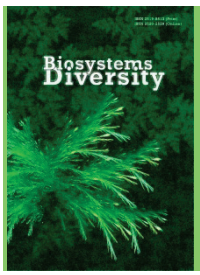

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Antifungal activity of the endophytic *Aspergillus* against *Candida albicans*

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Medicinal plants remain a reliable source of bioactive compound principles known for their proven therapeutic abilities against various infectious diseases. Endophytes, microorganisms residing within plant tissues, hold promise for producing novel metabolites with potential medical applications. This study analyzes the antagonism of endophytic fungi *Aspergillus* sp., isolated from medicinal plants, and their extract against *Candida albicans*, and their effectiveness was compared with that of a medical treatment, Phanazol 1% ointment. After isolating, purifying, and identifying endophytic fungi from the medicinal plants *Lavandula officinalis*, *Rosmarinus officinalis*, *Eucalyptus bicolor* and *Mentha piprita*, a total of ten endophytic fungi were obtained. These included two yeasts (yeast and *Rhodotorula* sp.), as well as eight moulds (*Aspergillus* sp., *Aspergillus niger*, *Nigrospora* sp., *Curvularia* sp., *Alternaria* sp., *Penicillium* sp.), and sterile mycelium. All these fungi were tested for their antagonism against *C. albicans*; using the cross-streak and disk diffusion methods for yeasts and moulds respectively, with the measurement of the diameter of the growth inhibition zone of the culture. Only the strain *Aspergillus* sp. and its ethyl acetate extract exhibited good activity against *C. albicans*, with inhibition zone widths of 27.5 and 20.3 mm, respectively. Its effectiveness is comparable to that of Phanazol 1% ointment. The use of gas chromatography mass spectrometry (GC/MS) unveiled the metabolite profiles of *Aspergillus* sp., enabling the recognition of 10 bioactive compounds, with butanedioic acid, kojic acid, and Cyclo L-prolyl-L-valine being the major ones, constituting 45.1%, 23.1%, and 5.1% of the total, respectively. These compounds serve as valuable platform chemicals that can be transformed into various other useful chemicals with various applications in agriculture, pharmaceuticals, food, cosmetics, and the healthcare industry. In addition to refining the active substances within this extract, it has the potential to open doors for creating novel bio-sourced medications aimed at addressing resistant opportunistic fungal or bacterial infections.

Keywords: *Aspergillus*; bioactive molecules; *Candida albicans*; endophyte; extraction; medicinal plants.

Introduction

Fungal diseases are on the rise in industrialized countries and are caused by microscopic fungi. This trend is attributable to advances in care for extremely fragile patients, such as those at the extremes of age and immunocompromised patients with cancer, autoimmune diseases, or undergoing immunosuppressive treatments. Bioprospecting for natural products is a route for the discovery of sources of new drugs via the isolation of bioactive metabolites from living organisms (Buatong et al., 2011; Alvin et al., 2014). Fungi are well-known for their capacity to produce secondary metabolites applied in medicine and agriculture (Janso & Carter, 2010). Fungal endophytes have been found in all plant families (including bryophytes and ferns), throughout the world, and in all kinds of climates (Zhang et al., 2006; Larran et al., 2016; Santoyo et al., 2016). Endophytes produce a wide variety of bioactive secondary metabolites that can influence the physiology, defense, and tolerance of the host plant against biotic and abiotic stresses while also promoting its growth (Martinez-Klimova & Sánchez, 2017).

Candida infections are the most common fungal diseases in humans, and their distribution has diversified. While *Candida albicans* remains the predominant etiological agent, the occurrence of other species such as *C. parapsilosis*, *C. glabrata*, and *C. auris* is on the rise. These pathogens often exhibit diminished susceptibility to widely employed antifungal agents, encompassing polyenes, triazoles, and echinocandins. Moreover, the frequency of emerging multi-drug-resistant strains within these species is increasing alarmingly. Consequently, the imperative to explore novel compounds that uniquely target these pathogenic species has become increasingly pressing (Sadeghi, 2018; Perez-Rodriguez et al., 2022). Due to advances in research and the integration of traditional medicine and modern medicine, endophytic fungi and microorganisms have been discovered and exhibit promising properties in the treatment of candidiasis. Endophytic fungi represent an underexplored reservoir of novel biological resources that can be harnessed in the pharmaceutical, industrial, and agricultural domains. These endosymbionts inhabit the interior of healthy plant tissues at various stages of their life cycle without causing apparent disease. They are found in all studied plant species and are present in internal tissues such as roots, stems, leaves, flowers, and fruits. They derive their habitat and nutrients from their plant hosts and chemically protect them against herbivores, insects, and phytopathogenic microorganisms. In this symbiotic relationship, endophytes produce a wide variety of bioactive secondary metabolites that can influence the host plant's physiology, defense, and tolerance against biotic and abiotic stresses, while promoting its growth. Plants constitute the primary source of secondary metabolites (80%), but they are also found in bacteria, fungi, and numerous marine organisms such as sponges, tunicates, corals, and snails. Endophytes from medicinal plants are garnering increasing interest and are considered an important and promising reservoir of new bioactive substances (Sadrati, 2021). With the aim of assessing the biological activities of bioactive secondary metabolites produced by endophytic fungi, endophytic fungi were isolated from four Algerian medicinal plants: *Lavandula officinalis*, *Rosmarinus officinalis*, *Eucalyptus bicolor* and *Mentha piprita*. The objectives to be achieved in this work are as follows: isolation, purification, and identification of endophytic fungi; determination of anti-*Candida albicans* activity; extraction and identification of bioactive molecules using GC/MS; and a study comparing the effectiveness of the extract and endophytic fungus exhibiting positive activity and that of Phanazol ointment 1%.

Material and methods

The *Candida albicans* strain used in this study was provided by the Applied Microbiology Laboratory at Ferhat Abbas University of Setif, Algeria. It was previously isolated from oral samples obtained from patients

with candidiasis. Samples of lavender, mint, eucalyptus, and rosemary plants were randomly collected from leaves, stems, and roots within the same area for each plant. The samples were selected to be asymptomatic for pathology. Collection was carried out in February 2023, with mint samples obtained from Saleh Deradjer, rosemary and lavender samples obtained from Saleh Boubnider University (Constantine 3), and eucalyptus samples from El Gerarem in Constantine, Algeria. The samples were individually stored in fully sterilized glass Petri dishes, covered with aluminum foil, and transported to the laboratory for use within a maximum of 24 hours (Zerroug, 2018).

All collected samples from each plant were pre-rinsed with tap water to remove adhering residues (dust and impurities), then subjected to a disinfection process to eliminate surface organisms, and subsequently cut into small pieces. Next, surface sterilization was performed following the protocols of Orole & Adejumo (2009) and Nacef et al. (2022). To eliminate all epiphytic microorganisms the fragments were sequentially immersed in 96% ethanol for 1 min, 2% sodium hypochlorite for 3 min, 96% ethanol for 30 s, and finally rinsed with sterile distilled water. The treated samples were cut into small pieces (0.5 × 0.5 cm) using a sterile pointed instrument and placed on sterile absorbent paper to air dry at room temperature. Five to seven pieces from each sample were cultured in Petri dishes containing PDA medium (Sadrati, 2020) supplemented with acetic acid (1% v/v) to inhibit bacterial growth. The Petri dishes were then incubated at 28 °C until the growth of endophytic fungi was detected. Emerging fungi were isolated, inoculated onto fresh PDA medium and incubated at 28 °C for 7 days. Each growing fungus was subcultured several times until a pure culture was obtained (Sadrati, 2020). The percentage of colonization was calculated according to Nacef et al. (2022).

The isolates were identified based on macroscopic and microscopic morphological characteristics using identification keys. The following features were considered: (i) mycelium morphology, including its colour and production of fruiting bodies and sclerotia, and (ii) spore shape, arrangement, and colour.

To assess the sensitivity of *C. albicans* to endophytic fungi, primary screening was conducted using the cross-streak method against the pathogenic yeast as described by Mohamed et al. (2017), and secondary screening was performed using the agar diffusion method on solid medium according to Djahra et al. (2015). Isolates exhibiting the highest anti-candidiasis activity were selected for further studies, the plates were incubated at 30 °C for 24 hours. Additionally, the agar diffusion method on Sabouraud medium was employed to measure the diameter of the inhibition zone (Sadrati, 2020). For this method, two disks of each 6 mm-diameter isolate were placed in Petri dishes containing Sabouraud medium, which were previously inoculated with *C. albicans* inoculum, then they were incubated at 37 °C for 48 hours. If the fungi exhibited an effect, an inhibition zone or transparent halo would form around the disk.

Two control plates were prepared: a negative control containing only *C. albicans* and a positive control containing *C. albicans* along with two disks of the antibiotic Actidion.

The degree of anti-candidiasis activity of the isolates was classified based on the average diameter of the inhibition zone. In this case, the diameter of the inhibition zone was categorized as follows: excellent activity (≥ 18 mm), good activity (12–15 mm), moderate activity (10–12 mm), and weak activity (≤ 9 mm). The experiment was conducted in duplicate (Mohamed et al., 2017).

A 6 mm disk from a pure culture of the endophytic fungus *Aspergillus* sp. was excised from a 7-day-old PDA plate. The disk was then placed into a 250 mL Erlenmeyer flask containing 100 mL of Yeast Extract Saccharose (YES) medium (comprising 15% w/v sucrose, 2% yeast extract, 0.05% $MgSO_4$, 0.1% metal trace ($ZnSO_4$*$7H_2O$ 1%, $CuSO_4$*$7H_2O$ 0.5%)). The flasks were incubated at 30 °C for 7 days. Upon completion of fermentation, the mycelium along with the medium was frozen at –20 °C for 2–3 hours. Subsequently, the frozen mycelium was pulverized and subjected to centrifugation at 15000 rpm for 15 minutes. Following this, samples were extracted twice with equal volumes of ethyl acetate. The organic layers were evaporated to dryness using a rotary evaporator at 40 ± 1 °C. The resulting dry crude extracts were reconstituted in 5% Dimethyl Sulfoxide (DMSO) and stored at 4 °C until further use (Nacef et al., 2020).

To determine the anti-*Candida albicans* activity of endophytic fungal extract, Sabouraud Agar plates were prepared by spreading the yeast suspension with concentration of 10^6 CFU/mL. After that, sterile disks of 6 mm diameter were put with 20 µL of the fungal extracts and placed on the inoculated agar. The plates were then incubated at 37 °C, for 48–72 h. The experiment was conducted in triplicate. One Petri dish was reserved to test 5% DMSO to ensure that it did not have an effect on *C. albicans*. All Petri dishes were incubated at 30 °C for 48 hours (Haddouchi et al., 2016).

The purpose of this step was to compare the anti-candidiasis effect between the treatment and the endophytic fungus. For this, Phanazol 1% ointment was selected, which is indicated for the treatment of certain skin mycoses (fungal skin conditions caused by fungi like *C. albicans*). The ointment to be tested was dissolved in 5% DMSO. In sterile Petri dishes, the inoculum was spread on Sabouraud medium, and 6 mm disks were impregnated with the ointment solution and placed on the surface of the Sabouraud medium. The Petri dishes were then incubated at 37 °C for 48 hours. The anti-candidiasis activity was determined by measuring the diameter of the inhibition zone of *C. albicans* around the disks (Selka et al., 2016).

GC-MS analysis was conducted to identify bioactive compounds present in the extract based on the growth of the studied fungus on Yeast Extract Saccharose Broth medium. A control was prepared using the same culture medium without the fungus. GC-MS analysis of the S1 extract was performed at the Biotechnological Research Centre Laboratory using a DB-5 column (30 m x 0.25 mm x 1 µm) made of 100% dimethylpolysiloxane. Helium (99.99%) was used as the carrier gas with a constant flow rate of 1 mL/min, and it was injected into the GC-MS instrument according to the program. The oven temperature was programmed as follows: 110 °C – 2 min hold; ramp up to 200 °C at a rate of 10 °C/min – no hold, further ramp up to 280 °C at a rate of 5 °C/min – 9 min hold. The injector temperature was set at 250 °C, and the total GC running time was 26 min. MS Program: mass spectrometry was conducted at 70 eV with a scan interval of 0.5 s and fragments analyzed in the range from 45 to 450 Da. The total MS running time was 26 min. Identification of bioactive constituents was achieved through interpretation of the Mass-Spectrum GC/MS using the database available at the Biotechnological Research Centre (CRBt) (Database/NIST11.L, Database/W9N11.L, Database/NIST11.L) (Chaudhary & Tripathy, 2015; Nacef et al., 2020).

The results were processed statistically using the SPSS Statistics 20 computer program (Armonk, NY: IBM Corp). Univariate analysis of variance was used (differences between mean values were calculated by ANOVA, they were considered significant at $P < 0.05$). The results are presented as mean ± standard deviation.

Results

All parts of the plants used were colonized by varying proportions of endophytic fungi, with a colonization rate of 29.2%. The distribution was as follows: two yeast isolates and eight mould isolates, with colonization rates of 7.1% and 92.9%, respectively.

The obtained results indicated that *L. officinalis* exhibited a high fungal colonization rate (36%), followed by *R. officinalis* and *E. bicolor* (25%), while *M. piperita* had a lower colonization rate (14%).

The identification of fungi was based on macroscopic and microscopic criteria. Ten fungal isolates were obtained, belonging to eight different genera (Fig. 1, 2).

The anti-candidiasis activity of endophytic fungal isolates was determined by measuring the diameter of clear zones around the fungal disks and comparing them with positive and negative controls after 5 days of incubation. Among the 10 fungal genera, only one fungus exhibited inhibitory activity against *C. albicans* with an inhibition zone diameter of 27.5 mm (excellent activity). This fungus was identified as *Aspergillus* sp., isolated from *L. officinalis*. However, no activity was observed for the other fungi (Fig. 3).

After 48 hours of incubation, the plates displayed inhibition zones around the disks impregnated with the ointment (which was dissolved in 5% DMSO) with a diameter of 29.1 mm. Based on this result, the degree of anti-candidiasis activity is classified as excellent.

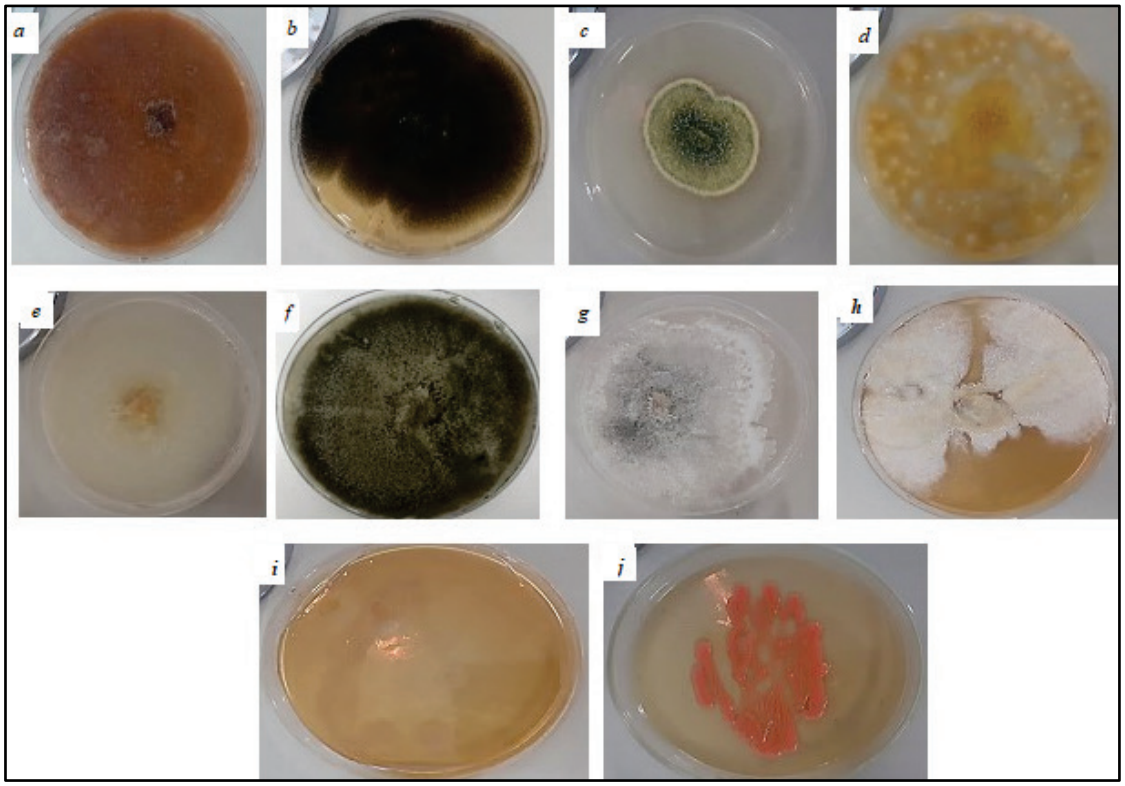

Fig. 1. Macroscopic appearance photographs of endophytic fungal isolates: *a* – *Curvularia* sp., *b* – *Aspergillus niger*, *c* – *Penicillium* sp., *d* – *Aspergillus* sp., *e* – steril mycelium, *f* – *Alternaria alternata*, *g* – *Nigrospora* sp., *h* – *Aspergillus* sp., *i* – yeast, *j* – *Rhodotorula* sp.

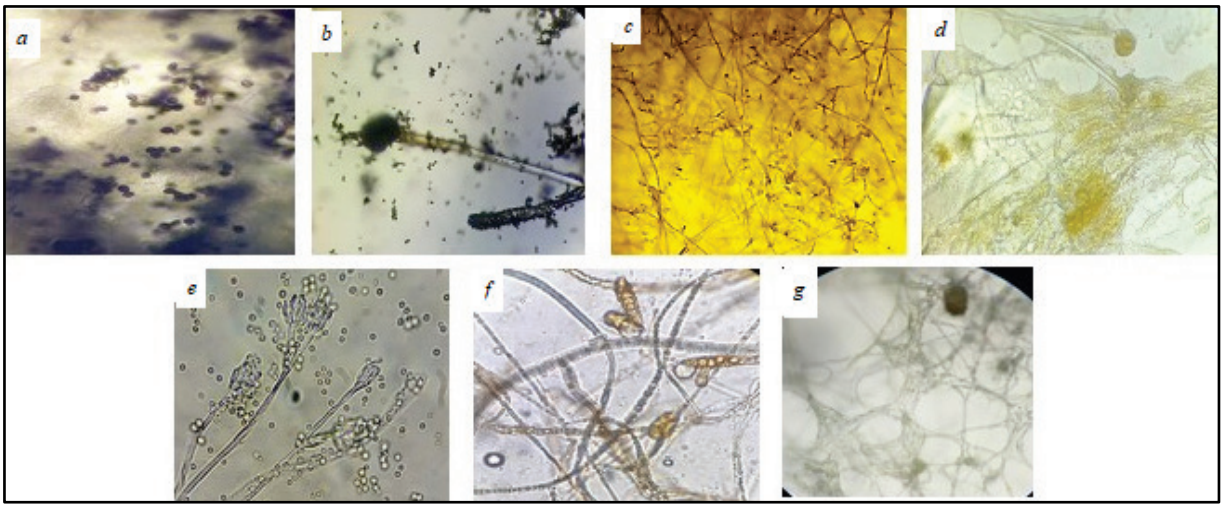

Fig. 2. Microscopic appearance photographs of endophytic fungal isolates: *a* – *Nigrospora* sp., *b* – *Aspergillus niger*, *c* – *Curvularia* sp, *d* – *Aspergillus* sp., *e* – *Penicillium* sp., *f* – *Alternaria alternata*, *g* – steril mycelium

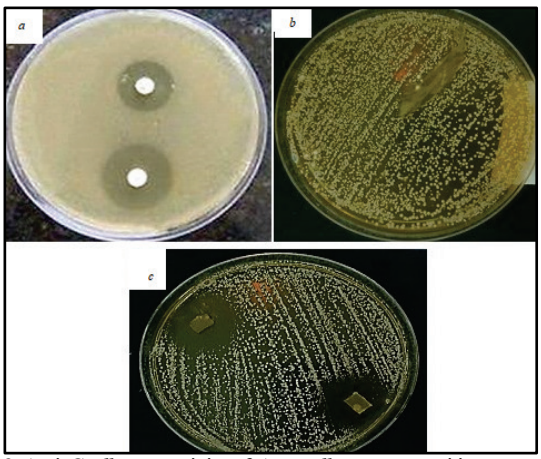

Fig. 3. Anti-*C. albicans* activity of *Aspergillus* sp.: *a* – positive control (*C. albicans* + actidion), *b* – negative control, *c* – *C. albicans* + *Aspergillus* sp.

After 48 hours of incubation, the plates containing the crude extract exhibited a distinct effect with a 20.3 mm inhibition zone (Fig. 4).

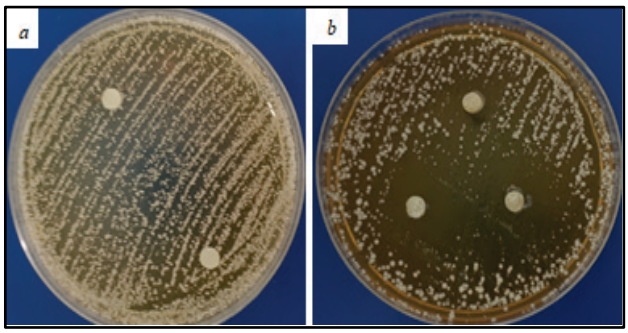

Fig. 4. Anti-*C. albicans* activity of *Aspergillus* sp. fungal extract: a – 5% DMSO control, b – fungal extract

The collected results allowed us to compare the anti-*C. albicans* activity of *Aspergillus* sp. and its extract with that of the ointment. The difference between the average diameters of the inhibition zones is presented in the results indicate that there is no significant difference ($P > 0.05$, Table 1).

Table 1
Anti-*C. albicans* effect of the endophytic fungus *Aspergillus* sp., its extract and Phanazol ($x \pm SD$, $n = 3$)

Endophytic fungus	Inhibition zone diameter (mm)
Aspergillus sp.	27.5 ± 2.1
Aspergillus sp. extract	20.3 ± 1.9
Phanazol	29.1 ± 2.3

Note: differences between mean values were calculated using the ANOVA test; there is no significant difference ($P > 0.05$).

The GC-MS chromatogram of the ethyl acetate extract obtained from the active endophytic fungus *Aspergillus* sp. reveals multiple peaks, with the predominant compounds being butanedioic acid (45.1%), and kojic acid (23.0%), and cyclo L-prolyl-L-valine (5.1%, Fig. 5, Table 2).

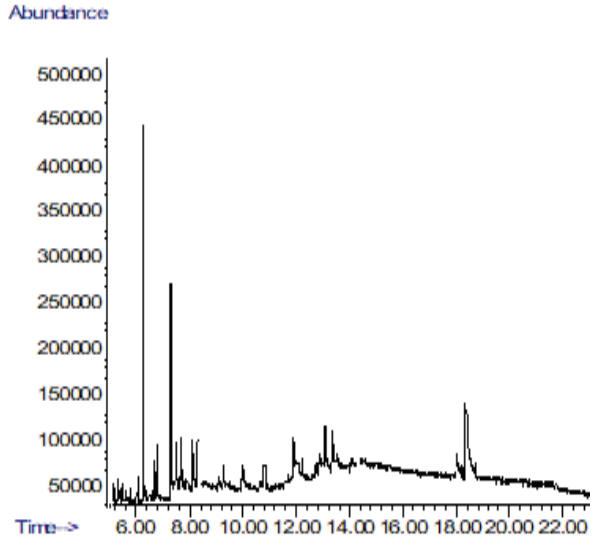

Fig. 5. GC-MS analysis of *Aspergillus* sp. ethyl acetate extract

Table 2
Components identified in *Aspergillus* sp. crude extract [GC-MS Study]

Series number	Retention time (min)	Name of the compounds	Total of percentage, %
1	6.21	Butanedioic acid*	45.07
2	7.57	Benzenemethanol,alpha-(1-aminoethyl)-(Methoxamine) 2,5-dimethoxy	0.25
3	7.60	Beta-Naphthoic acid ethyl ester	0.11
4	7.62	Glutaric acid	0.10
5	7.98	Kojic acid*	23.01
6	9.54	Acridine-3(10H)-one	0.29
7	12.42	Methoxy-2H-chromen-2-one-6	0.08
8	19.32	Cyclo L-prolyl-L-valine*	5.09
9	20.11	S-(tert-Butyl) 5-oxohexanethioate	0.36
10	22.68	2,2,6,9-tetramethyl-3,4,5,6-tetrahydro-1-benzoxocine-3,8-diol	0.13

Note: * means the highest percentage of the compounds in the extract.

Discussion

Candida albicans is responsible for the majority of human fungal infections. The resistance of *C. albicans* to antifungal agents continues to grow and evolve, complicating patient management, despite the introduction of new antifungal agents (Pfaller, 2012). The quest for novel antifungal molecules is ongoing, with many laboratories conducting research to combat serious fungal infections, particularly those caused by *Candida* species.

The diverse array of fungal endophyte species establishes an ecological niche within the internal tissues of plants. These pervasive fungi engage in beneficial interactions with their surroundings. Moreover, they represent a category of organisms with substantial potential for utilization in plant enhancement and disease management. The isolation of endophytic fungi from medicinal and other plants has yielded bioactive compounds that exhibit enhanced activity against a range of pathogenic microorganisms (Sandhu et al., 2014).

Out of a total of 105 segments from four medicinal plants, 28 distinct fungal isolates were obtained. The obtained results demonstrated that lavender (36%) exhibited a significant fungal colonization rate among the plants, followed by rosemary and eucalyptus (25%), while mint displayed a lower rate of colonization (14%). These percentages are notably higher compared to the findings of Li et al. (2007), who utilized *Saussurea involucrata*, reporting a colonization rate of 11.0%, and Khan et al. (2010), who studied the medicinal plant *Withania somnifera* and achieved a rate of 5.1%. The variation in these percentages can be attributed to differences in host species, sample size, and culture media employed (Gong & Guo, 2009).

According to the obtained results, a single endophytic fungus of the genus *Aspergillus* sp. exhibited anti-*C. albicans* activity. Phongpaichit et al. (2006) isolated fungal endophytes from five medicinal *Garcinia* plants and demonstrated that the metabolites produced by 70 fungal isolates, extracted with ethyl acetate, exhibited antimicrobial activity through the agar well diffusion method against *Staphylococcus aureus*, *Candida albicans*, *Cryptococcus neoformans*, and *Microsporum gypseum*. Phongpaichit et al. (2006) further identified several genera, including *Aspergillus*, *Botryosphaeria*, *Eutypella*, *Fusarium*, *Guignardia*, *Penicillium*, *Phomopsis*, and *Xylaria*, as exhibiting significant results in inhibiting microbial growth. Among these endophytes, three strains – *Phomopsis* sp., *Botryosphaeria* sp., and an unidentified fungus – displayed the most active production of secondary metabolites (Sandhu et al., 2014). The genus *Aspergillus* has been previously isolated as an endophyte and has shown activity against various pathogenic microorganisms, as demonstrated by studies conducted by Madki et al. (2010) and Maria et al. (2005). The inhibitory activity of the isolated endophytic fungi confirms the potential of these isolates to produce bioactive compounds that inhibit the growth of pathogenic organisms. These diverse secondary metabolites could find applications in the field of medicinal science and serve as a valuable source of potential drugs (Nacef et al., 2022).

Regarding the comparative study of anti-*C. albicans* activity between the Phanazol ointment and *Aspergillus* sp. and its extract, the results indicate no significant difference. These findings are in agreement with the work of Selka et al. (2016), who demonstrated antimicrobial activity against *E. coli* and *Proteus mirabilis* using extracts from *Vitis vinifera* L. with inhibition zone diameters of 12 and 20 mm, respectively. They also prepared an ointment from this extract with a zone of inhibition diameter of 18 mm against *C. albicans*. However, Orhan et al. (2009) reported the absence of antifungal activity against *C. albicans*. This information is noteworthy as studies on *C. albicans* have indicated its capacity to develop resistance to different antifungal agents (Akroum, 2021).

According to the product information, the active ingredient in Phanazol is Econazole, which is known for its antifungal activity against *Candida*, dermatophytes, *Malassezia furfur*, and gram-positive bacteria. This suggests that the appearance of the inhibition zone is due to the activity of Econazole against *C. albicans*. Thus, it is evident that this activity varies considerably based on the chemical composition of the antifungal agent in the extract. GC-MS analysis revealed that the major compounds were kojic acid, succinic acid, with traces of cyclo D-phenylalanyl-L-prolyl, which possess antibiotic characteristics. Cyclo L-prolyl-L-valine was predominantly found in the ethyl acetate extract of the *Pseudomonas aeruginosa* RKC1 (93.7%). This diketopiperazine (DKPs) exhibited quorum-sensing inhibition against the pathogen in liquid media during the active growth phase and regulated diverse metabolites of the pathogen (Kapadia et al., 2022). Initially, antibiotics produced by Aspergilli were referred to as aspergillin (Blumenthal., 2004), but recent studies have shown that *Aspergillus oryzae* can produce various secondary metabolites, such as Aspergillormarasmine, Cyclopiazonic acid (CPA), kojic acid, 3-nitropropionic acid, Maltoryzine, and Violacetine, which was reported as

an antibiotic produced on malt extracts (Bhumenthal, 2004). The observed anti-*C. albicans* activity in this study can also be attributed to the ability of the *Aspergillus* sp. strain to secrete enzymes that degrade fungal cell walls (Manjula & Podile, 2005), leading to direct physical alterations of cell walls (Praveen et al., 2012).

Conclusion

This study identified *Aspergillus* sp. isolated from lavender as the only endophytic fungus with anti-*C. albicans* activity, exhibiting a strong inhibitory effect that is comparable to that of the Phanazol ointment. This work serves as a preliminary exploration into the potential of Algerian medicinal plants for their antifungal properties. It would be intriguing to expand the study to encompass other microorganisms responsible for different infectious diseases and to assess the contribution of additional substances to the antimicrobial activity exhibited by the *Aspergillus* sp. endophyte and its extract. Further purification of the active compound from this extract could pave the way for the development of new potential bio-sourced medications to address resistant opportunistic fungal infections.

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There is no conflict of interest among the authors.

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