ENDOPHYTES FROM VIOLET PLANTS GROWING IN UZBEKISTAN AS A SOURCE OF PANCREATIC LIPASE INHIBITING COMPOUNDS

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https://doi.org/10.5281/zenodo.8397071

Abstract. *Obesity is one of the globally widespread diseases, accompanied by hypertension, hyperlipidemia, atherosclerosis, and type II diabetes. The most effective therapeutic approach for treating obesity is the inhibition of pancreatic lipase (PL). Eight isolates of endophytic fungi were obtained from Viola odorata growing in the park areas of Tashkent, Uzbekistan. Screening of PL inhibiting activity of secondary metabolites produced by isolates in vitro showed that the isolates expose inhibitory activity varying from 28.8 to 91,5 %. The extract of VO1R isolates from the root of V.odorata and identified as Aspergillus fisheri revealed the highest inhibition (91.5%), and a low IC50 value of 20 µg/ml is comparable with Xenical as standard. Phytochemical analysis showed that the VO1R extract contains alkaloids, terpenoids, tannins, and flavonoids.*

Keywords: *endophytic fungi, IC50, inhibition, pancreatic lipase.*

INTRODUCTION

Inhibitors of pancreatic lipase (PL) that disrupt pancreatic lipase activity are peripheral drugs that directly reduce fatty acid absorption into the systemic circulation and adipocytes (**Drent & Van der Veen, 1993**). Orlistat (Xenical) is currently the only drug approved for the long-term treatment of obesity (**Orlistat Information. 07.08.2015**). It is a saturated derivative of lipstatin isolated from the Gram-positive bacterium Streptomyces toxytricini (**Weibel *et al.*, 1987**). However, long-term use of orlistat appears to be associated with severe undesirable side effects, including hepatotoxicity, gallstones, kidney stones, and acute pancreatitis.

Plants with significant anti-obesity potential are reviewed for their ability to inhibit pancreatic lipase. Many studies report new compounds and natural products inhibiting lipase and having more significant potential than orlistat (**Seyedan *et al.*, 2015**). Some plant extracts show a pronounced effect on fat digestion and consist of compounds from various chemical classes, including polyphenols, saponins, and terpenes (**De la Garza *et al.*, 2011**). Although the list of plants used for treating obesity in traditional medicine is extensive, most species are still collected from the wild, making it difficult to scale up the production of plant anti-obesity drugs. In this aspect, endophytic microorganisms are an alternative to plant sources. Endophytes make an outstanding contribution to drug discovery by producing a variety of new chemical structures and biological activity (**Samantha *et al.*, 2021; Gupta *et al.*, 2019**). There are more and more reports of unique properties of endophytic microorganisms, including antimicrobial, antioxidant, enzyme inhibition, synthesis of growth-stimulating substances, and the ability to withstand environmental stress (**Shurigin *et al.*, 2022, Mukhammedov *et al.*, 2022, Kondrasheva *et al.*, 2022**). Their vast potential is determined by chemical innovations and their use in developing pharmaceutical agents (**Gupta *et al.*, 2019**).

Many studies have demonstrated the ability of endophytes to produce compounds with inhibitory properties against many enzymes (**Meshram *et al*., 2019**). According to studies on the inhibitory properties of endophytes, it was established that endophytic fungi produce compounds that have inhibitory effects on several enzymes that control the occurrence of diseases in the vast majority of vital human systems, including diabetes, hypercholesterolemia, Alzheimer's, and Parkinson's diseases, Inflammatory diseases, malignant degeneration of cells, gout and many others (**Meshram *et al*., 2019**).

The promise of endophytic fungi as possible producers of compounds for treating obesity was first shown by Gupta et al. (**Gupta *et al.*, 2014; Gupta *et al*., 2015**). Screening of culture filtrates of 70 endophytic fungi isolated from Aegle marmelos revealed the inhibitory potential of three isolates. The 57TBBALM isolate assigned to *Penicillium* showed an IC50 value of 3.69 mg/ml, comparable to the IC50 of Orlistat (2.73mg/ml) as a positive control (**Gupta *et al.*, 2014**). Lipase inhibitory activity was also studied in 39 endophytic fungi from medicinal plants in the Andaman Islands. Patil and Patil confirmed complete inhibition of pancreatic lipase by lemon endophyte extracts (**Patil & Patil, 2019**). Thus, when screening extracts of 18 endophytes from 6 local plants using phenol red agar with olive oil, a yellow halo was utterly absent under the CLL-2 extract. PL was partially inhibited by CLL-1 extract from Citrus lemon (**Patil & Patil, 2019**). Seven compounds, including a 13-angeloyloxy-ciclosporin, were isolated from endophytic Phomopsis sp.0391 cultivated with a histone deacetylase inhibitor. All of these isolates were evaluated for lipase inhibitory activity. For the first time, the cytosporon B and dothiorelone A compounds exhibited significant lipase inhibitory activity compared to the positive control (Orlistat, IC50 = 43 μg/mL) with IC50 values at 115 and 275 μg/mL, respectively (**Sheng *et al*., 2020**).

Thus, although there are few such studies, 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 development of new lipase inhibitors for the treatment of obesity.

One of the good plants in this aspect is *Viola odorata (Viola odorata L., Violaceae*), a herbaceous perennial plant common worldwide. This plant contains many chemical compounds, including phenols, coumarins, alkaloids, flavonoids, saponins, and vitamins (**Katoch *et al*., 2017, Aslam *et al*., 2021**). In addition, Katoch et al. showed that endophytic fungi possessing anticipated activity are present in Viola odorata endophytes (**Katoch *et al*., 2017**).

Considering that violet plants *Viola odorata* is widespread in Uzbekistan, both in gardens and the wild, this work aimed to study the inhibitory potential of endophytic fungi from *Viola odorata*.

MATERIAL AND METHODS

Isolation and identification of endophytic fungi

Endophytic fungi were isolated according to the standard isolation method from the roots, stems, leaves, and flowers of violets collected in the Tashkent region of Uzbekistan on Petri dishes with Czapek-Dox agar medium containing 50 mg/ml chlortetracycline and 250 mg/ml streptomycin to suppress bacterial growth **(Hazalin *et al*., 2009)**. The plates were incubated for 7-14 days at 28°C. Grown fungal isolates were subcultured on Czapek-Dox agar medium without antibiotics and stored at 2-4°C until use. The isolates were identified to the genus by studying the morphological features of seven-day-old cultures grown on Czapek-Dox medium in terms of the shape of colonies, the nature of growth on a nutrient medium, the shape of conidia and spores, and

colony pigmentation (**Litvinov, 1967**). Species identification was kindly carried out by specialists from «Syntol» (Moscow, Russia), using kits for DNA extraction and PCR from this company.

Cultivation of endophytes

For five days, the isolated endophytes were cultivated on a Czapek-Dox medium on an orbital shaker at 180 rpm, 28°C. The biomass of the cultures was separated by centrifugation at 6000 rpm and stored at -4°C.

Extraction of secondary metabolites

5 g of fungal biomass was homogenized and extracted with 50 ml of ethyl acetate, left for a day on a shaker at room temperature to obtain secondary metabolites. The mixture was then filtered (Whatman No. 1), and Na_2S_4 was added at a 40 µg/ml rate to remove the aqueous layer. Next, the mixture evaporated to dryness on a rotary evaporator and dissolved in one ml of dimethyl sulfoxide. The resulting extract was used as a stock solution and stored at +4°C (**Hazalin *et al.*, 2009**).

Lipase inhibition assay using tube method

The analysis was carried out according to **Patil and Patil, 2019**. First, PL enzyme (Sigma, 100 U/ml) at 10 mg/ml concentration in phosphate buffer (pH 7.4) was preincubated with the same extract volume. Xenical (Cheplapharm, Germany) was used as a positive control; distilled water was negative. All samples were incubated at 37°C for 20 minutes. Next, 100 ml of a suspension of olive oil in distilled water (pH 7.4) was added and again incubated at 37°C for 60 minutes. After incubation, phenol red was added to each tube (10 ml), and a color change was observed. The tests were performed in triplicate.

Quantitative PL inhibition assay

Determination of inhibitory PL activity of the fractions. 50 mg of lipase ("Sigma," 60 units/ml) was suspended in 10 ml Tris-HCl buffer containing 2.5 mM Tris and 2.5 mM NaCl, pH 7.4. The solution was shaken vigorously for 15 min and centrifuged at 4,000 rpm for 10 min, and the supernatant was collected. Initial solutions of the extracts and Xenical standard in DMSO with linear concentrations in the 2-2000 µg/ml range. The final reaction mixture consisted of 875 µl of buffer, 100 µl of the enzyme, and 20 µl of extract at various initial concentrations, preincubated for 5 min at 37 0 C, followed by the addition of 10 µl of the substrate (4-nitrophenyl palmitate in 10 mM in acetonitrile). The amount of DMSO in the final concentration did not exceed 2%. The optical mixture was measured on a spectrophotometer (UV-Vis model UV-5100) after 5 min at 405 nm. The percentage of inhibition was calculated using the formula:

% inhibition = [(Ae-At)/Ae x100

where Ae is the optical density of the enzyme control (without inhibitor), and it is the difference between the optical density of the test sample with and without the substrate. IC50 compounds were calculated by plotting a linear regression curve and compared with Xenical (**Bustanji *et al.*, 2011**).

Phytochemical analysis of the secondary metabolites composition

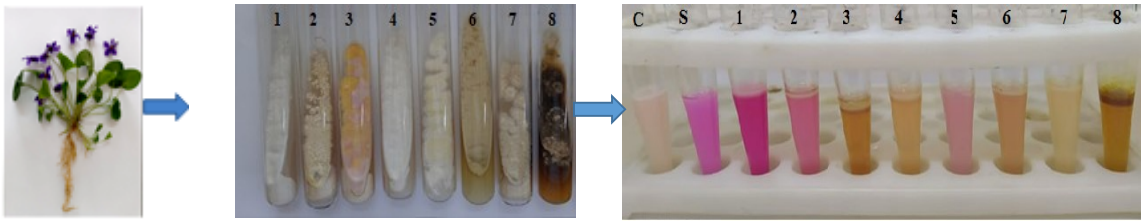
Conventional phytochemical methods determined the qualitative composition of the compounds in the extracts according to **Prabhavathi *et al.*, 2016.**

RESULTS AND DISCUSSION

Dietary fats play a central role in developing obesity; therefore, reducing fats available for metabolism is undoubtedly necessary for weight loss. That is why a real therapeutic target for obesity is PL, a critical enzyme for deleting dietary fat to free lipids and their absorption.

Therefore, medicinal plants with ethnopharmacological value are a sound source for isolating endophytes endowed with bioactive properties related to the host plant. Many endophytic fungi from different plants have been studied as sources of endophytes with various properties. However, there are few works on the study of endophytic fungi as possible lipase inhibitors, and the list of plants used for this purpose is also short (**Meshram *et al*., 2019; Gupta *et al*., 2014; Gupta *et al*., 2015**). The inhibitory activity of extracellular metabolites secreted into the culture medium is mainly considered.

In this study, we screened the bioactivity of intracellular metabolites against obesity in *Viola odorata* endophytes growing in Uzbekistan. Only eight isolates of endophytic fungi were isolated from various organs of *Viola odorata* growing in the Tashkent region of Uzbekistan (fig. 1(A, B)). Four isolates were obtained from the root and two isolates each from the leaves and flowers of the plant. According to morphophysiological features, isolates preliminarily identified as *Aspergillus, Penicillium*, and *Fusarium*, with representatives of the genus *Aspergillus* dominating.



A. **B.** **C.**

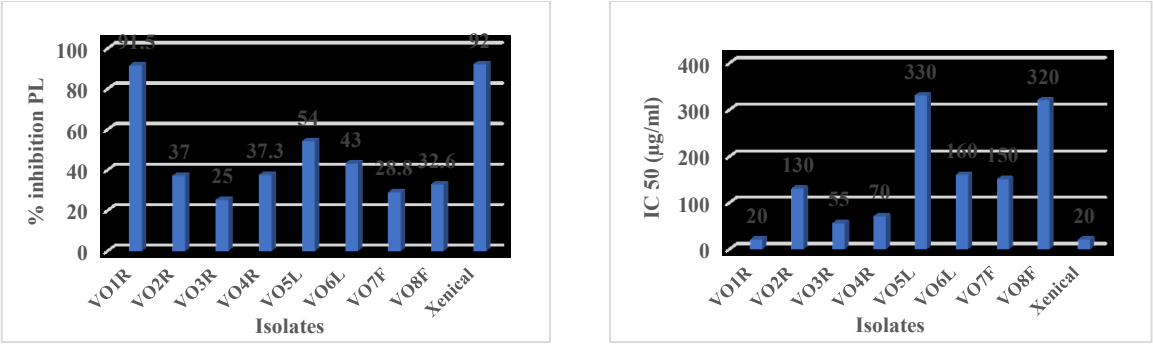
1-VO1R; **2**-VO2R; **3**-VO3R; **4**-VO4R; **5**-VO5L; **6**-VO6L; **7**-VO7F; **8**-VO8F, **C**-control; **S**-standard (Xenical)

Figure 1 Isolation of endophytic fungi from *Viola odorata* (A – *V. odorata*, B - pure cultures of endophytes) and PL Inhibitory activity of the endophytic extracts against Pl in phenol red tube test (C).

Previously, Katoch et al. isolated 27 endophyte morphotypes belonging to Ascomycota and Basidiomycota from *Viola odorata* (**Katoch *et al*., 2017**). However, the most remarkable diversity of endophytic fungi was also observed in roots, leaves, and buds.

During the initial screening of the inhibitory activity of the isolated isolates by the chromogenic method using a red phenol indicator after incubation with the substrate and pancreatic lipase, a change in the color of samples was observed in all extracts, but a characteristic purple color change, comparable to Xenical as a positive control, was observed in the presence of VO1R; VO2R; VO5L (fig.1 (C)).

At the same time, spectrophotometric measurement of lipase activity showed that biomass extracts of all eight isolates (VO1R, VO2R, VO3R, VO4R, VO5L, VO6L, VO7F, VO8F) reduce enzymatic activity to varying degrees. For example, the lowest inhibition of PL was 25-32.6% in isolates of VO3R, VO7F, and VO8F isolated from flowers and roots; moderate inhibition of PL from 37.3 to 43% was observed in VO4R and VO6L from violet leaves; the highest was 54.0 % in VO5L from leaves and 91.5 % in VO1R from the violet root (fig. 2(A)).



A. **B.**

Figure 2 Quantitative determination of the PL inhibitory activity of extracts(A) and IC50 values of violet endophyte extracts (B).

The VO1R isolate extract with the highest degree of PL inhibition also showed the lowest IC50 value of 20 µg/mL. Under the same experimental conditions, inhibition of PL by Xenical was 92%, and IC50 20 µg/ml, comparable to the values of the VO1R isolate (Fig. 2(B)).

Katoch et al. reported that extracts of seven *Viola odorata* endophytes showed inhibitory activity with low values (IC50 < 10 µg/mL), and the most active VOLF4 extract (*Aspergillus sp*.) showed inhibitory activity with an IC50 of 3.8 µg/mL (**Katoch *et al.*, 2017)**. Although the inhibitory activity of the isolates is lower than mentioned above, this allows us to conclude that violet endophytes, particularly the VO1R isolate, have sufficient potential as promising sources of anticipated compounds.

The isolate VO1R was identified as *Aspergillus sp.* based on morphological characteristics, and as *Aspergillus fisheri* VO1R (99.12%) by high throughput sequencing method. The strain was found first in Uzbekistan.

The *A. fisheri* VO1R extract, whose metabolites most strongly inhibited PL, was further tested for phytochemical composition. The phytochemical tests showed that the extracts contain terpenoids, tannins, flavonoids, and alkaloids. The data obtained are consistent with some reports indicating the presence of similar substances in plant extracts with high antilipase activity (**Seyedan *et al*., 2015; De la Garza *et al*., 2011**). Our data also suggest that the high anticipated activity of the endophytic fungus *A. fisheri* VO1R may be due to terpenoids, flavonoids, saponins, or alkaloids that require further investigation research related to the isolation, identification, and study of their interaction with PL.

CONCLUSION

Natural substances from endophytic fungi are recognized as good resources for developing various therapeutic compounds. The presented data on the isolation and screening of endophytic fungi from *V.odorata* growing in Uzbekistan have varied inhibitory effects on pancreatic lipase. The extract of *A. fisheri* VO1R containing terpenoids, tannins, flavonoids, and alkaloids reveals the highest inhibitory activity of 91,5% and IC50 20 mg, comparable with Xenical as standard. VO1R isolate identified as *A. fisheri* VO1R was selected as the most active strain and may be considered the promising source of antilipase compounds.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants performed by any authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Acknowledgments: We express our gratitude to the company "Synthol", Russia, for the identification of the active isolate *A. fisheri* VO1R.

REFERENCES

1. Aslam L, Kaur R., Sharma, V., Kapoor N., Mahajan R. (2021). Isolation and characterization of cyclotides from the leaves of *Viola odorata L.* using peptidomic and bioinformatic approach, *3 Biotech.*, **11(5)**: 211, https://doi.org/10.1007/s13205-021-02763-2
2. Bustanji Y., Al-Masri I.M., Mohammad M., Hudaib M., Tawaha K., Tarazi H., AlKhatib H.S. (2011). Pancreatic lipase inhibition activity of trilactone terpenes of Ginkgo biloba, *J. Enzyme Inhib. Med. Chem*., **26**: 453–459, https://doi.org/10.3109/14756366.2010.525509
3. De la Garza A.L., Milagro F.I., Boque N., Campión J., Martínez J.A. (2011). Natural inhibitors of pancreatic lipase as new players in obesity treatment. *Planta Med.*, **77(8):** 773–785, https://doi.org/10.1055/s-0030-1270924
4. Drent M.I., Van der Veen E.A. (1993) Lipase inhibition: a novel concept in the treatment of obesity, *Int. J. Obes. Rel. Metab. Disord*. **17:** 241–244.
5. Gupta M., Saxena S., Goyal D. (2015) Potential pancreatic lipase inhibitory activity of an endophytic Penicillium species. *J. Enzyme Inhib. Med. Chem*. **30**: 15–21, https://doi.org/10.3109/14756366.2013.871007
6. Gupta M., Saxena S., Goyal D. (2014) Lipase inhibitory activity of an endophytic fungal species of Aegle marmelos: a bioresource for potential pancreatic lipase inhibitors, *Symbiosis*, **64**: 149–157, https://doi.org/10.1007/s13199-015-0311-9
7. Gupta S., Chaturvedi P., Kulkarni M., Van Staden J. (2019) A critical review on exploiting the pharmaceutical potential of plant endophytic fungi, *Biotechnol. Adv*. **39:** 107462. https://doi.org/10.1016/j.biotechadv.2019.107462
8. Hazalin N.A., Ramasamy K., Lim S.M., Wahab I.A., Cole A.Lj., Majeed A.A. (2009) Cytotoxic and antibacterial activities of endophytic fungi isolated from plants at the National Park, Pahang, Malaysia. *BMC Complement Altern. Med*. **9**:46, https://doi.org/10.1186/1472-6882-9-46
9. Katoch M., Singh A., Singh G. (2017) Phylogeny, antimicrobial, antioxidant, and enzyme-producing potential of fungal endophytes found in *Viola odorata*. *Ann. Microbiol.*, **67:** 529–540, https://doi.org/10.1007/s13213-017-1283-1
10. Kondrasheva K., Egamberdiev F., Beshko N., Karimova F., Gulyamova T. (2022). Diversity and Halotolerance of Endophytic Fungi from Halophytes of Bukhara Region, Uzbekistan. *Acta Microbiologica Bulgarica*, **38(3):** 216-222.
11. Litvinov M.A., *Identification guide for microscopic fungi*, Nauka, Leningrad, 1967.
12. Meshram V., Uppal K., Gupta M. Endophytes: A Gold Mine of Enzyme Inhibitors. Microbial Bioprospecting for Sustainable Development. Chapter In*: Microbial Bioprospect. Sustainable Develop.,* 2019,
13. Mukhammedov I.I., Ruzieva D.M., Gulyamova, T.G. (2022) Comparative Evaluation of Antidiabetic and Antioxidant Activities of Methanol Fractions of *Penicillium brevicaule* alba CC200 and *Aspergillus egypticus* HT166S. *Moscow Univ. Biol.Sci. Bull*. **77**: 245–250. https://doi.org/10.3103/S0096392522040137

14. Orlistat (marketed as Alli and Xenical) Information. 07.08.2015. U.S. Food&Drug Administration. Retrieved from: https://www.fda.gov/drugs/postmarket-drug-safety-information-patients-and-providers/orlistat-marketed-alli-and-xenical-information
15. Patil M.P., Patil R.H. (2019) Data on the inhibitory effect of endophytic fungi of traditional medicinal plants against pancreatic lipase (PL). *Data Brief*, **27**:104797, https://doi.org/10.1016/j.dib.2019.104797
16. Prabhavathi R.M., Prasad M.P., Jayaramu M. (2016). Studies on Qualitative and Quantitative Phytochemical Analysis of *Cissus quadrangularis*. *Adv. Appl. Sci. Res.* **7(4)**: 11–17.
17. Samantha S, Ghosh S., Mandal N.C. Endophytic Fungi: A Source of Novel Pharmaceutical Compounds. In: *Endophytes,* Patil, R.H., Maheshwari, VL (eds) Springer, Singapore, 2021.
18. Seyedan A., Alshawsh M., Alshagga M.A., Koosha S. Zahurinm M. (2015) Medicinal Plants and Their Inhibitory Activities against Pancreatic Lipase: A Review. *Evid. Based Complementary Altern. Med.,* Article ID 973143, https://doi.org/10.1155/2015/973143
19. Sheng S.L., Li Y.P., Xiang H.Y., Liu Y., Wang Y.D., Kong L.P., Du G., Hu Q.F., Chen Y.J., Wang W.G. (2020) Histone Deacetylase Inhibitor Induced Lipase Inhibitors from Endophytic *Phomopsis* sp. 0391. *Rec. Nat. Prod*., **14(1)**: 42–47, https://doi.org/10.25135/rnp.134.19.01.1243
20. Shurigin V., Alimov J., Davranov K., Gulyamova T., Egamberdieva D. (2022). The diversity of bacterial endophytes from *Iris pseudacorus L*. and their plant beneficial traits. *Curr Res Microb Sci*. **12 (3)**:100133. https://doi.org/10.1016/j.crmicr.2022.100133
21. Weibel E.K., Hadvary P., Hochuli E., Kupfer E., Lengsfeld H. (1987) Lipstatin, an pancreatic lipase inhibitor, produced by *Streptomyces toxytricini.* I. Producing organisms, fermentation, isolation, and biological activity. *J. Antibiot.,* **40**:1081–5, https://doi.org/10.7164/antibiotics.40.1081