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Aspernolides F and G, new butyrolactones from the endophytic fungus *Aspergillus terreus*

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ABSTRACT

Two new butyrolactones: aspernolides F (**6**) and G (**7**), together with three stigmasterol derivatives: (22*E*,24*R*)-stigmasta-5,7,22-trien-3-β-ol (**1**), stigmast-4-ene-3-one (**2**), and stigmasta-4,6,8(14), 22-tetraen-3-one (**3**), two meroterpenoids: terretonin A (**4**) and terretonin (**5**), and a butyrolactone derivative: butyrolactone VI (**8**) have been isolated from the endophytic fungus *Aspergillus terreus* isolated from the roots of *Carthamus lanatus* (Asteraceae). Their structures were determined by spectroscopic means (1D, 2D NMR, and HRESIMS), as well as optical rotation measurement and comparison with literature data. The isolated compounds were evaluated for their anti-microbial, anti-malarial, anti-leishmanial, and cytotoxic activities. Compound **1** displayed a potent activity against MRSA and *C. neoformans* with IC_{50} values of 0.96 μg/mL and 4.38 μg/mL, respectively compared to ciprofloxacin (IC_{50} 0.07 μg/mL) and amphotericin B (IC_{50} 0.34 μg/mL), respectively. While, **6** showed good activity against *C. neoformans* (IC_{50} 5.19 μg/mL) and mild activity against MRSA (IC_{50} 6.39 μg/mL). Moreover, **1** and **2** exhibited very good anti-leishmanial activity towards *L. donovani* with IC_{50} values of 4.61 and 6.31 μg/mL, respectively and IC_{90} values of 6.02 and 16.71 μg/mL, respectively.

1. Introduction

Recently, attention has been focused on the biology and chemistry of the endophytic microorganisms (Geris dos Santos and Rodrigues-Fo, 2003). Endophytic fungi reside in most tissues of the living plants. They have proved to be one of the most important and underexplored resources available for biologically active natural products (Strobel et al., 2004; Schulz et al., 2002; Strobel, 2003). Although, the relationship between endophytic fungi and their host plants is still poorly investigated, highly frequent isolation of endophytic fungi from plants has contributed a number of secondary metabolites with diverse structures and interesting biological activities (Rateb and Ebel, 2011; Zhang et al., 2006; Gunatilaka, 2006). *Aspergillus* represents a large diverse genus, containing about 180 filamentous fungal species of substantial pharmaceutical and commercial values (Ibrahim et al., 2015; Lubertozzi and Keasling, 2009). Also, it is one of the major contributors to the secondary metabolites of fungal origin. *Aspergillus terreus* has been isolated from both marine and terrestrial sources. It is well known for the production of butenolides, which are a class of amino acid derivatives (Bai et al., 2014). Biogenetically, butyrolactones are derived from oxidative deamination of amino acids such as tyrosine and phenyl alanine (Bai et al., 2014; Rao et al., 2000; Nitta et al., 1983). Butyrolactones possess a wide range of biological activities such as cytotoxic (Wang et al., 2011; San-Martín et al., 2011; Parvatkar et al., 2009; Niu et al., 2008), anti-malarial (Haritakun et al., 2010), anti-H1N1 (Wang et al., 2011), anti-cholinesterase (Cazar et al., 2005), anti-microbial (Bai et al., 2014; San-Martín et al., 2011; Wang et al., 2011; Cazar et al., 2005), antioxidant (Sugiyama et al., 2010), and lipooxygenase and cyclin-dependent kinases inhibitors

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(Wang et al., 2011; Sugiyama et al., 2010; Fischer and Lane, 2000; Schimmel et al., 1998; Nishio et al., 1996; Kitagawa et al., 1993). As a part of our continuing interest in exploring bioactive metabolites from fungal source, we have isolated two new butyrolactones (**6** and **7**), along with six known compounds (**1**–**5** and **8**) from the endophytic fungus *A. terreus* isolated from the roots of *Carthamus lanatus* (Asteraceae) (Fig. 1). The structures of the isolated compounds were established on the basis of 1D, 2D NMR, and HRESIMS spectral data as well as optical rotation measurements. In addition, the anti-microbial, anti-malarial, anti-leishmanial, and cytotoxic activities of compounds **1**–**8** have been evaluated.

The fungus was cultured on rice solid medium. The metabolites from the rice culture were extracted with EtOAc. The later was concentrated and partitioned between *n*-hexane and 90% MeOH. The total 90% MeOH extract was subjected to VLC, silica gel, Sephadex LH-20, and RP-18 column chromatography to yield two new (**6** and **7**) and six known compounds (**1**–**5** and **8**).

2. Results and discussion

Compound **6** was obtained as yellow gum. Its molecular formula was assigned as $C_{26}H_{28}O_7$ on the basis of the HRESIMS pseudo-molecular ion peak at *m*/*z* 453.1916 $[M+H]^+$ (calcd for $C_{26}H_{29}O_7$, 453.1913), indicating 13 degrees of unsaturation. The ^1H and ^{13}C NMR spectral data of **6** revealed that eight of the thirteen units of unsaturation were attributed to two phenyl moieties. In addition, two carbonyls and four olefinic carbons account for another four degrees of unsaturation. Thus, the remaining unit indicated the presence of an aliphatic ring in **6**. The IR spectrum showed absorption bands at 3363 (hydroxyl), 1735 (ester/lactone carbonyl), and 1669 (C—H aromatic) cm^{-1}. The ^{13}C, DEPT, and HSQC spectra of **6** showed the presence of 26 carbons: three methyls, one methoxy (δ_C 53.6, 5-OCH_3), two methylenes, an oxymethylene (δ_C 60.3, C-1‴), eight methines, and 11 quaternary carbons, including two carbonyls at δ_C 170.7 (C-5) and 168.6 (C-1),

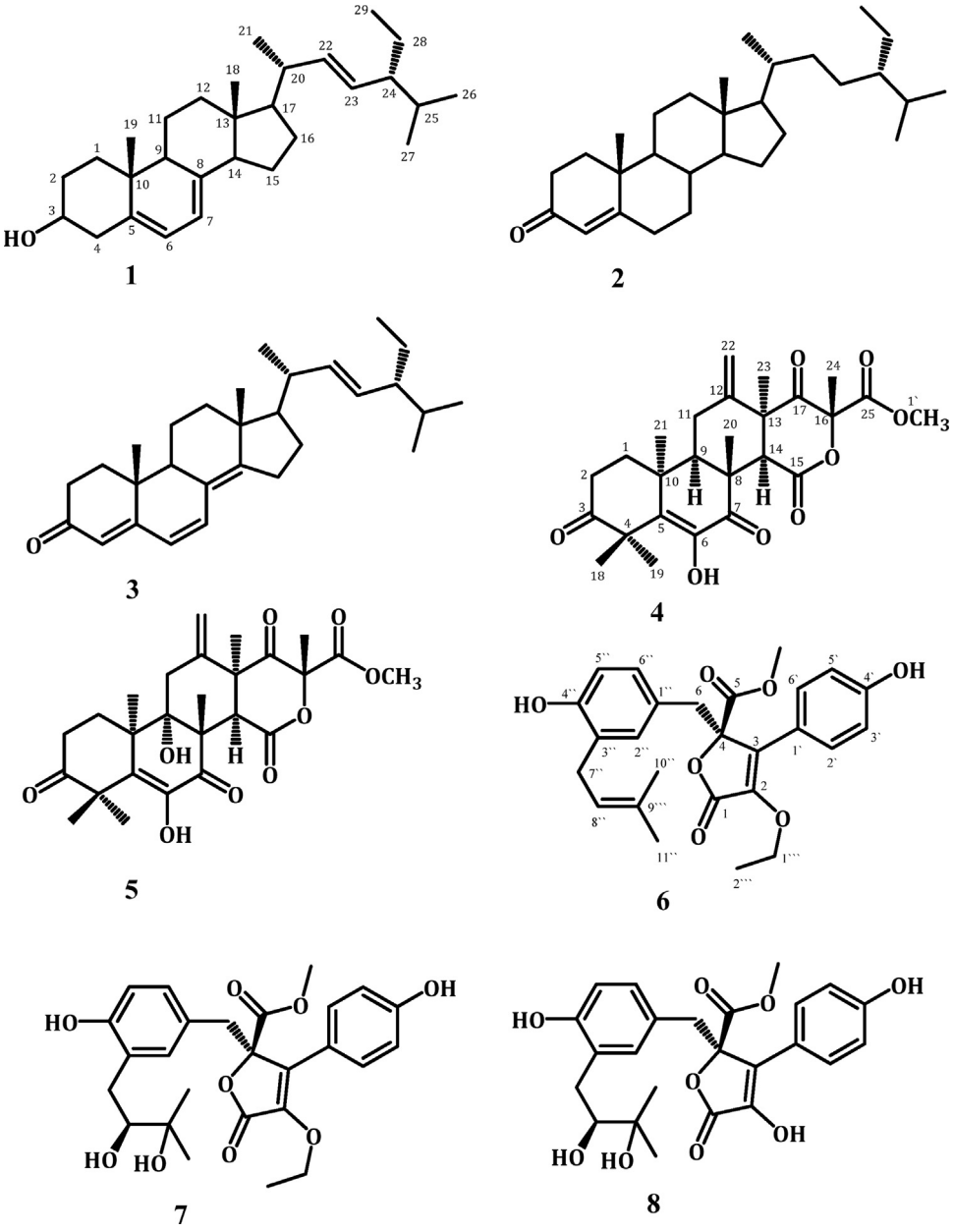

Fig. 1. Structures of the isolated compounds **1**–**8**.

two oxygen-bonded aromatic carbons at δ_C 158.4 (C-4′) and 154.2 (C-4″), and an oxygenated aliphatic carbon (δ_C 85.3, C-4). The ^{1}H NMR and ^{1}H–^{1}H COSY spectra showed four ortho-coupled aromatic protons of an A_2B_2 system at δ_H 7.65 (2H, brd, J = 8.4 Hz, H-2′, 6′) and 7.00 (2H, brd, J = 8.4 Hz, H-3′, 5′), indicating the presence of 1,4-di-substituted phenyl moiety, along with three aromatic signals at δ_H 6.52 (d, J = 2.0 Hz, H-2″), 6.65 (d, J = 8.0 Hz, H-5″), and 6.61 (dd, J = 8.0, 2.0 Hz, H-6″) characteristic for the presence of additional 1,3,4-tri-substituted benzene ring in the molecule. They were established by the HMBC cross peaks of H-2′ and H-6′/C-3′, C-5′, and C-4′, H-3′ and H-5′/C-1′, C-2′, C-4′, and C-6′, H-2″/C-4″ and C-6″, H-5″/C-1″, C-3″, C-4″, and C-6″, and H-6″/C-1″, C-2″, and C-4″ (Fig. 2). ^{1}H and ^{13}C NMR spectra of **6** displayed signals for tri-substituted olefinic double bond at δ_H 5.13 (1H, brt, J = 7.6 Hz, H-8″)/δ_C 122.8 (C-8″) and 131.5 (C-9″), methylene group at δ_H 3.01 (2H, d, J = 7.6 Hz, H-7″)/δ_C 28.0 (C-7″), and two olefinic methyls at δ_H 1.69 (3H, s, H_3-10″)/δ_C 25.8 (C-10″) and 1.60 (3H, s, H_3-11″)/17.7 (C-11″), suggesting the presence of isopentenyl moiety in **6**. This was established by the observed ^{1}H-^{1}H COSY cross peaks of H-7″/H-8″ and further confirmed by the HMBC correlations of H-7″/C-8″ and C-9″, H-8″/C-7″, C-10″, and C-11″, H_3-10″ and H_3-11″/C-8″ and C-9″, and H_3-10″/C-11″. Its connectivity at C-3″ was secured by the HMBC cross peaks of H-7″/C-2″, C-3″, and C-4″, H-8″/C-3″, and H-2″/C-7″. Additionally, a methylene group at δ_H 3.47 and 3.36 (2H, each d, J = 14.4 Hz, H-6)/δ_C 38.6 (C-6) was observed. The HMBC cross peaks of H-6/C-4, C-5, and C-1″ and H-2″ and H-6″/C-6 indicated that this methylene was connected to the quaternary carbon C-4 (δ_C 85.3) and C-1″ of the tri-substituted phenyl residue. Furthermore, an oxygenated methylene group at δ_H 4.15 (2H, q, J = 6.5 Hz, H-1‴)/δ_C 60.3 (C-1‴) and a triplet methyl δ_H 1.17 (J = 6.5 Hz, H_3-2‴)/δ_C 14.2 (C-2‴), indicating the presence of an ethoxy group. The ^{3}J and ^{4}J HMBC cross peaks of H-1‴ to C-2 and C-1, respectively, in addition to the ^{4}J HMBC cross peak of H-2‴ to C-2 confirmed the connectivity the ethoxy group at C-2. The stereochemistry at C-4 of **6** was deduced to be 4*R* based on biosynthetic considerations and similarity of its specific rotation ($[\alpha]_D$ + 39 (*c* 0.25, MeOH)) with those of previously reported butenolides (San-Martín et al., 2011; Nuclear et al., 2010; Haritakun et al., 2010; Roa et al., 2000). On the basis of these results and by comparison with literature, the structure of **6** was determined as (*R*)-methyl 4-ethoxy-2-(4-hydroxy-3-(3-methylbut-2-enyl) benzyl)-3-(4-hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate and named aspernolide F.

Compound **7** was also obtained as yellow gum with a molecular formula $C_{26}H_{30}O_9$ determined by its HRESIMS pseudo-molecular ion peak at *m/z* 487.1971 $[M+H]^+$ (calcd $C_{26}H_{31}O_9$, 487.1968), requiring 12 degrees of unsaturation. Compound **7** was 34 mass units more than **6** and one degree of unsaturation less than **6**, suggesting the presence of two additional hydroxyl groups in **7**. The 1D and 2D NMR spectral data (Table 1) revealed that the structure of **7** was very similar to those of **6** except the absence of the signals associated with the isopentenyl group at C-3″. Instead, a side chain, consisting of an oxygenated quaternary carbon δ_C 77.4 (C-9″), an oxymethine at δ_H 3.63 (1H, brt, J = 3.0 Hz, H-8″)/δ_C 68.4 (C-8″), a methylene at δ_H 2.71 (1H, m, H-7″A) and 2.48 (1H, m, H-7″B)/δ_C 31.4 (C-7″), and two methyl groups at δ_H 1.25 (3H, s, H_3-10″)/δ_C 26.0 (C-10″) and 1.24 (3H, s, H_3-11″)/δ_C 21.0 (C-11″) was observed. This was confirmed by the observed ^{1}H–^{1}H COSY and HMBC correlations (Fig. 2). The HMBC correlations of H-7″/C-2″ and C-4″, H-8″/C-3″, and H-2″/C-7″ indicated the attachment of the side chain at C-3″. It was reported that the stereochemistry at C-4 can assigned on the basis of the optical rotation's sign. Butyrolactones with *R*-configured C-4 possess positive optical rotation values such as butyrolactones I–VIII (Zhou et al., 2015; San-Martín et al., 2011; Nuclear et al., 2010; Haritakun et al., 2010; Rao et al., 2000), while those with negative optical rotation values possess *S*-configured C-4 as in isobutyrolactone II and V (Nong et al., 2014). The positive optical rotation of **7** ($[\alpha]_D$ + 68 (*c* 0.01, MeOH)) was consistent with the literature data, confirming the 4*R*-configuration. Due to the scarcity of the compound **7**, the configuration at C-8″ was assigned as *S* based on the coupling constant value (J = 3.0 Hz) and by comparison of ^{1}H and ^{13}C NMR chemical shifts with those of previously reported butyrolactones (San-Martín et al., 2011; Nuclear et al., 2010; Haritakun et al., 2010; Rao et al., 2000). This unambiguously led to the elucidation of **7** as (*R*)-methyl 2-(3-((*S*)-2,3-dihydroxy-3-methylbutyl)-4-hydroxybenzyl)-4-ethoxy-3-(4-

Table 1
NMR spectral data of **6** and **7** (DMSO-d_6, 400 and 100 MHz).

	6		7	
No.	δ_H [mult., J (Hz)]	δ_C (mult.)	δ_H [mult., J (Hz)]	δ_C (mult.)
1	–	168.6 (C)	–	168.5 (C)
2	–	138.5 (C)	–	138.6 (C)
3	–	128.0 (C)	–	128.2 (C)
4	–	85.3 (C)	–	85.2 (C)
5	–	170.7 (C)	–	170.8 (C)
6	3.47 d (14.4) 3.36 d (14.4)	38.6 (CH_2)	3.66 d (14.4) 3.45 d (14.4)	38.6 (CH_2)
1′	–	121.7 (C)	–	120.0 (C)
2′, 6′	7.65 brd (8.4)	129.4 (CH)	7.55 brd (8.4)	129.3 (CH)
4′	–	158.4 (C)	–	158.4 (C)
3′, 5′	7.00 brd (8.4)	116.3CH	6.93 brd (8.4)	116.2CH
1″	–	122.9 (C)	–	121.5 (C)
2″	6.52 d (2.0)	131.8 (CH)	6.58 brs	132.1 (CH)
3″	–	127.3 (C)	–	124.8 (C)
4″	–	154.2 (C)	–	152.2 (C)
5″	6.65 d (8.0)	114.7 (CH)	6.52 d (8.0)	116.3 (CH)
6″	6.61 dd (8.0, 2.0)	128.8 (CH)	6.51 brd (8.0)	129.3 (CH)
7″	3.01 d (7.6)	28.0 (CH_2)	2.71 m 2.48 m	31.4 (CH_2)
8″	5.13 brt (7.6)	122.8 (CH)	3.63 brt (3.0)	68.4 (CH)
9″	–	131.5 (C)	–	77.4 (C)
10″	1.69 s	25.8 (CH_3)	1.25 s	26.0 (CH_3)
11″	1.60 s	17.7 (CH_3)	1.24 s	21.0 (CH_3)
5-OCH_3	3.75 s	53.6 (CH_3)	3.76 s	53.8 (CH_3)
1‴	4.15 q (6.5)	60.3 (CH_2)	4.05 q (6.5)	60.2 (CH_2)
2‴	1.17 t (6.5)	14.2 (CH_3)	1.12 t (6.5)	14.4 (CH_3)
4′-OH	9.91 s	–	10.58 s	–
4″OH	9.08 s	–	9.97 s	–

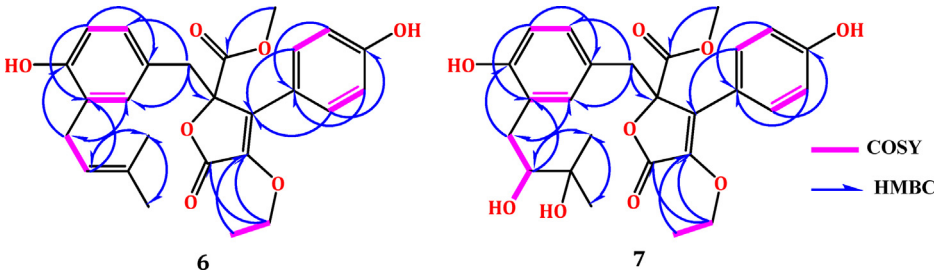

Fig. 2. ^{1}H–^{1}H COSY and HMBC correlations of **6** and **7**.

hydroxyphenyl)-5-oxo-2,5-dihydrofuran-2-carboxylate and named aspernolide G.

The known compounds were identified as (22*E*,24*R*)-stigmasta-5,7,22-trien-3-β-ol (**1**) (Ha et al., 1982; Toyama et al., 1952), stigmast-4-ene-3-one (**2**) (Barla et al., 2006), stigmasta-4,6,8(14), 22-tetraen-3-one (**3**) (Kobayashi et al., 1992), terretonin A (**4**) (Li et al., 2005), terretonin (**5**) (Springer et al., 1979; Liu et al., 2013), and butylrolactone VI (**8**) (San-Martín et al., 2011) by comparing their physical and NMR spectral data with literature.

The isolated compounds **1–8** were evaluated for their anti-microbial activity against *Candida albicans*, *Candida glabrata*, *C. krusei*, *Asperigillus fumigates*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Cryptococcus neoformans*, *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium intracellulare*, anti-leishmanial activity against *Leishmania donovani* promastigotes, and anti-malarial activity against chloroquine sensitive (D6, Sierraleon) and resistant (W2, Indo-china) strains of *Plasmodium falciparum* (Tables 2–4). Furthermore, they were tested for cytotoxicity against SK-MEL, KB, BT-549, SK-OV-3, LLC-PK_{11}, and VERO cell lines.

Compound **1** displayed a potent activity against MRSA with an IC_{50} value of 0.96 μg/mL and good antifungal activity against *C. neoformans* with an IC_{50} value of 4.38 μg/mL compared to ciprofloxacin (IC_{50} 0.07 μg/mL) and amphotericin B (IC_{50} 0.34 μg/mL), respectively. Also, **6** exhibited good against *C. neoformans* (IC_{50} 5.19 μg/mL) and mild activity against MRSA (IC_{50} 6.39 μg/mL). The rest of compounds (**2-5**, **7**, and **8**) showed no anti-microbial activity.

Compounds **1** and **2** exhibited very good anti-leishmanial activity towards *L. donovani* with IC_{50} values of 4.61 and 6.31 μg/mL, respectively and IC_{90} values of 6.02 and 16.71 μg/mL, respectively compared to pentamidine (positive control, IC_{50} 2.1 μg/mL). Furthermore, compounds **3–8** showed no activity. On the other hand, none of the tested compounds showed anti-malarial activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Additionally, they showed no cytotoxicity towards the tested cell lines.

3. Experimental

3.1. General experimental procedures

Melting points were carried out using an Electrothermal 9100 Digital Melting Point apparatus (Electrothermal Engineering Ltd., Essex, England). Optical rotation was measured on a JASCO DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan) at 25 °C at the sodium D line (589 nm). UV spectrum was recorded on a Hitachi 300 spectrometer (Hitachi High-Technologies Corporation, Kyoto, Japan). The IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer (Shimadzu, Kyoto, Japan). ESIMS spectra were obtained with a LCQ DECA mass spectrometer (Thermo Finnigan, Bremen, Germany) coupled to an Agilent 1100HPLC system equipped with a photodiode array detector. HRESIMS spectra measurements were performed on a Micromass Qtof 2 mass spectrometer (Bruker, Rheinstetten, Germany). 1D and 2D NMR spectra were determined on BRUKER AVANCE 400 instruments (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR) (Bruker BioSpin, Billerica, MA, USA) using $CDCl_3$ and DMSO-d_6 as solvents. Column chromatographic separations were performed on silica gel 60 (0.04–0.063 mm, Merck, Darmstadt, Germany), Sephadex LH-20 (0.25–0.1 mm, Merck, Darmstadt, Germany), and RP-18 (0.04-0.063 mm, Merck, Darmstadt, Germany). TLC analysis was performed on pre-coated TLC plates with silica gel 60 F254 (0.2 mm, Merck, Darmstadt, Germany). Spots were visualized by UV absorption at λ_{max} 255 and 366 nm followed by spraying with *p*-anisaldehyde/H_2SO_4. The solvent systems used for TLC analyses were *n*-hexane:EtOAc (85:15, S_1) and *n*-hexane:EtOAc (80:20, S_2).

Table 2
Anti-microbial activity results of compounds **1–8**.

Tested strains	Minimum inhibitory concentration, IC_{50} μg/mL							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
C. albicans	>20	>20	>20	>20	>20	>20	>20	>20
C. glabrata	>20	>20	>20	>20	>20	>20	>20	>20
C. krusei	>20	>20	>20	>20	>20	>20	>20	>20
A. fumigates	>20	>20	>20	>20	>20	>20	>20	>20
C. neoformans	4.38	>20	>20	>20	>20	5.19	>20	>20
S. aureus	11.7	>20	>20	>20	>20	7.49	>20	>20
MRS	0.94	>20	>20	>20	>20	6.39	>20	>20
E. coli	>20	>20	>20	>20	>20	>20	>20	>20
P.aeruginosa	>20	>20	>20	>20	>20	>20	>20	>20
M. intracellulare	>20	>20	>20	>20	>20	>20	>20	>20

Table 3
In vitro anti-leishmanial activity results of compounds **1–8**.

Compd no.	*L. donovani*	
	IC_{50} μg/mL	IC_{90} μg/mL
1	4.61	6.02
2	6.31	16.71
3	22.03	32.24
4	>40	>40
5	>40	>40
6	36.8	>40
7	>40	>40
8	>40	>40

IC_{50}: concentration causing 50% growth inhibition. IC_{90}: concentration causing 90% growth inhibition.

3.2. Isolation of the fungal material

A. terreus was isolated from the internal tissue of the healthy roots of *Carthamus lanatus* L. (Asteraceae), which collected from the wildly growing plant at Al-Azhar University campus in February 2013. The root was washed under running water and the surface was sterilized with 70% EtOH for 1 min. The root samples were rinsed in sterile water. To distinguish the remaining epiphytic fungi from endophytic fungi, an imprint of the root surface on biomalt agar was performed. The outer layer of the root was removed and small root tissue samples were cut aseptically and pressed onto agar plates containing an antibiotic to suppress bacterial growth. The plates were kept at 27 °C for the isolation of the endophyte. After incubation at room temperature, the fungal strain under investigation was found to grow exclusively out of the root tissues, but not on the agar plates taken from the imprint of the root surface. A fungal strain was maintained on potato-dextrose agar medium (Difco) at 25 °C. It was deposited at the Department of Microbiology, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut, Egypt under registeration number (AST No. Feb 2013).

3.3. Identification of the fungal material

The strain was identified from the observed morphological features of the fungus on potato-dextrose agar (PDA), sabroud dextrose, and nutrient agar as previously described by Watanabe (2002). The morphology was examined by light microscopy (CX31RBSF, Olympus) and identification was confirmed to be *A. terreus* by Prof. Mohamed Hosam Refaie Kotb, Prof. of Microbiology and Immunology, Department of Microbiology and Immunology, Animal Reproductive Research Institute, Giza, Egypt. From the

Table 4
Anti-malarial activity results of compounds **1**–**8**.

Compd no.	IC_{50} μg/mL				
	P. falciparum (D6 clone)	*P. falciparum* (D6 S1 clone)	*P. falciparum* (W2 clone)	*P. falciparum* (W2 S1 clone)	VERO
1	751.8	>6.3	997.7	>4.8	>4760
2	1831.9	>2.6	1877.2	>2.5	>4760
3	3931.5	>1.2	2763.9	>1.7	>4760
4	>4760	1	>4760	1	>4760
5	>4760	1	>4760	1	>4760
6	1938.3	>2.5	4109	1	>4760
7	1677.7	>2.8	1925.6	>2.5	>4760
8	>4760	1	>4760	1	>4760

growing cultures, the pure strain of *A. terreus* was isolated by repeated re-inoculation on PDA agar plates.

3.4. Cultivation

Mass growth of the fungus for the isolation and identification of its metabolites was carried out in 1 L Erlenmeyer flasks (each flask containing 100 mL of distilled water were added to 100 g commercially available rice and kept overnight prior to autoclaving). The fungus was grown on rice solid medium (8 flasks) at room temperature under septic conditions for 4 weeks.

3.5. Extraction and isolation

The metabolites from the rice culture were extracted with EtOAc. The obtained crude extract was concentrated under vacuum and partitioned between *n*-hexane and 90% MeOH. The total 90% MeOH extract (11.8 g) was subjected to normal phase vacuum liquid chromatography (VLC) using *n*-hexane, $CHCl_3$, EtOAc, and MeOH, which were separately concentrated to give AS-1 (1.5 g), AS-2 (2.9 g), AS-3 (2.55 g), and AS-4 (3.72 g), respectively. Fraction AS-2 (2.9 g) was subjected to VLC using *n*-hexane:EtOAc gradient elution to afford seven sub-fractions: AS-2-A (0.32 g), AS-2-B (0.49 g), AS-2-C (0.61 g), AS-2-D (0.29 g), AS-2-E (0.12 g), AS-2-F (0.41 g), and AS-2-G (0.36 g). Sub-fraction AS-2-A (0.32 g) was chromatographed over silica gel column (50 g × 50 × 2 cm) using *n*-hexane:EtOAc (98:2–85:15) to obtain **1** (21 mg, white needles). Silica gel column chromatography (80 g × 50 × 2 cm) of sub-fraction AS-2-B (0.49 g) using *n*-hexane:EtOAc (98:2–85:15) gave impure **2** (34 mg), which was further purified by RP-18 column (100 g × 50 × 2 cm) using MeOH:H_2O gradient to afford **2** (18 mg, white needles). Subfraction AS-2-C (0.61 g) was subjected to Sephadex LH-20 column chromatography (100 g × 70 × 3 cm) using MeOH:$CHCl_3$ (90:10) to afford impure **3**–**5**. They were further purified by RP-18 column (150 g × 50 × 3 cm) using MeOH:H_2O gradient to obtain **3** (23 mg, white needles), **4** (12 mg, colorless cubic crystals), and **5** (9 mg, colorless cubic crystals). Sub-fraction AS-2-D and E (0.41 g) were collected together based on TLC plates. Similarly, they were chromatographed over Sephadex LH-20 chromatography (100 g × 70 × 3 cm) using MeOH as an eluent to afford impure **6–8**. Purification was achieved by repeated silica gel column chromatography using $CHCl_3$:MeOH gradient to give **6** (6.7 mg, yellow gum), **7** (4.1 mg, yellow gum), and **8** (11 mg, yellow gum). The other fractions were retained for further investigation.

3.6. Spectral data

3.6.1. (22E,24R)-Stigmasta-5,7,22-trien-3-β-ol (**1**)

White needles; mp 151-152 °C; IR (KBr) ν_{max} 3435, 2850, 1665, 1585, 1460, 970 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H 2.30 (1H, m, H-1A), 1.36 (1H, m, H-1B), 1.91 (1H, m, H-2A), 1.80 (1H, m, H-2B), 3.62 (1H, m, H-3), 2.48 (1H, dd, *J* = 12.6, 11.4 Hz, H-4A), 2.44 (1H, dd, *J* = 12.6, 4.2 Hz, H-4B), 5.56 (1H, d, *J* = 6.2 Hz, H-6), 5.37 (1H, d, *J* = 6.2 Hz, H-7), 2.01 (1H, t, *J* = 5.8 Hz, H-9), 1.48 (1H, m, H-11A), 1.22 (1H, m, H-11B), 1.68 (1H, m, H-12A), 1.25 (1H, m, H-12B), 2.27 (1H, t, *J* = 7.2 Hz, H-14), 1.37 (1H, m, H-15A), 1.25 (1H, m, H-15B), 1.82 (1H, m, H-16A), 1.44 (1H, m, H-16B), 1.87 (1H, m, H-17), 0.62 (3H, s, H_3-18), 0.96 (3H, s, H_3-19), 2.07 (1H, m, H-20), 1.03 (3H, d, *J* = 6.8 Hz, H_3-21), 5.14 (1H, dd, *J* = 16.8, 6.9 Hz, H-22), 5.22 (1H, dd, *J* = 16.8, 7.2 Hz, H-23), 1.99 (1H, m, H-24), 1.78 (1H, m, H-25), 0.92 (3H, d, *J* = 6.3 Hz, H_3-26), 0.92 (3H, d, *J* = 6.3 Hz, H_3-27), 1.25 (2H, m, H-28), 0.82 (3H, t, *J* = 6.1 Hz, H_3-29); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 38.3 (C-1), 31.9 (C-2), 70.4 (C-3), 40.7 (C-4), 139.5 (C-5), 119.8 (C-6), 116.1 (C-7), 141.2 (C-8), 46.2 (C-9), 37.0 (C-10), 21.1 (C-11), 39.1 (C-12), 42.8 (C-13), 54.5 (C-14), 22.9 (C-15), 28.2 (C-16), 55.7 (C-17), 12.1 (C-18), 16.3 (C-19), 40.4 (C-20), 17.6 (C-21), 131.9 (C-22), 135.4 (C-23), 42.8 (C-24), 33.1 (C-25), 19.7 (C-26), 20.0 (C-27), 21.1 (C-28), 12.1 (C-29); ESIMS *m/z*: 411 $[M+H]^+$; HRESIMS *m/z*: 411.3629 $[M+H]^+$ (calcd for $C_{29}H_{47}O$, 411.3627).

3.6.2. Stigmast-4-ene-3-one (**2**)

White needles; mp 96-97 °C; IR (KBr) ν_{max} 2940, 1671, 1453 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.70 (1H, m, H-1A), 1.52 (1H, m, H-1B), 2.35 (2H, m, H-2), 5.70 (1H, s, H-4), 2.00 (1H, m, H-6A), 1.80 (1H, m, H-6B), 1.45 (1H, m, H-7A), 1.18 (1H, m, H-7B), 1.41 (1H, m, H-8), 1.15 (1H, m, H-9), 1.57 (1H, m, H-11A), 1.29 (1H, m, H-11B), 1.48 (1H, m, H-12A), 1.24 (1H, m, H-12B), 0.65 (1H, m, H-14), 1.65 (1H, m, H-15A), 1.35 (1H, m, H-15B), 1.60 (1H, m, H-16A), 1.31 (1H, m, H-16B), 0.87 (1H, m, H-17), 0.68 (3H, s, H_3-18), 1.15 (3H, s, H_3-19), 1.65 (1H, m, H-20), 0.89 (3H, d, *J* = 6.2 Hz, H_3-21), 1.48 (2H, m, H-22), 1.35 (2H, m, H-23), 1.22 (1H, m, H-24), 1.25 (1H, m, H-25), 0.83 (3H, d, *J* = 6.6 Hz, H_3-26), 0.83 (3H, d, *J* = 6.6 Hz, H_3-27), 0.76 (3H, t, *J* = 6.9 Hz, H_3-29); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 35.6 (C-1), 32.9 (C-2), 200.0 (C-3), 123.8 (C-4), 171.8 (C-5), 33.9 (C-6), 33.8 (C-7), 36.1 (C-8), 53.8 (C-9), 38.6 (C-10), 21.0 (C-11), 39.5 (C-12), 42.3 (C-13), 55.8 (C-14), 24.2 (C-15), 28.2 (C-16), 55.9 (C-17), 12.0 (C-18), 17.3 (C-19), 35.6 (C-20), 18.7 (C-21), 32.0 (C-22), 27.0 (C-23), 45.7 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 23.0 (C-28), 11.9 (C-29); ESIMS *m/z*: 413 $[M+H]^+$; HRESIMS *m/z*: 413.3780 $[M+H]^+$ (calcd for $C_{29}H_{49}O$, 413.3783).

3.6.3. Stigmasta-4,6,8(14), 22-tetraen-3-one (**3**)

White needles; mp 118-119 °C; ^1H NMR (400 MHz, $CDCl_3$): δ_H 2.02 (1H, m, H-1A), 1.82 (1H, m, H-1B), 2.54 (1H, m, H-2A), 2.48 (1H, m, H-2B), 5.72 (1H, brs, H-4), 6.58 (1H, d, *J* = 9.5 Hz, H-6), 6.00 (1H, d, *J* = 9.5 Hz, H-7), 2.13 (1H, m, H-9), 1.71 (1H, m, H-11A), 1.63 (1H, m, H-11B), 1.68 (1H, m, H-12A), 1.25 (1H, m, H-12B), 2.48 (1H, m, H-15A), 2.37 (1H, m, H-15B), 1.76 (1H, m, H-16A), 1.53 (1H, m, H-16B), 1.26 (1H, m, H-17), 0.94 (3H, s, H_3-18), 0.97 (3H, s, H_3-19), 2.11 (1H, m, H-20), 1.03 (3H, d, *J* = 6.0 Hz, H_3-21), 5.18 (1H, dd, *J* = 16.5, 6.3 Hz, H-22), 5.25 (1H, dd, *J* = 16.5, 6.7 Hz, H-23), 1.76 (1H, m, H-24), 1.48 (1H, m, H-25), 0.91 (3H, d, *J* = 7.0 Hz, H_3-26), 0.91 (3H, d, *J* = 7.0 Hz, H_3-27), 1.21 (2H, m, H-28), 0.82 (3H, t, *J* = 6.2 Hz, H_3-29); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 34.1 (C-1), 18.9 (C-2), 199.6 (C-3), 122.8 (C-4),

164.5 (C-5), 124.4 (C-6), 134.1 (C-7), 124.8 (C-8), 44.2 (C-9), 36.8 (C-10), 25.3 (C-11), 34.1 (C-12), 44.0 (C-13), 156.3 (C-14), 35.5 (C-15), 27.7 (C-16), 55.6 (C-17), 16.6 (C-18), 19.0 (C-19), 39.3 (C-20), 21.2 (C-21), 132.5 (C-22), 134.9 (C-23), 42.8 (C-24), 33.1 (C-25), 19.6 (C-26), 20.0 (C-27), 29.7 (C-28), 17.6 (C-29); ESIMS *m/z*: 407 $[M+H]^+$; HRESIMS *m/z*: 407.3311 $[M+H]^+$ (calcd for $C_{29}H_{43}O$, 407.3314).

*3.6.4. Terretonin A (**4**)*

Colorless cubic crystals, mp 233-234 °C; IR (KBr) ν_{max} 3369, 1736, 1712, 1683, 1105 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H 2.14 (1H, dd, *J* = 14.0, 9.0 Hz, H-1A), 1.72 (1H, dd, *J* = 14.0, 10.4 Hz, H-1B), 2.65 (1H, dd, *J* = 13.6, 9.0 Hz, H-2A), 2.50 (1H, m, H-2B), 1.54 (1H, dd, *J* = 12.2, 3.0 Hz, H-9), 2.42 (1H, dd, *J* = 13.8, 12.2 Hz, H-11A), 2.28 (1H, dd, *J* = 13.8, 3.0 Hz, H-11B), 2.75 (1H, brs, H-14), 1.38 (3H, s, H_3-18), 1.40 (3H, s, H_3-19), 1.76 (3H, s, H_3-20), 1.05 (3H, s, H_3-21), 5.05 (1H, brs, H-22A), 4.92 (1H, brs, H-22B), 1.36 (3H, s, H_3-23), 1.65 (3H, s, H_3-24), 3.76 (3H, s, H_3-1′), 6.18 (1H, s, 6-OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 34.2 (C-1), 32.6 (C-2), 214.1 (C-3), 47.9 (C-4), 137.1 (C-5), 138.9 (C-6), 197.7 (C-7), 45.7 (C-8), 52.8 (C-9), 36.8 (C-10), 29.0 (C-11), 142.7 (C-12), 50.6 (C-13), 49.1 (C-14), 166.8 (C-15), 85.6 (C-16), 201.1 (C-17), 20.9 (C-18), 23.6 (C-19), 16.5 (C-20), 16.8 (C-21), 112.5 (C-22), 23.5 (C-23), 22.0 (C-24), 168.2 (C-25), 53.8 (C-1′); ESIMS *m/z*: 473 $[M+H]^+$; HRESIMS *m/z*: 473.2176 $[M+H]^+$ (calcd for $C_{26}H_{33}O_8$, 473.2175).

*3.6.5. Terretonin (**5**)*

Colorless cubic crystals; mp 259-260 °C; IR (KBr) ν_{max} 3410, 2935, 1729, 1711, 1685, 1252, 1150 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ_H 2. 41 (1H, m, H-1A), 1.76 (1H, m, H-1B), 2.65 (1H, dd, *J* = 14.0, 8.0 Hz, H-2A), 2.44 (1H, m, H-2B), 2.91 (1H, brd, *J* = 15.0 Hz, H-11A), 2.27 (1H, brd, *J* = 15.0 Hz, H-11B), 3.53 (1H, s, H-14), 1.40 (3H, s, H_3-18), 1.37 (3H, s, H_3-19), 1.86 (3H, s, H_3-20), 1.15 (3H, s, H_3-21), 5.35 (1H, s, H-22A), 5.02 (1H, s, H-22B), 1.40 (3H, s, H_3-23), 1.64 (3H, s, H_3-24), 3.76 (3H, s, H_3-1′), 6.19 (1H, s, 6-OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 28.0 (C-1), 32.6 (C-2), 214.5 (C-3), 47.8 (C-4), 131.4 (C-5), 138.6 (C-6), 197.1 (C-7), 51.1 (C-8), 77.6 (C-9), 43.1 (C-10), 34.9 (C-11), 139.5 (C-12), 49.3 (C-13), 44.4 (C-14), 168.0 (C-15), 85.3 (C-16), 201.4 (C-17), 21.2 (C-18), 23.5 (C-19), 19.6 (C-20), 18.5 (C-21), 116.5 (C-22), 23.5 (C-23), 21.1 (C-24), 168.3 (C-25), 53.7 (C-1′); ESIMS *m/z*: 489 $[M+H]^+$; HRESIMS *m/z*: 489.2127 $[M+H]^+$ (calcd for $C_{26}H_{33}O_9$, 489.2125).

*3.6.6. Aspernolide F (**6**)*

Yellow gum; $[\alpha]_D$ + 39 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε): 216 (4.01), 254 (3.68), 299 (0.52) nm; IR (KBr) ν_{max} 3363, 2995, 1735, 1669, 1520, 1255, 1166 cm^{-1}; NMR see Table 1; ESIMS *m/z*: 453 $[M+H]^+$; HRESIMS *m/z*: 453.1916 $[M+H]^+$ (calcd for $C_{26}H_{29}O_7$, 453.1913).

*3.6.7. Aspernolide G (**7**)*

Yellow gum; $[\alpha]_D$ + 68 (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε): 235 (3.85), 307 (3.24) nm; IR (KBr) ν_{max} 3410, 2987, 1740, 1665, 1390, 1055 cm^{-1}; NMR see Table 1; ESIMS *m/z*: 487 $[M+H]^+$; HRESIMS *m/z*: 487.1971 $[M+H]^+$ (calcd for $C_{26}H_{31}O_9$, 487.1968).

*3.6.8. Butyrolactone VI (**8**)*

Yellow gum; $[\alpha]_D$ + 73 (*c* 0.5, MeOH); IR (KBr) ν_{max} 3440, 1735, 1660, 1055 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ_H 3.42 (1H, d, *J* = 16.0 Hz, H-6A), 3.32 (1H, d, *J* = 16.0 Hz, H-6B), 7.50 (2H, brd, *J* = 8.0 Hz, H-2′, 6′), 6.83 (2H, brd, *J* = 8.0 Hz, H-3′, 5′), 6.59 (1H, d, *J* = 2.0 Hz, H-2″), 6.41 (1H, d, *J* = 8.0 Hz, H-5″), 6.49 (1H, dd, *J* = 8.0, 2.0 Hz, H-6″), 2.63 (1H, dd, *J* = 16.0, 3.6 Hz, H-7″A), 2.28 (1H, dd, *J* = 16.0, 8.0 Hz, H-7″B), 3.25 (1H, dd, *J* = 8.0, 3.6 Hz, H-8″), 1.04 (3H, s, H_3-10″), 1.05 (3H, s, H_3-11″), 3.76 (3H, s, 5-OCH_3), 10.52 (1H, s, 4′-OH), 9.98 (1H, s, 4″-OH), 9.22 (1H, d, *J* = 5.6 Hz, 8″-OH); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 168.5 (C-1), 138.6 (C-2), 128.1 (C-3), 85.2 (C-4), 170.2 (C-5), 38.4 (C-6), 121.5 (C-1′), 129.2 (C-2′, 6′), 158.2 (C-4′), 116.2 (C-3′, 5′), 123.6 (C-1″), 133.4 (C-2″), 126.6 (C-3″), 154.8 (C-4″), 115.0 (C-5″), 129.0 (C-6″), 32.6 (C-7″), 68.4 (C-8″), 77.1 (C-9″), 26.2 (C-10″), 25.1 (C-11″), 53.8 (5-OCH_3); ESIMS *m/z*: 459 $[M+H]^+$; HRESIMS *m/z*: 459.1658 $[M+H]^+$ (calcd for $C_{24}H_{27}O_9$, 459.1655).

3.7. Anti-microbial assay

All the isolated compounds were tested for anti-microbial activity against *C. albicans* ATCC 90028, *C. glabrata* ATCC90030, *C. krusei* ATCC 6258, *A. fumigates* ATCC 90906, methicillin-resistant *S. aureus* ATCC 33591, *C. neoformans* ATCC 90113, *S. aureus* ATCC 2921, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, and *M. intracellulare* ATCC 23068 as described previously (Al-Musayeib et al., 2014; Ibrahim et al., 2012). Ciprofloxacin and amphotericin B were used as positive standards.

3.8. Anti-malarial assay

The isolated compounds were tested on chloroquine sensitive (D6, Sierraleon) and resistant (W2, Indo-China) strains of *P. falciparum* using previously reported method (Al-Musayeib et al., 2014; El-Shanawany et al., 2011). Artemisinin and chloroquine were included in each assay as anti-malarial drug controls.

3.9. Anti-leishmanial assay

The anti-leishmanial activity of the isolated metabolites was tested *in vitro* against a culture of *L. donovani* promastigotes as previously outlined (Al-Musayeib et al., 2014; El-Shanawany et al., 2011). Pentamidine and amphoterecin B were used as positive standards.

3.10. Cytotoxicity assay

The *in vitro* cytotoxic activity was determined against a panel of four human cancer cell lines (SK-MEL, KB, BT-549, SK-OV-3) and two noncancerous kidney cell lines (LLC-PK_{11} and VERO). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were seeded at a density of 25,000 cells/well and incubated for 24 h. Test samples were added at different concentrations and cells were again incubated for 48 h. At the end of incubation, the cell viability was determined using Neutral Red dye according to a modification of the procedure of Borenfreund et al. (Borenfreund et al., 1990). Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control.

4. Conclusion

Two new butyrolactones (**6** and **7**), along with six known compounds (**1–5** and **8**) have been isolated from the endophytic fungus *A. terreus*. Their structures were determined on the basis of extensive spectroscopic data analysis. Compound **1** displayed potent activity against MRSA and *C. neoformans*. While, **6** was active towards *C. neoformans*. Moreover, **1** and **2** exhibited very good anti-leishmanial activity towards *L. donovani*.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.09.006.

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