



DIVERSITY AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH THE ALGERIAN MEDICINAL PLANT *Artemisia herba alba*

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ABSTRACT. The study aimed to isolate, identify and investigate the antibacterial activity of endophytic fungi from the Algerian medicinal plant *Artemisia herba alba*. The isolation and identification allowed us to get ten (10) fungal strains belonging to four genera: *Fusarium*, *Penicillium*, *Alternaria*, and *Aspergillus*. The antibacterial activity of the fungi was evaluated by the agar-fungi disk diffusion method against five human pathogenic bacteria. All isolated fungal strains exhibited antibacterial activity against at least one of the bacteria tested with an inhibition zone (IZ) ranging from 7.5 to 25 mm. However, *Fusarium sp* and *Penicillium sp1* have the highest antibacterial activity with an IZ of 23.5 and 25 mm against *S. aureus* and *B. subtilis*, respectively. Therefore, the secondary metabolites of fungi were extracted after a fermentation by the butanol solvent. The antibacterial activity of the extracts was determined and results showed that *Penicillium sp2* has the highest effect against *Bacillus subtilis* with IZ of 27.3 mm. In conclusion, the present study is the first report about the antimicrobial activity of endophytic fungi residing in *A. herba alba*. This study helped to prove the use of *A. herba alba* in traditional medicine. In fact, the fungus *Penicillium* and *Fusarium* demonstrated the ability to produce bioactive agents with an antibacterial potential.

Keywords: *Antibacterial activity, Artemisia herba alba, Bioactive compounds, Endophytic fungi.*

INTRODUCTION

The phenomenon of bacterial resistance to antibiotics and the risks of toxicity associated with the massive use of drugs and synthetic antibiotics, have led researchers to find other natural sources providing new drugs that would be effective and with low toxicity [1, 2]. Endophytes are microorganisms that live in plant tissues, establishing mutual relationship, and are very different from those found on the surface of the plant. The most appropriate and collective of most definitions show endophytes as all the microorganisms that, during all or part of their life cycle, live in the internal tissues of host plants without causing any diseases [3, 4]. Several studies have been carried out regarding the biodiversity, taxonomy, ecology and the symbiotic relationship with the host. The symbiotic associations of endophytic fungi with plants produce beneficial substances for the host. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites [5, 6]. So far, studies reported a large number of bioactive compounds isolated from endophytic fungi which belonging to several structural classes like alkaloids, peptides, steroids, terpenoids,

phenols, quinones and flavonoids. Many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from the endophytic fungi, which being used for the treatment of a number of diseases [7, 8]. Therefore, most fungi-derived pharmaceuticals have been sourced from endophytic fungi, such as *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* [9, 10]. A large number of antibacterial metabolites are identified in many species of endophytic fungi throughout the world and some have been approved as drugs, such as cephalosporins and fusidic acid. Several mechanisms are often employed to explain the antibacterial potential of secondary metabolites of fungi such as inhibition of cell wall synthesis, depolarize the cell membrane, inhibition of protein synthesis, inhibition of nucleic acid synthesis, and metabolic pathways inhibition in bacteria [11, 12]. Over the last few decades, a great interest has developed in searching for antimicrobial drugs from natural products because of the belief that drugs derived from endophytic fungi are safe compared with synthetic drugs that may have adverse effects besides their high cost. Medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products. *Artemisia herba alba* is one of the oldest medicinal plants belonging to the genus *Artemisia* known for its therapeutic properties: antifungal, antibacterial, insecticidal and other biological activities [13]. In this context, we are interested in this study to the isolation, identification and antibacterial activity of the endophytic fungi from the Algerian medicinal plant *A. herba alba*.

MATERIALS AND METHODS

Materials

The plant material was collected in February, 2019 from the Maadid region, M'sila, Algeria. The plant was authenticated by Dr. Djamel SARRI (Department of biology and physiology of plants, University of Mohamed Boudiaf, M'sila, Algeria). After plant selection, disease-free parts of the stems were excised with a sterile scalpel and placed in sterile plastic bags for storage at 4°C until use.

Five pathogenic bacterial strains were used in the study that comprised of *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enterica* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633). All the strains were the American Type Culture Collection (ATCC), which were obtained from Laboratory of Biology, University of Mohamed Boudiaf, M'sila (Algeria).

Several chemicals are used in our study such as; Sodium hydroxide (NaOH), Hydrochloric acid (HCl), Agar, Sodium hypochlorite (NaOCl), Dimethylsulfoxide (DMSO), Mueller-Hinton agar (MHA), Potato dextrose agar (PDA) Potato dextrose broth (PDB). All chemicals and microbial culture mediums were purchased from ISOLAB GmbH Company.

Methods

Isolation of the endophytic fungus

The endophytes were isolated using a modified method described by Khan *et al.*, 2017 [14]. In order to ensure a good isolation of endophytic fungi, several mature, fresh and healthy plants were chosen. The samples were put in sterile plastic bags to transport them to the laboratory, where they were kept at a temperature of 4 °C until use, and must not exceed 24 hours [15].

To remove the epiphytes, the plant material (stems and leaves) was thoroughly washed using sterile water for 7-8 minutes, then the surface-disinfected by soaking in 70 % ethanol for 1 minute and 0.3 % hypochlorite sodium (NaOCl) solution for 4 min. Finally, the samples were putted back into 70 % ethanol for 30 seconds, rinsed three times with sterile water for 1 minute and dried on sterile filter paper. Then, small fragments of plant material were placed into petri dishes containing potato dextrose agar (PDA) culture medium supplemented with antibiotic gentamicin (10 mg/100 ml). The petri dishes were incubated at 25 °C and were monitored every day to check the growth of fungal colonies [16]. After 7-10 days of incubation, the fungi were transferred several times until obtaining pure isolates. The purity of each fungal culture was assessed by examination of colony morphology and the final pure cultures were transferred to PDA slant tubes. The percentage of colonization was calculated according to Dhayanithy *et al.*, 2019 [17], using the following formula:

Percentage of colonization (%) = (number of colonized segments) × 100/ (total number of segments).

Identification

The fungi were identified up to the genus level based on their morphological and microscopic characteristics such as colony topography, color, and growth pattern. Slides were prepared from cultures by hyphal tipping, stained with lactophenol aniline blue and viewed under microscope Fish *et al.*, 2015 [18].

Antibacterial activity of the endophytic fungi

The antibacterial activity of the endophytic fungi was determined against five pathogenic bacterial by agar-fungi disk diffusion method [19]. In this method, agar-fungi disks of 6 mm in diameter of young mushroom culture (7 days) cultured on the PDA medium were placed in the Mueller-Hinton agar (MHA) media plate inoculated with test bacteria. After incubation for 24 h at 37 °C, the inhibition zone (IZ) diameter around the cylinders was measured in mm. These measurements were interpreted according to the following categories (+++): IZ >20 mm as high activity, (++) : 10 mm >IZ >20 mm as moderate activity and (+): IZ<10 mm as low activity [20].

Fermentation and extraction of the secondary metabolites

Endophytic fungi which exhibited antibacterial activity in the preliminary screening were subjected to secondary metabolites extraction. The production of secondary metabolites was carried using liquid surface fermentation for 15 days at 28° C, where endophytic fungal colony was cultured in Erlenmeyer flasks (500 ml) containing 250 ml of potato dextrose broth (PDB) medium with intermediate shaking. After the incubation period, the culture broths were filtered through muslin cloth and the filtrates were centrifuged for 15 minutes at 5000 rpm. The supernatant was separated and extracted thrice with an equal volume of butanol (1:1, v/v). The filtrate extracts were concentrated using a rotary flash evaporator and dry crude secondary metabolites were obtained [22].

Antimicrobial activity of the extracts

The antibacterial activity of the extracts was performed by disk diffusion method according to Benslama *et al.*, (2017) [23]. Each fungal extract was dissolved in DMSO to give a concentration of 15 mg/ml. 10 µl of each extract were deposited using a micropipette on the sterile disks (6 mm in diameter) placed on the surface of the MHA media previously inoculated with the bacterial suspensions (0.5 McFarland). After

incubation for 24 h at 37 °C, the IZ diameters surrounding the disk was measured. Additionally, for comparative purposes, gentamicin antibiotic was used as positive control. After incubation of 24 h at 37 °C, the mean IZ diameters was recorded and standard error was calculated.

Statistical analysis

Experimental results were expressed as mean \pm SD of triplicate. The data were analysed by Student's *t*-test to determine statistical significance and $p < 0.05$ was considered as significant. All statistical analysis and graphing of data were performed using Graph pad prism 7.00 software.

RESULTS AND DISCUSSION

Isolation, purification and identification of the endophytic Fungi

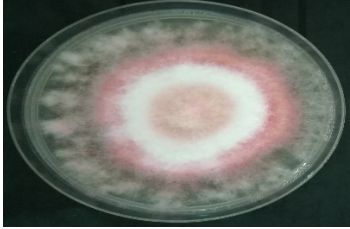
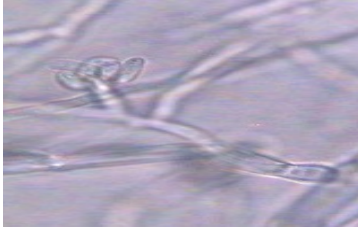
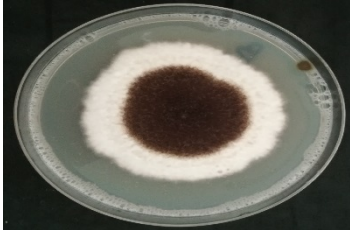
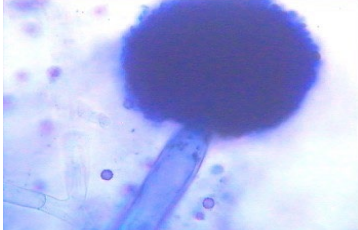






After 7 to 10 days of incubation, ten (10) different fungal strains were isolated from 22 segments of studied plant, which allowed us to get a colonization frequency of 22 %. This percentage close comparable to that obtained by Gashgari *et al.* (2016) [24], where they confirms that only one plant can be colonized by several endophytic fungi. The isolation and identification allowed to successfully identified ten fungal strains belonging to four genera: Fusarium, Penicillium, Alternaria and Aspergillus (Table 1). Two of the ten isolates (20 %) belong to the genus Aspergillus (isolates B and J). The Aspergillus genus is very diverse genus with 180 species, some of which have commercial and medical value [25]. This genus is appears as colonies of color first white, then yellow, then black, fluffy to powder, and flat relief. Under a microscope, it appeared partitioned thalli with a long, unclanged conidiophore bearing phialides on which globular conidia are inserted (Table 1). Aspergillus has already been isolated as an endophyte from *Artemisia nilagirica* by Myrchiang *et al.*, 2014 [26].



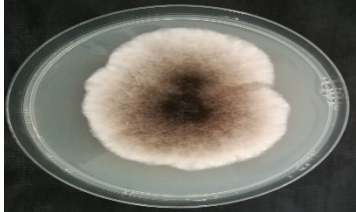

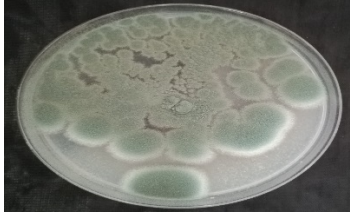
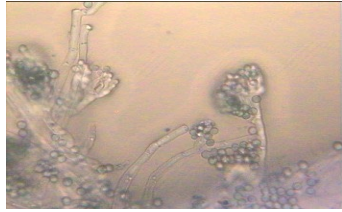
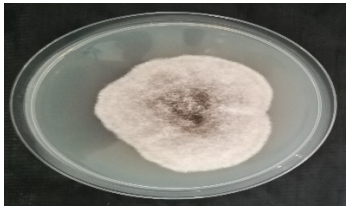

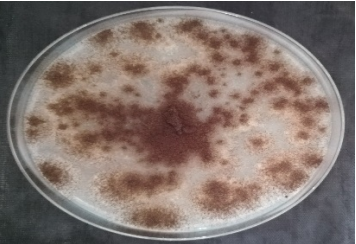
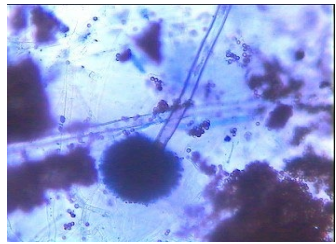
In addition, there are two species belong to the Penicillium genus (20%) (isolates F and H). This genus has colonies of white color then green blue or pinkish with a powdery texture. Under microscope, it shows septate thalli with a branched conidiophore where the phialides are arranged in whorls at their extremities. The Penicillium genus includes more than 200 species, some of which are used in the cheese fabrication; it has also been isolated from Indian medicinal plants *Nyctanthes arbor-tristis* and from other plants [27].

Four isolates (40 %) were identified from the Alternaria genus (Isolates C, D, G, and I). This genus shows conidia of ovoid and septate appearance, produced and borne by conidiophores in the form of thick, brown filaments. Their spores are multicellular and they are divided by septa (or septas) transverse and/or longitudinal. This ubiquitous genus has more than 299 species that are already isolated as endophytes from several plants such as *Nyctanthes arbor-tristis* and *Camptotheca acuminata* [27, 28].

Only one isolate (10 %) belongs to the Fusarium genus has been identified (isolate A). This genus includes more than 1000 species, such as Fusarium sp that been isolated from *Artemisia annua* and other plants [26, 27, 29]. The endophytic form of *Fusarium sp*, has also been isolated from *Artemisia annua* and other plants [28, 29]. The main morphological character of Fusarium is the presence of fusiform and septate macroconidia. The last isolate (10 %) is a sterile mycelium (E isolate), it did not form any form of sexual or asexual spores. Therefore, its identification is difficult. Many sterile fungi have been isolated as endophytes from different plants, e.g. from *Cinnamomum mercadoi* and even isolated from *Retama raetam* [29].

Table 1. Morphology, microscopic characters and classification of endophytic fungal strains.

Isolate	Macroscopic characters	Microscopic characters	Classification
A			Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Monaliaceae Order: Moniliales Genre: <i>Fusarium</i> Species: <i>Fusarium sp</i>
B			Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Moniliaceae Order: Moniliales Genre: <i>Aspergillus</i> Species: <i>Aspergillus sp1</i>
C			Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Dematiaceae Order: Moniliales Genre: <i>Alternaria</i> Species: <i>Alternaria sp1</i>
D			Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Dematiaceae Order: Moniliales Genre: <i>Alternaria</i> Species: <i>Alternaria sp2</i>
E			Sterile mycelium

F			<p>Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Moniliaceae Order: Moniliales Genre: <i>Penicillium</i> Species: <i>Penicillium sp1</i></p>
G			<p>Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Dematiaceae Order: Moniliales Genre: <i>Alternaria</i> Species: <i>Alternaria sp3</i></p>
H			<p>Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Moniliaceae Order: Moniliales Genre: <i>Penicillium</i> Species: <i>Penicillium sp2</i>.</p>
I			<p>Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Dematiaceae Order: Moniliales Genre: <i>Alternaria</i> Species: <i>Alternaria sp4</i></p>
J			<p>Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Moniliaceae Order: Moniliales Genre: <i>Aspergillus</i> Species: <i>Aspergillus sp2</i></p>

Antibacterial activity of the endophytic fungi

The antibacterial activity of endophytic fungi was determined and the results showed that all the isolated fungi have an antibacterial effect on at least one bacteria, with an IZ diameters ranged between 7.5 and 25 mm (Table 2). Endophyte *Penicillium sp1* showed significant antibacterial activity against *B. subtilis* with an IZ of 25 mm. Other endophytic fungi like *Aspergillus sp1* and *Alternaria sp3* recorded moderate antibacterial activity

(Table 2). The species *Alternaria sp2*, *Fusarium sp*, *Penicillium sp1* and *Penicillium sp2* showed an antibacterial effect against all the bacteria tested with an IZ ranging between 10.5 to 23.5 mm. Moreover, *Alternaria sp3* showed moderate antibacterial activity against one bacteria with IZ of 13.00 mm. Four active fungi were submitted to fermentation and secondary metabolites extraction and then to determine their antibacterial activity. However, the antibacterial activity of extracts was determined. The results showed that *Penicillium sp2* has the highest effect with an IZ of 27.3 mm against *Bacillus subtilis*. This result confirms the results obtained by Marcellano *et al.*, (2017) [29], who found the same effect of *Aspergillus* isolated from *Cinnamomum mercadoi* endemic plant of the Philippines. *Fusarium* genus was a rich source of various compounds with antibacterial activities. Among these compounds, Beauvericin, isolated from *F. oxysporum*, which is associated with *Cinnamomum kanihirai*, has been reported to be toxic to methicillin-resistant *S. aureus*, which is one of the agents against which there is an urgent need for new antibiotics [29]. *P. aeruginosa* seems the least affected by the ten fungi studied followed by *S. enterica* that it presents by its resistance a critical situation that threatens human health today. Endophytic fungi are excellent sources of new bioactive natural products with potential for exploitation in medical, agricultural and pharmaceutical fields [30].

Table 2: Antibacterial activity of fungi expressed as inhibition zone diameter (Values were expressed as mean±SD; n=3; (-): inactive).

Fungi	Bacteria				
	<i>E. coli</i> ATCC 8739	<i>S. aureus</i> ATCC 25923	<i>P. aeruginosa</i> ATCC 27853	<i>B. subtilis</i> ATCC 6633	<i>S. enterica</i> ATCC 14028
<i>Alternaria sp1</i>	7.5±0.71	12.5±0.71	-	18.0±2.83	8.5±0.71
<i>Alternaria sp2</i>	10.5±2.12	20.5±2.12	9.50±0.71	21.0±1.41	7.5±0.71
<i>Alternaria sp3</i>	-	-	-	13.0±1.41	-
<i>Alternaria sp4</i>	-	12.50±0.71	-	9.5±0.71	-
<i>Aspergillus sp1</i>	-	-	-	14.5±0.71	-
<i>Aspergillus sp2</i>	17.0±0.00	-	10.5±0.71	18.5±0.71	7.5±0.71
<i>Fusarium sp</i>	12.5±0.71	23.5 ±0.71	11.5±0.71	20.5 ±0.71	11.5±0.71
<i>Sterile mycelium</i>	11.5±0.71	16.5±0,71	-	17.5±0.71	9.5±0.71
<i>Penicillium sp1</i>	15.00±2.83	20.5±0.71	11.5 ±0.71	25.00±0.00	11.5±0.71
<i>Penicillium sp2</i>	15.50±0.71	23.0±1.41	11.5±0.71	23.5 ±0.71	10.5±0.71
Gentamicin	26.5	42.5	27.5	40.5	33.0

Antimicrobial activity of the extracts

The extracts of endophytic fungi showed a more or less important antibacterial activity, where the IZ ranging from 6.7-27.3 mm (Table 3). Interestingly, the extract of *Penicillium sp2* showed a strong antibacterial activity against on *B. subtilis* with an IZ of 27.3 mm. In addition, the antibiotic reference gentamicin has shown greater antibacterial effect compared with the extracts; with an IZ ranging from 26.5-42.5 mm. However, the extract of *Alternaria sp2* showed less antibacterial activity against *P. aeruginosa* and *S. enterica* with an IZ of 6.7 mm (Table 3). Moreover, it was remarkable that *Aspergillus sp1* hasn't any effect on the tested bacteria; exception *B. subtilis*.

Table 3. The antibacterial activity of fungi extracts expressed as inhibition zone diameter (Values were expressed as mean±SD; n=3; (-): inactive).

Fungi	Bacteria				
	<i>E. coli</i> ATCC 8739	<i>S. aureus</i> ATCC 25923	<i>P. aeruginosa</i> ATCC 27853	<i>B. subtilis</i> ATCC 6633	<i>S. enterica</i> ATCC 14028
<i>Alternaria sp2</i>	11.3±0.15	20.7±0.06	7.6±0.67	20.3±0.61	6.7±0.06
<i>Aspergillus sp1</i>	-	-	-	15.3±0.06	-
<i>Fusarium sp</i>	17.0±0.30	22.7±0.40	16.7±1.67	26.3±0.47	8.0±0.00
<i>Penicillium sp2</i>	11.7±0.15	17.0±0.17	11.3±1.13	27.3±0.31	8.7±0.06
Gentamicin	26.5	42.5	27.5	40.5	33.0

The results showed that the two genera *Fusarium* and *Penicillium* have an impressive antibacterial activity against Gram-positive bacteria than Gram-negative (**Fig. 01**), which seem more resistant, in the same results recorded by Marcellano *et al.*, (2017) [28]. The results showed the strongest antibacterial effect was recorded with the extracts of *Fusarium*, which confirms the results obtained by the agar-fungi disk method. Moreover, no-significant difference was recorded between the antibacterial effect of fungi and their extracts; except *Alternaria sp2*. The difference in the sensitivity between Gram-negative and Gram-positive bacteria may be due to the variation in their cell wall structure. The resistance of Gram-negative bacteria against antibiotics like penicillin originates from the secretion of the lactamase enzyme in the periplasmic space between the thin outer membrane and the cytoplasmic membrane [31]. Furthermore, many studies have shown that the anti-bacterial effect of the extracts relates mainly to the structure and the chemical properties of the components of these extracts [32].

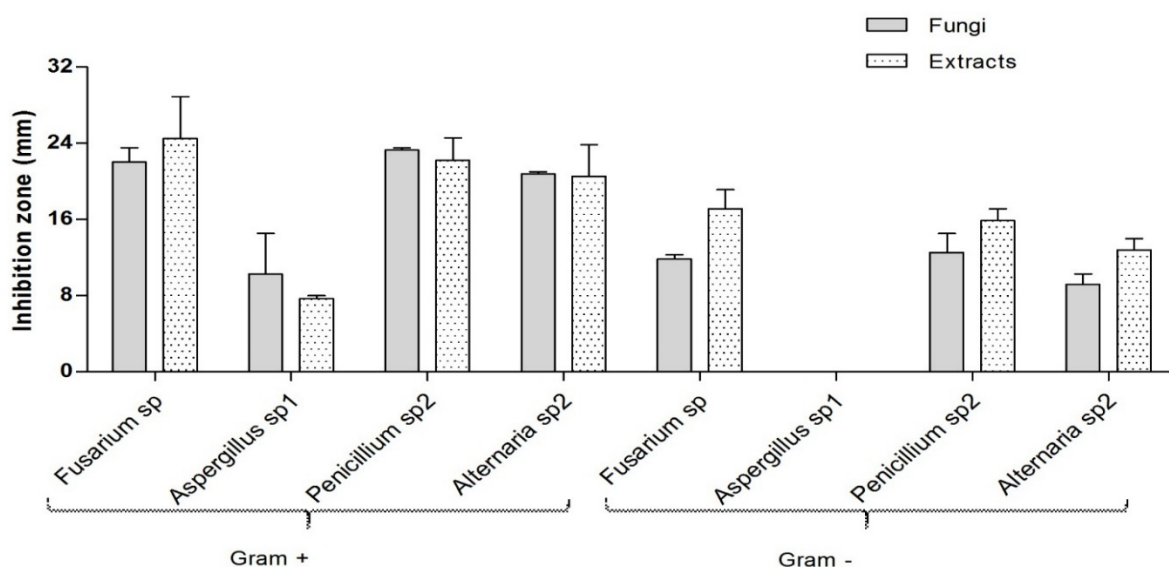


Fig.1. Comparison between the antibacterial effect of fungi and their extracts against Gram-negative bacteria (*E. coli* ATCC 8739, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853) and Gram-positive bacteria (*B. subtilis* ATCC 6633 and *S. enterica* ATCC 14028).

CONCLUSION

The present study is the first report about the antimicrobial activity of endophytic fungi residing in *A. herba alba*. This study helped to prove the use of *A. herba alba* in traditional medicine. In fact, the fungus *Penicillium* and *Fusarium* demonstrated the ability to produce bioactive agents with antibacterial potential, and may provide a new lead in the pursuit of new biological source of drug candidates.

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Conflict of Interest: The authors declare that there is no conflict of interests.

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