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Study of α-Amylase Inhibitory Activity of Fractions Purified by Chromatographic Methods of the Endophytic Fungus *Penicillium Brevicaule Alba-CC200*

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Abstract- In this study, the antidiabetic activity of purified fractions of the endophytic fungus *Penicillium brevicaule alba-CC200* isolated from the plant *Celosia cristata* was investigated. Antidiabetic activity was evaluated by inhibition of α-amylase enzyme. The secondary metabolites of *P. brevicaule alba-CC200* were partially purified by column chromatography and obtained fraction II inhibited α-amylase by 91.2%. High inhibitory activity was observed in fraction II and was determined to be cardiac glycosides and terpenoids by thin layer chromatography (TLC) and qualitative analysis. This requires a more precise study of the purified fractions using high-precision chromatographic methods.

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I. Introduction

According to the World Health Organization, there are more than 400 million diabetic patients in the world today. According to analysis, this figure may exceed 550 million by 2030. In Uzbekistan, 220,000 people have been registered with this disease [1]. In recent years, diabetes has taken the third place among the causes of death, and cardiovascular and oncological diseases are in the second place. The social consequences of diabetes are also negative, because the treatment of this disease requires huge costs from the health care system [2]. Diabetes mellitus is a disease characterized by the presence of chronic hyperglycemia accompanied by metabolic disorders of carbohydrates, lipids and proteins [3].

In the last decade, a number of new methods of diabetes treatment have been proposed, but three areas remain the main ones: a) diet, insulin for patients with type 1 diabetes, b) hypoglycemic drugs used for type 2 diabetes, c) general procedure and physical activity [2].

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There are many different therapeutic approaches in the treatment of type 2 diabetes. Inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase is one of the important approaches to lowering postprandial blood glucose levels. Such inhibitors, which are used in clinical practice for the treatment of diabetes mellitus, are known to be associated with various adverse gastrointestinal effects. Therefore, it is necessary to identify amylase inhibitors from natural sources with less adverse effects. *Caesalpinia bonducella* aqueous extract inhibited α-amylase activity by 87.26% [3]. The inhibitory effect on α-amylase was studied by ethanol extract of *Capparis spinosa*. A concentration of 25 mg/ml of extracts from roots and leaves inhibited α-amylase by 97.31-98.92% [4].

In vitro α-amylase inhibitory activity of secondary metabolites isolated from *S. persica* and the chemical composition responsible for its activity were studied. Aqueous extract of *S. persica* highly inhibited α-amylase activity by 72.39% and had an IC_{50} value of 376 μg/ml compared to acarbose with an inhibitory value of 65.99%. In the analysis of TLC, it was found that there are 15 different compounds [5].

Albidopyroneis synthesized by *Streptomyces sp.,* an actinomycete isolated from sediments at the bottom of the Atlantic Ocean. The chemical structure of albidopyrone is a six-membered aromatic lactone, with a methyl group in the 3rd position, an OH group in the 4th position, and an acetic acid aniline amide residue in the 6th position. Albidopyrone is not an antibiotic, but together with tyrosine kinases, it inhibits the activity of tyrosine phosphate B (TPB), which regulates tyrosine phosphorylation. TPB is the main negative regulator of insulin (controls the phosphor state of the insulin receptor). In this regard, TPB inhibitors are effective agents for the treatment of diabetes [6].

Botryodiplodia teobromae is an endophytic fungus isolated from the weed *Euphorbia hirta*. Fungi were treated with 1mM $AgNO_3$ to synthesize silver nanoparticles. The production of silver nanoparticles was confirmed using the cell filter and surface color

change from colorless to dark brown. Silver nanoparticles synthesized from *Botryodiplodia theobromae* fungi show the highest inhibitory percentage (94%) for α-amylase [7]. Endophytic fungi were isolated from the leaves of *Artocarpus heterophyllus*. The inhibitory activity of the obtained extracts on α-amylase was 89%, and 71% on standard acarbose [8].

In the treatment of patients with type 2 diabetes, α-glucosidase enzyme inhibitors are used. One of them, acarbose, is a pseudo tetrasaccharide that competes with mono- and disaccharides for digestive enzyme binding sites and slows the sequential fermentation of carbohydrates and absorption of carbohydrates in the small intestine [2, 4]. Butacarbose is mainly known as an α-glucosidase inhibitor and causes side effects such as abdominal distension, flatulence, and diarrhea [9, 10].

In pharmaceutics, natural products provide greater structural diversity than standard synthetic substances, offering great opportunities for the discovery of new compounds [11]. Lowering blood glucose is an effective tool in improving the survival rate of patients with diabetes. Endophytic fungi isolated from plants can be an alternative source of secondary metabolites for the treatment of diabetes [12].

Endophytic fungi are a hidden treasure trove of chemically important compounds with potential biological activity. Many natural compounds have been isolated from various endophytes [13]. Bioactive compounds produced by endophytic fungi include alkaloids, benzopyranones, quinones, cytochalazins, depsipeptides, enniatins, flavonoids, furandiones, isocoumarins, peptides, polyketones, phenols, hydroquinones, terpenoids, tetralones, and xanthones. Therefore, endophytes constitute a chemical reserve of new biologically active compounds, which are used in the pharmaceutical and agrochemical industries as antioxidant, antimicrobial, antidiabetic, antiparasitic, antiviral, insecticidal, antibiotic, immunosuppressive, and immunomodulatory agents [14, 15]. Endophytic fungi produce a variety of chemicals to allow them to live in plant cells. Due to their symbiotic relationship, they secrete pharmaceutically important biologically active compounds and enzyme inhibitors. 32 endophytes were isolated from the *Gymnema sylvestre* plant and their inhibition of α-amylase and α-glucosidase of *Fusarium equiseti* endophytic fungus was observed [16]. Asan α-amylase inhibitor, the IC_{50} value of the methanol extract of *Penicillium oxalicum* was 46.73 mg/ml, and the chloroform extract was 59.20 mg/ml [17]. Extracts from the endophytic fungus *Aspergillus awamori* isolated from *Acacia nilotica* inhibited α-amylase by 81% and α-glucosidase by 80% [18].

Secondary metabolites of the endophytic fungus *P. brevicaule alba-CC200* isolated from *Celosia cristata* were studied as inhibitors of pancreatic α-amylase enzyme. The ethylacetate extract of the obtained secondary metabolites showed 93% high inhibitory activity to α-amylase, and qualitative analysis revealed that the extract contained terpenoid, saponin, glycoside and phenolic components [19]. At the same time, to confirm which of the detected substances exert inhibitory activity, the method consists of elucidating through TLC, column chromatography and qualitative analysis methods.

II. Material and Methods

a) Cultivation of endophytic fungi

Both endophytic fungi were grown in the Czapek Dox nutrient medium on orbital shaker Elon 357 (Joanlab, Poland) at 180 rpm, 28°C, and for seven days. The biomass of the cultures was yielded by centrifugation at RS-6 (RHSnab, Russia) at 6,000 rpm and stored at +4°C.

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 [20]. 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: (A0-At)/A0x100%, where A0 is the absorption of the control sample, and T is the absorption of the experimental sample, respectively. As a comparison drug, acarbose from the commercial drug "Glucobay" (Bayer Pharma AG, Germany) was used.

c) Phytochemical analysis of extracts of secondary metabolites

Qualitative composition of compounds in extracts of endophytic fungi determined according to Prabhavathi et al. [21].

The tannins and phenolic substances was determined by adding 2-3 drops of 1% $FeCl_3$ solution to 2 ml of the extract. In the presence of iron ions, tannins give a black-blue or black-green color, and phenols - are purple.

The presence of saponins was established by diluting 1 ml of the extract with 5 ml of hot water (60 °C)

with intensive shaking for 5 minutes until the formation of a persistent foam. The foam volume was maintained for the next 30 minutes.

The terpenoids were determined by mixing 0.5 ml of the extract with 2 ml chloroform and 3 ml of H_2SO_4 (conc.). The formation between the phases of red-brown staining indicates the presence of terpenoids. 2 ml of the extract was mixed with 4 ml of hexane and shaken to determine the terpenoids. At the same time, the separation of the extract into 2 layers was observed. The upper layer was separated, 4 ml of 10% ammonia was added, and the lower layer's color was determined. The purple-pink color indicated the presence of anthraquinones.

The presence of cardiac glycosides was determined by mixing 1 ml of the extract with 1 ml of glacial acetic acid and then adding one drop of 3% ferric chloride in methanol. Then H_2SO_4 (conc.) was added along the tube wall, and the color of the lower layer was determined. Blue-green staining indicated the presence of cardiac glycosides.

To determine flavonoids, a few drops of 20% sodium hydroxide were added to 2 ml of each extract, and the formation of an intense yellow color was observed. Next, a few drops of 70% dilute hydrochloric acid was added, and the yellow color disappeared. The formation and disappearance of yellow color indicate the presence of flavonoids in the sample extract.

The alkaloids was determined by their ability to form compounds insoluble in water with complex iodides, which makes it possible to establish the presence of alkaloids even with their insignificant content. A solution of iodine in potassium iodide (Wagner reagent, Bouchard reagent) with alkaloids form brown, hardly soluble in water precipitates. Five drops of the reagent for precipitation of alkaloids are added to 1 ml of the extract. In the presence of alkaloids, a brown precipitate appears.

d) Thin layer chromatography (TLC)

In thin-layer chromatography, components are partitioned between stationary and mobile phases. Separation of the constituent parts moves at different speeds in the TLC plates depending on its polarity in stationary and mobile phases. SIGMA-ALDRICH Silicagelon TLCAL plates of size 10x10 cm were used for TLC. Plates were run in the following eluents: benzene: methanol (40:8) in a standard chromatographic chamber. 25µl of the extracts at a concentration of 25 mg/ml were dropped onto the starting line of the plates. The plates taken from the chromatographic chamber were dried and the following methods were used to determine the separated substances: under ultraviolet light at 254 nm; with iodine vapor in a desiccator; 20% solution of phosphorous-molybdic acid in alcohol; It was sprayed with a 10% solution of H_2SO_4 acid in methanol and dried at 110°C.

e) Column chromatography

Partial purification of the extracts was performed with modifications from Kaur et al. The extract was applied to a column (2 × 25 cm) of silica gel (size 100–250). The mobile phase used is chloroform: ethyl acetate: formic acid in a ratio of 5:4:1. Using this solvent system, fractions of 10 mL each were collected and designated as I and II, respectively. Activity was observed in fraction II. The chemical nature of the active fractions was determined using qualitative analysis and TLC methods [22].

Results were the mean of replicate analysis.

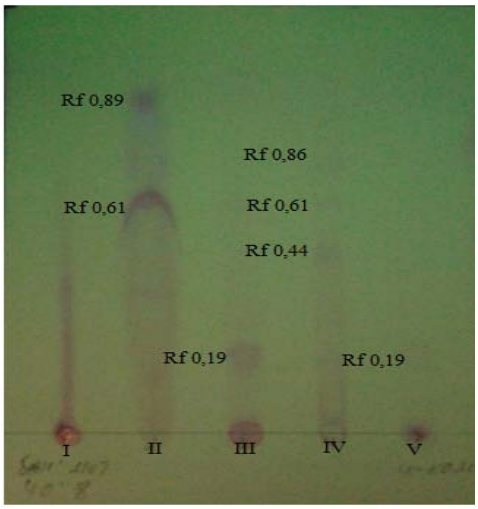
III. Results and Discussion

58 compounds that can inhibit α-amylase and α-glycosidase enzymes were identified in hyperglycemia. These substances mainly belong to flavones, flavone glycosides, triterpenes, alkaloids, tannins and other polyphenol compounds [23]. Partially purified enzyme inhibitors result in high activity, with maximal inhibitory activity determined based on morphological and molecular analysis [24].

Our results show that ethylacetate extract of *P. brevicaule alba–CC200* contains compounds responsible for the inhibition of amylase activity, so the extract was purified. Five fractions were obtained from the extracts by column chromatography, dissolved in DMSO and tested as an α-amylase inhibitor. From the data in Table 1, it can be seen that the highest inhibitory activity was 91.2% in fraction II, and the methanol fraction before chromatographic purification was 88.7%. Low inhibitory activity was 58.4% in fraction I, 26% in fraction III, 38.7% in fraction IV, and 12.4% in fraction V.

Table 1: Analysis of the inhibitory level of secondary metabolites of the endophytic fungus *P. brevicaule alba-CC200* and the analysis of fractions obtained by column chromatography.

Fractions	α-amylase % inhibitor	Rf
I	58,4	-
II	91,2	0,89
		0,61
III	26	0,19
IV	38,7	0,86
		0,61
		0,44
V	12,4	0,19



Eluent system: benzene:methanol (5:1). The volume of samples used was 25 μl. Determining reagent: 20% alcoholic solution of phosphorus-molybdic acid.

Fractions obtained as a result of column chromatography were checked for chemical purity of substances using TLC. When TLC plates were treated with a 20% alcoholic solution of phosphoro-molybdic acid, the spots were colored blue, which is due to the presence of sopanin and triterpenes. Jasleen Kaur et al., partially purified *Alternaria* endophytic fungus and when treated with $FeCl_3$ on TLC plates, the spots were stained purple, which can be attributed to the characteristic of phenolic compounds in the fraction [25]. Partially purified fraction III obtained from the endophytic fungus *Colletotrichum capsici* was found to have potent antidiabetic activity. In addition, determination by mass spectrometry showed that compounds in fraction III are mainly fatty acid and phenolic compounds [26]. Terpenoids, formaldehyde and eugenol biologically active substances were detected when ethylacetate extracts of endophytic bacteria isolated from *Mentha piperita* (mint) plant were examined using GC-MS spectrum [27]. Antioxidant, antidiabetic and anticholinesterase activity of biologically important phytochemicals contained in the methanol extract of *C. uredinicola* endophytic fungi was determined in vitro. The qualitative analysis of *C. uredinicola* extract shows the presence of flavonoids, tannins, alkaloids, glycosides, phenols, terpenoids and coumarins [28].

P. brevicale alba-CC200			
Fraction	α-amylase % inhibitor	Terpenoids	Cardiac glycosides
II	91,2		

Figure 1: Qualitative analysis of fraction II obtained by column chromatography

Also, fraction II with high inhibitory activity was tested by qualitative reactions for several chemical classes. The presence of cardiac glycosides and terpenoids was determined in the fraction with 91.2% α-amylase inhibitor. Figure 1.

Many studies suggest the use of triterpenes in the prevention of diabetic complications such as nephropathy, embryopathy, neuropathy or wound healing [29]. For example, oleanolic acid, betulinic acid and ursodeoxycholic acid are strong inhibitors of TGR5 receptors involved in energy metabolism [30]. The n-hexane fraction with the highest inhibitory activity of the plant *Nuxia oppositifolia* confirmed the presence of 3-oxolupenal and cathononic acid when examined using GC-MS analysis [31]. Many reports attribute the α-amylase inhibitory effect of the extracts to the terpenoids present in the extracts [32, 33].

IV. Conclusion

Fraction II of the endophytic fungus *P. brevicaule alba - CC200*, purified by column chromatography, inhibited α-amylase by 91.2%. TLC and quality analysis showed that the high inhibitory activity of fraction II is due to the content of cardiac glycosides and terpenoids. These natural bioactive compounds can inhibit α-amylase enzyme activity and regulate blood glucose levels. It can be an alternative source for hyperglycemic compounds.

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