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Pharmacological evaluation of endophytic *Penicillium pimiteouiense* SGS isolated from *Simarouba glauca* DC

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ABSTRACT

Medicinal plants are recently being recognized as resources of endophytes with interesting bioactive compounds. In the present study, pharmacological properties of the endophytic fungus *Penicillium pimiteouiense* SGS isolated from the medicinal plant *Simarouba glauca* DC were evaluated. Endophytes were isolated using surface sterilization procedure. The crude extract of cultured fungus was prepared in ethyl acetate and was evaluated for antimicrobial, antioxidant and anti hyperglycaemic activities. Phytochemical composition of the crude extract was also studied by standard qualitative assays. The extract of *P. pimiteouiense* SGS was found to have antimicrobial and antioxidant properties. Anti hyper glycaemic activity was also revealed by its inhibitory activities on alpha amylase and alpha glucosidase enzymes. Qualitative phyto chemical analysis of the crude extract showed presence of flavanoids, triterpenes, alkaloids and carbohydrates. The medicinal plant *S. glauca* needs to be explored further as a resource of rare endophytes with bio active compounds.

INTRODUCTION

The utilization of medicinal plants in the therapy of human disease is an age old practice. Phytochemical research has made much progress in the past few decades and has helped identifying the active principle involved in the therapeutic properties of medicinal plants. Almost all medicinal plants maintain endophytes; "microbes that colonize living, internal tissues of plants without causing any immediate over negative effects" (Bacon and White, 2000). Secondary metabolites produced by these endophytes have also received importance in recent years as they possess a wide variety of biological activities as that of plant metabolites. Endophytes can produce the same or similar secondary metabolites as their host plant (Alvin *et al*., 2014). Out of several thousands of medicinal plants, only a small percentage has been explored so far for endophytes. Thus investigation of endophytes in scarcely researched medicinal plants can open up an avenue for new leads in drug discovery.

Simarouba glauca (dysentery bark or paradise tree) has been in use to treat dysentery in many countries since long. The bark and leaf extract of *Simarouba* is well known for its different types of pharmacological properties like anticancerous, analgesic, antipyretic, antimicrobial, antioxidant, anti haemorrhagic etc (Manasi and Gaikwad,2011; Umesh,2015; Lakshmi *et al*., 2014). Quassinoids, which belong to the triterpene chemical family and alkaloids have been reported as active ingredients responsible for the properties of *S. glauca* (Polonsky *et al*., 1978; Rivero-Cruz *et al*., 2005). In the present study endophytic fungi isolated from *S. glauca* were evaluated for their bioactivities.

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MATERIALS AND METHODS

Isolation of endophytic fungi

Healthy leaves and stem of medicinal plant *S. glauca* were collected from Kottayam, Kerala and were brought to the laboratory within 2-3 hrs of collection. The collected plant materials were rinsed under running tap water and air-dried. Surface sterilization of the plant material was done by immersing them sequentially in 70% ethanol solution for 2 minutes and in 2 % sodium hypochlorite for 2 min. Thereafter the plant parts were rinsed thoroughly with sterile distilled water and air dried on sterile filter paper. The leaves and stem were cut under sterile conditions into 1 cm long fragments, thereby exposing the internal tissues. These fragments were placed onto petri dishes containing Potato Dextrose Agar (PDA). A control plate was maintained by making an impression of the surface sterilized plant part to verify the growth of epiphytes. The plates were incubated at 28 degree Celsius for 5 days. The same process was repeated with many samples to confirm the frequency of occurrence of the endophytes from different plant parts.

The hyphal tips of the fungi growing out from the cut edge of the fragments were transferred to a fresh PDA plate. One of the fungi which emerged recurrently from the stem fragments was selected for pure culture preparation and was given the code SGS. The pure cultures were then kept in 4 degree Celsius until further use. They were sub cultured at two weeks interval.

Mass culture of SGS and crude extract preparation

Mycelial blocks of 5 day old SGS was inoculated into 100 ml of potato dextrose broth and kept in a rotary shaker at 28 degree Celsius for 8-10 days. After filtering out the mycelia, the supernatant was extracted thrice with equal volumes of ethyl acetate. The ethyl acetate fraction was evaporated to dryness using rotary evaporator. The crude residue was weighed, dissolved in DMSO and was stored at 4 degree Celsius. This crude extract of known concentration was used as a stock for checking different pharmacological properties of the isolated endophyte SGS.

Antimicrobial activity

The antimicrobial activity of the fungal extract was evaluated on bacterial pathogens *Staphylococcus aureus,* and *Escherichia coli* . Well diffusion assay was carried out with different concentrations of SGS extract in Muller Hinton agar plates as per the protocol of Valgas *et al.* (2007)**.** DMSO and standard antibiotic Amikacin were used as negative and positive control respectively.

Antioxidant activity

The antioxidant activity of the fungal extract was evaluated by DPPH assay (Pandurangan *et al*., 2011). Ascorbic acid was used as a positive control. Percentage inhibition of DPPH radical by the extract was calculated using the formula:

Percentage inhibition=

[Abs control –Abs sample /Abs control] x 100

Concentration of extract resulting in 50% inhibition (IC50) was determined graphically.

Anti hyperglycaemic activity

Anti hyperglycaemic activity of the fungal extract was evaluated using alpha amylase and alpha glucosidase inhibition assays.

Alpha amylase inhibition was studied using iodine calorimetric assay as proposed by Xiao *et al*. (2006). Clinically available alpha amylase inhibitor Acarbose was used as positive control under same assay conditions. Inhibition of enzyme activity was calculated as:

Percentage inhibition = (A-C) X100/ (B-C), where, A is the absorbance of sample, B is the absorbance of blank (without enzyme), and C is the absorbance of control (without substrate).

Concentration of extract resulting in 50% inhibition of alpha amylase activity (IC50) was determined graphically.

Alpha glucosidase inhibition activity was determined by PNPG assay (Pavithra *et al*., 2014). Acarbose was used as the reference alpha glucosidase inhibitor. Percentage alpha glucosidase inhibition was calculated using the formula:

Percentage inhibition = [Abs control-Abs sample /Abs control] × 100.

Concentration of extract resulting in 50% inhibition of alpha glucosidase activity (IC50) was determined graphically.

Qualitative analysis for phytochemicals in crude extract

The ethyl acetate extract of SGS was tested for the presence of various phytochemicals as described by Devi *et al.* (2012).

Molecular characterization of SGS

DNA was isolated from the endophytic fungus SGS and ITS region was amplified in PCR using ITS1 and ITS4 primers (White et *al*., 1990). The PCR product was sequenced by Sanger's method of DNA sequencing (Sanger *et al*., 1977). The sequencing results were assembled and compared with NCBI data base. The program PhyML 3.0 aLRT was used for phylogeny analysis and Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*., 2008). The sequence was submitted in NCBI GenBank and accession number was obtained.

RESULTS AND DISCUSSION

Antimicrobial activity

SGS extracts used in well diffusion assay showed zone of inhibition in a concentration dependent manner. 100ug/ml of extract could not produce any zone of inhibition in both *S. aureus* and *E. coli* in well diffusion assay whereas SGS crude extract at a concentration of 1000µg/ml produced zone of inhibition comparable to that of standard antibiotic Amikacin (Figure 1 & 2). Considering the fact that it is a crude extract, it can be concluded that SGS extract holds promise as a potential antibacterial agent. Medicinal plants have by now opened up possibilities in the search

for new alternatives for antibiotics. A number of endophytic organisms, especially endophytic fungi isolated from medicinal plants have also been found to display significant antimicrobial activity. Endophytic fungi from *Ocimum* species was found to show antimicrobial activity against *Pseudomonas aeroginosa*, *Mycobacterium smegmatis*, *Salmonella typhimurium*, *Candida albicans* and *Penicillium chrysogenum* (Pavithra *et al*., 2012). Endophytic *Colletotrichum gloeosporioides* and *Fusarium oxysporum* isolated from *Plumeria acuminata* L. and *Plumeria obtusifolia* L. also showed inhibitory activities against *S. aureus* and *E.coli* (Ramesha and Srinivas, 2014).

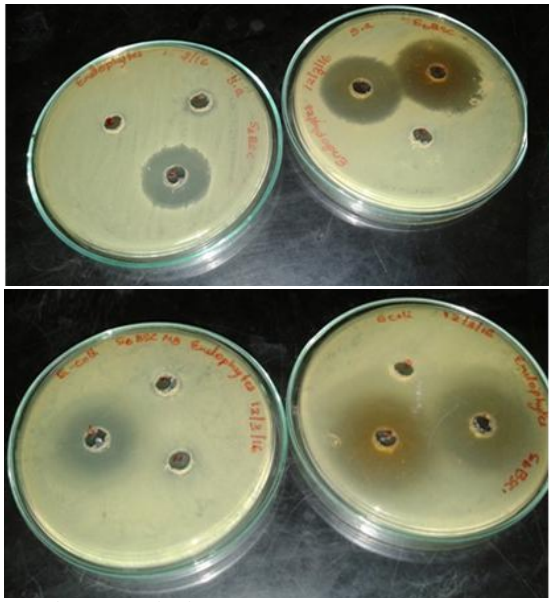

Fig 1: Zone of inhibition for fungal extract SGS against *S. aureus* and *E. coli* at different concentrations. SGS extract showed zone of inhibition in a concentration dependent manner in well diffusion assay.

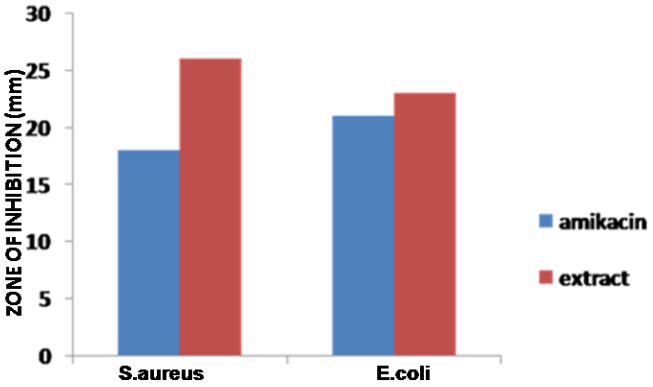

Fig 2: Comparison of Zone of inhibition of standard antibiotic Amikacin (30 mcg) and SGS extract (1000ug/ml). SGS extract showed comparable inhibition to that of standard Amikacin.

Antioxidant activity

Ethyl acetate extract of the endophytic fungus SGS at different concentrations showed antioxidant activity with an IC50 value of 100.88ug/ml (Figure 3). Standard antioxidant ascorbic acid showed an IC50 of 5.66ug/ml. Purification of active compounds in the fungal extract could increase its the IC50 value by many folds. Endophytic fungi like *Mortierella hyaline and Penicillium sp* isolated from medicinal plants *Osbeckia stellata* and *Schima khasiana* respectively (Bhagobaty and Joshi, 2012) showed high antioxidant activity with FRAP and DPPH assay . *Fusarium, Aspergillus* and *Mucor* isolated from *Lobelia nicotianifolia* have also been found show high antioxidant activity in a concentration dependant manner (Murthy *et al*., 2011). Antioxidants are able to protect the body from cancer, neurodegenerative disorders, atherosclerosis, inflammations, aging etc. The toxic side effects of synthetic antioxidants insist the search for natural free radical scavengers (Radulovic *et al*., 2007)

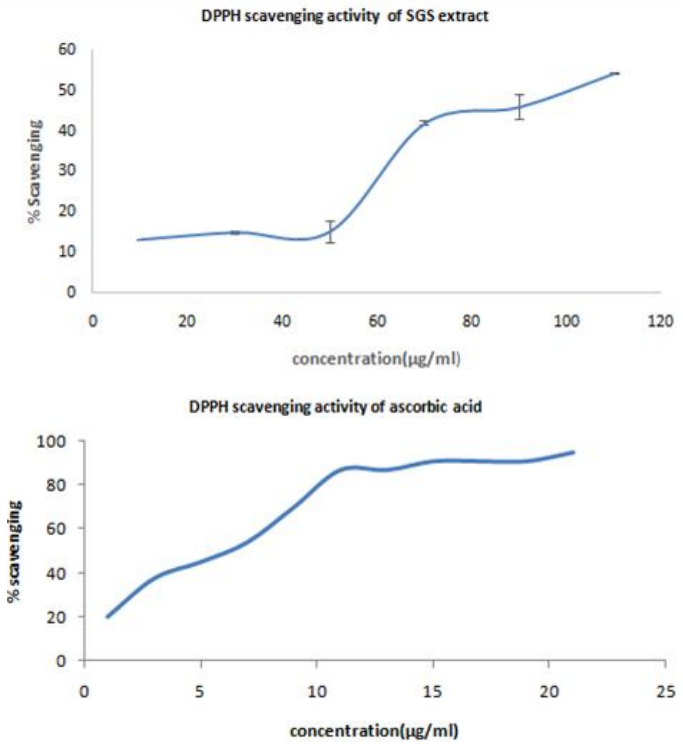

Fig 3: DPPH scavenging activity of SGS in comparison with ascorbic acid. SGS exhibited IC50 value of 100.88ug/ml while standard antioxidant Ascorbic acid exhibited IC50 value of 5.66ug/ml.

Alpha amylase and Alpha glucosidase inhibition activity

The ethyl acetate extract of the isolated fungus SGS showed inhibitory activity against alpha amylase and alpha glucosidase enzymes. The SGS extract showed an IC50 against alpha amylase at a concentration of 109.5µg/ml whereas the standard alpha amylase inhibitor acarbose showed an IC50 at a concentration of 164.02µg/ml under similar assay conditions (Figure 4).

The fungal extract SGS inhibited alpha glucosidase with an IC50 33µg/ml while the standard alpha glucosidase inhibitor acarbose exhibited IC50 at 22.19µg/ml (Figure 5). Thus the endophytic fungal extract SGS can be considered as a potentially good inhibitor of alpha amylase and alpha glucosidase. The inhibition of these enzymes decreases carbohydrate break down in the digestive tract and thus helps to control diabetes.

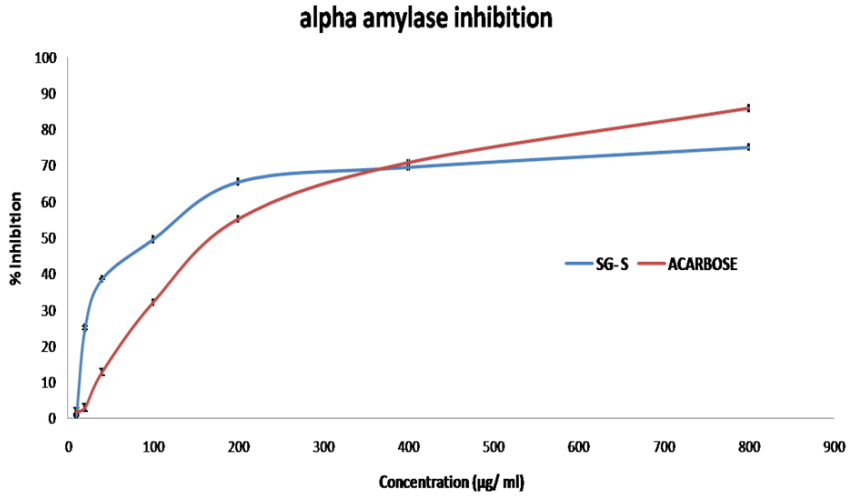

Fig 4: Alpha amylase inhibitory activity of SGS in comparison with Acarbose.
SGS extract showed IC50 at a concentration of 109.5µg/ml whereas Acarbose showed IC50 at 164.02µg/ml.

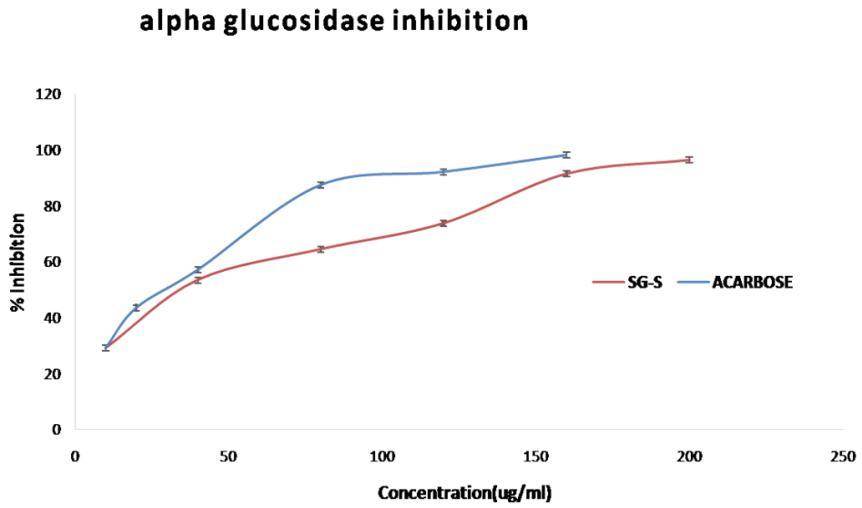

Fig 5: Alpha glucosidase inhibitory activity of SGS in comparison with Acarbose.
SGS extract inhibited alpha glucosidase with IC50 at 33µg/ml while acarbose exhibited IC50 at 22.19µg/ml.

Acarbose, the most commonly available synthetic alpha amylase /alpha glucosidase inhibitor has been shown to cause gastro intestinal side effects (Narkhede *et al*., 2011; Subramanian *et al*., 2008). Therefore, effective, nontoxic, natural product inhibitors of alpha amylase and alpha glucosidase have long been sought. Endophytic *Alternaria*, *Diaporthe*, *Trichoderma*, *Colletotrichum* and *Stemphylium* from menthya and bitter gourd were found to be potent anti diabetic agents with alpha amylase and alpha glucosidase inhibition properties (Pavithra *et al*., 2014). There are also other reports where extracts of endophytic fungi from *Hintonia latiflora*, *Swietenia macrophylla* and *Cassia siamea* were found to be strong alpha glucosidase inhibitors (Rivera-Chavez *et al*., 2013; Ramdanis *et al*., 2012; Munnim *et al*., 2013).

Phytochemical analysis

Standard qualitative tests performed for SGS extract showed positive results for flavanoids, triterpenoids, alkaloids and carbohydrates (Table 1). The antibacterial, antioxidant and anti diabetic potential of endophytic fungus SGS of the present study could be due to the active ingredients present in the crude extract. Like their host plant, endophytes isolated from many plants have been found to produce bioactive compounds such as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones (Rai *et al*., 2012). Flavones, flavonoids and flavonols synthesized by plants and endophytes are reported to show antibacterial properties by forming complex with extracellular proteins or with bacterial cell walls (Tsuchiya *et al*., 1996). Lipophilic flavonoids and terpenes can also disrupt membrane by virtue of their lipophilicity (Mendoza *et al*., 1997). Kovacevic (2004) has attributed the mechanism of antibacterial action of alkaloids to their ability to intercalate with DNA, inhibition of enzymes and inhibition of cell respiration. Many workers have elucidated a link regarding antioxidant activities of endophytic fungi and higher levels of phenol and flavonoid in their extract (Yadav *et al*., 2014; Li *et al*., 2015).

Table 1: Qualitative phytochemical analysis of SGS extract.

Phytochemical	Test	Result	Inference
Flavanoids	Shinoda's test	Reddish brown colour	Positive
Phenols	$FeCl_3$ test	No dark green/blue colour	Negative
Alkaloids	Mayer's test	Pale creamy precipitate	Positive
Cardiac glycosides	Keller -Kiliani test	No greenish blue colour	Negative
Triterpenoids	Salkowski's test	Reddish brown colour	Positive
Carbohydrates	Molish's test	Reddish violet ring	Positive
Saponins	Frothing test	No stable froth	Negative
Tannin	$FeCl_3$ test	No blue green or blue black colour	Negative

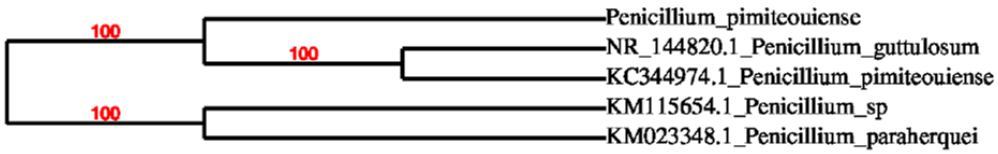

Fig 6: Phylogenetic tree showing relationship of SGS with closely related species. Fungus SGS showed 100% similarity with *Penicillium pimiteouiense.*

Similarly several previous studies have also indicated that flavonoids and terpenoids produce anti diabetic activity (Lu *et al*., 2010; Jung *et al*., 2006; Tan *et al*., 2008). It has been known that these compounds bind to the reactive sites of enzymes and alters catalytic activity (Payan et al., 2004; Mc Cue et al., 2004).

There are not many studies done to evaluate the pharmacological properties of *S.glauca* endophytes. The results of the present study thus draw attention to this medicinal plant which could be a novel source for isolation of endophytes with bioactive compounds.

Chracterization of endophytic fungus SGS

The isolated fungus SGS showed white to light greenish grey mycelium. During growth in PDA, brown colour pigments were produced at the bottom of the petri plate. On the upper side of the colony shining yellow coloured exudates were seen. Sequencing of the ITS region of the fungal DNA followed by similarity search using NCBI blast showed 100% similarity with *Penicillium pimiteouiense*. Phylogenetic tree is shown in figure 6. The sequence was deposited in NCBI GenBank with accession number KY611810.

Endophytic *P. pimiteouiense* isolated from Thai medicinal plant *Stemona tuberosa* has also been reported to show antioxidant activity (Theantana *et al*., 2012). To the best of our knowledge reports regarding pharmacological properties of endophytes of *S. glauca* as well as endophytic *P. pimiteouiense* are scarce, which grades the significance of the present study.

CONCLUSION

Endophytic *P. pimiteouiense* SGS isolated from *S. glauca* was found to show antibacterial, antioxidant and anti hyperglycaemic properties. Qualitative phytochemical analysis of the fungal crude extract showed the presence of flavanoids, triterpenes and alkaloids. Further purification of the extract could enhance revealing the specific compounds present in the fungus. Thus *S.glauca* could be sought as a potential source of many more rare endophytes with promising bio activities.

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