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Original Article

Biological activity of *Stemphylium Vesicarium*: An endophytic fungus isolated from *Portulaca Oleracea*

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ABSTRACT

Every plant species harbors endophytes that can produce novel bioactive compounds. In this present study, an attempt was made to isolate endophytic fungi from edible leaves of *Portulacaoleracea* (Pursley) to determine its antibacterial, antioxidant, and antidiabetic activity. The antibacterial activity was done against human foodborne pathogens (*Enterococcus faecalis, Escherichia coli, Salmonella typhi,* and *Shigella sonnei),* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)by microdilution broth method which, showed a good zone of inhibition against *Salmonella typhi* by *Stemphylium sp.* The MIC value between 10-20µg/ml and MBC value 20-40µg/ml was determined. The antioxidant activity was evaluated by DPPH, TPC, and FRAPassay, which showed considerable variations with reference to their positive control. The correlation among antioxidant assay indicated strong correlation between TPC and DPPH (R = 0.987, P < 0.01).While the antidiabetic assay by alpha-amylase inhibition was found to be 14.39-54.45%. The present study suggests the potential of endophytic fungi of *Portulaca oleracea*as as a source for antibacterial, antioxidant, and antidiabetic agents.

Keywords: Antibacterial, Antidiabetic, Antioxidant, Endophytic Fungi, *Portulaca oleracea* (Pursley), 18S rRNA

INTRODUCTION

A rich diversity of plants can be seen from place to place containing ethnobotanical important plants [1], wild plants [2], and pharmaceutically important plants [3]. Nowadays medicinal plants and their endophytes are important resources for the discovery of natural products, where every plant species harbors endophytes, endophytes are the organism that colonizes the internal of the plant parts, without causing any harmful effect on the host [4], and rather helping the plant by imparting resistance to host plant against biotic and abiotic stresses [5]. Thus, endophytes represent a microbial

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resource having novel sources for bioactive compounds which provide a wide range of activities against plant pathogens, also produce bio-molecules of pharmaceutical and agricultural importance. The reactive oxygen species and free radical-mediated stress contribute to aging, cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, and rheumatoid arthritis [6]. Also, the antioxidants are thought to be beneficial in the management of reactive oxygen species-mediated tissue injury [7]. Besides diabetes mellitus (DM) is a widespread and growing metabolic disorder where glucosidase breaks down disaccharides into glucose resulting in increased blood glucose level, distinguished by the lack of the body's ability to regulate glucose and insulin homeostasis [8]. Currently, DM has become a serious public health problem due to current lifestyle [9] hence studying DM-related mechanisms and the development of new antidiabetic drugs is important this is achieved by herbal medicine which is having fewer side effects compared to synthetic medicine.

Portulac aoleracea L commonly known as purslane, is an annual green herbaceous plant widely used as an edible plant as well as medicinal herbs. They are consumed raw as a vegetable or in salads or cooked in soups [10] also used traditionally worldwide as a folk medicine to cure a wide range of ailments and disorders such as skin diseases, fever, dysentery, diarrheal, bleeding piles, kidney and liver diseases, coughing, shortness of breath, and asthma [11,12]. The major bioactive components present in purslane are flavonoids followed by polysaccharides, coumarins, monoterpene glycoside, alkaloids, vitamins, and minerals [13]. Other components such as β-carotene, glutathione, melatonin, and high content of n-3 fatty acids which contribute to the antioxidant properties and free-radical scavenging activities have been shown in numerous in-vitro studies [14-16]. Some flavonoids (kaempferol and quercetin) and alkaloids (oleraceinA, oleracein B, and oleracein E) have been reported to be even higher than vitamin C and vitamin E [17]. Pharmacological investigations revealed antibacterial, hypolipidemic, anti-aging, anti-inflammatory, antioxidative [18], analgesic, and wound healing activities [19].Until now, the majority of studies on this herb have focused on the chemical compositions and bioactivities of small molecules. Although the antidiabetic, antiviral, and antioxidant potential of various polysaccharides has been documented. The leaves of this herb are used for flavoring and in preparation of traditional food [4] hence the present study was carried out to isolate endophytic fungi from *Portulaca oleracea* and to investigate their biological properties such as antibacterial, antioxidant, and antidiabetic activities.

MATERIALS & METHODS

Collection of sample: Leave samples of *Portulaca oleracea* (Pursley) Figure1 were collected from a local farm from Chikka Aluvara, Kodagu District. Karnataka, India. Collected fresh and symptomless leaves were transported to the laboratory, where they were processed for endophyte isolation in less than 24h.

Isolation of endophytic fungi: Endophytic fungi were isolated from the healthy plants of *Portulaca oleracea*as per the procedure described by [20] with minor modification in surface sterilization. The leave samples were surface sterilized with 70% ethanol for 1min followed by 4% sodium hypochlorite for 3min then by 70% ethanol for 30 seconds and finally washed thoroughly in sterile distilled water to remove the traces of alcohol and excess water dried in a sterile blotting sheet, chopped into small segments and transferred to Saborous Dextrose Agar (SDA) plates supplemented with streptomycin (100µg/ml) to suppress bacterial growth and incubated at 28°C for 7 days. The hyphal tips of the developing fungal colonies were transferred to fresh SDA slants to maintain the pure culture for further use.

Morphological characterization: Obtained fungal mycelium was strained using cotton blue and observed under the microscope. Morphological characterization including mycelia, sporangium, and spore morphology as well as the external form of the colony was identified using standard manuals described by [21,22].

Molecular characterization and phylogenetic analysis: Identify the Endophytic fungi were carried out by 18S rRNA gene sequence. Fungal isolates were cultured on Saborous dextrose agar (SDA) for 7 days at 26^0C, then the mycelium was scraped from SDA plate for DNA extraction by using EXpure Microbial DNA isolation kit according to manufacturer's protocol (Bogar Bio Bee stores Pvt Ltd.,). The quality and integrity of the DNA were estimated by electrophoresis on a 1% (w/v) agarose gel. The target rDNA region was amplified using primers ITS1(5' AGAGTTTGATCTGGCTCAG 3') and ITS4 (5' TACGGTACCTTGTTACGACTT 3') (Millipore) in a thermocycler. The reaction mixture contained 5 µL of isolated DNA in 25µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer), 5 µL of deionized water, and 12 µL of Taq Master Mix. Polymerase chain reaction (PCR) amplifications were performed in a thermal cycler with an initial denaturing step at 95º C for 2min,

followed by 25 amplification cycles of denaturation 95º C for 30sec; annealing 50°C for 30s; extension 72º C for 2 min and final extension 72º C for 10min. Obtained amplification products were separated by electrophoresis on 1% (w/v) agarose gel. The purified PCR product was then sent to sequencing by Sangers dideoxy method by using ABI PRISM® BigDyeTM terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The sequence was compared with National Centre for Biotechnology Information (NCBI) GenBank using BLAST search and CLUSTAL X software was used to generate phylogenetic tree alignment [23].

Metabolite extraction: The selected endophytic fungus was subjected to liquid surface fermentation for the production of bioactive compounds. Flasks contained 500ml Saborous Dextrose Broth (SDB) were inoculated with 7day old endophytic fungus. Flask was incubated at room temperature for 21days with periodical shaking at 150rpm. After 21days of incubation broth and mycelia mat were filtered through sterile muslin cloth, were bioactive compounds from the broth culture were extracted using an equal amount of ethyl acetate solvent. The mixture was mixed thoroughly for four hours at 100rpm and then left to soak overnight. After 24hrs, the ethyl acetate extract was separated using a separating funnel. The organic phase having bioactive compounds was dried by evaporation under reduced pressure using a rotary vacuum evaporator. The dried extract was re-dissolved in ethyl acetate or dimethyl sulphoxide (DMSO) for further purification and bioassays.

Assessment of antimicrobial activity: The antimicrobial activity was conducted using the agar well diffusion method [24]. The pathogens used in this test were food-borne human pathogenic bacteria *Enterococcus faecalis, Escherichia coli, Salmonella typhi,* and *Shigellasonnei.* To evaluate the antibacterial activity, bacteria were grown in nutrient broth and incubated for 24hr, adjusted to 0.5 McFarland turbidity standard corresponding to (~1.5×10^8 CFU/ml) and spread (100µl) on Petri dishes containing nutrient agar medium. Using sterile cork borer 6 mm wells were made in each plate and 100 µl of extracts were loaded on seeded plates. Negative control was maintained using DMSO and positive control was maintained by using standard antibiotics, viz Streptomycin (1mg/ml). The plates were then incubated at 37ºC for 24h. The experiment was carried out in three replicates and an inhibition zone was recorded.

Determination of minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs): Active extracts were further determined for their MIC and MBC by colorimetric microdilution test. The test organisms were grown for 24h at 37 C. 100µl of liquid culture media and 10µl of bacterial suspension adjusted to 0.5 McFarland turbidity standard (~1.5×10^8 CFU/ml) and test sample dissolved in DMSO were distributed to each well in different concentration ranges from 0 to 100mg/ml. chloramphenicol (1mg/ml) was used as a positive control, and sterile distilled water was used as a negative control for the test organisms, and the plates were incubated at 37 C for 24h. To detect the MIC 15 µl of 0.01% resazurin was added and re-incubated for 4h. The absence of bacterial growth with the lowest concentration was taken as the MIC. MBC was determined by removing a portion of liquid and sub-cultured on a nutrient agar plate and incubated at 37 C for 24h. The lowest concentration that yields no growth after this culturing was regarded as the MBC. All experiments were conducted in triplicate [25].

Phytochemical analysis: The ethyl acetate extract of the potent endophytic fungi was subjected to chemical constituent analysis such as phenols, flavonoids, alkaloids, terpenoids, saponins, tannins glycosides, and steroids using standard methods of [26].

DPPH Radical scavenging assay: DPPH assays were carried out to measure the scavenging activity of selected methanolic fungal extract with minor modification of a reported procedure [27]. Briefly, 3ml of the fungal extract was added to 3ml of 0.3mM methanolic solution of DPPH and shaken vigorously, the tubes were kept in dark for incubation at room temperature for 30min. Ascorbic acid was used as a positive control, and the absorbance was read against a blank at 517nm. Percent inhibition of DPPH was calculated according to the following equation:

$$\%\text{DPPH radical scavenging activity} = \frac{\text{Abs of control - Abs of test}}{\text{Abs of control}} \times 100$$

FRAP (Ferric reducing/ antioxidant power) assay: FRAP assay is based on the rapid reduction in free ic-tripyridyltriazine by antioxidants present in the samples forming ferrous-tripyridyltriazine, a blue-colored product [28]. Working FRAP solution was freshly prepared by mixing 15 µl of 0.1M sodium phosphate buffer, 15 µl of potassium ferricyanide (1%), and 10 µl of sample then incubate at 50°C for 20min. After incubation 15 µl of trichloroacetic acid, 55 µl of distilled water, and 110 µl of ferric chloride were added and mixed well. The absorbance was measure at 700nm if an increase in the reaction mixture indicates increased reducing capability. Ascorbic acid was taken as a

standard reference and the experiment was carried out in triplicates.

Determination of total phenolic content (TPC): TPC of the extract was determined using Folin-Ciocalteu assay [29]. In this assay, the reaction mixture containing 1ml of extract and 1ml of FolinCiocalteu phenol reagent was mixed well and allowed to stand for 5min. 2ml sodium carbonate solution was added and kept in the dark at room temperature for 30min. The absorbance was measured at 765nm using a UV-visible spectrometer against blank. Gallic acid was used as standard, and TPC was obtained from the regression equation: y = 0.026×+0.121 with R2 = 0.979 and expressed as mg/g gallic acid equivalent.

Alpha-amylase inhibition assay: Alpha-amylase inhibition assay was carried out by preparing reaction mixture using fungal extracts dissolved in DMSO, 150 µl of 0.02 M sodium phosphate buffer, and 100 µl of 1mg/ml alpha-amylase solution were incubated at 25° C for 10 min. About 100 µl of 1% starch solution in 0.02 M sodium dihydrogen phosphate buffer was added to tubes. The mixture obtained was then incubated for 10min at 25° C. The reaction was stopped by adding 200 µl of dinitrosalicylic acid solution, the tubes were heated in a boiling water bath for 5min and then cooled to room temperature. Then the mixture was made up by adding 2ml of distilled water. Where acarbose was a positive control and the absorption was recorded at 450 nm. The results were reported as the percent of inhibitory activity of the enzyme [30].

RESULTS AND DISCUSSION

To date, only a few plants have been extensively investigated for their endophytic biodiversity [31]. The study of endophytic fungi occupies an important part in fungal diversity as these endophytes play a very important and tremendous role in the production of secondary metabolites with pharmaceutical significance [32]. *Portulacaoleracea* an annual green herbaceous plant that we use in our day-to-day life is one of the best known and distributed species of the Portulacaceae family. *Portulacaoleracea* is a succulent plant eaten as leafy vegetables and has several medicinal properties as it contains the highest content of vitamin A among green leafy vegetables. Also plays a crucial role in vision healthy mucus membranes, and protects from lung and oral cavity cancer [33]. Therefore, the present work was initiated to find out endophytic flora associated with this widely used medicinal plant.

Isolation and purification of endophytic fungi: The pure strain of endophytes was identified and successfully isolated from the leaves of *Portulacaoleracea*. From the leaf segments four fungal endophytes belonging to different genera namely *Colletotrichum* sp., *Fusarium* sp., *Alternaria* sp., and *Stemphylium sp.* were isolated and identified through the morphological and microscopic method. Few studies were reported on *Portulacaoleracea* found by [34] they isolated eight endophytic species and identified them by morphological and molecular techniques. All four endophytes were then extracted using ethyl acetate which showed the significant distinction between the aqueous and the organic phase of the extract having high polarity.

Antibacterial potential of the metabolite: The crude ethyl acetate extract of the endophytic fungi displayed considerable antimicrobial activity shown in Table 1 that can be classified into poor activity (8-10mm), moderate activity (11-15mm), and good activity (16-23 mm). The result showed that fungal crude ethyl acetate extract inhibited gram-negative than gram-positive bacteria. Among the potent strains, a crude metabolite of an endophytic fungus, *Stemphyliumsp.* displayed significant antimicrobial activity against all tested pathogens. This showed the broad-spectrum nature of the metabolite. There are many reports about antimicrobial compounds produced by endophytic fungi in a culture that is active against plant and human pathogenic microorganisms [35]. Among them the active isolate *Stemphylium sp.* further determined for their MIC and MBC by colorimetric microdilution method shown in Table 2. The MIC values ranged between 10 and 20 mg/ml where *E. coli* and *Sh. sonnei* had the lowest MIC value (10mg/ml), followed by *E. faecalis* (15 mg/ml) and *S. typhi* (20 mg/ml). The results of MBC values ranged between 15 and 40 mg/ml. where *S. typhi* had the highest MBC value followed by *E. coli* and *Sh. sonnei* (20mg/ml) and *E. faecalis* (15 mg/ml). This suggests that *Stemphylium sp.* has a comparatively stronger antimicrobial activity against the four tested foodborne pathogens.

Phytochemical and Antioxidant assay: The ethyl acetate extract of endophytic fungi was evaluated for its phytochemical constituents using standard procedures Table 3. Nine bioactive secondary metabolites that included phenol, flavonoid, alkaloid, terpenoid, steroid, saponin, tannins, and glycosides were tested for phytochemical constituents, the results revealed the presence of phenol, flavonoids, alkaloids, terpenoids, and glycosides which are known to be biologically active and can be explored for generation of bioactive metabolites. The *Stemphylium sp*. extract revealed good phenolic content as well as radical scavenging activity among the other fungal species tested and suggested the phenolic content

represents a major group of compounds that act as primary free radicals scavengers. The total phenolic content of *Stemphylium sp*. isolate was determined using Folin-Ciocalteu reagent and the result was found to be 9.3 mg/ml GAE (Gallic acid equivalent) representing an approximate four-fold variation shown in Figure 2against gallic acid used as a standard.

The antioxidant potential was evaluated by DPPH and FRAP assay. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability [36,37]. A freshly prepared DPPH solution exhibits a deep purple color which disappears when an antioxidant is present in the medium. Antioxidant molecules can quench DPPH free radicals and convert them to a colorless product, resulting in a decrease in absorbance. The reducing capacity of compounds thus serves as an indicator of potential antioxidant property [38]. The crude isolate revealed high scavenging activity of 87.2 % represented in Figure 2 and the standard ascorbic acid exhibits at 95.06 % of scavenging activity. The result indicates that the metabolites from *Stemphylium sp.* could be potential agents in scavenging free radicals and treating diseases related to free radical reactions. The FRAP assay measures antioxidants in a sample at 593 nm compared to other assays measuring inhibition of free radicals exhibit 72.35 ± 0.16 (AAE mg/ml) indicating their electron-donating potential. Where the value of FRAP is directly proportional to its antioxidant capacity as in this assay, antioxidants act as reducing agents by donating electrons to ferric ions. The results obtained in the DPPH and FRAP assay indicated high activity presented in Figure. 2.

A strong correlation between total phenolic content and antioxidant activity was observed in our study. A significant positive correlation in Table 4was found between FRAP and TPC, DPPH and FRAP were 0.942 and 0.975 respectively. The correlation between TPC and DPPH free radical scavenging activity was also studied and it was observed to be highly positively correlated with $R = 0.987$. These results indicate that extract having more phenolic content tends to serve a strong antioxidant activity. Earlier studies on the screening of endophytic extracts for antioxidants from medicinal plants have revealed the chemical diversity of phenolic compounds from endophytes [39].

Alpha-amylase inhibition assay: A study on antidiabetic was performed to detect the inhibition by alpha-amylase assay. Alpha-amylase is an enzyme that hydrolyses carbohydrates into glucose, and easily absorbable [40]. The inhibition of alpha-amylase activity slows down the conversion of starch into glucose and effectively reduces glucose absorption [41,42]. Based on the data presented in Table 5, each isolate of the endophytic fungi inhibited the enzyme at different rates. Among the four isolates, *Colletotrichum*sp. had the lowest activity, where *Stemphylium sp.* had the highest percentage of inhibitory activity, but it was lower than acarbose.

Molecular characterization studies and sequence analysis: Based on molecular identification and BLAST similarities, the endophytic fungi *Stemphylium sp.* isolate (Fig.3) were identified as *Stemphyliumvesicarium*(Fig.4) (GenBank accession number MW391577 with 99% similarity to the type sequence LC512756). The identified G3 isolate depicted 100% query coverage with 0.0 E value respectively.

CONCLUSION

There is a vast diversity of microbes that remains untapped for evaluation of metabolite production that may possess valuable bioactivities. To the best of our knowledge, this study considers the first report conducted on the antimicrobial, antioxidant, and anti-diabetic activity of ethyl acetate extract of endophytic fungi isolated from *Portulacaoleracea*. Our study revealed both hosts, as well as its endophyte, are good sources of phytochemicals, antioxidants, antibacterial and antidiabetic activity. Further, elucidating the profile would reveal the presence of similar compounds both in host and endophyte which could be a promising therapeutic agent.

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Table 1: Antibacterial activity of endophytic fungi

Fungal isolates	Zone of inhibition (mm)			
	Escherichia coli	***Salmonella typhi***	***Shigellasonnei***	***Enterococcus faecalis***
*Colletotrichum*sp.	10.0 ± 0.0	14.0 ± 0.2	8.0 ± 0.1	11.0 ± 0.0
*Alternaria*sp.	12.0± 0.6	22.0 ± 0.2	10.0 ± 0.0	9.0 ± 0.4
Stemphylium sp.	13.0 ± 0.0	23.0 ± 0.0	17.0 ± 0.0	12.0 ± 0.0
Fusarium sp.	11.0 ± 0.1	18.0 ± 0.0	12.0 ± 0.1	10.0 ± 0.0
Streptomycin (1mg/ml)	25.0 ± 0.3	30.0 ± 0.4	20.0 ± 0.5	28.0 ± 0.3

Table 2: Minimum inhibitory and minimum bactericidal concentrations of extracts against foodborne bacteria

	Concentration (mg/mL)							
	Escherichia coli		***Salmonella typhi***		***Shigellasonnei***		***Enterococcus faecalis***	
Fungal Isolate	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Stemphylium sp.	10	20	20	40	10	20	15	15

Table 3: Phytochemical analysis

Fungal Isolates	Phenol	Flavonoids	Alkaloids	Terpenoids	Steroids	Saponin	Tannins	Glycosides
*Colletotrichum*sp.	-	-	+	-	-	-	-	+
*Alternaria*sp.	+	-	+	+	-	-	-	-
Stemphylium sp.	+	+	+	-	-	-	-	+
Fusarium sp.	+	-	-	-	-	-	-	-

+: present, -: absent

Table 4: Correlation matrix (Pearson's correlation coefficients).

Variable	TPC	FRAP	DPPH
DPPH	0.987**	0.975**	-
FRAP	0.942**	-	
TPC	-		
** Correlation is significant at the 0.01 level			

Table 5: Percentage of alpha-amylase inhibition by different endophytic fungi at the concentration of 50µg/ml.

Fungal isolates	Percentage of inhibition (% ± SD)
*Colletotrichum*sp.	14.39 ± 3.91
*Alternaria*sp.	35.78 ± 1.21
Stemphylium sp.	54.45 ± 2.51
Fusarium sp.	23.16 ± 1.32
Acarbose	68.50 ± 1.10

Figure 1:*Portulacaoleracea*(Pursley) grown in a field

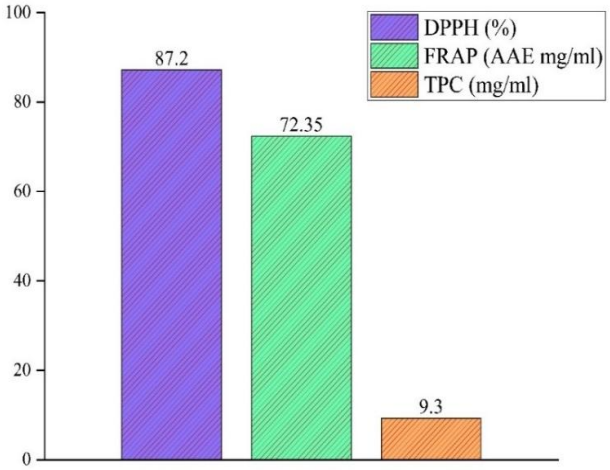

Figure 2: Antioxidant activity of *Stemphyliumsp.*

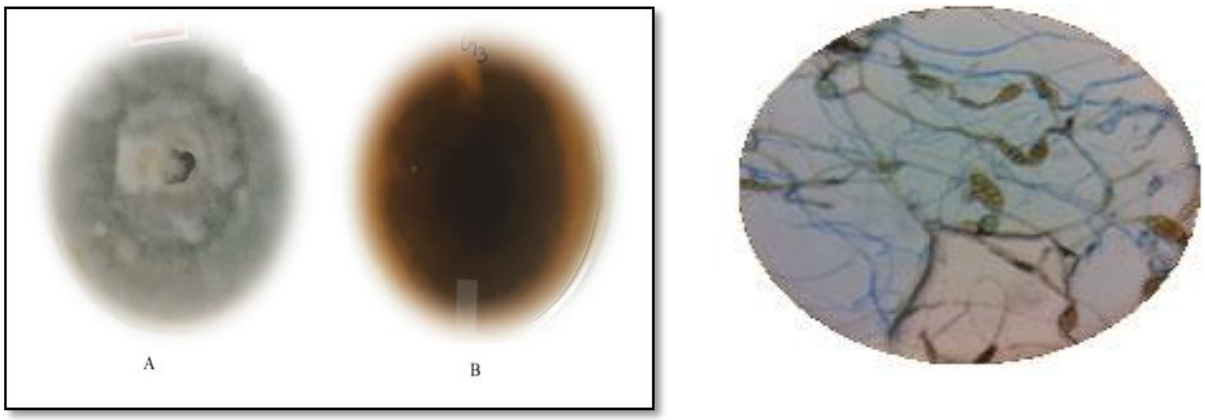

Figure3: Growth pattern A- Front view; B- Back view and microscopic view of endophytic fungi *Stemphyliumvesicarium*

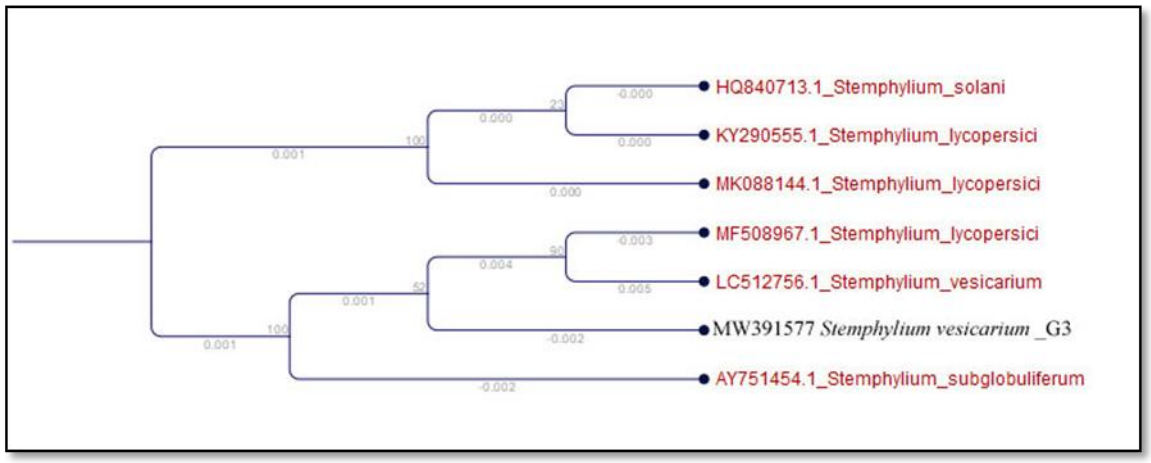

Figure 4: Evolutionary relationship between various species of *Stemphyliumvesicarium*by MEGA 7.0

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