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Bioactive extracts and chemical constituents of two endophytic strains of *Fusarium oxysporum*

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Abstract: Ethyl acetate extracts of cultures grown in liquid Czapek and on solid rice media of the fungal endophyte *Fusarium oxysporum* SS46 isolated from the medicinal plant *Smallanthus sonchifolius* (Poepp.) H. Rob., Asteraceae, exhibited considerable cytotoxic activity when tested *in vitro* against human cancer cells. Chromatographic separation yielded anhydrofusarubin (**1**) and beauvericin (**2**) that were identified based on their ^{1}H and ^{13}C NMR data. Compounds **1** and **2** showed the strongest cytotoxic activity against different cancer cell lines. Compound **2** also showed promising activity against *Leishmania braziliensis*. Hexanic extract of *F. oxysporum* SS50 grown on solid rice media also afforded a mixture of compounds that displayed cytotoxic activity against different cancer cell lines. Chemical analysis of the mixture of compounds, investigated by gas chromatography-mass spectrometry (GC-MS), showed that there was a predominance of methyl esters of fatty acids and alkanes.

Introduction

Microorganisms are recognized as prolific producers of bioactive natural products, many of them useful as clinical drugs (Gunatilaka, 2006; Newman & Cragg, 2012). Newman and Gragg (2012) recently emphasized that efforts to discover new bioactive natural products should focus on microorganisms living in interactions with their hosts. Endophytes are one type of such microorganisms, since they spend the whole or part of their lifespan inside the healthy tissues of the host inter- and/or intracellularly without causing discernible symptoms of plant disease (Borges et al., 2009). Natural products from fungal endophytes have a broad spectrum of biological activities, such as antimicrobial, immunosuppressant, anticancer, and also may act as biocontrol agents (Gunatilaka, 2006; Borges et al., 2009).

As part of our ongoing research on endophytic fungi found in association with Asteraceae species we have identified several bioactive extracts and compounds, as well as novel natural products (Gallo et al.; 2009; 2010; Guimarães et al., 2010; Borges et al., 2011).

In this paper, we report the chemical and biological of two fungal strains of *Fusarium oxysporum*, coded as SS46 and SS50, isolated as endophytes from the medicinal plant *Smallanthus sonchifolius* (Asteraceae).

Materials and Methods

General procedures

1D (regular ^{1}H and ^{13}C) and 2D nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on Bruker spectrometer (DPX-500), working at 500 MHz for ^{1}H, and at 125 MHz for ^{13}C. Tetramethylsilane (TMS) was used as internal reference. Column chromatographies were performed over silica gel 60, 230-280 mesh

(Ultra Chem) or Sephadex LH-20. Vacuum liquid chromatography (VLC) was carried out silica gel 60 H, 70-230 mesh (Merck). Thin-Layer chromatography (TLC) was performed on sílica gel 60 GF_{254} (Merck). Spots were located by spraying with anisaldehyde–sulfuric acid reagent, heated at 110 °C.

Isolation of strains

The endophytic fungal strains were isolated from the roots of *S. sonchifolius* according to the procedure described by Gallo et al. (2009). After collection, the plant material was washed with water and surface sterilized by immersion in 70% aqueous ethanol (2.5 min), followed by 2.5% aqueous sodium hypochlorite (30”, 60” or 90”), and finally with 70% aqueous ethanol (1 min). After these procedures, the leaves and roots were rinsed with sterilized water. This latter water was incubated in Petri dishes to ensure the elimination of all epiphytic microorganisms. Small pieces of the leaves and roots were excised and placed on agar in Petri dishes containing potato dextrose agar (PDA) medium at 30 °C. Individual hyphal tips of the emerging fungi were removed and placed on PDA slants.

Fermentation and extraction procedures

Both *F. oxysporum* strains were cultured in liquid Czapek and solid rice media. Liquid cultures were conducted in two steps. First, the silica stored fungi were inoculated onto PDA-containing medium in Petri dishes and incubated at 30 °C for seven days. After this period, the pre-inoculum was prepared by transferring the agar plugs (0.5 cm diameter) cut from the 7-day-old original cultures into 50 mL Falcon flasks containing 10 mL of rich medium (5 g tryptone, 10 g dextrose, 3 g yeast extract, 10 g malt extract, in 1000 mL final volume of H_2O, pH 6.2±0.2), and incubated for three days at 25 °C, 120 rpm. After this period, the pre-inoculum (20 mL) was transferred to a 1000 mL Erlenmeyer flask containing 200 mL of Czapek medium and incubated at 25 °C for 14 or 21 days (120 rpm). Nine Erlenmeyer flasks were used for a large scale extract production, aiming to isolate secondary metabolites. After 21 days 200 mL of ethanol were added to each flask, the resulting suspension was vacuum-filtered and the filtrate fractionated by liquid-liquid partition with ethyl acetate. The resulting organic layer was evaporated under reduced pressure to produce the ethyl acetate extracts (EaL). Fermentations of *F. oxysporum* SS46 and *F. oxysporum* SS50 carried out for fourteen days yielded, respectively, 1.4289 g (coded SS46-EaL_{14}) and 1.0884 g (coded SS50-EaL_{14}) of EaL extracts. After 21 days of incubation, *F. oxysporum* SS46 yielded 0.8082 g of EaL extract (coded SS46-EaL). *F. oxysporum* SS50 was cultured only for 14 days. A Falcon flask containing seed medium without inoculum was submitted to the same preculture conditions. The resulting pre-inoculum was transferred to Czapek medium and treated in the same way for control purposes.

The solid fermentation experiments were carried out in ten 500 mL Erlenmeyer flasks containing 90 g of parboiled rice in 90 mL distilled water per flask, previously twice autoclaved at 120 °C for 40 min for each fungal strain. Agar plugs (about 2 x 2 cm) cut from the 7-day-old original cultures on PDA agar were used for inoculation. One flask, without inoculum, was kept for control use. After 21 days incubation in a BOD (Biological Oxygen Demand) device at 30 °C, 150 mL of methanol were added to each flask and the contents were allowed to stand overnight at room temperature. The methanol was filtered and evaporated under reduced pressure yielding the methanol extract, which was submitted to the liquid-liquid partition with hexane and ethyl acetate. The resulting organic layer was evaporated under reduced pressure to produce the hexanic and ethyl acetate extracts. Solid culture of *F. oxysporum* SS46 afforded 0.41 g of the hexanic extract (SS46-HxS) and 2.89 g of the ethyl acetate extract (SS46-EaS). Fermentation of *F. oxysporum* SS50 yielded 0.16 g of the hexanic extract (SS50-HxS) and 0.84 g and ethyl acetate extract (SS50-EaS).

Isolation of compounds

The SS46-EaS extract (2.77 g) was chromatographed by vacuum liquid chromatography (VLC) on silica gel and eluted with hexane, hexane/ethyl acetate (gradient), ethyl acetate/methanol (gradient) and methanol to give ten fractions (F1-F10). Fraction F5 (100 mg) was purified by chromatography on a Sephadex LH-20 column using methanol as eluent to give **1** (5.2 mg). The SS46-EaL extract (89 mg) was purified by chromatography on a column packed with silica gel 60. Elution with hexane/ethyl acetate 8:2 and 2:8, (crescent gradient), ethyl acetate/methanol 19:1 and methanol 100% afforded twenty fractions, which were combined in nine sub-fractions according to TLC analysis. The sub-fraction 7 (6.1 mg) was identified as **2**. The SS50-HxS extract (155.4 mg), obtained after 21 days on rice medium, was investigated by gas chromatography-mass spectrometry (GC-MS).

Gas chromatography-mass spectrometry (GC-MS)

The SS50-HxS extract was analyzed on a Shimadzu QP-2010 gas chromatograph interfaced to a mass spectrometer (GC-MS). The following conditions were used: DB-5MS column (30 m x 0.25 mm x 0.25 µm); helium (99.999%) carrier gas at a constant flow of 1.1 mL/min; 1 µL injection volume; injector split ratio of 1:40; injector temperature 240 °C; electron impact

mode at 70 eV; ion-source temperature 280 ºC. The oven temperature was programmed from 100 ºC (isothermal for 5 min), with an increase of 10 ºC/min to 250 ºC (isothermal for 5 min), and 10 ºC/min to 280 ºC (isothermal for 15 min). A mixture of linear hydrocarbons (C_9H_{20}-$C_{40}H_{82}$) was injected under the same conditions as standards, and identification of constituents performed by comparing the spectra obtained with those of the equipment's database (Wiley 7 lib), and by using the Kovats Index, calculated for each constituent as previously described (Adams, 1995; Van den Dool & Kratz, 1963).

Cytotoxic bioassay

Human tumor cell lines MDA-MB435 (melanoma), HCT-8 (colon), and SF295 (brain) were obtained from National Cancer Institute (Bethesda, MD, USA). All cell lines were cultured in RPMI1640 medium, supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin). Cultures were maintained in a humidified incubator at 37 °C and 5% CO_2 atmosphere. The cytotoxic effect was assessed using the MTT-dye reduction assay for cell viability described by Mosmann (1983) according to an established protocol. Briefly, tumor cells were placed in 96-well plates at the following densities: 0.7×10^5 (HCT-8), 0.6×10^5 (SF295), and 0.1×10^6 (MDA-MB435) cells/mL. Extracts were tested in a single concentration (50 µg/mL) and pure compounds in a range between 0.39 and 25 µg/mL during 72 h. Control groups received the same amount of vehicle and doxorubicin (Doxolem®, Zodiac Produtos Farmacêuticos S/A, Brazil), which was used as a positive control. Growth inhibition rates were quantified as the percentage of control absorbance by the reduced dye at 550 nm in accordance with the following equation:

Inhibition rate = [(OD control well – OD treated well) x (OD control well)$^{-1}$] x 100.

Each sample was tested in two independent experiments performed in triplicates. The results consisted of the average value for each experimental unit. The IC50 values and their 95% confidence intervals were obtained by non linear regression using the *GraphPad Prism* program (Intuitive Software for Science, San Diego, CA). An activity scale was utilized to appraise the cytotoxic potential of the tested samples: inactive samples (I), samples with low activity (LA, cell growth inhibition between 1-50%), moderated activity (MA, cell growth inhibition between 50-75%), and high activity (HA, cell growth inhibition between 75-100%).

Leishmanicidal assay

The *L. braziliensis* strain H3227 (MHOM/BR/94/H-3227) used in this study was kindly provided by Prof. Dr. Aldina Barral (Professor of Immunology, Centro de Pesquisas Gonçalo Moniz, FIOCruz-Bahia, Brazil). The parasites were maintained *in vitro* in M199 medium (GIBCO, Grand Island, NY) at 26 ºC supplemented with 10% heat inactivated fetal calf serum, 2% human urine, 20 mM HEPES, 4 mM $NaHCO_3$, 10 U/mL of penicillin and 100 µg/mL of streptomycin (GIBCO).

The anti-promastigote assay was carried out according to the method of Dutta et al. (2005). Beauvericin (**2**) was serially diluted from 0.02 to 10 µM in Schneider's medium (supplemented with 10% FBS and 2% human urine) containing 2 x 10^5 parasites/mL. A total of 4 x 104 parasites were seeded per well in 96-well microplates incubated at 26 °C until the end of the log phase. After, 100 µg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in 10 µL of PBS was added per well, and the plates were incubated at 37 °C for 4 h. Following incubation, the plates were centrifuged at 3000 x *g* for 5 min, the supernatant was removed, and precipitated formazan was dissolved in 100 µL of dimethyl sulfoxide. The absorbance was measured at 492 nm, and the data for two independent assays performed in triplicate were analyzed by non-linear regression with variable slope (Graph Pad Prism 5.0 software). The EC50 was defined as the effective drug concentration that inhibits parasite proliferation by 50% compared with a non-treated culture. Geneticin (G418) was used as a positive control (EC50 0.007 µM).

Results and Discussion

Extracts obtained after fourteen days of incubation of both endophytic fungi in liquid Czapek medium did not show any relevant cytotoxic activity. The best incubation time for production of cytotoxic compounds was 21 days, in both liquid and solid media. The most active extracts were found to be SS50-HxS and SS46-EaL, which showed high cytotoxic activities against the three cancer cell lines (Table 1). Extract SS46-EaS also showed high activity against HCT-8 cells and moderate activity against SF295 cells. Interestingly, *F. oxysporum* SS46 produced bioactive ethyl acetate extracts in both liquid and solid media. On the other hand, *F. oxysporum* SS50 produced bioactive compounds of lower polarity only in solid medium, as observed for the high activities of SS50-HxS extract. In our previous investigation, *F. oxysporum* SS46 had already produced high cytotoxic ethyl acetate extracts; however no cytotoxic activity had been detected for *F. oxysporum* SS50 (Gallo et al., 2009). This result can be explained by the different extraction methods, because in that previous screening all the fungal solid cultures extracts were partitioned only with ethyl acetate. Chemical investigation was focused on the most active extracts: SS46-EaS, SS46-EaL and SS50-HxS.

Table 1. *In vitro* cytotoxic activity of the extracts obtained from the endophytes *F. oxysporum* SS46 and *F. oxysporum* SS50 after 21 days of fermentation (50 µg/mL).

Crude extract*	Inhibition of cell growth (%)		
	HCT-8	MDA-MB435	SF295
SS46-HxS	55.83	16.53	43.61
SS50-HxS	97.72	90.53	83.78
Solid medium control Hx-extract**	35.41	I	I
SS46-EaS	84.82	36.23	67.02
SS50-EaS	43.45	I	0.77
Solid medium control Ea-extract**	31.53	5.74	I
SS46-EaL	100.00	99.32	100.00
Liquid medium control extract	I	I	I
Doxorubicin	97.30	98.27	96.94

* SS: fungal strain; Hx: hexane; Ea: ethyl acetate; S: solid rice medium; L: liquid Czapek medium for 21 days; **Extract from culture media without fungus; HCT-8: colon cancer cells; MDA-MB435: melanoma cells; SF295: brain cancer cells; high activiy: >75%; moderated activity: 50 to 75%; low activity: <50 %; I: inactive.

The SS50-HxS extract was analyzed by GC-MS. Twenty-seven components were detected in this extract, and twelve of them (76.8%) were identified by the GC-MS data (Table 2). According to our results, there is a predominance of alkanes and fatty acid methyl esters in this extract and the main constituents are: (9Z,12*Z*)-octadecadienoic acid methyl ester (54.45%), methyl hexadecanoate (9.73%) and (9*Z*)-octadecenoic acid methyl ester (4.70%). Fifteen compounds remained unidentified because the spectral data could not be associated with any compound database system (Wiley lib 7). The analysis of hexane rice-control extract (without fungus), showed that there is little correlation in their chemical composition. There are only five compounds in common in the extracts: 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, methyl hexadecanoate, (9*Z*)-octadecenoic acid methyl ester, clionasterol and stigmast-4-en-3-one. Therefore, seven compounds differentiate the SS50-HxS extract from the culture medium control extract: (2*E*, 4*E*)-decadienal, pentadecane, hexadecane, heptadecane octadecane, (9*Z*,12*Z*)-octadecadienoic acid methyl ester and dehydroergosterol. All extracts analyzed in this study differed considerably in their secondary metabolites. The SS50-HxS showed pronounced cytotoxicity against the three human cancer cell lines. As the major components of the hexane extract are a mixture of three methyl esters (methyl hexadecanoate, (9*Z*)-octadecenoic acid methyl ester and (9*Z*,12*Z*)-octadecadienoic acid methyl ester), which represents 68.88% of the extract, presumably they should be responsible for the high cytotoxic activity of the crude extract. The culture medium control extract did not show cytotoxic activity (Table 1). This extract also contains a fatty acid methyl esters mixture, but a mixture of two and only a lesser amount, methyl hexadecanoate or methyl palmitate, (9*Z*)-octadecenoic acid methyl ester, representing only 17.41% crude extract.

Table 2. Chemical composition of hexane extract of the culture of *F. oxysporum* SS50.

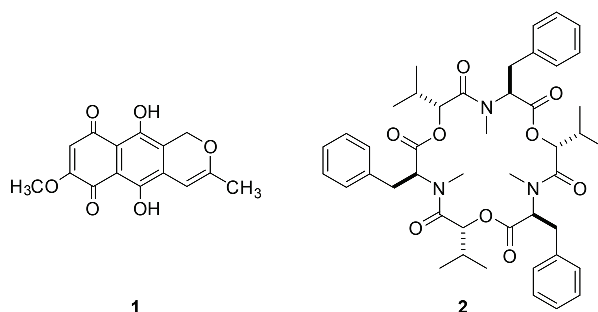
peak	RT (min)	Compound	(%)	KI exp.[a]	KI lit.[b]
1	9.248	(2*E*,4*E*)-decadienal	0.32	1320	1319
2	15.191	pentadecane	0.37	1500	1500
3	18.770	hexadecane	0.62	1599	1600
4	22.337	heptadecane	0.51	1699	1700
5	25.821	octadecane	0.68	1800	1800
6	27.688	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.62	1855	*
7	30.004	methyl palmitate or methyl hexadecanoate	9.73	1925	1927
9	35.285	(9*Z*,12*Z*)-octadecadienoic acid methyl ester		2092	2092
11	35.461	(9*Z*)-octadecenoic acid methyl ester	4.70	2097	*
22	61.520	dehydroergosterol	1.94	3133	*
23	64.693	clionasterol	1.51	3283	*
24	67.826	stigmast-4-en-3-one	1.36	3407	*

[a]KI exp: Kovats indices using the experimental equation Van den Dool; [b]KI lit.: Kovats indices according to the literature (Adams, 1995). *Kovats index not found in the literature.

Previously reported data found that another mixture of three fatty acid methyl esters (methyl myristate, methyl palmitate and methyl stearate), isolated from the ascidian *Didemnum psammatodes*, also showed high cytotoxic activity and antiproliferative effects against four cell lines leukemia. The mechanism of action of these fatty acid methyl esters was found to involve inhibition of DNA synthesis and induction of both necrosis and apoptosis (Takeara et al., 2008). The major constituent of this mixture is the methyl palmitate or methyl hexadecanoate, also identified as one of the three constituents of the mixture of methyl esters present in the SS50-HxS extract. Still according to the literature, when each methyl ester blend was tested separately, the cytotoxic activity decreased, suggesting that a synergistic effect of the mixture of fatty acid methyl esters (Takeara et al., 2008).

The SS46-EaS extract exhibited high cytotoxicity against the colon cancer cells (84.82%) and moderate cytotoxicity against central nervous system cancer cells (67.02%) (Table 1). This extract was subjected to vacuum liquid chromatography, and subsequent purification of the resulting fractions by chromatographic techniques resulted

in the isolation of **1**. The SS46-EaL extract showed the higher cytotoxicities against the three human cancer cell lines: colon (100.0 %), melanoma (99.32%) and central nervous system (100.0%). The SS46-EaL extract was fractionated by column chromatography resulting in the isolation of compound **2**. Analysis of the ^{1}H NMR, ^{13}C NMR, HMQC and HMBC spectra, together with HRESIMS data and comparison to literature data, led to the identification of compounds **1** and **2** as the naphthoquinone anhydrofusarubin and the cyclo-hexadepsipeptide beauvericin, respectively (Kurobane et al., 1980; Zhan et al, 2007). The occurrence of naphthoquinone **1** and cyclo-hexadepsipeptide **2** has been previously reported in the genus *Fusarium* (Tatum & Baker, 1983).



The cytotoxic activities of compounds **1** and **2** were evaluated *in vitro* against the three cancer cell lines. Anhydrofusarubin (**1**) and beauvericin (**2**) presented significant cytotoxicity against all the evaluated cancer cells, and the IC50 values ranged from 2.39 to 9.85 µg/mL (Table 3). Anhydrofusarubin (**1**) was recently isolated from a marine *Fusarium* sp. strain and showed selective cytotoxicity against human oral carcinoma cell lines (KB cells) and breast cancer (MCF-7) (Trisuwan et al., 2010). It has been reported previously in the literature *in vitro* cytotoxic activity of beauvericin (**2**), also isolated from an endophytic *F. oxysporum* strain, in other cell lines of human cancer (Zhan et al., 2007). Compound **2** is an ionophore able to transport cations across the membranes, especially calcium, leading to the cytotoxic effects (Zhan et al, 2007; Wang & Xu, 2012). As part of our research interest in finding antiparasitic compounds, beauvericin (**2**) was tested in vitro against promastigotes of *Leishmania braziliensis*, and showed EC50 1.86 µM. Therefore, compound **2** can be considered an interesting leishmanicidal natural product for further structure diversification. Beauvericin (**2**) had already been reported as a bioactive compound against other cancer lines, HIV1 virus and bacterial strains (Wang & Li, 2012). We now report the activity of beauvericin against different cancer lines and, for the first time, against *L. braziliensis*.

This study demonstrated that strains of fungal endophytes, identified as the same species and isolated from the same plant tissue, may vary considerably in the class of secondary metabolites produced. They may also produce different compounds depending on culturing conditions. *F. oxysporum* SS46 produced compound **1** in solid medium, and the cyclo-hexadepsipeptide **2** in liquid medium, both compounds extracted with ethyl acetate after 21 days of fungal fermentation. These compounds have important antiproliferative properties selective for cancer cells that might explain the cytotoxicities observed for the extracts. There was no production of these compounds by *F. oxysporum* SS50, which synthesizes predominantly fatty acid methyl esters and alkanes in solid medium.

Table 3. *In vitro* cytotoxic activity of compounds **1** and **2**, IC50.

Compound	Cell lines - IC50 µg/mL (IC95)[a]		
	HCT-8	MDA-MB435	SF295
Compound 1	9.85 (7.59-12.79)	6.23 (4.99-7.78)	6.32 (4.38-9.11)
Compound 2	3.02 (2.73-3.34)	3.17 (2.56-3.93)	2.39 (1.89-3.02)
Doxorubicin (positive control)	0.04 (0.02-0.06)	0.2 (0.1 – 0.34)	0.04 (0.01-0.005)

[a]IC95: 95% confidence interval.

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References

Adams RP 1995. *Identification of essential oil components by gas chromatography/mass spectrometry.* Illinois: Allured Publ.

Borges WS, Borges KB, Bonato PS, Said. S, Pupo MT 2009. Endophytic fungi: natural products, enzymes and biotransformation. *Curr Org Chem 13*: 1137-1163.

Borges WS, Mancilla G, Guimarães DO, Duran-Patron R., Colado IG, Pupo MT 2011. Azaphilones from the endophyte *Chaetomium globosum. J Nat Prod 74*: 1182-1187.

Dutta A, Bandyopadhyay S, Mandal C, Chatterjee M 2005. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. *Parasitol Int 54*: 119-122.

Gallo MBC, Chagas FO, Almeida MO, Macedo CC, Cavalcanti BC, Barros FWA, Moraes MO, Costa-Lotufo LV, Pessoa C, Bastos JK, Pupo MT 2009. Endophytic fungi found in association with *Smallanthus sonchifolius* (Asteraceae) as resourceful producers of cytotoxic bioactive natural products. *J Basic Microbiol 49*: 142-151.

Gallo MBC, Cavalcanti BC, Barros FWA, Moraes, MO, Costa-Lotuffo LV, Pessoa CO, Bastos JK, Pupo MT 2010. Chemical constituents of *Papulaspora immersa*, an endophyte from *Smallanthus sonchifolius* (Asteraceae), and their cytotoxic activity. *Chem Biodivers 7*: 2941-2950.

Guimarães DO, Borges WS, Vieira NJ, Oliveira LF, Silva CHTP, Lopes NP, Dias LG, Duran-Patron R, Collado IG, Pupo MT 2010. Diketopiperazines produced by endophytic fungi found in association with two Asteraceae species. *Phytochemistry 71*: 1423-1429.

Gunatilaka AAL 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J Nat Prod 6*9: 509-526.

Kurobane I, Vining L, Mcinnes AG, Walter JA 1980. Use of ^{13}C in biosynthetic studies. The labeling pattern in dihydrofusarubin enriched from [^{13}C]- and [^{13}C, ^{2}H] acetate in cultures of *Fusarium solani. Can J Chem 58*: 1380-1385.

Mosmann T 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods 65*: 55-63.

Newman DJ, Cragg GM 2012. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod 75*: 311-335.

Takeara R, Jimenez PC, Wilke DV, Moraes MO, Pessoa C, Lopes NP, Lopes JLC, Lotufo TMC, Costa-Lotufo LV 2008. Antileukemic effects of *Didemnum psammatodes* (Tunicata: Ascidiacea) constituents. *Comp Biochem Physiol Part A 151*: 363-369.

Tatum JH, Baker RA, 1983. Naphthoquinones produced by *Fusarium solani* isolated from citrus. *Phytochemistry 22*: 543-547.

Trisuwan K, Khamthong N, Rukachaisirikul V, Phongpaichit S, Preedanon S, Sakayaroj J 2010. Anthraquinone, cyclopentanone, and naphthoquinone derivatives from the sea fan-derived fungi *Fusarium* spp. PSU-F14 and PSU-F135. *J Nat Prod 73*: 1507-1511.

Van den Dool H, Kratz PD 1963. A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. *J Chromatogr 11*: 463-471.

Wang Q, Lijian, X 2012. Beauvericin, a bioactive compound produced by fungi: a short review. *Molecules 17*, 2367-2377.

Zhan, J, Burns AM, Liu MX, Faeth SH, Gunatilaka AAL 2007. Search for cell motility and angiogenesis inhibitors with potential anticancer activity: beauvericin and other constituents of two endophytic strains of *Fusarium oxysporum. J Nat Prod 70*: 227-232.

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