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Screening of Pancreatic Lipase Inhibitors of Endophytic Fungi of Medicinal Plants in Uzbekistan

T.G. Gulyamova, D.M. Ruzieva, M. Yoldosheva, G.A. Rasulova and K.V. Kondrasheva *

Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan, A. Kadyry street, 7B, 100128, Tashkent, Uzbekistan.

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Search for pancreatic lipase (PL) inhibitors is essential for obesity and associated chronic disease therapy. PL inhibitors significantly reduce enzyme activity and prevent the absorption and hydrolysis of triglycerides into free fatty acids. This study provides new evidence of screening PL inhibitory activity of 15 endophytic fungi from 6 medicinal plants in Uzbekistan. In general, the extracts of almost all twelve endophytes exposed the inhibitory potential, but only five of them had inhibition levels above 50%. The highest inhibitory activity, amounting to 73.7% and 65.2%, and comparable to the activity of Xenical as a standard, was established for Fusarium sp.-AL142R and F.sambucinum - AL135L, respectively, isolated from the endemic plant Allium longicuspis. The values of IC50 inhibitory activity are 20.7 and 8.01, respectively, compared with Xenical as a standard with IC50 20.6 μg/ml. The inhibitory extracts of both endophytes contain alkaloids, terpenoids, flavonoids, and tannins. The data obtained for the first time indicate the potential of Uzbekistan plant endophytes as possible sources of PL inhibitors

Keywords: Endophytic Fungi; Pancreatic Lipase Inhibitory Activity.

Obesity is a cardiometabolic risk factor that affects serum lipids by increasing triglyceride levels and lowering high-density lipoprotein (HDL) cholesterol levels [1, 2]. Being one of the most common health diseases, obesity is a global issue and leads to the development of various health disorders including dyslipidemia, metabolic syndrome, hypertension, and an increased risk of cardiovascular mortality as well as several types of cancer[3, 4]. And in this regard, sufficient attention is paid to the search for new ways to combat obesity and associated diseases. Ali et al reported on the use of glutamic acid derivatives as an alternative chemotherapy to platinum-based drugs that have an antagonistic effect on cancer cells[5, 6].

The main therapeutic approach to preventing obesity is to slow the absorption of fatty acids by inhibiting lipase in the digestive tract[7]. Pancreatic lipase (PL) is the primary digestive enzyme that catalyzes the hydrolysis of fats. PL inhibitors interfere with the activity of pancreatic lipase. They belong to drugs of peripheral action, mediating a direct decrease in the absorption of calories in the gastrointestinal tract and affecting the absorption of lipids[8, 9]. The only anti-obesity drug of this type approved is the lipase inhibitor Orlistat[10, 11]. It is a saturated derivative of lipstatin, a potential natural PL inhibitor derived from *Streptomyces toxytricini*[12].

*Corresponding author E-mail: kseniya81@yandex.ru

Still, recent evidence reveals that long-term and systematic intake of Orlistat can lead to severe side effects, including hepatotoxicity, requiring the development of new safe, and effective drugs for the treatment of obesity[13]. That is why research based on endophytic natural products may be the key to developing drugs for these types of health problems[14]. Endophytes provide a significant contribution to the process of drug discovery and development, as they produce natural products with a variety of new chemical structures and biological activity[15-17] There is much evidence on the inhibitory properties of endophytes regarding enzymes, which control the occurrence of most diseases of all vital human systems[18, 19].

The promise of endophytic fungi as potential producers of lipase inhibitors was first reported by Gupta *et al* 2014 when screening culture filtrates of 70 endophytic fungi[20, 21]. Only one isolate, 57 TBBLAM (*Penicillium*), showed an IC50 value of 3.69 ìg/ml, comparable to an IC50 of 2.73 ìg/ml Orlistat as a positive control[20]. Katoñh *et al* 2017 evaluated the inhibitory activity of 27 endophyte isolates isolated from *V.odorata*[12]. According to their findings, extracts of seven endophytes exhibited inhibitory activity with an IC50 value of <10ìg/mL, and VOLF4 identified as *Aspergillus sp.* was the most active. Sarkar *et al 2017* studied the potential inhibitory activity of the culture fluid of 39 endophytic fungi from medicinal plants of the Andaman Islands, of which two strains isolated from *Citrus lemon* and *Aegle marmelos* inhibited lipase activity by 75% and 83%, respectively[22]. Complete inhibition of pancreatic lipase by lemon endophyte extracts was confirmed by Patil *et al* 2019[23].

Endophyte biodiversity has tremendous potential for chemical innovation and its potential use in developing pharmaceutical agents[14]. A studies on inhibitors of PL quite limited, although the available reports strongly suggest that endophytic fungi can quite effectively suppress the activity of pancreatic lipase and therefore can serve as a potential source of metabolites for the obtaining of new lipase inhibitors for the treatment of obesity. The study of endophytes of local plants as PL inhibitors producers is of particular importance for Uzbekistan, where every fifth resident of Uzbekistan suffers from obesity. Our data may open up opportunities for the development of a domestic drug for the treatment of obesity based on local raw materials. These studies are conducted by us for the first time in Uzbekistan.

For the past couple of years, endophytic fungi of nearly 30 plants growing in Uzbekistan exhibited the perspective potential of local strains as producers of metabolites with cytotoxic, antibiotic, antioxidant activity, and inhibitory activity against pancreatic amylase[24-26]. This study aimed to screen pancreatic lipase inhibitors in 15 strains of endophytic fungi isolated from 6 plants growing in Uzbekistan.

MATERIALS AND RESEARCH METHODS

Cultivation of endophytes.

Endophytes were grown submerged on a Chapek-Dox medium at a temperature of 28°C for seven days on an orbital shaker at 120 rpm and rest. The biomass was separated by centrifugation at 6000 rpm and stored at +4°C.

Extraction of secondary metabolites from the biomass of endophytic fungi

The extraction of metabolites from the biomass of endophytes was carried out according to Hazalin *et al* 2009[27]. 5 g of mycelial mass was homogenized, transferred into a conical flask with 50 ml of ethyl acetate, and left for a day on a shaker at room temperature. The mixture was filtered through a paper (Whatman # 1), and Na_2SO_4 was added (40 ìg/ml) to remove the aqueous layer. Then the mixture was evaporated to dryness, and 1 ml of dimethyl sulfoxide (DMSO) was added. The obtained extract was used as a stock and stored at +4°C.

Determination of PL inhibition in chromogenic assays

Chromogenic assays were used for indicating lipase inhibitory activity by orange halo decrease on agar dishes containing the indicator dyes rhodamine B and phenol red, in the presence of olive oil as a substrate and pork pancreatic lipase (PPL), as described by Patil *et al* 2019[23].

For the rhodamine assay, Petri dishes with 2.5% olive oil, 1.3% agarose, and 0.3% rhodamine. After adding 35 µl of a solution containing 15 µl of PPL (Sigma, 40 U/ml) preincubated with 20 µl dishes were incubated at 37°C for 24 hours. Blank samples contained 15 µl of PPL and 20 µl of DMSO. A decrease in the diameter of the halo

compared to the blank indicated the inhibition of PL. Xenical was used as standard (active substance-Orlistat, CHEPLAFARM, Germany).

For the phenol red assay, Petri dishes filled with 2% agar containing 2.5% olive oil as substrate, 0.01% phenol red, 0.01% Tween 80, pH 7.0. 35 il of a solution having the same composition as in the rhodamine assay was added, and incubation at 37°C for 24 hours. The decrease in the halo size compared to the control indicates the inhibitory lipase potential of the extract.

The formula to estimate the inhibitory activity is as follows:

$$\% \text{ inhibition} = C\text{-}T/C \times 100\%$$

where

C - the diameter of the blank halo (lipase+DMSO);

T - the diameter of the sample halo (lipase+extract)

Determination of PL inhibitory activity by titration of free fatty acids

The approach is based on determining the rate of the enzymatic hydrolysis of a 40% emulsion of olive oil by the amount of formed fatty acids[28]. Their content was found in the medium by titration with 0.05 N sodium hydroxide. In a conical flask (100 ml), 5 ml of the substrate emulsion, 0.5 ml of the extract, and 4 ml of phosphate buffer with pH 7.0 were added and incubated at 37°C for 5 minutes. Then, 1 ml of PPL (Sigma, 100 U/ml) was added to the mixture and mixed well. Incubation at 37°C for one hour. Then the mixture was added with 30 ml of ethanol to inactivate the enzyme. The resulting solution was titrated with 0.05N NaOH solution in the presence of phenolphthalein indicator until pink in color. The blank sample contained DMSO instead of extract.

The percentage of enzyme inhibition was estimated by the following formula:

$$\% \text{ inhibition} = (A_1 - A_2) / A_1 \times 100\%$$

where

A_1 - the volume of NaOH used in blank titration (mL);

A_2 - the volume of NaOH used in sample titration (mL).

IC50 values of extracts were calculated by constructing a linear regression curve and compared with Xenical.

Phytochemical Analysis

An affiliation of secondary metabolites to chemical classes of compounds was determined by using differential coloring[29].

Alkaloids were determined by mixing 1 ml of each extract, 1 ml of marquis reagent, 2ml of concentrated sulphuric acid, and then a few drops of 40% formaldehyde. The appearance of dark orange or purple color indicated the presence of alkaloids.

For the determination of terpenoids, 0.5 ml of extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulfuric acid. The formation between the phases of red-brown staining indicated the presence of terpenoids.

Flavonoids were determined by mixing 2 ml of 20% NaOH and 3 ml of methanol fraction. The appearance of yellow color positively indicated the presence of flavonoids.

Saponins were revealed by dilution of 2 ml of each extract with 6 ml of distilled water and shaking vigorously; the formation of bubbles or persistent foam indicates the presence of saponins.

To determine tannins, 10% of alcoholic ferric chloride to 2 ml of each extract was added, with the formation of a brownish-blue or black color indicating the presence of tannins.

Proteins were determined in 2 ml of each extract by adding 1 ml of 40% sodium hydroxide and a few drops of 1% copper sulfate. The formation of a violet color indicated the presence of peptide linkage molecules in the sample extract.

To determine cardiac glycosides to 1 ml of each extract, 0.5 ml of glacial acetic acid and 3 drops of 1% aqueous ferric chloride solution were added, formation of a brown ring at the interface indicated the presence of cardiac glycosides in the sample extract.

Anthraquinones' presence was determined by mixing 2 ml of a fraction with 4 ml of hexane and the subsequent shaking. The extract gave two layers. The upper layer was separated, 4 ml of diluted 10% ammonia was processed, and the lower layer's color was determined. The pink coloring indicated the presence of anthraquinones.

For phenols determination to 2 ml of each extract, 2 ml of 5% aqueous ferric chloride was added. The formation of blue color indicated the presence of phenols in the sample extract.

All experiments were performed in triplicate

Statistical analysis

Statistical analysis was performed by carrying out Student's t-test. Values were expressed

as mean± SD. Experiments were conducted in triplicates and the replicates were considered for calculating the mean.

RESULTS AND DISCUSSION

Qualitative screening for PL inhibitors

Several plants growing in Uzbekistan have already been studied for endophytes[25, 26], but this is the first study on the potential of local strains of endophytic fungi against obesity.

The screening involved 15 endophyte strains previously isolated from various native plant species. For the primary observation of the effect of raw extracts of fungal secondary metabolites on lipase activity by the halo size on chromogenic dishes with two indicators. The study finds that most of the extracts have inhibitory activity. In the rhodamine assay, the inhibitory activity is exhibited by extracts of 12 endophytes, and 11 strains in the phenol red (Table 1)

It should be noted that the inhibitory activity values are comparatively higher in the presence of the phenol red indicator. The findings of the differences in inhibition values are in line with the study of Gupta *et al.,* 2015[21].

The highest activity over 50% and comparable to Xenical (65.6%), was observed in the extracts of *S.minorum-VM83R* (55.1%), *C.tenuissimum-AF183* (51.7%); *F.sambucinum-AL135L* (58.6%), *U. consortiae-FF155L* (51.7%) and *Fusarium sp.-AL142R* (62%) (Fig.1).

Quantitative screening of PPL inhibition

The data obtained on chromogenic assays were quantitatively confirmed by direct hydrolysis of olive oil by PPL in vitro by following titration of free fatty acids (Table 2).

The data presented in Table 2 reveal that the level of lipase inhibition by both the standard and experimental samples in this assay is noticeably higher than in the chromogenic assays. The best inhibition values were 65% and 73.7% in *F. sambucinum – AL135L* and *Fusarium sp. – AL142R*. The concentrations at which the 50% inhibition (IC50) of porcine PL activity in the presence of *F. sambucinum – AL135L* and *Fusarium sp. – AL142R* extracts and Xenical standard were found to be 8.01 ± 1.06 ìg/mL, 20.7 ± 2.42 ìg/mL and 20.6 ± 1.05, respectively. Patil et al 2021 reported that pentacyclic triterpenoids from endophytic Colletotrichum gigasporum were found to show inhibitory activity against PL with IC50 of 16.62 ± 1.43 ìg/mL.

The endophytic *F. sambucinum – AL135L* and *Fusarium sp. – AL142R* are isolated from the root and leaf of *A. longicuspis*, an endemic specie considered to be the ancestor of *Allium sativum* (garlic). Sometimes *A. longicuspis* is given as a synonym of *A. sativum*. Garlic is a well-known dietary plant, which for many centuries has been used also in traditional medicine for the prevention and treatment of different diseases[30]. *C. tenuissimum-AF183* with inhibitory activity of 52.7% was also isolated from the endemic bulbous

Tàble 1. Effect of secondary metabolites from endophytes on PPL activity in rhodamine and phenol red assays

	Endophytes	Host plant	Halo size, mm (Rhodamine)	Enzyme inhibition, %	Halo size, mm (Phenol red)	Enzyme inhibition, %
1	*S. minorum -VM83R*	*Vinca minor*	7.0±0.09	53.3	13±0.04	55.1
2	*A. terreus -AF104S*	*Allium filidens*	12.0±0.02	20	20±0,02	31
3	*C.tenuissimum – AF183*		8.0±0.09	46.6	14±0.09	51.7
4	*F. sàmbuñinum – AL135L*	*Allium longicuspis*	7.5±0.06	50	12±0.08	58.6
5	*Fusarium sp. – AL142R*		6.5±0.07	56.6	11±0.06	62
6	*U. consortiae – FF155L*	*Ferula foetida*	7.0±0.08	53.3	14±0.09	51.7
7	*M. trigonosporus – 186*	*Lagochilusolga*	12.0±0.03	20	21±0,08	27.6
8	*A. egypticus – HT166S*	*Helianthus tuberosus*	14.0±0,08	6.6	30±0.07	-
9	*N. sphaerica – HT189L*		14.5±0.08	3.3	28±0.05	3.2
10	*D.purpureofuscus – HT182*		16.0±0.04	-	30±0.04	-
11	*M. verrucaria – HT190R*		9.0±0.03	40	16±0.02	44.8
12	*A. roseovilutinum – 170*	*Achillea filipendula*	13.0±0.04	13.3	26±0.03	10.3
13	*P. brevicompactum– 171*		14.0±0.01	6.6	21±0.02	27.5
14	*P. brevicaule alba – CC200*	*Celosia cristata*	15.0±0.03	-	30±0.07	-
15	*A. repens- 202*		15.0±0.04	-	32±0.08	-
16	Control		15.0±0.02	0	29±0.05	0
17	Xenical (as standard)		6±0.01	60	10±0.03	65.5

The tests were carried out in triplicate.

plant *A. filidens*. Yang *et al* 2018 reported on the hypolipidemic properties of garlic and onion oils against obesity[31].

S. minorum-VM83R (55.1%) from *V. minor* and *U. consortiae – FF155L* (51.7%) from *F. foetida* also have high inhibitory activity. Abdulmyanova *et al* 2015[25], and Ruzieva *et al* 2017[26] have shown that endophytes isolated from A. *longicuspis, A. filidens, V. minor*, and *F. foetida* produce compounds with antibacterial, antidiabetic, and cytotoxic properties, indicating their potential as a new source of secondary metabolites with therapeutic value. Some studies have shown promising activities of *F asafoetida,* including anti-obesity[31, 32]. *Azizian et al* 2012 reported that the resin of *F. asafoetida* reduces the lipids level and demonstrates hepatoprotective properties[33]. Thus, given the beneficial properties of *A. longicuspis, A. filidens, V. minor*, and *F. foetida*, it can be assumed that their endophytes may be expected producers of anti-obesity metabolites.

The extracts of *Fusarium sp-AL142R* and *F. sambucinum-AL135L*, the most strongly inhibited PL, were further tested for phytochemical composition (Table 3). At the same time, extracts of both endophytes contained four of the nine classes of chemicals studied – alkaloids, terpenoids, flavonoids, and tannins.

Tàble 2. PPL inhibitory activity of endophytes extracts

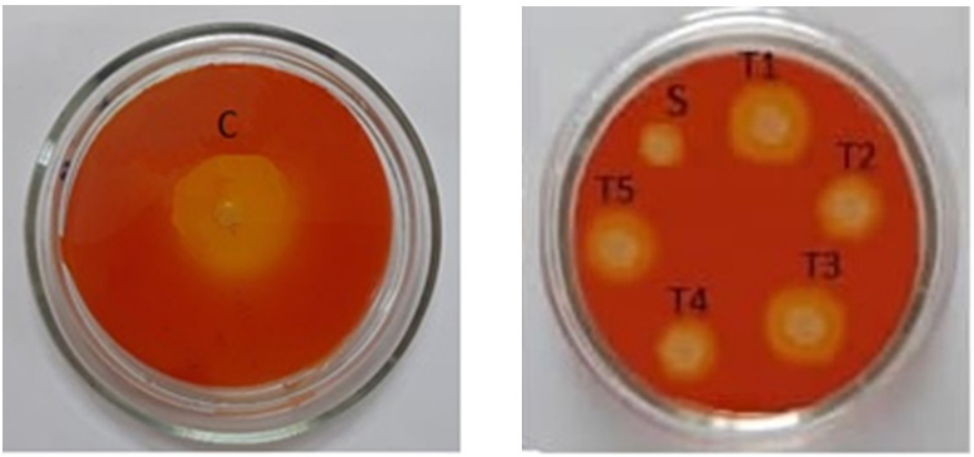
	Endophytes	Enzyme inhibition, %	IC50, μ/ml
1	*S. minorum-VM83R*	58.7±1.71	
2	*A. terreus-AF104S*	38.4±2.18	
3	*F. sambucinum-AL135L*	65.2±3.72	8.01±1.06
4	*Fusarium sp.-AL142R*	73.7±3.92	20.7±2.42
5	*C. tenuissimum-AF183*	51.7±2.56	
6	*U. consortiae-FF155L*	52.7±1.89	
7	*M. trigonosporus-186*	26,6±2.42	
8	*A. egypticus-HT166S*	13.7±2.13	
9	*N. sphaerica–HT189L*	3.2±1.21	
10	*D. purpureofuscus-HT182*	5.3±2.32	
11	*M. verrucaria-HT190R*	44.2±2.52	
12	*A. roseovelutinum-170*	10.6±1.39	
13	*P. brevicompactum–171*	18.2±3.52	
14	*P. brevicaule alba-CC200*	-	
15	*A. repens-202*	23.8±2.52	
16	Xenical (as standard)	80.5±1.81	20.6±1.04

The tests were carried out in triplicate

Table 3. Phytochemical characterization of the inhibitory extracts

	Compounds	*F. sàmbuñinum-AL135L*	*Fusarium sp.-AL142R*
1	Alkaloids	+	+
2	Ñardiac glycosides	-	-
3	Terpenoids	+	+++
4	Saponins	-	-
5	Flavonoids	+	++
6	Anthraquinone	-	-
7	Tannin	+	+
8	Phenols	-	-
9	Proteins	-	-

+ sign denotes the severity of the presence of the compound.
The tests were carried out in triplicate.



C – control;
S - Xenical;
Ò1 - C. tenuissimum-AF183;
T2 – F. sambucinum-AL135L;
T5 - S. minorum -VM83R;
T3 – U. consortiae-FF155L;
T4 - Fusarium sp.-AL142R.

Fig. 1. Decrease of halo size in phenol red assay by extracts of most active endophytes

In general, the phytochemistry of compounds capable to inhibit PL is well studied only in plant objects, which is presented in several reviews[34, 35] reporting on the anti-lipase properties of various classes of compounds, including polyphenols, saponins, terpenes, and terpenoids. There are still few studies on the chemical nature of endophytic inhibitors. It is reported that anti-lipase extracts of endophytes isolated from ginger rhizome contain terpenoid compounds, phenols, tannins, flavonoids, alkaloids, and saponins[36]. A phenol-containing fraction from endophyte *D. arengae* is capable, like Orlistat, competitively inhibiting PL and thus represents a potential drug compound[37].

CONCLUSION

The findings of this study provide new evidence that the endophytes associated with medicinal plants of Uzbekistan produce secondary metabolites with significant PL inhibitory activity. The data obtained allow to conclude that endophytic fungi of local medicinal plants have a sufficiently high PL inhibitory potential. Selected endophytes *F. sambucinum – AL135L* and *Fusarium sp. – AL142R* due to their relatively high activity against PL and low IC50 can be used as a source of PL inhibitors to develop antiobesity drugs.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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