δ 2.42 assigned to methyl group attached to aromatic ring. The signals at δ 1.50-1.80 (3H x 4) were assigned to the protons of four olefinic methyl groups. Analysis of the ^{13}C-NMR data for emericellin (1) showed quaternary carbon at δ 182.2 assigned to carbonyl group in armomatic ring. Most of the correlations in emericellin (1) were established by ^{1}H, ^{1}H Cosy and HMBC spectrum **(Figure 1)**. The structure of emericellin was confirmed by means of the X-ray diffraction method [9].

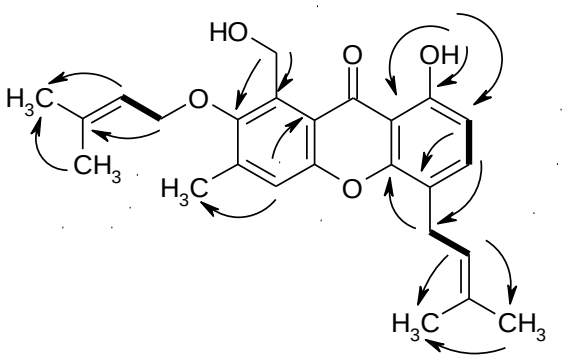
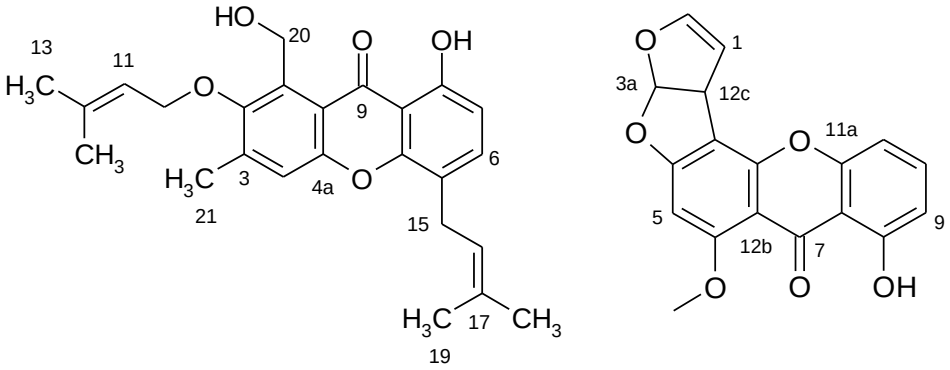


Figure 1: Some selected ^{1}H, ^{1}H COSY (▬) and HMBC (→) correlations of emericellin (1)

Sterigmatocystine (2):

Fraction II was subjected to sephadex LH-20 column using methanol which gave sterigmatocystine (2) as colourless crystals. It showed UV absorbing zone on 254 nm. Sterigmatocystine (2) was isolated with a molecular weight (*m/z* 324) corresponding to the molecular formula $C_{18}H_{12}O_6$. The ^{1}H-NMR spectrum of sterigmatocystine (2) exhibited the singlet signal at δ 13.17 assigned to the hydrogen bonded phenolic hydroxy proton and the protons at δ 7.48, 6.78 and 6.68 assigned to 1,2,4 trisubstituted aromatic ring while the singlet at δ 6.38 revealed to benzene pentasubstituted. The singlet protons at δ 3.93 may be methoxy protons. Analysis of the ^{13}C-NMR data for sterigmatocystine showed quaternary carbon at δ 181.4 assigned to carbonyl group in armomatic ring. A search in the Chemical Abstract with these data suggested sterigmatocystin (2), It was further confirmed by the literature data and comparing with authentic spectra [10].



(1) **(2)**

Figure 2: The chemical structure of emericellin (1) and sterigmatocystine (2).

Table 1: ^{1}H and ^{13}C NMR shifts of emericellin (1) and sterigmatocystine (2).

Position	δ_C [a.b]	mult.	δ_H [a,c] (mult.)
1	116.9	C_q	
2	153.6	C_q	
3	137.3	C_q	
4	119.8	CH	7.25(s)
4a	152.4	C_q	
5a	152.9	C_q	
5	118.9	C_q	
6	133.9	CH	7.42(d,8.4)
7	110.5	CH	6.72(d,8.4)
8	158.3	C_q	
8a	109.1	C_q	
9	182.2	C_q	
1a	118.1	C_q	
10	63.2	CH_2	4.72, 4.61 (m)
11	114.1	CH	5.21(m)
12	138.2	C_q	
13	25.8	CH_3	1.76(s)
14	18.2	CH_3	1.73(s)
15	27.4	CH_2	3.47(m)
16	121.4	CH	5.31(m)
17	134.2	C_q	
18	17.9	CH_3	1.80(s)
19	25.7	CH_3	1.82(s)
20	57.2	CH_2	4.60(s)
21	16.2	CH_3	2.42(s)
OHAr			12.63(s)
$OHCH_2$			4.35(s)

Position	δ_C [a.b]	mult.	δ_H [a,c]
1	102.5	CH	5.37 (dd)
2	145.4	CH	6.40(d)
3a	113.3	CH	7.19(s)
4a	154.0	C_q	-
5	90.2	CH	6.38 (s)
6	162.5	C_q	-
6a	105.9	C_q	-
7	181.4	CO	-
7a	108.9	C_q	-
8	154.9	C_q	-
9	105.8	CH	6.68 (d)
10	135.7	CH	7.48(t)
11	111.2	CH	6.78(dd)
12a	164.6	C_q	-
12b	106.5	C_q	-
12c	48.26	CH	4.77(d)
OCH_3	56.8		3.93(s)
OH	-		13.17(s)

[a] $CDCl_3$; [b] 150 MHz; [c] 400 MHz

Sterigmatocystine (2) is a fungal secondary metabolite produced by fungi of the genera *Aspergillus* and *Penicillium*. ST is the end product of a biosynthetic pathway in some fungal species such as *A. versicolor* and *A. nidulans*, but is also a well-known precursor of aflatoxin B1 synthesis in various other fungal species [11]. ST has been shown to be genotoxic and potentially carcinogenic in studies with experimental animals and exerts teratogenic effects at higher exposure levels [12]. The biosynthesis of polyketides resembles that of fatty acids, although keto groups in the latter are most often reduced. While many secondary metabolites can be used as pharmaceutical agents, there has been considerable effort to control production and reduce contamination by these mycotoxins [13]. sterigmatocystin was screened for its *in-vitro* cytotoxicity against two type of leukaemia (murine L1210 and human CCRF-CEM), four solid tumours (murine colon 38, human colon HCT-116, human lung H-125, human liver HEP-G2), as well as human normal cells (CFU-GM) using the disc diffusion assay [14]. The biological activity of sterigmatocystin towards mice, ducklings, and some microorganisms was determined. The LD_{50} of sterigmatocystin for mice was in excess of 800 mg/kg. Aflatoxin B_1 was 125 times more toxic in the ducklings tests than was sterigmatocystin. The antibiotic disc assay demonstrated that 100 µg of sterigmatocystin did not inhibit any of the microorganisms tested [15].

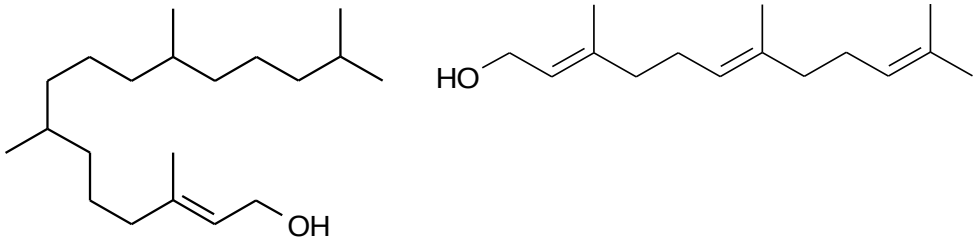
Gas Chromatography – Mass Spectrometry Analysis (*GC-MS*):

Chemical constituents of the n-hexane fraction by using GC-MS analysis was performed by Assiut University GC-MS (7890 GC system/ 5975Binert XL EI/CI MSD Column: DB-5 m s (30 m X 0.25 mm X 0.25 µm) EI MS at 70eV with Varine MAT 731, high resolution with perflurokerosine as standard. ESI MS was recorded on a Finnigan LCQ.

Table 2: GC-MS analysis of the non-polar fraction of *Emericella nidulans*

Compound	R.T. (min.)	Value %	M. Formula	M. Weight
n-Octadecane	21.154	3.158	$C_{18}H_{38}$	254.49
1-Nonadecene	23.194	2.733	$C_{19}H_{38}$	266.51
9-Tricosene	26.175	0.945	$C_{23}H_{46}$	322.61
9,12-Octadecadienoic acid ethyl ester	31.581	7.355	$C_{20}H_{36}O_2$	308.50
Oleic acid ethyl ester	31.721	4.231	$C_{20}H_{38}O_2$	310.52
Palmitic acid butyl ester	32.123	5.675	$C_{20}H_{40}O_2$	312.53
9,12-Octadecadienoic acid(z, z)-methyl ester	35.527	14.148	$C_{19}H_{34}O_2$	294.47
Oleic acid	35.609	8.854	$C_{18}H_{34}O_2$	282.47
Phytol (3)	36.291	3.095	$C_{20}H_{40}O$	296.54
Farnesol (4)	40.383	1.834	$C_{15}H_{26}O$	222.37

In this fraction we found two terpenoid compounds phytol (3) and farnesol (4), fatty acid as oleic acid, three hydrocarbons and the others are fatty acid esters. Phytol (3) is a diterpene alcohol that can be used as a precursor for the manufacture of synthetic forms of vitamin E and vitamin K1 [16]. In ruminant, the gut fermentation of ingested plant materials liberates phytol, which is then converted to phytanic acid and stored in fats. Phytol and/or its metabolites have been reported to bind to and/or activate the transcription factor [17]. Farnesol (4) is a natural 15-carbon organic compound which is an acyclic sesquiterpene alcohol which is under standard conditions it is a colourless liquid. It is hydrophobic and thus insoluble in water, but miscible with oils. Farnesol (4) is produced from 5-carbon isoprene compounds in both plants and animals.



(**3**) (**4**)

Figure 3: Selected structural formula for non-polar fraction by GC-MS analysis

Conclusion:

The current study found 10 chemical constituents from n-hexane fraction of *E. nidulans* by (GC-MS) analysis and 2 secondary metabolites isolated from crude extract, To prove that fungi are still in the focus of interest because their high productivity of a wide range of bioactive compounds and interesting molecules.

References:

1. Kralj, A., S. Kehraus, et al. (**2006**). "Arugosins G and H: Prenylated Polyketides from the Marine-Derived Fungus Emericella nidulans var. a cristata." Journal of natural products 69(7): 995-1000.

2. Wei, Y., C.-M. Ma, et al. (**2009**). "Synthesis of dammarane-type triterpene derivatives and their ability to inhibit HIV and HCV proteases." Bioorganic & medicinal chemistry 17(8): 3003-3010
3. Pinto, M., M. Sousa, et al. (**2005**). "Xanthone derivatives: new insights in biological activities." Current medicinal chemistry 12(21): 2517-2538.
4. Gonzalez, M. J., M. S. J. Nascimento, et al. (**1999**). "Immunomodulatory activity of xanthones from Calophyllum teysmannii var. inuphylloide."Planta medica 65(04): 368-371.
5. (a)Lu, Z. X., M. Hasmeda, et al. (**1998**). "Inhibition of eukaryote protein kinases and of a cyclic nucleotide-binding phosphatase by prenylated xanthones." Chemico-biological interactions 114(1-2): 121-140. (b) Jinsart, W., B. Ternai, et al. (**1992**). "Inhibition of wheat embryo calcium-dependent protein kinase and other kinases by mangostin and γ-mangostin." Phytochemistry 31(11): 3711-3713.
6. Ho, C.-K., Y.-L. Huang, et al. (**2002**). "Garcinone E, a xanthone derivative, has potent cytotoxic effect against hepatocellular carcinoma cell lines." Planta medica 68(11): 975-979.
7. Nakatani, K., N. Nakahata, et al. (**2002**). "Inhibition of cyclooxygenase and prostaglandin E2 synthesis by γ-mangostin, a xanthone derivative in mangosteen, in C6 rat glioma cells1." Biochemical pharmacology 63(1): 73-79.
8. Hawas, U. W., L. T. El-Kassem, et al. (**2012**). "In-vitro bioassays on the metabolites of the fungus Emericella nidulans isolated from the Egyptian Red Sea algae." Egyptian Pharmaceutical Journal 11(2): 124.
9. Fukuyama, K., T. Tsukihara, et al. (**1975**). "The Crystal and Molecular Structure of Emericellin, A Metabolite of Aspergillus nidulans." Bulletin of the Chemical Society of Japan 48(10): 2947-2948.
10. Ashley, D. L., D. L. Orti, et al. (**1987**). "Proton nuclear magnetic resonance evidence for two configurations of the hemiacetals of aflatoxin B1 and sterigmatocystin." Journal of agricultural and food chemistry 35(5): 782-785.
11. Veršilovskis, A. and S. De Saeger (**2010**). "Sterigmatocystin: occurrence in foodstuffs and analytical methods–an overview." Molecular nutrition & food research 54(1): 136-147.
12. Kusunoki, M., J. Misumi, et al. (**2011**). "Long-term administration of the fungus toxin, sterigmatocystin, induces intestinal metaplasia and increases the proliferative activity of PCNA, p53, and MDM2 in the gastric mucosa of aged Mongolian gerbils." Environmental health and preventive medicine 16(4): 224.
13. Yu, J.-H. and T. J. Leonard (**1995**). "Sterigmatocystin biosynthesis in Aspergillus nidulans requires a novel type I polyketide synthase." Journal of Bacteriology 177(16): 4792-4800.
14. Valeriote, F., C. K. Grieshaber, et al. (**2002**). "Discovery and development of anticancer agents from plants." Journal of Experimental Therapeutics and Oncology 2(4): 228-236.
15. Lillehoj, E. and A. Ciegler (**1968**). "Biological activity of sterigmatocystin." Mycopathologia et mycologia applicata 35(3-4): 373-376.
16. Daines, A. M., R. J. Payne, et al. (**2003**). "The synthesis of naturally occurring vitamin K and vitamin K analogues." Current Organic Chemistry 7(16): 1625-1634.
17. Gloerich, J., N. van Vlies, et al. (**2005**). "A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARα-dependent and-independent pathways." Journal of lipid research 46(4): 716-726.