ABSTRACT

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by high blood sugar caused due to lack of insulin action or secretion from pancreatic β-cells. Diabetes can be controlled by medicines, balanced diet and changes in lifestyle. Diabetes is increasing at an alarming rate and India has been declared as the “Diabetes capital of the world”. Therefore there is a huge demand for the development of antidiabetic drugs. Several synthetic drugs and insulin are available for the treatment of diabetes but still it has been a failure to attain normal sugar levels without adverse side effects. Therefore there is a need for phytotherapy and alternative medicine. In this context enzymes play a major role in biochemical process such as cell signalling, transduction and metabolic functions. Enzymes such as aldose reductase (AR) and carbohydrate metabolizing enzymes (α-amylase and α-glucosidase) are associated with type II DM. Elevation of post prandial hyperglycemia is caused due to action of glucosidases which are involved in breakdown of complex sugars into simple sugars. Therefore inhibition of alpha amylase and alpha glucosidase helps in diabetes management. Conventionally available glucosidase inhibitors such as acarbose and miglitol competitively and reversibly inhibits α-glucosidase enzyme from intestine as well as pancreas. But these drugs possess gastrointestinal side effects such as abdominal pain, flatulence and diarrhoea in the patients. Therefore there is a need for natural sources which are less expensive and have lesser side effects.

Most of the drugs have been targeted towards the control of blood sugar levels, but secondary complications of diabetes is also an another major threat to the health sector. Long term exposure to hyperglycemia may lead to secondary complications of diabetes that includes nephropathy, neuropathy and retinopathy. It has been found that
in case of diabetic patients more than 30% of glucose is converted to sorbitol which leads to sorbitol accumulation that causes damage to nerve cells, retinal cells and kidney. Therefore inhibition of aldose reductase will play an important role in control of secondary complications of diabetes. Currently available aldose reductase inhibitors such as (zopolrestat, ponalrestat, and tolrestat) has low tissue permeability and sorbinil has better tissue permeability but possess side effects like skin allergy and liver damage. Thus there is a need for development of aldose reductase inhibitors which are highly effective and safe.

Many of the plant extracts have been used in the treatment of diabetes. Medicinal plants are considered as the primary source of therapeutically important secondary metabolites. Medicinal plants are used for various disease treatment because of their safety, low cost, more effective and availability. About 200 compounds have been purified from plants that are found to be hypoglycaemic. If the medicinal plants are utilized continuously for the natural treatment of diabetes there will be a threat for these herbs due to over exploitation. Therefore endophytic fungi are one of the alternative natural resources for new antidiabetic compounds. Endophytic fungi are defined as microbes that inhabit healthy plant tissues in their life cycle without causing apparent harm to their host. Endophytes can be used as a source of machinery for the production of novel drugs as they are fast growing, easy recovery of products and culture condition can be optimized inside the lab. Endophytes are also known to produce secondary metabolites similar to that of their host plant secondary metabolites. In the present study we have chosen three important medicinal plants namely Ocimum sanctum L. (Tulsi), Momordica charantia L. (Bitter gourd) and Trigonella foenum-graceum L. (Methi). The focus of this study was to isolate endophytic fungi from these medicinal plants and screen for the alpha amylase and
aldose reductase inhibitors. If any of the endophytic fungi isolated from these plants have the capability to produce these inhibitors then medicinally important plant species can be saved from becoming endangered. As these endophytic fungi are easy to grow and it requires limited space, exploration of endophytic fungi for the production of novel drugs would be beneficial.

2. OBJECTIVES

1. Isolation of endophytic fungi from Ocimum sanctum (Tulsi), Momordica charantia (Bitter gourd) and Trigonella foenum-graceum (Methi).

2. Screening of isolated endophytic fungi for the production of alpha amylase inhibitors and aldose reductase inhibitors.

3. Identification of endophytic fungi producing inhibitors of our interest.

4. Purification, identification and characterization of alpha amylase inhibitors and aldose reductase inhibitors

3. METHODOLOGY

Isolation of endophytic fungi from Ocimum sanctum L. (Tulsi), Momordica charantia L. (Bitter gourd) and Trigonella foenum-graceum L. (Methi) was carried out by surface sterilizing the plant materials with 70% ethanol, 1% sodium hypochlorite followed by washing with two sets of sterile distilled water. The plant material was then placed on potato dextrose agar media (PDA) supplemented with streptomycin to avoid bacterial contamination. After 3-5 days of incubation at room temperature, mycelium grown from plant material was subcultured onto new PDA plate without antibiotic. Mycelial discs were then inoculated into YPF (Yeast extract, peptone and fructose) media for the extraction of secondary metabolites. After 21 days of
incubation, secondary metabolites were extracted using ethyl acetate as a solvent system. Ethyl acetate was evaporated and residue obtained as dry powder was stored at 4°C for future studies. Fungal ethyl acetate extract was tested for alpha amylase, alpha glucosidase and aldose reductase inhibition activity. Alpha-amylase inhibition assay was performed using the dinitrosalicylic acid (DNS) method and acarbose was used as standard drug. Alpha glucosidase inhibition assay was carried out using substrate p-nitro phenyl α –D-glucopyranose and acarbose was used as standard. Aldose reductase inhibition assay was carried out by measuring the oxidation of NADPH spectrophotometrically. In-vitro glucose uptake study was carried out using commercial baker’s yeast spectrophotometrically by DNS method.

Morphological characteristics of fungal colony grown on PDA at 25±2°C in dark were studied for the bioactive endophytic fungal isolates.

The endophytic fungal isolates which have shown inhibitor activity for at least one of three enzymes α-amylase, α-glucosidase or aldose reductase inhibitors were identified by rDNA molecular method. Molecular identification of fungi was carried out by isolating DNA, amplifying ITS region and sequencing method. BLASTn search was performed for the DNA sequences to find the closest match in the GenBank database.

Activated silica gel (200-400 mesh size) was packed onto a glass column using ethyl acetate solvent and it was equilibrated with respective solvents. Fungal ethyl acetate extract was loaded on top of the silica gel and eluted stepwise: chloroform (100%), ethyl acetate (100%), methanol (100%) and water. About 20 fractions measuring 2 mL each were collected and each fraction was tested separately for activity.

The functional groups present in column purified fraction were identified by FT-IR spectrophotometer (Bruker- alpha).
4000 cm\(^{-1}\) with a scan rate of 32. The UV visible spectra of column purified fraction was analysed at 200-700 nm range to find out absorption peaks of active compounds. Biochemical analysis of the purified compounds was made as similar to phytochemical screening methods.

The Agilent 6540 Ultra High Definition (UHD) Accurate Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system was used for characterization of secondary metabolites of *A. carthami*. Agilent 6540 UHD Q-TOF coupled to Agilent 1290 Infinity LC system consisting of (autosampler with DAD system) equipped with reverse phase eclipse plus C18 column (3mm X 100 mm) was used for analysis. The mobile phase used for HPLC analysis was 0.1 % formic acid in methanol (positive mode) and 100 % methanol (negative mode) at a flow rate of 0.5 mL/min. Five microlitre of sample was injected. The data was recorded and processed using Agilent Masshunter Acquisition and Masshunter Qualitative analysis software. The LC-MS (Q-TOF) results suggested the possibility of existence of multiple compounds in the column purified extract. Based on the abundance of the compound score, based on their mass-comparison with known natural compounds and based on the related literature, we have selected dihydronorwoginin, alternariol and tenuazonic acid as potential inhibitors of either porcine pancreatic alpha amylase (PPA) or human aldose reductase (AR) present in the column purified fraction. The hypothesize is that, out of these three selected molecules the one that is inhibiting above enzymes should have the highest binding affinity. Considering this we performed molecular docking simulations for all three molecules with AR and PPA independently. Molecular docking was performed using the software AutoDock tools and Autodock Vina
v.1.1.2. The coordinates for porcine pancreatic alpha amylase (PDB code: 1OSE) and human aldose reductase (PDB code: 2R24) were obtained from protein data bank.

GC system (Thermo GC Ultra) connected with DSQ II (Thermo) was used for analysis of column purified extract of T. atroviride. Thermo TR-wax column (30m X0.25mm ID with 0.25 µm film thickness) was used. Nitrogen was used as a carrier gas. The MS was operated in electron ionization mode (70 eV) with a scan range of m/z 50–600. Identification of compound was carried out using NIST library.

4. RESULTS AND DISCUSSION

A total of 69 endophytic fungal isolates from 140 pieces of twigs and 15 fungal isolates from 140 pieces of leaves were isolated using 14 Ocimum sanctum L. (Tulsi) plants. These isolates were screened for the morphological characteristics and those having similar characteristics were grouped. Out of 84 endophytic fungal isolates only 22 fungal isolates having different culture characteristics were screened for enzyme inhibitors. A total of 11 endophytic fungi were isolated from bittergourd and 11 endophytic fungal isolates from menthya leaves. The endophytic fungal isolates were transferred into a new agar plate and subcultured every 15 days. These endophytic fungal cultures were maintained by sub-culturing into new agar plates regularly. The endophytic fungal isolates were also stored at 4°C in agar slants and sterile water.

Out of 22 fungal isolates from Tulsi, ethyl acetate extract of seven endophytic fungal isolates inhibited both porcine pancreatic α-amylase and α-glucosidase enzymes. Three out of 11 endophytic fungi from bittergourd were shown positive for both PPA and glucosidase inhibitors. Similarly ethyl acetate extract of 11 endophytic fungal isolates from menthya were tested for PPA and glucosidase inhibitors and seven endophytic fungi was found to be positive.
Aldose reductase enzyme inhibition was shown by five endophytic fungi (POST034, POST047, POST053, POST060 and POST067) isolated from Tulsi, three endophytic fungi (PMCF001, PMCF003 and PMCF004) isolated from bittergourd were found to be positive for aldose reductase inhibitors. None of the endophytic fungi isolated from Menthya showed significant aldose reductase inhibition.

Ethyl acetate extract of eight endophytic fungal isolates from Tulsi, four from Bittergourd and seven from Menthya inhibiting one or more enzymes were identified. The DNA sequence was submitted to the GenBank and obtained GenBank accession number for each of the fungal sequence. Based on the sequence similarity fungal isolates of Tulsi plant with code POST034 was identified as *Alternaria tenuissima*, POST047 identified as *Trichoderma sp*, three isolates POST053, POST060 and POST067 were identified as *Alternaria alternata*, *A. carthami* and *A. porri* respectively, isolate POST075 identified as *Diaporthe sp* and POSL083 identified as *Colletotrichum gloesporioides*.

All the four endophytic isolates PMCF001, PMCF003, PMCF004 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 were identified as *Stemphylium globuliferum* having sequence similarity of 99-100% with the existing GenBank database. The isolate PTFL003 was identified as *Alternaria sp.* with 100% sequence homology. Both fungal isolates PTFL001 and PTFL004 identified as *Stemphylium lycoperici*.

Present study showed that *Stemphylium globuliferum* isolated from menthya 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 fungal ethyl acetate extract (crude) having the α-amylase inhibition activity more than the present
available drug acarbose is of very interesting and promising result. The $\alpha$-glucosidase inhibition activity of the same fungal species is also very near to the standard acarbose value indicates the better hope in optimization of secondary metabolite production and purification of compounds for the pharmaceutical applications. This is the first study on *Stemphylium sp* isolated from menthya and proved to have a potent antidiabetic agent.

The crude ethyl acetate extract of endophytic fungi *Trichoderma* sp have shown very good aldose reductase enzyme inhibition activity with very less IC$_{50}$ value against a purified standard quercetin. It can be stated that the antidiabetic activity of crude ethyl acetate extract of endophytic fungi (*Alternaria tenuissima, Alternaria carthami, Colletotrichum gloesporioides, Diaporthe* sp. and *Trichoderma* sp.) isolated from *Ocimum sanctum* proved to be the most promising due its low IC$_{50}$ value which is very close to acarbose and quercetin standard. The endophytic fungi isolated from bitter gourd have shown the best aldose reductase inhibition activity at crude extract level. The best aldose reductase inhibition is shown by the endophytic fungi *Trichoderma atroviride* isolated from Bitter gourd.

*Alternaria carthami* isolated from *O.sanctum* and *T.atroviride* from *M.charantia* was chosen for purification, characterization of active metabolites and further studies due to its highest activity for alpha amylase and aldose reductase. Secondary metabolites characterised in column purified extract of *A.carthami* as dihydronorwogonin, cerebroside C, alternariol, tenuazonic acid and stearamide possesing antidiabetic and anticancer activity. From the literature review, it has been found that secondary metabolites isolated from *Alternaria sp* has been studied extensively for anticancer activity and very few studies have been carried out on antidiabetic activity. Crude ethyl acetate extracts of *A.carthami* showed effective inhibition of rat lens aldose
reductase (RLAR) activity with an IC$_{50}$ value of 15 µg/mL which proved to efficient when compared to commercially available purified inhibitors. Crude ethyl acetate extract and column purified extract of *A. carthami* used at a concentration range of 50-500 µg/mL showed highest glucose uptake at 500 µg/mL with 87.70 and 55.92 % respectively. Hence study of antidiabetic activity of *A. carthami* metabolite may lead to exploration of this fungal source as an alternative for drug development from natural source. *Alternaria carthami* proving to be the most effective anticancer agent against C6 and MCF-7. Alternariol isolated from *A. carthami* may be responsible for anticancer activity of this extract. Alternariol has been widely studied for its cytotoxic activity in previous studies. Based on abundance and database score, compounds with relative peaks were chosen. In this context, major compound eluted with peak at retention time (RT) 1.645 min in LC plot was dihydronorwogonin which belongs to the class of flavonoids. The second highest compound was cerebrosides C. Other lipid constituents were also observed. Alternariol and Tenuazonic acid which is predominanintly produced by *Alternaria sp* as shown by previous studies is also present in column purified extract studied. Autodock analysis results have shown that for aldose reductase, dihydronorwogonin showed the highest binding affinity with best fit conformer having a calculated affinity of -10.4 kcal/mol among three molecules followed by alternariol (-8.0 kcal/mol) and tenuazonic acid (-6.8 kcal/mol). This difference in affinity suggests that dihydronorwogonin is the possible inhibitor of AR. We also attempted to dock the known AR inhibitor quercitin. The binding affinity for quercetin calculated using the same program is found to be -10.3 kcal/mol suggests that the binding affinity of dihydronorwogonin is comparable to that of quercitin. Dihydronorwogonin is also showing strong binding energy of -8.4 kcal/mol for porcine pancreatic $\alpha$- amylase which is comparable to the standard drug acarbose.
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(-10.3 kcal/mol). Similarly, binding affinity for alternariol (-8.2 kcal/mol) is near to that of dihydronorwogonin suggests that the possibility of both molecules acting as inhibitors of PPA. Tenuazonic acid showed comparatively less binding affinity of -6.4 kcal/mol for PPA. Based on all the experimental support dihydronorwogonin is the most probable molecule present in the extract which is inhibiting the AR.

From the literature it is evident that *Trichoderma atroviride* produces a wide range of bioactive secondary metabolites. In the present study we have found that column purified fractions of crude ethyl acetate extracts of *T. atroviride* inhibits α-amylase and aldose reductase with very less IC₅₀ value than standard drug acarbose and quercetin respectively. There are no studies available on production of aldose reductase or α-amylase inhibitors from *T.atroviride*. Crude ethyl acetate extract of *T.atroviride* was used at a concentration range of 50-300 µg/mL and it showed highest glucose uptake at 300 µg/mL. Therefore proving to be a strong antidiabetic agent in near future. There are no studies on antidiabetic activity of Pyrrolo (1, 2-a) pyrazine 1, 4-dione, hexahydro and *Trichoderma atroviride*. Therefore these findings have explored wide advantages of *Trichoderma atroviride* and its secondary metabolite Pyrrolo (1, 2-a) pyrazine 1, 4-dione, hexahydro as antidiabetic and anticancer agent. In the present study we have identified bioactive compounds which are reported for the first time as an antidiabetic agent. This study has also reported for the first time that *A.carthami* and *T.atroviride* may be used as the potential natural sources for development of antidiabetic compounds.

5. **Summary and conclusion**

The present study focussed on isolation of endophytic fungi from medicinal plants such as Tulsi, bittergourd and menthya, isolated endophytic fungi was screened for
alpha amylase, alpha glucosidase and aldose reductase inhibitors and the active compound responsible for enzyme inhibition and anticancer activity was identified. A total of 84 endophytic fungi from Tulsi, 11 from bittergourd and 11 from Menthy were isolated. Seven endophytic fungi from Tulsi, three from bittergourd and seven from Menthy were found to be active against porcine pancreatic alpha amylase and alpha glucosidase. Five endophytic fungi from Tulsi, three from bittergourd showed aldose reductase inhibition. Only three endophytic fungi isolated from Menthy showed moderate aldose reductase inhibition. Two important fungi namely *Trichoderma atroviride* and *A.carthami* were extensively studied for the compounds responsible for enzyme inhibition activity. In the present study dihydronorwogonin from *A.carthami* (Tulsi) and Pyrrolo 1, 2 a pyrazine hexahydro from *T.atroviride* (Bittergourd) are found to be the most probable alpha amylase and aldose reductase inhibitors. Both active compounds have anticancer activity. Presence of dihydronorwogonin has been reported for the first time in endophytic fungi. Dihydronorwogonin and Pyrrolo 1, 2 a pyrazine hexahydro reported for the first time as an alpha amylase and aldose reductase inhibitors.