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Antibacterial activity of endophytic fungus, *Penicillium griseofulvum* MPR1 isolated from medicinal plant, *Mentha pulegium* L.

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In the present study, an endophytic fungus with a considerable antimicrobial activity was isolated from the medicinal plant *Mentha Pulegium* L. The morphological and molecular identification revealed that this fungus is a *Penicillium griseofulvum*. A preliminary screening was done to choose the suitable culture medium for a maximum production of the bioactive compounds using the dual-culture agar diffusion assay. The malt extract agar (MEA) and potato dextrose agar (PDA) media were the suitable, and the MEA was selected for the further study. Extraction was done with three solvents, n-Hexane, dichloromethane and ethyl acetate, and the crude extracts were tested against Gram-positive and negative bacteria. A high activity was found with ethyl acetate and dichloromethane crude extracts against all bacteria with a maximal inhibition zones of 45.5 and 41 mm respectively obtained against *Escherichia coli*. The minimum inhibitory and bactericidal concentrations (MIC, MBC) of ethyl acetate crude extract were evaluated using the broth micro-dilution method. A MIC of 50 µg / ml on Gram-negative bacteria and of 100 µg / ml on Gram-positive bacteria was found. The MBC_S ranged from 50 and 200 µg/ml. The time kill study has revealed a bactericidal activity of the *Penicillium griseofulvum* crude extract. At 24 h and for all concentrations (MIC× 2 and MIC× 4), 100% killing of the bacterial cells was achieved. These results prove that the extract of *Penicillium griseofulvum* can be a promising source of important bioactive molecules.

Keys words: Antibacterial activity, endophytic fungi, *Mentha pulegium* L., *Penicillium griseofulvum.*

INTRODUCTION

Medicinal plants like all plants interact continously with different microorganisms, like fungi (both pathogenic and beneficial), living on the surface of plants (epiphytes) and their roots (mycorhizes) and even inside (endophytes)

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(Passari et al., 2015). Endophytic fungi live asymptomatically in the intercellular and/or intracellular spaces of healthy plants for at least a part of their life (Dos Banhos et al., 2014). All parts (leaves, stems, roots, barks, etc) of all plants examined till date are colonized by endophytic fungi (Shen et al., 2014). The relationships between the endophytic fungi and their hosts can change between neutral association to mutualistic, pathogenic or saprophytic interactions depending on genetic dispositions of the two partners, environmental factors and time (Martín et al., 2013).

The increasing resistance of pathogenic micro-organisms to antibiotics in the world, and recently in Algeria, leading to the resurgence of more complex infections (Baba Ahmed-Kazi Tani and Arlet, 2014), has pushed researchers to discover new bioactive molecules. The capacity of endophytic fungi to biosynthesize bioactive secondary metabolites (more than 20.000 bioactive molecules) (Marson Ascêncio et al., 2014) and their various biological activities such as antimicrobial, anti-viral, anticancer, antioxidant, antidiabetic, and anti-inflammatory activity (Sadrati et al., 2013; Bashyal et al., 2014; Shah et al., 2015; Jouda et al., 2016a; Pan et al., 2016; Chen et al., 2016; Singh and Kaur 2016; Yao et al., 2017) have made this fungus to be important, as an important source of new bioactive secondary metabolites potentially useful for human medicine and other domains.

Medicinal plants have been well studied because they harbor rare microorganisms, that imitate the chemistry of their hosts, and making the same molecules or derivate more active than those synthesized by their hosts. It is also probable that the bioactivity of medicinal plants may be due to endophytes living inside plants by genetic exchange (Tayung et al., 2011).

Mentha pulegium L., commonly known as pennyroyal, belongs to the Lamiaceae family. It is native to America, Europe, North Africa and in Asia Minor and Near East (Mahboubi and Haghi, 2008). It is widely used in traditional medicine (food poisoning, bronchitis, diuretic, flatulence, intestinal colic), food like culinary herb, cosmetics and aromatherapy (Hajlaoui et al., 2009; Aires et al., 2016). Only Debbab et al. (2009) and Teiten et al. (2013) have studied the endophytic fungi of this plant, and have determined the cytotoxicity and anticancer activity of the bioactive metabolites of the endophytic fungus, *Stemphylium globuliferum*. However, the antibacterial activity of endophytic fungus of this plant has not been examined; for this reason we selected this plant for isolation of this endophytic fungus in order to investigate its antibacterial activity.

MATERIALS AND METHODS

Sample collection and fungal isolation

Endophytic fungi were isolated from the healthy medicinal plant *Mentha pulegium* L., collected from the mountain of Megriss Setif Algeria in June 2014. They were identified by faculty botanists, according the following method. Different tissues (leaves, stems and roots) were washed with tap water, cut into small pieces (leaf pieces size of approximately 5.0 × 5.0 cm; stems and root pieces of approximately 5.0 cm long); they were surface-sterilized by sequential immersions in 70% (v/v) ethanol for 1 min, 3% sodium hypochlorite for 4 min and 70% (v/v) ethanol for 1 min and then rinsed thrice in sterilized distilled water for 1 min each (Petrini, 1986; Pimentel et al., 2006; Xie et al., 2016). After drying on sterile filter paper, each segment was cut into 0.5 cm and aseptically transferred onto Petri-dishes containing Potato Dextrose Agar (PDA) (Sigma-Aldrich; pH 5.6 ± 0.2) supplemented with 100 mg/l of Gentamicine to suppress bacterial growth. The dishes were incubated at 28°C and checked continuously for the growth of endophytic fungi colonies. Each fungus emerging from the segments was transferred to fresh PDA plates and PDA slants for conservation at 4°C.

Initial screening of antibacterial activity

Tested bacteria

The endophytic fungi were screened for their antibacterial activity using the pathogenic bacteria, *Bacillus cereus* ATCC 10876, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922. Each bacterium was grown in nutrient agar (NA) at 37 °C for 24 h, and diluted until the concentration got to 10^8 c.f.u. / ml by spectrophotometry ($OD_{=}$ 0.08-0.1 at 625 nm) (Powthong et al., 2013).

Preliminary antibacterial assay

Antibacterial activity of the endophytic fungi was performed using the method of agar plug diffusion explained by Powthong et al. (2013) and Sahani et al. (2017), with some modification. Briefly, pure cultures of the endophytic fungi were cultivated on the surface of PDA at 28°C for 14 days. Then, a small disk of fungal colony was cut (6 mm diameter) using a sterile cork borer and placed on previously inoculated Muller Hinton Agar (MHA) (Sigma- Aldrich; 7.3±0.2) plates. A disk of PDA without fungi was used as negative control. The Petri dishes were refrigerated at 4°C for 4 h for complete diffusion of antibacterial compounds from the fungal disks, then these plates were incubated at 37°C for 24 h. Antibacterial activity was determined by measuring zones of inhibition produced by the endophytic fungi. The endophytic fungus MPR1 displaying good antibacterial activity was selected for further study.

Identification

The fungus was initially identified based on morphological characteristics using the standard mycological manuals and microscopic examination (Rajeswari et al., 2017). For molecular identification, isolate was grown on PDA plate for 7 days at 25°C. DNA was extracted from culture using the Ultraclean Microbial DNA Isolation Kit (MoBio, Solana Beach, CA, USA) following the manufacturer's protocols.

Molecular characterization of the isolates was performed using sequencing of the internal transcribed spacer (ITS) region that is standard gene regions. For this aim, ITS gene regions were amplified with the primers V9G, 5'-TTACGTCCCTGCCCTTTGTA-3' (forward) and LS266, 5'-GCATTCCCAAACAACTCGACTC-3' (revers) (Chen et al., 2016; Kadaifciler and Demirel, 2017) by polymerase chain reaction [Veriti® 96-Well ThermalCycler (Applied

Biosystems®)].

Amplification was carried out with a total volume of 25 µl containing 1 µl of genomic DNA, 2.5 µl of 2.5 µM forward and reverse primers, 2.5 µl of 10 × Taq buffer + KCl-$MgCl_2$ (Bioline, UK), 2.5 µl of 25 µM $MgCl_2$ (Fermentas, CA, USA), 2µl of 2.5 µM dNTP mix, 0.25 µl of 5 U / µl Taq DNA polymerase (Bioline, UK), and 11.75 µl of nuclease free water under the following condition: Initial denaturation step for 5 min at 95°C, followed by 35 cycles of denaturation for 45 s at 95°C, annealing for 30 s at 56 °C and extension of 2 min at 72°C, followed by a final extension at 72°C for 6 min (Kadaifciler and Demirel, 2017). PCR product was separated by agarose gel electrophoresis (1% W/v in 1xTAE) and visualized by GelRed staining. PCR products were purified using EXOSAP-IT (Affimetrix) and sequenced using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) following the method of Demirel et al. (2013).

Data analyses

The sequence was compared with those deposited in the NCBI GenBank Database via BLAST searches (Altşchul et al., 1990), and aligned using the Muscle in MEGA7.0 software package together with the other sequences of morphologically and phylogenetically related type species that were obtained from NCBI GenBank (Kumar et al., 2016). The aligned data sets were analyzed using Maximum Likelihood analysis based on the Tamura Nei model (Tamura and Nei, 1993) as implemented in the MEGA 7.0 with 1000 bootstrap replications. Gaps and missing data in all the position were eliminated. *Penicillium crustosum* (AF033472) was used as out group. The obtained sequence data were deposited in NCBI GenBank, and an accession number was obtained.

Selection of suitable culture media

To select the best medium in which the isolate exhibited maximum inhibition zone, the maximum antibiotic production was then used for further study; MPR1 was cultivated on the surface of different culture media, potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA) (dextrose, 40 g/l; peptone, 10 g/l; Agar, 15 g/l; pH 5.6 ± 0.2), yeast extract agar (YEA) (yeast extract, 3 g/l; Peptone, 5 g/l; Agar, 15 g/l; pH 7.2 ± 0.2), malt extract agar (MEA) (malt extract, 20 g/l; dextrose, 20 g/l; peptone, 6 g/l; agar, 15 g/l; pH 5.4 ± 0.2), yeast malt extract agar (YMEA) (yeast extract, 3 g/l; Malt extract, 3 g/l; dextrose, 10 g/l; peptone, 5 g/l; agar, 15 g/l; pH 6.2 ± 0.2) at 28°C for 14 days. The agar plug diffusion method was done as described above against four Gram-positive bacteria *B. cereus* ATCC 10876, *E. faecalis* ATCC 49452, *S. aureus* ATCC 25923, Methicillin-resistant *S. aureus* ATCC 43300 and four Gram-negative bacteria *Citrobacter freundii* ATCC 8090, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922.

Fermentation and extraction

The endophytic fungus was cultivated on Malt Extract Broth (MEB) (Malt extract, 20 g/l; Dextrose, 20 g/l; Peptone, 6 g/l; pH 5.4 ± 0.2) by inoculating agar blocks of actively growing pure culture (6 mm in diameter) in 250 ml Erlenmeyer flasks containing 100 ml of the medium MEB for 21 days at 28 ± 2°C and 150 rev / min. Fungal mycelia were separated using Whatman filter paper and further centrifuged at 4000 rev / min / min for 5 min (Mai et al., 2013; Astuti et al., 2014).

In order to choose the best extraction solvent, three organic solvents were used as n-Hexane, dichloromethane and ethyl acetate. The extraction method was performed based on the method explained by Saraswaty et al. (2013). Briefly, the supernatant of liquid fermentation was extracted with an equal volume of organic solvent starting with the non-polar organic solvent (n-Hexane). The solution was then mixed for 10 min and allowed to stand. The layer of n-Hexane was then collected and evaporated under vacuum using a rotary evaporator (BÜCHI). The remaining aqueous phase was re-extracted by the other solvents using the same method. The crude extracts were then dissolved in dimethyl sulphoxide (DMSO) and kept at 4°C.

Evaluation of antibacterial activity of crude extracts

Antibacterial activity of secondary metabolites extracted from MPR1 was done following an agar well diffusion method described by Fatima et al. (2016) against *B. cereus* ATCC 10876, *S. aureus* ATCC 25923, Methicillin-resistant *S. aureus* ATCC 43300, *C. freundii* ATCC 8090, *S. typhimurium* ATCC 13311 and *E. coli* ATCC 25922.

100 µl (10^8 c.f.u. / ml) of bacterial inoculums was spread on MHA plates; then wells (6 mm diameter) were made and 25 µl of each extract was poured into these wells. DMSO was used as negative control. After incubation of 24 h at 37°C, the diameter of each zone of clearance on plates was measured and used as an indicator of antibacterial activity of the extract.

Determination of minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

The minimum inhibitory concentrations (MICs) and the minimal bactericidal concentrations (MBCs) were determined using broth micro-dilution method following the protocol established by CLSI (2012). The ethyl acetate extract was diluted in DMSO to have an initial concentration of 1600 µg / ml and 2-fold serial dilution was done (1600, 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 µg / ml), 90 µl of sterile Mueller Hinton Broth (MHB) (Sigma-Aldrich; pH 7.4 ± 0.2); the wells were then inoculated with standard size of bacterial suspension of 5× 10^6 c.f.u. / ml. The DMSO and the standard drugs (Imipenem and Gentamycin) were used as negative and positive control, respectively.

After 24 h of incubation at 37°C, 20 µl of 0.5% of 2, 3-5 Triphenyltetrazolium chloride aqueous solution was added to each well. After a second incubation for 30 min at 37°C, the lowest concentration of ethyl acetate crude extract which did not show any visual color change was recorded as the MIC. All wells that showed growth inhibition were subcultured on MHA. After incubation of 24 h at 37°C, the lowest concentration that exhibited no visible growth was considered to be the MBC.

Time kill study

To determine the bactericidal or bacteriostatic effect of *Penicillium griseofulvum* ethyl acetate extract, the time kill test was done against Methicillin-resistant *S. aureus* ATCC 43300. Into three tubes of MHB medium with different concentrations of extract (MIC, MIC× 2 and MIC× 4), the bacterial suspension was transferred to obtain an initial inoculum cell density of approx. 5 × 10^5 c.f.u. / ml; a fourth tube was used as the growth control containing the MHB and the bacterial suspension. These four tubes were then incubated at 37°C in a rotary shaker at 150 rev / min for varied time intervals (0, 1, 2, 4, 6, 12 and 24 h). For viable cells counts, in each time interval, a 0.1 ml sample was removed, diluted and spread onto

Table 1. Preliminary antibacterial activity of endophytic fungus (n=2, means).

Parameter	Inhibition diameter zone (mm)			
	Bc	***Sa***	***Pa***	***Ec***
MPR1	33.5±0.71	31.5±0.71	16.5±2.12	47.5±0.71
Negative control	00±00	00±00	00±00	00±00

Ba, *Bacillus cereus* ATCC 10876; Sa, *Staphylococcus aureus* ATCC 25923; Pa, *Pseudomonas aeruginosa* ATCC 27853; Ec, *Escherichia coli* ATCC 25922.

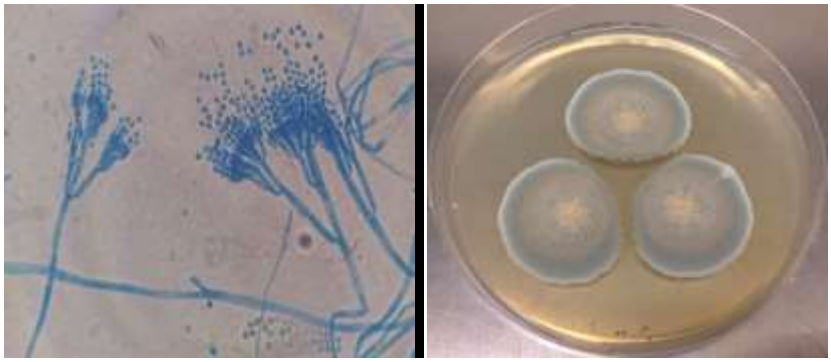

Figure 1. Morphological characteristics of MPR1 isolate.

MHA plates and these plates were then incubated at 37°C for 24 h and the bacterial colonies were counted.

Time kill curve (log c.f.u. / ml vs. time) was drawn for each concentration of extract and control culture, and percentages of dead cells were calculated as the following equation:

Reduction (%) $= V_{0-} V_z / V_0 \times 100$

Where, V0 is the initial viable cell count and V_z is the viable cell count at time z (Ibrahim et al., 2015).

Generally, the bactericidal effect is obtained with a lethality percentage of 90% for 6 h, which is equivalent to 99.99% of lethality for 24 h (Balouiri et al., 2016).

Statistical analysis

All experiments were performed in duplicates, and statistical analysis was carried out using SAS/STAT® 9.2 software. Group comparisons were performed using the two-way ANOVA followed by Student-Newman-Keuls multip-rang test. Results are represented as mean ± standard deviation (SD) and significant effects of treatments were determined by F values (P≤ 0.05).

RESULTS AND DISCUSSION

Isolation and preliminary antibacterial assay of endophytic fungi

The 16 endophytic fungi emerging from the surface sterilized plant segments were preliminary screened against four clinical pathogenic bacteria; this permitted a rapid and qualitative selection of the bioactive micro-organism.

According to the results of this screening, we focused our work on a single fungal isolate MPR1 that inhibited all of these pathogenic bacteria with a good activity (Table 1).

The isolate showed maximum activity against *E. coli* ATCC 25922 with an inhibition diameter zone of 47.5 mm followed by *B. cereus* ATCC 10876, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 with inhibition diameters zones of 33.5; 31.5 and 16.5 mm, respectively.

The results of this preliminary screening are in agreement with the results obtained with endophytic fungi isolated from two medicinal plants *Ocimum sanctum* and *Aloe vera* by Sahani et al. (2017), where all isolated endophytic fungi had broad-spectrum of antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria.

Identification of endophytic fungus MPR1

The fungal isolate MPR1 was characterized by observing colony morphology, pigmentation, growth pattern and sporulation structure, and the fungus has been preliminarily identified up to genus level as *Penicillium* sp. (Figure 1).

Best-scoring maximum likelihood tree based on the Tamura–Nei model calculated using MEGA 7.0 based on ITS sequences showing the relationships of the newly

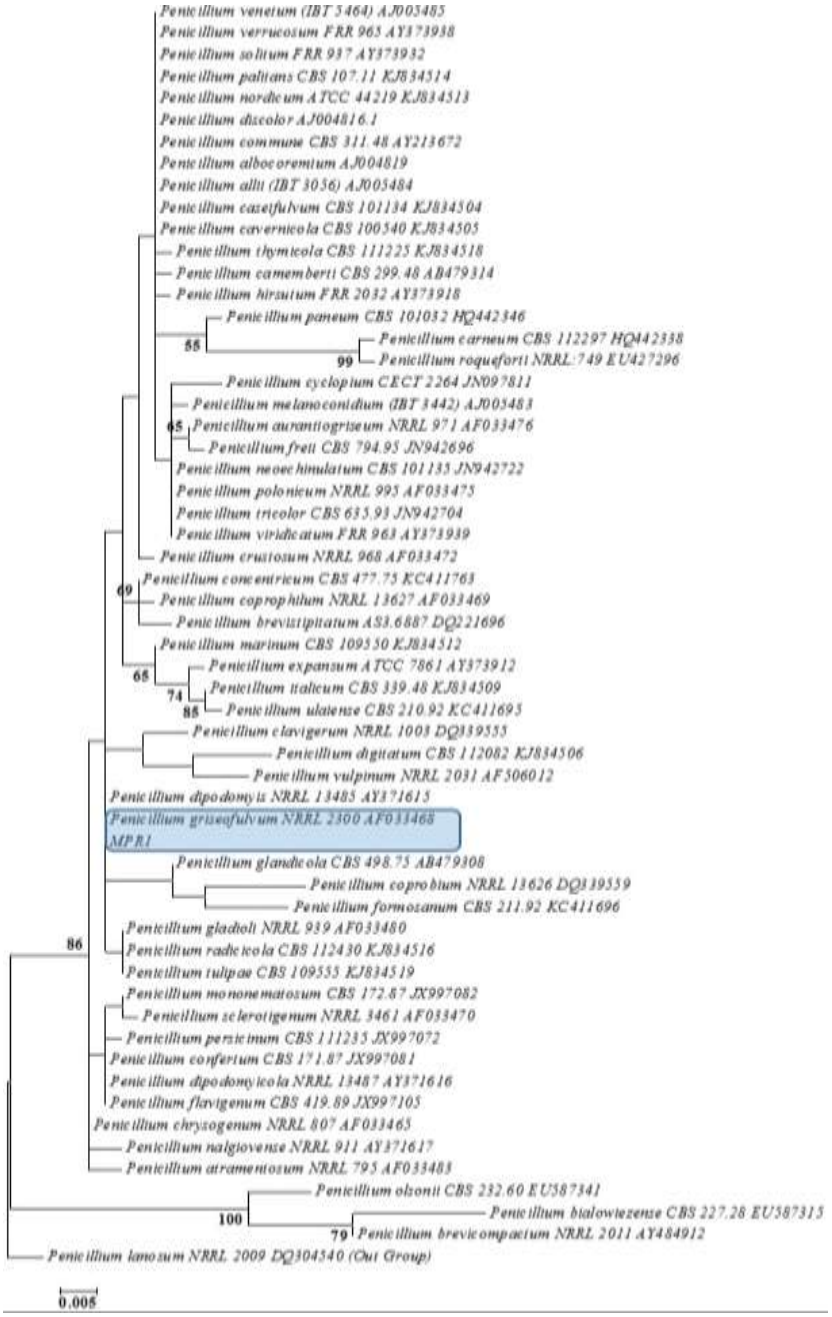

Figure 2. Best-scoring Maximum likelihood tree calculated using MEGA 7.0 based on ITS sequences showing the relationships of the newly generated sequence in this study with previously known taxa in NCBI GenBank.

generated sequences (GenBank accession number MH006592) in this study with previously known taxa in the NCBI GenBank. The scale bar denotes 0.002 substitutions per position. The tree with the highest log likelihood (–1444.9347) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 58 nucleotide sequences. There were a total of 451 positions in the final dataset. Figure 2 shows phylogenetic

Table 2. Antibacterial activity assay from *Penicillium griseofulvum* grown on five culture media (n = 2, means).

Pathogenic bacteria	Inhibition diameter zones (mm)							
	Media							
	Bc	Ef	Sa	MRSA	Cf	St	Pa	Ec
PDA	32.3±1.2	25.3±0.6	39.7±0.6	40.3±0.6	33.7±2.1	35.0±0.0	15.3±1.5	33.0±0.0
NC	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
SDA	0.0	0.0±0.0	21.0±1.0	23.3±0.6	23.0±0.0	28.3±0.6	0.0±0.0	27.7±0.6
NC	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
YEA	25.0±0.0	10.3±0.6	24.3±1.5	26.3±0.6	27.0±1.0	25.3±0.6	0.0±0.0	23.0±0.0
NC	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
MEA	33.0±1.7	29.0±1.0	41.7±0.6	42.7±0.6	38.0±0.0	38.7±0.6	18.3±0.6	38.0±0.0
NC	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
YMEA	18.7±0.6	9.0±0.0	14.7±0.6	15.0±0.0	20.3±0.6	20.0±0.0	0.0±0.0	25.0±0.0
NC	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Bc, *Bacillus cereus* ATCC 10876 ; Ef, *Enterococcus faecalis* ATCC 49452; Sa, *Staphylococcus aureus* ATCC 25923; MRSA, Methiciline-resistant; *Staphylococcus aureus* ATCC 43300 ; Cf: *Citrobacter freundii* ATCC 8090, Sa, *Salmonella typhimurium* ATCC 13311; Pa, *Pseudomonas aeruginosa* ATCC 27853; Ec, *Escherichia coli* ATCC 25922; NC, Negative control.

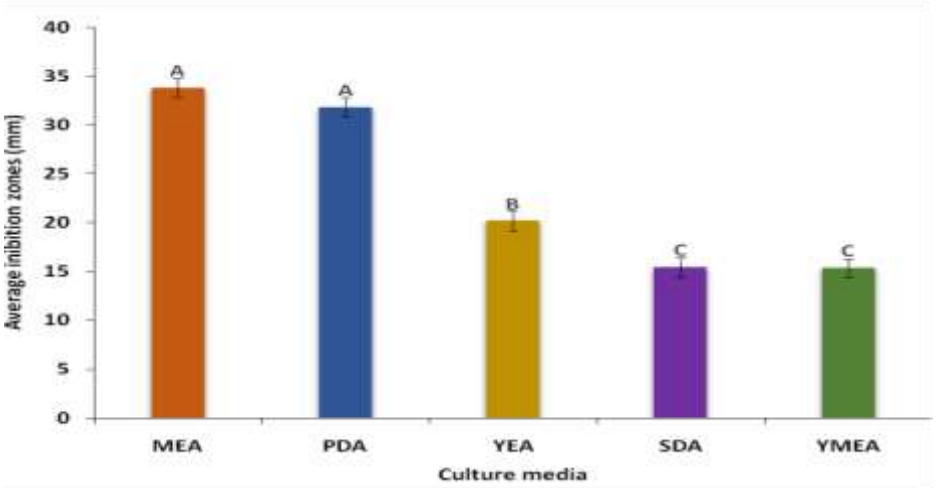

Figure 3. Effect of different culture media on antibacterial activity of *Penicillium griseofulvum* against all pathogenic bacteria.

tree belong to investigated taxa. The endophytic fungus MPR1 was found to be a homolog of type culture *Penicillium griseofulvum NRRL 2300* (AF033468).

The tree is rooted with *Penicillium lanosum NRRL 2009* (DQ304540) (Bootstrap 1000). The species *P. griseofulvum* has been isolated as endophyte from different host plants in different studies. For example, Conti et al. (2012) isolated *P. griseofulvum* from the leaves of *S. verticillata* (L.). Another study resulted in the isolation of *P. griseofulvum* from *Palicourea Tetraphylla* Cham. and Schltdl (Rosa et al., 2010). D'Souza and Hiremath (2015) isolated *P. griseofulvum* from seven different medicinal plants of India.

Selection of suitable culture media

For the optimization of the production of the endophytic fungi bioactive molecules of five different basal media PDA, SDA, YEA, MEA, YMEA were used (Table 2).

According to the statistical analysis (Figure 3), MEA and PDA (no significant differences) were found to be the best media for maximum production of antibacterial bioactive compound with average inhibition zones of 33.8 mm and 31.8 mm, respectively and the best activity was observed against Methiciline-resistant *S. aureus* ATCC 43300 (42.7 mm and 40.3 mm respectively). YEA comes in second position (20.2 mm of average inhibition zones)

Table 3. Antibacterial activity of different crude extracts of *Penicillium griseofulvum* (n = 2, means).

Pathogenic bacteria	Inhibition diameter zone (mm)			Negative control (DMSO)
	Solvents			
	Ethyl acetate	Dichloromethane	n-Hexane	
Bc	40.5±0.7	35.5±0.7	0.0	0.0±0.0
Sa	37.5±0.7	31.5±0.7	0.0	0.0±0.0
MRSA	41.5±0.7	33.0±0.0	0.0	0.0±0.0
Cf	43.0±1.4	36.5±0.7	0.0	0.0±0.0
St	43.5±0.7	36.5±0.7	0.0	0.0±0.0
Ec	45.5±0.7	41.0±1.4	0.0	0.0±0.0

Bc, *Bacillus cereus* ATCC 10876; Sa, *Staphylococcus aureus* ATCC 25923; MRSA, Methiciline-resistant *Staphylococcus aureus* ATCC 43300; Cf, *Citrobacter freundii* ATCC 8090; St, *Salmonella typhimurium* ATCC 13311; Ec, *Escherichia coli* ATCC 25922.

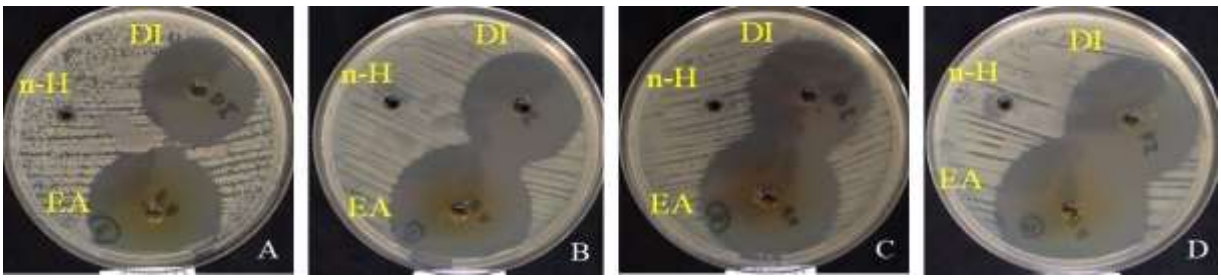

Figure 4. Antibacterial activity of ethyl acetate crude extract of *Penicillium griseofulvum* against pathogenic bacteria. **A.** *Bacillus cereus* ATCC 10876; **B.** *Citrobacter freundii* ATCC 8090; **C.** *Salmonella typhimurium* ATCC 13311; **D.** *Escherichia coli* ATCC 25922; **EA.** Ethyl acetate; **DI.** Dichloromethane; **n-H,** n-Hexane.

with maximal inhibition zone of 27.0 mm obtained against *C. freundii* ATCC 8090, followed by SDA and YMEA (no significant differences), with average inhibition zones of 15.4, 15.3 and 28.3 mm against *S. typhimurium* ATCC 13311, and 25.0 mm against *E. coli* ATCC 25922, respectively.

Data were presented as mean ± SD (n=2), means with the same letter are not significantly different ($p > 0.05$).

Anwar and Iqbal (2017) also studied the effect of growth media on antibacterial activity, and suggested that secondary metabolites are highly affected by the composition of the growth medium. Verma et al. (2017) found that starch was the source of carbon allowing the endophytic fungus to produce the highest amount of bioactive molecules followed by glucose. This is in agreement with the results obtained with MPR1 on PDA and MEA that contain the appropriate concentration of starch and glucose. The same results have been obtained with Mathan et al. (2013), where the PDA was the medium giving the best activity for *Aspergillus terreus.*

Evaluation of antibacterial activity of crude extracts

Table 3 and Figure 4 show the antibacterial activity of different crude extracts of *P. griseofulvum* on agar well diffusion method. All the pathogenic bacteria were inhibited by ethyl acetate and dichloromethane crude extracts with the maximal activity showed against *E. coli* ATCC 25922 (45.5 and 41.0 mm respectively), unlike n-Hexane crude extract which did not inhibit any pathogenic bacteria.

The statistical analysis (Figure 5) showed that ethyl acetate was the best extraction solvent allowing the extraction of bioactive molecules (41.9 mm of average inhibition zones), followed by dichloromethane (35.7 mm of average inhibition zones). These can be explained that the bioactive molecules produced by this fungus are better extracted by polar solvents ethyl acetate and also dichloromethane than apolar like n-Hexane.

Data were presented as mean ± SD (n=2), means with the same letter are not significantly different ($p > 0.05$).

These results correspond to those obtained by Tong et al. (2014) where antimicrobial compounds of *Phomopsis* sp. ED2 and the endophytic fungus of medicinal herb *Orthosiphon stamineus* were mainly present in the ethyl acetate extract. Another study showed that the ethyl acetate extracts of two endophytic fungi isolated from *Ocimum citriodorum* Vis. show better activity than the other extracts (Mu'azzam et al., 2015).

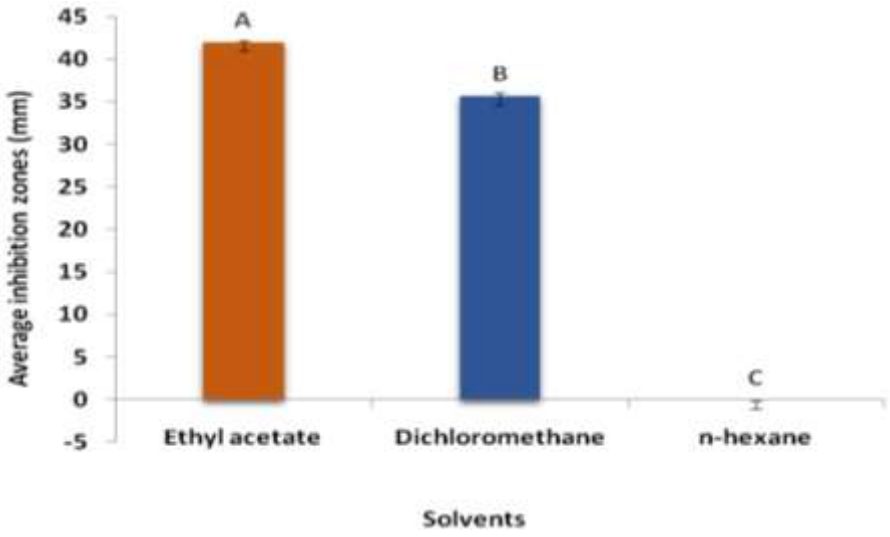

Figure 5. Effect of different extraction solvents on antibacterial activity of *Penicillium griseofulvum* against all pathogenic bacteria.

Table 4. MIC and MBC values (µg / ml) of endophytic fungus *Penicillium griseofulvum* against different pathogenic bacteria.

Pathogenic bacteria	*Penicillium griseofulvum*		Imipenem	Gentamycin	DMSO
	MIC	MBC	MIC	MIC	MIC
Bc	100±0.0	100±0.0	/	0.25±0.0	-
Sa	100±0.0	100±0.0	/	0.50±0.0	-
MRSA	100±0.0	100±0.0	/	0.25±0.0	-
Cf	50±0.0	200±0.0	0.25±0.0	/	-
St	50±0.0	50±0.0	1.00±0.0	/	-
Ec	50±0.0	200±0.0	0.50±0.0	/	-

Bc, *Bacillus cereus* ATCC 10876; Sa, *Staphylococcus aureus* ATCC 25923; MRSA, Methiciline-resistant *Staphylococcus aureus* ATCC 43300; Cf, *Citrobacter freundii* ATCC 8090; St, *Salmonella typhimurium* ATCC 13311; Ec, *Escherichia coli* ATCC 25922; -, inactive.

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC)

MIC and MBC values (Table 4) were recorded for the crude ethyl acetate extract from *P. griseofulvum;* the MIC of this crude extract was of 50 µg / ml for the three Gram-negative pathogenic bacteria (*C. freundii* ATCC 8090, *S. typhimurium* ATCC 13311, *E. coli* ATCC 25922) and of 100 µg / ml for the three Gram-positive pathogenic bacteria (*B. cereus* ATCC 10876, *S. aureus* ATCC 25923, Methiciline-resistant *S. aureus* ATCC 43300).

According to Klepser et al. (1998), if the MBC/MIC ratio is ≤ 4, the extract is bactericidal and if > 4, it is bacteriostatic; and since MBC/MIC ratios obtained for all the test bacteria were ≤ 4 we can say that *P. griseofulvum* crude extract has a bactericidal effect against all pathogenic bacteria.

Broad spectrum antibacterial activity obtained by this endophytic fungus extract has already been observed with extracts of different endophytic fungi such as the endophytic fungi isolated from *Calotropis procera* (Rani et al., 2017).

Gram-negative bacteria are generally more resistant than Gram-positive bacteria and are therefore more difficult to contend, because their outer membrane protects the peptidoglycan cell wall. The results obtained in our study reveal the opposite, and this can be explained by the fact that the mode of action of the active molecules produced by *P. griseofulvum* MPR1 is not on the cell wall but possesses a different mode of action (Yenn et al., 2014). These results correspond to the results obtained with the extracts of other *Penicillium* such as *Penicillium* sp., an endophytic fungus of *Garcinia nobilis* that has been a source of new molecules with antibacterial activity mainly on Gram-negative bacteria (Jouda et al., 2014, 2016b). *Penicillium minioluteum* ED

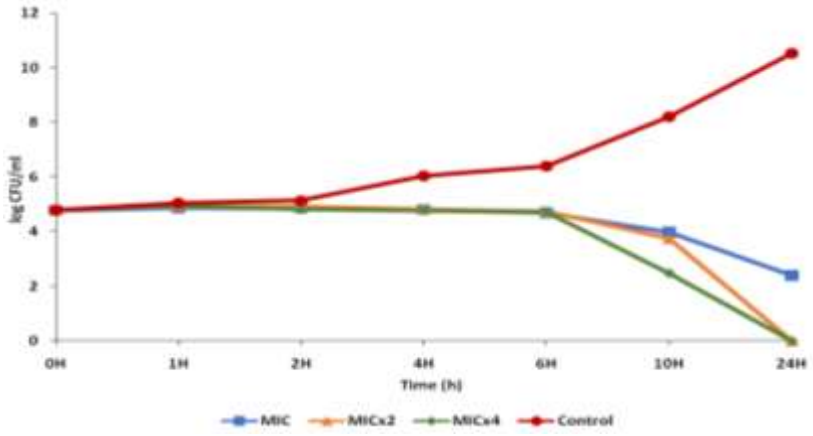

Figure 6. Time kill curve of *Penicillium griseofulvum* ethyl acetate crude extract against Methiciline-resistant *Staphylococcus aureus* ATCC 43300.

24 isolated from *Orthosiphon stamineus* Benth. also show better activity against Gram-negative compared to Gram-positive bacteria (Yenn et al., 2014).

Time kill

The time-kill dynamic process is used for the evaluation of bactericidal or bacteriostatic effect of bioactive molecules; bactericidal activity is indicated by a 3-$\log_{10}$ reduction, the equivalent of 99.9% cell death, and the number of viable cells in the presence of the antibacterial compound (Balouiri et al., 2016).

Killing growth profile of *P. griseofulvum* ethyl acetate crude extract against Methicilline-resistant *S. aureus* ATCC 43300 shows a bactericidal effect at 24 h of incubation; 100% killing of the bacterial cells was achieved for MIC × 2 and MIC × 4 concentrations (Figure 6).

Previously, Ibrahim et al. (2015) reported the bactericidal effect of endophytic fungus *N. sphaerica* CL-OP 30 against Methicilline-resistant *S. aureus* at 24 h. The silver nanoparticules of *Penicillium polonicum* has also demonstrated bactericidal activity against a multidrug-resistant bacterium, *A. baumanii* (Neethu et al., 2018).

Conclusion

In this study, we highlighted the antibacterial potency of endophytic fungi isolated from the medicinal plant, *M. pulegium* L. With preliminary screening we selected one isolate *P. griseofulvum* with a good antibacterial activity. This was confirmed after extraction, where ethyl acetate was the best solvent used for the extraction of bioactive secondary metabolites. The MIC/MBC and time kill of this extract show that it has a bactericidal effect against several bacteria. These promising results push us to want to determine the composition of this extract as well as its active fraction.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Aires A, Marrinhas E, Carvalho R, Dias C, Saavedra MJ (2016). Phytochemical composition and antibacterial activity of hydroalcoholic extracts of *Pterospartum tridentatum* and *Mentha pulegium* against *Staphylococcus aureus* isolates. BioMed Research International pp. 1-11.

Altşchul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Anwar J, Iqbal Z (2017). Effect of growth conditions on antibacterial activity of *Trichoderma harzianum* against selected pathogenic bacteria. Sarhad Journal of Agriculture 33(4):501-510.

Astuti P, Wahyono, Nababan, OA (2014). Antimicrobial and cytotoxic activities of endophytic fungi isolated from *Piper crocatum* Ruiz & amp; Pav. Asian Pacific Journal of Tropical Biomedecine 4(Suppl 2):S592–S596.

Baba Ahmed-Kazi Tani Z, Arlet G (2014). New of antibiotic resistance among Gram-negative bacilli in Algeria. Pathologie Biologie 62(3):169-178.

Balouiri M, Sadiki M, Ibnsouda SK (2016). Methods for *in vitro* evaluating

antimicrobial activity: A review. Journal of Pharmaceutical Analysis 6(2):71-79.
Bashyal BP, Wellensiek BP, Ramakrishnan R, Faeth SH, Ahmad N, Gunatilaka AA (2014). Altertoxins with potent anti-HIV activity from *Alternaria tenuissima* QUE1Se, a fungal endophyte of *Quercus emoryi*. Bioorganic Medicinal Chemistry 22(21):6112-6116.
Chen C, Verkley GJ, Sun G, Groenewald JZ, Crous PW (2016). Redefining common endophytes and plant pathogens in Neofabraea, Pezicula, and related genera. Fungal Biology 120(11):1291-1322.
Chen L, Zhang QY, Jia M, Ming QL, Yue W, Rahman K, Qin LP, Han T (2016). Endophytic fungi with antitumor activities: Their occurrence and anticancer compounds. Critical Reviews in Microbiology 42(3):454-473.
Clinical and Laboratory Standards Institute (CLSI) (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Ninth Edition M07A8. Wayne, PA, USA.
Conti R, Cunha IGB, Siquerira VM, Souza-Motta CM, Amorim ELC, Araújo JM (2012). Endophytic microorganisms from leaves of Spermacoce verticillata (L.): Diversity and antimicrobial activity. Journal of Applied Pharmaceutical Science 2(12):017-022.
Debbab A, Aly AH, Edrada-Ebel R, Wray V, Muller WE, Totzke F, Zirrgiebel U, Schachtele C, Kubbutat MH, Lin WH, Mosaddak M, Hakiki A, Proksch P, Ebel R (2009). Bioactive metabolites from the endophytic fungus *Stemphylium globuliferum* isolated from *Mentha pulegium*. Journal of Natural Products 72(4):626-31.
Demirel R, Sariozlu NY, İlhan S (2013). Polymerase chain reaction (PCR) Terverticillate Penicillium species agricultural soils in Eskişehir Province. Brazilian Archives of Biology and Technology 56(6):980-984.
Dos Banhos EF, De Souza AQL, De Andrade JC, De Souza ADL, Koolen HHF, Albuquerque PM (2014). Endophytic fungi from *Myrcia guianensis* at the Brazilian Amazon: Distribution and bioactivity. Brazilian Journal of Microbiology 45(1):153-161.
D'Souza MA, Hiremath KG (2015). Isolation and bioassay screening of medicinal plant endophytes from Western Ghats forests, Goa, India. International Journal of Advanced Research in Biological Sciences 2(8):176-190.
Fatima N, Mukhtar U, Ul-Haq I, Qazi MA, Jadoon M, Ahmed S (2016). Biological evaluation of endophytic fungus *Chaetomium* sp. NF15 of Justicia adhatodaL.: A potential candidate for drug discovery. Jundishapur Journal of Microbiology 9: e29978.
Hajlaoui H, Trabelsi N, Noumi E, Snoussi M, Fellah H, Riadh K, Bakhrouf A (2009). Biological activities of the essential oils and methanol extract of tow cultivated mint species (*Mentha longifolia* and *Menthapulegium*) used in the Tunisian folkloric medicine. World Journal of Microbiology and Biotechnology 25(12):2227-2238.
Ibrahim D, Chai Lee C, Yenn TW, Zakaria L,Sheh-Hong L (2015). Effect of the extract of endophytic fungus, *Nigrospora sphaerica* CL-OP 30, against the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumonia* cells. Tropical Journal of Pharmaceutical Research 14(11): 2091-2097.
Jouda JB, Kusari S, Lamshöft M, Mouafo Talontsi F, Douala Meli C, Wandji J, Spiteller M (2014). Penialidins A-C with strong antibacterial activities from Penicillium sp.; An endophytic fungus harboring leaves of Garcinia nobilis. Fitoterapia 98(2014):209-214.
Jouda JB, Tamokou JD, Mbazoa CD, Douala-Meli C, Sarkar P, Bag PK, Wandji J (2016a). Antibacterial and cytotoxic cytochalasins from the endophytic fungus *Phomopsis* sp. harbored in *Garcinia kola* (Heckel) nut. BMC Complementary and Alternative Medicine 16:1-9.
Jouda JB, Tamokou JD, MbazoaCD, Sarkar P, Bag PK,WandjiJ (2016b). Anticancer and antibacterial secondary metabolites from the endophytic fungus *Penicillium* sp. CAM64 against multi-drug resistant Gram-negative bacteria. African Health Sciences 16:734-743.
Kadaifciler DG, Demirel R (2017). Fungal biodiversity and mycotoxigenic fungi in cooling-tower water systems in Istanbul, Turkey. Journal of Water and Health 15(2):308-320.
Klepser ME, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA (1998). Influence of test conditions on antifungal time-kill curve results: Proposal for standardized methods. Antimicrobial Agents and Chemotherapy 42(5):1207-1212.
Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7):1870-1874.
Mahboubi M, Haghi G (2008). Antimicrobial activity and chemical composition of *Mentha pulegium* L. essential oil. Journal of Ethnopharmacology 119(2): 325-327.
Mai N, Matainaho T, Rai PP, Barrows LR (2013). Antimicrobial activity of endophytes in six medicinal plants collected in the central province, Papua New Guinea. Pacific Journal of Medical Sciences 11(2):57-69.
Marson Ascêncio PG, Ascêncio SD, Aguiar AA, Fiorini A, Pimenta RS (2014). Chemical assessment and antimicrobial and antioxidant activities of endophytic fungi extracts isolated from *Costus spiralis* (Jacq.) Roscoe (Costaceae). Evidence-Based Complementary and Alternative Medicine 2014:2-10.
Martín JA, Witzell J, Blumenstein K, Rozpedowska E, Helander M, Sieber TN, Gil L (2013). Resistance to Dutch Elm disease reduces presence of xylem endophytic fungi in Elms (Ulmus spp.). PLoS One 8(2):e56987.
Mathan S, Subramanian V, Nagamony S (2013). Optimization and antimicrobial metabolite production from endophytic fungi Aspergillus terreus KC 582297. European Journal of Experimental Biology 3(4):138-144.
Mu'azzam KAAR, Taufiq MMJ, Darah I (2015). Isolation and screening of antibacterial activity of endophytic fungi isolates from *Ocimum citriodorum* Vis. leaves. Australian Journal of Basic and Applied Sciences, 9(28):155-161.
Neethu S, Midhun SJ, Radhakrishnan EK, Jyothis M (2018). Green synthesized silver nanoparticles by marine endophytic fungus *Penicillium polonicum* and its antibacterial efficacy against biofilm forming, multidrug-resistant *Acinetobacter baumanii*. Microbial Pathogenesis 116:263-272.
Pan F, Liu ZQ, Chen Q, Xu YW, Hou K,Wu W (2016). Endophytic fungus strain 28 isolated from *Houttuynia cordata* possesses wide-spectrum antifungal activity. Brazilian Journal of Microbiology 47(2):480-488.
Passari AK, Mishra VK, Gupta VK, Yadav MK, Saikia R, Singh BP (2015). *In vitro* and *in vivo* plant growth promoting activities and DNA fingerprinting of antagonistic endophytic actinomycetes associates with medicinal plants. PLoS One 10(9):1-18.
Petrini O (1986). Taxonomy of endophytic fungi of aerial plant tissue. In: Fokkema NJ, Heuvel J Van Den (Eds.). Microbiology of the Phyllosphere. Cambridge: University Press pp. 175-187.
Pimentel IC, Glienke-Blanco C, Gabardo J, Stuart R M, Azevedo JL (2006). Identification and colonization of endophytic fungi from soybean (*Glycine max* (L.) Merril) under different environmental conditions. Brazilian Archives of Biology and Technology 49(5):705-711.
Powthong P, Jantrapanukorn B, Thongmee A, Suntornthiticharoen P (2013). Screening of antimicrobial activities of the endophytic fungi isolated from *Sesbania grandiflora* (L.) Pers. Journal of Agricultural Science and Technology 15(20):1513-1522.
Rajeswari S, Revathi R, Arvind Prasanth D (2017). Isolation and identification of endophytic fungi from *Terminalia chebula* of eastern Ghats, Tamilnadu. Asian Journal of Pharmaceutical and Clinical Research 10(4):21-23.
Rani R, Sharma D, Chaturvedi M, Yadav JP (2017). Antibacterial activity of twenty different endophytic fungi isolated from *Calotropis procera* and time kill assay. Clinical Microbiology 6:1-6.
Rosa LH, Gonçalves VN, Caligiorne RB, Alves TM, Rabello A, Sales PA, Romanha AJ, Sobral ME, Rosa CA, Zani CL (2010). Leishmanicidal, trypanocidal, and cytotoxic activities of endophytic fungi associated with bioactive plants in Brazil. Brazilian Journal of Microbiology, 41(2):420-430.
Sadrati N, Harzallah D, Zerroug A, Dahamna S, Bouharati S (2013). Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (*Triticum durum*). Journal of Plant Protection Research 53(2):128-136.
Sahani K, ThakurD, Hemalatha KPJ, Ganguly A (2017). Antibacterial activity of endophytes from selected medicinal plants. International Journal of Advanced Research 5(3):2076-2086.

Saraswaty V, Srikandace Y, Simbiyani NA, Jasmansyah, SetiyantoH, Udin Z (2013). Antioxidant activity and total phenolic content of endophytic fungus *Fennellia nivea* NRRL 5504. Pakistan Journal of Biological Sciences 16(22):1574-1578.

Shah M, Deshmukh SK, Verekar SA, GohilA, Kate AS, Rekha V,Kulkarni-Almeida A (2015). Anti-inflammatory properties of mutolide isolated from the fungus *Lepidosphaeria* species (PM0651419). Springerplus 4:1-10.

Shen XY, Cheng YL, Cai CJ, Fan L, Gao J, Hou CL (2014). Diversity and antimicrobial activity of culturable endophytic fungi isolated from Moso bamboo seeds. PLoS One 9(4):e95838.

Singh B, Kaur A (2016). Antidiabetic potential of a peptide isolated from an endophytic *Aspergillus awamori*. Journal of Applied Microbiology 120(2):301-311.

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10(3):512-526.

Tayung K, Barik BP, Jha DK, Deka DC (2011). Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from bark of Himalayan yew. Mycosphere 2(3):203-213.

Teiten MH, Mack F, Debbab A, Aly AH, Dicato M, Proksch P, Diederich M (2013). Anticancer effect of altersolanol A, a metabolite produced by the endophytic fungus *Stemphylium globuliferum*, mediated by its pro-apoptotic and anti-invasive potential via the inhibition of NF-kB activity. Bioorganic and Medicinal Chemistry 21(13):3850-3858.

Tong WY, Zaadah Nurul J, Nurhaida, Tan WN, Melati K, Latiffah Z, Darah I (2014). Antimicrobial activity of Phomopsis sp. ED2 residing in medicinal plant *Orthosiphon stamineus* Benth. Annual Research and Review in Biology 4:1490-1501.

Verma SKR, Lal M, Debnath M (2017). Optimization of process parameters for production of antimicrobial metabolites by an endophytic fungus *Aspergillus* sp. CPR5 isolated from *Calotropis procera* root. Asian Journal of Pharmaceutical and Clinical Research 10(4):225-230.

Xie L, Usui E, Narisawa K (2016). A endophytic fungus, *Ramichloridium cerophilum*, promotes growth of a non-mycorrhizal plant, Chinese cabbage. African Journal of Biotechnology 15(25):1299-1305.

Yao YQ, Lan F, QiaoYM, Wei JG, Huang RS, LiLB (2017). Endophytic fungi harbored in the root of Sophora tonkinensis Gapnep: Diversity and biocontrol potential against phytopathogens. Microbiologyopen 6(3):1-17.

Yenn TW, Ngim AS, Ibrahim D, Zakaria L (2014). Antimicrobial activity of *Penicillium minioluteum* ED24, an endophytic fungus residing in Orthosiphon stamineus Benth. World Journalof Pharmacy and Pharmaceutical Sciences 3(3):121-132.