Research Article

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Antimicrobial activity of secondary metabolites produced by *Aspergillus neobridgeri* isolated from *Pistacia lentiscus* against multi–drug resistant bacteria

Nouari Sadrati[1], Amina Zerroug[1], Rasime Demirel[2], Sabrina Bakli[1] and Daoud Harzallah[1]

[1]Laboratory of Applied Microbiology, Faculty of Natural and Life Sciences, University Ferhat Abbas Sétif 1, 19000, Sétif, Algeria; [2]Department of Biology, Faculty of Sciences, Eskişehir Technical University, Eskişehir, Turkey.

Article Info

Cite this article:
Sadrati N, Zerroug A, Demirel R, Bakli S, Harzallah D. Antimicrobial activity of secondary metabolites produced by *Aspergillus neobridgeri* isolated from *Pistacia lentiscus* against multi-drug resistant bacteria. Bangladesh J Pharmacol. 2020; 15: 82-95.

Abstract

The *in vitro* antibacterial activity of the endophytic fungus *Aspergillus neobridgeri* PLR9 (isolated from the plant *Pistacia lentiscus)* against a wide range of tested bacteria were studied. The maximum inhibitory activity was found on malt extract agar medium with a mean inhibitory zone of 11.3 mm (p<0.05). The ethyl acetate extract showed a significant inhibitory zone of 13.4 mm (p<0.05), followed by dichloromethane 9.1 mm. Whereas, *n*-hexane extract showed no activity. The largest inhibition zones were observed against *Staphylococcus aureus* 3, *S. aureus* 5 and *Escherichia coli* 1 with 21.0 mm of diameter. The MIC's were ranged between 312.5 and 1,250 µg/mL. The MBC's were ≤2500 µg/mL for most Gram positive bacteria and ≥2500 µg/mL for most Gram negative bacteria. Time kill assay study against the *Enterococcus faecium* showed a bactericidal effect at 4x MIC and bacteriostatic against *Acinetobacter baumannii* at different concentrations. Moreover, it is the first report, PLR9 isolated as endophyte and studied for their antimicrobial activity.

Introduction

Endophytic fungi are present in all plant species studied so far and they can be found in the internal tissues of roots, stems, leaves, flowers, fruits or seeds (Tanapichatsakul et al., 2018). They received habitat and nutrients from their hosts and protect their hosts chemically from browsers, insects, biotic and abiotic stresses and improve plant growth of their host (Ibrahim et al., 2018; Malhadas et al., 2017).

Endophytes produce bioactive compounds similar to those in the host plant (Yu et al., 2014). In addition, novel chemical skeletons of secondary metabolites have antimicrobial, antiparasitic, cytotoxic, anti-inflammatory, antitumor, antioxidant and neuroprotective activities (Malhadas et al., 2017).

Due to their ability to produce of a wide range biologically active compounds and their ability to inhibit several pathogens in the plant, animal and human; endophytes from medicinal plants are becoming more interesting to be explored and are considered a promising resource for new bioactive compounds (Atiphasaworn et al., 2017).

Several studies have shown the ability of different species of endophytic fungi to inhibit pathogenic microorganisms, for example; *Aspergillus terreus* and *Aspergillus tubingensis* (Yadav et al., 2016), *Diaporthe terebinthifolii* LGMF907 (de Medeiros et al., 2018), *Phomopsis prunorum* (Qu et al., 2020), *P. griseofulvum* TPL25 (Luo et al., 2015), *Alternaria* GFAV15 (Yadav et al., 2020), *Alternariatenuissima* OE7 (Chatterjee et al., 2019) and *Trichoderma koningiopsis* QA-3 (Shi et al., 2020).

Among 16 fungal strains isolated from the *Erigeron canadensis*, the ethyl acetate extract of strains NPR003 and NPR005 showed the strongest antifungal inhibitory effects (Bai et al., 2017). Bai et al. (2019) isolated six endophytic fungal strains from *Rumexmadaio,* strains L1 and R1 belonged to the *Colletotrichum* and *Fusarium* genera respectively had stronger inhibitory effects on *Staphylococcus aureus*, and the R1 isolate was the source of two known antimicrobial agents dibutylphthalate and beauvericin. Another known metabolite named resorcinol type of lipid (compound 1) was purified from extract of the endophytic fungus *Chaetomium cupreum* isolated from the ornamental plant *Mussaenda luteola.* This compound showed significant antibacterial against *Mycobacterium* with MIC of 6.3 µg/mL, and also exhibited a strong inhibition against both *E. coli* (ATCC 25922) as well as *S. aureus* (ATCC 25923) (Shylaja et al., 2018). The endophytic fungi *Aspergillus terreus* and *Trichoderma virens* associated with Sri Lankan plants have the capacity to produce the compounds butyrolactone I and 9-epiviridol respectively. Both compounds exhibited MIC values in the range 128-256 µg/mL against Gram positive *Bacillus subtilits*, *S. aureus*, methicillin resistant *S. aureus* (MRSA) and *Escherichia coli* (Ratnaweera et al., 2018).

The species of *Pistacia lentiscus* belong to the family Anacardiaceae composed of more than eleven species. Fruits and leaves parts of *P. lentiscus* tree are commonly used in traditional Algerian medicine for the treatment of eczema, paralysis, diarrhea, throat infection, jaundice and asthma. The plant oil is used for the treatment of small wounds, burns, coughs and erythema, respiratory problems of allergic origin and ulcers.

The leaf extracts of *P. lentiscus*, and *P. atlantica* have good antifungal activity and low antibacterial activity (Benhammou et al., 2008).

Endophytic fungi of the plant *P. lentiscus* are not yet studied for their antimicrobial activity. For these reasons, this paper is focused on the screening of antimicrobial activity of bioactive secondary metabolites produced by endophytic fungus PLR9 isolated from *P. lentiscus.*

Materials and Methods

Sampling of P. lentiscus

The fresh leaf, stem and root of *P. lentiscus* were collected from the Mansoura mountain located in the east of Bordj Bou Arreridj (36.052538 N, 4.491563 E), Algeria in March 2014. The identification of the plant was carried out in the Botanic Laboratory, Farhat Abbas University. The plant samples were kept in a sealed plastic bag and returned to the laboratory on the same day and the isolation of the endophytic fungi commenced within 24 hours of the collection of plant samples (Zerroug et al., 2018).

Isolation and preservation of endophytic fungus PLR9

The fungus PLR9 was isolated as a symptomless endophyte from the *P. lentiscus*. The plant samples were washed with running tap water, allowed to dry at room temperature. Before surface sterilization, the plant material was cut into small pieces (approximately 3 cm). The sample fragments were successively surface sterilized by immersion into 70% ethanol (1 min) and 2% sodium hypochlorite (3 min), 70% ethanol for 30 sec and then finally rinsed with sterilized distilled water three times for 2 min. The roots were aseptically placed on the petri dishes containing solid culture medium potato dextrose agar supplemented with penicillin (30 mg/L) and gentamicin (30 mg/L) to suppress the bacterial growth and incubated at 28°C until the outgrowth of endophytic fungi was discerned. Emergent fungi were isolated and inoculated into the fresh potato dextrose agar antibiotic-free medium and incubated at 28°C for 7 days. This step was repeated several times until the pure endophytic fungal stain with uniform colony was obtained. The water washing from the surface-sterilized samples showed no microbial growth on the potato dextrose agar after 10 days of incubation under the same condition. The isolate was maintained by a periodic transfer onto the potato dextrose agar at 4°C for short-term preservation and in sterile distilled water containing 30% v/v of glycerol for long-term preservation (Campos et al., 2015).

Morphological characteristics

The fungus PLR9 was identified initially based on the colony morphology inoculated onto the malt extract agar and potato dextrose agar media and incubated at 28°C for 10 days, after that all characteristics macroscopic and microscopic were noted. The microscopic identification of fungus was carried out by the lactophenol staining technique (Arevalo et al., 2009).

DNA extraction, PCR amplification and sequencing

The PLR9 isolate was inoculated onto the malt extract agar and incubated at 28°C for 5 days. Genomic DNA was extracted after that using the ultraclean microbial DNA isolation kit (MoBio, USA) as described in the manufacturer's protocol, and then the extracted DNA was stored at -20°C. Standard gene regions ITS, BenA and calmodulin were used for molecular identification. ITS region was amplified using primers V9G, 5'-TTACGT-CCCTGCCCTTTGTA-3' (forward) and LS266, 5'-GCATTCCCAAACAACTCGACTC-3' (reverse), while BenA region was amplified using the primers Bt2a-F 5'-GGTAACCAAATCGGTGCTGCTTTC-3' (forward) and Bt2b-R 5'-ACCCTCAGTGTAGTGA-CCCTTGGC-3'(reverse), for the calmodulin the primers CMD5-F 5'-CCGAGTACAAGGARGCCTTC-3' and CMD6-R 5'-CCGATRGAGGTCATRACGTGG-3' were used. Polymerase chain reactions (PCR) were performed using Veriti 96-well thermal cycler (Applied Bio-

systems, USA). The final reaction volume that used was 25 µL, which containing 1 µL of genomic DAN, 2.5 µL of 2.5 µM forward and reverse primers, 2,5 µL of Taq buffer + KCl-MgCl$_2$ (Fermentas), 2.5 µL of 25 mM MgCl$_2$ (Fermentas), 2 µL of 2.5 mM dNP mix, 0,25 of 5 U/µL Taq DNA polymerase (Fermentats), and 11.75 µL of sterile deionized water. The thermal cycler program for ITS was as follows: start step of 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C annealing temperature, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. β-Tubulin and calmodulin, the thermocycling conditions for amplifications had an initial denaturing step of 94°C for 10 min, 36 cycles of 94°C for 5 sec, 58°C (β-tubulin) and 56°C (calmodulin) for 45°C, 72°C for 1 min, followed by a final elongation step at 72°C for 7 min (Visagie et al., 2013; Demırel, 2016).

The PCR products were separated by electrophoresis and then purified and sequenced. The ITS locus was sequenced using V9G, LS266, ITS1–5' TCCGTAGGTG-AACCTGCGG-3' and ITS4–5'TCCTCCGCTTATTGA-TATGC-3', while β-tubulin and calmodulin were sequenced using the same primers as those for the PCR amplification. Sequencing reactions were performed in an automated DNA Sequencer CEQ 8000 Genetic Analyses System.

Phylogenetic analysis

The ITS, β-tubulin and calmodulin gene sequences were disposed into GeneBank Data Library with the acession numbers (MK600510), (MK680531) and (MK-680532) respectively. Thereafter, consensus sequence data of the three loci were blasted against the NCBI GeneBank database. A sequence dataset was created for each locus using newly generated sequences and those loaded from GeneBank. For phylogenetic analysis, the multiple sequence alignment was carried out using MUSCLE software in the MEGA 7 package, followed by manual adjustments. Maximum likelihood analyses were applied to all individual and combined ITS, β-tubulin and calmodulin alignment using MEGA 7 with the Tamurai-Nei model. Support of the internal branches of the trees was assessed by the bootstrap method with 1000 replications. The phylogram is rooted in the *Aspergillus flavus* CBS 100927 (Visagie et al. 2013; Houbraken et al., 2014; Siqueira et al., 2017).

Optimization of optimal culture medium for the production of antimicrobial substances

To optimize the production of antibiotics, five culture media recommended were tested: malt extract agar, potato dextrose agar, Sabouraud dextrose agar, yeast extract sucrose agar and yeast extract malt extract agar. The antimicrobial activity test was carried out by the agar cylinder technique described above against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Citrobacter freundii* ATCC 8090, *Salmonella typhimurium* ATCC

13311, *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC 49452, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, *B. cereus* ATCC 49452 and one yeast *C. albicans* ATCC 1024. After collecting the results, a comparison of the inhibition zone averages was made to determine the best production medium that was used in the next fermentation (Devaraju and Satish, 2011).

Fermentation and crude extracts preparation

The crude extracts of endophytic fungus were obtained using the modified method of Sharma et al. (2016). The mycelial agar plugs with 1 cm in diameter were excised from the periphery of 7-days-old PLR9 culture, and the two plugs were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of malt extract broth medium selected in the previous step. The flasks were incubated at 28°C for 21 days with periodical shaking at 150 rpm. After the complete growth, the cultures were taken out and filtered through sterile cheesecloth to separate the mycelial mass from the fermented broth. The resulting filtrates were extracted three times by sequential solvent extraction procedure using three solvents with different polarity: *n*-hexane, dichloromethane and ethyl acetate. An equal volume of solvents was added to the filtrate, and the flask that contains the mixture was placed on the shaker for 2 hours to allow complete extraction. The filtrate and the solvents were taken in a separation funnel and then kept for one hour till the two immiscible layers formed. Then, the organic layer was recovered and evaporated in a rotavapor ((R-215, BÜCHI Labortechnik AG, Flawil, Switzerland). The fungal extracts were then dissolved in dimethyl sulfoxide, passed through 0.22 µm filtration membrane and kept at 4°C.

Choice of the best solvent for extraction

In order to determine the best solvent for extraction which was used in the following steps: the obtained extracts in the solvents (ethyl acetate, dichloromethane and *n*-hexane) were tested for their antimicrobial activities. The obtained results were then processed statistically. The antimicrobial activity was performed using wells diffusion method against 8 bacteria, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. freundii* ATCC 8090, *S. typhimurium* ATCC 13311, *S. aureus* ATCC 25923, *E. faecalis* ATCC 49452, methicillin-resistant *S. aureus* ATCC 43300, *B. cereus* ATCC 49452 and one yeast *C. albicans* ATCC 1024. Each bacterial strain was grown in plate count agar at 37°C for 18-24 hours to obtain a freshly grown pure culture. Some colonies from each bacterium were suspended in 0.8% NaCl and were mixed for 15 sec to ensure homogeneity. The turbidity was adjusted to match of a 0.5 Mac-Farland standard to have optical density of 0.08-0.1 at 625 nm corresponding to 1 x 10^8 CFU/mL using a spectrophotometer (Jenway 7310, Cole-Parmer). The bacterial suspension was swabbed with a sterile cotton swab evenly in the plate in which 30 µL of sterilized

fungal extract was loaded. Dimethyl sulfoxide was used as a negative control. Petri dishes were incubated at 37°C for 24 hours and the diameters of the clear inhibition zones surrounding the holes were measured with a ruler and recorded in mm (Abiala et al., 2016; Mefteh et al., 2018).

Large-scale fermentation and semi-purification

After the selection of ethyl acetate as the best solvent for extraction, the preparation of the semi-purified ethyl acetate extract was carried out in a similar manner as that was used in the above step. But before concentrating, the ethyl acetate fraction was washed with an equal volume of water to remove the remaining polar constituents and salts. The dried ethyl acetate extract was further partitioned between *n*-hexane and 90% methanol (1:1) to remove fatty acids and other non-polar constituents. The remaining 90% methanol phase was concentrated using a rotary evaporator and finally dissolved in dimethyl sulfoxide (Kumar and Kaushik, 2013).

Antibacterial activity of ethyl acetate extract against multi-drug resistance

Semi-purified ethyl acetate extract fraction was tested for its ability to inhibit the growth of human pathogenic bacteria using the agar well diffusion assay as described above. A wide range consisting of 25 bacteria were used. Dimethyl sulfoxide was used as a negative control, vancomycin and imipenem as a positive control for Gram positive and Gram negative bacteria respectively (Abiala et al., 2016).

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of antimicrobial compound, which is identified as the lowest concentration of this compound at which there is no visible growth of bacteria of fungi. MIC values of PLR9 ethyl acetate extract were determined by broth microdilution technique in 96-well microliter plates with minor modification, following the recommendations of the Clinical and Laboratory Standards Institute (Rani et al., 2017). Muller Hinton broth was made and sterilized using an autoclave. 90 µL of sterile Muller Hinton broth was added to each well of the microliter plate followed by the addition of fungal extract in dimethyl sulfoxide (stock solution 5,000 µg/mL dissolved in $1/10^{th}$ diluted dimethyl sulfoxide in sterile Muller Hinton broth) in the first row of the microliter plate. And then, serial dilutions were performed to get final concentrations ranging from 4.9 to 2,500 µg/mL using the micropipette (A1-A10). The test microorganisms were grown in Muller Hinton broth at 37°C for 18-24 hours and adjusted to 0.5 McFarland standard corresponding to the optical density (OD_{625} 0.08–0.1). The adjusted cultures were then diluted 100 times in Muller Hinton broth and used as inoculum at a final concentration to approximately 10^5 colony forming units (CFU). Each plate had a negative control contained Muller Hinton broth with test microorganisms in column A11 and blank control to check the sterility consisted only of Muller Hinton broth in the column A12. $1/10^{th}$ diluted DMSO was used as a negative control. The plates were incubated at 37°C for 24 hours. After incubation, 10 µL of 0.2% 2,3,5-triphenyl tetrazolium chloride solution was added to each well and then plates were incubated at 37°C for 1 hour. A visible change of color purple to pink indicated the growth of microorganisms.

Determination of minimum bactericidal concentration (MBC) and MIC index

The minimum bactericidal concentration was determined using the results of the MIC assay. The concentrations showing no visible growth of bacteria were identified, 10 µL of the sample from each well was taken and streaking on agar plates to judge the viability. All the plates were then incubated at 37°C for 24 hours, the lowest concentrations of the extract that did

not produce any bacterial growth (prevention the growth of >99.9% microbial cells) on the solid medium were regarded as MBC values for this extract. To determine the efficacy and the effect bactericidal of bacteriostatic on the bacterial growth of ethyl acetate extract, MIC index (MBC/MIC) was calculated. If MBC/MIC is ≤ 4, then it is bactericidal and if MBC/MIC is >4, then it is bacteriostatic (Rakholiya et al., 2015).

Time kill curve studies

Time kill analysis is a method used to measure the change in the number of bacterial colonies in the culture medium upon the addition of the sample over time. The lethal effect of PLR9 extract was performed using time kill assay as described elsewhere (Rani et al., 2017; Perim et al., 2019) with some modifications. Bacterial density of *Enterococcus faecium* 1 and *Acinetobacter baumannii* cultures was adjusted to a 0.5 McFarland standard, after that suspensions were diluted 1/10 time in Muller Hinton broth medium. A volume of 200 µL of bacterial suspension was introduced into 15 mL sterile falcon tubes containing 1800 µL of Muller Hinton broth, with different concentrations of extract fixed at 1× MIC, 2× MIC, and 4× MIC. The final concentration of bacterial suspension yielded was approximately 5×10^5 CFU/mL. Falcon tubes containing 1% dimethyl sulfoxide, bacterial inoculum and Muller Hinton broth without PLR9 extract were used as a positive control. All samples were then incubated at 37°C in a rotary shaker at 150 rpm. A volume of 100 µL of samples was pipetted out from each tube at 0, 1, 2, 4, 6, 12 and 24 hours, diluted 4 serially and 25 µL of diluted samples were spread over freshly prepared Muller Hinton agar and incubated at 37°C for 24 hours. After the incubation period, the emergent bacterial colonies were counted and compared with those of control. The reduction percentage in the total viable count of CFU was counted using the following formula:

$$\%Reduction = \frac{Initial\ count - Count\ at\ x\ interval}{Initial\ count} \times 100$$

The logarithm of the viable counts (CFU/mL) was plotted against time to determine the killing rate. Generally, the bactericidal effect is obtained with a lethality percentage of 90% for 6 hours, which is equivalent to 99.9% (≥3 log_{10}) of lethality after 24 hours of incubation, but if the original inoculum is maintained or the reduction less than 99.9% (≥3 log_{10}) of the total number of CFU/mL in the original inoculum, the effect is bacteriostatic.

Statistical analysis

Statistical analysis was done by SAS/STAT® 9.2 software. Results of the antibacterial activities analysis were done by the one-way ANOVA followed by the Student-Newman-Keuls MULTIP-rank test to compare the averages of inhibition zones. The results were expressed as means ± SD. The difference was considered statistically significant when p<0.05.

Results

Isolation and identification of the endophytic fungus

PLR9 colony exhibited a rapid growth on malt extract agar and potato dextrose agar. The colony diameters after 7 days of incubation were 36-38 and 38-40 mm respectively. Mycelium white and no production of spores on both culture media after 7 days of incubation, the colony reversed yellowish to light brownish on potato dextrose agar and brown on malt extract agar. Extensive sporulation with yellowish-brown color was observed after prolonged incubation (since the 8th day of incubation), Conidia were globose and smooth, in short chains and vesicles spathulate. After 7th day, the fungus begins to produce reddish-brownish pigment in both culture media and became brown at the age on malt extract agar. Exudates secretion was observed only on malt extract agar and no sclerotia detected on both culture media (Figure 1).

The sequenced genes (internal transcribed spacer rDNA: 605 pb), β-tubulin (BenA: 520 pb) and calmodulin (CaM: 600 pb) of PLR9 isolate were disposed in GenBank database under accession numbers MK-600510, MK680531 and MK680532 respectively. The BLAST search using GenBank database of the internal transcribed spacer, BenA and calmodulin sequences showed that PLR9 isolate shared 99% similarities with *A. neobridgeri* NRRL 13078.

The aligned data sets of internal transcribed spacer, BenA and calmodulin sequences for 30 strains including, PLR9 isolate and type or reference strains of related species and an out-group, were used for phylogenetic analysis. Maximum likelihood analyses were applied to all individual and combined genes.

Single and combined genes analyses of sequences revealed similar topologies with minor differences in the support values of the internal nodes. The internal transcribed spacer marker was the least informative, being unable to discriminate between closely related species. Maximum likelihood of tree based on the concatenated genes was used for presenting phylogenies (Figure 2), the tree was rooted in *A. flavus* CBS 100927, bootstrap support from 1000 replicates is shown at the nodes. The phylogeny based on the single and combined gene sequences shows that PLR9 is closely related to the species *A. neobridgeri* NRRL 13078 supported by bootstrap values for likelihood (ML> 99%).

Morphological identification allowed to identify PLR9 isolate at the genus level as belonging to the *Aspergillus* genus. The identification at the species level was then
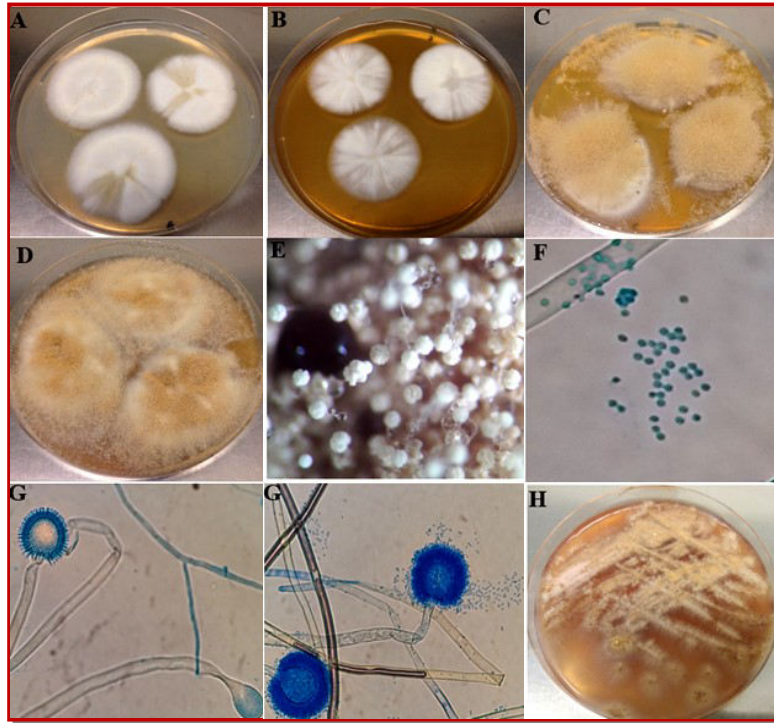
Figure 1: Morphological characteristics of PLR9 isolate: Colony surface on potato dextrose agar (A), malt extract agar (B) after 7 days, potato dextrose agar (C), malt extract agar (D) after 10 days of incubation at 28 °C. Conidiophore + exudates under zoom stereo binocular microscope (E). Conidia (F), Conidiophore, mycelium (G) under microscope, and reddish/brownish pigments production (H)

confirmed by the molecular identification. Based on the morphological and molecular identification, the PLR9 isolate was identified as *A. neobridgeri* belongs to the Circumdati section.

Preliminary antimicrobial screening

The fungus showed a wide spectrum, inhibiting both Gram positive and Gram negative bacteria. PLR9 isolate exhibited moderate activity against *B. cereus* ATCC 49452 with a zone of inhibition of 10 mm, however, against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. The activity was stronger with inhibition zones of 15 and 16 mm respectively. On the other hand, the endophytic isolate showed weak to no ability to inhibit the growth of *P. aeruginosa ATCC 27853* and *C. albicans* ATCC 1024 tested in this study respectively (Table I).

Choice of optimal culture medium and solvent for the production and extraction of antimicrobial substances

To improve and select the best culture medium that allows the best production of the molecule bioactives, five culture media were tested; malt extract agar, potato dextrose agar, Sabouraud dextrose agar, yeast extract sucrose agar and yeast extract malt extract agar. The antimicrobial activity test was conducted by the agar cylinder technique. The maximum of the average of inhibition zones (11.3 mm) was recorded in malt extract agar medium and it was significantly higher than the other used media (p<0.05). In the second position, the

potato dextrose agar medium showed an average of inhibition zones of 5.3 mm followed by yeast extract sucrose agar medium with an average of 1.2 mm. While Sabouraud dextrose agar and yeast extract malt extract agar showed the lowest activity (Figure 3).

Based on the preliminary screening of medium selection, only one medium was selected for metabolites production. PLR9 was fermented in malt extract broth and the produced secondary metabolites were extracted using three solvents, ethyl acetate, dichloromethane and *n*-hexane. Crude extract from ethyl acetate has shown the highest mean of inhibition zone of 13.4 mm with a significant difference at p<0.05, followed by dichloromethane that had a moderate antibacterial activity of 9.11 mm (Figure 4). The yeast *Candida albicans* is still resistant to both extracts. Whereas, using *n*-hexane extract, no activity had been shown against all the tested microorganisms.

Antibacterial activity of ethyl acetate extract against multidrug-resistant bacteria

Ethyl acetate crude extract of PLR9 isolate was found to have significant antibacterial activities against Gram positive bacteria followed by moderate activity against Gram negative. The largest inhibition zones were observed against *S. aureus* 3, *S. aureus* 5 and *E. coli* 1 with 21 mm of diameter. In contrast, 9 mm was observed against *K. pneumoniae* 1 and *K. pneumoniae* 2 as the
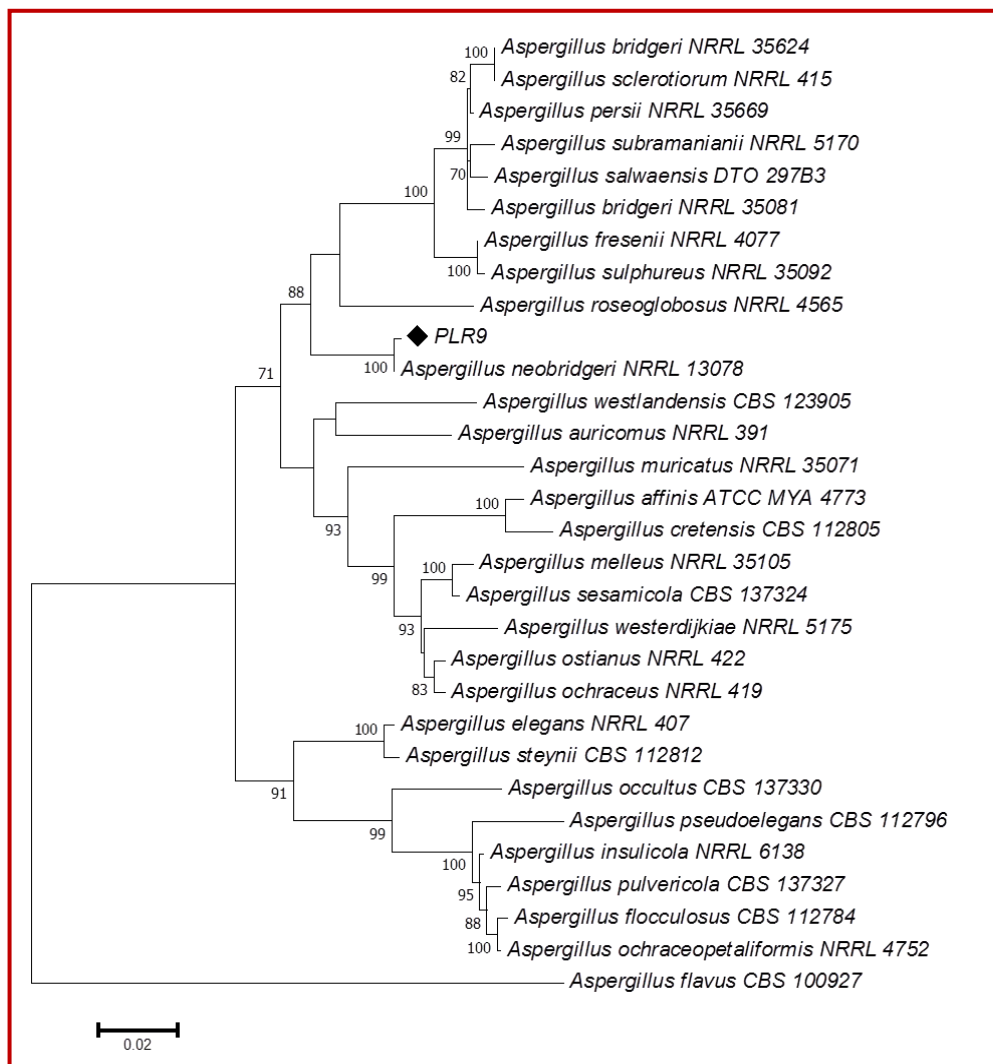
Figure 2: Phylogenetic tree generated by Maximum Likelihood analysis based on the combined alignment of ITS, BenA, and CaM sequences data. The tree is rooted with *A. flavus* CBS 100927. Maximum likelihood bootstrap values MLBS≥70 % are given at the nodes

weakest zone. However, the strains *P. aeruginosa* 2 and *P. aeruginosa* 3 are the most resistant bacteria (Table II). The comparison of means of inhibition zones shows that the Gram positive have a greater sensitivity to the ethyl acetate extract (17.96 mm) with respect to that of Gram negative bacteria (11 mm).

The MIC's obtained in this study were ranged between 312.5 and 1250 µg/mL. The MBC's were ≤ 2500 µg/mL for most Gram positive bacteria, but for most Gram negative bacteria were ≥2500 µg/mL. The MBC/MIC ratio was found always ≤4 for all Gram positive bacteria, which showed that the effect of ethyl acetate extract was bactericidal. On other hand, the extract had bacteriostatic effect (MBC/MIC ≥ 4) against most Gram negative bacteria, in particular *K. pneumoniae* 1, *K. pneumoniae* 2, *P. aeruginosa* 2 and *P. aeruginosa* 3 which confirms the resistance founded in the precedent part (Table III).

Time kill curve studies

Time kill assay was performed over a period of 24 hours with the *E. faecium* 1 and *A. baumannii* being exposed to MIC, 2x MIC and 4x MIC values of ethyl acetate extract of PLR9 isolate. A graph was plotted between the logarithmic number of CFU/mL and time (Figure 5). The bactericidal effect is obtained with a percentage of 99.9% of lethality for 24 hours, but if the original inoculum is maintained or the reduction of less than 99.9% of the total number of CFU/mL in the original inoculum, the effect is bacteriostatic. For *E. faecium* 1, and after 24 hours of contact, time kill assay showed >99.9% of reduction at 4x MIC, but no significant reduction observed at MIC and 2x MIC concentrations. The time kill assay for *A. baumannii*, any reduction was observed at the MIC and 2x MIC concentrations, the maximum reduction noted was 23.7% at 4x MIC value. Therefore, the effect of fungal

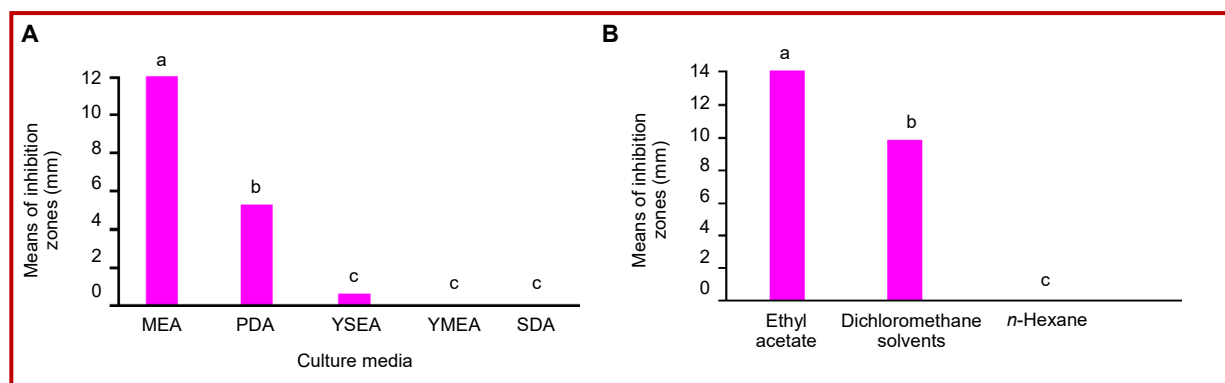| Table I	
Zone of inhibition of endophytic fungi PLR9 against test microorganisms	
Test microorganism	Inhibition zones (mm)
E. coli	16
P. aeruginosa	8
B. cereus	10
S. aureus	15
C. albicans	0

Figure 3: Effect of culture media (A) and solvent used (B) on antibacterial activity of PLR9 isolate, means with the same letter are not significantly different at (p<0.05); MEA (malt extract agar), PDA (potato dextrose agar), YSEA (Yeast extract sucrose agar), YMEA (yeast extract malt extract agar), SDA (Sabouraud dextrose agar); superscript "a, b, c" means with the same letter are not significantly different at p<0.05

extract at 4x MIC concentration was bactericidal for *E. faecium* 1 and bacteriostatic against *A. baumannii* at all different concentrations, this confirms the resistance of Gram negative bacteria found during the first and second screening and MIC part.

Discussion

Based on the morphological identification, the PLR9 isolate was identified as *Aspergillus* sp. Phylogenetic analysis of sequence parts of ribosomal RNA, BenA and CaM genes indicated that PLR9 is a member of Circumdati section and very close to the *A. neobridgeri* species supported by bootstrap values for likelihood (ML >99 %).

In all previous studies, *A. neobridgeri* has never been isolated from plants as endophyte. This is the first time, and in this study, where it was isolated from *P. lentiscus*. This species was first characterized in 2004 by Frisvad et al. (2004), it was isolated from soil in Nebraska, U.S.A., and has been described and validated as new species. However, various species of the *Aspergillus* genus have been isolated from different plants. *Aspergillus flavipes* Y-62 from *Suaeda glauca* (Akhter et al., 2018), *Aspergillus tennesseensis* from inner tissue of an unidentified marine alga (Li et al., 2018).

Aspergilus flavus QQSG-3 isolated from *Kandelia obobata* (Wu et al., 2018), *Aspergillus* sp. from *Trpterygium wilfordii* (Qi et al., 2018). *Aspergillus nidulans* from *Nyctanthes arbor-tristis* (Sana et al., 2018). Cheng et al. (2018), they are found that the genus *Aspergillus* was among the most dominating endophytic fungi isolated from *Paeonia lactiflora*.

Endophytic fungi have been isolated from almost every plant species (over 400,000) examined to date (Mmbaga et al., 2018). In this study, we used all parts of *P. lentiscus* for isolation of endophytic fungi, but PLR9 isolate was isolated only from roots. *A. neobridgeri PLR9* isolate may be of origin from the soil of the rhizospheric part and has some degree of specificity to both plant and the organ (James et al., 2012).

For the choice of the optimum culture medium, the malt extract agar was the best medium allowing the highest production of bioactive secondary metabolites. These results support the finding of Singh et al. (2012) and Kumari et al. (2018) who found malt extract agar as the best medium. On the other hand, Bhagat et al. (2016), (Thamilvanan et al. (2018), Deka and Jha, (2018) and Toghueo et al. (2018) reported the potato dextrose agar as the best medium for the maximum growth and production of metabolites. Kalyani et al. (2016) found that the maximum growth and metabolite production

Table II			
Antibacterial evaluation of PLR9 ethyl acetate extract against test bacteria			
Zone of Inhibition (mm)			
Test microorganisms	PLR9 extract	Positive control	Negative control
Gram positive bacteria		Vancomycin 30	DMSO
Bacillus cereus	16.5 ± 2.1	19	0
Micrococcus luteus	19.5 ± 0.7	27	0
Microbacterium yannicii	16.5 ± 0.7	46	0
Staphylococcus aureus 1	18.5 ± 0.7	20	0
Staphylococcus aureus 2	19 ± 0	21	0
Staphylococcus aureus 3	21 ± 0	21	0
Staphylococcus aureus 4	19.5 ± 0.7	20	0
Staphylococcus aureus 5	21 ± 0	19	0
Enterococcus faecalis 1	17 ± 0	19	0
Enterococcus faecalis 2	14 ± 0	18	0
Enterococcus faecalis 3	12 ±0	20	0
Streptococcus pyogenes	21 ± 0	Non-detectable	0
Gram negative bacteria		Imipenem 10	DMSO
Citrobacter freundii	16.5 ± 2.1	30	0
Acinetobacter baumannii	12.5 ± 0.7	0	0
Pseudomonas aeruginosa 1	9.5 ± 0.7	24	0
Pseudomonas aeruginosa 2	0 ± 0	0	0
Pseudomonas aeruginosa 3	0 ± 0	0	0
Proteus mirabilis	18 ± 0	0	0
Salmonella typhimurium	0 ± 0	22	0
Escherichia coli 1	21 ± 1.4	30	0
Escherichia coli 2	16 ± 1.4	31	0
Escherichia coli 3	16 ± 0	29	0
Escherichia coli 4	15.5 ± 0.7	21	0
Klebsiella pneumoniae 1	9 ± 0	18	0
Klebsiella pneumoniae 2	9 ± 0	17	0
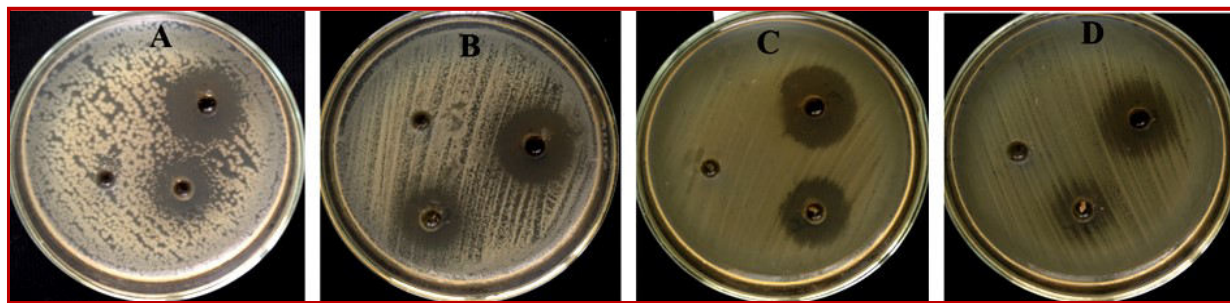
Figure 4: Antibacterial activity of extracts of PLR9 isolate, *B. cereus* (A), Methicillin-resistant *Staphylococcus aureus 2* (B), *E. coli* (C) and *C. freundii* (D)

by *Aspergillus niger* when Sabouraud dextrose agar is used. In the present study, ethyl acetate was found to be the best suitable solvent for maximum extraction of bioactive molecules. Many similar results were reported earlier which correlates with this work. Goutam et al. (2016) reported that among the three organic solvents used, the crude metabolites of *Aspergillus terreus* (JAS-2) extracted using ethyl acetate

Table III		
MIC and MBC of ethyl acetate fraction against bacteria		
Pathogenic bacteria	Ethyl acetate extract of PLR9 isolate		
	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
Gram-positive bacteria			
Bacillus cereus	312.5	1250	4
M. luteus	625	625	1
Microbacterium yannicii	156.3	312.5	2
Staphylococcus aureus 1	625	625	1
Staphylococcus aureus 2	625	2500	4
Staphylococcus aureus 3	625	1250	2
Staphylococcus aureus 4	625	625	1
Staphylococcus aureus 5	625	625	1
Enterococcus faecalis 1	625	625	1
Enterococcus faecalis 2	625	1250	2
Enterococcus faecalis 3	625	2500	4
Enterococcus faecalis 4	625	625	1
Enterococcus faecium 1	625	2500	4
Enterococcus faecium 2	625	2500	4
Streptococcus pyogenes	312.5	312.5	1
Streptococcus australis	625	625	1
Gram-negative bacteria			
Citrobacter freundii	312.5	1250	4
Acinetobacter baumannii	312.5	1250	4
Pseudomonas aeruginosa 1	625	1250	2
Pseudomonas aeruginosa 2	1250	>2500	>2
Pseudomonas aeruginosa 3	625	>2500	>4
Pseudomonas mirabilis	312.5	2500	8
Salmonella typhimurium	625	>2500	>4
Escherichia coli 1	312.5	1250	4
Escherichia coli 2	312.5	2500	8
Escherichia coli 3	312.5	2500	8
Escherichia coli 4	312.5	1250	4
Klebsiella pneumoniae 1	625	>2500	>4
Klebsiella pneumoniae 2	625	>2500	>4

showed maximum cell inhibition against pathogenic bacteria. Similarly, results reported by Singh et al. (2015) regarding antibacterial activity of the endophytic fungus *Alternaria* sp., crude extract from ethyl acetate showed very good results compared to other solvents.

The antibacterial activity obtained by plug agar method for the PLR9 isolate may be due to the secretion of diffusible extracellular metabolites in the agar medium, these compounds having a specific effect against bacteria but no against yeast. This technique is easy and widely used to detect non-volatile compounds produced by microorganisms (Sadrati et al., 2013; Hamzah et al., 2018). It has been clearly reported that the antagonistic properties are largely influenced by the quality of the medium and the type of organisms. The biosynthesis of antibiotic substances was found to be largely influenced, both quantitatively and qualitatively by the composition of the medium (Selvamohan et al., 2016; Abdelghani, 2017). The maximum zone of inhibition that was recorded with malt extract agar medium indicate that their ingredients induced and which may serve as precursors for the biosynthesis of bioactive secondary metabolites by the isolate PLR9. The selection of the suitable solvent system highly depends on the specific nature of the bioactive compound targeted. Different solvents have ability to extract the bioactive compounds from culture broth, where; hexane and diethyl ether to isolate non-polar compounds, while ethyl acetate, dichloromethane, and chloroform were used to extract semi-polar compounds (Djamaan et al., 2018). Ethyl acetate was recognized as the best organic solvent which could be used to extract the fungal bioactive metabolites (Bhagat et al., 2016). These results indicate that the antibacterial bioactive compounds are semi-polar in nature and can be extracted using ethyl acetate and dichloromethane, but none of the antimicrobial compounds present in the non-polar fraction (*n*-hexane).

Ethyl acetate extract of *A. neobridgeri PLR9* showed significant activity against Gram positive and Gram negative bacteria. No study has been done on the
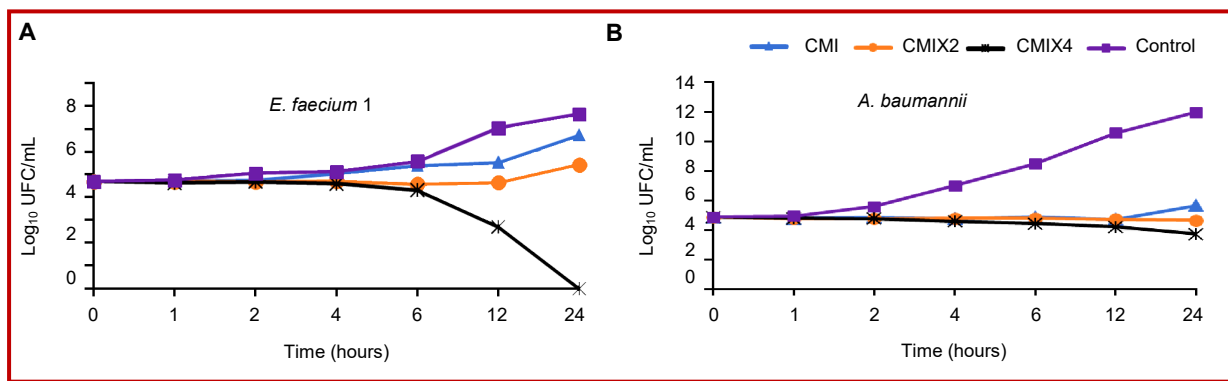

Figure 5: Time kill curve of ethyl acetate crude extract of PLR9 against *E. faecium* 1 (A) and *A. baumannii* (B) at MIC, 2x MIC and 4x MIC

antibacterial activity of *A. neobridgeri* species. However, several works have been reported on the isolation and antibacterial activity of different species of endophytic fungi belonging to the genus *Aspergillus*. The extracts of these fungi were exhibited broad spectrum activity against Gram positive and Gram negative bacteria including multi-drug-resistant strains similarly with the results obtained in the present study. Bezerra et al. (2015) found that the endophytic *Aspergillus ochraceus* exhibited a high range of antibacterial activity, and inhibited the growth of four out of 10 pathogenic bacteria. Six different species of *Aspergillus* were isolated from *Calotropis procera* exhibited a good antibacterial activity against all tested bacteria but it was lower against Gram negative bacteria (Rani et al., 2017; Murali et al., 2017). Yadav et al. (2016) reported that extract of two endophytic fungi *A. terreus* and *A. tubingensis* are able to inhibit the growth of both *K. pneumoniae* and *P. aeruginosa* multi-drug resistant strains. Results of MIC values of the present study are in agreement with those obtained from the previous study of *Aspergillus* sp. extract, where MIC values ranged from 500 to 4000 µg/mL (Leong et al., 2018). The resistance phenomenon of Gram negative bacteria has generally been observed and which results in a bacteriostatic effect of the extracts during the time kill kinetics study, this has been shown by previous studies made on extracts of *A. nomius* (Rani et al., 2017) and those of *A. terreus* MP15 (Yin et al., 2015)

The reason for the different sensitivity between Gram positive and Gram negative bacteria could be ascribed to the morphological and chemical structure differences of their cell wall (Sadrati et al., 2013). Cell wall of Gram negative bacteria have an outer phospholipid membrane, lipopolysaccharide components and periplasmic space. This constitutes makes the cell wall less permeable to lipophilic solutes and provide an addition degree of protection against antibiotics targeting the cell wall (Yin et al., 2015). Whereas, the cell wall of Gram positive bacteria is less complex and can be more susceptible, this due to the presence of only outer peptidoglycan layer which is not an effective permeability barrier, and may facilitate the infiltration of hydrophobic compounds (Zainuddin et al., 2010). *Aspergillus* species are able to synthesize several molecules active against pathogenic bacteria (Ma et al., 2016 ; Gombodorj et al., 2017; Monggoot et al. 2018 ; Yang et al. 2018). *A. neobridgeri* and according to the results obtained by Frisvad et al. (2004), Visagie et al. (2014) and Varga et al. (2015) regarding the mycotoxins production using standards, this species has been found able to synthetizing penicillic acids, xanthomegnins, insulicolides, aspochracin and sclerotides. The significant antibacterial activity obtained in the present study may be due to the presence one or more these molecules in the crude extracts especially penicillic acid that which has been found active against bacteria (Ezzat et al., 2007; Varga et al., 2015; Nguyen et al., 2016), or due to other molecules that not yet characterized.

Conclusion

A. neobridgeri PLR9, an endophytic fungus isolated from the plant *P. lentiscus* was able to inhibit the growth of broad number of human pathogenic bacteria.

Financial Support

Conflict of Interest

Authors declare no conflict of interest

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Author Info
Daoud Harzallah (Principal contact)
e-mail: harzallahdaoud@univ-setif.dz