Antifungal activity of *Bacillus*sp. Gn-A11-18isolated from decomposing solid green household waste in water and soil against *Candida albicans* and *Aspergillus niger*

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Abstract. *Candida albicans* an opportunistic pathogenic fungus causes many infections in humans. Whereas *Aspergillus niger* is a fungus that can produce ochratoxins, a group of extremely dangerous secondary metabolites that are classified as potentially carcinogenic to humans and also causing deterioration in grapes, strawberries, etc. The purpose of the current study is to isolate, purify, identify and characterize new microorganisms associated with solid green household waste for the control of *C. albicans* and *A. niger*. The antifungal activity of bacterial isolates was carried out in vitro by the agar plug diffusion method, the disk and well diffusion method. The isolate that showed promising activity has been identified by those macroscopic, microscopic and biochemical characteristics. The results obtained in the course of this study showed the isolation of an isolate named Gn-A11-18, which was shown to have significant inhibitory activity with a 42.66% inhibition percentage against *A. niger* and a 44.66 mm inhibition diameter against *C. albicans* compared to the controls. The identification of Gn-A11-18 isolate has shown that this isolate belongs to the genus *Bacillus* with a similarity to *Bacillus subtilis* and *Bacillus tequilensis.* In the light of the results of this study, we can suggest that the bioactive compound of *Bacillus* sp. Gn-A11-18 could become a biological alternate that could have an important role to fight against *C. albicans* and *A. niger.*
Keywords:*A. niger,* Antifungal activity*, C. albicans,* green household waste, recovery

1. Introduction

The quantity of household waste in Morocco is increasing due to population growth, rapid urbanization, a growing economy, and rising living standards [1]. This increase may have a negative impact on the environment and human health [2]. Currently the recovery of solid waste is the greatest challenge for a large part of the social, scientific and also economic class. In the literature, there are two main solid waste recovery mechanisms including composting (aerobic composting and vermi-composting) [3] and energy (incineration, granulation, biomethanization) [4-6]. However, until now, information on the recovery of microorganisms associated with the digestion of solid householdwaste in the natural environment remains unknown. In addition, the valorization of these microorganisms to control *A. niger* and *C. albicans* to our knowledge has not been done before.

A. niger is a fungus that is capable of producing ochratoxins, a group of extremely dangerous secondary metabolites which are classified as potentially carcinogenic to humans [7, 8]and also causing deterioration in grapes, strawberries, apples, sweet oranges and cherry tomatoes [9,10,7]. While *C. albicans* is a commensal organism and is frequently found in the gastrointestinal tract, urogenital tract, mouth, skin and also in the respiratory tract in humans and other mammals [11,12]. *C. albicans* is also a pathogenic fungus responsible for about 30% of patients with nosocomial infection and candidiasis, especially of the pulmonary type, which causes high morbidity and mortality in immunocompromised people [12].

Several current research studies have been focused on the control study against *C. albicans* and *A. niger*, including Xu *et al.*[13]which were used to control *C. albicans* by the use of coumarin, Kim and Kang[14]were used cell-free supernatant of a probiotic strain, *Pediococcus acidilactici* HW01 to control *C. albicans*, other researchers have been shown that probiotic bacteria have significant antifungal effects against *C. albicans*[15]. Whereas Mustapha *et al.*[16]have been controlling *A. niger* with biopolymer films containing turmeric oil, An *et al.*[17]have been shown that α-terpineol and terpene-4-ol, the critical compounds in tea tree oil, exert antifungal activities in vitro and in vivo against *A. niger*, Devipriya and Roopan [18] use the plant extract from *Cissus quadrangularis* to fight against *A. niger*. Most of these research studies have focused on the use of essential oils from some plants and/or their extracts, while the control of *C. albicans* and *A. niger* by the use of microorganisms has been poorly documented.

The resistance of fungal microorganisms, especially *C. albicans* and *A. niger*, to commercially available antifungals is a very relevant factor because it is often associated with high morbidity and mortality [19]. Therefore, research efforts must be intensified to find new bioactive molecules with a minimum of side effects to

control pathogenic and phytopathogenic fungi in general, *C. albicans* and *A. niger* in particular.
The present study was designed to recover microorganisms associated with solid green household waste (banana, pomegranate and tangerine wastes) decomposing in water and soil. The principal objective of which was to isolation, purification, characterization and identification of new bacteria producing bioactive molecules effective against *C. albicans* and *A. niger*.

2. Materials and methods

2.1. Pathogens and phytopathogens

The two species pathogenic *C. albicans* ATCC 10231 and phytopathogenic *A. niger* have been provided by Biotechnology Laboratory, Faculty of Sciences Dhar El Mahraz, Sidi Mohammed Ben Abdellah University, Fez-Atlas, Morocco. These two species have been verified by their macroscopic (appearance, shape, storyteller, odor, color, etc.) and microscopic (mycelial filaments, presence or absence of anastomosis loops and partitions, special structures, fruiting, spore and conidiophore diameter, etc.) characteristics using lactophenol cotton blue for *A. niger*[20]and methylene blue for *C. albicans*[21].

2.2. Isolation of antagonistic bacterial strains

Antifungal bacterial isolates have been isolated from solid green household waste (banana, pomegranate and tangerine waste) decomposing in water and soil. Briefly, 10 g of waste was collected in accordance with the Pochon and Tardieux[22], cut and then crushed in 100 ml of sterile spring water. The suspension is then shaken for 2 hrs in order to release as much microbial load as possible [1]. Isolation was carried out by the suspension-dilution technique, 0.1 ml of each dilution (10^{-1} to 10^{-6}) was separately sown on the surface by spreading on the MEA medium (Malt Extract Agar) modified by enrichment with 7% (v/v) waste extract. After spreading and incubation at 30 °C in the dark for 48 hrs. The bacterial colonies have been purified by the striation method in the same medium and under the same isolation condition.

2.3. Screening for in vitro inhibitory effects of Gn-A11-18 isolate

2.3.1. Agar plug diffusion method

The screening test for new antifungal bacterial isolates have been performed using the agar plug diffusion method [23]. The pure bacterial isolates have been cultured on the MEA medium. After 7 days of incubation at 30°C in the dark, plugs (6 mm in diameter) were cut by a die and placed on the MEA medium surface which was seeded with *C. albicans* ATCC 10231. In the case of *A. niger*, the screening of new antifungal bacterial isolates was also carried out on the MEA medium, but by means of direct confrontation in accordance with the [24]. The inoculated petri dishes have been incubated at 30°C in the dark and in an atmosphere saturated with moisture. The inhibition diameter and inhibition percentage have been determined after 48 hrs and 7 days of incubation for *C. albicans* and *A. niger* respectively [25,26].

2.3.2. Disk and well diffusion method

The Gn-A11-18 isolate which showed promising inhibitory activity against *C. albicans* and *A. niger* has been cultured in three replicates in Erlenmeyer (100 ml) each containing 50 ml of sterile malt extract medium. After 7 days of incubation at 30°C in the dark and in a humidity saturated atmosphere. The suspensions have been centrifuged at 5000 rpm for 10 min, then filtered by wattman paper and millipore filters (0.45 then 0.22 µm in diameter) under aseptic conditions. The evaluation of the filtrate of Gn-A11-18 isolate has been performed by the disk diffusion method [27], and by the well diffusion method [28]. Petri dishes containing the MEA medium have been sown with *C. albicans* and inoculated with *A. niger*. Wattman paper disks (6 mm in diameter) have been deposited on the surface of the inoculated media and impregnated with 20 µl filtrate [25]and wells (6 mm in diameter) have been made by a sterile die on the inoculated media, then filled with 100 µl filtrate [28]. The inoculated petri dishes have been incubated at 30°C in the dark. The inhibition diameter and inhibition percentage have been determined after 48 hrs and 7 days of incubation for *C. albicans* and *A. niger* respectively [25, 26].

2.4. Characterization of optimized antifungal activity conditions of Gn-A11-18 isolate

The production of the antifungal metabolites of Gn-A11-18 isolate has been performed using four culture media; Malt extract (ME), Sabourant (SB), Yeast and Malt extract (YM) and nutrient broth (NB). Briefly, Erlenmeyer (50 ml) containing 25 ml of each culture medium have been inoculated with the culture of Gn-A11-18 isolate 48 hrs. old. After incubation at 30°C for 48 hrs, 10 ml aliquots have been sampled, centrifuged at 5000 rpm for 10 min [19]. The supernatants have been filtered through Millipore filters (0.45 and 0.22 µm in diameter) and tested using the well diffusion method [28]. By the same process, the production of the antifungal metabolites of Gn-A11-18 isolate has been carried out using the liquid malt extract medium at different pHs from 1 to 10, the pH has been adjusted by KOH 1N and H_3PO_4 1N and incubated for 48 hrs at different temperatures (25, 30, 37 and 44 °C).

2.5. Gn-A11-18 isolate identification

Gn-A11-18 isolate with high antifungal activity against *A. niger* and *C. albicans* has been identified on the basis of macroscopic, microscopic and biochemical characteristics. Staining of Gram, spore and catalase tests have been carried out for the first time. Then, the biochemical characteristics of the Gn-A11-18 isolate were tested using API 20E gallery according to the method described by Logan and Berkeley [29]. Growth in the NB medium at different pHs from 4 to 10, pH has been adjusted by KOH

1N and H_3PO_4 1N, growth at different temperatures (25, 30, 37, 44 and 50 °C) and growth in the NB medium containing different NaCl concentrations (0, 2, 4, 6, 8 and 10% (w/v)) have also been tested [30].

2.6. Statistics analysis

All tests and experiments have been repeated three times. The results have been designed and processed using GraphPad prism 8 software. The statistical analysis of the results obtained was carried out using IBM SPSS 20 Statistics for Windows software, the Duncan's Multiple Range Test method, a student *t-test* and an analysis of variance (ANOVA I) at the threshold of $\alpha = 5\%$ [1].

3. Results and discussion

3.1. Isolation and identification of Gn-A11-18 isolate

All bacterial isolates associated with green household waste (tangerine, banana and pomegranate waste) decomposing in water and soil over time (from February to June 2018) have been purified and grown in the same isolation medium, we have isolated 226 isolates (Fig. 1), where the highest number (51 and 49 isolates) have been isolated from the pomegranate and tangerine waste digested in the soil respectively (Fig. 1). The antifungal activity of these 226 isolates has been evaluated by the agar plug diffusion method, only 5 isolates that have shown antifungal activity, and especially the isolate named Gn-A11-18 which has been shown a promising activity against *C. albicans* ATCC 10231 and *A. niger*. The results of the cultural and biochemical studies of isolate Gn-A11-18 are presented in table (1). This isolate is a gram positive, capped, rod-shaped bacillus grouped into pairs and chains, forming the endospores (Fig. 2), it has been shown a positive reaction to the catalase test, for β-galactosidase, gelatinase, acetoin production, Citrate utilization, Nitrate reduction and for the use of carbonaceous substrate (D-Glucose, D-Mannitol, Inositol, D-Sorbitol, D-Sucrose, D-Melibioses and Tonsil), while it had been negative for the use of carbonaceous substrate (L-rhamnose and L-arabinose), Arginine dihydrolase, Lysine decarboxylase, Ornithine decarboxylase, H_2S production, Urease, Tryptophan deaminase and Indole production. In addition, it has been able to grow at a temperature of 50°C, and over a wide pH range of 4 to 10 and a NaCl concentration between 0 and 4% (Table. 2). Based on the macroscopic, microscopic and biochemical characteristics that have been performed during the course of this study, Gn-A11-18 isolate belongs to the genus *Bacillus* with a great similarity to *Bacillus subtilis* and *Bacillus tequilensis*.

The Gn-A11-18 isolate is gathered at *Bacillus* spp isolated and characterized by Balouri et *al.*[19], but there are deficiencies especially for the use of carbonaceous substrates and also our isolate cannot grow at a concentration of NaCl above 4%. It is also resembling *B. subtilis* described by Bouhairi[31], however Gn-A11-18 isolate it has been negative for the use of carbon substrate L-arabinose. Which suggests that the Gn-A11-18 isolate must be a subspecies of *B. subtilis*.

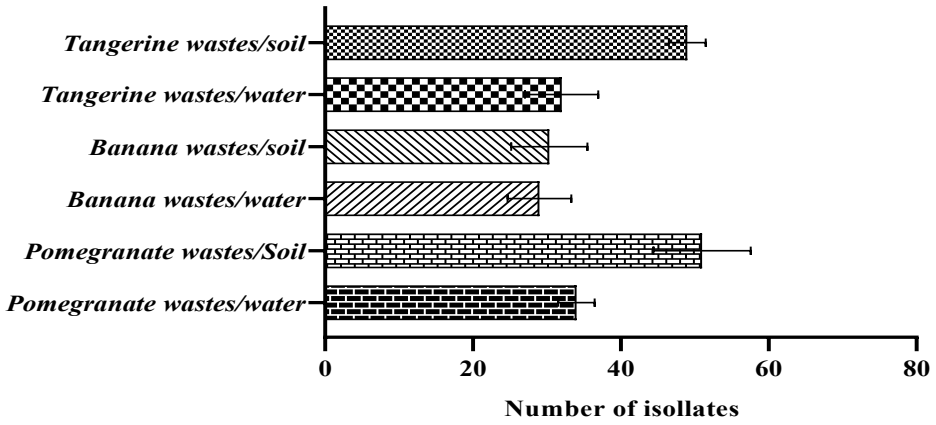

Fig.1. Number of isolates isolated from three types of decomposing waste in water and soil.

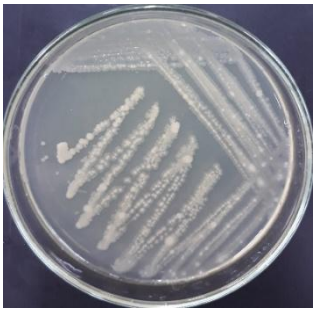

Fig. 2. Gram staining and cultural aspect of Gn-A11-18 isolate

Table 1. Cultural and biochemical characteristics of Gn-A11-18 isolate.

Tests	Results
Biochemical tests	
β-galactosidase	+
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate utilization	+
H_2S production	-
Urease	-
Tryptophan deaminase	-
Indole production	-
Acetoin production	+
Gelatinase	+
D-glucose	+
D-Mannitol	+
Inositol	+
D-sorbitol	+
L-rhamnose	-
D-Sucrose	+
D-Melibiose	+
Amygdalin	+
L-arabinose	-
Nitrate reductase	+
Catalase	+
Growth at	
50°C	+
44 °C	+
37°C	+
30 °C	+
25°C	+
Growth at PH	
4	+
5	+
6	+
7	+
8	+
9	+
10	+
Growth in NaCl%	
0	+
2	+
4	+
6	-
8	-
10	-

3.2. In vitro inhibitory effects of Gn-A11-18 isolate

In vitro evaluation of the antifungal activity by using agar plugs of Gn-A11-18 isolate against *C. albicans* and *A. niger* showed very high antifungal activity with an inhibition percentage of 42.66% against *A. niger* compared to the control (Fig. 3A and 3B) and also with an inhibition diameter of 44.66 mm against *C. albicans* compared to the control (Fig. 3C and 3D). Based on the promising results of the use of agar plugs from Gn-A11-18 isolate against *C. albicans* and *A. niger*, the evaluation of the filtrate of this isolate has been performed using the disk and well diffusion method. The results obtained are shown in figure (4) and table (2), these results show that the filtrate of Gn-A11-18 isolate has significant antifungal activity with an inhibition diameter of 31.33 mm and 42.33 mm against *C. albicans* and an inhibition percentage of 29.66% and 40.33% against *A. niger* by using the Disk and Well diffusion method, respectively. The filtrate of Gn-A11-18 isolate which has been autoclaved at 120°C for 30 min shows high activity against *C. albicans* and *A. niger* (Table. 2), which shows that the bioactive substance which has been present antifungal activity of the Gn-A11-18 isolate is thermoresistant.

Several scientific research studies have focused on the control of *C. abicans* and *A. niger* by the use of essential oils and substances of bacterial and fungal origin including Moussaid *et al.*[25], which have shown the isolation of an F27 isolate, the filtrate of this isolate has an antifungal activity with an inhibition diameter of 14.7 mm against *C. albicans*.Balouiri *et al.*[19]have been shown in this research that *Bacillus* spp isolated from Calotropis Procera Ait rhizosphere has shown an antifungal activity against *C. albicans* ATCC 102031 with an inhibition diameter of 36.33 mm on the YM (Yeast and Malt extract) medium. The study by Bamidele *et al.*[28]showed that the six strains of lactic acid bacteria showed varying degrees of anti-*Candida* activity, especially *Pediatric pentosaceus* BTA 51 cucumber showed the largest inhibition zone of 14 mm at neutral pH. Bulgasem *et al.*[32]have been found that the free cell supernatant produced by *L. plantarum* isolated from salad vegetables has significant antifungal activity against *C. albicans* with a 25 mm inhibition zone. Pudake *et al.*[33]in their studies evaluated seven biological control agents *Trichderma hamatum*, *T. lignorum*, *T. koningi*, *T. harzianum*, *T. viride*, *Pseudomonas fluorescence* and *Gliocladium virens* for their efficacy against *A. niger*, of

these seven fungal antagonists tested, *Trichoderma harzianum* has been shown to be most efficient and has significantly recorded the highest proportion of mycelial inhibition (99.93%). The current study shows that our *Bacillus* sp. Gn-A11-18 has the largest inhibition, especially against *C. albicans*, compared to other published studies.

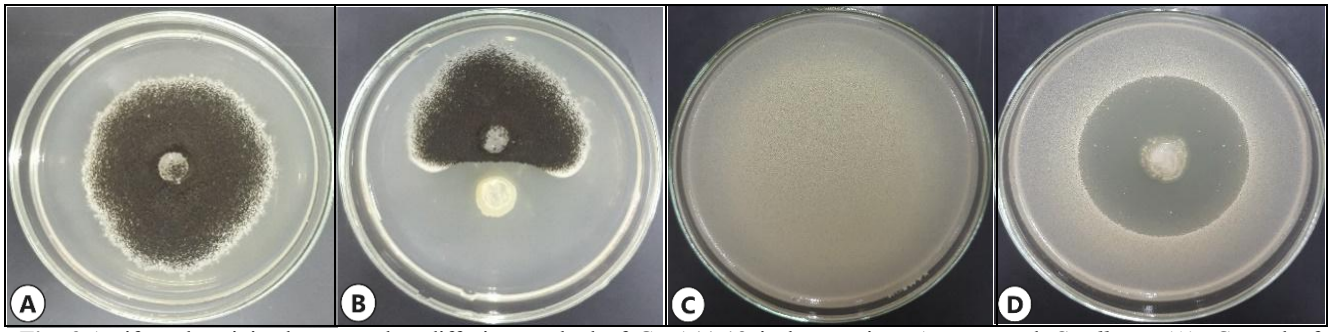

Fig. 3. Antifungal activity by agar plug diffusion method of Gn-A11-18 isolate against *A. niger* and *C. albicans***(A).** Control of *A. niger***(B).** Gn-A11-18 Isolate against *A. niger***(C).** Control of *C. albicans***(D).** Gn-A11-18 isolate against *C. albicans*.

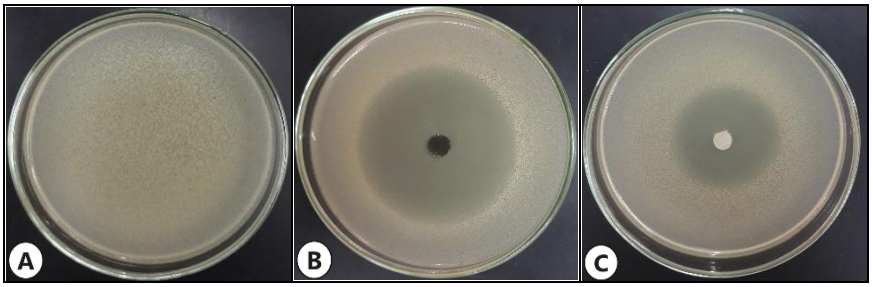

Fig. 4. Antifungal activity by disk and well diffusion method of filtrate from Gn-A11-18 isolate against *C. albicans***(A).** Control of *C. albicans***(B).** Gn-A11-18 isolate filtrate against *C. albicans* (well diffusion) **(C).** Gn-A11-18 isolate filtrate against *C. albicans* (disk diffusion).

Table 2. Percentage and inhibition diameter of the Gn-A11-18 isolate filtrate against *A. niger* and *C. albicans*

	***A. niger* (%)**	***C. albicans* (mm)**
Agar plug of Gn-A11-18 isolate	42.66±1.15	44.66±0.58
Gn-A11-18 isolate filtrate (disk diffusion)	29.66±0.57	31.33±0.58
Gn-A11-18 isolate filtrate (well diffusion)	40.33±0.57	42.33±0.58
Gn-A11-18 isolate filtrate autoclaved at 120°C (disk diffusion)	27.66±0.57	27.33±1.15
Gn-A11-18 isolate filtrate autoclaved at 120°C (well diffusion)	38.00±1.0	41.00±1.73

3.3. Effect of culture medium, temperature and pH on the antifungal activity of Gn-A11-18 isolate against *A. niger* and *C. albicans*

The different culture media, pH and temperatures have been examined to determine the culture medium, pH and optimal temperature of the antifungal activity of Gn-A11-18 isolate against *A. niger* and *C. albicans*. The antifungal activity of isolate filtrate has been tested by the well diffusion method. The results obtained are summarized in table (3), according to which the highest antifungal activity was observed in the MEA medium compared to the NB, YM and SB media. With regard to pH, the best pH of the antifungal activity which has been the neutral pH which gives the highest activity, 42.33 mm of inhibition against *C. abicans* compared to the control and a percentage of inhibition in the order of 40.33% against *A. niger* compared to the control. While the optimal temperature for the production of antifungal substances by Gn-A11-18 isolate is 30°C compared to the temperatures tested.

Bacillus species are capable of producing several antifungal metabolites on NB (Nutrient Broth), LB (Luria Bertani) and other simple and complex culture media. However, this is the first time the production of antifungal metabolites by *Bacillus* spp against *C. albicans* and *A.*

niger has been reported with high antifungal activity in the MEA medium. Moreover, in contradiction to the results obtained by Balouiri *et al.*[19], which show that the YMA medium (Yeast Malt extract Agar) gives the highest antifungal activity of *Bacillus* spp. against *C. albicans* and also contrary to the study by Kumar *et al.*[34]which have been shown that the TSB medium gives the best production of the antifungal activity of *B. subtilis*, we have shown that MEA gives the best antifungal activity of the Gn-A11-18 isolate against *A. niger* and *C. albicans*. Neutral pH is the optimal pH of the production of antifungal metabolites of our isolate, this is in line with the studies of Bamidele *et al.*[28]which have shown that the neutral pH gives the best antifungal activity against *C. albicans*. The temperature of 30°C is the optimal temperature for the production of antifungal metabolites of Gn-A11-18 isolate against *C. albicans* and *A. niger*, our results are in accordance with those of [19, 25]which showed that the optimal temperature of antifungal activity is 30°C, and as opposed to Bamidele *et al.*[28]which showed in their studies that the temperature 37°C is the optimal temperature for the production of antifungal substances from lactic acid bacteria to control *C. albicans.*

Table 3. Characterization of culture medium, temperature and pH of the antifungal activity of Gn-A11-18 isolate against *A. niger* and *C. albicans*

Parameters		***A. niger*(%)**	***C. albicans*(mm)**
pH	1.0	00±0.00	00±0.00
	2.0	00±0.00	00±0.00
	3.0	00±0.00	00±0.00
	4.0	19.66±0.58	29.66±0.58
	5.0	25.5±0.50	28.66±0.58
	6.0	40.33 ±0.58	42.33±0.58
	7.0	40.33±0.58	42.33±0.58
	8.0	34.67±0.58	32.33±1.52
	9.0	35.67±1.15	32.66±1.52
	10.0	35±1.00	33.66±1.15
Temperature (°C)	25	36±2.00	39.33±0.58
	30	40.33±0.57	42.33±0.58
	37	31.33±0.58	36.67±0.58
	44	32.33±1.53	32.67±1.15
Culture media	ME	40.33±0.57	42.33±0.58
	NB	26.33±2.52	27.67±1.53
	SB	25.67±1.15	26.67±1.15
	YM	34.5±0.87	36.33±0.58

ME. Malt extract, **NB.** Nutrient broth, **SB.** Sabourand, **YM.** Yeast and Malt extract

4. Conclusion

The present study shows promising antifungal activity of Gn-A11-18 isolate against *A. niger* and *C. albicans*. This isolate has been identified as *Bacillus* sp isolated from solid household waste digested in water and soil. This discovery suggests that the bioactive substance of this isolate may be new antifungal drugs to control pathogenic and phytopathogenic fungi and especially *C. albicans* and *A. niger*. However, further research is needed to complete the molecular identification of this isolate, the extraction of bioactive fractions, the physicochemical characterization of bioactive fractions, the purification and physicochemical characterization of antifungal compounds.

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