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Detection of α-amylase inhibitors using thin-layer chromatography

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Abstract. There are many natural compounds in the treatment of type 2 diabetes mellitus, and the nature of most of them has not yet been studied. Because the diversity and sources of natural compounds are large. Also, their examination requires a lot of time and expense. We carried out experiments on endophytic fungi *Penicillium brevicaule alba-CC200* and *Aspergillus egypticus-HT166S*, isolated from local plants *Celosia cristata* and *Helianthus tuberosus*. Secondary metabolites of both strains were separated into fractions using polar and non-polar solvents. Eluent systems were optimized to ensure complete separation of metabolites using thin-layer chromatography (TLC). The optimal separation of secondary metabolites was provided by an eluent system with a ratio of benzene:methanol (5:1). In particular, the methanol fraction of *P. brevicaule alba-CC200* yielded 6 Rf values (0.83; 0.74; 0.59; 0.57; 0.49; 0.45) and *A. egypticus-HT166S* yielded 12 Rf values (0.97; 0.86; 0.74; 0.60; 0.54; 0.52; 0.48; 0.44; 0.37; 0.30; 0.22; 0.13). The spots on the chromatographic plates were then incubated with the enzyme and the plates were placed in a starch medium. The α-amylase inhibitory activity of the substances on the TLC plate was determined by washing with iodine. Qualitative reactions were performed to study the nature of the blue spots on the surface of the plates by isolating the Rf values. It was found that saponins were present in *P. brevicaule alba-CC200* and flavonoids in *A. egypticus-HT166S*. This method is very simple, requires little time and expense. At the same time, it allows for rapid and visual screening of the inhibitory activity of many natural compounds.
Keywords: type 2 diabetes mellitus, endophytic fungi, α-amylase inhibitor, thin layer chromatography, secondary metabolites.
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ОРИГИНАЛЬНЫЕ СТАТЬИ

Определение ингибиторов α-амилазы с помощью тонкослойной хроматографии

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Аннотация. В лечении сахарного диабета 2 типа используется множество природных соединений, и природа большинства из них до сих пор не изучена. Поскольку разнообразие и источники природных соединений велики, их изучение также требует больших затрат времени и средств. Мы провели эксперименты на эндофитных грибах *Penicillium brevicaule alba-CC200* и *Aspergillus egypticus-HT166S*, выделенных из местных растений *Celosia cristata* и *Helianthus tuberosus*. Вторичные метаболиты обоих штаммов были разделены на фракции с использованием полярных и неполярных растворителей. Элюентные системы были оптимизированы для обеспечения полного разделения метаболитов с помощью тонкослойной хроматографии (ТСХ). Оптимальное разделение вторичных метаболитов было

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обеспечено элюентной системой с соотношением бензол:метанол (5:1). В частности, метанольная фракция *P. brevicaule alba-CC200* дала 6 значений Rf (0.83; 0.74; 0.59; 0.57; 0.49; 0.45), а *A. egypticus-HT166S* – 12 значений Rf (0.97; 0.86; 0.74; 0.60; 0.54; 0.52; 0.48; 0.44; 0.37; 0.30; 0.22; 0.13). Пятна на хроматографических пластинках затем инкубировали с ферментом, после чего пластинки помещали в крахмальную среду. Ингибирующую активность веществ в отношении α-амилазы на пластинке для тонкослойной хроматографии определяли промыванием йодом. Качественные реакции проводили для изучения природы синих пятен на поверхности пластинок путем выделения значений Rf. Было обнаружено наличие сапонинов в *P. brevicaule alba-CC200* и флавоноидов в *A. egypticus-HT166S*. Этот метод очень прост, не требует много времени и затрат. В то же время он позволяет быстро и визуально оценить ингибирующую активность многих природных соединений.

Ключевые слова: сахарный диабет 2 типа, эндофитные грибы, ингибитор α-амилазы, тонкослойная хроматография, вторичные метаболиты.

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Introduction

According to the International Diabetes Federation, approximately 463 million people were diagnosed with type 2 diabetes worldwide in 2019, and this figure is expected to increase to 642 million in 2040 [1]. These figures may increase the risk of a type 2 diabetes pandemic [2]. An estimated 422 million people worldwide currently suffer from type 2 diabetes, the majority of whom live in low- and middle-income countries [3]. It has become a tradition to treat patients with type 2 diabetes with hypoglycemic drugs [4]. Currently, drugs such as acarbose, voglibose and miglitol are used as α-glucosidase and α-amylase inhibitors in the treatment of type 2 diabetes mellitus, but their cost is high and they have negative clinical effects. Therefore, attention has been focused on natural α-amylase and α-glucosidase inhibitors [5]. It is known that secondary metabolites of endophytic fungi isolated from plants can be an alternative source in the treatment of diabetes mellitus [6]. Polar and non-polar solvents are used for the extraction of secondary metabolites depending on the nature of the compound being extracted [7]. However, when the nature and polarity of the active compounds are unknown, secondary metabolites are usually isolated and solvent screening is carried out [8]. Thin layer chromatography (TLC) method is also used for purification of secondary metabolites. TLC is considered to be a simple, easy and less labor-intensive method of analysis. It is also used in extraction of biologically active compounds [9]. TLC method can be estimated as a less labor-intensive visual method for screening of α-amylase inhibitors [10]. Our studies were conducted on local strains of *P. brevicaule alba-CC200* and *A. egypticus-HT166S* isolated from *Celosia cristata* and *Helianthus tuberosus* plants stored in the laboratory of the Collection of Industrially Important Microorganisms of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. In our previous studies, ethyl acetate extracts of secondary metabolites of both endophytic fungi inhibited α-amylase by 81% and 76% [11, 12, 13, 14, 15]. In the present studies, the active fractions of *P. brevicaule alba-CC200* and *A. egypticus-HT166S* strains were focused on the determination of the nature of the α-amylase inhibiting compound using TLC method.

Materials and methods

Endophyte cultivation. The two endophytic fungi obtained were grown on a Czapek-Dox nutrient medium in an Elpon 357 shaker (Poland) at 180 rpm and 28°C for 7 days. The biomass of the cultures was separated in a RS-6 centrifuge (Russia) at 6000 rpm and stored at +4°C [16].

Biomass drying. Biomass drying was carried out according to the method of Jane et al. [17]. A 5-10 g sample of biomass was weighed three times in a desiccator and the samples were dried at 105°C. The moisture

content of the biomass was determined by cooling it in a desiccator at room temperature.

Extraction of secondary metabolites from endophytic fungal biomass. Extraction of metabolites from endophytic fungal biomass to determine biological activity using the method of Hazalin et al., with modifications by Lang et al. [18]. For this purpose, 5 g of biomass was homogenized in a Potter homogenizer, placed in a conical flask, 25 ml of polar and non-polar solvents were added - methanol, ethanol, water, n-butanol, ethyl acetate, acetonitrile and hexane and mixed at room temperature in an Elpon 357 shaker (Poland) at 180 rpm. Then the mixture was filtered through filter paper (Whatman paper No. 1) and 40 µg/ml Na_2SO_4 was added to remove the aqueous layer. The mixture was then dried in a Heideolph HB Digitel vacuum evaporator (Germany) and dissolved in 1 ml of dimethyl sulfoxide (DMSO). The resulting extract was used as a stock solution and stored at +4°C.

Stepwise purification of the active extract by differential fractionation in different solvents. The ethyl acetate extract of the endophytic fungal biomass was subjected to differential fractionation according to the scheme proposed by Kumar et al. [19]. Ethyl acetate was added to the 7-day-old endophyte biomass in a ratio of 1/5 and mixed at 180 rpm in an Elpon 357 shaker (Poland) at room temperature. The resulting ethyl acetate extract was successively extracted with water. The separated ethyl acetate extract was extracted with methanol. The separated aqueous extract was divided between solutions with butanol and a methanol extract with n-hexane (1:1). The extracts of n-hexane, butanol, water and methanol were dried on a Heideolph HB Digitel vacuum evaporator (Germany) and dissolved in 1 ml DMSO. The obtained extracts were stored at +4°C for reuse.

Determination of α-amylase inhibitory activity of endophytic fungal extracts. Determination of α-amylase activity was carried out according to the method used for plant extracts [20]. For this, a starch solution of 1 g/10 ml of water was prepared as a substrate, boiled for 2 min, distilled water was added to a volume of 100 ml. The prepared solution can be used for 2-3 days. 2 ml of the prepared starch solution were incubated for 10 minutes. After incubation, the reaction was stopped by adding 10 ml of the working iodine reagent and the optical density was measured at a wavelength of 630 nm on a UV-5100 spectrophotometer (China). Unlike the experimental sample, the extract was not added to the control sample. The level of inhibitory activity was calculated using the formula [Ao-At]/Ao×100%, where Ao is the level of light absorption of the control sample, At is the level of light absorption of the experimental sample. A modern medicinal inhibitor, acarbose, was used as a comparative inhibitor.

Thin Layer Chromatography (TLC). In thin layer chromatography plates, substances move between the stationary and mobile phases at different speeds depending on their polarity. For TLC, a silica gel matrix was used, L × W 10 × 20 cm, fluorescent indicator 254 nm (Sigma-Aldrich, Germany). TLC was carried out in a standard chromatographic chamber with the following combination of eluent systems:

For polar compounds: butanol: water: acetic acid. (40:50:10), butanol: acetic acid: water (40:12:28), ethyl acetate: acetic acid: water (70:10:20), ethyl acetate: formic acid: acetic acid: water (15: 1:1:2). For weakly polar compounds: chloroform: methanol:water (60:28:5), chloroform:methanol (8:2), chloroform:ethanol (15:2). For non-polar compounds: chloroform:ethanol (14:1), benzene:methanol (5:1), benzene:methanol (5:0.5).

25 µl of extracts at a concentration of 25 mg/ml were applied to the starting line of the plates. The plates taken from the chromatographic chamber were dried and the following detection methods were used: in ultraviolet light at 254 nm; sprayed with 20% phosphomolybdic acid in alcohol, 10%

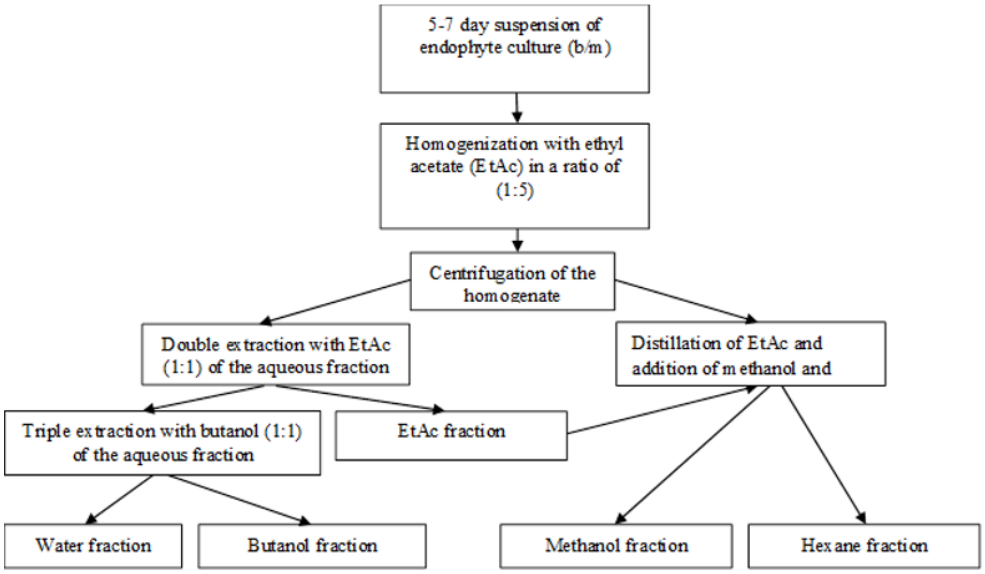


Fig. 1. Scheme of purification of secondary metabolites of primary ethyl acetate extract by differential fractionation

Рис. 1. Схема очистки вторичных метаболитов первичного этилацетатного экстракта дифференциальным фракционированием

H_2SO_4 acid solution in methanol and dried in a muffle furnace at 110°C [21].

Inhibition assay using thin-layer chromatography. The inhibition assay using thin-layer chromatography was carried out according to the method of Sonkamble et al., with minor modifications. The experiment was performed on a silica gel matrix, L×W 10×20 cm, plates with a 254 nm fluorescent indicator (Sigma-Aldrich, Germany).

An elution system with a benzene:methanol ratio (5:1) was chosen as the optimal mobile eluent. The spots on the plates were observed under UV light at 254 nm and 366 nm. Then the plates were incubated with the α-amylase enzyme for 30 minutes.

After incubation, the plates were prepared with 1% starch substrate in a phosphate buffer with a pH of 6.9 and incubated with the enzyme at 37°C for 10 minutes. It was then dried at 100°C for 5 minutes and washed with Gram's iodine solution. Each experiment was repeated three times [10].

Results and discussion

Ethyl acetate extracts of *P. brevicaule alba-CC200* and *A. egypticus-HT166S* biomass were fractionated with methanol, hexane, water and butanol solvents to isolate metabolites with the highest inhibitory activity (see Figure 1).

The initial amount of ethyl acetate extract before differential fractionation and the inhibitory activity towards α-amylase were 7.2±0.23 g and 81±0.26% for *P. brevicaule alba-CC200* and 2±0.24 g and 76±0.22% for *A. egypticus-HT166S*. As a result of differential fractionation, it was found that the main part of the extract amount and inhibitory activity was transferred to the methanol fraction. In this case, 6.24±0.21 g and 80.7±0.25% for *P. brevicaule alba-CC200*, 1.33±0.26 g and 75.4±0.27 for *A. egypticus-HT166S*. The amount of hexane fraction and inhibitory activity for both strains were 0.54±0.23 g and 5.2±0.22%, 0.56±0.29 g and 7±0.21%, respectively.

In the butanol and water fractions, inhibitory activity was not detected. This is due to the fact that secondary metabolites with inhibitory activity contained in the ethyl acetate extract do not pass into water and butanol (see Table 1).

The purpose of differential fractionation of secondary metabolites is to purify previously unknown active metabolites using solvents. This facilitates the purification of metabolites by subsequent chromatographic

Table 1. Yield and inhibitory activity of *P. brevicaule alba-CC200* and A. *egypticus-HT166S* extracts by differential fractionation

Таблица 1. Выход и ингибирующая активность экстрактов P. brevicaule alba-CC200 и A. egypticus-HT166S путем дифференциального фракционирования

Strains	Biomass in 1 liter of suspension		Initial activity of ethyl acetate		Water fraction		Butanol fraction		Methanol fraction		Hexane fraction	
	Biomass, (g)	Humidity, (%)	Extract (g)	α-amylase inhibitor (%)	Extract (g)	α-amylase inhibitor (%)	Extract (g)	α-amylase inhibitor (%)	Extract (g)	α-amylase inhibitor (%)	Extract (g)	α-amylase inhibitor (%)
P. brevicaule alba-CC200	80±0.19	85±0.26	7.2±0.23	81±0.26	0.3±0.27	-	0.12±0.29	-	6.24±0.21	80.7±0.25	0.54±0.23	5.2±0.22
A. egypticus-HT166S	70±0.17	82±0.29	2±0.24	76±0.22	-	-	0.12±0.27	-	1.33±0.26	75.4±0.27	0.56±0.29	7±0.21

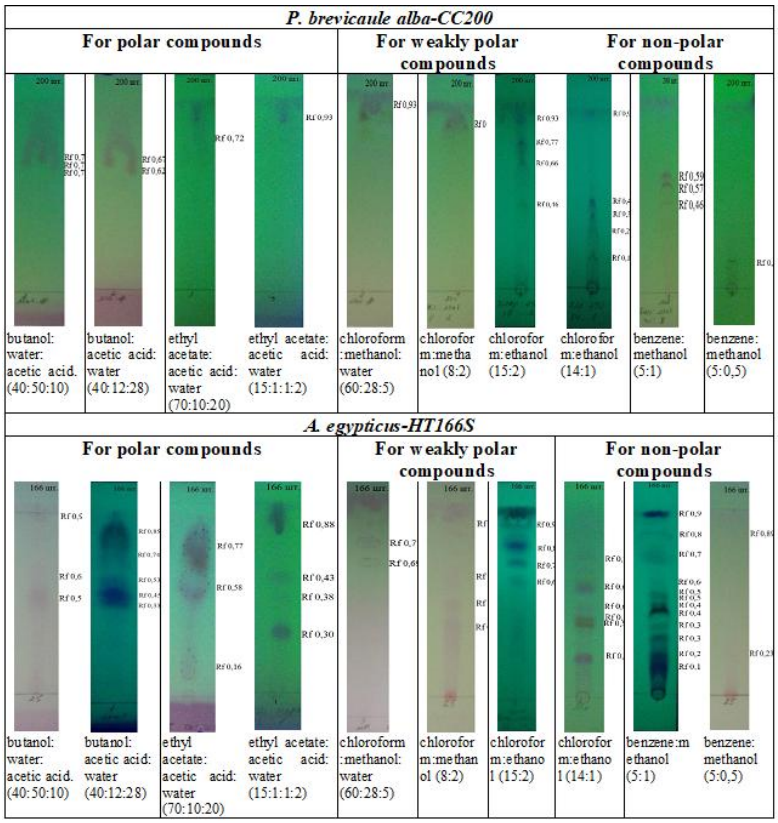


Fig. 2. Separation of secondary metabolites of *P. brevicaule alba-CC200* and *A. egypticus-HT166S* in different eluent systems.

Рис. 2. Разделение вторичных метаболитов P. *brevicaule alba-CC200* и *A. egypticus-HT166S* в различных системах элюирования.

methods. When using the thin-layer chromatography (TLC) method, the eluent system for secondary metabolites is first optimized [22].

A combination of different eluent systems was used to separate inhibitory substances in the methanol fractions of *P. brevicaule alba-CC200* and *A. egypticus-*

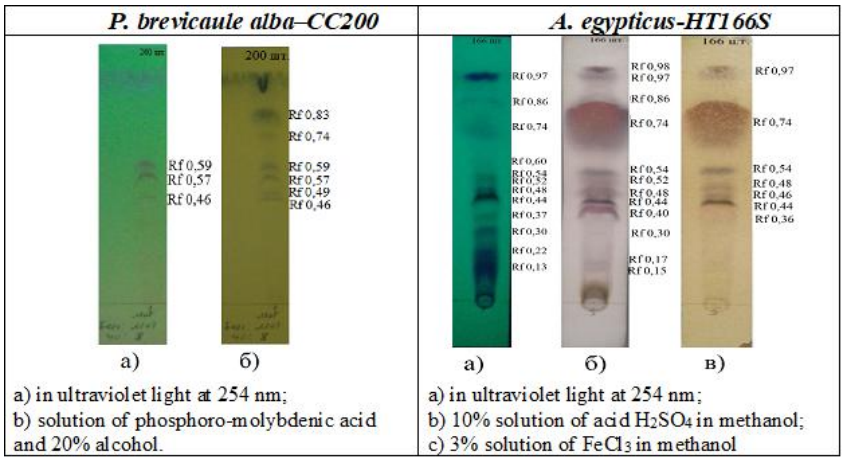


Fig. 3. Staining of *P. brevicaule alba-CC200* and *A. egypticus-HT166S* strains with tracers of isolated Rf values on plates.

Рис. 3. Окрашивание штаммов *P. brevicaule alba-CC200* и *A. egypticus-HT166S* индикаторами изолированных значений Rf на планшетах.

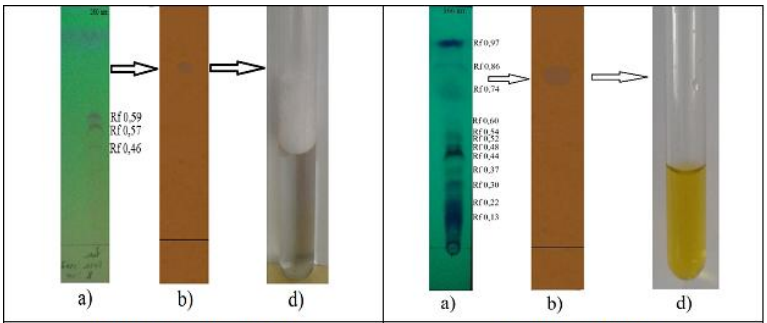
HT166S using the chromatographic method. As can be seen from the data in Figure 2, satisfactory separation of the methanol fractions of *P. brevicaule alba-CC200* and *A. egypticus-HT166S* on plates of eluent systems was observed in a mixture of chloroform:ethanol (14:1) and benzene:methanol (5:1). However, the benzene:methanol (5:1) eluent system provided optimal separation of secondary metabolites in both strains. In particular, the methanol fraction of *P. brevicaule alba-CC200* had six - 6 Rf (0.83; 0.74; 0.59; 0.57; 0.49; 0.45) and 12 Rf (0.97; 0.86; 0.74; 0.60; 0.54; 0.52; 0.48; 0.44); 0.37; 0.30; 0.22; 0.13) (see Fig. 2).

For better determination of Rf values of *P. brevicaule alba-CC200* chromatographic plates, which are well separated in the eluent system in the benzene:methanol ratio, a 20% solution of phosphomolybdic acid in alcohol was used. (5:1). In this case, the detection reagent allowed us to detect secondary metabolites at a higher level compared to ultraviolet light with a wavelength of 254 nm. The blue color of the R_f values when processing substances on TLC plates with a phosphomolybdenum solution is due to the presence of saponins.

Also, isolated Rf values of *A. egypticus-HT166S* on plates were processed with a 10% solution of H_2SO_4 acid in methanol and a 3% solution of $FeCl_3$ in methanol. Purple coloration of Rf values on the sorbent indicates the presence of flavonoids in secondary metabolites. At the same time, Rf values differed when processed with different detection reagents, which is due to the different chemical nature of the secondary metabolites isolated on *A. egypticus-HT166S* plates (see Fig. 3).

Thus, the benzene:methanol (5:1) system was recognized as the optimal eluent for both endophytic fungi. To determine the inhibitory activity of substances on chromatographic plates, they were incubated with the enzyme. Then the plates were placed in a starch medium. The α-amylase inhibitory activity of substances on the TLC plate was determined after washing with gram-iodine (Fig. 4).

The formation of blue spots on the surface of substances on chromatographic plates is associated with their inhibitory activity. This reduces the time and costs associated with the identification of substances with inhibitory activity. We isolated the substances that showed inhibitory activity in the plates and determined their nature using qualitative reactions. As a result of qualitative reactions, the active substances on the surface of the plates showed positive test results for saponins in *P. brevicaule alba-*



P. brevicaule alba–CC200
a) In ultraviolet light at 254 nm;
b) After incubation of the enzyme with the substrate, staining with iodine solution according to Gram;
d) Qualitative reaction specific to saponins.

A. egypticus-HT166S
a) In ultraviolet light at 254 nm;
b) After incubation of the enzyme with the substrate, staining with iodine solution according to Gram;
d) Qualitative reaction specific for flavonoids.

Fig. 4. Qualitative reactions and inhibition of active substances on chromatographic plates by α-amylase

Рис. 4. Качественные реакции и ингибирование активных веществ на хроматографических пластинках α-амилазой

CC200 and flavonoids in *A. egypticus-HT166S*. It should be noted that the results of our studies are consistent with scientific sources on the inhibitory properties of saponins in medicinal plants. For example, Fang Dou et al., studied antidiabetic medicinal plants in vitro for the inhibitory activity of α-glucosidase and α-amylase. Moreover, the bark extracts of *Araliataibaiensis* exhibited inhibitory activity against α-glucosidase and α-amylase. The main inhibitory activity was exhibited by glucuronic acid on the C-3 aglycone [23]. Hanh et al., isolated three different saponins from the methanol extract of the leaves of *Polyscias fruticosa (L.) Harms*. Saponin 1 inhibited the activity of α-amylase and α-glucosidase, but saponins 2 and 3 were inactive. Saponin 1 and low concentrations of acarbose exhibited inhibitory activity against α-amylase. The obtained data showed that *P. fruticosa* and its saponin 1 can be used for the prevention and treatment of diabetes and its complications [24]. The α-amylase inhibitory activity of secondary metabolites of *A. egypticus-HT166S* is associated with several compounds, most of which are flavonoid substances. Over the past 20 years, natural flavonoids have attracted attention as antidiabetic agents.

One of the most studied flavonoids is quercetin, which has strong α-amylase inhibitory activity [25]. For example, flavonoid and phenolic compounds were determined from the endophytic fungi *Aspergillus nidulans* and *Aspergillus oryzae* isolated from the plant *Ginkgo biloba L*. [26].

Conclusion

Both endophytic fungi were studied as an alternative source of hypoglycemic drugs. According to the results of differential fractionation and TLC methods, the strain *P. brevicaule alba-CC200* was more effective than *A. egypticus-HT166S*. In this case, the strain *P. brevicaule alba-CC200* can produce highly active metabolites. It can also be concluded that the structural diversity of the metabolites of *P. brevicaule alba-CC200* facilitates the isolation of compounds with inhibitory activity from it.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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