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Structural Elucidation and Bioactivity Studies of Secondary Metabolites from Endophytic *Aspergillus niger*

KEYWORDS: Endophytic Fungi; Taxonomy; Secondary Metabolites; Structural Elucidation; Bioactivity

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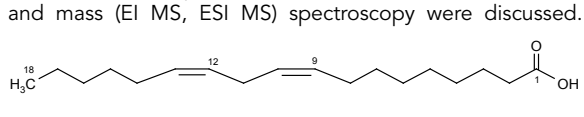
ABSTRACT *During the research for bioactive secondary metabolites from microorganisms, the endophytic fungi Aspergillus niger sp. isolate R8 was found to produce eight diverse bioactive compounds (1-8) by applying to large scale fermentation on M2-medium followed by working up and purification using a series of chromatographic techniques. Structural elucidation of the yielded compounds was achieved using intensive studies of their NMR (1H, ^{13}C & 2D NMR) and mass (EI MS, ESI MS) spectrometry. Structures of the isolated compounds were assigned aslinoleic acid (1), R(-)-glycerol monolinoleate (2), ergosta-7,22-dien-3β,5α,6α-triol (3), aurasperone B (4), aurasperone C (5), aurasperon F (6), asperamide A (7) and cerebroside C (8). In this article, taxonomical characterization, fermentation, structural characterization of the obtained metabolites were reported together with their in vitro antimicrobial assay against Bacillus subtilis, Staphylococcus aureus, Streptomyces viridochromogenes, Escherichia coli, Candida albicans, Mucor miehi, Chlorella vulgaris, Chlorella sorokiniana, Scenedesmus subspicatus, Rhizoctonia solani and Pythium ultimum, and cytotoxic activities against brine shrimp.*

Introduction

In recent years, numerous metabolites possessing uncommon structures and potent bioactivity have been isolated from strains of bacteria and fungi collected from diverse environments, such as soils, animals, plants and sediments[1-3]. Therefore, several research groups and pharmaceutical companies were motivated to start sampling and screening large collections of fungal strains for antibiotics [4], antimycotics[5], antivirals [6], anticancers [7] and pharmacologically active agents [8]. Approximately, more than 30000 diseases are clinically described today, less than one-third of these can be treated symptomatically, and even a fewer can be cured. Therefore, there is vital need for new therapeutic agents with infectious disease control [9]. Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host[10,11]. Fungi have been widely investigated as a source of bioactive compounds[12,13]. An excellent example of this is the anticancer drug, taxol, which had been previously supposed to occur only in the plants[14-17]. They are among of the highly interesting productive microorganisms for novel metabolites, exhibiting a variety of biological activities against different diseases and are so rarely handled [18,19]. Hence, endophytic fungi might be helpful to treat some of the recently explored diseases[20-22].

During our continual search for bioactive secondary metabolites from microorganisms, extracts (supernatant and cells) of the endophytic fungal strain *Aspergillus niger* sp. isolate R8 exhibited high cytotoxicity against brine shrimps (100%), and high anti-algal activity, along with weak-moderate antibacterial and antifungal activities. Additionally, interest diverse chemical bands being of different compounds, some of them showed violet staining on spraying with anisaldehyde/sulphuric acid, and the others are of UV-greenish yellow fluorescence were observed during TLC. In accordance, the strain was applied to large scale fermentation using M_2 medium and shaking, which after working up and purification with the aid of numerous chromatographic means, afforded eight diverse compounds: linoleic acid (**1**), R(-)-glycerol monolinoleate (**2**), ergosta-7,22-dien-3,5,6-triol (**3**), aurasperone B (**4**), aurasperone C (**5**), aurasperon F (**6**), and asperamide A (**7**) and cerebroside C (**8**). In the present study, taxonomical characterizations of the strain together with the antimicrobial and cytotoxic activities, structural elucidation of the afforded compounds using NMR (^{1}H, ^{13}C& 2D NMR) and mass (EI MS, ESI MS) spectroscopy were discussed.



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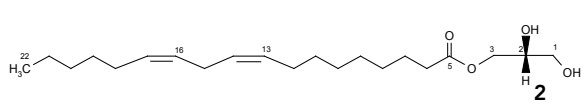

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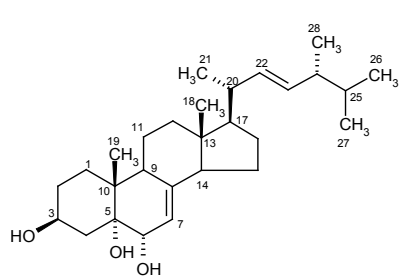

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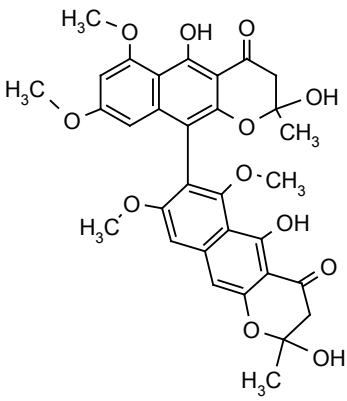

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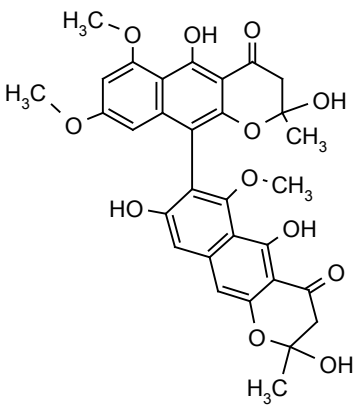

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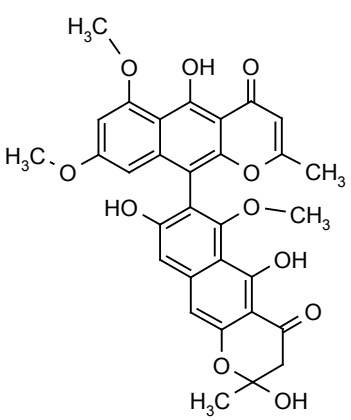

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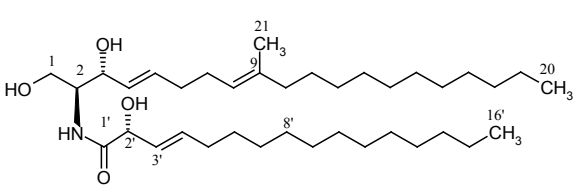

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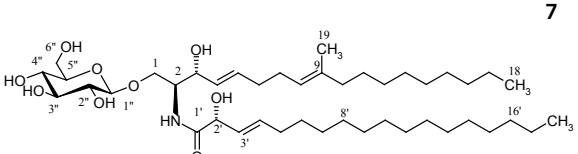

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2 Results and Discussion

2.1 Taxonomical Characterization and Pre-screening

The endophytic *Aspergillus niger* sp. R8 was isolated from the leaves of sweet potato; *Ipomoea batatas* using the reported methods of Petrini[23] and Khan[24]. *Aspergillus niger* grew rapidly on Czapek's agar at 25–30°C, showing good rate of growth even up to 45°C on CDA. Colonies were carbon black in color. It produced abundant submerged mycelia in the medium. According to microscopic studies of the fungus (Fig. 1), conidiophores were smooth with thick walls, unseptate, 200 -1000 µm long and 7-10 µ thick. They were uncolored near the vesicle. Conidial heads were fuscous black, globose, up to 300-500 µm in diameter. Vesicles were colorless and globose, thick-walled up to 35 µm in diameter. Conidial chains were present over the entire surface of vesicles. Conidia were rough, globose and 3-4 µm in diameter. Based on these typical features, the fungus isolate 8 has been identified as *Aspergillus niger* [25,26]

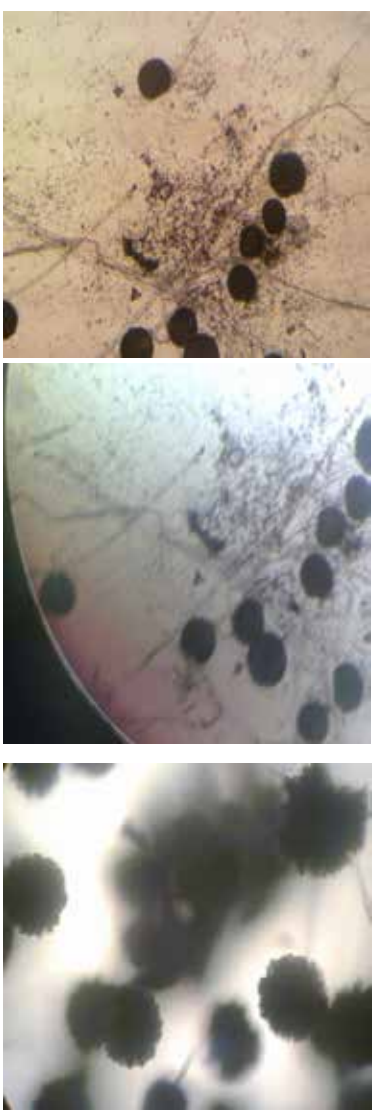

Figure1: Colonies and Microscopic characterization of the endophytic *Aspergillus niger* **sp. R8**

According to a carried out pre-screening, extracts (filtrate and cells) of the endophytic isolate *Aspergillus niger* R8 showed a weak-moderate antibacterial and antifungal activ-

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ity against a number of test microorganisms (Table 5). Moreover, the strain extracts showed high activity against microalgae, and potent cytotoxicity (100%) against brine shrimp. In alternative way, chemical screening of the extracts during TLC exhibited numerous bands of different polarities; some of them were UV inactive, which showed a violet-blue staining on spraying with anisaldehyde/sulphuric acid and heating. In addition, some middle polar yellow and as UV-green fluorescence bands were exhibited, which turned brown on exposing to conc. sulphuric acid, however, they are negatively affected by aqueous sodium hydroxide, as indicative of xanthone analogues (naphtho-γ-pyrone systems) [27].

2.2. Isolation and Structure Elucidation

Large scale fermentation of the fungus strain was carried out on Malt Extract (ME) medium showing yellow culture broth. After harvesting and working up, the afforded crude extract was applied to purification using a series of chromatographic techniques to deliver the desired bioactive secondary metabolites (**1**-**8**); their structural features are discussed below.

Linoleic acid

Compound **1** was obtained as low polar colourless oil, exhibiting UV non-absorbance on TLC, which was detected as violet-blue by anisaldehyde/sulphuric acid. Based on its chromatographic properties, study of its NMR (^{1}H&^{13}C) spectral data as well as molecular weight (280 Dalton) by EI mass spectrum and the corresponding molecular formula $C_{18}H_{32}O_2$, search in AntiBase[3] and comparison with authentic spectra, and literature[28,29], structure of **1** was deduced as linoleic acid. Conjugated linoleic acid (CLA) was reported to inhibit carcinogenesis and atherosclerosis, enhance immunologic function while protecting against the catabolic effects of immune stimulation, affect body composition (reducing body fat gain while enhancing lean body mass gain)[30]. In addition, linoleic acid and lenolenic acids and their methyl esters proved potent antimalarial activities[31].

R(-)-glycerol monolinoleate

Compound **2**, further colourless oil was obtained, showing similar chromatographic properties to **1**. The molecular weight of **2** was deduced as 354 Dalton, having the corresponding formula $C_{21}H_{38}O_4$ according to HRESIMS. Based on its chromatographic properties and the intensive study of its 1D (^{1}H&^{13}C) and 2D (HSQC, HMBC and H,H COSY) NMR data (Fig.2, Table 1), structure of **2** was definitely interpreted as R(-)-glycerol monolinoleate, which we have recently reported as well [3,29]. (R) and (S)-Glycerol-monolinoleate forms were reported to exhibited inhibitory activities with IC_{50} values of 45.0 and 52.0 μM, respectively, against lipoprotein-associated phospholipase A2 [Lp-PLA2]; the latter is a specific marker of vascular inflammation associated with atherosclerosis[32].

Table 1: ^{13}C and ^{1}H NMR (CD_3OD) spectral data for R(-)-glycerol monolinoleate (2)

No.	dc	δ_H (mult.; *J* in [Hz])	No.	dc	δ_H (mult.; *J* in [Hz])
1	63.1	3.62 (dd,3.6, 11.7), 3.51 (dd, 6.0, 11.6)	13	127.9	5.26 (m)
2	69.9	3.85 (m)	14	130.0	5.34 (m)
3	64.8	4.09 (d, 5.6)	15	25.3	2.72 (t, 5.8)
5	174.1	-	16	129.7	5.34 (m)
6	33.9	2.29 (t, 7.6)	17	127.6	5.26 (m)
7	24.7	1.57 (t,6.6)	18	27.0	2.00 (t, 6.7)
8	28.9	1.26-1.22 (m)	19	28.8	1.22 (m)
9	29.1	1.26-1.22 (m)	20	31.2	1.26-1.22 (m)
10	29.4	1.26-1.22 (m)	21	22.3	1.26-1.22 (m)
11	29.5	1.26-1.22 (m)	22	13.9	0.85 (t, 6.9)
12	26.9	2.00 (t, 6.7)			

Ergosta-7,22-dien-3β,5α,6α-triol

As middle polar colourless solid, compound **3** was obtained exhibiting a blue staining on spraying with anisaldehyde/sulphuric acid and heating which later turned to brown. This is an indicative to a steroidal nature of **3** [33,34]. The molecular weight of **1** was determined as 430 Dalton and the corresponding molecular formula as $C_{28}H_{46}O_3$ according to ESI and HR-ESI, mass spectrometry, respectively. This deduced the existence of six double equivalents. An intensive study of the 1D (^{1}H &^{13}C) and 2D (H,H COSY, HMQC and HMBC) NMR spectroscopy (Fig. 3 and Table 2) confirmed certainly the structure of **3** as ergosta-7,22-dien-3,5,6-triol; cerevisterol [35,36]. Compound **3** exhibited potent fish toxicity causing embryo notochord malformation of zebrafish with EC_{50} value of 7.83 μg·mL^{-1} after 72 hrs[37], while it did not show any activity (IC_{50} > 128 μg/mL) against KB cells growth[38].

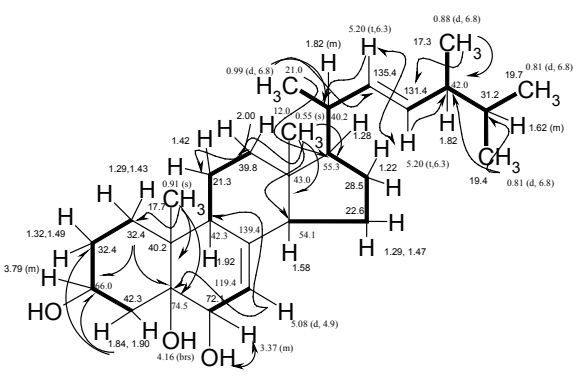

Figure 3: Selected HMBC (→) and H,H COSY (—) experiments of ergosta-7,22-dien-3,5,6-triol (3)

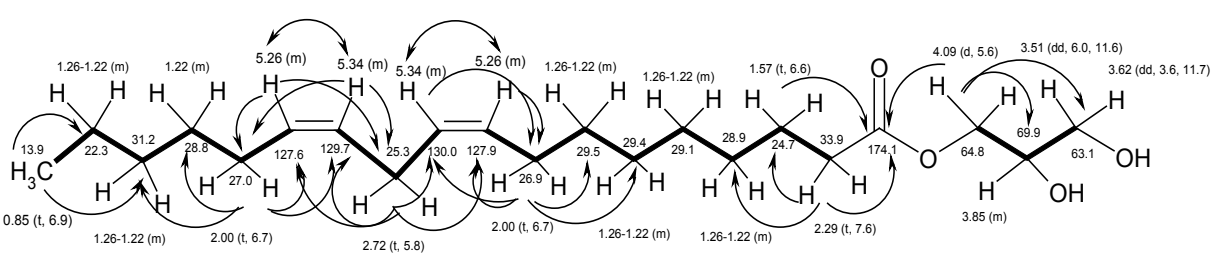

Figure 2: H,H COSY and HMBC connectivities of R(-)-glycerol monolinoleate (2)

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Table 2: ^{13}C and ^{1}H NMR (DMSO-d_6) spectral data for ergosta-7,22-dien-3,5,6-triol (3)

No.	dc	δ_H (mult.; *J* in [Hz])	No.	dc	δ_H (mult.; *J* in [Hz])
1	32.4	1.29 (m), 1.43 (m)	15	22.6	1.29 (m), 1.47 (m)
2	32.4	1.32 (m), 1.49 (m)	16	28.5	1.22 (m)
3	66.0	3.79 (m)	17	55.3	1.28 (m)
4	42.3	1.84 (m), 1.90 (m)	18	12.0	0.55 (s)
5	74.5	-	19	17.7	0.91 (s)
6	72.1	3.37 (m)	20	40.2	1.82 (m)
7	119.4	5.08 (d, 4.9)	21	21.0	0.99 (d, 6.8)
8	139.4	-	22	135.4	5.20 (t, 6.3)
9	42.3	1.92 (m)	23	131.4	5.20 (t, 6.3)
10	40.2	-	24	42.0	1.82 (m)
11	21.3	1.42 (m)	25	31.2	1.62 (m)
12	39.8	2.00 (m)	26	19.7	0.81 (d, 6.8)
13	43.0	-	27	19.4	0.81 (d, 6.8)
14	54.1	1.58 (m)	28	17.3	0.88 (d, 6.8)

Aurasperones B, C and F

Compound **4** is yellow solid, showing an UV yellowish green fluorescence with a molecular weight of 606 Da, corresponding to the molecular formula $C_{32}H_{30}O_{12}$ (HRESI MS). The ^{1}H NMR spectrum established **4** as a dimeric pattern of fonsecin B[39]. Further intensive study of the NMR data and comparison with our recently published data assigned **4** as aurasperone B[39].

As second dimeric xanthone, compound **5** was obtained exhibiting typical chromatographic properties of aurasperone B, with molecular weight of 592 Da and a corresponding molecular formula $C_{31}H_{28}O_{12}$. The ^{1}H NMR spectrum displayed the same pattern as in aurasperone B (**4**), except that the methoxy signal (δ 3.78) of 8-OCH_3 in **4** was replaced by a phenolic hydroxyl group, referring to aurasperone C (**5**), which has been recently published by us as well[39]. Compound **6**, a third dimeric xanthone, with a molecular formula $C_{31}H_{26}O_{11}$ was obtained; The ^{1}H NMR spectra displayed five sp^2 methines (δ 6.87-6.08), one higher than **4** and **5**, due to the replacement of one of the AB signals of methylene groups by an α-aromatic methine. Accordingly, one of the β-bounded methyls of the lactones was down-field shifted. As similar to **5**, three methoxy signals were visible, while the fourth one was replaced by a phenolic OH. Based on these spectroscopic features, structure **6** was deduced as aurasperone F [39,40].

Bis-naphtho-γ-pyrones (dimeric naphtho-γ-pyrones) represent a vital group of aromatic polyketides derived from fungi, exhibiting a variety of biological activities including cytotoxic, antitumor, antimicrobial, tyrosine kinase and HIV-1 integrase inhibition properties, demonstrating their potential applications in medicine and agriculture. Up to date, approximately 59 bis-naphtho-γ-pyrones from fungi have been reported in the past few decades[41-45] .

Asperamide A

As colourless solid, compound **7** was obtained, showing UV non-absorbance, while it was stained as violet on spraying with anisaldehyde/sulphuric acid and heating. The ESI mass spectrum deduced the molecular weight as 592 Dalton, with a corresponding molecular formula $C_{37}H_{69}NO_9$. The ^{1}H NMR spectrum showed signals for five olefinic protons (δ 5.81-5.08), two oxymethines (δ 4.55: H-2′ and 4.28: H-3), however, it is missing the existence of a sugar, pointing to an aglycone characteristic of 7. Two multiplets of an oxymethylene group (δ 3.94, H_2-1) and amide-attached methine signal (δ 3.72, H-2) were visible. In the region of δ 1.96 –2.04, broad multiplet signals of four methylene groups, in addition to a further broad signal of 19 CH_2 (δ 1.26 -1.31), were remarked. Two triplet methyl signals (δ 0.89: CH_3-18, 16′) and one singlet 3H (δ 1.58: CH_3-9) of an olefinic-attached methyl group, were observed. The ^{13}C NMR spectrum of **8** (Table 8) showed 37 carbon signals, among them five olefinic methines (δ 133.9~123.1) and two quaternary carbons (δ 136.1, 173.9), the last of them (δ 173.9) is characteristic for an amide/ acid carbonyl. Three sp^3 oxygenated carbons, implying two methines and one methylene, at δ 73.6, 73.1, 61.7, repectively. Additionally, twenty-three methylenes (δ 39.7-22.8), one methine (δ 54.6), and three methyl carbon signals (δ 16.0~14.1) were visible. Based on the revealed spectral data and search in AntiBase and comparison with corresponding literature [46], structure **7** was established as asperamide A.

Asperamides A (**7**) was reported previosly along with and Asperamides B, representing sphingolipids, were characterized previously from the culture extract of *Aspergillus niger* EN-13, an endophytic fungus isolated from marine brown alga *Colpomenia sinuosa*[46]. In the antifungal assay, asperamide A (**7**) was reported to display moderate activity against *Candida albicans*, which has been confimed in our reported work.

Table 3: ^{13}C and ^{1}H NMR shifts (125, 300 MHz) of asperamide A (7) in $CDCl_3$

No.	δ_C	δ_H (mult.; *J* in [Hz])	No.	δ_C	δ_H (mult.; *J* in [Hz])
1	61.7	3.94 (dd, 11.2, 3.4), 3.72 (dd, 11.2, 3.2)	3′	126.8	5.53 (dd, 15.3, 7.2)
2	54.4	3.88 (m)	4′	135.3	5.89 (dd, 15.3, 7.2)
3	73.6	4.28 (dd, 7.7, 3.9)	5′	32.3	2.04 (m)
4	128.5	5.55 (dd, 15.2, 6.3)	6′	28.9	1.38 (m)
5	133.9	5.81 (dd, 15.2, 6.3)	7′	29.72	1.31 – 1.26 (brs)
6	32.6	2.08 (m)	8′	29.71	1.31 – 1.26 (brs)
7	27.6	2.07 (m)	9′	29.7	1.31 – 1.26 (brs)
8	123.1	5.08 (t, 6.6)	10′	29.6	1.31 – 1.26 (brs)
9	136.1	-	11′	29.5	1.31 – 1.26 (brs)
10	39.7	1.96 (m)	12′	29.4	1.31 – 1.26 (brs)
11	28.1	1.36 (m)	13′	29.3	1.31 – 1.26 (brs)
12	29.7	1.31 – 1.26 (brs)	14′	31.9	1.31 – 1.26 (brs)
13	29.6	1.31 – 1.26 (brs)	15′	22.7	1.31 – 1.26 (brs)
14	29.5	1.31 – 1.26 (brs)	16′	14.1	0.89 (t, 6.9)
15	29.4	1.31 – 1.26 (brs)			
16	29.3	1.31 – 1.26 (brs)			
17	29.0	1.31 – 1.26 (brs)			
18	31.9	1.31-1.26 (brs)			
19	22.8	1.31-1.26 (brs)			
20	14.1	0.89 (t, 6.9)			
CH_3-9	16.0	1.58 (s)			
1′	173.9	-			
2′	73.1	4.55 (d, 7.2)			

Cerebroside C

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As colourless solid, compound **8** was obtained, showing high similarity in the chromatographic properties to those of **7**, however, with higher polarity. The ESI mass spectrum deduced the molecular weight as 753 Dalton, with a corresponding molecular formula $C_{43}H_{79}NO_9$, having two CH_2 groups more than those of our recently reported cerebroside A. The ^{1}H NMR spectrum showed a strongly similar pattern to those cerebroside A. In accordance, five olefinic proton signals (δ 5.82-5.16), two oxymethines (δ 4.45: H-2′ and 4.13: H-3) along with a typical anomeric proton (δ 4.29: H-1′′, *J*~7.6 Hz), revealing the presence of a sugar moiety. Further five oxygenated proton signals (δ 3.89-3.22: H-2′′–H-6′′) were exhibited, confirming the sugar unit. Two multiplets of an oxymethylene group (δ 4.15, H_2-1) and amide-attached methine signal (δ 3.96, H-2) were visible. In the region of δ 1.98 –2.11, broad multiplet signals of four methylene groups attached to sp^2 carbons of olefinic systems, in addition to a further broad signal of 19 CH_2 (δ 1.26 -1.30), were remarked. Two triplet methyl signals (δ 0.91: CH_3-18, 18′) and one singlet 3H (δ 1.61: CH_3-9) of an olefinic-attached methyl group, were observed. The ^{13}C NMR spectrum of **8** (Table 8) showed 43 carbon signals, among them five olefinic methines (δ 134.6~124.7) and two quaternary carbons (δ 136.7, 175.4), the last of them (δ 175.4) is characteristic for an amide/acid carbonyl. Seven sp^3 oxygenated carbons, implying six methines and one methylene, were visible between δ 77.9-62.5, together with an anomeric carbon of a sugar unit (δ 104.6). Additionally, twenty-three methylenes (δ 40.8-23.8), one methine (δ 54.5), and three methyl carbon signals (δ 16.2~14.5) were visible. Based on the revealed spectral data and search in AntiBase and comparison with corresponding literature [46,47], structure **8** was established as cerebroside C.

Cerebrosides; glycosphingolipids, were reported in several phytopathogens as elicitors that induce the disease resistance in e.g. rice plants [48]. Sphingolipids are ubiquitous components of the membranes of all eukaryotic cells and are particularly abundant in plasma membranes[47]. In animals, they play important roles in general membrane function, cell-to-cell contact, cell recognition, and regulation of cell growth, differentiation, and apoptosis[47]. Sphingolipids modulate transmembrane signal transduction via their effects on protein kinases associated with growth factor receptors and on protein kinase C, thereby regulating cell proliferation and inducing cell differentiation and apoptosis[47]. In fungi, sphingolipids are known to function as inducers of cell differentiation[47]. Cerebrosides are know of thier antifungal activities at low concentrations[49,50]. They are characterized bythier moderate antibacterial activities against Gram negative species, however, they reported weak[50] or no activities[49] agianst Gram positives species.

Table 4: ^{13}C and ^{1}H NMR shifts (125, 300 MHz) of cerebroside C (8) in CD_3OD

No.	δ_C	δ_H (mult.; *J* in [Hz])	No.	δ_C	δ_H (mult.; *J* in [Hz])
1	69.6	3.72 (dd, 10.5, 5.4), 4.15 (dd, 10.5, 3.2)	4′	134.6	5.82 (dt, 15.3, 7.7)
2	54.5	3.96 (m)	5′	33.4	2.03 (m)
3	72.8	4.13 (dd, 7.2, 5.3)	6′	30.2	1.42 (m)
4	131.0	5.44 (dd, 15.4, 7.3)	7′	30.66	1.26 – 1.36 (brs)
5	134.4	5.72 (dd, 15.3, 6.5)	8′	30.61	1.26 – 1.36 (brs)
6	33.7	2.11 (m)	9′	30.59	1.26 – 1.36 (brs)
7	28.6	2.08 (m)	10′	30.4	1.26 – 1.36 (brs)
8	124.7	5.16 (t, 6.7)	11′	30.30	1.26 – 1.36 (brs)
9	136.7	-	12′	30.28	1.26 – 1.36 (brs)
10	40.8	1.98 (m)	13′	30.20	1.26 – 1.36 (brs)
11	28.9	1.36 (m)	14′	30.28	1.36 – 1.30 (brs)
12	30.8	1.36 – 1.30 (brs)	15′	30.20	1.36 – 1.30 (brs)
13	30.7	1.36 – 1.30 (brs)	16′	32.8	1.36 – 1.30 (brs)
14	30.6	1.36 – 1.30 (brs)	17′	23.5	1.36 – 1.30(brs)
15	30.5	1.36 – 1.30 (brs)	18′	14.6	0.91 (t, 6.9)
16	30.4	1.36 – 1.30 (brs)	1′′	104.6	4.29 (d, 7.6)
17	23.8	1.36 – 1.30 (brs)	2′′	74.9	3.22 (dd, 9.2, 7.6)
18	14.5	0.91 (t, 6.9)	3′′	77.9	3.36 (dd, 9.2)
CH_3-9	16.2	1.61 (s)	4′′	71.5	3.30 (m)
1′	175.4	-	5′′	77.8	3.30 (m)
2′	74.1	4.45 (d, 5.8)	6′′	62.5	3.89 (dd, 11.1, 1.2), 3.71 (dd, 11.1, 4.3)
3′	129.0	5.50 (dd, 15.3, 6.0)			

2.3 Biological Activities

Diverse antimicrobial activity testing for the crude extract of endophytic fungi *Aspergillus niger* sp. isolate R8 was carried out in comparison (40 mg/disc) with the whole isolated compounds (**1-8**) (10 mg/disc) against eleven microbial tests on the bases of agar diffusion method (Table 5). The crude extract showed high cytotoxic activity (100%) and moderate to high anti-microalgal activity (12-18 mm) against *Chlorella sorokiniana, Scenedesmus subspicatus,* and *Rhizoctonia solani*. In contrast, compounds **1**-**6** showed no antibacterial or antifungal activities against the tested microorganisms; while asperamide A (**7**) and cerebroside C (**8**), exhibited weak-moderate antibacterial activities (11-17 mm), such that cerebroside (**8**) exhibited higher activity than those of the non-glycosylated asperamide A (**7**). Furthermore, both compounds 7,8 displayed moderate -high activities against *Candida albicans* (13-15) and *Mucor miehi* (16-18), and the microalgae *Chlorella vulgaris* (13-14), *Chlorella sorokiniana* (12-14), and *Scenedesmus subspicatus* (13-15). Cytotoxic examination of the isolated compounds (**1-8**) against the brine shrimp, confirmed their activities to be ranged between moderate (28-35%) and weak (9-11%) (Table 5).

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Table 5: Antimicrobial (40 µg/disc (∅ 9 mm; [mm]) and cytotoxic (10 µg /ml) activities of compounds 1-8.

Compouds	Diameter of inhibition (mm)											Brine Shrimp
	BS[a]	SA[b]	SV[c]	EC[d]	CA[e]	MM[f]	CV[g]	CS[h]	SS[i]	PS[j]	PU[k]	(%)
Crude extract filtrate	11	10	11	10	12	11	18	13	14	00	00	100
Crude extract cells	10	00	00	00	00	00	17	12	13	00	00	100
Linoleic acid **(1)**	00	00	00	00	00	00	00	00	00	00	00	11
R(-)-glycerol monolinoleate **(2)**	00	00	00	00	00	00	00	00	00	00	00	11
Ergosta-7,22-dien-3,5,6-triol **(3)**	00	00	00	00	00	00	00	00	00	00	00	10
Aurasperone B **(4)**	00	00	00	00	00	00	00	00	00	00	00	7
Aurasperone C **(5)**	00	00	00	00	00	00	00	00	00	00	00	10
Aurasperon F **6**	00	00	00	00	00	00	00	00	00	00	00	9
Asperamide A **(7)**	11	11	12	15	13	16	13	12	13	00	00	28
Cerebroside C **(8)**	13	14	15	17	15	18	14	14	15	00	00	35

[a]*Bacillus subtilis*, [b]*Staphylococcus aureus*, [c]*Streptomyces viridochromogenes* (Tü 57), [d]*Escherichia coli*, [e]*Candida albican*, [f]*Mucor miehi*, [g]*Chlorella vulgaris*, [h]*Chlorella sorokiniana*, [i]*Scenedesmus subspicatus*, [j]*Rhizoctonia solani*; [k]*Pythium ultimum*

3. Experimental

The NMR spectra were measured on Varian Unity 300 (300.145 MHz) and Varian Inova 600 (150.820 MHz) spectrometers. ESI MS was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). EI mass spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV). Flash chromatography was carried out on silica gel (230-400 mesh). R_f values were measured on Polygram SIL G/UV_{254} TLC cards (Macherey-Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany). All chemicals served in the biological study were of analytical grade, which were purchased from Sigma, Merck and Aldrich.

3.1. Endophytic fungus *Aspergillus niger* R8

3.1.1. Plant Asecertained as a Source of the Endophyte

Red leaves of Seat Potato were collected from from the herbarium of National Research Centre for Agriculture, Cairo, during October 2009. Immediately after the collection, plant parts were washed with tap water and processed for isolation of endophytic fungi. To improve the sensitivity and specificity of routine culture approach for identification of *Aspergilli* in the level of species, we used four differential media including (CZA), (CZYA) and (MEA).

3.1.2 Media preparation

Potato dextrose agar (PDA) medium (potato infusion 200 g, glucose 20 g, agar 20 g distilled water 1 liter) was used for isolation and purification of endophytic fungi. Antibiotic, ampicilin and streptomycin 200 µg/L of the medium was added to the media to avoid bacterial contamination. Czapek agar (CZA) medium (sucrose 30 g, K_2HPO_4 1 g, agar 25 g, distilled water 1 liter), Czapek yeast agar (CZYA) medium (Sucrose 30 g, K_2HPO_4 1.0 g, yeast extract 5 g, agar 25 g and distilled water 1 liter) and Malt extract agar (MEA) medium (glucose 20 g, malt extract 20 g, peptone 10 g, agar 25 g and distilled water 1 liter). Malt extract agar medium (malt extract 15 g; agar 15 g; pH: 7.5) was used for small scale multiplication of endophytic fungi, being used for extracting metabolite.

3.1.3 Isolation and Taxonomy of the Endophytic Fungi

The endophytic fungi *Aspergillus niger* sp. R8 was isolated from the red leaves of sweet potato; *Ipomoea batatas*, collected from the herbarium of National Research Centre for Agriculture, Cairo. Leaves of sweat potato were cut into small segments and surface-sterilized by sequential washes in 95% ethanol (30 s), 5% sodium hypochlorite (5 min) 95% ethanol (30 s) and rinsed with sterile water. The strain was cultivated on PDA medium, Antibiotic, ampicillin and streptomycin 200 µg/L of the medium was added to the media to avoid bacterial contamination [51]. Plates were incubated at 28°C for 1 week. Furthermore, the endophytic nature of the isolated strain was checked daily until within 21 growing days. Individual fungal colonies were transferred onto other plates with PDA. Fungal spore formation was encouraged by placing the endophytes onto autoclaved carnation leaves. The plates were continuously monitored for spore formation by stereo and light microscopy. Identification of the endophytic *Aspergillus* species during our investigation was carried out using the morphological characteristics and microscopic features were examined by optical light microscope (10 ×90) Olympus CH_{40} according to Raper & Fennell (1965)[52] and Klich (2002)[53].

Fermentation and working up

Small pieces (1 cm^{-1}) of well grown subcultures of endophytic *Aspergillus niger* sp. isolate R8 were inoculated into hundred 1 L Erlenmeyer flasks, each containing 250 mL of sterilized ME broth medium. The inoculated flasks were incubated for 7 days at 28 °C. After harvesting, the resulting yellow culture broth was mixed with ca. 1 kg diatomaceous earth (Celite) and filtered during a filter press. The filtrate was extracted using XAD-16 resin followed by elution with MeOH/H_2O, and collected aqueous methanolic extract was concentrated *in-vacuo.* The remaining water residue was then extracted with ethyl acetate. The mycelium cake was first extracted with ethyl acetate (3×), and then by acetone (3×). The acetone extract was evaporated *in-vauo*, and the residual aqueous solution was re-extracted by ethyl acetate. According to TLC monitoring, ethyl acetate extracts of mycelium and supernatant showed high similarity and were combined and followed by concentration *in-vacuo* to afford 8.3 g as greenish-brown crude extract.

Isolation

The crude extract (7.4 g) was applied to column chromatography on silica gel (40 × 10 cm) and eluted with cyclohexane-CH_2Cl_2-MeOH gradient. According to TLC, four fractions were obtained; FI (1.4 g), FII (2.8 g), FIII (1.9 g) and FIV (0.7 g). An application of the fast fraction to a further silica gel column (2 × 60 cm) followed by Sephadex LH-20 (DCM/40% MeOH) delivered two colourless oils of linoleic acid (**1**, 15 mg) and R(-)-glycerol monolinoleate (**2**, 50 mg). FII was purified by PTLC chromatogram (20 × 40 cm, DCM/7% MeOH, double elution) and then by Sephadex LH-20 (MeOH) yielding a colourless solid of ergosta-7,22-dien-3,5,6-triol (**3**, 16 mg) along with three yellow solids of Aurasperones B (**4**, 8 mg), C (**5**, 10 mg) and F (**6**, 11 mg). A combination of fractions FIII and FIV and purification using PTLC chromatogram (20 × 20 cm, DCM/15%

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MeOH) and Sephadex LH-20 (MeOH) gave two colourless solids of asperamide A (**7**, 18 mg) cerebroside C (**8**, 17 mg).

Linoleic acid; (9Z,12Z)-9, 12-octadecanoic acid (1)
$C_{18}H_{32}O_2$ (280.45), UV non absorbing colourless oil, which turned blue by anisaldehyde/sulphuric acid and heating. – R_f = 0.88 (CH_2Cl_2/MeOH, 10%). – ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.97 (s, br, 1 H, COOH), 5.42-5.27 (m, 4 H, 9,10,12,13-CH), 2.77 (t, *J* = 6.0 Hz, 2 H, 11-CH_2), 2.37 (t, *J* = 7.2 Hz, 2 H, 2-CH_2), 2.07 (m, 4 H, 8,14-CH_2), 1.62 (m, 2 H, 3-CH_2), 1.41-1.22 (m, 14 H, 4,5,6,7,16,17-CH_2), 0.84 (m, 3 H, 18-CH_3). – ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 180.0 (CO, C_q), 130.0 (CH-13), 129.8 (CH-9), 127.9 (CH-10), 127.7 (CH-12), 31.4 (CH_2-2), 29.5 (CH_2-16), 29.44 (CH_2-11), 29.42 (CH_2-14), 29.3 (CH_2-8), 29.1 (CH_2-7), 29.0 (CH_2-6), 29.0 (CH_2-5), 27.0 (CH_2-4), 25.5 (CH_2-3), 24.6 (CH_2-15), 22.4 (CH_2-17), 14.0 (CH_2-18). – EI MS (70 eV): *m/z* (%) = 280.4 (77), 264 (27), 137 (8), 124 (14), 110 (25), 95 (55), 81 (82), 67 (100), 55 (91), 41 (90).

R(-)-glycerol monolinoleate (2)
$C_{21}H_{38}O_4$ (354) Colourless oil, UV absorbance, turned violet with anisaldehyde/sulphuric acid. – R_f = 0.21 (CH_2Cl_2/5% CH_3OH). – ^{1}H NMR (CD_3OD, 300 MHz). -^{13}C NMR (CD_3OD, 75 MHz) see Table 1. – (+)-ESIMS: *m/z* (%) = 731 $[M+Na]^+$, 100), 377 $[M+Na]^+$, 42). – (+)-HRESIMS: *m/z* = 377.1151 (calcd. 377. for $C_{21}H_{38}NaO_4$).

Ergosta-7,22-dien-3β,5α,6α-triol (3)

$C_{28}H_{46}O_3$ (430), UV non absorbing colourless solid, which turned blue by anisaldehyde/sulphuric acid and heating and changed later to brown. –R_f = 0.13 (CH_2Cl_2/MeOH, 5%), 0.36 (CH_2Cl_2/MeOH, 7%). $[a]^{20}_D$(MeOH) -12°C (c =0.1). – ^{1}H NMR (DMSO-d_6, 300 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) are listed in Table 2. –(+) ESI MS: *m/z* (%) = 453 ($[M+Na]^+$, 28), 883 ($2M+Na]^+$, 11). (+)-HRESI-MS: *m/z* = 453.3339 [M+Na] (calcd. 453.3339 for $C_{28}H_{46}O_3Na$ [M+Na]), 883.6785 [2M+Na] (calcd. for 883.6785 for $C_{56}H_{92}O_6Na$ [2M+Na]).

Aurasperone B (4)
Yellow solid, UV-green fluorescence (365 nm), turned orange with anisaldehyde/sulphuric acid; R_f = 0.47 (CH_2Cl_2/5% MeOH). - ^{1}H NMR (300 MHz, $CDCl_3$): δ = 14.51 (brs, 1H, 5'-OH), 14.08 (brs, 1H, 5-OH), 6.84 (s, 1H, H-9), 6.72 (s, 1H, H-9), 6.37 (d, 1H, *J* = 1.1 Hz, H-7'), 6.14 (d, 1H, *J* = 1.1Hz, H-9'), 3.99 (s, 3H, 6'-OCH_3), 3.78 (s, 3H, 8-OCH_3), 3.63 (s, 3H, 8'-OCH_3), 3.39 (s, 3H, 6-OCH_3), 3.02, (d, 2H, *J* = 16.3 Hz, 3-H_2), 2.89 (m, 2H, 3'-H_2), 1.79 (s, 3H, 2-CH_3), 1.46 (s, 3H, 2'-CH_3). -EI MS m/z (%) = 570.3 ($[M-2H_2O]^+$, 100), 539.4 ($[M-(2H_2O+OCH_3)]^+$, 74), 524.3 (5), 299.2 (12), 272.2 (13), 269.7 (24), 230.2 (18), 193.1 (12), 154.2 (14), 149.1 (19), 130.1 (48), 91.1 (54), 57.1 (30), 43.1 (57). - (+)-HRESI MS m/z 607.18100 ([M +H]+, calcd: 607.18100 for $C_{32}H_{31}O_{12}$), m/z 629.16294 ($[M+Na]^+$, calcd: 629.16295 for $C_{32}H_{30}O_{12}Na$).

Aurasperone C (5)
Yellow solid, UV-green fluorescence (365 nm), turned orange with anisaldehyde/sulphuric acid; R_f = 0.25 (CH_2Cl_2/5% MeOH). - ^{1}H NMR (300 MHz, CD_3OD): δ = 6.84 (s, 1H, H-10), 6.57 (s, 1H, H-9), 6.38 (d, 1H, *J* = 1.2 Hz, H-9'), 6.20 (d, 1H, *J* = 1.2 Hz, H-7'), 3.93 (s, 3H, 6'-OCH_3), 3.60 (s, 3H, 8'-OCH_3), 3.50 (s, 3H, 6-OCH_3), 3.30-3.29 (m, 4H, 3,3'-H_2), 1.69 (s, 3H, 2-CH_3), 1.49 (s, 3H, 2'-CH_3); EI MS m/z (%) = 574.3 ($[M-H_2O]^+$, 6), 556.3 ($[M-2H_2O]^+$, 42), 525.3 ($[M-(2H_2O+OCH_3)]^+$, 32), 264.2 (7) 58.2 (28), 43.1 (100). -(+)-HRESI MS m/z 615.14779 ($[M+Na]^+$, calcd: 615.14729 for $C_{31}H_{28}O_{12}Na$), m/z 593.16570 ($[M+H]^+$, calcd: 593.16534 for $C_{31}H_{29}O_{12}$).

Aurasperone F (6)
Yellow solid, UV-green fluorescence (365 nm), turned orange with anisaldehyde/sulphuric acid; R_f = 0.54 (CH_2Cl_2/5% MeOH). -^{1}H NMR (300 MHz, CD_3OD): δ = 6.87 (s, 1H, H-10), 6.55 (s, 1H, H-9), 6.51 (d, 1H, *J* = 1.1Hz, H-9'), 6.36 (brd, 1H, *J* = 1.1 Hz, H-7'), 6.08 (s, 1H, H-3'), 3.95 (s, 3H, 6'-OCH_3), 3.63 (s, 3H, 8'-OCH_3), 3.43 (s, 3H, 6-OCH_3), 3.35-3.25, (m, 2H, 3-H_2), 2.16 (s, 3H, 2'-CH_3), 1.65 (s, 3H, 2-CH_3). - EI MS m/z (%) = 556.5 ($[M-H_2O]^+$, 5), 286.3 (8), 84.1 (12), 57.2 (10), 44.1 (100). -(+)-ESI MS *m/z* (%) = 1172 ($[2M+Na+H]^+$, 19), 575 ($[M+H]^+$, 100). - (-)-ESI MS *m/z* (%) = 1721 ($[3M-H]^-$, 31), 1147 ($[2M-H]^-$, 22), 573 ($[MH]^-$, 100).

Asperamide A (7)
$C_{37}H_{69}NO_4$ (591.4) White solid, UV non-absorbing, violet colour by anisaldehyde/sulphuric acid and heating. – R_f = 0.37 (CH_2Cl_2/10% MeOH). – ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 3. – (+)-ESIMS: *m/z* (%) = 614 ($[M + Na]^+$, 100), 1182 ($[2 M + Na]^+$, 7). – (+)-HRESI MS *m/z* 592.52991 $[M + H]^+$ (calcd. 592. 529915 for $C_{37}H_{70}NO_4$).

Cerebroside C (8)
$C_{43}H_{79}NO_9$ (753.4) White solid, UV non-absorbing, violet colour by anisaldehyde/sulphuric acid and heating. – R_f = 0.15 (CH_2Cl_2/10% MeOH). – ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 4. – (+)-ESIMS: *m/z* (%) = 776 ($[M + Na]^+$, 100), 1529 ($[2 M + Na]^+$, 9). – (+)-HRESI MS *m/z* 754.58273 $[M + H]^+$ (calcd. 754.582735 for $C_{43}H_{80}NO_9$).

3.2 Biological Activity
3.2.1 Antimicrobial activity
Antimicrobial assays were conducted utilizing the disc-agar method (Burkholder et al., 1960) against diverse sets of microorganisms include Bacillus subtilis, Staphylococcus aureus, Streptomyces viridochromogenes (Tü 57), Escherichia coli, Candida albicans, Mucor miehi, Chlorella vulgaris, Chlorella sorokiniana, Scenedesmus subspicatus, Rhizoctonia solani and Pythium ultimum. Both bacterial and algal strains were grown on nutrient agar medium, while, the fungal strains were grown on (CZA) medium. The fungal extract and pure compounds (1-8) were dissolved in CH2Cl2/10% MeOH at a concentration of 1 mg/mL. Aliquots of 40 µL were soaked on filter paper discs (9 mm , no. 2668, Schleicher & Schüll, Germany) and dried for 1 h at room temperature under sterilized conditions. The paper discs were placed on inoculated agar plats and incubated for 24 h at 38 °C for bacteria and 48 h (30°C) for the fungi , while the algae test strains were incubated at ~ 22°C in day light for 8~10 days.

3.2.2 Cytotoxicity
The cytotoxic assay was performed according to Takahashi et al method (Takahashi et al, 1989)[54] and Sajid *et al*. screening (Sajid et al., 2009)[55].

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