



Identification of Bioactive Compounds of the Endophytic Fungus *Aspergillus egypticus*-HT166S Inhibiting the Activity of Pancreatic α -Amylase

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ABSTRACT

Objectives: *Diabetes mellitus* (DM) is a worldwide increasing problem, associated with development of hyperlipidemia, coronary heart disease, 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 produced by *Aspergillus egypticus*, isolated from *Helianthus tuberosus*.

Materials and Methods: The PA inhibitory activity of the secondary metabolites determined using iodometric method. Isolation of inhibitory compounds was carried out by column chromatography, thin layer chromatography and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

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

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

Key words: *Diabetes mellitus*, endophyte, diabetes, secondary metabolites, inhibitory activity, column chromatography, 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 DM.² The number of patients with diabetes is growing dramatically worldwide. According to World Health Organization forecasts, by 2040, the number of patients with diabetes will be 642 million. Simultaneously, 90% of the total number of patients are with type 2 DM.³

DM therapy is aimed 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 type 2 DM is reducing postprandial blood glucose, caused by delayed glucose absorption by inhibition of polysaccharide breakdown to mono- and disaccharides by α -amylase and α -glucosidase in the intestine.^{2,4-7} 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, and 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.

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

For example; *Colletotrichum capsici* isolated from *Eugenia cumini* L. has strong antibacterial efficacy and antidiabetic action and contains fatty acids and phenolic compounds.¹¹ Similar results were reported by Govindappa et al.¹² who *in vitro* determined the antidiabetic, antioxidant, and anticholinesterase activities of the methanolic extract of the endophyte *Cladosporium uredinicola* isolated from endemic plant *Calophyllum tomentosum* Wight. Phytochemical analysis of the fungal extract showed presence of flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids, and coumarins.¹²

In our previous studies of the roots, stems, leaves, and tubers of *Helianthus tuberosus* L. growing in Uzbekistan, there were obtained 17 endophytic fungal isolates related to different genera.¹³ The most active *Aspergillus egypticus*-HT166S inhibited α -amylase activity for more than 80%.¹⁴

The 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 were recovered in the methanol fraction.¹⁵

In this regard, this work aims to separate and study of inhibitory compounds in the methanol extract of the endophytic fungus *A. egypticus*-HT166S.

MATERIALS AND METHODS

Cultivation of A. egypticus-HT166S endophytes

The endophytic fungus *A. egypticus*-HT166S, previously isolated from the stem of *H. tuberosus*, was grown submergely in Czapek-Dox medium on an orbital shaker at 160 rpm for 7 days.¹⁴ 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.⁵, 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 dimethyl sulfoxide was added. The resulting dry methanol extract was stored at 4°C for reuse.¹⁵

Column chromatography

The methanol extract (500 mg) was applied to a column (2 x 25 cm) filled with 20 g of silica gel (100/250, LaChema) and eluted in chloroform: methanol 50:1 ~ 1:1 graduated solvent

system to yield fractions at a flow rate of a mobile phase 1.5 mL/min. Those fractions with the same R_f value after thin layer chromatographic analysis were pooled together and evaporated till the dried fraction (A1-M12) was obtained.¹¹

Thin layer chromatography

Samples of 25 μ L were loaded onto plates (Sigma-Aldrich, Germany) and chromatographed in the chloroform: Methanol (5:1) system. The plates were scanned with ultraviolet light at a wavelength of 254 nm. Samples with the same R_f values were pooled and dried.

Liquid chromatography-tandem mass spectrometry (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 positive ion mode, positive ion electrospray method, drying gas flow rate of 5 L/min, drying gas temperature of 300°C, ion acceleration voltage on the skimmer 35 V, fragmented 175 V, 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 μ m, 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 Cap pump at 15 μ L/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.¹⁶ The starch solution prepared as a substrate in an amount of 1 g/10 mL of water, boiled for 2 min, the sample volume was adjusted to 100 mL with distilled water. 100 mL of pancreatic α -amylase (0.1 M Na-acetate buffer is 13 mL at pH 7.2), 100 μ g of endophyte extract, 2 mL of acetate buffer were incubated for 10 min at 30°C for 2 mL 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: $(A_0 - A_t)/A_0 \times 100\%$, where A_0 is the absorption of the control sample, and A_t is the absorption of the experimental sample, respectively. As a comparison drug, acarbose was used from a commercial drug "Glucobay" (Bayer Pharma AG, Germany) was used.

The concentration causing 50% inhibition of pancreatic α -amylase (IC_{50}) by the test samples was quantified as described by Murado et al.¹⁷

Calculation of results

The values are expressed as the mean value of \pm standard deviation (n: 3). Statistical analysis has not been performed for evaluation of the results.

RESULTS AND DISCUSSION

As mentioned above, for the 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 was extracted by methanol.¹⁵

Figure 1 demonstrates the total ion chromatogram of the initial methanol fraction of *A. egypticus*-HT166S. As can be seen from the chromatographic data, the methanol fraction contained many substances, three of which are represented by relatively high peaks.

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%. Simultaneously, the highest level of inhibitory activity was noted in the K-10 fraction with an R_f value of 0.74 and the content of secondary metabolites constituting 10% of the initial weight of the dry methanol fraction (Table 1).

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

Note 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

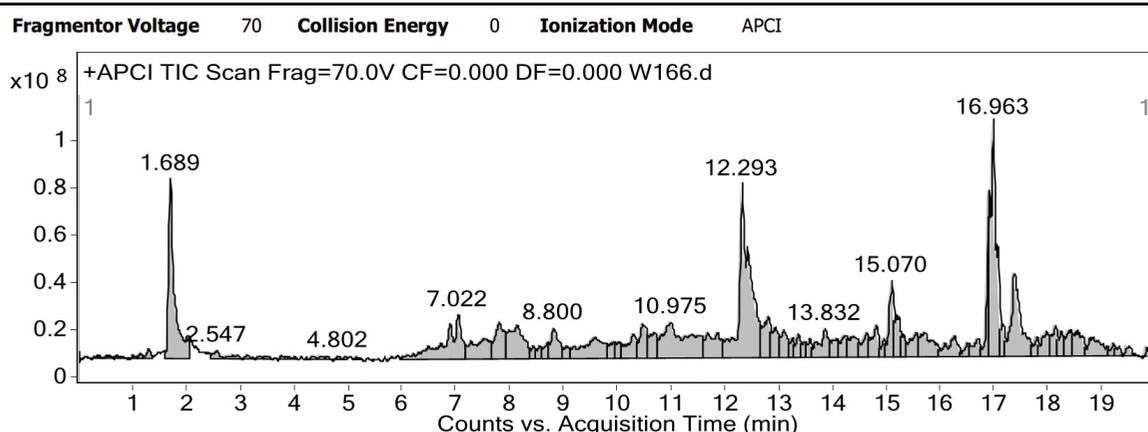


Figure 1. Total ion chromatogram of the total methanol fraction of the biomass *Aspergillus egypticus*-HT166S.

Because 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 obtained fraction of metabolites was further evaluated by inhibition of pancreatic amylase

Table 1. Content and inhibitory activities of samples obtained from purification on a column of the total methanol fraction of *Aspergillus egypticus*-HT166S

Fractions	R_f	Dry weight, %	α -Amylase inhibition, %
A-1	0.13	3.8 \pm 0.02	14.6 \pm 0.29
B-2	0.22	4.5 \pm 0.03	15.2 \pm 0.29
C-3	0.30	7.8 \pm 0.37	28.6 \pm 0.30
D-4	0.37	4.1 \pm 0.35	17.7 \pm 0.27
E -5	0.44	3.2 \pm 0.33	25 \pm 0.28
F-6	0.48	2.7 \pm 0.13	15 \pm 0.34
G-7	0.52	3.1 \pm 0.04	24.3 \pm 0.29
H-8	0.54	6.5 \pm 0.34	7.0 \pm 0.26
J-9	0.6	5.2 \pm 0.24	24 \pm 0.30
K-10	0.74	10 \pm 0.17	76.2 \pm 0.29
L-11	0.86	6.6 \pm 0.14	18.8 \pm 0.30
M-12	0.97	2.3 \pm 0.03	-
Total methanol fraction	-	100	75.4 \pm 0.27

Each value is the average of three analyses \pm standard deviation

in vivo studies have shown that they can prevent diabetes and its complications.¹⁹ In identifying flavonoids, we referred to the experimental data of Zhang et al.²⁰, 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.

The mass spectral analysis of the bioactive K-10 sample showed compounds with molecular ions $[M + H]^+$ with m/z 359.0, m/z 345.0, and m/z 327.0 (Figure 2).

On comparative analysis of our results with the literature data, the compounds were assigned as polymethoxylated flavones (PMF).

PMF is a subclass of flavonoids in which all or almost all hydroxyls are blocked by methylation, have high oral bioavailability, exhibit anti-allergic, antioxidant, antibacterial, antiproliferative, anti-inflammatory, and anti-cancer activities.²¹ The literature provides information on PMFs, mainly nobiletin, tangeretin, sinensetin, and isosinensetin from citrus plants, and discusses their antidiabetic effects *in vitro*.²² For example; nobiletin, the polymethoxylated flavonoid, reduces the inflammation associated with gestational DM (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.²³ Sundaram et al.²⁴ 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 revealed 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.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_{50} values of 4.82 mg/mL and 4.74 mg/mL, respectively, compared to IC_{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 that of the reference drug (acarbose), and indeed contains bioactive compounds with potential inhibitory activity against α -amylase (Figure 3).

CONCLUSION

Natural bioactive compounds can inhibit α -amylase, which are the best and most useful substances to lower the blood

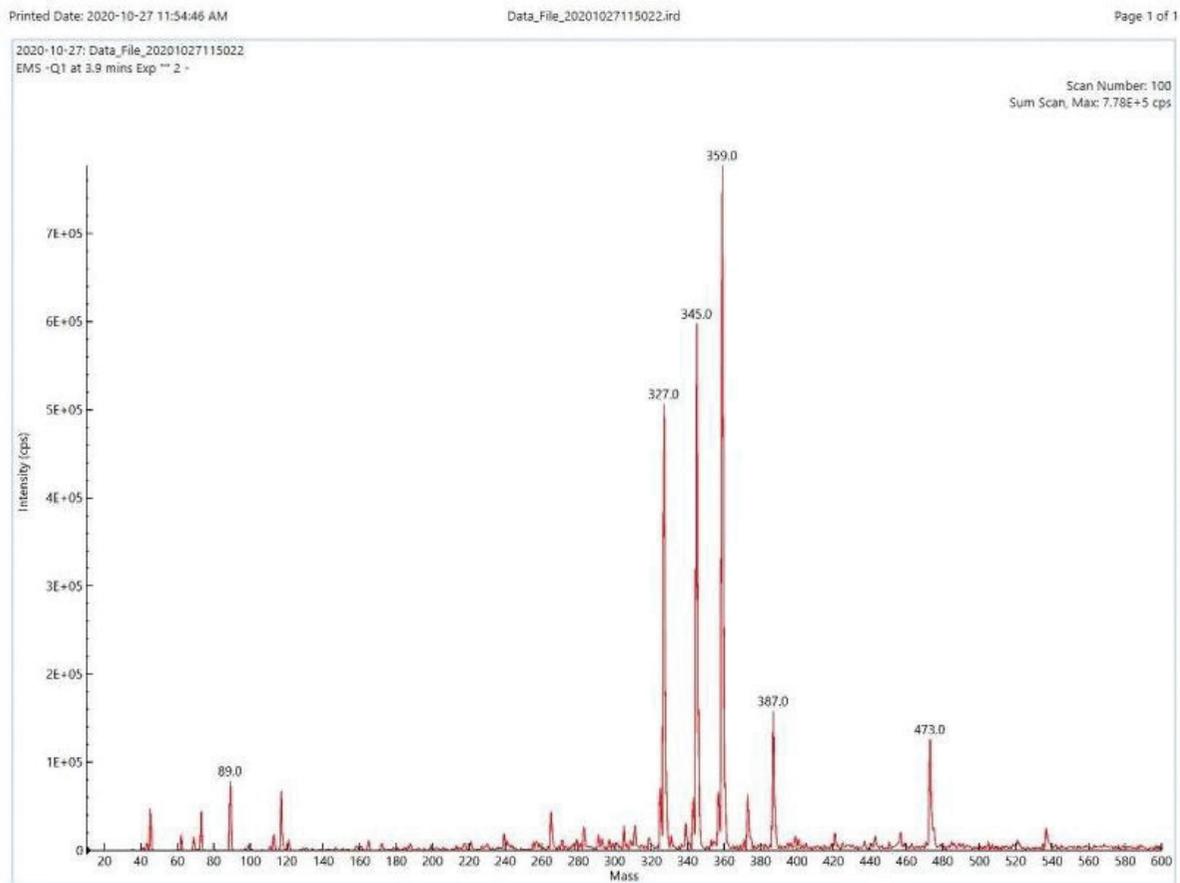


Figure 2. Mass spectral analysis of the fraction K-10

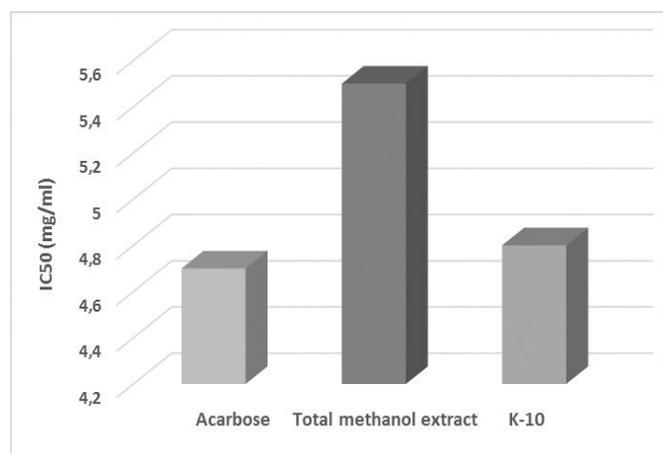


Figure 3. IC₅₀ (in µg/mL) of total methanol extract and K-10 fraction compared with acarbose

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 developing new technologies for the production of microbial amylase inhibitors.

The presented studies show that the *A. egypticus*-HT166S endophyte from *H. tuberosus* produces PMF with high inhibitory activity against pancreatic α -amylase, comparable to the activity of the commercial drug, acarbose. However, to establish the structure of inhibitory PMF, it is necessary to conduct further studies using analysis - infrared and nuclear magnetic resonance spectroscopy.

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

Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: I.M., G.R., Concept: S.N., Design: S.N., Data Collection or Processing: D.R., Analysis or Interpretation: T.G., Literature Search: D.R., Writing: D.R.

Conflict of Interest: No conflict of interest was declared by the authors.

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