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Composition of Secondary Metabolites of Endophytic Fungus *Aspergillus egypticus* HT-166S isolated from *Helianthus tuberósus*

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A B S T R A C T

The component composition of the secondary metabolites *Aspergillus egypticus* HT-166S isolated from stem of *Heliánthus tuberósus* plant, which has the ability to strongly inhibit the activity of pancreatic α-amylase, was studied. Qualitative analysis of phytocompounds showed that among metabolites there were terpenoids, tannins, flavonoids, glycosides, saponins and alkaloids. The highest inhibitory activity was shown by ethyl acetate and benzene extracts of *A. egypticus* HT-166S. 42 components related to hydrocarbons and their functional derivatives, fatty acids, terpenoids, alkaloids, phenol carboxylic acids and their derivatives were identified by gas-liquid chromatography of benzene extraction.

Introduction

The use of dietary plants and preparations developed on their basis is considered as an alternative medicine and has become of particular importance in many countries. Currently, many studies are focused on the scientific evaluation of dietary plants and natural products for the control of various diseases (Ang-Lee *et al.*, 2001; Coman *et al.*, 2012). In particular, various substances of plant origin belonging to different classes of phytochemicals have the ability to inhibit the activity of pancreatic α-amylase and are used in the practice of diabetes treatment (de Sales *et al.*, 2012). At the same time plants used in traditional medicine play a very important role as a source of new bioactive strains of endophytes, perhaps because of their beneficial properties in certain degree are the result of metabolites produced by the endophytic community inhabiting them (Kaul

et al., 2012; Kamana *et al.,* 2017). *Helianthus tuberósus* (Jerusalem artichoke or sunflower tuberous), a perennial tuberous plant of the *Asteraceae* family, often used as a dietary product in diabetes as a rich source of biologically active terpenoids, phenolic compounds, carbohydrates and fatty acids (Helmi *et al.,* 2014; Li *et al.,* 2009; Otmar, 2009). A number of bacterial endophytes with growth-stimulating, antimicrobial action were isolated from *Helianthus tuberósus* (Akshatha *et al.,* 2014).

From the roots, stems, leaves and tubers of *Helianthus tuberósus* growing in Uzbekistan we obtained 17 endophytic fungal isolates related to *Acremonium, Alternaria, Aspergillus, Gliocladium, Fusarium, Penicillium, Trichoderma* and *Ulocladium* genera (Ruzieva *et al.,* 2016).

Moreover, it has been shown that the extracts of isolated endophytic fungi can strongly inhibit the activity of pancreatic α-amylase and can be considered as possible producers of inhibitory compounds (Ruzieva *et al.,* 2017). As the most active strain inhibiting α-amylase activity for more than 80% *Aspergillus egypticus* HT166S isolated from plant stem was selected.

In this regard, in order to identify the antidiabetic compounds the objective of this work was to study the component composition of secondary metabolites of *A. egypticus* HT166S.

Materials and Methods

A. egypticus were grown by submerged fermentation in 500 ml flasks containing 100 ml of Chapek-Dox liquid medium for 5 days at 26 °C.

For extraction of secondary metabolites 5 g of biomass of isolate was milled in a Potter homogenizer, transferred to a cone flask containing 50 ml of ethyl acetate, and left for 24 hours on the rotary shaker at room temperature. The mixture was filtered through filter paper (Whatman #1) and Na_2SO_4 (40 µg/ml) was added. After 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.

Determination of α-amylase activity was carried out by the modified method used for measurements in the plant extracts (Visweswari *et al.,* 2013). For this purpose a solution of starch as a substrate was prepared at the rate of 1 g /10 ml water, boiled for 2 minutes, adjusted to 100 ml with distilled water and used within 2 – 3 days. To 2 ml of the prepared starch solution 100 µl pancreatic α-amylase (13 u/ml in 0.1 M Na-acetate buffer pH 4, 7), 100 µl of the extract endophyte (20 mg/ml), 2 ml of acetate buffer were added and incubated for 10 minutes at 300ºC. In blank sample as a control the extract was not added. Incubation was terminated by adding 10 ml of iodine reagent and the absorbance was measured at a wavelength of 630 nm. For the preparation of iodine reagent 0.5 g of crystalline iodine, 5 g of potassium iodide were dissolved in 250 ml water; to obtain a working solution 2 ml of this reagent was adjusted to 100 ml by 0.1M HCL. All the assays were carried out in triplicates and average inhibition was calculated using the following formula: $(A_0–A_t)/A_0x100\%$, where A_0 - absorption of control sample, A_t - absorption of test sample.

The qualitative composition of the components in the extracts was determined as described by Visweswari *et al.,* (Visweswari *et al.,* 2013).

Antioxydant activity was determined by method described by Boboev *et al.,* (Boboev

et al., 2012). The reaction mixture (10 ml) contained 0.1 m acetate buffer, pH 4.2, 20 vol % ethanol, 4 mM (+)-catechin and 10 mg/l $FeCl_3$. To determine antioxidant activity 20 mg of ethyl acetate extract *A. egypticus* HT-166S was added to the experimental sample. Incubation was performed at 40 °C for 14 days. The degree of oxidation of (+)-catechin was determined daily by optical density at 440 nm on photoelectric colorimeter KFK-2 (Russia).

For gas-liquid chromatography the biomass of *A. egypticus* HT-166S (50.125 g) was mixed with silica gel (33 g) and dried at room temperature under vacuum. The dried residue (36.524 g) was placed in a chromatographic column containing 10 g of silica gel (brand "KSK") and firstly eluted three times with benzene, then with chloroform. The resulting benzene and chloroform extracts were separately concentrated under vacuum at 25-30 °C. The yield of benzene extraction was 72 mg, and chloroform extraction - 103 mg.

The determination of the composition of extracts was performed using Agilent 7890A GC gas chromatograph with a quadrupole mass spectrometer Agilent 5975C inert MSD as detector and a quartz capillary column HP-5MS (30 m × 250 μm × 0.25 μm), grafted stationary phase of 5% feniletilamine in the temperature regime: 50 °C (2 min) – 10 °C/min up to 200 °C (6 min) – 15 °C / min up to 290 °C (15 min). The amount of sample 1 μl, flow rate of mobile phase 1.3 ml/min. Components were identified on the basis of comparison of mass spectra with the data of digital libraries W8N05ST.L and NIST08.

Results and Discussion

From stem of *Helianthus tuberósus* it was firstly isolated endophytic fungus *Aspergillus egypticus* which is one of rarely isolating species for the territory of Uzbekistan. Preliminary screening of the components of the extracts of secondary metabolites of this endophytic fungus named as *A. egypticus* HT-166S showed the presence a number of constituents such as terpenoids, tannins, alkaloids, glycosides, etc. (Table 1).

As shown in the results (Fig. 1) all used solvents except water and hexane release high inhibitory activity in the range from 60 to 93%, and the highest inhibitory activity have ethyl acetate extracts (93%) followed by benzene (80%) and acetonitryl extracts (79%), containing alkaloids, terpenoids, and phenols.

Since the antidiabetic properties of the compounds are, to some extent, also related to antioxidant activity (Saini and Gangwar, 2017), the effect of ethyl acetate extract *A. egypticus* HT-166S on the oxidation rate (+)-catechin was studied.

As can be seen from the obtained data, in the presence of the extract, (+)-catechin oxidation rate is significantly reduced, indicating the presence of compounds with antioxidant properties as well.

To determine the component composition, secondary metabolites were also extracted from the biomass of *A. egypticus* HT-166S by benzene and chloroform, and gas-liquid chromatography of the extracts was carried out as mentioned above.

As can be seen from the data presented in Table 2, 42 components are identified in benzene and chloroform extraction, which belong to different classes and groups of natural compounds, including hydrocarbons (aliphatic, cyclic, aromatic) and their functional derivatives (1-4, 6-11, 13-15, 17, 19-21, 24, 25, 27-34), fatty acids (5, 12, 16, 23, 36, 39, 42), mono- and triterpenoids (18, 38. 41), alkaloids (26, 35), phenol carboxylic acids and their derivatives (22, 37, 40).

Fig.1 Effect of ethyl acetate extract *A. egypticus* HT-166S on oxidation rate (+) – catechin

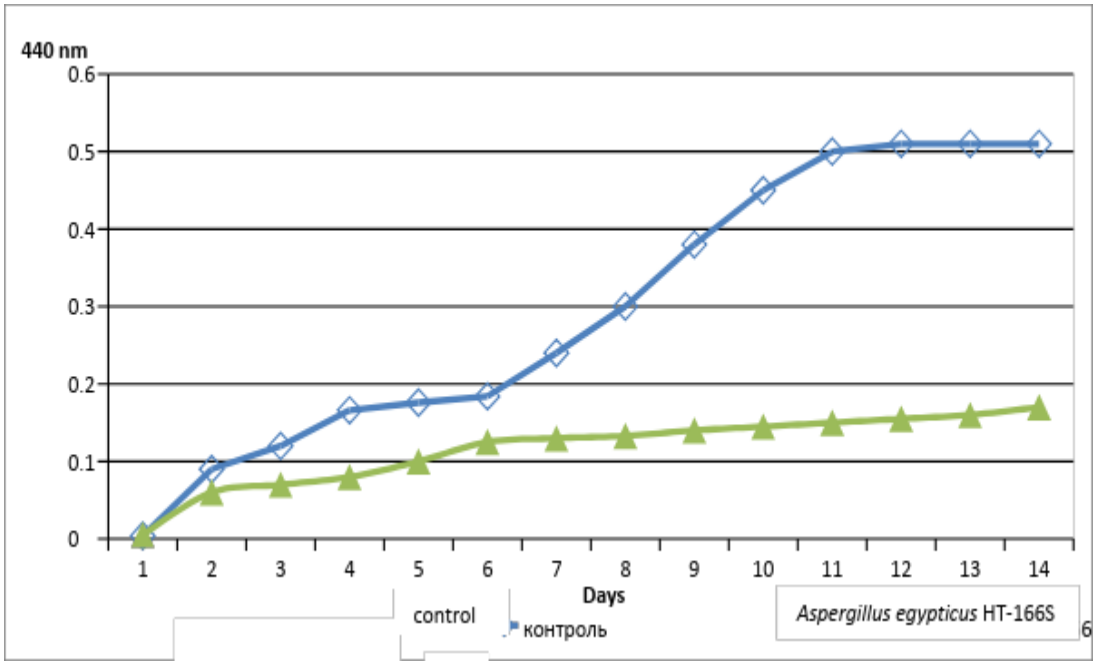

Table.1 Preliminary screening of *A. egypticus* HT166S secondary metabolites extacted by different solvents

Amylase Inhibition (%)	Methanol	Water	n-Butanol	Ethylacetate	Acetonitryl	Benzene	Hexane	Ethanol
	60	20	69	93	79	80	26	75
Alkaloids	+	+	-	+	+	+	-	+
Flavonoids	+	-	+	-	-	-	-	-
Terpenoids	-	-	-	+	+	+	+	-
Saponins	+	+	+	-	-	-	+	+
Tannins	-	+	-	+	+	-	-	-
Phenols	+	-	+	+	+	+	+	+
Glycosides	+	+	+	-	-	-	_	+

"+" – presence of compound; "-" –absence of compound

Table.2 Component composition of benzene and chloroform extraction

№	Compounds	*RT, min*	*RI*	Benzene extraction	Chloroform extraction
1	(*E*)-2-Heptenal	5.138	929	0.22	1.45
2	2-Pentyl furan	6.165	1000		1.73
3	Hexanoate	7.413	1044		8.12
4	1,2-Diethylbenzene	7.481	1047	0.03	
5	2-Propylmalonicacid	7.745	1056		5.47
6	(E)-2-Octenal	7.911	1062		0.74
7	Undecane	8.920	1098	0.05	
8	α-Buthylcyclopropene methanol	9.000	1101		6.15
9	1-Nonanal	9.104	1104	0.13	4.42
10	(*E*)-2-Decenal	10.955	1160		0.25
11	Dodecane	12.080	1195	0.05	
12	Octanoic acid	12.271	1201		0.49
13	Decenal	12.394	1205		0.65
14	2,4-Nonadienal	12.707	1214		0.17
15	1,3,5-Triethylbenzene	12.843	1219	0.03	
16	2-Octenoic acid	13.568	1241		0.79
17	(Z)-2-Decenial	14.140	1259	0.25	3.15
18	2,6-Dimethylocta-1,7-diene-3,6-diol	14.675	1276	0.07	
19	2,4-Decadienal	15.204	1292	0.09	
20	Tridecane	15.314	1296	0.05	
21	(E,E)-2,4-Decadienal	15.942	1315	0.18	0.28
22	Isobenzofuran-1,3-dion	16.144	1322		0.26
23	2-Nonanic acid	16.446	1331		0.17
24	2-Undecenal	17.429	1362	0.04	
25	Tetradecane	18.499	1396	0.05	0.25
26	1-(4-Bromobutyl)-2-piperidinone	18.856	1408		0.46
27	(*E*)-2Decenol	19.981	1444		0.11
28	2,6-Di(n-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadiene-1-on	20.836	1473	0.04	
29	Pentadecane	21.580	1497	0.03	0.24
30	Hexadecane (Cetane)	24.562	1595	0.07	1.17
31	3,6-dimethyl decan	25.933	1643		0.07
32	1-Ethenyl-cyclododecanol	27.728	1706		0.13
33	Docozane-1-ol	27.864	1711		0.15
34	Octadecane	30.206	1793		0.28
35	1-Acetyl-19,21-epoxy-15,16-dimethoxy-aspidospermidine-17-ol	33.133			0.31
36	Methyl ester hexadecanoic acid	33.982			0.39
37	Dibutylphthalate	35.261	1906	0.08	1.80
38	14-β-Pregnane	36.109			0.46
39	Palmitate	44.717			6.53
40	Mono-(2-ethylhexyl) phthalic acid ester	51.843		**14.57**	
41	6-Fluoro-7-dehydrocholesterol	51.942		**59.63**	
42	**Stearate**	**62.327**			**10.49**

It should be noted that a number of compounds we found, previously were identified in extracts of *Colletotrichum gloeosporioides* from *Phlogacanthus thyrsiflorus* plant and in extracts of *Pestalotiopsis neglecta* BAB-5510 isolated from the leaves of *Cupressus torulosa*, including pentadecane, tetradecane dodecane, hexadecane, octadecane and their derivatives, as well as derivatives of phthalic acid and a number of others (Devi and Singh, 2013; Sharma *et al.*, 2016).

It was also reported that a significant decrease in blood glucose levels cause extracts of two strains of endophytes pp. *Aspergillus* and *Phoma* isolated from *Salvadora oleoides Decne (Salvadoraceae*), with the main active substance in these extracts being phenolic derivatives (Dhankhar *et al.*, 2013).

Recent studies indicate that many of the metabolites found in extracts of *Aspergillus egypticus* have bioactive properties. For example, the antimicrobial activity of phthalic acid derivatives (Devi and Singh, 2013; Sharma *et al.*, 2016), many bioactive properties exposed by octadecanoic acid derivatives, identified in the extracts of endophytic fungi from *Ocimum sanctum* (Chowdhary and Kaushik, 2015). Gas-liquid chromatography of methanol extracts of endophytic fungi p. *Penicillium* from *Tabebuia argentea*, which inhibit the activities of alpha-amylase, beta-glucosidase and peptidyl peptidase IV, revealed 18 different phytocompounds (Murugan *et al.*, 2017). Similar to our data, the composition of *Penicillium* extracts contains derivatives of phthalic acid, functional derivatives of octadecane and hexadecane, derivatives of phenolcarboxylic acid, and it is shown that the antidiabetic effect of the extracts is associated with octadecanoic acid (Murugan *et al.*, 2017). In a number of reports it is also mentioned that fatty acids of some endophytes have antidiabetic properties (de Sales *et al.*, 2012; Kamana *et al.*, 2017).

Thus, our studies have shown that the endophytic fungus *A. egypticus* HT-166S, isolated from the stem of *Helianthus tuberósus*, produces a number of important bioactive secondary metabolites. Assuming that the bioactivity of *A. egypticus* HT-166S can be caused by different compounds, obtained data indicate a rich potential of this endophyte as a producer of biotechnologically valuable bioactive metabolites.

To confirm the antidiabetic activity of *A. egypticus* HT-166S, future research should focus on isolation and purification the inhibitor substance for *in vivo* testing on experimental animals.

According to our information it is first report on the component composition of metabolites *A. egypticus* HT-166S isolated from the *Helianthus tuberósus*.

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