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Bioactivity of Fungal Endophytes associating with *Allium* Plants growing in Uzbekistan

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ABSTRACT

Keywords

Allium species, endophytic fungi, cytotoxicity, antibacterial activity, antidiabetic activity.

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Sixteen endophytic fungi were isolated from endemic *Allium* species which were collected from selected locations in Uzbekistan: south-western KyzylKum, Kashkadarya region, Pap hills, foothills of Kuramin range, foothills of the Nurata and Chatkal nature reserves. The isolates were tested for antibacterial, antidiabetic and cytotoxic activity. Obtained data showed that endophytes produce compounds with antibacterial, antidiabetic and cytotoxic properties that indicate the potential of endophytic fungi associated with *Allium* plants as a source of new secondary metabolites with therapeutic value.

Introduction

Endophytes are the microorganisms that live in the intercellular space of a healthy plant tissues without causing symptoms of disease (Padhi *et al.*, 2013; Strobel and Daisy, 2003). The symbiosis between plants and microorganisms is well known. In particular, the plant protects and feeds the endophyte which in response produce bioactive substances that enhance the growth and resistance of plants in environment (Nair and Padmavathy, 2014). Moreover, some endophytic microorganisms are characterized by their ability to synthesize the same metabolites as the host plant.

So, systematic investigation of endophytic fungi associated with different plants is necessary, and will not only provide with genetic information, but also may allow for new natural products with higher bioactivity to be found with potential application in medicine, agriculture and industry (Gutierrez *et al.*, 2012; Pimentel *et al.*, 2011).

The genus *Allium* which includes garlic (*Allium sativum*), leek (*A. porrum*), onion (*A. cepa*), shnit-onions (*A. schoenoprasum*) consist of more than 700 other species that have been described in Afghanistan,

Kazakhstan, Kyrgyzstan, Pakistan, Tajikistan, Turkmenistan, Uzbekistan, and Northern Iran (Augusti, 1996; Brewster, 2008; Maaß and Klaas, 1995). Ancient Chronicles reports about common onion (*Allium cepa* L.) and garlic (*A. sativum* L.) are coming out of this area to Europe during the times of antique civilization. Onion and garlic possess antidiabetic, antibiotic, hypocholesterolemic, fibrinolytic and other properties. Except for industrial crops, the local people use in diet a number of wild species but their therapeutic purposes are rarely reported (Jin-long *et al.*, 2011; Keusgen *et al.*, 2006; Navruzshoev, 1994). Research on endophytes from plants of the family Allium is a few. So, endophytic fungi isolated from garlic has a pronounced inhibitory effect on phytopathogens *Rhizoctonia solani* and *Botrytis cinerea* (Khassanov, 1996).

Several rare wild species of this plant inhabit in the territory of Uzbekistan (Keusgen *et al.*, 2006; Rabinowitch and Currah, 2002). A systematic study of endophytic microorganisms as potential sources of new compounds relevant to the assessment of bioactivity of secondary metabolites, thus studies of endophytes of Allium plants are of great interest. In this context, the aim of this work was the isolation of endophytic fungi from two endemic Allium species and characterization of their antibacterial, cytotoxic and antidiabetic activity.

Materials and Methods

Study Area and Material Sampling

Plant material was collected from March to May 2012, 2013 and 2014 on the territory of the south-western KyzylKum (foothills of Mount Kulzhuntog), Kashkadarya region, Pap hills, foothills of Kuramin range (Uygurca), foothills of the Nurata and

Chatkal nature reserves and kindly provided by Dr. Tojibaev (Institute of Flora and Fauna Gene Pool of the Academy of Sciences RU). All plants were kept at a temperature of +40°C. All plants were preserved at +4°C temperature. Plant samples were identified and stored in a herbarium.

Isolation of Endophytic Fungi

Endophytic fungi were isolated by the method as described previously by Hazalin *et al.* (Hazalin *et al.*, 2009). Roots, stems and leaves were respectively washed in tap water, sterilized in 70% ethanol for 1 min followed by 0.1% HgCl₂ for 7 min, rinsed three times in de-ionized water, cut into segments approximately 5 mm in diameter and placed in 90 mm Petri dishes containing Czapek-Dox agarized medium with 50 mg/ml chlortetracycline and 250 mg/ml streptomycin sulfate to inhibit bacterial growth. The plates were incubated for 7-14 days at 28 °C. Different mycelia growing out of the segments were sub-cultured and individually maintained on antibiotics-free Czapek-Dox-agar medium. Colony morphology and growth and spore formation of the isolates were then studied on Potato-Dextrose-agar medium.

Endophytic Fungi Identification

Isolated strains were identified by classical methods on the basis of morphology using pertinent monographs (Litvinov, 1967). Isolated strains were deposited at the Institute of Microbiology of the Uzbekistan Academy of Sciences where they were maintained at +4 °C.

Fermentation

To accumulate biomass for further extraction and determination of biological activity, endophytes were grown by

submerged fermentation in 500 ml flasks containing 100 ml of Chapek-Dox liquid medium for 5 days at 28°C.

The Extraction of Secondary Metabolites of Endophytic Fungi

To determine the antibacterial and antifungal activities, metabolites from biomass were extracted by methods as described previously by Lang *et al.* (Lang *et al.*, 2005) with modifications described previously by Hazalin *et al.* (Hazalin *et al.*, 2009). 5 g of biomass of each isolate was milled in a Potter homogenizer, transferred to a cone flask containing 50 ml of ethyl acetate, and left for extraction at night on a shaker at room temperature. The mixture was filtered through filter paper (Whatman #1) and Na₂SO₄ (40 µg/ml) was added. After the filtration, the extract was striped to dryness on a rotary evaporator and mixed with 1 ml of dimethyl sulfoxide (DMSO). The resulting extract was used as a stock solution and stored at +4 °C.

Antimicrobial Assay

The fungal extracts were screened using the agar diffusion method for antimicrobial activity against potentially pathogenic bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Antimicrobial activity was assessed by the size (diameter in mm) of the inhibition zones. Gentamicin sulphate at a concentration of 10 µg/ml was used as standard, while sterilized water was used as the negative control. Each inhibition experiment was repeated three times. Testing cultures were grown on beef-extract agar for bacteria ("HiMedia", India). The Petri dishes with corresponding nutrient solutions were inoculated with daily testing culture suspension in physiological solution with a concentration of 1x10⁶, dried, cut 5

mm diameter wells in agar, embedded with 300 l of extract and incubated at a temperature of 37°C for 24 48 hours.

Cytotoxicity Assay

To evaluate the cytotoxic activity of the extracts there were used verified cultures of cancer cells carcinoma of the cervix (HeLa), larynx (HEp-2) obtained from the Bank of cell cultures of the Institute of Cytology RAS (Saint-Petersburg, Russia), and primary culture of healthy hepatocytes. The growth of cancer cells was determined according to a previously described protocol (Mossman, 1983) by the ability of viable cells to reduce yellow staining of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) with the formation of blue formazan. For MTT assay cells were washed in phosphate buffer and collected by trypsinization, were placed in a 96 well cell plate, incubated, and treated with various concentrations of extracts -100, 10 and 1µg/ml, stock solutions of the extracts were diluted with culture medium to the final concentration of DMSO of 0.1%. After 72 hours incubation, the medium in each well was replaced by MTT solution (5mg/ml in phosphate buffer), cups were incubated for 4 hours under 5% CO₂ and 95% air at 37 °C. Then MTT reagent was removed and the formazan crystals produced by viable cells were dissolved in DMSO and gently shaken. The absorption was determined at 492 nm. Experiments were repeated three times. The percentage growth inhibition was calculated using following formula: % cell inhibition = 100-[(At-Ab)/(Ac-Ab)]x 100, where At - absorption of the sample, Ab - absorption of the blank and Ac - absorption of the control. The effects of the extracts were expressed by IC₅₀ values (the concentration of a substance that reduces the absorption of the treated cells by 50% with respect to

untreated cells). "Cisplatin" (India) was used as the comparison drug, intact untreated cells was used as the control.

α -Amylase Inhibition Assay

100 μ l of the porcine pancreatic amylase (PPA) solution (1.1 U) in 0.1 M Na-acetate buffer pH7.2 was added to 100 μ l of the extract and incubated at 30 °C for 10 minutes. The reaction was initiated by the addition of 100 μ l of 1% soluble starch solution and incubated at 37°C. The reaction was arrested after 10 min of incubation by the addition of 5 ml of iodine color reagent and absorbance was measured at 630 nm. For the preparation of iodine reagent 0.5 g of crystalline iodine and 5 g of potassium iodide were dissolved in 250 ml of water; for a working solution of 2 ml of this reagent was adjusted to 100 ml by 0.1 M HCL. Acarbose was used as the standard PPA inhibitor, and solution of PPA without extract sample was used as negative controls. All the assays were carried out in triplicates and average percent inhibition of enzymes by the fungal extract was calculated using the following formula: $(A_0 - A_t)/A_0 \times 100\%$, where A_0 - absorption of control sample, A_t is the absorption of test sample, respectively.

Result and Discussion

About 200 different *Allium* species were reported for the mountainous regions of Middle and South-West Asia (Hazalin *et al.*, 2009). We have investigated endophytic fungi inhabited in endemic species *A. filidens* Regel and *A. longicuspis* Regel.

A. filidens Regel (piozi diona) refers to the section *Allium* of the subgenus *Allium* (Khassanov, 1996). Although *A. filidens* taxonomically quite distant from garlic, it is often used in the same way for medicinal purposes, in particular, the bulbs of this

plant the locals used for headaches (Keusgen *et al.*, 2006). *A. longicuspis* Regel, collected mainly in Central Asia, presumably is the ancestor of garlic (Khassanov, 1996).

As shown in table 1, 16 isolates of endophytic fungi in total were obtained from these plants. Of these, 7 isolates classified as *Aspergillus*, 3 isolates – as *Penicillium*, 3 isolates - as *Alternaria*, 2 isolates - as *Fusarium*, 1 isolate - as *Cladosporium*. The most number of isolates obtained from bulbs of *A. filidens* and leaves of *A. longicuspis*. The dominant endophytic fungi in *A. filidens* are *Penicillium* representatives, while in *A. longicuspis* various species of *Aspergillus* are dominated.

Three isolates obtained from bulbs and buds, belong to the rare species - *Alternaria tenuissima*, *Cladosporium tenuissimum* and *Aspergillus spectabilis*, firstly observed in the conditions of Uzbekistan. There is information about *Alternaria tenuissima* isolated from the desert plant *Tribulus terrestris* L. producing enterotoxin with immune modulating and cytotoxic activity (Bashyal *et al.*, 2014; Navruzshoev, 1994; Wu *et al.*, 2014).

Recent studies indicate that many compounds produced by endophytes possess the antimicrobial activity. It is evident that the use of antimicrobial substances of endophytes is a promising way to overcome the increasing resistance of plants and human pathogens. Antimicrobial metabolites isolated from endophytes belong to diverse structural classes, including alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids (Pimentel *et al.*, 2011; Shentu *et al.*, 2014; Yu *et al.*, 2010).

Study of antibacterial activity of endophytes from *Allium* using the test cultures *E. coli*,

S. aureus and *P. aeruginosa*, showed that 13 from 16 isolates of endophytes produce metabolites inhibiting the growth of pathogens in the range from 2 to 10 mm (Table 2). Moreover, 5 strains of endophytes exhibit antibiotic activity against two pathogens, the strains *Alternaria* sp. – AL136L and *A. versicolor* – AL140R isolated from *A. longicuspis*, and *Penicillium* sp. - AF106 from *A. filidens*, are able to suppress the growth of three pathogens simultaneously. Although the antibacterial activity is observed mainly in the endophytes of *A. longicuspis*, the largest zone of inhibition observed for extract of *Penicillium* sp. - AF106 isolated from *A. filidens* (Fig. 1).

Thus the study of inhibiting properties of *Allium* associated endophytes against bacterial pathogens revealed their promising antibacterial potential.

Antidiabetic properties of endophytes were studied by in vitro inhibition of pancreatic α -amylase as target enzyme. The results showed that endophytes isolated from *A. filidens* produce compounds with inhibitory activity. As can be seen from the presented data (Fig.2), in the range of concentrations 5-100 mg extracts of *A. terreus* - AF104S isolated from the stem, *Penicillium* sp. - AF106 and *Penicillium* sp. - AF120 inhabiting plant bulbs, inhibited amylase activity very similar to inhibition by acarbose.

Determination of half-maximal inhibitory concentration of extracts also showed values close to IC₅₀ of acarbose (Fig.3). Moreover, the data suggests that the strain *A. terreus* - AF104S produces compounds with a lower IC₅₀ compared with acarbose.

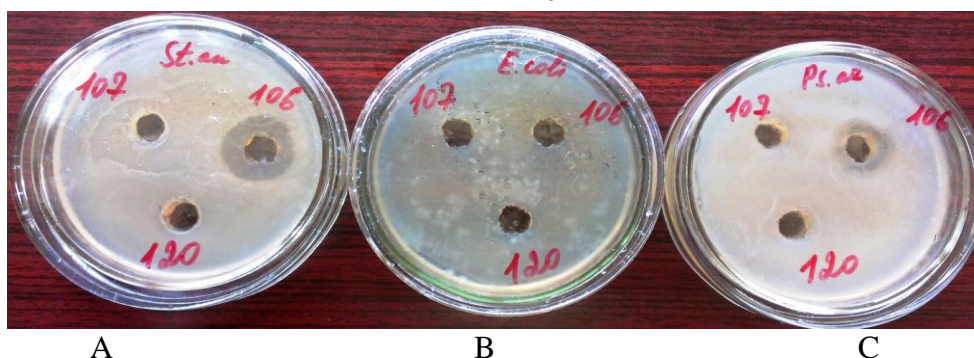
Table.1 Endophytic Fungi Isolated from Endemic *Allium* Species

<i>Allium filidens</i>		
1	<i>Aspergillus terreus</i> - AF104S	stem
2	<i>Penicillium</i> sp. - AF105	bulb
3	<i>Penicillium</i> sp. - AF106	
4	<i>Aspergillus terreus</i> - AF107	
5	<i>Penicillium</i> sp. - AF120	
6	<i>Cladosporium tenuissimum</i> - AF183	
7	<i>Alternaria tenuissima</i> – AF180	bud
<i>Allium longicuspis</i>		
8	<i>Fusarium sambucinum</i> - AL135L	leaf
9	<i>Alternaria</i> sp. - AL136L	
10	<i>Aspergillus terreus</i> - AL138L	
11	<i>Aspergillus flavus</i> - AL139L	
12	<i>Alternaria</i> sp. - AL141L	
13	<i>Aspergillus ochraceus</i> - AL137R	
14	<i>Aspergillus versicolor</i> - AL140R	
15	<i>Fusarium</i> sp. - AL142R	
16	<i>Aspergillus spectabilis</i> - AL184	bulb

Table.2 Antibacterial Activity of Endophytes from Allium

#	Test cultures	<i>E. coli</i>	<i>S.aureus</i>	<i>P.aeruginosa</i>
	Isolates	Pathogens' growth inhibition zone, mm		
<i>Allium longicuspis</i>				
1	<i>Fusarium subbusinum</i> – AL135L	5,0	-	-
2	<i>Alternaria sp.</i> – AL136L	5,0	5,0	5,0
3	<i>Aspergillus terreus</i> – AL138L	-	2,0	7,0
4	<i>Aspergillus flavus</i> – AL139L	-	-	4,0
5	<i>Alternaria sp.</i> – AL141L	-	-	6,0
6	<i>Aspergillus ochraceus</i> – AL137R	-	3,0	7,0
7	<i>Aspergillus versicolor</i> – AL140R	4,0	7,0	6,0
8	<i>Fusarium sp.</i> – AL142R	2,0	-	-
9	<i>Aspergillus spectabilis</i> – AL184	-	3,0	5,0
<i>Allium filidens</i>				
10	<i>Aspergillus terreus</i> - AF104S	-	1,0	2,0
11	<i>Penicillium sp.</i> - AF106	10,0	7,0	7,0
12	<i>Aspergillus terreus</i> - AF107	-	2,0	-
13	<i>Alternaria tenuis</i> – AF180	-	2,0	2,0

Fig.1 Pathogens' Growth Inhibition Zone By Extracts of *Penicillium sp.* – af 106 isolated from bulb of *A. filidens*



A - *Staphylococcus aureus*; B - *Escherichia coli*; C - *Pseudomonas aeruginosa*

Fig.2 α -Amylase Inhibition by Extracts of endophytes from *Allium filidens*

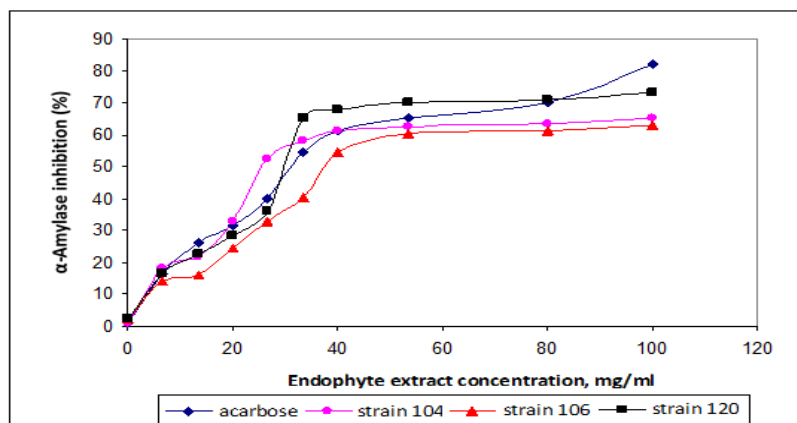
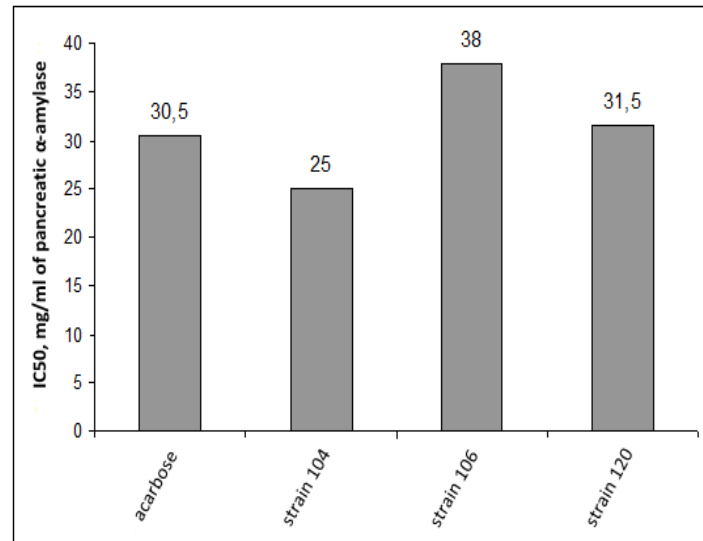


Table.3 Cytotoxic Activity of Endophytes from *Allium filidens*

Isolate	Hepatocytes			HeLa			HEp-2		
	100 µg/ml	10 µg/ml	1 µg/ml	100 µg/ml	10 µg/ml	1 µg/ml	100 µg/ml	10 µg/ml	1 µg/ml
<i>Aspergillusterreus</i> -AF104S	100±9,0	98±5,5	69±5,2	8±0,05	3±0,01	0±0,01	13,5±1,5	11,5±0,5	0±0,05
<i>Penicillium sp.</i> - AF105	12±0,02	0±0,05	0±0,05	66±5,9	51±1,5	25±1,5	53±1,6	16,5±0,7	0±0,05
<i>Penicillium sp.</i> - AF106	23±1,4	4±0,01	0±0,05	31±0,9	18,5±0,5	7±0,5	23±0,2	18,5±0,5	5,5±0,05
<i>Aspergillusterreus</i> - AF107	62±5,6	23±0,9	19±0,5	37±3,5	13±0,5	10±0,7	35±1,5	17±0,5	12,5±0,5
<i>Penicillium sp.</i> - AF120	100±9,8	83±9,8	24±0,7	69±5,5	33±1,3	25±0,5	64±5,6	43,5±4,5	22,5±2,1
Cisplatin	100±13,0	66±11,2	29±1,4	95±11,4	72±6,1	25±7,2	87±9,3	49±6,5	36±1,2

Fig.3 IC₅₀ of Extracts of endophytes from *Allium filidens*



It should be noted that in the literature there are a number of reports of endophytic fungi with antidiabetic activity. Thus the extracts of the mycelium of 17 endophytic fungi isolated from the plant *Salvadora oleoides* were tested on animals with alloxan-induced diabetes. Significant reduction of blood glucose was caused by methanolic extracts of *Aspergillus* sp. JPY2 and *Aspergillus* sp. JPY1, and acetone extracts of *Phoma* sp. (Dhankhar *et al.*, 2013). The ability to antidiabetic activity due to the presence of an inhibitor of α -amylase was detected in *Colletotrichum* sp., isolated from *Taxus sumatrana* (Artanti *et al.*, 2012). The isolation and identification of bioactive compounds revealed inhibitory activity of α -amylase showed that this compound is unsaturated fatty acid (Santiago *et al.*, 2014).

Obtained data on significant inhibitory activity of crude extracts of endophytes from *A. filidens* suggest further investigation of the nature of inhibitory compounds.

It is known that fungi are a rich source of chemotherapeutic metabolites (Sujkowska-Ziala *et al.*, 2005). The study of metabolites of endophytic fungi opens up new opportunities to obtain new anticancer agents. Research in this direction is currently being developed intensively in different types of medicinal plants. So, a few endophytic fungi were selected from several plants that produce Taxol (Gangadevi and Muthumary, 2009; Kumaran *et al.*, 2008). 23 endophytes with anti-cancer activity against 5 different cancer cell lines were isolated from *Aquilaria sinensis* (Jin-long *et al.*, 2011). Endophytic fungus *Phoma* sp. isolated from *Cinnamomum mollissimum* was found to produce three well-known

polyketides, one of which has a strong (97,3%) and two moderate cytotoxic effect on the culture of leukemic cells P388 (Santiago *et al.*, 2014).

It was observed that *Penicillium* sp. - AF105 and *Penicillium* sp. - AF120 isolated from the bulbs of *Allium filidens* expose notable cytotoxic activity towards both cancer cell lines. The extracts of *Penicillium* sp. - AF105 on the line of HeLa cells exposed cytotoxicity close to cisplatin cytotoxicity at all three tested concentrations and no cytotoxicity against healthy hepatocytes, while extract of *Penicillium* sp. - AF120 affecting cytotoxic on HEP-2 cell line and hepatocytes as cisplatin. Thus obtained data indicated that endophytic fungi *A. filidens* produce secondary metabolites with cytotoxic properties.

It should be noted that the bioactivity of related endophytic fungi quite differed even though they are associated with the same species of the host plants. So, *Alternaria* sp. – AL136L isolated from the leaves of *A. longicuspis* exhibited antibacterial activity to *E. coli*, *S. aureus*, and *P. aeruginosa*, while *Alternaria* sp. – AL141L - only to *P. aeruginosa*. Three strains of *Penicillium* sp. isolated from bulbs of *A. filidens* strongly differed in cytotoxic, antibacterial and anti-amylase activity.

The obtained data showed that endophytic fungi of wild *Allium* species produce compounds with antibacterial, antidiabetic and cytotoxic properties, apparently playing a certain physiological role in the metabolism of endophyte and plant in the process of symbiosis. However, the bioactive properties of the investigated *Allium* endophytes suggests their high biotechnological potential as sources of

new secondary metabolites with therapeutic value.

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