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BIOACTIVE METABOLITES OF THE ENDOPHYTE *KHUSKIA ORYZAE* ISOLATED FROM THE MEDICINAL PLANT *BIDENS BIPINNATA*

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ABSTRACT

Endophytes are considered a promising source of new bioactive secondary metabolites and are found in almost all plants. The medicinal plant Bidens bipinnata Lin. (Asteraceae) that is known for its anti-inflammatory, antiseptic and antifungal effects has been chosen for investigation of its endophytes for the search of bioactive secondary metabolites. An endophytic fungus was isolated from the plant and was identified as Khuskia oryzae on the basis of ITS sequence comparison. The ethyl acetate extract of the fungal isolate obtained from the stem parts of the plant exhibits antibacterial activity in agar diffusion assays as well as cytotoxic and antiproliferative effects. Activity-guided chromatographic fractionation resulted in the isolation of the active metabolites which were identified by different spectroscopic techniques. A new bioactive oxylipin called 8-oxo-(9E, 11E) octadecadienoic acids and the oxygenated bioactive fatty acid 9-oxo-(10E, 12E) octadecadienoic acids as well as the mycotoxin sterigmatocystin have been identified. A cytotoxic assay was performed for all compounds and revealed the first to be of cytotoxic activity against HeLa cell lines with a CC_{50} value of 43.4 µg mL^{-1} and cytostatic activity against HUVEC and K-562 cancer cell lines with GI_{50} values of 47.0 µg mL^{-1} and 43.2 µg mL^{-1} respectively. The cytotoxic assay performed on 8-oxo-(9E, 11E) octadecadienoic acid showed cytotoxicity against HeLa cell lines with a CC_{50} value of 30.3 µg mL^{-1} and also cytostatic activity against HUVEC and K-562 cell lines with a GI_{50} value of 27.8 µg mL^{-1}. In the antibacterial assay performed against B. subtilis moderate antibacterial activity was observed for 9-oxo-(10E, 12E)-octadecadienoic acid (inhibition zone 24 mm at a conc. of 250 µg mL^{-1}), while higher antibacterial activity was detected for 8-oxo-(9E, 11E)-octadecadienoic acid (inhibition zone 24 mm at a conc of 250 µg mL^{-1}). A cytotoxic assay was carried out on sterigmatocystin where it proved to be of weak cytotoxicity and also weak cytostatic activity with CC_{50} and GI_{50} values of more than 50 µg mL^{-1}. This is the first report of bioactive metabolites other than griseofulvin from Khuskia oryzae.

KEYWORDS: *Oxylipin, sterigmatocystin, endophyte, Khuskia oryzae, Bidens bipinnata.*

INTRODUCTION

Over the years, drug discovery has focused on microbial sources from which nearly 80% of the world`s antibiotics have been discovered. These sources have almost entirely been from soils collected from around the world, but new microbial origins need to be examined for the production of useful bioactive compounds [1]. Although it appears that the pharmaceutical industry has completely exhausted the hidden treasures of the microbial world to find solutions for the infectious diseases of the last century, a report by the American Academy of Microbiology estimates that less than 5% of fungal species are currently known and only 1% of these microbes have been cultured and characterized. Thus challenges to find novel microbes remain. Endophytes have been identified as a promising source of new pharmacologically active secondary metabolites that might be suitable for medicinal or agrochemical applications.

Several antimicrobial agents have been discovered from microbial sources since the discovery of penicillin G in 1928 [2]. The advantage of using microorganisms for the production of bioactive natural products is the possibility of producing large quantities with reasonable costs by large scale fermentations. Cephalosporin C is one of the famous antibacterial agents discovered after penicillin G [3], while griseofulvin is one of the first antifungal agents obtained from fungi [4]. *Bidens bipinnata* is an annual weed distributed widely in the tropical and subtropical regions of the world. It is well-known as hairy beggar ticks or Spanish needles and is reported to be a weed of 31 crops in more than 40 countries [5]. It is an herbaceous plant widely distributed in Africa, America, China, and Japan that is used in traditional medicines for treatment of inflammation and various diseases, including hepatitis and diabetes[6]. Furthermore, the ethanolic crude extract from the roots of *B. bipinnata* contains polyacetylenes and flavonoids that exert in vitro antimalarial activity against *Plasmodium falciparum* [7].

MATERIALS AND METHODS

Strain isolation and taxonomic classification

Samples of *B. bipinnata* were collected near Cairo, Egypt. After surface sterilization of the fresh, healthy, aerial plant parts an endophytic fungal strain was isolated. The strain was cultivated in four different culture media a malt extract (M4), a caseine–flesh peptone (M5), a cornsteep (M25) and a dextrose–yeast (M26) medium both as a shaken and as a stationary culture and was then subjected to antimicrobial activity screening. Results of the agar diffusion assay performed showed moderate antibacterial activity against *Bacillus subtilis.*

The strain was identified as *Khuskia oryzea* by ITS sequence comparison. Literature data showed that the only known metabolite of this rarely investigated strain was the antifungal agent griseofulvin [8]. In the following three active constituents of this strain are presented which have never been reported from it before.

Endophyte fermentation, extraction and isolation

Large scale fermentation (30 L) in medium M25, where it showed the highest antimicrobial activity. Total extraction of culture broth and mycelium with ethyl acetate yielding 7 g of dried crude extract after solvent evaporation was carried out. Chromatographic fractionation of the extract was performed on silica gel using a solvent system of hexane: ethyl acetate starting with a proportion of 9:1 and then gradually increasing the proportion of ethyl acetate till final elution with 100% ethyl acetate. After combining the similar fractions three main fractions were obtained. Activity guided fractionation resulted in the isolation of three pure compounds after several purification steps on Sephadex LH-20 using methanol as a solvent and finally isolation of the pure compounds using RP silica on the preparative HPLC.

General

NMR spectra were recorded on a Bruker DPX-300 and a Bruker DRX-500 at 300 MHz and 500 MHz for ^{1}H, and 125 MHz for ^{13}C NMR, respectively; chemical shifts are given in δ values (ppm) and were measured relative to tetramethylsilane as standard. IR spectra were recorded on a Bruker FT-IR (IFS 55) spectrometer. UV spectra were recorded on a Cary 1 Bio UV vis spectrophotometer (Variant). HPLC-MS measurements were recorded on an Agilent high performance 1100 series LC/MSD Trap module with an API - electrospray source, PC printer and LC/MSD chemstation software for data acquisition and data analysis. HRESIMS were recorded on a Finnigan TSQ Quantum Ultra AM Thermo Electron. Open column chromatography was performed on silica gel 60 (Merck, 0.04-0.063 mm, 230-400 mesh ASTM) and Sephadex LH-20 (Pharmacia). TLC: silica gel plates (silica gel 60 F_{254} on aluminum foil or glass, Merck), spots were visualized by spraying with anisaldehyde/sulfuric acid followed by heating. Analytical HPLC was conducted on a Shimadzu HPLC system using a Nucleosil 100-5 C_{18} column (5 µm, 125 x 4.6 mm) with

MeCN/0.1% TFA-H_2O as eluent (flow rate 1 mL/min, 15/85 to 100% MeCN in 30 min) and UV detection at 254 nm. Preparative HPLC was performed on a Shimadzu HPLC system using a Nucleosil 100-5 C_{18} column (5 µm, 250 x 16 mm, pore diameter 100 Å) using a flow rate of 10 mL/min starting elution with 25% MeCN and ending with 100% MeCN in 45 min with a UV detector. All solvents used were spectral grade or distilled prior to use.

Antimicrobial assay

Antifungal activities were studied qualitatively by agar diffusion tests according to the literature [9, 10].

Antiproliferative and cytotoxic assays

Cells and culture conditions

Cells of HUVEC (ATCC CRL-1730), K-562 (DSM ACC 10) and HeLa (DSM ACC 57) were cultured in DMEM (CAMBREX 12-614F), RPMI 1640 (CAMBREX 12-167F) and RPMI 1640 (CAMBREX 12-167F) respectively. All cells were grown in the apropriate cell culture medium supplemented with 10 mL/L ultraglutamine 1 (Cambrex 17-605E/U1), 500 µL/L gentamicin sulfate (CAMBREX 17-518Z), and 10% heat inactivated fetal bovine serum (PAA A15-144) at 37°C in high density polyethylene flasks (NUNC 156340).

Antiproliferative assay

The assay was carried out according to previously described method (Dolezal, 2009). The test substances were dissolved in DMSO before being diluted in DMEM. The adherent cells were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L 2163). For each experiment, approximately 10 000 cells were seeded with 0.1 mL culture medium per well of the 96-well microplates (NUNC 167008).

Cytotoxic assay

For the cytotoxic assay, HeLa cells were pre-incubated for 48 h without the test substances. The dilutions of the compounds were carried out carefully on the subconfluent monolayers of HeLa cells after the pre-incubation time. Cells were incubated with dilutions of the test substances for 72 h at 37°C in a humidified atmosphere and 5% CO_2.

Method of evaluation

For estimating the influence of chemical compounds on cell proliferation of K-562, the numbers of viable cells present in multiwall plates were determined via CellTiter-Blue® assay. The indicator dye resazurin was used to measure the metabolic capacity of cells as an indicator of cell viability. Viable cells of untreated control retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. Under our experimental conditions, the signal from the CellTiter-Blue® reagent is proportional to the number of viable cells. The adherent HUVEC and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gentle washing the stain was eluted with 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN). The GI_{50} and CC_{50} values were defined as being where the dose response curve intersected the 50% line, compared to untreated control. The comparisons of the different values were performed with software Magellan (TECAN).

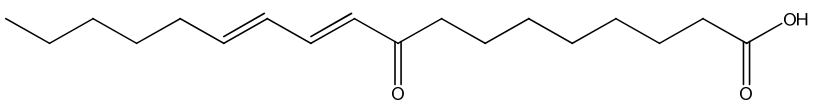

Figure 1. Chemical structure of 9-oxo-(10*E*, 12*E*) octadecadienoic acid

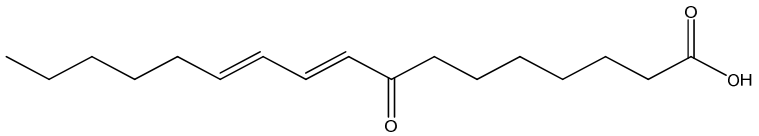

Figure 2. Chemical structure of 8-oxo-(9*E*, 11*E*)-octadecadienoic acid

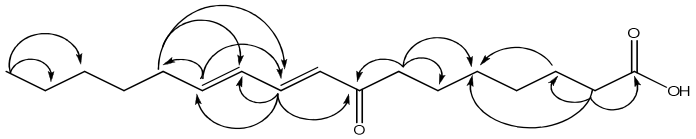

Figure 3. Key HMBC correlations of 8-oxo-(9*E*, 11*E*)-octadecadienoic acid

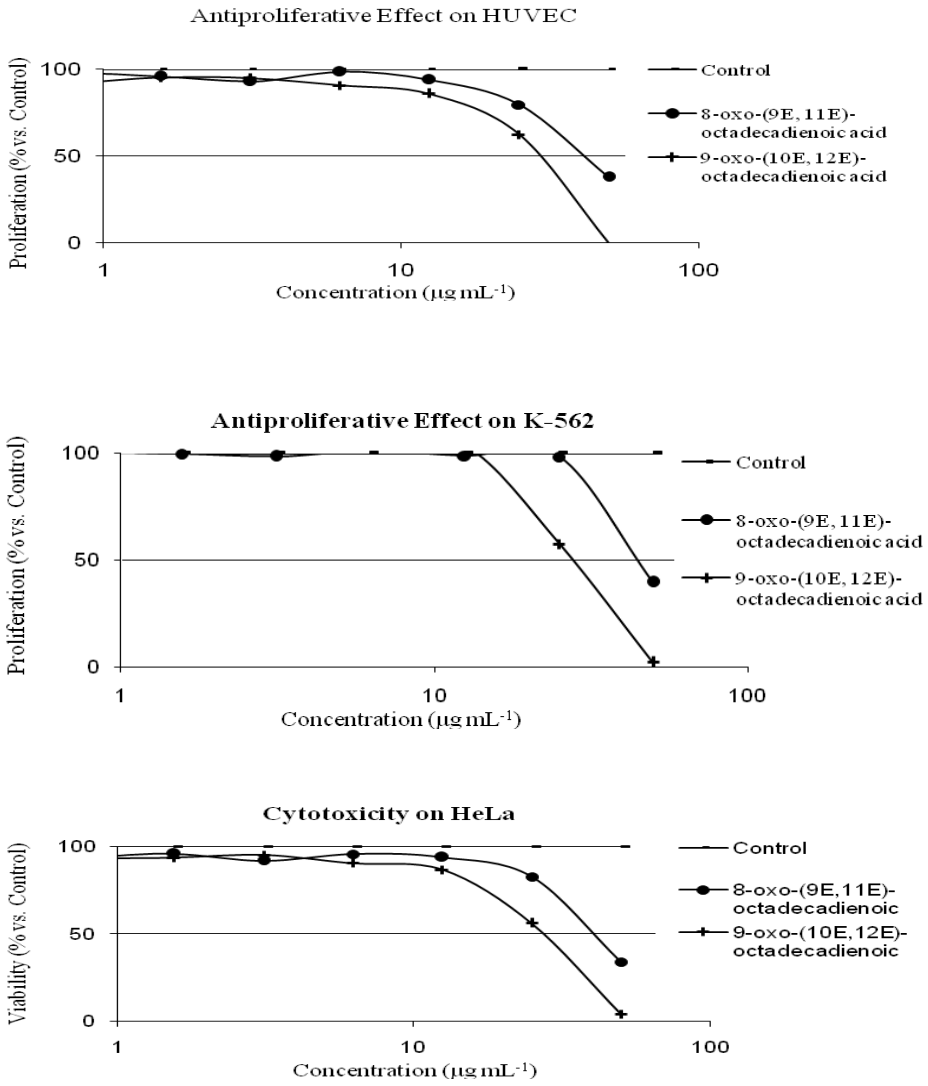

Figure 4. Antiproliferative and cytotoxic activities of 9-oxo-(10*E*, 12*E*)-octadecadienoic acid and 8-oxo-(9*E*, 11*E*)-octadecadienoic acid against HUVEC, K-562 and HeLa cell line

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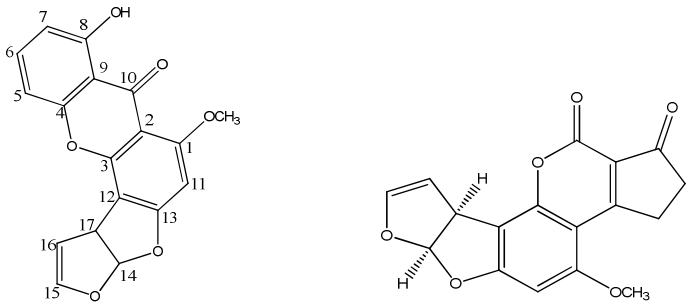

Sterigmatocystin Aflatoxin B1

Figure 5. Chemical structures of sterigmatocystin and aflatoxin B1

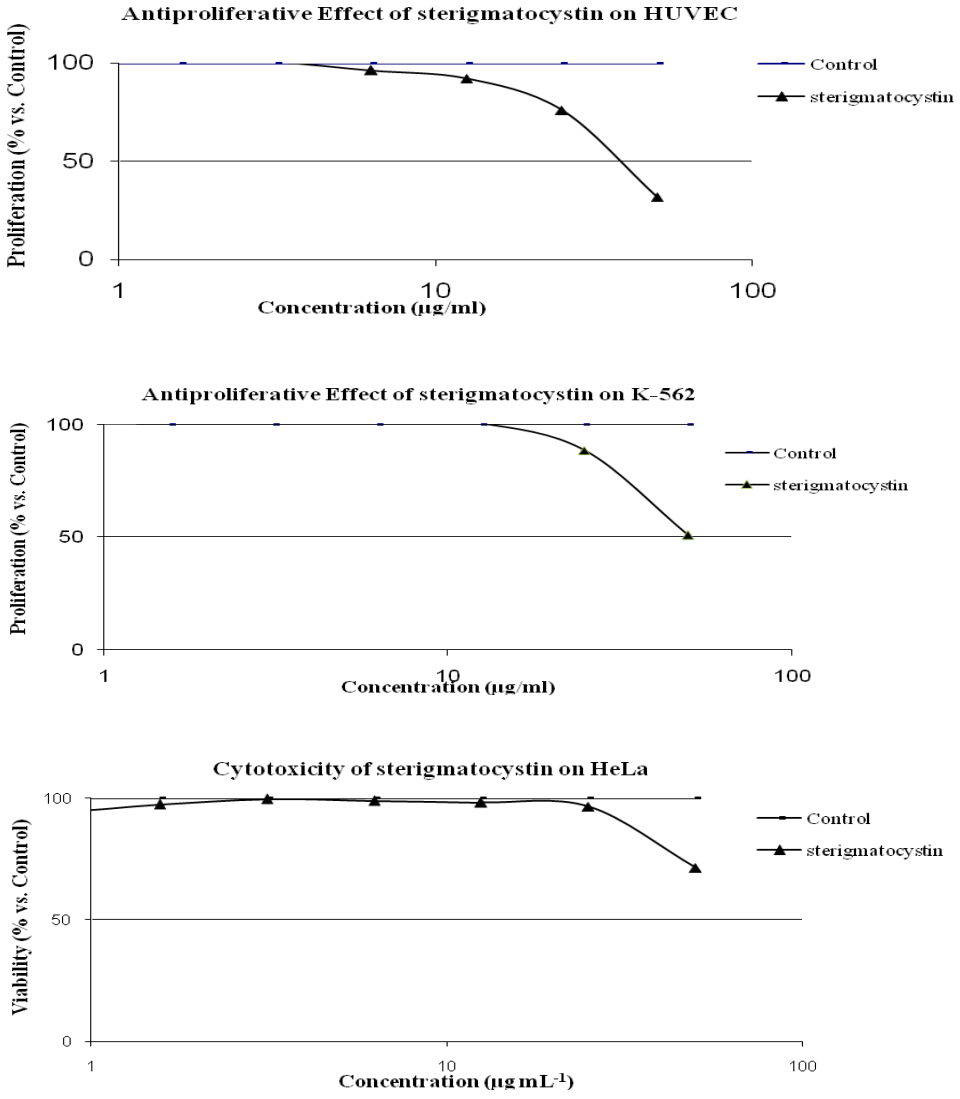

Figure 6. Antiproliferative and cytotoxic activities of sterigmatocystin on HUVEC, K-562 and HeLa cell lines

RESULTS

A molecular formula of $C_{18}H_{31}O_3$ (*m/z* 295.2056 $[M+H]^+$) was suggested by HRESIMS for the first isolated compound thus indicating four degrees of unsaturation. The ^{13}C NMR spectrum revealed the presence of 18 carbons two of which represented a carbonyl and a carboxyl group, respectively. The presence of two olefinic bonds was deduced from the appearance of signals at chemical shift values of δ 128.07, 142.78, 128.97, and 145.36 ppm in the ^{13}C NMR spectrum. The pattern of ^1H NMR chemical shifts of the four olefinic protons at δ 6.1 ppm (d, *J* = 15.5 *Hz*), 6.2 ppm (d, *J* = 15.5 *Hz*), 6.3 ppm (d, *J* = 15.5 *Hz*), 7.2 ppm (dd, *J* = 2.8, 8.8, 15.5 *Hz*) suggested the presence of a diene system in the compound and the coupling constants indicated a *trans* configuration of the olefinic protons being thus in clear agreement with a 10*E*, 12*E* diene system. The HMBC experiment showed correlations between H-2 and C-1, C-5 and C-6 as well as between H-10 and C-9 and C-12. Furthermore HMBC correlations were observed between H-11 and C-13 as well as between H-12 and C-11 which served to establish the structure as 9-oxo-(10*E*, 12*E*) octadecadienoic acids (Fig 1).

For the second compound a molecular formula of $C_{17}H_{29}O_3$ (as *m/z* 281.1055 $[M+H]^+$) was deduced from HRESIMS, indicating four degrees of unsaturation. The ^{13}C NMR spectrum revealed the presence of 17 carbon signals with one representing a carbonyl, a carboxyl and four sp^2 carbons. The NMR data (Table 1) of the compound showed great similarity to those of 9-oxo-(10*E*, 12*E*)-octadecadienoic acid, the only difference observed was the absence of one methylenic carbon signal. The presence of a diene system in the structure was supported by the appearance of four olefinic protons in the ^1H NMR spectrum. The pattern of ^1H NMR chemical shift values of the four olefinic protons and their coupling constants were also characteristic for a 9*E*, 11*E* diene system. The structure of the compound (Fig 2) was established by detailed 2D NMR spectroscopic studies including COSY, HMQC and HMBC experiments (Fig 3), which revealed the compound as a new oxylipin called 8-oxo-(9*E*, 11*E*)-octadecadienoic acid. A cytotoxic assay was performed for both compounds and revealed the first to be of cytotoxic activity against HeLa cell lines with a CC_{50} value of 43.4 µg mL^{-1} and cytostatic activity against HUVEC and K-562 cancer cell lines with GI_{50} values of 47.0 µg mL^{-1} and 43.2 µg mL^{-1} respectively (Fig 4). The cytotoxic assay performed on 8-oxo-(9*E*, 11*E*) octadecadienoic acid showed cytotoxicity against HeLa cell lines and also cytostatic activity against HUVEC and K-562 cell lines with a CC_{50} value of 30.3 µg mL^{-1} for the cytotoxic activity and a GI_{50} value of 27.8 µg mL^{-1} for the cytostatic activity against HUVEC and K-562 cell lines (Fig 4). Compared to the first oxylipin isolated from this strain 8-oxo-(9*E*, 11*E*)-octadecadienoic acid exerts higher cytostatic and cytotoxic activities. In the antiacterial assay performed against *B. subtilis* moderate antibacterial activity was observed for 9-oxo-(10*E*, 12*E*)-octadecadienoic acid (inhibition zone 24 mm at a conc. of 250 µg mL^{-1}), while 8-oxo-(9*E*, 11*E*) octadecadienoic acid showed higher antibacterial activity (inhibition zone 18 mm at a conc. of 250 µg mL^{-1}). For the third bioactive compound isolated a molecular formula of $C_{18}H_{13}O_6$ (*m/z* 325.2506 $[M+H]^+$) was determined by HRESIMS. The ^1H-NMR spectrum showed eight proton signals, a hydroxyl proton appearing at δ 13.28 ppm indicating the presence of a chelated phenolic hydroxyl group and a methoxy group at δ 56.88 ppm. The ^{13}C-NMR data revealed the presence of 18 carbon signals and thus together with the ^1H-NMR confirmed the suggested molecular formula. The HMBC correlations clearly revealed the first part of the structure as being a benzo-pyrone. The downfield shift of C-7 and C-2 indicates their connection to oxygen atoms and thus reveals this part of the structure as being a tetrahydrodifurano ring system. By comparing the obtained structure with literature data it was found to be the mycotoxin sterigmatocystin (Fig 5), which has been previously isolated from several *Aspergillus* species like *Aspergillus versicolor* and *Aspergillus multicolor* [11]. The xanthone nucleus attached to a bifuran structure closely resembles the aflatoxins and has

similarly been shown to be toxic to mice [12], rats [13] monkeys [14], ducklings [12] and is carcinogenic and mutagenic when injected or fed to rats [15]. A cytotoxic assay was carried out on sterigmatocystin to examine its cytotoxic activity against HeLa cell lines and its cytostatic activity on HUVEC and K-562 cell lines (Fig 6) where it proved to be of weak cytotoxicity and also weak cytostatic activity with CC_{50} and GI_{50} values of more than 50 µg mL^{-1}.

Table.1 Antiproliferative and cytotoxic activities of sterigmatocystin on HUVEC, K-562 and HeLa cell lines

Position	$\delta^{13}C$	$\delta^{1}H$ (*J* in *Hz*)	HMBC
1	174.9, qC		
2	33.8, CH_2	2.15, *m*	1, 3, 5
3	24.5, CH_2	1.45, *m*	5
4	28.0, CH_2	1.35, *m*	
5	28.5, CH_2	1.20, *m*	
6	23.9, CH_2	1.40, *m*	
7	40.0, CH_2	2.55, *m*	5, 6, 8
8	200.7, qC		
9	128.2, CH	6.10, *d* (15.5)	
10	143.2, CH	7.15, *dd* (2.7; 8.9; 15.5)	8, 11, 12
11	129.2, CH	6.26, *d* (15.5)	13
12	145.8, CH	6.25, *d* (15.5)	10, 13
13	32.6, CH_2	2.12, *m*	10, 11
14	28.5, CH_2	1.25, *m*	
15	31.0, CH_2	1.22, *m*	
16	22.1, CH_2	1.25, *m*	
17	14.1, CH_3	0.85, *m*	15, 16

DISCUSSION

The oxygenated derivatives of fatty acids, known as oxylipins to which two of the isolated secondary metabolites of *K. oryzae* belong, are important signaling molecules in animals and terrestrial plants [16]. In animal systems eicosanoids regulate cell differentiation, immune response and homeostasis. In contrast terrestrial plants use derivatives of C18 and C16 fatty acids as developmental or defense hormones. The oxylipin 9-oxo-(10*E*, 12*E)* octadecadienoic acid was previously found in the marine red alga *Chondrus crispus* and was found to be involved in induction of innate immunity of this alga [16]. Furthermore it was found in another study to have good activity against the bacterial plant pathogens *Phytophthora parasitica* and *Cladosprium herbarum* which is why it was suggested to contribute to plant protection not only by induction of defensive responses but also by direct antimicrobial activity in some cases [17]. More recently a study was conducted on the fungitoxic constituents of the basidiomycete *Gomphus floccosus* and detected 9-oxo-(10*E*, 12*E*)-octadecadienoic acids together with two other oxylipins as the active constituents with predominant antifungal activity against *Phomopsis* species [18].

Sterigmatocystin and cultures which are capable of its production have been detected in wheat [19] and coffee beans [20]. It belongs to the main 20 mycotoxins that are known to occur in foodstuffs at significant levels and frequency to

be of food safety concern [11]. These mycotoxins have been reported to be produced by five fungal genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* [11]. This is the first report of sterigmatocystin isolation from *K. oryzae.*

CONCLUSION

In conclusion, a fungal endophyte has been isolated from the important plant *B. bipinnata* and identified it as a *K. oryzae* strain. Through bioactivity-guided fractionation the isolation and full characterization of a new oxylipin which has not been isolated from a natural source before was accomplished. Furthermore, the fungus produces the oxylipin 9-oxo-(10*E*, 12*E*) octadecadienoic acid and the mycotoxin sterigmatocystin which have never been reported from *K.oryzae* before.

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REFERENCES

1. Verma, V.C., R.N. Kharwar, and G.A. Strobel, *Chemical and functional diversity of natural products from plant associated endophytic fungi.* Nat Prod Commun, 2009. **4**(11): p. 1511-32.
2. Strobel, G. and B. Daisy, *Bioprospecting for microbial endophytes and their natural products.* Microbiol Mol Biol Rev, 2003. **67**(4): p. 491-502.
3. Newton, G.G.F. and E.P. Abraham, *Cephalosporin C, a new antibiotic containing sulphur and D-_-aminoadipic acid.* Nature, 1955. **175**(4456): p. 548.
4. Grove, J.F., et al., *Griseofulvin. Part IV. Structure.* J. Chem. Soc., 1952: p. 3977-3987.
5. Horiuchi, M. and Y. Seyama, *Antiinflammatory and Antiallergic Activity of Bidens pilosa L. var. radiata SCHERFF.* J. Health Sci., 2006. **52**(6): p. 711-717.
6. Ming-mei Zhong1, F.-h.C., *Li-ping Yuan1, Xiao-hua Wang2, Fan-rong Wu1,Feng-lai Yuan1, Wen-ming Cheng, *Protective effect of total flavonoids from Bidens bipinnata L. against carbon tetrachloride-induced liver injury in mice.* Journal of Pharmacy and Pharmacology, 2007. **59**(7): p. 1017-1025.
7. Attia H. Atta, S.M.M., *Evaluation of some medicinal plant extracts for antidiarrhoeal activity* Phytotherapy Research, 2005. **19**(6): p. 481-485.
8. Broadbent, D.H., H. G.; Lehan, M., *Production of Griseofulvin by Khuskia oryzae.* Trans. Br. mycol. Soc., 1974. **62**(3): p. 625-626.
9. Afonin, S., et al., *4-fluorophenylglycine as a label for 19F NMR structure analysis of membrane-associated peptides.* ChemBioChem, 2003. **4**(11): p. 1151-1163.
10. Heinisch, L., et al., *Highly antibacterial active aminoacyl penicillin conjugates with acylated bis-catecholate siderophores based on secondary diamino acids and related compounds.* Journal of Medicinal Chemistry, 2002. **45**(14): p. 3032-3040.
11. Steyn, P.S., *Mycotoxins, general view, chemistry and structure.* Toxicol Lett, 1995. **82-83**: p. 843-51.
12. Lillehoj, E.B. and A. Ciegler, *Biological activity of sterigmatocystin.* Mycopathol Mycol Appl, 1968. **35**(3): p. 373-6.
13. Purchase, I.F. and J.J. van der Watt, *Acute toxicity of sterigmatocystin to rats.* Food Cosmet Toxicol, 1969. **7**(2): p. 135-9.

14. van der Watt, J.J. and I.F. Purchase, *Subacute toxicity of sterigmatocystin to rats.* S Afr Med J, 1970. **44**(6): p. 159-60.
15. Dickens, F., H.E. Jones, and H.B. Waynforth, *Oral, subcutaneous and intratracheal administration of carcinogenic lactones and related substances: the intratracheal administration of cigarette tar in the rat.* Br J Cancer, 1966. **20**(1): p. 134-44.
16. Bouarab, K., et al., *The innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways.* Plant Physiol, 2004. **135**(3): p. 1838-48.
17. Prost, I., et al., *Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens.* Plant Physiol, 2005. **139**(4): p. 1902-13.
18. Cantrell, C.L., et al., *Isolation and identification of antifungal fatty acids from the basidiomycete Gomphus floccosus.* J. Agric. Food Chem., 2008. **56**(13): p. 5062-8.
19. Scott, P.M., et al., *Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products.* J Agric Food Chem, 1972. **20**(6): p. 1103-9.
20. Purchase, I.F. and M.E. Pretorius, *Sterigmatocystin in coffee beans.* J Assoc Off Anal Chem, 1973. **56**(1): p. 225-6.