SUPPLEMENTARY MATERIAL

Anticancer activity of Ophiobolin A, isolated from the Endophytic fungus *Bipolaris setariae*

Dimple R. Bhatia [a]*, Payal Dhar [a], Varun Mutalik [b], Sunil Kumar Deshmukh [b], Shilpa A.Verekar [b], Dattatraya C. Desai [c] , Rajendra Kshirsagar [c], Padma Thiagarajan [d] and Veena Agarwal [a]

[a]*Department of Pharmacology, Piramal Enterprises Limited, Mumbai, Maharashtra, India;* [b] *Department of Natural Products, Piramal Enterprises Limited, Mumbai, Maharashtra, India;* [c] *Department of Chemistry, Piramal Enterprises Limited, Mumbai, Maharashtra, India;* [d] *School of Biosciences and technology, VIT University, Vellore, Tamil Nadu, India.*

*Corresponding author. *Email: dimplebhatia2009@gmail.com

The present work describes the anticancer activity of Ophiobolin A isolated from the endophytic fungus *Bipolaris setariae.* Ophiobolin A was isolated using preparative HPLC and its structure was confirmed by HRMS, ^{1}H NMR, ^{13}C NMR, COSY, DEPT, HSQC and HMBC. It inhibited solid and haematological cancer cell proliferation with IC_{50} of 0.4-4.3 µM. In comparison IC_{50} against normal cells was 20.9 µM. It was found to inhibit the phosphorylation of S6 (IC_{50} = 1.9 ± 0.2 µM), ERK (IC_{50} = 0.28 ± 0.02 µM) and RB (IC_{50} =1.42 ± 0.1 µM) the effector proteins of PI3K/mTOR, Ras/Raf/ERK and CDK/RB pathways respectively. It induced apoptosis and inhibited cell cycle progression in MDAMB-231 cancer cells with concomitant inhibition of signalling proteins. Thus, this study reveals that anticancer activity of Ophiobolin A is associated with simultaneous inhibition of multiple oncogenic signalling pathways namely PI3K/mTOR, Ras/Raf/ERK and CDK/RB.

Keywords: Ophiobolin A, *Bipolaris setariae*, PI3K/mTOR, Ras/ERK, CDK/RB

Experimental

Fungal identification

The endophytic fungus was isolated from fresh, healthy leaves of *Parthenium*

hysterophorus Linn. Family Asteraceae (Suryanarayanan et al. 1998) collected in September 2010 from Mumbai, India. The leaves were rinsed twice in sterile distilled water. Surface sterilization was done by immersing the leaves in 70% ethanol for 2 min (two times) followed by rinsing twice in sterile distilled water. The leaves were then cleaved aseptically into small segments (approximately 1 cm in length). The material was placed on 9 cm plates containing potato dextrose agar (PDA) supplemented with chloramphenicol (50 mg/L) to suppress bacterial growth and incubated at 25 ± 1 °C. After several days, hyphae growing from the plant material were transferred to fresh plates with PDA medium, and incubated for 10 days. The culture was periodically checked for purity and subsequently maintained on PDA slants. The culture was assigned the institutional (Piramal enterprises limited) code PM1055406.The fungus was identified as *Bipolaris setariae* using partial sequence analysis of the internal transcribed spacer region (ITS) of rDNA using ITS1 and ITS4 primers (White et al. 1990). A nucleotide to nucleotide BLAST (Altschul et al. 1990) query of the gene bank database (http://www.ncbi.nlm.nih. Gov/BLAST) recovered EF452444.1 *Bipolaris setariae* as the closest match to the ITS rDNA of PM1055406 (99%). Evolutionary analyses (Figure S5) were performed using MEGA6 (Tamura et al. 2013). The partial sequence of the ITS of rDNA Culture No. PM1055406 has been deposited with NCBI with the accession number (NCBI No. KP722593).

Isolation and characterisation of Ophiobolin A from *Bipolaris setariae*

The separated mycelium from fermented broth of PM1055406 was soaked in methanol for 12 h, filtered and the filtrate was concentrated using rotavapor at 45 ºC to yield 3G of crude extract. The crude extract was suspended in 500 mL demineralised water and partitioned with equal volume of ethyl acetate in a separating funnel and the procedure was repeated for three times. The organic layer was concentrated to get 1.2 g of the enriched ethyl acetate extract. The final purification to obtain ophiobolin A was achieved by using preparative HPLC (Column: Waters, X-bridge RP-18, 250 × 10 mm, 5µ). The mobile phase used was an isocratic mixture of acetonitrile and water (1:1) with a flow rate of 5mL/min. The isolated compound was analysed by analytical HPLC for purity (Column: Merck, Lichrosphere, RP-18, 125 × 4 mm; Mobile phase: water and acetonitrile, 98/0, 0/15, 0/20, 98/21, 98/25 (% water/time in min) and was confirmed as Ophiobolin A by different spectroscopic analyses like HRMS, ^{1}H NMR, ^{13}C NMR,

HSQC, COSY, DEPT and HMBC. The HRMS data showed the molecular ion peak at m/z 423 for M+Na corresponding to the molecular formula $C_{25}H_{36}O_4Na$. The spectroscopic data of the isolated compound (Figure S6) was compared with the reported spectral data for Ophiobolin A (Tsuna et al. 2011) and was found to be identical.

Cell lines and antibodies

Cancer cell lines A2780, PC3, MDAMB-231, MCF-7, MM1R, RPMI8226, U266B1 and Jurkat were obtained from the American Type Culture Collection (ATCC). Cell lines were cultured in ATCC recommended media supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37 ºC and 5% CO_2. Antibodies directed against pAKT (S473), AKT, pS6 (S235/236), S6, pERK, (Y42/44), ERK, cyclin D1 and actin were obtained from cell signaling technology. Peroxidase labeled anti-rabbit or anti-mouse secondary antibody were obtained from Santacruz biotechnology.

ELISA Assays

All ELISA assays were done in 96 well plates. For pS6 ELISA assay PC3 Cells were seeded at density of 5000 cells per well in RPMI1640 medium containing 10% FBS. After 24 h, cells were serum starved for 16 h. Serum starved cells were treated with Ophiobolin A for 2 h followed by serum stimulation for 30 minutes. At the end of incubation period, cells were fixed and probed with antibodies to pS6 (S235/236).For measurement of pERK levels, MDAMB-231 cells were seeded at a density of 25000 cells per well in RPMI 1640 medium containing 10% FBS. After 24 h, medium was replaced with RPMI1640 with 2% FBS and treated with Ophiobolin A for 1 h. At the end of incubation period, cells were fixed and probed with antibodies to pERK (Y42/44). pS6 (S235/236) and pERK (Y42/44) levels were detected using HRP substrate and plates were read at 490 nm using spectramax spectrophotometer (molecular devices, USA). For pRB assay, MDAMB-231 cells were seeded at a density of 20000 cells per well in RPMI1640 medium containing 10% FBS. After 24 h of incubation, cells were treated with Ophiobolin A for 24 h. At the end of the incubation period, cells were fixed and probed with antibodies to pRB (S780). pRB levels were detected with dylight 549 stained secondary antibody and the plates were read on

Cellomics Array Scan® VTI HCS Reader. IC_{50} value for all ELISA assays was determined by plotting percentage cell growth inhibition versus drug concentration using Graph pad Prism software (USA).

Western blot analysis

Cells were seeded at a density of $1X10^6$ and serum starved for 16 h. Serum starved cells were treated with the compound for 2 h or 24 h. Cells were lysed on ice with cell lytic buffer (Sigma C3228) supplemented with protease and phosphatase inhibitors. Proteins were separated on 10-12.5% SDS-PAGE gels and analysed by immunoblotting. Blots were probed with primary antibody for respective proteins at 4 ºC for 16 h. Peroxidase labeled anti-rabbit or anti-mouse antibodies (Santacruz) were used as the secondary antibody. Following incubation with secondary antibodies, proteins were detected using super west chemiluminiscence substrate (Pierce). Images were acquired on Bio-Rad gel doc system (USA). Densitometric analysis was done by Image J analysis software (USA). Statistical validation was done with Graph pad prism software. Data were compared by one-way ANOVA followed by Turkey test analysis for multiple comparisons. Differences with respect to control were considered statistically significant at $P < 0.05$ (n=3).

Cell growth assay

For cell growth effect assay, cells were seeded at a density of 3000 cells per well of appropriate medium containing 10% FBS in a 96 well white plate (Nunclon). After 24 h of incubation in humidified 5% CO_2 incubator at 37 ºC, cells were treated with Ophiobolin A for 48 h. At the end of incubation period, the plates were assayed for cell growth inhibition effect using propidium Iodide (PI) for solid tumor cell lines and MTS for haematological cell lines and human peripheral blood mononuclear cells. IC_{50} value was determined by plotting percentage cell growth inhibition versus drug concentration using Graph pad Prism.

Cell cycle analysis

MDAMB-231 cells were seeded in 6-well plates at a density of $0.5x10^5$ cells. After 16 h of incubation, cells were treated with varying concentrations of Ophiobolin A for 48 h.

After treatment, cells were trypsinised, washed and fixed in 70% ethanol in PBS and stored at 4 °C for 24 h. Cells were subsequently suspended in PBS and treated with 1µg/µL of RNAse A and 1µg/µL of PI in PBS for 45 minutes for staining of DNA. The cell cycle distribution was performed using a BD FACS Calibur flow cytometer and analyzed using Cell Quest software (BD Biosciences, USA).

Caspase-3/7assay

Caspase-3/7 activity was measured using Caspase-Glo 3/7 assay Kit according to the manufacturer's instructions (Promega, USA). MDAMB-231 cells were seeded at a density of 3000 cells/well in a 96-well plate. After 24 h of incubation, cells were treated with varying concentrations of Ophiobolin A for 48 h. At the end of treatment period, luminescence was measured to calculate caspase-3/7 activitiy.

Spectral Data

Ophiobolin A: Yellow solid

m.p: 167-170 °C.

^{1}H NMR (500 MHz, $CDCl_3$): 9.24 (1H, s), 6.83 (1H, dd, *J* = 6.5, 8.5), 5.10 (1H, d, *J* = 7.5), 4.47 (1H, ddd, *J* = 8.0, 8.5, 5.0), 3.17 (1H, d, *J* = 10.5), 3.14 (1H, br), 2.93 (1H, d, *J* = 19.0), 2.47 (1H, d, *J* = 17.0), 2.27–1.75 (10H, m), 1.71 (3H, d, *J* = 1.2), 1.62 (3H, d, *J* = 1.2), 1.53 (1H, m), 1.46 (1H, m), 1.24 (3H, s), 1.19 (1H, dd, *J* = 14, 12), 1.14 (3H, d, *J* = 6.5), 0.84 (3H, s).**^{13}C NMR** (300 MHz, $CDCl_3$): 216.9, 193.8, 163.3, 142.4, 136.8, 125.5, 94.1, 76.5, 71.0, 60.5, 54.8, 49.7, 48.4, 43.1, 42.5, 41.3, 35.1, 34.9, 31.5, 25.8, 25.7, 23.5, 18.3, 17.9, 17.7; HRMS (FAB) [M+Na]+ calculated for $C_{25}H_{36}O_4Na$: 423.2511, found: 423.2520.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215(3): 403-10.

Suryanarayanan TS, Kumaresan V, Johnson JA. 1998. Foliar fungal endophytes from two species of the mangrove Rhizophora. Canadian Journal of Microbiology. 44(10): 1003-1006.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.

Tsuna K, Noguchi N, Nakada M. 2011. Convergent Total Synthesis of (+)-Ophiobolin A. Angewandte Chemie International Edition 50 (4): 9452-9455.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics in PCR protocols. A guide to methods and amplifications. (eds.) Innis MA, Gelfand DH, Sninsky JJ and White TJ; Academic Press San Diego, CA, USA. 315-322.

Tables

	Activity at 1 µg/mL		
Sample	% pERK Inhibition	% pS6 Inhibition	% pRB Inhibition
Mycelial extract	71 ± 6.9	66 ± 7.6	60 ± 7.5
Aq. extract	10 ± 1.3	5 ± 0.7	12 ± 1.2
EtOAc extract	84 ± 7.1	75 ± 6.8	80 ± 6.9
Pure compound Ophiobolin A	91 ± 8.9	88 ± 9.6	85 ± 8.9
PI-103 (0.15 µM)	-	50 ± 4.9	-
Sorafenib (0.1 µM)	50 ± 8.3	-	-
Staurosporine (0.1 µM)	-	-	50 ± 9.7

Table S1: Bioactivity of isolated fractions at 1µg/mL. PI-103, Sorafenib and Staurosporin were used as assay standards for pERK, pS6 and pRB respectively at concentrations mentioned in the parenthesis.

Figures

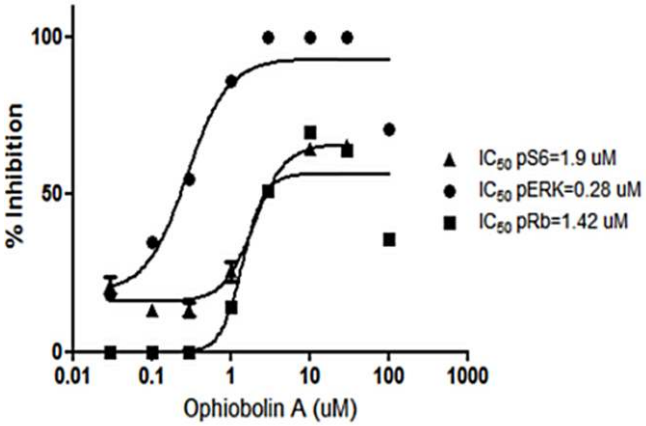


Figure S1 Effect of Ophiobolin A in pS6, pERK and pRB ELISA assays.

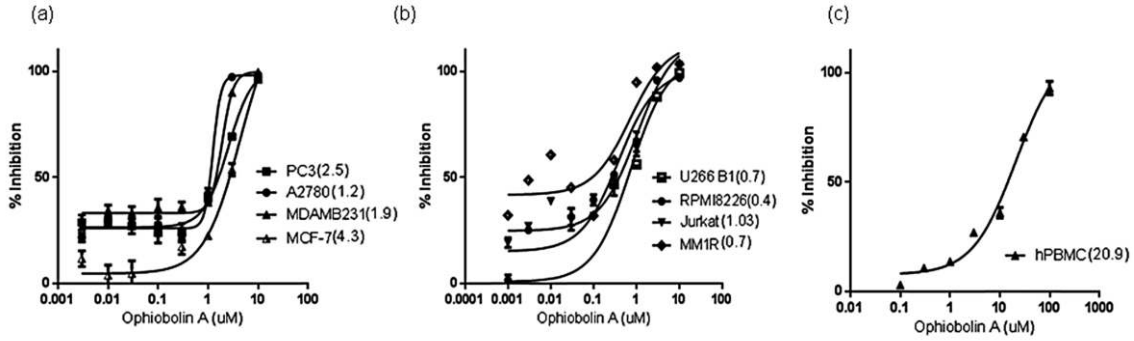


Figure S2 Cell growth inhibition activity of Ophiobolin A against (a) solid cancer (b) haematological cancer and (c) Normal cells. IC_{50} (μM) values are given in parenthesis.

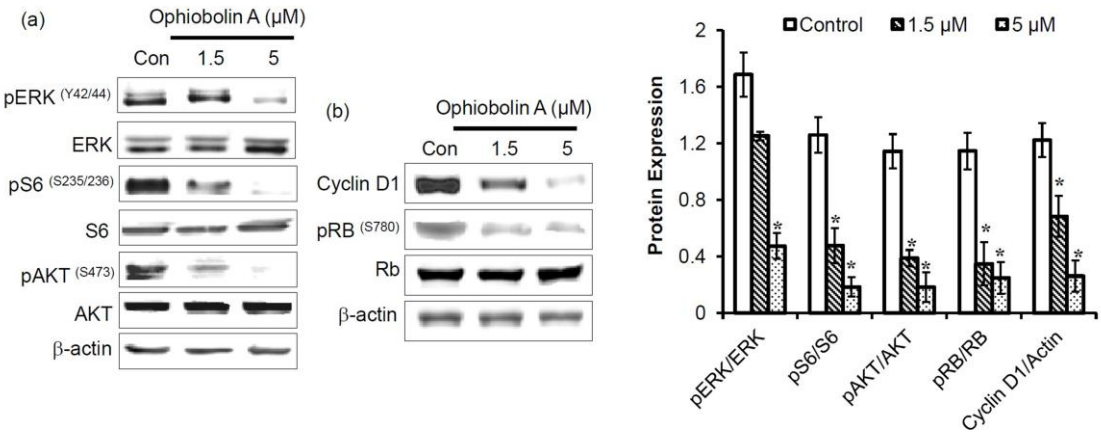
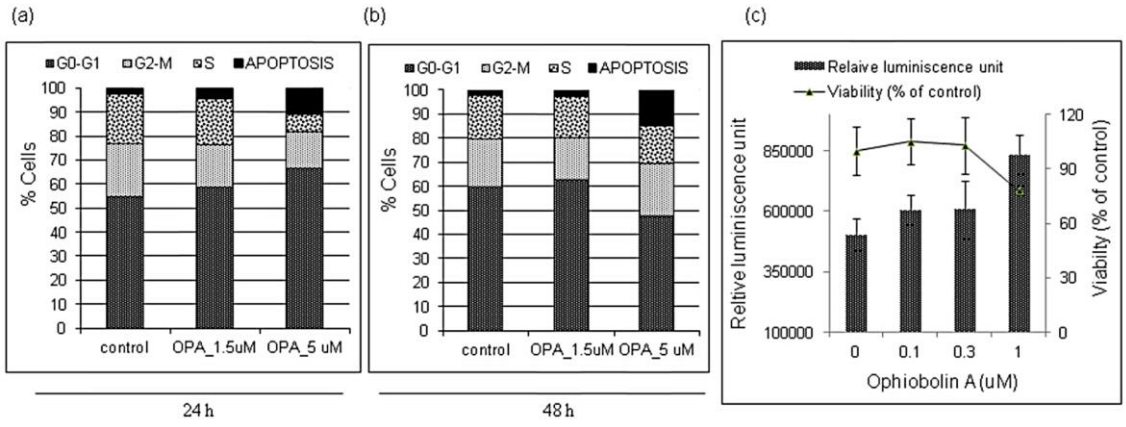


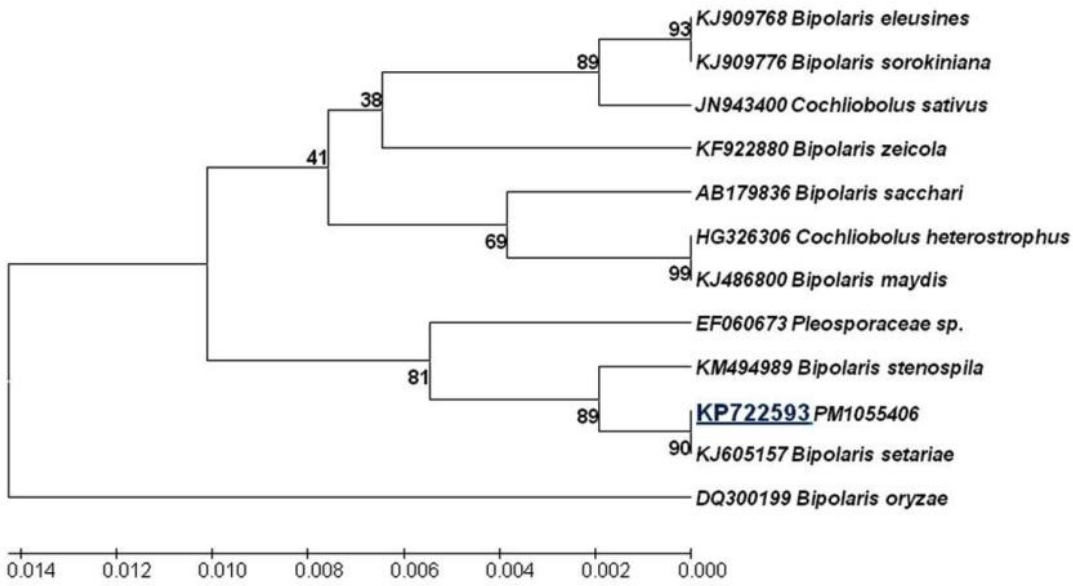
Figure S3 Effect of Ophiobolin A on oncogenic proteins in triple negative breast cancer cell line MDAMB-231 (a) 2 h and (b) 24 h. (c) Densitometry analysis results are shown as the mean ±SEM of 3 independent experiments.*, P<0.05.



176

177 Figure S4 Effects of Ophiobolin A on cell cycle kinetics (a) 24 h and (b) 48 h. (c)
178 Apoptosis induction in MDAMB-231 cells after 48 h.

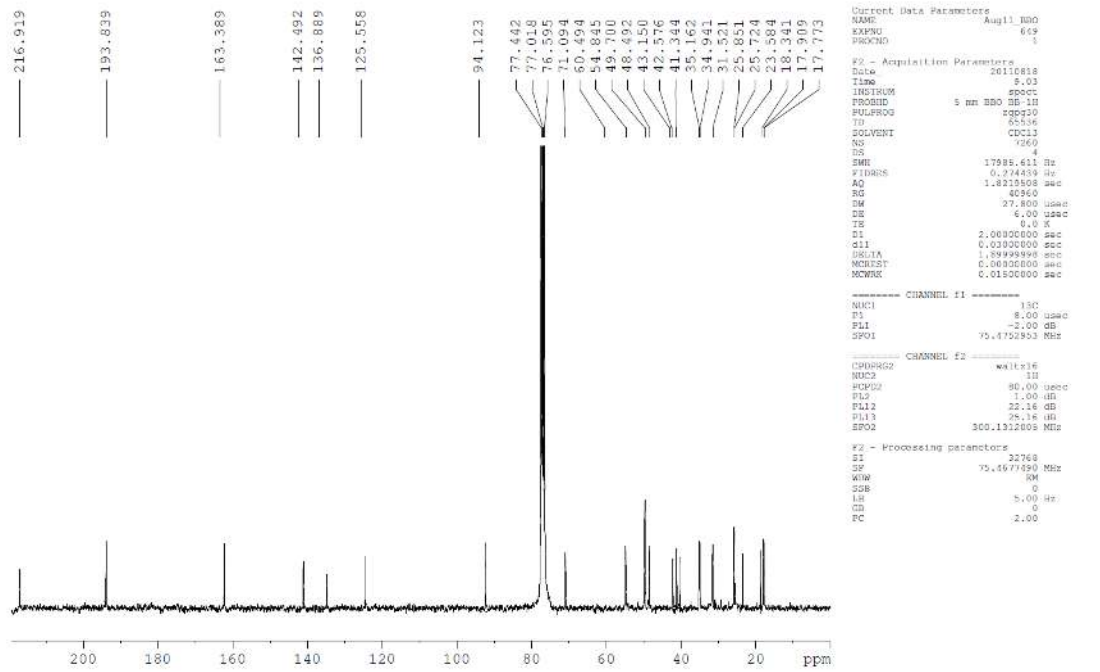
179



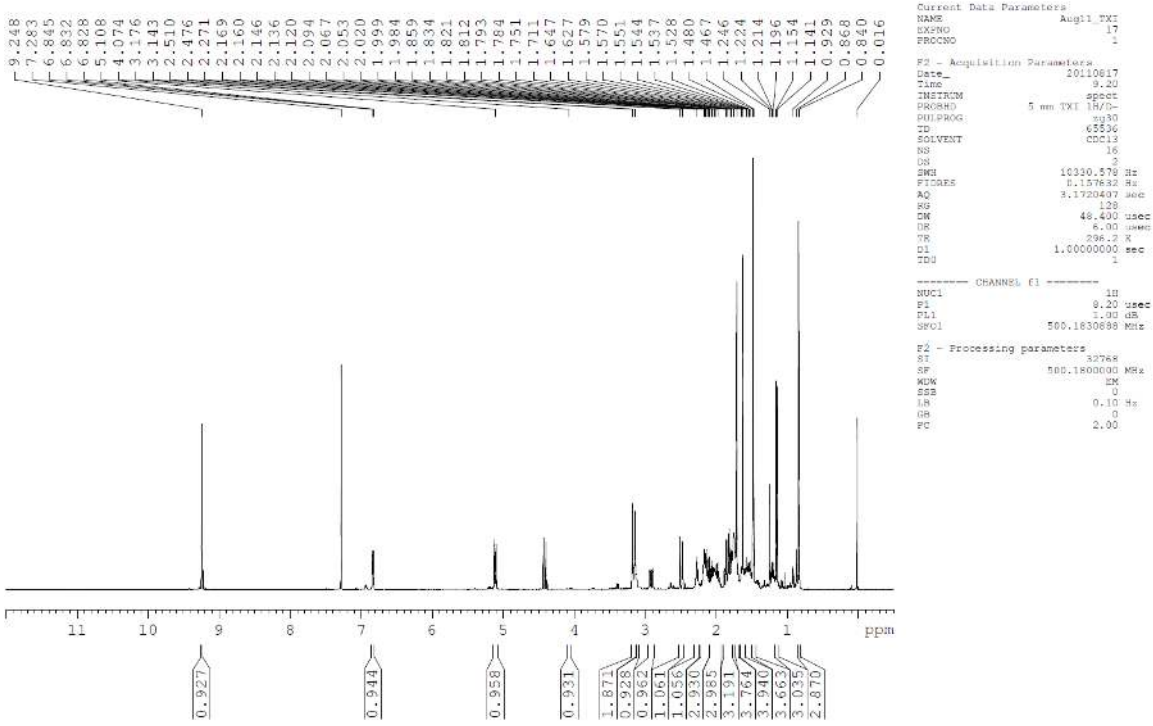
180
181 Figure S5 Phylogenetic tree based on ITS region sequences for PM1055406 isolate with
182 reference strains. Numbers at the respective nodes are percentage of 1000 bootstrap
183 replicates. Bar indicates genetic distance due to sequence variation.

184

185



186



187

188 Figure S6 ^{13}C NMR and ^{1}H NMR of Ophiobolin A.

189