BIOACTIVE SECONDARY METABOLITES FROM ENDOPHYTIC *ASPERGILLUS FUMIGATUS*: STRUCTURAL ELUCIDATION AND BIOACTIVITY STUDIES

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ABSTRACT

During the research for bioactive secondary metabolites from microorganisms, the endophytic fungi *Aspergillus fumigatus* sp. isolate R7 was found to produce a set of promising bioactive compounds (**1-10**) after its large scale fermentation, working up and purification using a series of chromatographic techniques. Structural elucidation of the yielded compounds using intensive studies of their NMR (^{1}H, ^{13}C& 2D NMR) and mass (EI MS, ESI MS) spectrometry confirmed them as linoleic acid (**1**), R(-)-glycerol monolinoleate (**2**), bis-dethio-(bis-methyl-thio)-gliotoxin (**3**), fumiquinazoline-F (**4**), fumiquinazoline-D (**5**), (Z,Z)-N,N'-[1-[(4-Hydroxy-phenyl)-methylene]-2-[(4-methoxy-phenyl)-methylene]-1,2-ethanediyl]-bis-formamide (**6**), pyrazoline-3-one trimer (**7**), Tricho-9-ene-2α,3α,11α,16-tetraol (**8**), 2'-deoxy-thymidine (**9**), and cerebroside A (**10**). In this article, taxonomical characterization, fermentation, structural characterization of the obtained metabolites were reported together with their antimicrobial and cytotoxic activities. *www.relaquim.com*

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

RESUMEN

En la investigación de metabolitos secundarios bioactivos de microorganismos, después de la fermentación a gran escala, el procesamiento y la purificación, utilizando una serie de técnicas cromatográficas, del hongo endofito Aspergilius fimigatus sp. aislado R7 se encontró la producción de una serie de compuestos bioactivos prometedores (1-10). La elucidación estructural de los compuestos producidos, utilizando estudios de RMN (^{1}H, ^{13}C y 2D RMN) y espectrometría de masas (EM IE, EM ESI) permitió identificarlos como ácido linoléico (**1**), R(-)-monolinoleato de glycerol (**2**), bis-destio-(bis-metil-tio)-gliotoxina (**3**), fumiquinazolina-F (**4**), fumiquinazolina-D (**5**), (Z,Z)-N,N'-[1-[(4-Hidroxifenil**)-**metilen]-2-[(4-metoxi-fenil)-metilen]-1,2-etanediil]-bis-formamida (**6**), trimero de pyrazolin-3-ona (**7**), Trico-9-en-2α,3α,11α,16-tetraol (**8**), 2'-desoxi-timidina (**9**), and cerebrosida A (**10**). En

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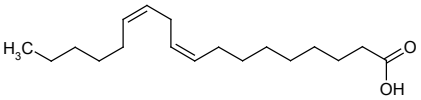
este artículo se reporta la caracterización taxonómica, fermentación, caracterización estructural de los metabolitos obtenidos junto con su actividad antimicrobiana y citotóxica. *www.relaquim.com*

Palabras clave: Hongos endófitos; Taxonomía; Metabolitos secundarios; Elucidación estructural; Bioactividad.

1 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 (Faulkner, 2000; Laatsch, 2006; Laatsch, 2010). Therefore, many pharmaceutical companies and research groups were motivated to start sampling and screening large collections of fungal strains for antibiotics (Butler, 2004), antimycotics (Li & Strobel, 2001), antivirals (Singh *et al.*, 2003), anticancers (Zhang *et al.*, 2006) and pharmacologically active agents (Song *et al.*, 2004). Even though 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. The increasing occurrence of multiresistant pathogenic strains has limited the effect of traditional antimicrobial treatment. Hence, there is an urgent need for new therapeutic agents with infectious disease control (Larsen *et al.*, 2005). Endophytic fungi were originally defined as all fungi that live asymptomatically within living plant tissues (Saikkonen *et al.*, 1998). They were generally considered as commensalistic symbionts, receiving nutrients and habitat from their hosts, which mostly provided the host with chemical protection from insects and browsers (Stierle *et al.*, 2000). In the past ten years, the biology of endophytic fungi in aerial plant tissues has become an important area for study, however, the chemistry of these organisms is only beginning to be explored (Stierle *et al.*, 2000). There is growing evidence that bioactive substances produced by microbial endophytes may not only be involved in the host-endophyte relationship, but may also ultimately have applicability in medicine, agriculture and industry (Strobel, 2002). According to recent reported literatures, endophytic fungi were confirmed as unusual productive sources of bioactive metabolites (Hassan, 2007; Chen, 2011; Shiono, 2011), which might be helpful to treat some of the recently explored diseases.

During our continual program for searching of bioactive secondary metabolites from microorganisms, the endophytic fungal strain *Aspergillus fumigatus* sp. isolate R7 exhibited high antimicrobial activity and interest chemical bands being of diverse compounds, which mostly turned between orange-violet on spraying with anisaldehyde/sulphuric acid during TLC. Therefore, the strain was applied to large scale fermentation (using M_2 medium on shaker), working up and purification by numerous chromatographic means (see experimental part) affording ten diverse compounds, namely, linoleic acid (**1**), R(-)-glycerol monolinoleate (**2**), bis-dethio-(bis-methyl-thio)-gliotoxin; FR-49175 (**3**), fumiquinazoline-F (**4**), fumiquinazoline-D (**5**), (Z,Z)-N,N'-[1-[(4-Hydroxyphenyl)-methylene]-2-[(4-methoxy-phenyl)-methylene]-1,2-ethanediyl]-bis-formamide (**6**), pyrazoline-3-one trimer (**7**), Tricho-9-ene-2α,3α,11α,16-tetraol (**8**), 2'-deoxy-thymidine (**9**), and cerebroside A (**10**). In the present study, taxonomical characterization of the strain together with the antimicrobial and cytotoxic activities, structural elucidation of yielded secondary metabolites with the aid NMR (^{1}H, ^{13}C& 2D NMR) and mass (EI MS, ESI MS) were discussed.



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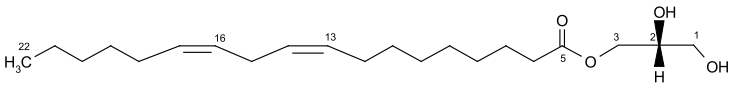

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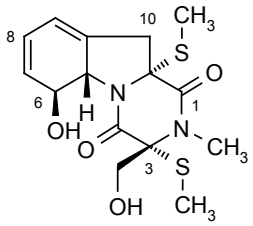

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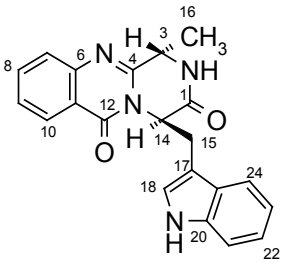

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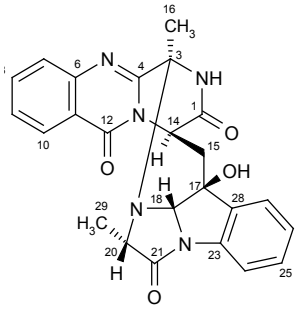

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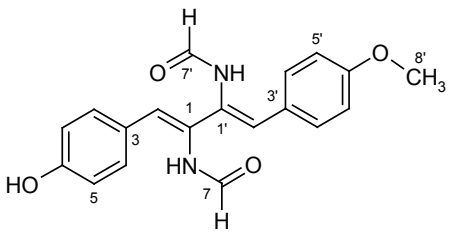

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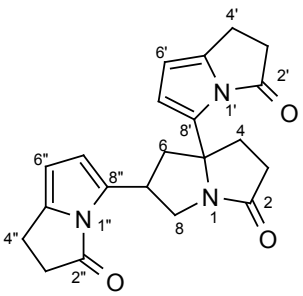

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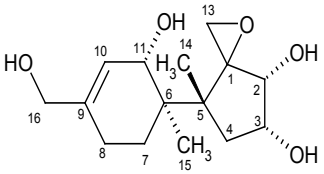

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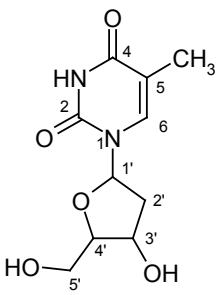

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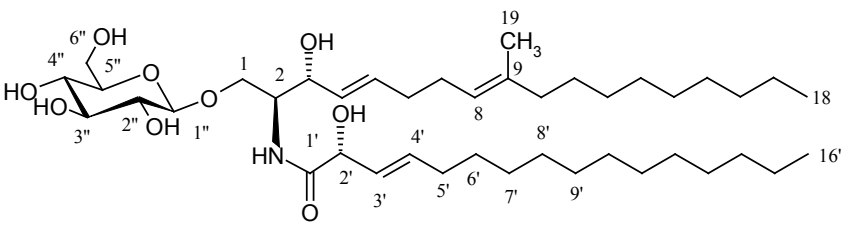

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2 RESULTS AND DISCUSSION

2.1 TAXONOMICAL CHARACTERIZATION AND PRE-SCREENING

The endophytic *Aspergillus fumigatus* sp. R7 was isolated from the leaves of sweat potato; *Ipomoea batatas* using the reported methods of Petrini (Petrini, 1986) and Khan (Khan *et al.*, 2007). The fungus was shown to produce surficial and submerged hyphae on potato dextrose agar (PDA) medium. The growth was being most prominent on czapek-dox agar (CDA) at 25–30°C, showing good rate of growth even up to 45°C on CDA. The colony on CDA is typically bluish-green. The mycelium is colourless and inconspicuous. Microscopic studies of the fungus (Fig. 1) has shown the conidiophores as smooth to finely rough walled, 200–300µm long, up to 7µm in diameter, enlarging gradually into vesicles of 18–20µm diameter. Metulae is absent; phialides are ampulliform, 7–9µm long, with a short neck. The conidia are mostly subglobose-globose to ellipsoidal, 2.5–3µm in length, echinulate, adhering in long compact columns. Based on these typical features, the fungus has been identified as *Aspergillus fumigatus* (Klich, 2002; Raper & Fennell, 1965).

According to a carried out pre-screening, the endophytic isolate *Aspergillus fumigatus* R7 showed high antibacterial activity against Gram positive (*B. subtilis* [16 mm], and *St. aureus* [15 mm]) and Gram negative bacteria (*P. aeruginosa* [19 mm], and *E. coli* [16 mm]). In contrast, the strain extract showed no activity against pathogenic fungi; *A. niger, A. flavus*, *C. albicans*. Alternatively, the strain extract showed numerous bands during TLC of different polarities, some of them were UV in active, while the others are UV absorbing during TLC, which were mostly turned pink-orange on spraying with anisaldehyde/sulphuric acid and heating.

2.2. ISOLATION AND STRUCTURE ELUCIDATION

Large scale fermentation of the strain was carried out on M_2 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 mentioned ten secondary metabolites (**1**-**10**). Structures of the afforded compounds were confirmed on the bases of different spectroscopic means (NMR, and MS) and comparison, and identified as linoleic acid (**1**) (Shaaban, 2004), R(-)-glycerol monolinoleate (**2**) (Laatsch, 2010), bis-dethio-(bis-methyl-thio)-gliotoxin; FR-49175 (**3**) (Zhang, 2011; Abdel Rahim, 2011), fumiquinazoline-F (**4**) (Takahashi *et al.*, 1995; Larsen *et al.*, 1998; Silva *et al.*, 2004; Abdel Rahim, 2011), fumiquinazoline-D (**5**) (Zhang, 2011; Abdel Rahim, 2011), (Z,Z)-N,N'-[1-[(4-Hydroxy-phenyl)-methylene]-

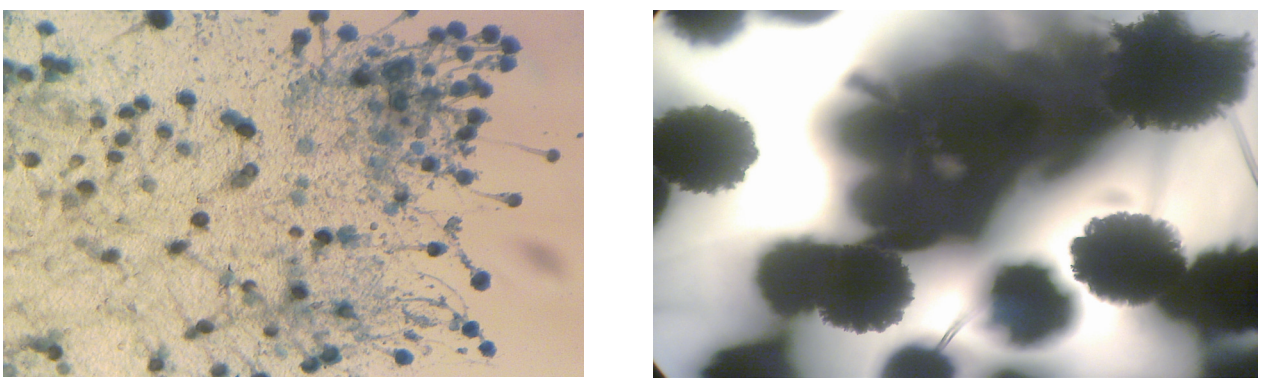

Figure1: Colonies and Microscopic characterization of the endophytic *Aspergillus fumigatus* sp. R7

2-[(4-methoxy-phenyl)-methylene]-1,2-ethanediyl]-bis-formamide (**6**) (Breinhold *et al.*, 1996; Abdel Rahim, 2011), pyrazoline-3-one trimer (**7**) (Davidson& Schumacher, 1993; Ridley& Simpson, 1981; Fotso *et al.*, 2006; Abdel Rahim, 2011), Tricho-9-ene-2α,3α,11α,16-tetraol (**8**) (McCormick *et al.*, 1989), 2'-deoxy-thymidine (**9**) (Shaaban, 2004) and cerebroside A (**10**) (Koga *et al.*, 1998; Sitrin, 1988; Mohamed, 2010).

Bis-dethio-(bis-methyl-thio)-gliotoxin (**3**) was isolated recently from endophytic *Penicillium* sp. BCC16054 (Intaraudom *et al.*, 2013), showing a very strong antitubercular activity against *Mycobacterium tuberculosis* with MIC value of 48.8 ng/mL (0.14 lM) (Intaraudom *et al.*, 2013). These results supported the fact that gliotoxin has been considered as a potential antitubercular drug (McMahon *et al.*, 2011).

Fumiquinazoline F (**4**) had been previously isolated from the fungus *Aspergillus fumigatus*, which was isolated from the marine fish *Pseudolabrus japonicus* (Takahashi *et al.*, 1995). Fumiquinazoline F (**4**) was also isolated from *Aspergillus lentulus* (Larsen *et al.*, 2007), *Penicillium thymicola* (Larsen *et al.*, 1998) and *Penicillium corylophilum* (Silva *et al.*, 2004). Biologically, fumiquinazoline F (**4**) was reported to show high antitumor activity (Han *et al.*, 2007; Zhang *et al.*, 2007). Similarly, fumiquinazoline-D (**5**) was produced previously by the fungus *Aspergillus fumigatus*, which was isolated from the marine fish *Pseudolabrus japonicus,* exhibiting moderate cytotoxicity against cultured P388 cells (Takahashi *et al.*, 1995). Fumiquinaozolines were as well reported recently from the pathogenic *Aspergillus sydowii* and other *Aspergillus* species (Cai & Lu, 2012). Alternatively, (Z,Z)-N,N'-[1-[(4-Hydroxy-phenyl**)-**methylene]-2-[(4-methoxy-phenyl)-methylene]-1,2-ethanediyl]-bis-formamide (**6**) was previously isolated from the fungus *Hamigera avellanea* and reported to exhibit a marginal activity against a variety of pathogenic fungi and bacteria (Breinhold *et al.*, 1996; Abdel Rahim, 2011).

Pyrrolizin-3-one trimer (**7**) was previously isolated from marine-derived *Streptomyces* sp. strain QD518 as an artifact obtained from the unstable 5,7-dihydroxy-5,6,7,8-tetrahydrooazocin-2(1H)-one (Fotso *et al.*, 2006). In contrast, compound **7** is having the 1-azabicyclo[3.3.0]octane skeleton (pyrrolizidine), was found frequently in plants (Robins, 1989) and insects (Boppre, 1986), and is a part of pheromones, defensive agent, or growth determinant. Mammals convert many of these pyrrolidizine alkaloids into dehydro-pyrrolizidines, which exhibit e.g. hepatotoxic, mutagenic, and carcinogenic activities (Fotso *et al.*, 2006).

Tricho-9-ene-2α,3α,11α,16-tetraol (**8**) is belonging to trichothecenes, sesquiterpene metabolites, possessing an olefinic bond and an epoxide group, which are produced by several genera of fungi, including Fuisarium (McCormick *et al.*, 1989), Trichothecium, Trichoderma, Myrothecium, Cephalosporium, Stachybotrys, Verticimonosporium, and Cylindocarpon (Matsumoto *et al.*, 1977; Minato *et al.*, 1975; Mirocha *et al.*, 1977; Ishii & Ueno, 1981). The biochemical basis of the toxicity of the trichothecenes is their inhibition of protein biosynthesis (Apsimon *et al.*, 1985; Bennett *et al.*, 1980).

Finally, cerebroside A (**10**) is an antifungal agent against *Candida albicans*. Cerebrosides; glycosphingolipids, were reported in several phytopathogens as elicitors that induce the disease resistance in e.g. rice plants (Umemura *et al.*, 2002). Cerebrosides are a kind of important bioactive substances isolated mainly from sea cucumber (Xu *et al.*, 2011). Cerebrosides are mostly composed of three different structural units; a polar head group (monosaccarides, such as glucose or galactose), an amide-linked fatty acid, and a long-chain base (LCB) which is also called a sphingoid base (Xu *et al.*, 2011). Recent studies indicated that cerebrosides have antitumor, immunomodulatory, antibacterial and cytotoxic activities (Xu *et al.*,

2011). Due to their moderate bioactivities and smaller side-effects, cerebrosides have been developed to prevent and cure chronic diseases.

2.3 BIOLOGICAL ACTIVITIES

Diverse antimicrobial activity testing for the crude extract of endophytic fungi *Aspergillus fumigatus* sp. isolate R7 was carried out in comparison with the whole isolated compounds (**1--10**) against eleven microbial tests on the bases of agar diffusion method (40 µg/disc). The crude extract showed high cytotoxic activity (100%) which was attributed reasonably to fumiquinazoline-F (**4**, 85%) and fumiquinazoline-D (**5**, 85%). The strain extract showed high antibacterial activity against the Gram-positive *Bacillus subtilis* (18 mm) and *Streptomyces viridochromogenes* (Tü 57, 18 mm), and the microalgae *Chlorella vulgaris* (15 mm), *Chlorella sorokiniana* (13 mm) and *Scenedesmus subspicatus* (15 mm). Compounds **4** and **5** exhibited further activity against the Gram-positive *Bacillus subtilis* (12, 15 mm), *Staphylococcus aureus* (12, 15 mm) and fungi (*C. albicans* [11, 11 mm] and *M. miehi* [12, 13 mm]). Except compounds **4** and **5**, cytotoxic examination of the remaining compounds (**1-3**, **6-10**) against the brine shrimp, confirmed their activities to be ranged between moderate and weak. Activity of the whole compounds (**1-10**) are listed in Table 1.

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).

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

Comp. No.	BS[a]	SA[b]	SV[c]	EC[d]	CA[e]	MM[f]	CV[g]	CS[h]	SS[i]	PS[j]	PU[k]	Brine Shrimp
Crude extract	18	12	18	0	0	0	15	12	15	0	0	100 %
1	0	0	0	0	0	0	0	0	0	0	0	11 %
2	0	0	0	0	0	0	0	0	0	0	0	10 %
3	15	0	16	0	0	0	0	0	0	0	0	8.8 %
4	12	12	0	0	11	12	0	0	0	0	0	85 %
5	15	11	0	0	11	13	0	0	0	0	0	80 %
6	12	0	13	0	12	14	0	0	0	0	0	33 %
7	11	13	12	0	0	0	0	0	0	0	0	45 %
8	12	13	13	0	0	0	0	0	0	0	0	25 %
9	0	0	0	0	0	0	0	0	0	0	0	3 %
10	11	11	0	0	0	0	0	0	0	0	0	28%

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

3.1 ENDOPHYTIC FUNGUS *ASPERGILLUS FUMIGATUS* R7

3.1.2 ISOLATION

The endophytic fungi *Aspergillus fumigatus* sp. R7 was isolated from the red leaves of sweat 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 Potato dextrose agar (PDA) medium [Potato infusion 200 g; Dextrose 20 g; Agar 20 g, Distilled water 1 liter], Antibiotic, ampicillin and streptomycin 200 µg/L of the medium was added to the media to inhibit the bacterial growth until the mycelium or colony originating from the newly formed surface of the segments appeared (Phongpaichit *et al.*, 2006). 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.

3.1.2 FERMENTATION AND WORKING UP

A 30-liter shaker culture of the endophytic fungi *Aspergillus fumigatus* sp. isolate R7 was incubating at 28 °C using M_2 medium for 7 days. 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 vacuo*, 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.

3.1.3 ISOLATION

The crude extract (8.3 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 (1.6 g), FII (3.2 g), FIII (2.2 g) and FIV (0.6 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**, 45 mg) and R(-)-glycerol monolinoleate (**2**, 120 mg). FII was purified by PTLC chromatogram (20 × 40 cm, DCM/7% MeOH, double elution) and then by Sephadex LH-20 (MeOH) yielding three colourless oils of bis-dethio-(bis-methyl-thio)-gliotoxin (**3**, 25 mg), fumiquinazoline-F (**4**, 8 mg) and fumiquinazoline-D (**5**, 12 mg). Fraction III was purified by PTLC chromatogram (20 × 40 cm, DCM/7% MeOH, double elution) and then by Sephadex LH-20 (MeOH) delivering two colourless solids of (Z,Z)-N,N'-[1-[(4-Hydroxy-phenyl)-methylene]-2-[(4-methoxy-phenyl)-methylene]-1,2-ethanediyl]-bis-formamide (**6**, 11 mg) and pyrazoline-3-one trimer (**7**, 9 mg). A final purification of fraction FIV using PTLC chromatogram (20 × 20 cm, DCM/15% MeOH) and Sephadex LH-20 (MeOH) gave three colourless solids of Tricho-9-ene-2a,3a,11a,16-tetraol (**8**, 7 mg), 2'-deoxy-thymidine (**9**, 23 mg) and cerebroside A (**10**, 18 mg). Spectroscopic data of the isolated compounds (**1**-**10**) are present in attached file "Supplementary Data".

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. The fungal extract was dissolved in CH_2Cl_2/10% MeOH at a concentration of 1 mg/mL. Aliquots of 40 µl were soaked on filter paper discs (9 mm Ø, 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 bacterial and 48 h (30°C) for the fungal isolates, while the algal test strains were incubated at ~ 22°C in day light for 8~10 days. The fungal extract was examined against the following test microorganisms: *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*.

For the fungal extract examination, representative test microbes; *Aspergillus niger, Aspergillus flavus, Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were served. Both bacterial and fungal strains were grown on nutrient agar medium (g/l): Beef extract 3; peptone,10; and agar, 20. The pH was adjusted to 7.2. The fungal strain was grown on Czapek-Dox medium (g/l): Sucrose, 30; $NaNO_3$, 3; $MgSO_4.7H_2O$, 0.5l; KCl, 0.5; $FeSO_4$, 0.01; K_2HPO_4, 1; and agar, 20. The pH was maintained at 6.0. The disc diffusion test has been done according to Collins (Collins *et al.*, 1985). Filter paper discs (5 mm diameter) were saturated with 200 µ*g* from the culture extract, and located on the surface of the agar plates (150 mm diameter containing 50ml of solidified media). The paper discs were placed on inoculated agar plats and incubated for 24 h at 38 °C (bacteria and yeast) and 48 h at 30°C (fungi).

3.2.2 CYTOTOXICITY

The cytotoxic assay was performed according to Takahashi method (Takahashi *et al.*, 1989) and Sajid *et al.* screening (Sajid *et al.*, 2009).

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