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Isolation, Characterization and Antibacterial Activity of Endophytic Fungi from *Marrubium vulgare* L.

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ABSTRACT

The aim of this work is the isolation, identification and evaluation of the antibacterial activity of endophytic fungi from the Algerian medicinal plant *Marrubium vulgare* L. The isolation of endophytic fungi is carried out by the method of sterilization of surface to eliminate epiphytes then incubation of the fragments treated according to a precise protocol. The identification of the grown isolates allowed us to obtain three fungal strains belonging to three genera: Fusarium, Alternaria and Chaetomium, which are generally of the Deuteromycete phylum. The antibacterial capacity of the fungi was tested against five human pathogenic bacteruim using the agar-fungi disk diffusion method. With an inhibition zone (IZ) spanning from 7.5 to 25 mm, all isolated fungal strains showed antibacterial activity against at least one of the bacteria tested.. However, *Fusarium sp* has the highest antibacterial activity with an IZ of 19 and 24 mm against *S. aureus* and *B. subtilis*, respectively. Finally, our results clearly confirm that the medicinal plant *M. vulgare* L. presents a reservoir of endophytic fungi, which can be used in various fields, especially pharmaceutical fields.

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Introduction

The emergence of antibiotic resistance in bacteria, as well as the dangers of toxicity associated with the widespread use of medicines and synthetic antibiotics, has prompted researchers to look for new natural sources of effective and low-toxic medications (Sevindik et al., 2017; Korkmaz et al., 2021). The increase in the resistance of bacteria to antibiotics is a serious worldwide problem which has directed the search for the identification of new biomolecules with broad antibacterial activity and possessing low toxicity (Sagar et al., 2019). The development of antibiotic resistance has become a global problem as bacteria have acquired a variety of resistance mechanisms to cope with harsh environmental conditions (Sagar et al., 2019). Resistance sites are variable between bacterial species and they are classified into several pathways. Within the same bacterial strain, several different resistance mechanisms can be found (Bouyaha et al., 2017). Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissue without causing symptoms/diseases known to occur anywhere in plants. Endophytes have been shown to enhance the ability of plants to tolerate abiotic and biotic stresses existing in mutuality with host plants

Endophytes are microorganisms that live in plant tissues, establishing relationships with each other, and are very different from those found on the surface of the plant. The most appropriate and collective of most definitions shows endophytes as the set of microorganisms which, during all or part of their life cycle, live in the internal tissues of host plants without causing disease (Padhi et al., 2013; Praptiwi et al., 2018). Several studies have been conducted regarding biodiversity, taxonomy, ecology and symbiotic relationship with the host. The symbiotic associations of endophytic fungi with plants produce substances beneficial to the host. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites (Padhi et al., 2013; Praptiwi et al., 2018). So far, studies have reported a large number of bioactive compounds isolated from endophytic fungi belonging to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinones and flavonoids. Many valuable bioactive compounds with antimicrobial, insecticidal, antioxidant, cytotoxic and anticancer activities have been successfully discovered from endophytic fungi, which are used for the treatment of a number of diseases (Joseph and Priya, 2011;

Gouda et al., 2016; Kına et al., 2021; Uysal et al., 2021; Akgül et al., 2022). A large number of antibacterial metabolites are identified in many species of endophtic fungi around the world and some have been approved as drugs, such as cephalosporins and fusidic acid (Ebrahimi et al., 2010; Reygaert, 2018).

In recent decades, great interest has grown in the search for antimicrobial drugs from natural products due to the belief that drugs derived from endophytic fungi are safe compared to synthetic drugs which may have adverse effects in more than their high cost. In the interest of natural product drug discovery, the exploration of medicinal plants for endophytic fungi is of great interest. Statistics over the past three decades reveal that approximately 50% of new drugs have been formulated from derivatives of natural products (Sevindik, 2021; Adeleke and Babalola, 2021). Medicinal plants are known to harbor endophytic fungi that are thought to be associated with the production of pharmaceuticals. Bioactive natural compounds produced by endophytes show promising potential utility in the fields of human health and safety, although there is still a large demand from the pharmaceutical industry for synthetic products for economic and time-consuming reasons. Human health issues, such as the development of drug resistance in human pathogenic bacteria, fungal infections and life-threatening viruses, claims of new therapeutic agents for the effective treatment of diseases in humans, plants and animals that are currently unsatisfied (Pimentel et al., 2011; Pehlivan et al., 2021).

M. vulgare is one of the oldest Algerian medicinal plants belonging to the genus *Marrubium* known for its therapeutic properties: antifungal, antibacterial, insecticide and other biological activities (Aćimović et al., 2020). In this context, we are interested in this study to: Isolation, purification, identification and antibacterial activity of endophytic fungi from the medicinal plant *M. vulgare.*

Materials and Methods

Materials

The plant materials were collected in February, 2019 from the from the region of Berhoum M'sila, Algeria. The plant was authenticated by Dr. SARRI Djamel (Department of biology and physiology of plants, University of 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.

Bacteria

Five pathogenic bacterial species were used in the study, which includes two Gram-positive bacteria and three Gram-negative bacteria. The used bacterial strains are referenced as American Type Culture Collection (ATCC). The strain are: *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 8739, *Salmonella enterica* ATCC 14028, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923.

For the chemicals; several chemicals are used in our study such as; Butanol, Ethanol, 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.

For the equipment: Spectrophotometer (SHIMADZUUVmini-1240), Incubator (Memmert), Water bath (Memmert), Autoclave (Pbinternational), Centrifuge (SIGMA), Rotary evaporator (BUCHI R-210).

Figure 1. *Marrubium vulgare* L.

Methods

Isolation of endophytic fungus

In order to ensure a good isolation of endophytic fungi, it is necessary to choose matured, fresh and healthy plant material. For this purpose, several healthy and mature plants were chosen and the samples were taken randomly from different locations on the plants, and put in sterile plastic bags to transport them to the laboratory. The samples thus collected are preserved at a temperature of 4°C while waiting to be used, and must not exceed 24 hours (Chirane et al., 2020).

The endophytes were isolated using a modified method described by Khan et al., (2017). The material was thoroughly washed in sterile water for 7-8 minutes, the surface-disinfected by soaking in 70% ethanol for 1 minute and 0.3% hypochlorite sodium (NaOCl) solution for 4 min. The samples are putted back into 70% ethanol for 30 seconds, rinsed three times with sterile demineralized water for 1 minute each time and dried on sterile filter paper to remove the epiphytes. Small fragments of inner tissues and needles were placed into petri dishes containing aqueous PDA, supplemented with antibiotic gentamicin (10 mg/100 mL) and incubated at 25 °C until fungal growth was initiated. To verify the effectiveness of the sterilization and to confirm that the isolates came from the internal tissues, aliquots from the third wash were spread on the PDA agar, the absence of fungi on the media indicates that the surface sterilization was well done and that any epiphytic fungi was eliminated (de Siqueira et al., 2011). After several days of incubation, the purity of each fungal culture was assessed by examination of colony morphology. After purifying the isolates several times as described above, the final pure cultures were transferred to PDA slant tubes. As controls, uncut, surface-disinfected,

and non-disinfected pieces were also placed on the same agar to check for contaminated fungi.

Finally, after incubation each mushroom grows will be transplanted several times until obtaining pure cultures. The percentage of colonization is calculated according to Dhayanithy et al., 2019, using the following formula:

PC= (NCS) ×100/(TNS)

PC : Percentage of colonization
NCS : number of colonized segments
TNS : total number of segments

Identification of isolated fungi

The fungi were identified based on morphological characteristics of fungi isolated in their pure state according to the methods described by Fish et al., (2015), taking into account their macroscopic or morphology characters (color, colony appearance) from 7-day cultures on PDA medium and microscopic (thallus form and spores).

Antibacterial activity of isolated endophytic fungi

To assess the antibacterial activity of endophytic fungi, the agar cylinder method (also known as the double diffusion culture technique) was used. All isolated endophytic fungi were screened for their antibacterial activity based on the diffusion of their metabolites on MH agar.

The selected bacterial strains were revived in tubes containing nutrient broth using a flamed Pasteur pipette and incubated at 37° C. for 24 hours before being used in the potency tests. From each tube of nutrient broth showing cloudiness, a Petri dish containing nutrient agar was streaked and incubated at 37°C for 24 hours. After incubation, a few well-isolated and perfectly identical colonies were scraped off with the aid of a platinum loop, then they were discharged into 5 ml of sterile physiological water. The bacterial suspension must be well homogenized and the turbidity afterwards has been adjusted to 0.5 McFarland, which corresponds to an absorbance between 0.08 and 0.1 nm at a wavelength of 620 nm (Devraju and Satish, 2011). The bacterial suspension was inoculated using a sterile swab onto Petri dishes containing the MH medium. The swab was soaked in the bacterial suspension, then it is wrung out by rotating it on the internal wall of the tube in order to discharge it as much as possible. The MH medium was rubbed with serrated streaks over their entire agar surface from top to bottom, each time turning the dish 60° (Devraju and Satish, 2011). Then, agar-fungi disks of 6 mm in diameter of young mushroom culture (7 days) cultured on the PDA medium were placed in the MHA media plate inoculated with test bacteria. After incubation for 24 h at 37 °C, the IZ diameter around the cylinders were 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 (Chirane et al., 2020).

Results and Discussion

Isolation, purification and identification of endophytic fungi

After a culture of 7 days or more than a few days, the verification of the sterilization test was positive; no fungus has grown in the boxes; which shows that any fungus emerging from the segments comes from the internal tissues of the plant. While the culture of the segments made it possible to obtain 4 fungal isolates of different aspects, which allowed us to estimate a colonization percentage of 88%, a percentage confirms that a single plant can be colonized by several endophytic fungi.

The isolates obtained were subcultured several times on PDA agar in order to obtain pure cultures allowing their identification, which was possible thanks to the identification key of Philippe Dufresne (2018).

The identification of fungi is based on macroscopic, microscopic criteria after isolation and culture on culture media. The macroscopic criteria are based on the observation of the colonies and their front and back color, their size, their relief, their appearance (filamentous, sticky), their transparency (opaque, translucent), the shape of the contours and the pigmentation. The microscopic criteria are based on the morphological aspect of the different fungal structures: the type of thallus (septate or not), the color of the hyphae (dark or light), the shape of the spores, the origin of the spores (endogenous or exogenous), the shape of the heads (brush-shaped, aspergillus) (Aouati and Chebil, 2018). By taking into account their macroscopic and also microscopic characteristics according to 7-day cultures, the results were found to be appropriate (Table 1).

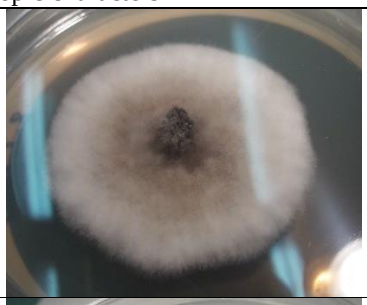
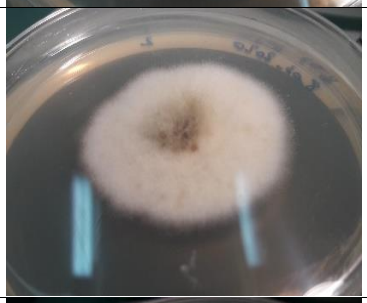
Two of the four isolates obtained belong to the genus Altenaria (isolate A and B) which is a genus of deuteromycete fungi of the Dematiaceae family. This genus contains a large number of species (more than 60) parasites or saprophytes.

Only one isolate (C) belongs to the genus Fusarium has been identified from the family Monaliaceae, a genus that has more than 1000 species. The endophyte form of *Fusarium sp.* has also been isolated from *Artemisia annua* and *Aleppo pine* (Zhang et al., 2012; Abdellaoui and Debih, 2019). Many endophytic fungi have been recognized as sources of novel metabolites of pharmaceutical importance. They also have the potential to synthesize various bioactive metabolites can be used directly or indirectly as a therapeutic agent against many diseases (Vasundhara et al., 2019).

A number of metabolites related to endophytic fungi have been discovered from various sources which are known to possess a range of activities as antibacterial, antiviral and anticancer agents (Deshmukh et al., 2018).

A single isolate belongs to the genus Chaetomium, which comprises between 80 and 100 species according to the documents consulted: the taxonomic data vary considerably for this genus. The best known and most studied species is *Chaetomium globosum.*

Table 1. The fungal strains identification

Isolate	Macroscopic characters	Classification
A		Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Dematiaceae Order: Moniliales Genre: Altenaria Species: *Alternaria sp1*
B		Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Dematiaceae Order: Moniliales Genre: Altenaria Species: *Alternaria sp2*
C		Kingdom: Mushrooms Division: Deuteromycotina Class: Hyphomycetes Family: Monaliaceae Order: Moniliales Genus: Fusarium Species: *Fusarium sp* .
D		Kingdom: Mushrooms Division: Ascomycete Class: Euascomycetes Family: Chaetomiaceae Order: Sordariales Genus: Chaetomium Species: *Chaetomium sp*

Conclusion

Unfortunately, the misuse of antibiotics has led to a very worrying emergence of increasingly resistant bacteria, which today constitutes one of the most serious threats to global health. However, in many parts of the world, this problem is not yet taken with the seriousness that infectiologists and microbiologists give it.

The consequence of the development of resistance is simple to predict, but the serious thing is that it can promote infectious processes, in particular by reducing the capacity of the immune system, which leaves the body powerless against bacterial attacks. Some of these effects could sometimes be corrected preventively by respecting the dose to be taken, the times of taking and the duration of the treatment.

Endophytic fungi are excellent sources of new bioactive natural products with potential for exploitation in a wide variety of medical, agricultural and industrial fields. They are also capable of producing a very large number of secondary metabolites with extremely different chemical structures and possessing a very broad spectrum of pharmacological activity.

The isolation and identification of these endophytes, based on their macroscopic and microscopic characters allowed us to obtain four different fungal isolates belonging to three genera: Alternaria, Fusarium and Chaetomium

Conflict of interest

The authors state that no conflicts of interest exist regarding this publication and dissemination of the information provided here.

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