Identification of bioactive compounds of the endophytic fungus *Aspergillus egypticus-HT166S* inhibiting the activity of pancreatic α-amylase

**Pankreatik α-amilaz aktivitesini inhibe eden endofitik mantar *Aspergillus egypticus-HT166S*‘nin biyoaktif bileşiklerinin tanımlanması**

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**ABSTRACT**

**Objectives:** Diabetes mellitus (DM) is worldwide increasing problem, associated with development of hyperlipidemia, coronary heart diseases, hypertension and other chronic diseases. Decreasing of glucose absorption by inhibition of α-amylase is one of the therapeutic approaches to retard diabetes type 2. Pancreatic α-amylase (PA) inhibition widely studied mechanism for determination of potential of natural compounds as antidiabetic agents. The aim of this work was identification of inhibitory secondary metabolites producing by *Aspergillus egypticus*, isolated from *Helianthus tuberosus*.

**Materials and Methods:** The PA inhibitory activity of the secondary metabolites determined by iodometric method. Isolation of inhibitory compounds was carried out by column chromatography, TLC and LC-MS/MS analysis.

**Results:** It was found that inhibitory concentration of a compound K-10 (Rf = 0.74) isolated from metanolic extract of *A. egypticus* was 4.82 mg/ml. LC-MS/MS analysis of K-10 showed the presence of polymethoxylated flavones.

**Conclusion:** The fungal endophyte *A. egypticus-HT166S* can be considered as a source of polymethoxylated flavones as potential agents for the development of new PA inhibitors.

**Keywords:** diabetes mellitus, endophyte, diabetes, secondary metabolites, inhibitory activity, column chromatography, LC-MS/MS.

**ÖZ.**

**Amaç:** Diabetes mellitus (DM), hiperlipidemi, koroner kalp hastalıkları, hipertansiyon ve diğer kronik hastalıkların gelişimi ile ilişkilii dünya çapında artan bir problemidir. α-amilazın inhibisyonu ile glukoz emiliminin azaltılması, diyabet tip 2’yi geciktirmek için terapötik yaklaşımlardan biridir. Pankreatik α-amilaz (PA) inhibisyonu, antidiyabetik ajanlar olarak doğal bileşiklerin potansiyelinin belirlenmesi için geniş çapa çalıslan mekanizmalarıdır. Bu çalışmanın amacı, Helianthus tuberosus’tan izole edilen *Aspergillus egypticus* tarafından üretilen inhibitör sekonder metabolitlerin tanımlanmasıdır.
Gereçler ve Yöntemler: İyodometrik yöntemle belirlenen ikincil metabolitlerin PA inhibitör aktivitesi. Inhibitör bileşiklerin izolasyonu kolon kromatografisi, TLC ve LC-MS/MS analizi ile gerçekleştirildi.

Sonuçlar: A.egypticus'un metanolik ekstraktından izole edilen bir K-10 bileşiğinin (Rf =0.74) inhibitör konsantrasyonunun 4.82 mg/ml olduğu bulundu. K-10'un LC-MS/MS analizi, polimetoksile flavonların varlığını gösterdi.

Sonuç: Mantar endofiti A.egypticus-HT166S, yeni PA inhibitörlerinin geliştirilmesi için potansiyel ajanlar olarak bir polimetoksile flavon kaynağı olarak düşünülebilir.

Anahtar kelimeler: diabetes mellitus, endofit, diyabet, sekonder metabolitler, inhibitör aktivite, kolon kromatografisi, LC-MS/MS.

INTRODUCTION
Diabetes mellitus (DM) is a metabolic syndrome characterized by hyperglycemia and abnormalities in the metabolism of carbohydrates, fats, and proteins, leading to insulin secretion or/and sensitivity.1 The consumption of a high-carbohydrate diet causes postprandial hyperglycemia with the development of a complete symptomatic picture of type 2 diabetes.2 The number of patients with diabetes is growing dramatically worldwide. According to WHO forecasts, by 2040, the number of patients with diabetes will be 642 million. At the same time, 90% of the total number of patients are type 2 diabetes.3 DM therapy is aiming to prevent hyperglycemia and subsequent complications associated with cardiovascular factors, and in general, to improve the quality of life.

One of the treatment approaches of diabetes type 2 is reducing postprandial blood glucose, caused by delayed glucose absorption by inhibition of polysaccharides breakdown to mono- and disaccharides by α-amylase and α-glucosidase in the intestine.4,5,6,7 Inhibitors of these enzymes prolong the total carbohydrate digestion time, contributing to a decrease in the rate of glucose absorption, followed by blocking the postprandial increase in glucose levels.8 However, most known to date inhibitors (acarbose, miglitol, voglibose) have severe undesirable side effects - abdominal pain, bloating, diarrhea, kidney cancer, liver damage, and acute hepatitis.6,9 The development of new natural inhibitors of pancreatic α-amylase and α-glucosidase that can restore normoglycemia without side effects requires appropriate research in herbal medicine and alternative medicine.

Some secondary metabolites of the antidiabetic plants successfully demonstrate the properties of inhibitors of carbohydrate degrading enzymes, which may serve to control type 2 diabetes.2,4,5,7 In recent years, endophytes of medicinal plants have been considered the most attractive source of natural product sources with a high structural diversity and bioactivity and have several advantages over plant raw materials.10 The endophytes of antidiabetic plants are of particular interest since they can probably produce compounds that mediate the antidiabetic properties of the host plants.4,5,6

For example, Colletotrichum capsici isolated from Eugenia cuminii L have strong antibacterial efficacy and antidiabetic action and contain fatty acids and phenolic compounds.11 Similar results were reported by Govindappa M. et al., which in vitro determined the antidiabetic, antioxidant, and anticholinesterase activity of the methanolic extract of the endophyte Cladosporium uredinicola isolated from endemic plant Calophyllum tomentosum. Phytochemical analysis of the fungal extract showed flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids and coumarins.12

In our previous works from the roots, stems, leaves, and tubers of Helianthus tuberosus growing in Uzbekistan there were obtained 17 endophytic fungal isolates related to different genera.13 The most active A.egypticus-HT166S inhibited α-amylase activity for more than 80%.14 At fractionation of crude ethylacetate extract of A.egypticus-HT166S metabolites by the stepwise extraction with polar and non-polar solvents, it was found that the metabolites with the highest inhibitory activity are recovered in the methanol fraction.15
In this regard, the purpose of this work is to separate and study inhibitory compounds in the methanol extract of the endophytic fungus *A. egypticus-HT166S*.

**MATERIALS AND METHODS**

*Cultivation of Aspergillus egypticus-HT166S endophyte*

The endophytic fungus *A. egypticus-HT166S*, previously isolated from the stem of the Helianthus tuberosus, was grown submergely in Czapek-Dox medium on a orbital shaker at 160 rpm for 7 days. The biomass was separated from the culture liquid by centrifugation at 6000 rpm.

*Fractionation of secondary metabolites*

Fractionation of secondary metabolites of *A. egypticus-HT166S* biomass was carried out according to the scheme proposed by Kumar et al, including sequential extraction with water, methanol:hexane (1:1), and butanol. As a result, a methanol extract was obtained with an inhibitory activity of 75.4%. The extract was dried on a rotary evaporator and 1 ml of DMSO was added. The resulting dry methanol extract was stored at 4 °C for reuse.

*Column chromatography*

500 mg of a methanol extract was applied to a column (2 × 25 cm) filled with 20 g of silica gel (100/250 μm, LaChema) and eluted in a chloroform: methanol 50: 1 ~ 1: 1 graduated solvent system to yield fractions at a flow rate of a mobile phases 1.5 ml / min. Those fractions with same Rf value after TLC analysis were pooled together and evaporated till dried fraction (A1-M12) was obtained.

*Thin layer chromatography*

Samples of 25 μL were loaded onto plates (Sigma–Aldrich, Germany) and chromatographed in the chloroform: methanol (5:1) system. The plates were scanned in UV light at a wavelength of 254 nm. Samples with the same Rf values were pooled and dried.

*LC-MS analysis*

The mass spectra of the fractions obtained on a Q-TOF LC-MS Agilent Technologies 6520V device under the following conditions: ESI+ ion source, positive ion electrospray method, drying gas flow rate of 5 l/min, drying gas temperature of 300°C, ion acceleration voltage on the skimmer 35V, fragmenter 175V, range MS 150-1000 m/z, target MS-MS 50-1000 m/z, collision energy - 30, 40, 50, 65. Samples injected onto a Zorbax SB C18. 3 microns, 150 x 0.5 mm column (Agilent Technologies 1200) with a mobile phase: A-0.1% formic acid, B-acetonitrile + 0.1% formic acid. Elution on the Agilent Technologies 1260 Gp pump at 15 μl/min: 5 min-60%, 15-20 min - 90%, 25 min - 60% of the mobile phase B.

*Determination of the inhibitory activity*

Each sample obtained after the separation of the methanol fraction on the column was examined for inhibitory activity. The activity of the α-amylase fractions was determined according to the method used in plant extracts. The starch solution prepared as a substrate in an amount of 1 g/10 ml of water, boiled for 2 min, the sample volume adjusted to 100 ml by distilled water. 100 ml of pancreatic α-amylase (0.1 M Na-acetate buffer is 13 ml at pH 7.2), 100 mcg of endophyte extract, 2 ml of acetate buffer were incubated for 10 minutes at 30° C for 2 ml of starch prepared from the preparation. The incubation reaction was then stopped and immersed in 10 ml of an aqueous reagent, and the optical density was measured at 630 nm on a SPECOl-1300. To prepare the iodine reagent, 0.5 g of crystalline iodine, 5 g of potassium iodide, and 250 ml of dissolved in water were taken; 2 ml of this reagent was added to 100 ml of 0.1 M HCl to obtain a working solution. The inhibitory activity was expressed by the formula: (Ao-At)/Ao×100%, where Ao is the absorption of the control sample, and At is the absorption of the experimental sample, respectively. As a comparison drug, acarbose from the commercial drug "Glucobay" (Bayer Pharma AG, Germany) was used.

The concentration causing 50% inhibition of pancreatic α-amylase (IC50) by the test samples was quantified as described in Murado et al. There were conducted 3 replications. The values are expressed as the mean value of ± SD (n = 3).

**RESULTS AND DISCUSSION**
As mentioned above, for isolation of bioactive substances with high inhibitory activity, the total ethyl acetate extract biomass of *A. egypticus-HT166S* was fractionated in solvents of different polarities, and the highest inhibitory activity is extracted by methanol.\(^5\)

Figure 1 shows the TIC chromatogram of the initial methanol fraction of *Aspergillus egypticus-HT166S*. As can be seen from the chromatographic data, the methanol fraction contains a number of substances, three of which are represented by relatively high peaks.

![Figure 1. TIC chromatogram of the total methanol fraction of the biomass of *Aspergillus egypticus-HT166S*](image)

As a result of fractionation of the methanol extract by column chromatography in a gradient concentration of chloroform:methanol 50: 1 ~ 1: 1, twelve fractions (A1-M12) were obtained, which were dried on a rotary evaporator. Each of the obtained fractions of metabolites was further evaluated by inhibition of pancreatic amylase.

As can be seen from the data in Table 1, the inhibitory activity of the obtained metabolite samples varies widely from 7.0 to 76.2%. At the same time, the highest level of inhibitory activity was noted in the K-10 fraction with an Rf value of 0.74 and the content of secondary metabolites constituting 10% of the initial weight of the dry methanol fraction (Table 1).

### Table 1. Content and inhibitory activity of samples obtained by purification on a column of the total methanol fraction of *A. egypticus-HT166S*

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Rf</th>
<th>Dry weight, %</th>
<th>α-amylase inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>0.13</td>
<td>3.8±0.02</td>
<td>14.6±0.29</td>
</tr>
<tr>
<td>B-2</td>
<td>0.22</td>
<td>4.5±0.03</td>
<td>15.2±0.29</td>
</tr>
<tr>
<td>C-3</td>
<td>0.30</td>
<td>7.8±0.37</td>
<td>28.6±0.30</td>
</tr>
<tr>
<td>D-4</td>
<td>0.37</td>
<td>4.1±0.35</td>
<td>17.7±0.27</td>
</tr>
<tr>
<td>E-5</td>
<td>0.44</td>
<td>3.2±0.33</td>
<td>25±0.28</td>
</tr>
<tr>
<td>F-6</td>
<td>0.48</td>
<td>2.7±0.13</td>
<td>15±0.34</td>
</tr>
<tr>
<td>G-7</td>
<td>0.52</td>
<td>3.1±0.04</td>
<td>24.3±0.29</td>
</tr>
<tr>
<td>H-8</td>
<td>0.54</td>
<td>6.5±0.34</td>
<td>7.0±0.26</td>
</tr>
<tr>
<td>J-9</td>
<td>0.6</td>
<td>5.2±0.24</td>
<td>24±0.30</td>
</tr>
<tr>
<td>K-10</td>
<td>0.74</td>
<td>10±0.17</td>
<td>76.2±0.29</td>
</tr>
<tr>
<td>L-11</td>
<td>0.86</td>
<td>6.6±0.14</td>
<td>18.8±0.30</td>
</tr>
<tr>
<td>M-12</td>
<td>0.97</td>
<td>2.3±0.03</td>
<td>-</td>
</tr>
<tr>
<td>Total methanol fraction</td>
<td>-</td>
<td>100</td>
<td>75.4±0.27</td>
</tr>
</tbody>
</table>
Each value is the average of three analyses ± standard deviation.

Qualitative phytochemical analysis of the K-10 fraction showed a positive reaction to flavonoids, as evidenced by the formation of an intense yellow color by 20% NaOH and disappearance of color by 70% hydrochloric acid.\textsuperscript{18}

It should be noted that over the past 20 years, scientific attention has been paid to natural compounds, such as flavonoids, which serve as antidiabetic agents. Flavonoids improve the pathogenesis of diabetes and its complications by regulating glucose metabolism, liver enzyme activity, and lipid profile. In vitro and in vivo studies have shown that they can prevent diabetes and its complications.\textsuperscript{19} In identifying the nature of flavonoids, we referred to the experimental data of Zhang et al., who developed a fast and efficient analytical method of tandem mass spectrometry with high-performance liquid chromatography for the structural characterization of flavonoids from complex extracts of traditional Chinese medicines.\textsuperscript{20}

Mass spectral analysis of the bioactive K-10 sample showed the presence of compounds with molecular ions (M+H) with m/z 359.0, m/z 345.0, and m/z 327.0. (Fig.2).

![Mass spectral analysis of the fraction - K-10](image)

Comparative analysis of our results with the literature data, the compounds were assigned to polymethoxylated flavones (PMF). PMF is a subclass of flavonoids in which all or almost all hydroxyls are blocked by methylation, have high oral bioavailability, exhibiting anti-allergic, antioxidant, antibacterial, antiproliferative, anti-inflammatory, and anti-cancer activity.\textsuperscript{21} The literature provides information on PMFs, mainly Nobiletin, Tangeretin, Sinensetin, and Isosinensetin from Citrus plants, and discusses their antidiabetic effects \textit{in vitro}.\textsuperscript{22} For example, the polymethoxylated flavonoid- Nobiletin reduces the inflammation associated with gestational diabetes mellitus – (GDM), a condition in which pregnant women suffer from carbohydrate intolerance during pregnancy. Nobiletin improved glucose metabolism in animal and human GDM models and may be a novel therapeutic agent for preventing GDM.\textsuperscript{23} Sundaram et al. evaluated the antihyperglycemic potential of PMF Tangeretin on the activity of key enzymes of carbohydrate and glycogenic metabolism in control rats and rats with streptozotocin-induced diabetes. Studies have shown that Tangeretin modulates the activity of liver enzymes due to increased insulin secretion and reduces blood glucose levels in rats with streptozotocin-induced diabetes due to its antioxidant potential.\textsuperscript{24}

Comparative analysis of the inhibitory activities of the purified sample K-10 and acarbose as a reference standard showed almost the same low IC\textsubscript{50} values of 4.82 mg/ml and 4.74 mg/ml, respectively, compared to the IC\textsubscript{50} of the total methanol extract (5.53 mg/ml).
The results obtained indicate that the inhibitory activity of the purified fraction K-10 is comparable to the standard sample-acarbose, and indeed contains bioactive compounds with potential inhibitory activity against α-amylase. (Fig. 3)

**Figure 3.** IC\textsubscript{50} μg/mL of total methanol extract and K-10 fraction when compared with acarbose.

**CONCLUSION**

Natural bioactive compounds can inhibit α-amylase, which are the best and most useful substances to lower the blood sugar. Inhibition of α-amylase is a successful manner in the prevention and therapy of diabetes. Therefore, the search for new sources of bioactive compounds, in particular, endophytic fungi, is an alternative way for the development of new technologies for the production of microbial amylase inhibitors.

The presented studies show that the *Aspergillus egypticus-HT166S* endophyte from the *Helianthus tuberosus* plant produces polymethoxylated flavones with high inhibitory activity against pancreatic α-amylase, comparable to the activity of the commercial drug Acarbose. However, to establish the structure of inhibitory polymethoxylated flavones, it is necessary to carry out further studies using analyzes - IR and NMR spectroscopy.

Based on the data obtained, it can be concluded that the endophytic fungus *Aspergillus egypticus-HT166S* can be considered as a new source of pancreatic amylase inhibitors for the development of hypoglycemic drugs.

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