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Bioactive Secondary Metabolites from *Arthrinium Phaeospermum* KM 668704, An Endophytic Fungus of The Medicinal Plant *Andrographis Paniculata* Corda M.B. Ellis

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Abstract: *An endophytic fungus was isolated from the leaves of the medicinal plant Andrographis paniculata. The fungus was identified as Arthrinium phaeospermum based on the rRNA sequence analysis, and was cultivated on rice solid medium. By bioassay guided fractionation, four secondary metabolites were isolated from A. phaeospermum KM 668704. In vitro antifungal, antibacterial assay showed that compound 2 displayed clear inhibition of 3 plant pathogenic fungi as well as 5 pathogenic bacteria. Compounds 1, 3, 4 exhibited only weak antifungal activity, and the chemical structure of compound 2 was determined to be 5-(1-Hydroxy-ethyl)-6-methyl-cyclopenta[b]furan-2-one on the basis of spectroscopic analyses.*

Keywords: *Endophytic fungus Arthrinium phaeospermum, secondary metabolites, Terpene, antifungal, antibacterial.*

1. INTRODUCTION

Endophytes are the microorganisms that colonize living, internal tissues of plants without causing any immediate negative effects (Bacon *et al*., 2000). They reside internal tissues of all healthy plants. Many substances found in plants were extracted from their endophytes (Azevedo *et al*., 2000). Through the endophyte - plant, interaction microorganisms can produce several substances of biotechnological interest, including bioactive secondary metabolites with pharmaceutical application (Strobel and Daisy 2003; Schulz and Boyle, 2005; Strobel, 2003; Strobel, 2006), being used in the production of antimicrobials that inhibit the development of pathogens (Chareprasert *et al*., 2006; Strobel *et al*., 1999; Weber *et al*., 2007). The studies are more focused on the Isolation and or application of antibiotic substances from endophytes of medicinal plants in recent times (Garcia *et al*., 2012; Visalakchi and Muthumary, 2009; Li *et al.*, 2005). The crude extracts of the endophytic fungus *A. phaeospermum* KM 668704 isolated from *Andrographis paniculata* showed antifungal and anti bacterial activities. We report the antibiotic compounds involved and the structure of one of the main antifungal and antibacterial compound as revealed by the spectroscopic data in this paper.

2. MATERIALS AND METHODS

2.1 Isolation and purification of the endophytic fungi

The endophytic fungus strain KM 668704 was isolated from leaves of *Andrographis paniculata* collected in Bhadrachalam forests, Khammam Dist. Telangana. The freshly collected leaf samples were washed thoroughly in running stream of tap water to remove the dust particles present on the surface of leaves. The leaves were surface sterilized by sequentially dipped into 0.5% sodium hypochlorite (2 min) and 70% ethanol (2 min), and rinsed with sterile water, then allowed to surface-dry under sterile conditions (Prathyusha *et al*., 2014). The leaf samples were cut into 2-5 mm and six of them were placed on each plate containing potato dextrose agar (PDA), Malt Extract agar (MEA) and Water Agar medium (WA). The Petri dishes were sealed with parafilm and incubated at 27C. After 1 week, the emerging

hyphae from segments were cut and transferred into new PDA Petri dish for purification. The isolated endophytic fungi were stored on PDA slants

2.2 Identification of the endophytic fungal strain KM 668704

One of the endophytic fungal strains (OU E- 38) was identified based on the morphology of the fungal culture colony, the characteristics of the spores, and reproductive structures using standard

identification manuals (Barnet & Hunter, 1998) and characterized with molecular methods. Further by fungal strain (OU E- 38) was characterized by amplifying the 18S rDNA using primers ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) and ITS 4 (with base sequences TCCTCCGCTTATTGATATGC) (White *et al.,* 1990). The rDNA was sequenced and the sequencing result obtained was pasted in Basic Local Alignment Search Tool (BLAST) software supported by National Institute of Health (NIH) using the National Centre for Biotechnology Information (NCBI) database (http//: www. NCBI.NLM.NIH.GOV) to compare and align with the sequences of known fungi. The rDNA sequence was deposited with NCBI and the accession number assigned was KM 668704

2.3 Fermentation and metabolites isolation

The endophytic fungus, *Arthrinium phaeospermum* KM 668704 was grown on rice solid medium for the extraction of secondary metabolites. It was carried out by transferring fresh fungal cultures into 1L Erlenmeyer flasks containing 100 g rice medium (100 g rice in 100 ml distilled water). The cultures were then incubated at room temperature for 30 days (No shaking). After incubation period, 250 ml ethyl acetate (EtOAc) was added to each flask with culture and left overnight. Culture media were then cut into pieces to allow complete extraction and left for 3–5 days. After filtration, the culture filtrate was extracted three times with equal volumes of EtOAc. The solvent phase and aqueous phase were separated by separating funnel. Solvent phase was collected and concentrated using rotary vacuum evaporator (150 rpm; 25C). The crude metabolite (3.50 g) is mixed with 7 g silica gel, dried at 50C, and then loaded on a silica gel column containing 200 g silica gel (200–300 mesh). Solvent system consisting of *n*-Hexane, EtOAc (80:20) was used for elusion. Fractions were collected in 20 ml conical flask with a flow rate of 2-3 drops per minute. A total 120 fractions, each consisting of 10 ml were collected. TLC of each fraction was analyzed for purity. From the above fractions a total of four compounds were isolated (Fig. 1).

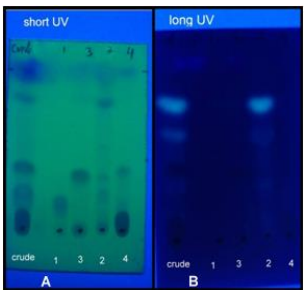


Figure1. *Chromatogram of A. phaeospermum (A irradiated at 254 nm, B when irradiated at 366 nm).*

2.4 Structure elucidation

Structures were elucidated using nuclear magnetic resonance (NMR) techniques and various mass spectrometry (MS) methods like LC-MS and Infrared (IR) spectra.

H^1 NMR and ^{13}CNMR spectra were performed on 400 MHz and 100 MHz Bruker Ultra shield (Avance-ш) Nano Bay spectrometer. All the spectra were recorded at 298 K. H^1 NMR data are reported as follows: s: singlet, d: doublet, t: triplet, bs: broad singlet. Infrared spectra were obtained employing Bruker FT-Infrared, Tensor-27 using KBr pellets.

2.5 Antimicrobial Activity

Test organisms include five bacterial and three fungal pathogens. Gram-positive bacteria: *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus* and Gram-negative bacteria: *Escherichia coli*. The plant pathogens are *Fusarium oxysporum*; *Colletotrichum dematium* and *Macrophomina phaseolina* were evaluated for the antimicrobial activity.

3. RESULTS AND DISCUSSION

The genomic DNA of the fungus isolated from the leaves of *Andrographis paniculata*, was amplified by internal transcribed spacer ITS1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) primers to obtain rRNA sequences was subjected to BLAST analysis for alignment and compared with NCBI database. on phylogenic tree analysis the rRNA sequence was deposited in NCBI database and the accession number given is **KM668704**. The rRNA sequences up on BLAST analysis for alignment and comparison with NCBI database, **OU E 38 (KM668704)** is showing close similarity (99%) with *Arthrinium phaeospermum* (Fig. 2).

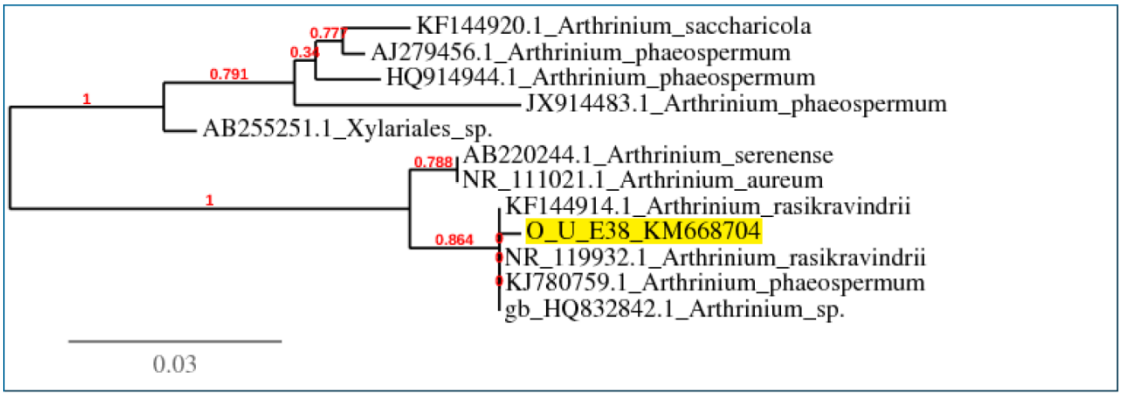


Fig2. *Phylogenetic tree of the endophytic fungus, Arthrinium phaeospermum inferred by neighbor-joining analysis of 18S rDNA sequences.*

The EtOAc extract of the fermentation culture was isolated and purified by column chromatography, yielded four compounds. The bioactive compounds were identified by chromatographic (TLC, HPLC) and spectroscopic Techniques (LC-MS and NMR).

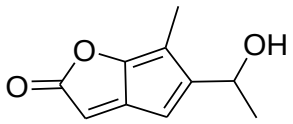
Compound 2

IR Cm^{-1}: 3383, 2971, 2930, 2865, 1788, 1738, 1459, 1375, 1248, 1167, 1124, 1046, 951.

^{1}H-NMR (400 MHz, $CDCl_3$**): δ** 8.3 (S, 1H), 6.5(S, 1H), 6.3 (S, 1H), 5.6(quartet, 1H), 1.62 d, 3.7 H) 1.25(S, 4.3H).

^{13}CMR (100.6 MHz, $CDCl_3$**) δ**: 177.7, 165.5, 161.9, 148.9, 132.5, 129.8, 100.0, 98.2.75.01, 17.817.

The number of carbons derived from the intensity of m+1 peak, 11.7% when the intensity of molecular ion is 100% at m/z 178 amu, are ten. Based on mass and IR spectra, the molecular formula of the compound 2 is tentatively assigned as $C_{10}H_{10}O_3$. Based on the ^{1}HNMR and ^{13}C NMR data the proposed structure for the compound 2 is **5-(1-Hydroxyethyl)-6-methyl cyclopenta [b] furan-2-one (Fig.3).**



$C_{10}H_{10}O_3$; Mol. Wt.: 178

5-(1-Hydroxyethyl)-6-methyl-2*H*-cyclopenta[*b*]furan-2-one

Fig3. *Structure of compound 2 obtained from solid state fermentation of endophytic fungus A. **phaeospermum.***

Compounds based on 1, 3 and 4 exhibited IR spectrum similar to that of Compound 2. As the fractions 1, 3 and 4 were not purified their structure is not investigated. Based on IR spectrum of slightly impure compounds they may be identified as **lactones** similar to that of fraction 2. Compound 2 is identified as a bicyclic monoterpinoid γ-lactone. Compounds 1, 3 and 4 may also be monoterpinoid γ-lactones.

Compound 2 showed much antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli* and also showed inhibition against three pathogenic fungi i.e. *Fusarium oxysporum*; *Colletotrichum dematium* and *Macrophomina phaseolina* (Table-1, Fig. 4,5)

Table1. *Antimicrobial activities of pure compounds isolated from A. phaeospermum*

Compound name	Zone of inhibition by test compound (mm)				
	B. c	B. s	E. coli	S. a	P.a
1	6.0	7.5	0	3.5	0
2	5.5	10	6.0	10	3.5
3	6.0	0	0	0	0
4	7.5	5.5	6.0	4.0	0

B. c= *Bacillus cereus*, B. s= *Bacillus subtilus*, E. coli= *Escheresia coli*,

S. a = *Staphylococus aureus*, P.a =*Pseudomonas aeruginosa*

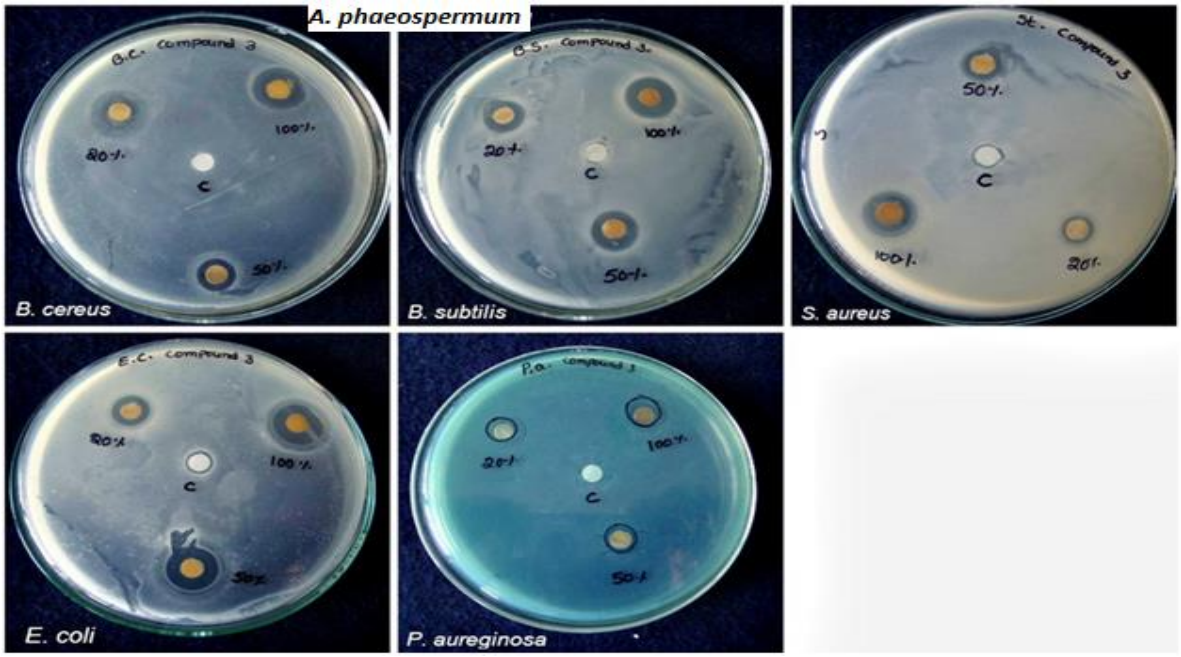


Fig4. *Antibacterial activity of compound 2 obtained from solid state fermentation of endophytic fungus*

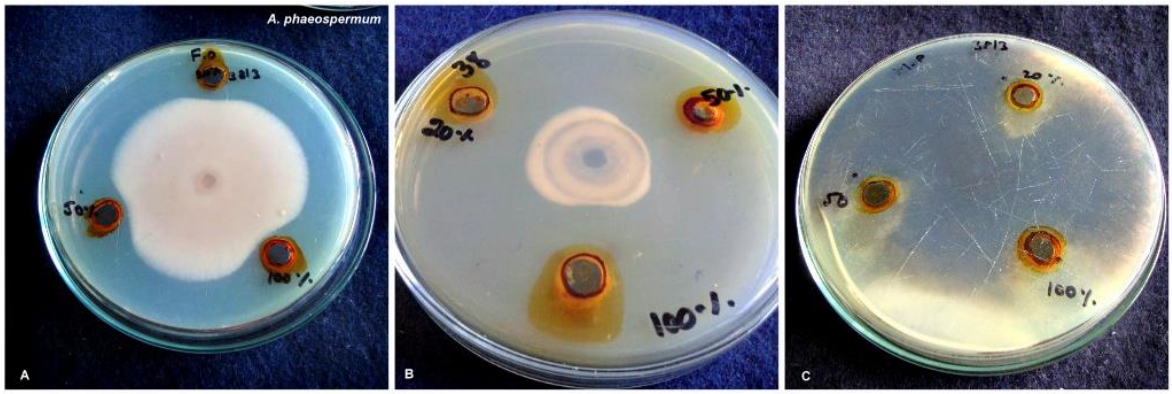


Fig4. *Antifungal activity of compound 2 obtained from solid state fermentation of endophytic fungus A. phaeospermum isolated from A. paniculata (A= F, oxysporum, B= C. dematium C= M. phaseolina).*

4. DISCUSSION

Ethyl acetate extracts of *Arthrinium* state of *Apiospra montagnei* have shown high levels of antimicrobial activity against *E. coli*, *P. aeruginosa* and *A. fumigatus* on sucrose supplemented Czapek dox medium (Ramos and Said, 2011). Rosa *et al.* (2010) reported cytotoxic effects of crude extracts of endophytic *Arthrinium* strains against human cancer cells.

The endophytic fungus *A. phaeospermum* isolated from *A. paniculata* has effectively inhibited all the five bacterial pathogens and most of the fungal pathogens in this study. The results of the present study

have reiterated the earlier findings with reference to the effective solvent ethyl acetate as it has shown high levels of antibacterial and antifungal activity.

In the present study also the secondary metabolites extracted in crude form have shown antimicrobial activity against bacterial pathogens, such as *B. subtilis, B. cereus, and S. aureus.* The crude extract of *A. phaeospermum*, after purification, yielded 4 compounds. Based on chromatographic and spectroscopic similar spectra data, their super imposability suggests that all the four metabolites belong to the same class of secondary metabolites. These were tentatively identified as hydroxy lactones. Natural products from endophytic fungi have a broad spectrum biological activity and grouped into several categories, including alkaloids, steroids, terpenoids, isocoumarins, quinines, phenylpropanoids and lignans, phenol and phenolic acids, aliphatic metabolites, lactones etc. (Zhang *et al*., 2006). Brady *et al*. (2001) reported guanacas terpenes, a highly diverse family of diterpenoid natural products from an unidentified endophytic fungus CR115. A novel antibiotic, phomol was isolated from a species of *Phomopsis* in the medicinal plant *Erythrina cristagalli* (Weber *et al*., 2004). Dai *et al.* (2005) have isolated six novel compounds from a fungal endophyte *Phomopsis* sp. on *Adenocarpous foliosus.*

An important growth regulator, Gibberellin, a diterpene, has been identified in the endophytic *Arthrinium phaeospermum* KACC43901 (Khan *et al*., 2009) isolated from the roots of *Carex kobomugi*. The secondary metabolites of endophytic fungus *Pestalotiopsis virgatula* VN2 have shown strong cytotoxic activity against MCF-7 and MDAMB-231 cell lines. The fraction E produced an effective antibacterial activity against multidrug resistant *S. aureus* (Arivudainambi *et al*., 2014). The compound 2 isolated from *A. phaeospermum* in this study, has been identified as a monoterpenes, hydroxy lactone and it has shown a broad spectrum antimicrobial activity like other diterpenoid guanacasterpenes against *S. aureus* and others.

5. CONCLUSION

On the isolation of Endophytic *Arthrinium phaeospermum* forms a new report from *Andrographis paniculata*. The compounds from the strain **KM668704** displayed strong or weak antifungal and antibacterial activity, which indicated that endophytic *Arthrinium phaeospermum* as a promising source of natural bioactive and novel metabolites with great potential for further study.

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