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Penicillium citrinum Thom: A potential antihyperglycemic endophyte from *Gymnema sylvestre*

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Endophytic fungi producing bioactive compounds from medicinal plants have enormous applications as, antibiotics, anti-parasitic, agrochemical, antioxidants and anticancer agents. In the present research, aimed to investigate the qualitative and quantitative analysis of secondary metabolites of an endophyte isolated from *Gymnema sylvestre* and to evaluate its antidiabetic potential. The frequently occurring fungal isolate VSQ-11 was selected for in detailed research work. Based on the morphological and molecular characteristics, the isolate was identified as *Penicillium citrinum Thom*. Myco-chemical studies exhibit the maximum presence of flavonoids (4 mg/g) followed by phenols (1 mg/g), tannins (1.178 mg/g), saponins (1.175 mg/g), steroids (0.96 mg/g), glycosides (0.6 mg/g) and alkaloids (0.34 mg/g) in crud fungal extract. The Albino mice treated with fungal ethyl acetate extract (250 mg/kg body weight) has shown a reduction in blood glucose level as compared to the negative control group.

Keywords: Antihyperglycemic activity, Endophytes, *Gymnema sylvestre*, *Penicillium citrinum* Thom, Phytochemicals.

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Introduction

Ayurveda, an Indian system of medicine stresses the use of plant-based herbs to cure disorders. *Gymnema sylvestre* is a woody climber belonging to the family Asclepiadaceae, traditionally known for its therapeutic properties in treating several human ailments. *G. sylvestre* is distributed all over the world including Indian localities such as Western Ghats, Banda, Konkan and Deccan Peninsula[1]. The plant is commonly called Gurmar (Hindi) which means the destroyer of sugar, causing loss of sweet taste on chewing the leaves. *G. sylvestre* is an important anti-diabetic and diuretic herb experimentally/clinically proved to be anti-hyperglycemic[2] which regenerate and repair beta cells in Type 2 diabetic patients[3]. The plant is also extensively used in the treatment of asthma, inflammations, eye complaints, snake bite, chronic cough, hepatosplenomegaly, and stomach problems[4]. Gymnemic acid, one of the important metabolites of *G. sylvestre* has its receptor on the outer layer of the intestine, which prevent absorption of sugar molecules in the intestine and leads to a decrease in the blood sugar levels[5]. Gymnemagenin, the triterpenoid found in this medicinal plant is used in the pharmaceutical industry as an anti-diabetic agent[6].

Diabetes mellitus is a chronic disorder of metabolism followed by an abnormal rise in plasma glucose levels, as a consequence of unequilibrated insulin production and/or insensitivity to the effect of this hormone. Type 2 diabetes is the most common type, associated with overweight and obesity caused by insulin resistance and often remains undiagnosed for many years because the hyperglycaemia is not severe enough to provoke noticeable symptoms of diabetes[7] (ADA 2016) meanwhile change in lifestyle is the simplest way of delaying or preventing Type 2 Diabetes[8]. Currently used anti-diabetic drugs have adverse side effects such as Neuropathy, Anaemia (Metformin), Upper RTI infection (Saxagliptin and Vildagliptin), Ketoacidosis, Genital mycosis (Canagliflozin), Lipoatrophy and lipohypertrophy (At the site insulin injection), Bladder cancer (Rosiglitazone) which can be prevented by replacing available drugs to the bioactive compounds[9].

Endophytes are microorganisms, which resides in the internal living tissues of plants without causing any apparent symptoms and injuries to the host and can protect the host plant from infectious agents by

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secreting bioactive secondary metabolites[10]. Although enormous natural products have been identified so far from plants and microbes, the discovery of powerful bioactive metabolites from the endophytes is a slightly untouched area that needs to be explored for novel active compounds against human ailments. In view of this, the present research focused on isolation and identification of potent endophytic fungus from *G. sylvestre* useful in reducing blood glucose levels in hyperglycemic conditions.

Materials and Methods

Collection of plant material

The leaves of *G. sylvestre* were collected from the Medicinal Garden of the Department of Botany, Gulbarga University, Kalaburagi, Karnataka and authenticated using the flora of Gulbarga district (HGUG 58)[11].

Isolation of fungal endophyte from *G. sylvestre*

The leaves were washed with running tap water thoroughly to remove debris, washed with sterile distilled water and surface sterilized using 3% Sodium hypochlorite. The surface-sterilized leaves were cut into 2-4 mm segments and aseptically transferred into Petri plates containing PDA medium. Plates were incubated at 28±2 °C for 10 days. The plates were monitored regularly to check the growth of endophytic fungal colonies from leaf segments. After the growth, the potential endophyte was transferred to the PDA plate for further studies[12].

Morphological and molecular characterization of endophyte VSQ-11

The morphological identification of endophyte VSQ-11 was done by microscopic observations of colony colour, appearance, texture, mycelia structure, pigmentation pattern, arrangement of conidiospores and conidia were identified and confirmed by the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute (ARI), Pune. The photomicrographs were obtained with the help of Carl Zeiss, AXIO Imager 2, (Germany). The measurements were also confirmed with the software available with the Carl Zeiss, AXIO Imager 2, Germany.

For molecular characterization, the seven days old fungus culture was filtered through a 10 mL syringe containing glass wool. Then the culture was placed in a tube containing 60-80 mg sterile glass beads (425-600 µM) and lysis buffer (100 Mm Tris HCl [pH-8.0], 50 mM EDTA, 3% SDS), homogenized at 6 M/S for 60 sec. The homogenate was rotated at 13,000 rpm for 10 min and moved the supernatant to a new microcentrifuge tube containing 2 µL of RNase A (10 mg/mL) and incubated at 37 °C for 15 min. An equivalent volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 13000 rpm for 10 min. the collected supernatant was transferred to a microcentrifuge tube and precipitated the DNA by adding 100% ethanol at -20 °C for 30 min. Then, centrifuged the content at 12,000 rpm for 10 mins to collect the DNA and the pellet was washed with 70% ethanol and re-centrifuged at 12,000 rpm for 5 min. The DNA pellet was air-dried and dissolved in 1X TE buffer. The fungal ITS region was amplified using primer pairs of ITS4 (Reverse-5' TCCTCCGCTTATTGATATC 3') and ITS5 (Forward-5' GGAAGTAAAAGTCGTAACAAGG 3'). PCR reaction mixture was set for 25 µL by adding 10x PCR buffer-2.5 µL, 2mM dNTPs -1 µL, Taq polymerase (1U/µL)-0.2 µL, primers ITS4 (10 PM/µL)-1µL, ITS5 (10 PM/µL)-1µL, template (10 ng/µL)-2.5 µL and made the final volume 25 µL by adding 16.8 µL of distilled H_2O. Then the thermal PCR cycler was set to the conditions. The PCR products were visualized on 1.2% agarose gel containing ethidium bromide (EtBr) and purified DNA products then sent for DNA sequencing[13]. The sequence was analyzed by using the BLAST program and submitted to the NCBI Gene Bank database. A phylogenetic tree was constructed with MEGA 6 software by the neighbour-joining method[14]. The culture VSQ-11 has been deposited in NFCCI-ARI, Pune with deposition number NFCCI-4673.

Extraction of crude compound

The fungus VSQ-11 was grown in a 100 mL Erlenmeyer flask containing the PDB medium. The inoculated flasks were incubated at 28±1 °C for 21 days. After incubation, culture was filtered to remove the mycelial mat. The mycelia were soaked in an equal volume of ethyl acetate for 24 h then crushed in a pestle-mortar and filtered extract. The filtrate thus obtained was allowed to evaporate to the dryness. The dried crude extract was collected and dissolved in organic solvents such as methanol, petroleum ether and chloroform for qualitative and quantitative analysis of secondary metabolites[15].

Qualitative phytochemical screening of secondary metabolites

Qualitative analysis of secondary metabolites in different solvent extracts has been carried out by adopting standard methods adopted by Suryawanshi and Vidyasagar[16] and Tiwari *et al.*[17].

Quantitative estimation of secondary metabolites

The quantitative estimation of alkaloids, phenols, flavonoids, saponins, tannins, steroids and glycosides were carried out by using standard methods adopted by Harborne[18] and Chukwuma *et al*.[19].

Oral glucose tolerance test

Drugs and chemicals

Glimepiride (Amaryl® 1 mg) and Acarbose (Glucobay 50) as standard, glucose (2%), Gum acacia, VSQ – 11 crude extract (250 mg/kg Bodyweight), Glucometer.

Animals

Albino mice were used for the study of glucose tolerance tests. These experimental animals were acclimatized to laboratory conditions at the animal house, Luqman Pharmacy College, Kalaburagi, Karnataka. Animals were maintained at 22 to 24 °C and 60-70% humidity. The animals were supplied with water and commercial feed. These animals were grouped according to their body weight and marking was made as Head, Body, Tail, and Head-body. The animals were grouped into 5 groups. Group 1: control, Group 2: Glimepiride, Group 3: Acarbose, Group 4: negative control, Group 5: fungal extract (VSQ-11).

Preparation of suspension

The prescribed dose of Glimepiride (6 mg), Acarbose (150 mg) and fungal extract (7 mg) mixed with an equal amount of gum acacia and homogenized in a pestle and mortar one by one and made the final volume up to 5 mL with distilled water. About 2% glucose was prepared as a negative control. The dosage suspension was prepared in a 1:2:1 ratio (drug: distilled water: gum acacia) and stored in culture tubes.

Collection of blood

The animals were kept at fasting before the collection of blood. The blood sample was collected from the tail vein method at 1, 2, 4, and 8 hours after drug treatment.

Estimation of blood glucose level in albino mice

The blood glucose level (BGL'S) was estimated using Glucometer-one[20].

Results and Discussion

A fungal endophyte VSQ-11 was isolated from *G. sylvestre* during Feb. 2019 and recorded its morphological characters (dull green, velvety, reverse pale yellow) appeared on the PDA medium. Microscopic structure (Fig. 1) revealed unbranched to branched conidiophores with smooth wall measures up to 176×4 µm, Metulae measures 11.82-21.57×2.6-3.3 µm in size and were 2-5 in number, unequal in length containing numerous, ampulliform and hyaline Philalides (11.15×2.70 µm). Conidia (2.58×2.58 µm) globose smooth-walled structure present on the Philalides. These results indicate that the fungus VSQ - 11 belongs to *Penicillium genus*. Based on molecular studies, the endophyte was identified as *Penicillium citrinum* Thom (Fig. 2). The nucleotide sequence thus obtained was submitted to the NCBI gene bank (Accession number MH856132.1). The phylum Ascomycota with several species of *Penicillium* is the most common representative of the endophytic fungal community[21]. *Penicillium crustosum* isolated from *Phoenix dactylifer*[22], *Penicillium citrinum* isolated from

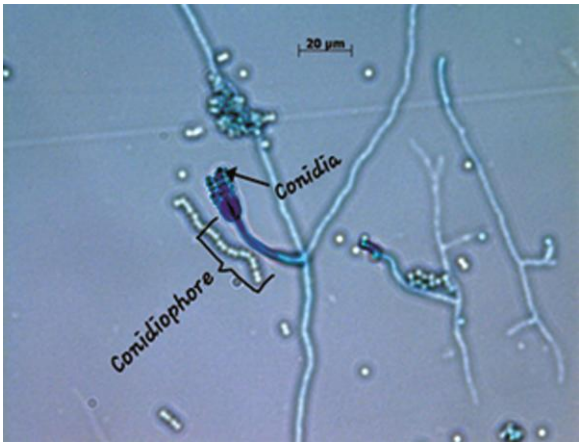

Fig. 1 — Microscopic structure of fungus with conidiophores and conidia.

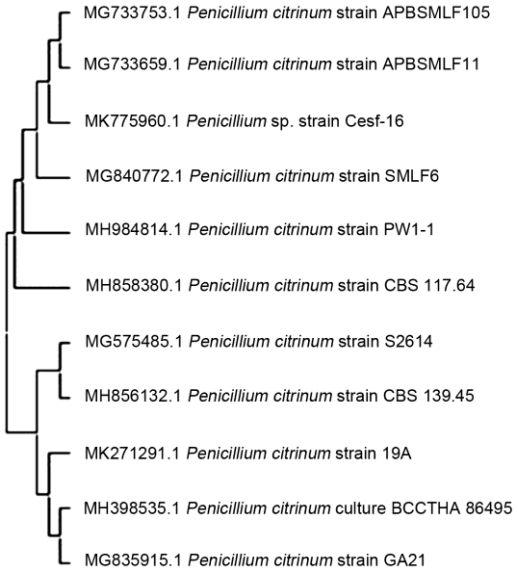

Fig. 2 — Phylogenic tree of endophyte VSQ-11.

Boswellia sacra[23], *Penicillium concentricum* from *Trichocolea tomentella*[24] and *Penicillium sclerotiorum* was isolated from *Cassia fistula* L.[25]. Endophytic *Penicillium* spp. have been isolated from unique niches such as Rhizome[26], marine sponge[27] and mangrove[28] and can be directly employed for its agricultural importance, as it serves as a source for phytoremediation, plant growth-promoting compound, and biocontrol agent against plant pathogen, moreover, it is also emerging as a precursor for biotechnological application[6]. Endophytic *Penicillium* is reported to be a source of structurally diverse bioactive compounds such as antidiabetic[28], antiobesity[29], antiviral[30], anticancer[25], anti-inflammatory[31], antifibrotic[32], immunosuppressive[33], neuroprotective[34], and antibacterial[35].

The myco-chemical studies of *Penicillium citrinum* Thom in different solvents extracts revealed the presence of alkaloids, flavonoids, phenols, tannins, saponins, glycosides, and steroids (Table 1). Among the solvent extracts studied, chloroform extract has shown positive results for the presence of all secondary metabolites except the glycoside however the reducing sugar has been detected only in chloroform extract. Similarly, glycoside has been detected only in pet ether extract.

Table 1 — Preliminary screening of secondary metabolites in *Gymnema sylvestre*

Phytochemical tests	Methanol	Chloroform	Petroleum ether
Alkaloids			
Dragendroff's test	+	+	+
Mayer's test	-	-	-
Wagner's test	-	-	-
Phenols			
$FeCl_3$ test	+	+	-
Ellagic test	-	-	-
Flavonoids			
Shinoda test	-	-	-
$FeCl_3$ test	+	+	-
Zn-HCL test	-	-	-
NaOH test	-	-	-
Lead acetate test	-	-	-
Triterpenoids			
LB test	-	-	-
Salkowaski test	-	+	-
Saponins			
Foam test	-	+	+
Steroids			
LB test	+	+	+
Salkowaski test	-	-	-
Tannins			
$FeCl_3$ test	+	+	-
Glycosides			
Kellar-killiani test	-	-	+
Reducing Sugars			
Fehling's test	-	+	-
Benedict's test	-	-	-

Quantitative estimation of secondary metabolite from *Penicillium citrinum* Thom extract determined maximum amount of flavonoids (4 mg/g) followed by tannins (1.178 mg/g), saponins (1.175 mg/g), phenols (1 mg/g), steroids (0.96 mg/g), glycosides (0.6 mg/g) and alkaloids (0.34 mg/g) (Table 2). Flavonoids have been shown to have a wide range of biological and pharmaceutical property including hypoglycemic activity as reported through peripheral glucose uptake and enzyme expression for carbohydrate metabolism triggered by flavonoids[36]. Tannin has also been shown to possess α-amylase and α -glycosidase inhibition capability[37]. α-amylase and α-glucosidase catabolize polysaccharides into simpler form then hydrolyze into absorbable monosaccharides and finally increase the glucose level. Saponins help in blood glucose reduction by stimulating beta cells and pancreatic islets in the pancreas[38]. Alkaloid possesses antioxidant and anti-diabetic activity through antioxidant potential, ROS production, glucose uptake and PTP-1B inhibition[39].

The blood glucose levels in all groups of animals studied were measured from 0 hours to 8 hours after drug administration (Fig. 3). In the animal group treated with fungal extract, the blood glucose level was raised initially after loading the glucose, however, after 2 h of drug administration, a significant reduction in blood glucose level was observed as compared to the negative control animal group. Then maximum blood glucose level in treated animals was recorded in the 2nd hour (158.3±29.67*). The standard drugs Glimepiride and Acarbose were proved to be more effective in reducing blood glucose than the fungal extract. In negative control animals, blood glucose levels increased continuously till 4 h (153.3±5.608**). Similar results were reported[40] by the extract of fungal endophyte isolated from *Salvadora oleoides* Decne after 180 min of the glucose load. The standard drug glimepiride was most effective in reducing blood glucose levels.

Table 2 — Quantitative estimation of secondary metabolites

Photochemical constituents	Quantity (mg/g)
Alkaloids	0.34
Phenols	1.0
Flavonoids	4.0
Saponins	1.175
Tannins	1.178
Steroids	0.96
Glycosides	0.6

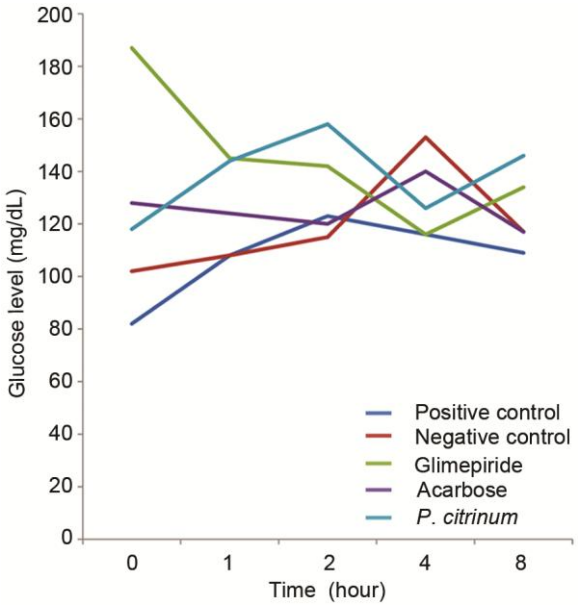

Fig. 3 — Effect of *P. citrinum* extract on glucose tolerance in albino mice.

Glimepiride and acarbose treated groups produced hypoglycemic effects due to the rapid release of insulin and increase sensitivity of pancreatic β-cell to glucose[41]. Most of the endophytic fungi such as *Xylariaceae* sp. QGS 01, *Alternaria* sp. *Aspergillus* sp., *Penicillium* sp. were reported to exhibit antidiabetic potential through the inhibition of alpha glycosidase enzyme[28,42,43].

The present study reports ethyl acetate as the most suitable solvent to recover the bioactive compounds from fungal endophytes. A similar study is reported on ethyl acetate extract of endophyte isolated from five different medicinal plants[44]. Methanol was also found good as an extraction solvent to recover the active anti-diabetic molecule[45,46].

Conclusion

Secondary metabolites such as alkaloid, flavonoid, tannin, and saponin have been detected from endophytic *Penicillium citrinum* Thom isolated from *G. sylvestre*. The fungal crude extract showed anti-hyperglycemic activity in the animal model, which proves that endophytes *P. citrinum* Thom isolated from the ethnomedicinal plant can be used as an alternative to the *G. sylvestre* for the isolation of bioactive molecules with antidiabetic potential. Further research on the characterization of the active molecules and its efficacy against hyperglycemic conditions has been recommended.

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Conflict of interest

Nil.

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