

Phytochemical analysis of α -amylase-inhibiting secondary metabolites of endophyte *Penicillium brevicaula* Thom.

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Abstract: *The present work was aimed to identify phytochemicals in methanol extract *P. brevicaula* alba Thom - CC200, obtained from *C. cristata*, by qualitative and TLC analysis and evaluation of α -amylase inhibitory activity in vitro. It was determined the presence in the extract of glycosides, terpenoids, and saponins. The partitioning of the extract by TLC showed that inhibition of enzyme activity at 50,3% and 37,6% is associated with two compounds of saponin nature.*

Keyword: *diabetes mellitus, endophytic fungi, *Celosia cristata*, methanol, α -amylase inhibition, phytochemicals, saponin*

Diabetes mellitus (DM) is a disease caused by endocrine and metabolic disorders, characterized by chronic hyperglycemia, impaired lipid, and protein metabolism, and having a serious impact on health, quality, and life expectancy (1). Diabetes of all types can lead to complications in many parts of the body and can increase the overall risk of dying prematurely. Possible complications include heart attack, stroke, kidney failure, leg amputation, vision loss, and nerve damage. In pregnancy, poorly controlled diabetes increases the risk of fetal death and other complications (1-3). Currently, there is a dramatic increase in the number of diabetic patients worldwide. According to WHO statistics (2016), 1.5 million people died as a result of diabetes in 2012, 2.2 million people died as a result of diabetes-related diseases such as cardiovascular and renal impairment (4). Statistics on the global spread of diabetes suggests a steady increase in the number of patients to 366 million people by 2030 (1,2). In a few decades, it will be the world's common disease and one of the biggest public health problems with an estimated minimum of half a billion cases (5). The basic strategy in the treatment for diabetes mellitus is the maintenance of blood glucose levels in normal conditions. The most common is insulin-independent type 2 diabetes (type 2 diabetes), which accounts for 90-95% of the total number of patients with diabetes (6). The growing trend towards the development of type 2 diabetes is of serious concern throughout the world and requires research to find new therapeutic agents. One strategy for treating type 2 diabetes is to lower postprandial blood glucose. This may be due to a delay in glucose absorption through inhibition of α -amylase and α -glycosidase, which are responsible for the breakdown of oligo- and polysaccharides into mono- and disaccharides (7). Known commonly available in market α -glycosidase and α -amylase inhibitors such as acarbose, miglitol, and voglibose prolong the total digestion of carbohydrates, which reduces the rate of glucose absorption, followed by the prevention of postprandial hyperglycemia. However, all inhibitors have serious side effects - abdominal pain, flatulence, diarrhea, kidney cancer, liver

damage, and acute hepatitis (7,8). In this regard, it is necessary to search for intestinal inhibitors of α -glycosidase and pancreatic α -amylase, free of the main side effects. There are several reported antidiabetic properties of medicinal plants till now (9-11). In the last decades, endophytes have been considered as important microbial resources of bioactive compounds (12-14). Endophytes are a unique class of microorganisms that live inside plant tissue in a symbiotic relationship with plants. Endophytes characterized by the production of a large variety of substances contributing to the resistance of host plants under various biotic and abiotic stresses. Moreover, some endophytes can synthesize the characteristic metabolites of the host plants (13). Starting from 2002, endophytic strains have generated nearly half of the newly discovered metabolites derived from fungi. Endophytic fungi have been reported to produce secondary metabolites possessing antibacterial, cytotoxic, neuroprotective, and antioxidant activities some of which are under clinical trials (11). Such metabolites show anti-inflammatory, antihypertensive, anticancer, antifungal, immunomodulatory properties (12-14). Along with that, secondary metabolites of endophytes successfully exhibit the properties of enzyme inhibitors, including degradation of carbohydrates, and therefore can be considered as a means of controlling type 2 diabetes (15). Thus, endophytes producing α -amylase and α -glycohydrolase inhibitory compounds, are isolated from many different plant species (16-21,26). In vitro inhibition of α -amylase and a significant decrease in blood glucose was shown by extracts of two endophytes species *Aspergillus* and *Phoma* isolated from *Salvadora oleoides* Decne (Salvadoraceae) (16). Inhibition of α -amylase is shown in all endophyte isolates isolated from bay leaves (17). Pavitra et al (18) reported that ethyl acetate extracts of nine endophytic fungi out of 22 strains isolated from *Momordica charantia* and *Trigonella foenum-graceum* were found to be positive for α -amylase and α -glucosidase inhibitors. Two isolates PTF005 and PTF006 showed promising inhibition activity on α -amylase with better IC 50 value in comparison with acarbose (18).

High in vitro inhibitory activity was established in the extract of *Penicillium* isolated *Tabebuia argentea* plant (19). Five endophytic fungi were obtained from *Tulsi* and *Aloe vera* plants, which extracts inhibited α -amylase, and the maximum activity was noted in the endophytic strain *Nigrospora* (20). Screening of antidiabetic activity by amylase-assay was performed for 8 strains from 3 different plants, *Mangifera indica*, *Azadirachta indica*, and *Syzygium cumini* L. showed that all isolates could inhibit the activity of α -amylase with different degrees of inhibition (6).

At screening of 36 endophytic fungi were isolated from *Acacia nilotica*, inhibitory activity against both α -amylase (81%) and α -glucosidase (80%) was exhibited in an isolate, identified as *Aspergillus awamori*. The inhibitor was characterized to be proteinaceous in nature with an approximate molecular mass of 22 kDa. (21).

The abovementioned reports suggest that endophytes can be harnessed as new α -amylase inhibitors for the better management of diabetes.

In our previous studies, it was shown that endophytic fungus *Penicillium brevicaulis* Thom - CC 200 isolated from *Celosia cristata* produces metabolites exposing a high (up to 90%) amylase inhibitory activity (22). Considering that endophytes are capable to produce bioactive metabolites belonging to various classes of chemical compounds the aim of the study was preliminary identification of the nature of inhibitory compounds by partitioning of *P. brevicaulis* Thom - CC200 metabolites in different solvents and TLC analysis.

Methods

Fermentation. Endophytic fungus *P. brevicaulis* Thom - CC200 was grown under submerge fermentation in 500 ml flasks containing 100 ml of Chapek-Dox medium for 5 days at 26 °C on an orbital shaker.

Secondary metabolite extraction was carried according to Kumar et.al (23). The mycelial mass of endophyte separated from the broth by filtration was ground after overnight soaking in ethyl acetate and filtered. The filtrate was partitioned with ethyl acetate and dried under a vacuum evaporator. After extraction with ethyl acetate, aqueous residue was extracted thrice with butanol. The dried ethyl acetate extract was further partitioned between hexane and 90% methanol. The yield of the extracts in the respective solvents is given in Table 1.

Phytochemical screening of fungal extracts was carried out for the presence of alkaloids, flavonoids, tannins, phenols, saponins, terpenoids and cardiac glycosides using standard methods (24,25).

Chromatographic detection and partial purification of the bioactive metabolite from the methanolic extract was performed by thin-layer chromatography (TLC). For this, the methanolic fraction was spotted (50 μ L) on the TLC plate («Sigma –Aldrich», Germany) and chromatography was performed by employing solvent system benzene: methanol (40:8). 20% ethanolic solution of phosphoric molybdenum acid was used as detecting reagent. Silica residue was extracted and centrifuged and the supernatant was transferred to a microcentrifuge tube. The silica-free supernatant was checked for inhibitory activity and phytochemical identification. Inhibition of α -amylase activity.

The determination of α -amylase activity was performed by modified iodine method protocol (26). 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 30^oC. In contrast to the test sample the extract was not added to a control. After incubation, the reaction was terminated by adding 10 ml of iodine reagent, and the absorbance was recorded at 630 nm. Iodine reagent was 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. Each experiment was done in triplicates and the average percent of inhibition was calculated by the formula: $(A_0 - A_t)/A_0 \times 100\%$, where A_0 - absorption of a control sample, A_t - the absorption of a test sample, respectively.

Results and discussion.

It is known that bioactivity against hyperglycemia could be associated with a wide range of substances related to alkaloids, glycosides, glucomannan resins, polysaccharides, guanidines, steroids, glycopeptides, terpenoids, etc. (15). Depending on the nature of the extractable compound, various polar and non-polar solvents are used to extract secondary metabolites (23). However, when the nature and polarity of the active compound are unknown, given the large variety of secondary metabolites, it is usually to screen solvents to select the conditions for the extraction of substances with high desired bioactivity. In this study, fungal endophyte *P. brevicaulis* Thom - CC200 associated with plant *C. Cristata*, exposing high α -amylase activity was studied to determine the nature of the inhibitory secondary metabolite. In this regard metabolite with the highest inhibitory activity was obtained by partitioning of primary ethyl acetate extract of fungal mycelium with ethanol, water, hexane, and butanol. Obtained four extracts were screened for the presence of phytochemicals and its inhibitory activity, results are depicted in Table 1.

Table 1.: Phytochemical composition, yield and inhibitory activity of *P. brevicaulis* Thom – CC200 extracts in different solvents

Phytochemical	Methanol	Hexane	Water	Butanol
Alkaloids	–	–	–	–
Flavonoids	–	–	–	–
Terpenoids	+	–	–	–
Saponins	+	–	+	–
Tannins	–	–	–	–
Phenols	–	–	+	+
Cardiac glycosides	+	–	–	–
Anthraquinones	–	–	–	–
Yield mg/g of biomass	25	5,8	3,6	3,1
α -Amylase inhibition %	88,7	26,2	15,3	6,5

As can be seen from the data, partitioning of ethyl acetate extract allowed to obtain metabolites with inhibitory activity of 88.7% in the methanolic extract, metabolites with a lower inhibitory activity are fractionated with hexane (26.2%), and the lowest activity is maintained in the water and butanoic fractions at 15.3% and 6.5%, respectively.

It should be noted that similar high amylase inhibition was reported for the biomass extract (89.01%) and cultural broth (93.91%) of the endophytic fungus *B. Os.1F* isolated from the plant *Orthosiphons picatus* BBS (27). Screening for the antidiabetic activity of 8 endophyte strains isolated from *Mangifera indica*, *Azadirachta indica*, and *Syzygium cumini* L showed high inhibitory activity of 5 strains. The highest activity was 62% in the aqueous extract of the JCO strain from *Syzygium cumini* L., in strains NTH, NGU, MB, and MAN it was 59.5%, 51%, 49.3%, and 49.6%, respectively (6).

A phytochemical analysis of the obtained fractions of *P.brevicaule alba* showed that of the eight determined chemical classes (terpenoids, saponins, phenols, alkaloids, anthraquinones, cardiac glycosides, and flavonoids), only terpenoids, saponins, phenols, and cardiac glycosides are found. It was observed that the bulk of bioactive metabolites with inhibitory activity of 88.7% fractionated with methanol and associated with terpenoids, saponins, and glycosides. Saponins and phenols were found in the water fraction, and phenols were detected in the butanol fraction with the least inhibitory activity. It should be noted that a qualitative analysis of the hexane fraction did not show any of the tested compounds. The small inhibitory activity of this fraction could be associated with components unidentified by methods used in this study.

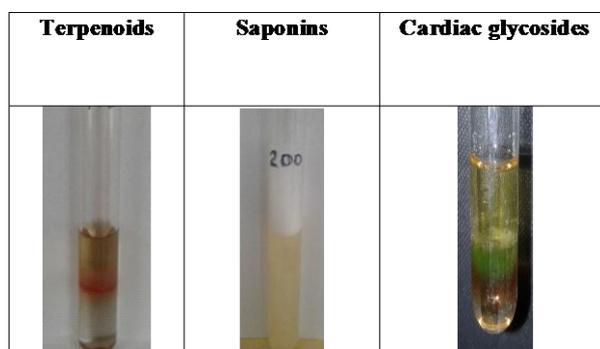


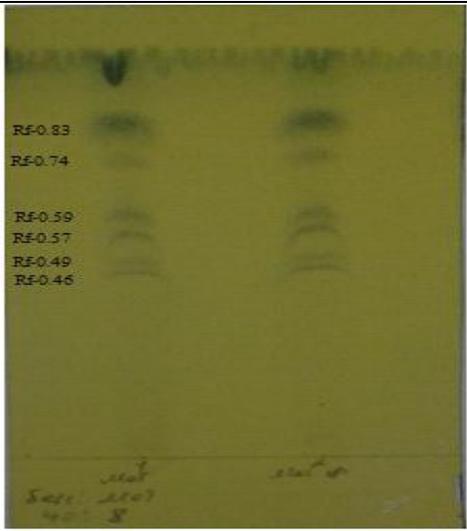
Figure 1. Composition of methanolic extract of *P. brevicaule alba* Thom - CC20

Table 2 presents a qualitative analysis of the methanol fraction, indicating a rather high content of terpenoids, which is convincingly proved by the formation of red-brown staining between the phases. The formation of saponins is evidenced by the formation of a thick and resistant foam during agitation, which lasts more than 30 minutes. Blue-green staining between phases indicates the presence of cardiac glycosides.

There are several reports on the phytochemical nature of the anti-amylase activity of secondary metabolites of endophytic fungi. Significant α -amylase inhibition of $61.76 \pm 1.07\%$ exhibited by methanolic extract of endophytic fungus *Cochliobolus* sp. It was considered that the inhibition potential might be due to the occurrence of phenolic content (28). In vitro inhibition of α -amylase is shown in all endophyte isolates from bay leaves, the highest inhibition in the three isolates was 14.4%, 12.9%, and 39.2% (17). It was established that the main inhibiting substance in the two most active extracts is associated with phenolic compounds. Authors concluded that fungal endophytes from bay leaves have the potential as an alternative source for obtaining secondary metabolites for the treatment of diabetes (17). High in vitro inhibitory activity was established in the methanolic extract of the endophytic fungus *Penicillium* from the *Tabebuia argentea* plant (19). A study of the phytochemical profile of the extract showed the presence of 18 different compounds that maximally inhibit the activity of α -amylase, β -glucosidase, and dipeptidyl peptidase IV. It was concluded that octadecanoic acid is responsible for antidiabetic activity (19). The α -amylase inhibition level was determined to evaluate the anti-diabetic activity of *Azadirachta indica* endophytes (29). The results revealed that there was an α -amylase inhibition activity of 38.52% at the concentration of 150 $\mu\text{g/ml}$. A good correlation was observed between the level of total phenol, antioxidant, and anti-diabetic ability for all studied extracts (29).

Table 2.

TLC fractionation of *P.brevicaule alba Thom -CC200* methanolic extract

	No	Rf	α -amylase inhibition (%)
	1	0,83	15,3
	2	0,74	14,63
	3	0,59	6,93
	4	0,57	10,93
	5	0,49	50,3
6	0,45	37,6	

1, 2 -methanolic extract of *P. brevicale alba Thom* – CC 200
Solvent system: benzene:methanol (40:8).
The volume of sample 250 μl .
Detecting agent: 20% ethanolic solution of phosphoric-molybdenum acid

To determine the nature of the most active metabolite, we analyzed the methanol fractions of *P. brevicale alba Thom* - CC 200 by thin-layer chromatography. TLC analysis in the

benzene: methanol system (40: 8) showed a clear separation of the methanolic extract for 6 bands with indicated Rf values (Table 2).

For identification of the most active metabolites, each band was scraped off from chromatographic plates, the dry weight of metabolites and their inhibitory activity was determined. It was found that the anti-amylase activity of each of the excised bands varied and in total sum activity of individual fractions exceeds the initial activity of the crude methanol extract. Obviously, crude methanolic extract yielded more potent compounds once they had undergone some purification. The highest inhibitory activity values exposed components of bands No. 5 and No. 6 - 50.6% and 37.6%, respectively (Fig.2).

Qualitative analysis of active bands No.5 and No.6 showed the presence of saponin compounds, as evidenced by the formation of abundant and persistent foam when the samples are dissolved in hot, up to 60°C, water. The solution foamed well with shaking and had copious foam even in very large dilutions. The chemical composition of saponins is known to be glycosides with steroid and triterpene aglycones. By foaming, the group affiliation of saponins is tentatively determined (30). For the reaction, the aqueous solution of saponins was divided into two parts, the first was acidified to pH = 1, the second was alkalinized to pH = 13. Both solutions in the test tubes were shaken and observed the formation of columns of foam. In our case, in both test tubes foam columns of approximately equal size and resistance were formed, which indicated that the studied saponins belong to the triterpene group.

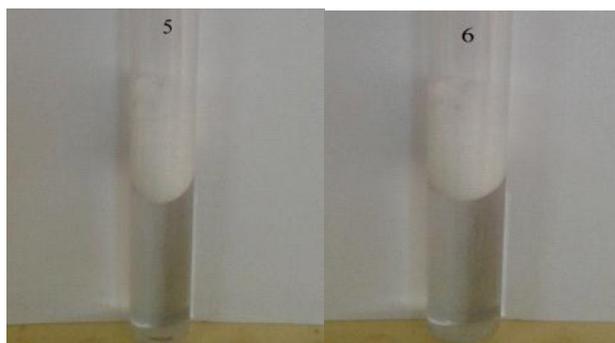


Figure 2. α -Amylase Inhibiting saponins from *P.brevicaule alba* Thom -CC200

It should be noted that the results of our studies are consistent with literature data, which support the inhibitory properties of triterpene saponins of medicinal plants. In general, the fundamental role of saponin as an antidiabetic agent is known from studies of plants. Saponins from various plants have been reported to have hypoglycemic activity, regulate blood glucose levels and prevent diabetic complications due to their antioxidant activity (31,32).

There are also reports that many endophytic fungi produce saponins, but their antidiabetic activity has not been established. So, the highest concentration of saponins 2.049 mg/mL was found in G22 (*Penicillium* sp.) at study of the diversity and capacity to produce saponins of endophytic fungi of *Aralia elata* distributed in Northeast China (33). Two novel fungal endophytes, *Fusarium* sp. PN8 and *Aspergillus* sp. PN17, producing saponins were isolated from traditional Chinese medicinal herb *P. notoginseng*. The saponin extracts exhibited moderate to high antimicrobial activity against pathogens tested (34).

On the basis of our data we can conclude that the high inhibitory activity of the methanol fraction of *P. brevicaula alba* - CC 200 is associated with saponin compounds, which could be commercially produced and exploited for better management of diabetes. Thus endophyte

P. brevicaula alba - CC 200, isolated from the plant *Celosia cristata*, can be a promising source of saponins as antidiabetic drugs. This is the first report on α -amylase inhibition property of saponins from endophytic fungi.

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