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Aspergillus niger strain HA106-H2: An endophyte from *Gymnema sylvestre* for anti-hyperglycemic potential

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ABSTRACT

***Gymnema sylvestre* is an important medicinal herb traditionally used as anti-hyperglycemic agent. The present study was aimed to use the fungal endophytes as an alternate source to the plant. Ten endophytic fungal strains were isolated from the leaves of *G. sylvestre*. Of which the frequently recovered strain VSS -23 was identified as *Aspergillus niger* strain HA106-H2 and selected for further investigation. The crude solvent extracts of *A. niger* strain HA106-H2 employed in phytochemical screening showed the presence of alkaloids, phenols, flavonoids, tannins and saponins. The anti-hyperglycemic activity of the ethyl acetate extract at 250mg/kg of body weight in animal model (albino mice) exhibited 9.15% reduction in blood glucose level as compared to 6.25% reduction in standard drug acarbose at 50mg/kg concentration in 120 min after drug administration.**

Keywords: Endophyte, *Gymnema sylvestre, Aspergillus niger*, Anti-hyperglycemic activity

INTRODUCTION

Gymnema sylvestre is an important herb belonging to family Ascelpidaceace, widely distributed in Western and Eastern part of India, Malaysia, Srilanka, Tropical Africa and Australia. The plant is commonly called as Madhunashini (Sanskrit), Gurmar (Hindi), Sannagerasehambu (Kannada), Periploca of the woods (English). *G. sylvestre* is known for its anti-diabetic (Laha and Paul, 2019), anti-inflammatory (Kumar *et al.,* 2012), dental caries (Devi and Ramasubramaniaraja, 2009), anti-viral (Selim *et al.,* 2018), anti-obesity (Kaushik *et al.,* 2011) and hypolipidemic properties (Kumar *et al.,* 2015). Gymnemic acid extracted from the plant has a receptor on the surface of the outer layers of intestine play an important role in preventing absorption of sugar molecules in intestine and decreasing the blood sugar levels (Rao *et al.,* 2010). As *G. sylvetsre* is a slow growing plant listed in rarely available medicinal plant and used in the treatment of several human ailments its availability in near future may be under threat. Under such situation, the endophytes could serve the purpose as an alternate source for the production of pharmacologically similar drug. In ayurveda, the plant is primarily used in the treatment of diabetes and its related disorders for several years. Bioactive metabolite of *G. sylvestre* such as alkaloids, phenols, flavonoids, tannins, saponins, glycosides, steroids, terpenoids etc. have been used as drugs or lead compounds in the production of drug (Porchezhian and Dobriyal, 2003). Diabetes is chronic disease caused by hyperglycemic condition characterized by dysfunction or destruction of pancreatic β-cells (Oshima *et al.,* 2006). Recent report reveals approximately 463 million adults were living with diabetes; by 2045 this will rise to 700 (IDF, 2019), the elevated blood glucose is the third uppermost risk factor for premature mortality, following high blood pressure and the use of tobacco (Tafesse, 2017). Complications of diabetes include renal failure, neuropathy and peripheral vascular disease lead to loss of limbs, retinopathy with increased risk of blindness, increased risk of cardiovascular disease and stroke (Basit *et al.,* 2012). Before the discovery of insulin, the disease was treated traditionally using medicinal plants (Tahraoui *et al.,* 2007; Kazi, 2014; Preuttiporn and Preedanon, 2019). Each plant may contain its specific active compound responsible for the reduction of hyperglycemia. The available modern therapeutic administrations have serious side effects such as hypoglycemia (sulphonylureas), lactic acidosis, folate and B_{12} malabsorption (metformin), gastrointestinal symptom (acarbose), weight gain (sulphonylureas and thiazolidinediones), and edema (thiazolidinediones) (Campbell, 2007). Therefore, the treatment of diabetes using natural sources without adverse side effect is in demand.

Endophytes co-exist with plants without causing any disease to the host plant and protect them from infectious agents and adverse climatic conditions by producing bioactive compounds (Sandhu *et al.,* 2014)). Several endophytes have been reported to produce therapeutically useful molecule, such as anti-cancer (Uzma *et al.,* 2018), antibacterial (Deshmukh *et al.,* 2015) and antiviral compounds (Selim *et al.,* 2014). Therefore, the present work was carried out to identify a potential fungal endophyte capable of producing anti-hyperglycemic molecule.

MATERIALS AND METHODS

Collection of plant material

The leaves of *G. sylvestre* were collected from Medicinal garden of Department of Botany, Gulbarga University, Kalaburagi, Karnataka and authenticated using the flora of Gulbarga district (HGUG 58) (Seetharam *et al.,* 2000).

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 them into petriplates 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 PDA plate for further studies (Ambika and Vidyasagar, 2018).

Morphological and molecular characterization of endophyte VSS-23

The morphological and molecular characterization of endophyte VSS-23 was done at NFCCI (National Fungal Culture Collection of India) Agharkar Research Institute, Pune. The sequence was analyzed by using BLAST program and submitted to the NCBI Gene Bank database. A phylogenetic tree was constructed with MEGA 6 software by the neighbor-joining method (Tamura *et al.,* 2011).

Extraction of crude compound

The fungus VSS-23 was grown in 100 ml Erlenmeyer flask containing 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 equal volume of ethyl acetate for 24 hrs then crushed in a pestle-mortar and filtered the extract. The filtrate thus obtained was allowed to evaporate to dryness. 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 (Vineeta *et al.,* 2018).

Qualitative phytochemical screening of secondary metabolites

Qualitative analysis of secondary metabolites in different solvent extracts has been carried out by adopting standard methods (Harbone,1998).

Quantitative estimation of secondary metabolites

The quantitative estimation of alkaloids, flavonoids (Ikan, 1981), phenols (Swain and Hillis, 1959), tannins (Schanderi, 1970) and saponins (Sanchez *et al.,* 1979) were carried out by using standard methods.

Pharmacological activity

Oral glucose tolerance test

Drugs and chemicals: Glimepiride (amaryl) 1 mg) and acarbose (glucobay 50) as standard, glucose (2%), gum acacia, VSS 23 crude extract (250 mg/kg body weight), glucometer.

Animals: Albino mice were used for the study of glucose tolerance test. These experimental animals were acclimatized to laboratory condition at animal house, Luqman Pharmacy College, Kalaburagi, Karnataka. Animals were maintained at 22°C 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: glimepride, group 3: acarbose, group 4: negative control, group 5: fungal extract (VSR-24).

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

Collection of blood at time intervals: The animals were kept at fasting before 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 (Srinivasulu *et al.,* 2018). The percentage reduction of plasma glucose level was determined using the following formula.

Percentage reduction of plasma glucose level = (a b)/ a × 100

Where, a- indicates initial plasma glucose level and b- indicates plasma glucose level at time 't'. The data were then recorded in a chart to measure and compare the percentage reduction of plasma glucose level between the standard and sample.

RESULTS

Isolation and identification of endophytic fungi

In total, ten fungal endophytes were isolated from *G. sylvestre* during Feb. 2019. Of which, based on frequent recovery, isolate VSS-23 was selected for further studies. The morphological characters of endophyte (carbon black, velvety, reverse cream wrinkled colonies) on PDA medium were displayed (Fig. 1). Microscopic structure revealed smooth walled brown, pigmented, conidiophores, upto 721 x 19.40 µm long, with globose to subglobose vesicles (57.65 x 51.5 µm). Sterigmata were biseriate; primary sterigmata (28.5 x 8.42 µm), spatulate, septate, brown whereas, the secondary sterigmata (9.70 x 25 µm) appeared brown, ampulliform and olivaceous. Sterigmata bore globose to subglobose conidia which were rough-walled, olivaceous brown to dark brown, measured up to 5.0 x 5.0 µm. These results indicate that the fungus VSS 23 belongs to *Aspergillus* group. Based on the molecular studies, the strain was identified as *A. niger* strain HA106-H2 (accession No. MH 935986, Fig 2).

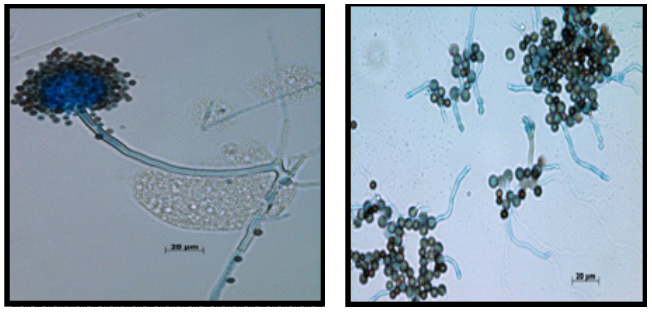

Fig 1: Morphology of *A. niger* strain HA106-H2

Qualitative and quantitative analysis of secondary metabolite

Qualitative analysis of secondary metabolite from different solvent extracts confirms the presence of alkaloids, phenols, flavonoids, saponins and tannins (Table 1), while reducing sugars and glycosides have displayed negative result. The secondary metabolites detected were almost similar in

methanol, petroleum ether and chloroform extracts, except the saponin, which was detected in only chloroform extract. The quantitative estimation of secondary metabolite from *Aspergillus* strain revealed maximum alkaloids (4 mg/g) and flavonoids 4 mg/g followed by phenols (1 mg/g), tannins (0.9 mg/g) and saponins (0.5 mg/g) (Table 2).

Fig. 2: Phylogenetic tree of endophyte VSS-23

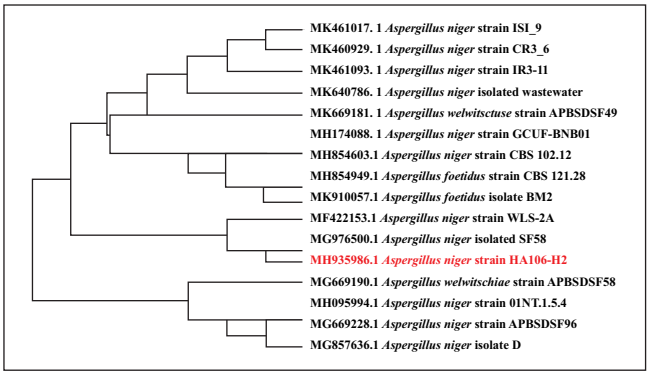

Table 1: Qualitative analysis of secondary metabolites

S. no.	Phytochemical tests	Methanol	Petroleum ether	Chloroform
1.	**Alkaloids**			
a.	Mayer's test	-	-	-
b.	Dragendorff test	+	+	+
c.	Wagner's test	-	-	-
2.	**Phenols**			
a.	$FeCl_3$ test	+	+	+
b.	Ellagic test	-	-	-
3.	**Flavonoids**			
a.	Shinoda test	-	-	-
b.	$FeCl_3$ test	+	+	+
c.	Pew's test	-	-	-
d.	NaOH test	-	-	-
e.	Lead acetate test	-	-	-
4.	**Triterpenoids**			
a.	L-B test	-	-	-
b.	Salkowaski test	-	-	-
5.	**Saponins**			
a.	Foam test	-	-	+
6.	**Sterols**			
a.	L – B test	-	-	-
b.	Salkowaski test	-	-	-

+ Positive - Negative

Table 2: Quantitative estimation of secondary metabolites

Phytochemical constituents	Quantity (mg/g)
Alkaloids	4.0
Phenols	1,0
Flavonoids	4.0
Tannins	0.9
Saponins	0.5

Pharmacological studies for anti-hyperglycemic activity

The mean blood glucose levels of normal untreated (negative control) and treated with standard drug and fungal extracts mice were subjected to glucose tolerance (Table 3). The animals treated with fungal extract exhibited anti-hyperglycemic action after 60 minute (min) of glucose loading. At 60 min, the blood glucose level reached to the maximum of 161.7±15.60** mg/decilitre (dl) in fungal extracts treated animals and significant reduction of 139.0±16.26* mg/dl was recorded till 120 min. The negative control showed continuous increase in blood glucose level of 153.3±5.60 mg/dl at 240 min. The fungal extract showed significant reduction in percent blood glucose level (9.15%) which is better than the standard acarbose (6.25%) (Fig 3).

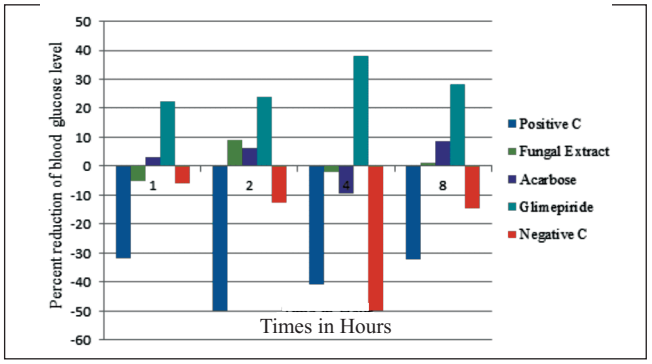

Fig. 3: Percent reduction of plasma glucose level

DISCUSSION

Isolation and identification of endophytic fungi

G. sylvestre is an important anti-diabetic and diuretic plant experimentally/clinically proved to be anti-diabetic (Tiwari *et al.,* 2014) which regenerates and repairs beta cells in Type 2 diabetic patients (Baskaran *et al.,* 1990). In the present research a total ten endophytes were isolated from *G. sylvestre*, of which one endophyte based on its frequent recovery was chosen for further studies and identified as *Aspergillus niger* strain HA106-H2. The phylum *Ascomycota* is reportedly the most common representative of endophytic fungi community (Hamzaah *et al.,* 2018). *A. niger* CSR3 has been reported from *Cannabis sativa* (Lubnaa *et al.,* 2018), A. terreus JS-2 from *Achyranthus aspera* (Goutam *et al.,* 2016), *A. ochraceus* from *Bauhinia forficata* (Bezerra *et al.,* 2015). Dhankhar *et al.* (2013) isolated 17 endophytes from *Salvadora oleoides* Dense for its anti-diabetic potential. The endophytes were also derived from unique environment such

as mangrove ecosystem (Hamzah *et al.,* 2018), wind fallen trees (Koukol *et al.,* 2012), marine algae (Sarasan *et al.,* 2017) and marine Sponge (Chaidir *et al.,* 2010).

Quantitative estimation of secondary metabolite

The quantitative analysis of secondary metabolites from *Aspergillus niger* strain HA106-H2 revealed the presence of alkaloid (4mg/g) and flavonoid (4mg/g) in highest amount followed by phenol (1mg/g), tannin (0.9mg/g) and saponin (0.5mg/g). These metabolites reduce the blood glucose level through their antioxidant property. The mechanism of anti-hyperglycemia might also be due to the oxidative stress caused by flavonoids (Sharma *et al.,* 2008). Alkaloid possesses antioxidant and anti-diabetic activity through antioxidant potential, ROS production, glucose uptake and PTP-1B inhibition (Tiong *et al.,* 2013). Phenolics (Asgar, 2013) and saponins (Duo *et al.,* 2013) play potential role in controlling blood sugar level by inhibiting the activity of α-glycosidase and α-amylase.

Pharmacological studies for anti-hyperglycemic activity

Anti-hyperglycemic activity of fungal extract treated mice in terms of decrease in percent blood glucose level was prominent at 120 min (9.15%) of drug administration which is significantly effective than the standard drug acarbose (6.25%). Acarbose is an alph-glycosidase inhibitor used for treating type II diabetics, can cause critical side effects, such as, liver disorders. This indicates, the fungal extract is able to metabolize glucose efficiently in mice. In support of present study, aqueous extract of endophytic fungi isolated from Jambolana has reported to possess 62% α- amylase inhibition (Khan *et al.,* 2019) Similarly, endophytes from Amla fruit noted with 15-38% α-amylase and sucrose inhibition (Singh *et al.,* 2017). The animals treated with fungal extract showed reduction in mean blood glucose levels after 60 min of drug administration. At 60 min, the blood glucose level reached the maximum (161.7±15.60**mg/dl) in fungal extract treated group and significant reduction was observed till 120 min whereas, the mean blood glucose level in the negative control group has increased continuously till 240 min (153.3± 5.608** mg/dl). Similar results were reported (Dhankhar *et al.,* 2013) by the extract of fungal endophyte isolated from *Salvadora oleoides* Decne with a significant reduction in blood glucose level after 180 min of glucose load. Standard drug glimepiride is most effective in reducing blood glucose level than acarbose. Glimepiride and acarbose treated groups produced hypoglycemic effect due to rapid release of insulin and increases the sensitivity of pancreatic β-cell to glucose (Korytkowski *et al.,* 2002). Many endophytic fungi have already reported as a source of a bioactive compound with alpha-glycosidase inhibition potential. *Xylariaceae* sp. QGS 01, *Alternaria* sp. *Aspergillus* sp., *Penicillium* sp. are reported to possess significant anti-diabetic activity through the alpha glucosidase inhibition compared to standard acarbose (Indrianingsiha *et al.,* 2017; Murugan *et al.,* 2017; Kaur *et al.,* 2018). Solvent used for extraction of crude fungal metabolite is equally important for recovering active compound. In support of present research, ethyl acetate extract of endophyte isolated from five different medicinal plants reported to possess anti-diabetic potential by inhibiting alpha amylase enzyme (Patel and Shah, 2019). Similarly, methanol as extraction solvent is reported to have potential for recovery of active anti-diabetic molecule (Govindappa *et al.,* 2013; Bisht *et al.,* 2016).

In total, the study revealed the use of *Aspergillus niger* strain HA106-H2 isolated from *G. sylvestre* as a source of novel anti-diabetic agent which can prevent severe side effects caused by currently available drug. Further study on anti-diabetic drug purification and identification from *Aspergillus niger* strain HA106-H2 is recommended for future perspective.

Table 3: Glucose Tolerance Test

Groups	Blood glucose level (mg/dl)				
	0hrs	1hrs	2hrs	4hrs	8hrs
Positive Control	82±7.234** P 0.0077	108.0±13.58* P 0.0154	123.0±11.00** P 0.0079	116.3±8.876** P 0.0058	109.3±8.950** P 0.0066
Negative control	102.0±12.34* P 0.0143	108.7±20.10* P 0.0326	115.0±20.01* P 0.0290	153.3±5.608** P 0.0013	117.7±33.38 P 0.0719
Glimepiride	187.0±9.074** P 0.0023	145.0±16.46* P 0.0126	142.7±8.686** P 0.0037	116.3±21.88* P 0.0182	134.3±6.33** P 0.0022
Acarbose	128.3±12.24** P 0.0090	124.7±4.333** P 0.0012	120.7±21.36* P 0.0299	140.7±28.69* P 0.0392	117.7±8.413** P 0.0051
***Aspergillus niger* strain HA106 -H2**	153.3±12.77** P 0.0069	161.7±15.60** P 0.0092	139.0±16.26* P 0.0134	156.3±2.963** P 0.0004	151.3±25.20* P 0.0266

Values are presented as mean ± SD. ***: P < 0.001, **: P < 0.01, *: P < 0.05 compared to control

CONCLUSION

Endophytic *Aspergillus niger* strain HA106-H2 isolated from *G. sylvestre* has been shown to possess different phytochemicals such as alkaloid, flavonoid, tannin and saponin. Significant anti-hyperglycemic activity of fungal crud extract in animal model confirms its efficacy in developing drug against hyperglycemic condition. The fungus can serve as a substitute to currently available drugs and reduce the indiscriminate use of *G. sylvestre*.

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