



Received on 07 May, 2014; received in revised form, 14 July, 2014; accepted, 18 August, 2014; published 01 December, 2014

EVALUATION OF α -AMYLASE, α -GLUCOSIDASE AND ALDOSE REDUCTASE INHIBITORS IN ETHYL ACETATE EXTRACTS OF ENDOPHYTIC FUNGI ISOLATED FROM ANTI-DIABETIC MEDICINAL PLANTS

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Keywords:

Hypoglycaemic,
enzyme inhibitors,
endophytic fungi,
medicinal plants
Secondary metabolites,

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ABSTRACT: Twenty two endophytic fungi were isolated from anti-diabetic plants *Momordica charantia* and *Trigonella foenum-graceum*. Ethyl acetate extracts of all fungal isolates were tested for inhibition of aldose reductase and carbohydrate metabolizing enzymes. Ethyl acetate extracts of nine endophytic fungi were found to be positive for α - amylase and α - glucosidase inhibitors. Crude extracts of fungal isolates PTFL005 and PTFL006 showed promising inhibition activity on α - amylase with an IC₅₀ value of 15.48 and 13.48 μ g/mL respectively. Whereas, the control acarbose had 22.38 μ g/mL of IC₅₀ value for α - amylase at similar experimental condition. Fungal isolates PTFL006 and PTFL011 were found to have potent α -glucosidase inhibitors with an IC₅₀ value of 17.37 and 10.71 μ g/mL which was close to the standard acarbose (6.53 μ g/mL). The only one fungal isolate PMCF003 showed reasonably better inhibition for aldose reductase with an IC₅₀ value of 40 μ g/mL as compared to standard control quercetin (15 μ g/mL). All the active endophytic fungal isolates were identified by DNA sequencing method. The most active isolates identified as *Trichoderma atroviride* (PMCF003) and *Stemphylium globuliferum* (PTFL005, PTFL006 and PTFL011) were reported for the first time as endophytes in these plants from India.

INTRODUCTION: Hyperglycemia defined as a heterogeneous disease caused due to deficiency of insulin secretion and action¹. International Diabetes Federation has reported that 8.3 % (366 million) of adults were infected with diabetes in the year 2011 and this number may rise up to 552 million people by the year 2030². Post prandial hyperglycemia is caused due to action of two enteric enzymes (α -amylase and α -glucosidase) that are attached to the brush border of the intestinal cells.

Therefore inhibition of these two enzymes leads to slow rate of carbohydrate digestion and glucose absorption^{3, 4}. The known glucosidase inhibitors commercially available are acarbose and miglitol that competitively and reversibly inhibits α -glucosidase enzyme from intestine as well as pancreas, but these synthetic drugs possess side effects such as liver disorders, flatulence, abdominal pain, renal tumours, hepatic injury, acute hepatitis, abdominal fullness and diarrhoea⁵⁻⁷.

About 26.1 % of Indian population are found to be suffering from diabetic neuropathy which is an important complication of diabetes mellitus⁸. Aldose reductase is the first and rate-limiting enzyme in the polyol pathway that reduces glucose to sorbitol, inhibition of aldose reductase would

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.5(12).5334-41</p>
<p>Article can be accessed online on: www.ijpsr.com</p>	
<p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(12).5334-41</p>	

delay or substantially prevent these secondary diabetic complications as studied in animal models and also evaluated in clinical trials⁹. High levels of aldose reductase have been detected in the cornea, lens, kidney and myelin sheath¹⁰.

The medicinal plants such as *Momordica charantia* (MC) commonly known as Bitter gourd and *Trigonella foenum-graceum* (TF) commonly known as Mentha has been used in Indian herbal medicine for diabetic control.

Recent studies had shown that endophytic fungi which are living inside the plants have the ability to produce identical or similar bioactive compounds as their host plants^{11, 12}. Endophytic fungi are found to be promising sources of secondary metabolites and they also play an important role in drug discovery¹³. In recent years endophytes are considered as outstanding source of secondary metabolites with pharmaceutically important bioactive compounds. The aim of present study was to isolate endophytic fungi from medicinal plants MC and TF to evaluate the antidiabetic activity of secondary metabolite.

MATERIALS AND METHODS:

Isolation of Endophytic fungi

Fresh samples of *Momordica charantia* (fruit) and *Trigonella foenum-graceum* (leaf and twig) were collected from the nearby market located at Devanahalli, Bangalore, India. Plant materials were collected in a sterile polythene bag and processed within one hour of sample collection to avoid contamination. Samples were thoroughly washed under running tap water to remove debris and cut into 1 cm long.

In case of bittergourd, seeds were removed and pericarp is used for the isolation. Surface of samples sterilized sequentially with 70% ethanol for 1.5 min followed by 1.0 % sodium hypochlorite (NaOCl) (v/v) for 2 min and further cleaned by passing through two sets of sterile distilled water. The sterile samples were placed on potato dextrose agar (PDA) media containing 200mg/L of streptomycin antibiotic in petriplate. The parafilm wrapped petriplates were incubated at room temperature till the fungal mycelia starts growing from the sample. The isolated endophytic fungi was

transferred into a new agar slants and stored at 4°C for the further studies¹⁴.

Fermentation and extraction of secondary metabolites

Two or three discs of fresh mycelium grown from mother culture was inoculated into 500 mL Erlenmeyer flask containing 300 mL YPF (Yeast extract- 3g, Peptone- 10g, Fructose- 20g, Distilled water -1000mL, pH-6) and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. After incubation, ethyl acetate (1:1 ratio) was added to flask containing media and kept on shaker (150 rpm) for overnight. The mycelial mass was separated from the broth by filtration and macerated with ethyl acetate to get intracellular secondary metabolites. The organic phase of extraction mixture was separated and evaporated to dryness before storing at 4°C for further analysis.

α -amylase inhibition Assay

100 μ L of the porcine pancreatic amylase (PPA) solution (1.1 U) was added to 100 μ L of test substance and incubated at room temperature (RT) for 20 minutes. The reaction was initiated by the addition of 100 μ L of 1% soluble starch solution and incubated at 37°C. The reaction was arrested after 10 min of incubation by the addition of 200 μ L of dinitrosalicylic acid colour reagent. The tubes were kept in a boiling water bath for 10 minutes, cooled and diluted with distilled water and absorbance was measured at 470 nm¹⁵. Acarbose was used as the standard alpha-amylase inhibitor and vehicle was used as negative controls. All the assays were carried out in triplicates and average percent inhibition of enzymes by the fungal extract was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

α -Glucosidase inhibition Assay

Alpha glucosidase inhibition activity of ethyl acetate extract of fungal isolates were measured in microplate wells using the method explained by Suneel et al¹⁶. α -glucosidase enzyme solution was prepared in PBS (pH 7.0) and diluted to 0.4 U/mL of working solution. The test solution was prepared by mixing 50 μ L of enzyme with 10 μ L of fungal extract and diluted to 150 μ L with 100mM

phosphate buffer pH 7.0 and mixed well. The reaction mixture was incubated at RT, after 5 min 50 μ L of substrate p-nitro phenyl α -D-glucopyranose (20mM) was added. The reaction mixture was mixed well before incubating for 15 min at RT, later 30 μ L of sodium carbonate solution (200mM) was added to stop the reaction. The optical density of a yellow coloured product measure at 405nm using a microplate reader. Control and test blank OD's were obtained by replacing enzyme with buffer¹⁷.

Preparation of rat lens aldose reductase (ALR2)

Crude aldose reductase was prepared from 2-3 month old WNIN male rat lens by dissecting its eye balls. The dissected lenses were homogenized and diluted 10 times using 100 mM Phosphate buffer pH6.2. the homogenized sample was centrifuged at 15000 x g for 30 min at 4°C and the supernatant was used as enzyme source (ALR2)¹⁸. The protocol was followed as per the animal ethical committee approval in NIN, Hyderabad, India.

Aldose reductase enzyme assay

Aldose reductase enzyme activity in the lens extract was carried out as described by Hayman and Kinoshita¹⁹. The 1 mL of assay mixture contained 50 μ mol of potassium phosphate buffer pH 6.2, 0.2 M lithium sulfate, 5 μ mol β -mercapto ethanol, 10 μ mol DL-glyceraldehyde and enzyme preparation (rat lens). The assay mixture was incubated at 37°C and initiated reaction by the addition of 0.1 μ mol of NADPH. The rate of oxidation of NADPH was measured at 340nm using a spectrophotometer (Lamda 35, Perkin-Elmer, Shelton, U.S.A.). Specific activity of ALR2 was expressed in μ moles of NADPH oxidized/h/100 mg protein.

Aldose reductase inhibition assay

For inhibition studies concentrated stocks of crude extract was prepared in DMSO were used and the final concentration of DMSO was not more than 1%. Various concentrations of extracts were added to assay mixtures of ALR2 and incubated for 5 min before initiating the reaction by NADPH as described above. The percentage inhibition was calculated considering the activity in the absence of extract as 100%. The IC₅₀ values were determined by non-linear regression analysis of the plot of percentage inhibition versus log concentration²⁰.

Identification of Endophytic fungi:

Endophytic fungi were transferred to potato dextrose broth and incubated at 30°C for 7 days. After 7 days of incubation, mycelium was collected by filtration and DNA was extracted as the protocol described by Lu Y et al²¹. Purified DNA was subjected to PCR amplification using primers ITS1 and ITS4. PCR was done in 25 μ l reaction containing 50ng genomic DNA, 10 x PCR buffer (2.5 μ L), 10 mM dNTP (0.5 μ L), 25 mM MgCl₂ (3.5 μ L), 10 μ M each primer (0.5 μ L), 250 unit Taq DNA polymerase (0.2 μ L). PCR was carried out in a thermal cycler (TECHNE) with an initial denaturation of 95° C for 5 min, followed by 35 cycles of 95° C for 30 s, 52° C for 45 s, 72° C for 90 s and a final extension at 72° C for 10 min.

Amplicon DNA was purified using Qiagen columns and submitted to Eurofins, Bangalore, India. Automated DNA sequencing was performed using an ABI 3730xl 96-capillary DNA Analyzer Machine. The sequence data was analyzed using the BLAST software (BLASTN) available at the National Center of Biotechnology information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/>) to determine the identity of the endophyte. Gene sequences were submitted to genebank and accession numbers were obtained.²²

RESULTS:

Isolation of endophytic fungi and extraction of secondary metabolites

A total of 11 endophytic fungi were isolated from bitter gourd fruits and 11 from mentha leaves. All the isolates of mentha are from leaves and none from any of the twigs part. All the endophytic fungi are subcultured in to new agar plates and stored in multiple methods such as agar plates, agar slants and sterile distilled water. Twenty two fungal isolates were grown in large scale separately and extraction of secondary metabolites in ethyl acetate solvent yield about 0.5 to 1.0gm/L of dry powder per fungi.

Alpha-amylase and α -glucosidase inhibition assay:

All the 22 endophytic fungal extracts were tested for PPA inhibitors and only 9 of them have shown significant inhibition for both enzymes. Percentage inhibitions of PPA by different fungal isolates at various concentrations was calculated and plotted

in the **Figure 1** for both MC and TF. Crude extract of nine endophytic fungi isolate PTFL005 and PTFL006 have IC₅₀ values of 15.48 and 13.48 μg/mL respectively. Both highl active isolates are isolated from the leaf part of the menthya plant. The IC₅₀ value of these two fungal extract was less than IC₅₀ value of standard drug acarbose (22.38 μg/mL) when assayed under similar condition. The IC₅₀ values for all the fungal extract was calculated along with the standard anti-diabetic drug acarbose as control and shown in the **Figure 2**.

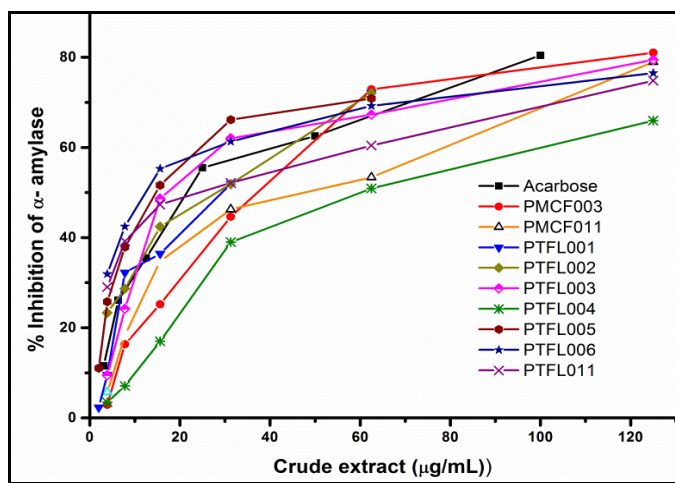


FIGURE 1: PERCENT INHIBITION OF α-AMYLASE WITH DIFFERENT CONCENTRATION OF FUNGAL EXTRACT.

Fungal isolates PMCF003, PMCF011 and PTLF001 to PTLF011 compared with standard Acarbose

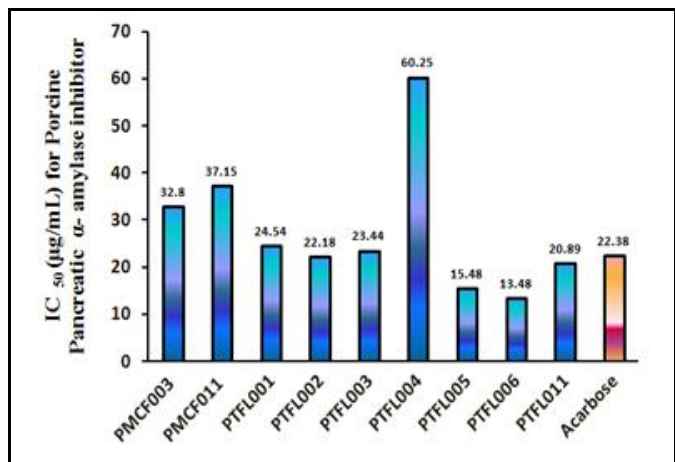


FIGURE 2: IC₅₀ VALUES OF FUNGAL EXTRACT AGAINST α AMYLASE ENZYME.

Fungal isolates PMCF003, PMCF011 and PTFL001 to PTLF011 compared with standard Acarbose.

The same set of 22 endophytic fungal extracts tested for α-amylase inhibitors were also tested for α-glucosidase inhibitors. Ethyl acetate extracts of

nine isolates had shown very good inhibition activity for α-glucosidase enzyme. The percentage inhibition of glucosidase enzyme by the different concentrations of crude extract was measured and plotted against the concentration as shown in **Figure 3**.

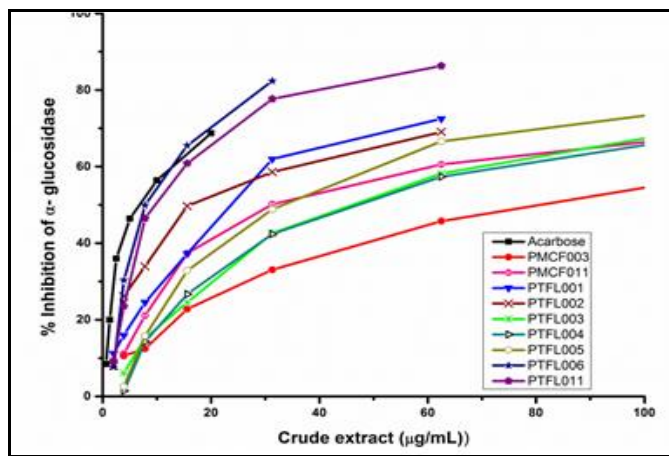


FIGURE 3: PERCENT INHIBITION OF α -GLUCOSIDASE WITH DIFFERENT CONCENTRATION OF FUNGAL EXTRACT.

Fungal isolates PMCF003, PMCF011 and PTLF001 to PTLF011 compared with standard Acarbose

Two isolates PTFL006 and PTFL011 had IC₅₀ values of 17.37 and 10.71 μg/mL respectively when compared to acarbose IC₅₀ value (6.53 μg/mL) under similar experimental condition. These two most active isolates were also from leaves of the menthya plant. The IC₅₀ values calculated for all the active fungal extracts along with the standard acarbose and plotted against concentration of crude extracts as shown in the **Figure 4**.

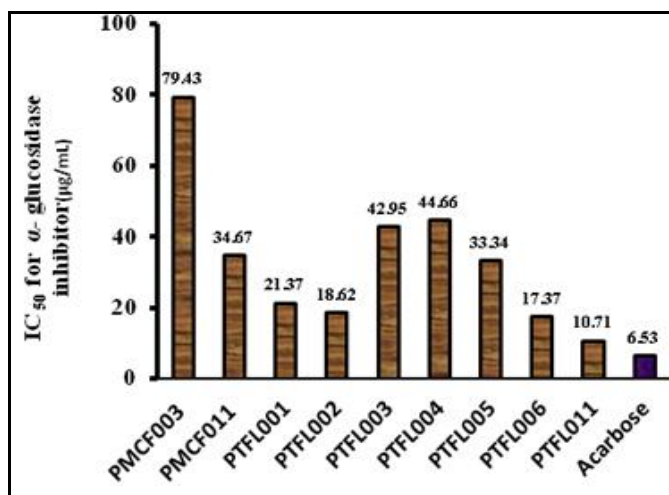


FIGURE 4: IC₅₀ VALUES OF FUNGAL EXTRACT AGAINST α-GLUCOSIDASE ENZYME.

Fungal isolates PMCF003, PMCF011 and PTFL001 to PTLF011 compared with standard Acarbose

Aldose reductase inhibition assay

Out of 22 endophytic fungi isolated, ethyl acetate extracts of ten endophytic fungi (PMCF001, PMCF002, PMCF003, PMCF006, PMCF008, PMCF011, PTFLO05, PTFLO06 and PTFLO11) were tested for aldose reductase inhibitors (ALR2). Only three extracts (PMCF001, PMCF003 and PTFLO06) were found to be positive for aldose reductase inhibitors. Percentage inhibition of rat lens aldose reductase enzyme by fungal extract at different concentrations estimated and plotted against concentration along with the quercetin as standard (Figure 5).

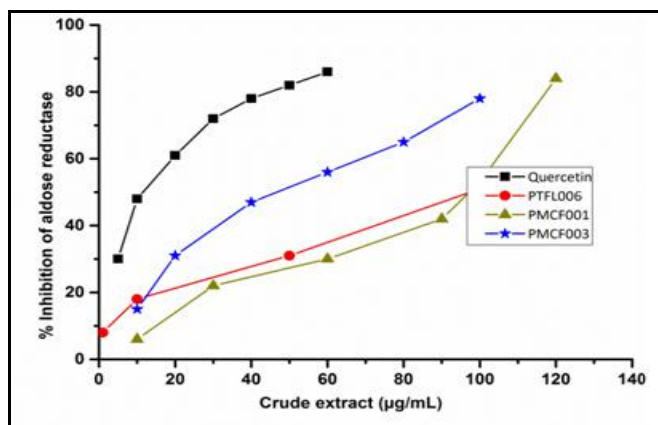


FIGURE 5: PERCENT INHIBITION OF ALDOSE REDUCTASE ENZYME WITH DIFFERENT CONCENTRATION OF FUNGAL EXTRACT.

Fungal isolates PMCF001, PMCF003 and PTFLO06 compared with standard Quercetin

The IC₅₀ values for these endophytic fungi against ALR2 was determined and represented in the Figure 6. PTFLO06, PMCF001 and PMCF003 showed aldose reductase inhibition with an IC₅₀ value of 100, 78 and 40 µg/ml respectively. PTFLO05, PTFLO06 and PTFLO11 have shown

percentage inhibition against aldose reductase at the concentration range 0-100 µg/mL with 46, 51 and 35 % respectively.

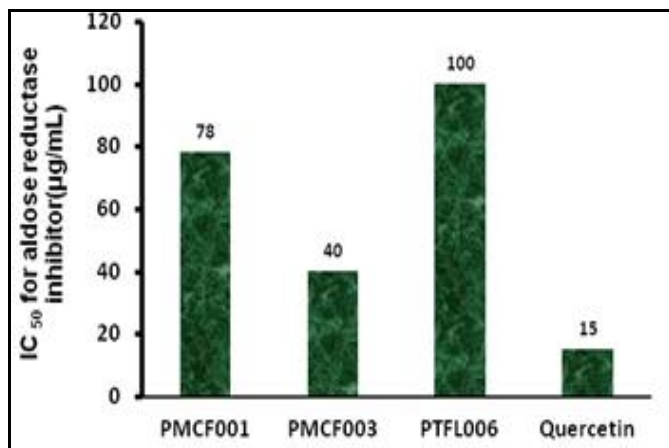


FIGURE 6: IC₅₀ VALUES OF FUNGAL EXTRACT AGAINST ALDOSE REDUCTASE ENZYME.

Fungal isolates PMCF001, PMCF003 and PTFLO06 compared with standard Quercetin

Molecular identification of endophytic fungi

The endophytic fungal isolates which showed inhibition for at least one of the three enzymes α-amylase, α- glucosidase or aldose reductase inhibitors were identified by rDNA molecular method. Conserved DNA region for all these fungal isolates was purified, amplified and sequenced for their identification. The sequence were submitted to the Gene Bank and obtained Gene Bank accession number. Based on the sequence similarity closest matching in the blast search was selected and identified the fungal isolates. The identified species, gene bank accession number and their percent nucleotide homology with the existing data base are represented in the Table 1.

TABLE 1: LIST OF ENDOPHYTIC ISOLATES IDENTIFIED BY rDNA METHOD

Sl No.	Endophytic fungi code	Identified species	Gene bank accession number	Molecular Identity (closest match in GenBank)	Accession number (closest match in GenBank)	Nucleotide homology (%)
1	PMCF001	<i>Trichoderma atroviride</i>	KC702783	<i>Trichoderma atroviride</i>	AB633204.1	100
2	PMCF003	<i>Trichoderma atroviride</i>	KC702784	<i>Trichoderma atroviride strain TUCIM N154</i>	JN387049.1	99
3	PMCF011	<i>Trichoderma atroviride</i>	KF911101	<i>Trichoderma atroviride T-26</i>	KC884783.1	100
4	PTFL001	<i>Stemphylium lycopersici</i>	KF911102	<i>Stemphylium lycopersici</i>	AB704312.1	99
5	PTFL002	<i>Stemphylium globuliferum</i>	KF911103	<i>Stemphylium globuliferum</i>	KF479193.1	100
6	PTFL003	<i>Alternaria sp PEGT001</i>	KF911104	<i>Alternaria sp PEGT001</i>	KC707558.1	100
7	PTFL004	<i>Stemphylium lycopersici</i>	KF911105	<i>Stemphylium lycopersici</i>	AB704312.1	97
8	PTFL005	<i>Stemphylium globuliferum</i>	KF911106	<i>Stemphylium globuliferum</i>	KF479193.1	100
9	PTFL006	<i>Stemphylium globuliferum</i>	KF911107	<i>Stemphylium globuliferum</i>	KF479193.1	100
10	PTFL011	<i>Stemphylium globuliferum</i>	KF911108	<i>Stemphylium globuliferum</i>	KF479193.1	100

All the three endophytic isolates PMCF001, PMCF003 and PMCF011 from *M. charantia* were identified as *Trichoderma atroviride* with 99-100% sequence homology. The fungal isolates PTFL002, PTFL005, PTFL006 and PTFL011 from Menthya plant are identified as *Stemphylium globuliferum* having sequence similarity of 99-100% with the existing genebank data. The isolate PTFL003 which was identified as *Alternaria sp.* showed 100% sequence homology. PTFL001 and PTFL004 identified as *Stemphylium lycopersici* with 99% and 97% sequence homology respectively.

DISCUSSION: Several studies have reported the use of herbal extracts as antidiabetic, however these extracts have not proved to be successful as medicines due to lack of specific standards being prescribed for herbal medicines and supportive animal/clinical trials²³. Aqueous methanol extracts of *M.charantia* seeds have shown an α -glucosidase inhibitory activity²⁴. There was a significant decrease ($p<0.01$) in the fasting blood glucose levels in streptozotocin (STZ) induced diabetic rats treated with the MC extract, as compared to the untreated diabetic rats.

M. charantia extract known to exhibit anti-hyperglycemic effect in the streptozotocin or alloxan-induced diabetic rats^{25 26}. The compound 4-hydroxyleucine present in the seeds of TF have proven to stimulate insulin production²⁷. It has been reported that there was a significant reduction in blood glucose level in alloxan-diabetic rats treated with aqueous extract of fenugreek leaf as compared to healthy rats²⁸.

In the present study we have isolated endophytic fungi from these two plants and evaluated their antidiabetic activity. Endophytic fungi have the capability to produce bioactive compounds such as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones²⁹. In a study six endophytic fungi were isolated from *Swietenia macrophylla* King seeds and five ethyl acetate extracts had showed better glucosidase inhibition activity than acarbose with lowest IC₅₀ values was 73.64 $\mu\text{g/mL}$. These α -glucosidase inhibitor was found to be belongs to flavonoid group³⁰. Structure activity relationship studies have proved that ganoderma acids isolated from *Ganoderma lingzhi* as a potent α -glucosidase inhibitor³¹. In the

present study crude ethyl acetate extract of two isolates belonging to *Stemphylium globuliferum* species isolated from Menthya leaf showed highest PPA inhibitory activity with an IC₅₀ values of 15.48 and 13.48 $\mu\text{g/mL}$. These two crude extracts IC₅₀ values are far below the IC₅₀ value of standard drug acarbose (22.38 $\mu\text{g/mL}$) under similar experimental condition, which indicates these isolates has higher potential for production of pharmaceutically important drug if purified further. There are three isolates belonging to the *Stemphylium globuliferum* species which also showed very good α -glucosidase inhibition activity. The IC₅₀ value for these are ranging from 10-18 $\mu\text{g/mL}$ which is very near to the standard acarbose (6.53 $\mu\text{g/mL}$) tested under similar *in-vitro* condition.

Endophytic fungus B.Os.1F isolated from Kumis kucing (*Orthosiphon spicatus* BBS) showed highest inhibition percentage with 93.91% from filtrate extract and 89.01% from biomass extract. Endophytic fungi isolated from Mengkudu (*Morinda citrifolia* L.), Sirih Merah (*Piper crocatum* L.), Sirih Hitam (*Piper ornatum* L.) have shown alpha glucosidase inhibition activity³². Aqueous extracts of bitter gourd and fenugreek have shown aldose reductase inhibitory activities with IC₅₀ values $<0.5 \text{ mg/mL}$ ³³.

As per the proposal of this study, the fungi isolated from Bitter gourd have shown the best aldose reductase inhibition activity at crude extract level. The best aldose reductase inhibition was shown by the endophytic fungi *Trichoderma atroviride* isolated from Bittergourd.

To our present knowledge there are no reports on *Stemphylium* sp having α -amylase and α -glucosidase inhibitors. This is the first study on *Stemphylium* sp isolated from menthya and proved to have a potent antidiabetic agent. *Trichoderma* sp isolated from bittergourd found to be the potent aldose reductase inhibitors with an IC₅₀ value of 40 $\mu\text{g/mL}$. The endophytic microorganisms are excellent sources of bioactive natural products having great demands in health care sector³⁴. Therefore exploration of these endophytic fungi having the capability to produce antidiabetic compounds will lead to revolution in biotechnology industry.

CONCLUSIONS: Present study concludes that *Stemphylium globuliferum* is one of the best endophytic fungi having both α -amylase inhibition and α -glucosidase inhibition activity and this is the first report from India. The crude extract having the α -amylase inhibition activity more than the standard available drug acarbose is of very interesting and promising result. The α -glucosidase inhibition activity of the same fungal species were also very near to the standard acarbose value indicates that there is a better hope in optimization of secondary metabolite production and purification of compounds for the pharmaceutical applications.

ACKNOWLEDGEMENTS: Authors PN, SL and AK would like to thank Admar Mutt Education Foundation for the financial support in conducting this research. SL and PN are thankful to Manipal University for their PhD registration.

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How to cite this article:

Pavithra N, Sathish L., Babu N, Venkatarathanamma V, Pushpalatha H, Reddy GB and Ananda K: Evaluation of α -Amylase, α -Glucosidase And Aldose Reductase Inhibitors in Ethyl Acetate Extracts of Endophytic Fungi Isolated from Anti-Diabetic Medicinal Plants . *Int J Pharm Sci Res* 2014; 5(12): 5334-41.doi: 10.13040/IJPSR.0975-8232.5 (12).5334-41.

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