

ISOLATION OF α -AMYLASE INHIBITORS FROM METHANOL FRACTION OF THE ENDOPHYTIC FUNGUS *PENICILLIUM BREVICAULE* ALBA THOM

Saodat Nasmetova, Tashkhan Gulyamova, Dilorom Ruzieva, Iqbol Mukhammedov, Lilia Abdulmyanova

Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan

Imb-anruz@mail.ru

Abstract. *Postprandial hyperglycemia is a significant risk factor involved in type 2 diabetes. The elevated level of postprandial hyperglycemia is caused by carbohydrate-hydrolyzing enzymes, α -amylase, and α -glucosidase. Inhibition of these enzymes appears to be an effective means for diabetes management. Recent studies suggested that endophytic fungi of some medicinal plants produce α -glucosidase and α -amylase inhibitors. The endophytic fungus *P. brevicaule alba-CC 200*, which exposes inhibition of α -amylase, was isolated from the *Celosia cristata* plant. This article presents data on the purification of inhibitory compounds from the methanolic extract of *Penicillium brevicaule alba Thom-CC200*. Fraction of G-7 with the highest inhibitory activity of 76.5% isolated by adsorption column chromatography. The yield of dry samples was more than 18 weight % (w/w) with more than 60% purity. The HPLC and LC-MS of fraction G-7 indicated that the inhibitory activity is these fractions associated with the triterpene saponins.*

Keywords: *diabetes mellitus, endophytic fungi, *Penicillium brevicaule alba Thom-CC200*, α -amylase inhibitors, extract, secondary metabolite, chromatography.*

Diabetes mellitus is the endocrine and metabolic disorder characterized by chronic hyperglycemia, impaired lipid, and protein metabolism and severely impacts health, quality, and life expectancy. According to the International Diabetes Federation, about 415 million people have diabetes. Of the total number of diabetes patients, insulin-independent type 2 diabetes (DM2) accounts for 90-95% and leads to severe complications such as nephropathy, neuropathy, retinopathy, and cardiovascular diseases [1].

Control of the kinetics of carbohydrate digestion and monosaccharide absorption used to treat diabetes. Inhibitors of the digestive enzymes α -glycosidase and α -amylase used for the treatment of diabetes. Acarbose and voglibose inhibitors prolong the total digestion of carbohydrates and absorption in the small intestine [2-4]. However, acarbose is primarily known as an α -glucosidase inhibitor and causes side effects such as bloating, flatulence, and diarrhea [5, 6].

Natural products offer a broad structural diversity compared to standard synthetic substances, providing more significant pharmaceutical searching opportunities for new molecules [7]. For example, 58 compounds of medicinal plants can inhibit the enzymes α -

amylase and α -glycosidase. These substances mainly belong to flavones, flavone glycosides, triterpenes, alkaloids, tannins, and other polyphenolic compounds [8].

Endophytic fungi of medicinal antidiabetic plants due to their ability to synthesize the same substances as the host plant, including α -amylase inhibitors, are an auspicious object as alternative producers of hypoglycemic compounds with minimal side effects [9].

In scientific literature, several reports indicate the ability of endophytic fungi to produce compounds that exhibit hypoglycemic activity by inhibiting α -amylase [10-14]. For example, secondary metabolites of ethyl acetate fractions of endophytic fungi *Colletotrichum capsici*, isolated from the plant *Eugenia cumini* L, have a potent antibacterial efficacy, antidiabetic action and are fatty acids and phenolic compounds [16].

Similar results were reported by Govindappa M. et al., which in vitro determined the antioxidant, antidiabetic, and anticholinesterase activity of biologically relevant phytochemical compounds in methanol extract of endophytic fungi *Cladosporium uredinicola*. Qualitative analysis of the extracts showed flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids, and coumarins [17]. Extracts of eleven antidiabetic medicinal plants also studied for the activity of inhibition of α -glucosidase and α -amylase in vitro. The extracts of *Aralia taibiensis* bark surpassed other extracts by IC50 values and showed high inhibitory activity against α -glucosidase and α -amylase [18].

Earlier, we isolated the endophytic fungus *P.brevicaule alba Thom-CC200* from the plant *Celosia cristata*, belonging to the family *Amaranthaceae*, with pronounced inhibitory activity to pancreatic α -amylase. As a result of studies on the composition of intracellular metabolites of endophyte, *P. brevicaule alba-CC200* biomass by step fractionation, the highest degree of inhibition of α -amylase found the methanol fraction - 88.7 %. This work aimed to purify and identify bioactive compounds of the methanol fraction of the endophytic fungus *P.brevicaule alba-CC200* that inhibit the activity of α -amylase by column chromatography, HPLC and LC-MS / MS analysis.

Materials and methods

Cultivation.

The endophytic fungus *P. brevicaule alba Thom-CC 200*, isolated from the inflorescence of the plant *Celosia cristata*, was grown on Czapek–Dox medium on a rocker at 160 rpm for seven days.

Extraction of secondary metabolites of P. brevicaule alba Thom - CC 200

Isolation of secondary metabolites from the ethyl acetate extract of *P. brevicaule alba Thom - CC 200* carried out according to the scheme proposed by Kumar et al.[19], which includes sequential extraction in water, a mixture of methanol with hexane (1:1), and butanol. As a result, a methanolic extract obtained with a high - 88.7% inhibitory activity. The extract dried on a rotary evaporator, and 1 ml of dimethyl sulfoxide added the resulting methanol extract stored at 4 ° C before use.

Inhibition α -amylase activity.

The determination of α -amylase activity was carried out by the modified iodine method protocol (25). To 2 ml of starch solution, 100 μ l pancreatic α -amylase (13 u/ml in 0.1 M Na-acetate buffer pH4,7), 100 μ l of the extract endophyte (20 mg/ml), 2 ml of acetate buffer were added and incubated for 10 minutes at 30⁰ C. Control sample was without the

extract. After incubation, the reaction terminated by adding 10 ml of iodine reagent, and the absorbance recorded at 630 nm. Iodine reagent prepared from 0.5 g of crystalline iodine, 5 g of potassium iodide dissolved in 250 ml of water; to obtain a working solution of 2 ml of reagent adjusted to 100 ml by 0.1 M HCL. The experiment was in triplicates, calculated by the formula: $(A_0 - A_t)/A_0 \times 100\%$, A_0 - absorption of a control sample, A_t - the absorption of a test sample. Acarbose used as standard.

Column chromatography

An amount of 500 mg extracts were homogenized with Celite 545, dried and fractionated using chloroform:EtOAc (50:1~1:1) graduated solvent system to yield some fractions. Those fractions with same R_f value after TLC analysis were pooled together and evaporated till dried fraction (A1-J9) was obtained.

HPLC analysis

HPLC analysis performed on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6*150 mm, 5 μ m), with a mobile phase of a mixture of acetonitrile and 0.1% phosphoric acid (60:40 v/v) as an eluent. The volume of the injected sample is 20 μ l. The flow rate is 1.5 ml/min at a wavelength of 238 nm.

LC-MS analysis

Mass spectra of extracts taken on Q-TOF LC-MS (Agilent Technologies 6520B) under the following conditions: ion source ESI+, electrospray positive ion method, the drying gas flow rate 5 l/min, drying gas temperature 300°C, the ion acceleration voltage on skimmer 35V, fragmentor 175V, MS range 150 – 1000 m/z targeted MS-MS 50 – 1000 m/z, collision energy 30, 40, 50, 65. Samples were injected on Zorbax SB C18 column, 3 μ m, 150x0,5 mm (Agilent Technologies 1200) with mobile phase: A - 0.1% formic acid, B – acetonitrile + 0,1 % formic acid. Elution on Agilent Technologies 1260 Cap Pump at 15 μ l/min: 5 min - 60%, 15-20 min – 90%, 25 min – 60% of mobile phase B in three replications.

The values expressed as an average value of \pm S.D (n = 3).

Results and discussion

Previously from the Jerusalem artichoke plant, which is widely used in the diet of diabetic patients, we have isolated endophytes producing secondary metabolites with significant inhibitory activity to pancreatic α -amylase [21]. *P. brevicaule alba*'s inhibitory activity, one of the most active strains, was 88,7%. Phytochemical and TLC analysis of *P. brevicaule alba*- CC200 methanol extracts showed saponins, terpenoids, and cardiac glycosides [22].

In this work, to clarify the nature of the inhibitory metabolite, we carried out the separation of the methanol extract by adsorption chromatography on silica gel, followed by HPLC and mass spectral analysis of the isolated active fractions.

As a result of chromatography of 500 mg of dry methanol fraction, 9 eluates selected. The eluates tested for α -amylase inhibitory activity at a concentration of 5 mg/ml of the eluent. The tested samples showed different degrees of inhibition, from 12.4% to 76.5%.

As can be seen from data in Table 1, the highest level of inhibitory activity, which is 76.5%, belong to eluate G-7. Drying of the samples by vacuum evaporation caused the formation of a white amorphous powder. The yield of dry substances of active fraction contains 18.6 % (w/w) of the dry methanol fraction (Table 1)

Table 1. Separation of bioactive compounds of the methanol fraction of *P. brevicaula alba*-CC200 that inhibit the activity of α -amylase by column chromatography

| Nº | fractions | Yield, % | α -amylase inhibition, % |
|------|-----------|----------|---------------------------------|
| I | A-1 | 16.1 | - |
| II | B-2 | 12.2 | - |
| III | C-3 | 6.7 | 12.4 |
| IV | D-4 | 7.16 | - |
| V | E -5 | 11 | 15.6 |
| VI | F-6 | 3.04 | - |
| VII | G-7 | 18.6 | 76.5 |
| VIII | H-8 | 8.2 | 27.2 |
| IX | J-9 | 1.4 | - |

Purity of the selected active sample G-7 by HPLC. The presented data indicate a relatively high homogeneity of the samples purified on the column (Fig1).

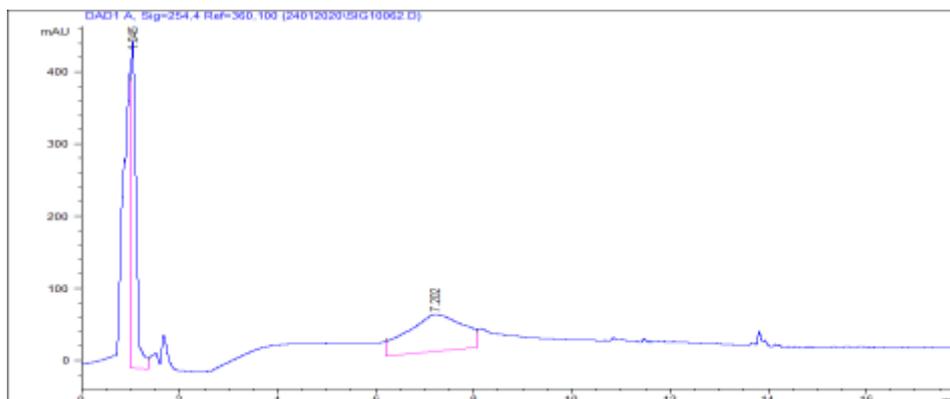
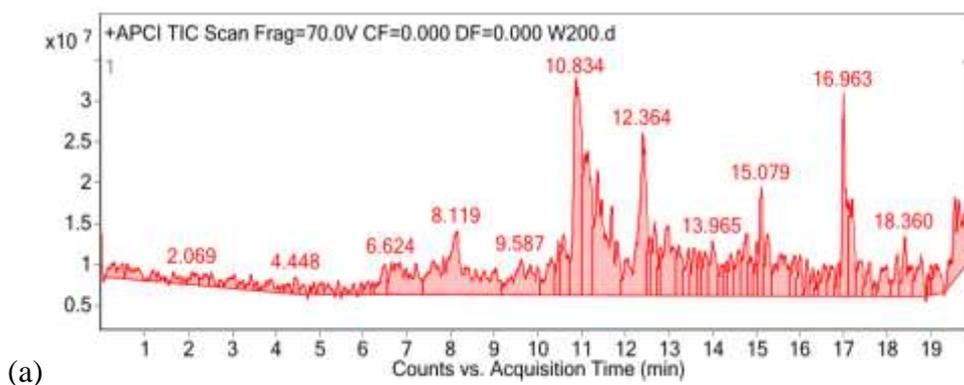
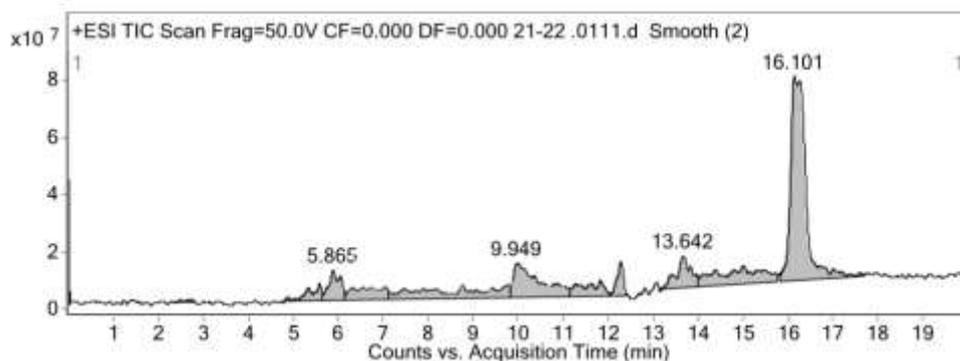


Fig. 1. HPLC profile of bioactive fractions G-7 isolated from *P. brevicaula alba*-CC200 methanolic extract

As can be seen from the data of TIC chromatograms of the total methanol fraction contains several substances represented by different peaks in height (Fig. 3, a). The fraction G-7 contains four compounds, three of which as minor peaks, while the main component corresponds to the highest peak at 16.1 minutes of the chromatogram profile (Fig. 3, b).



(a)



(6)

**Fig. 2. TIC-chromatogram of *P. brevicaule alba* – CC200 secondary metabolites:
a) the total methanol extract; b) fraction G-7**

MS-analysis of the main compound at 16.1 min in the fraction G-7 gives a molecular ion (M + H) with m / z 803.7 and fragment ions with m / z 391.3 and m / z 605.7 (Fig. 3).

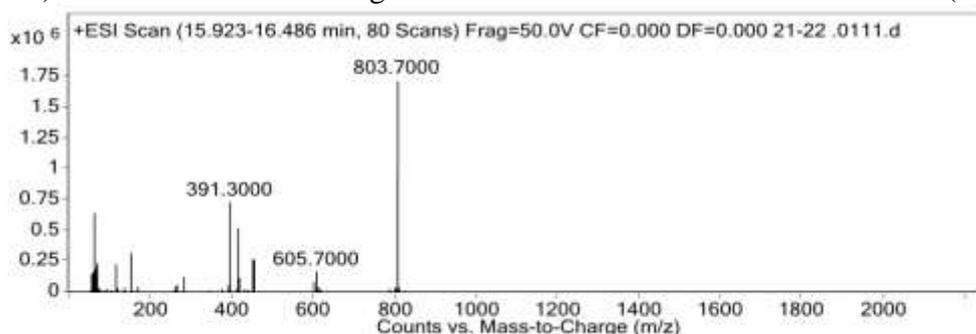


Fig. 3. MS - analysis of the main peak of fraction G-7 isolated from methanolic extract of *P. brevicaule alba*-CC200.

It emphasized that the phytochemical analysis of the fraction G-7 showed a positive reaction to saponins, as evidenced by the formation of thick and persistent foam in an aqueous solution. By foaming, the group belonging of saponins was determined and roughly attributed to triterpene saponins [23] (Fig. 4)

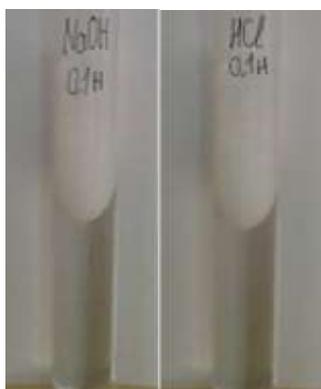


Fig.4. Determination of the triterpene saponins in fraction G-7 isolated from methanol extract of *P.brevicaule alba* – CC200.

Three drops of an aqueous solution (1:10) of fraction G-7 added to test tubes with 5 ml of 0,1N HCl and 0,1 N NaOH and shook for 30 min. The formation of equal height and stability foam column indicates the presence of the triterpene saponins.

Triterpene saponins are known to have a relatively high molecular weight. For example, glycyrrhizic acid, which used as a food sweetener and a drug in licorice preparations, has a molecular weight of 822.94 g / mol. According to the ion spectrum m/z 803.7 (M⁺) readings, it assumed that the active fraction's main component most likely represented by saponin and, possibly, a derivative of glycyrrhizic acid.

The studies of triterpene saponins have increased dramatically due to their diverse and potentially attractive biological activity. The literature contains data on the chemical structures of several hundred triterpenoid saponins of plant and animal origin. Triterpenoid saponins consist of a triterpene aglycone with one or more sugar residues attached. However, due to similar physical and chemical properties, the isolation and identification of a large variety of triterpenoid saponins remain difficult [24].

There are also reports on the antidiabetic properties of triterpene saponins of medicinal plants [25,26]. Many studies suggest using triterpene saponins to prevent diabetic complications such as nephropathy, embryopathy, neuropathy, or degenerative wounds [27]. Four triterpenoid saponin compounds detected in the methanolic extract of the leaves of the *Piper auritum* plant based on bioactivity. These compounds significantly reduced serum glucose, total cholesterol, and triglycerides in vivo in mice compared to controls [28]. There are different patterns of occurrence of triterpene saponins in almost 30 species belonging to the Amaranthaceae family, including *Celosia cristata* L. [29]. The bioactivity of saponins mixtures or individual saponins in vitro and in vivo indicates that they can be used as antidiabetic, cytotoxic, immunomodulatory, hepatoprotective, hypolipidemic, and anti-inflammatory agents.

Kumar BS et al. studied the structure and pharmacological properties of triterpene saponins of Amaranth family plants [30]. Determination of the antioxidant and inhibitory activity to α -amylase of methanol extract of *Amaranthus Caudatus* Linn showed high inhibitory activity (IC₅₀ 19.2 mcg/ml). The extract contained pentacyclic triterpene saponins, represented by oleanane, ursane, and lupane derivatives, and others with a vast range of structures [31].

In this regard, the results of our research and existing reports can be an indirect indication that the inhibitory activity to α -amylase of endophytic fungus *P. brevicaulis* CC200 isolated from *Celosia cristata* due to triterpene saponins peculiar to the host plant itself. However, further studies by IR and NMR spectroscopy and comparison with electronic libraries' data are necessary to determine the inhibitory compound structure.

Thus, the endophytic fungus *P. brevicaulis* CC200 can be considered a promising source of α -amylase inhibitors, and further research will lead to obtaining new natural antidiabetic drugs.

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