Journal of Research in Pharmacy

Research Article

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Biological activities assessment of secondary metabolites derived from *Aspergillus* species

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Received: 21 June 2024 / Revised: 26 August 2024 / Accepted: 27 August 2024

ABSTRACT: *Aspergillus* species are valuable sources of bioactive compounds with potential therapeutic, industrial, and agri-food applications. Therefore, the aim of this study was to evaluate different biological activities including; antioxidant, anti-inflammatory, antidiabetic, antifungal, and antiproliferative exhibited by secondary metabolites produced by three fungal species; *Aspergillus quadrilineatus* (MH109538), *Aspergillus niveus* (MH109544) and *Aspergillus wentii* (MH109545). The obtained results showed that the *Aspergillus wentii* extract exhibited significant activity in the DPPH and Phenanthroline assays, with an IC_{50} values of 131.85 ± 0.72 µg/mL, and an $ABS_{0.5}$ value of 55.58 ± 1.08 µg/mL respectively. While *Aspergillus niveus* extract demonstrated considerable antioxidant activity in the ABTS test, with an IC_{50} value of 78.15 ± 1.41 µg/mL. In addition, the assessment of anti-inflammatory activity revealed a significant effect of the *Aspergillus quadrilineatus* extract, with an IC_{50} value of 280.00 ± 0.43 µg/mL. Furthermore, the three extracts exhibited significant anti-diabetic activity compared to the reference molecule (acarbose). Regarding antifungal activity, evaluated by well method against three fungal strains, the *Aspergillus niveus* extract showed an important effect against the yeast *Candida albicans* with an inhibition zone diameter of 24.6 mm. Moreover, the MTS assay indicated cytotoxicity of the three fungal strains against the used cell lines recording 100 % mortality for *Aspergillus quadrilineatus* at 0.5 mg/mL and 0.25 mg/mL concentrations, and for *Aspergillus niveus* and *Aspergillus wentii* at 0.5 mg/mL against the MCF-7 tumor cell line.

KEYWORDS: *Aspergillus quadrilineatus*; *Aspergillus niveus*; *Aspergillus wentii*; secondary metabolites; biological activities.

1. INTRODUCTION

Microorganisms are a valuable source of therapeutically bioactive compounds, particularly through the production of low molecular weight secondary metabolites [1]. Unlike primary metabolites, these metabolites are not directly necessary for organism growth [2]. Among microorganisms, fungi include a surprisingly diverse collection of about 1.5 million species that can potentially generate a wide range of metabolites such as alkaloids, benzoquinones, flavanoids, phenols, steroids, terpenoids, tetralones and xanthones. Due to their richness in natural compounds, fungi are extensively exploited in the food, pharmacological and medicinal industries [3].

Currently, 45 % of known microbial metabolites originate from fungi, with filamentous fungi, including *Penicillium*, *Trichoderma*, and *Aspergillus*, which represent almost 99% of these fungal metabolites [4]. However, more than 492 distinct species of the vast genus *Aspergillus* can be found worldwide [5]. The ability

How to cite this article: Bramki A, Bertrand V, Makhlouf FZ, Latifi R, Salahouali A, Bensouici C, Mebirouk R, De Pauw-Gillet MC. Biological activities assessment of secondary metabolites derived from Aspergillus species. J Res Pharm. 2025; 29(4): 1562-1572.

ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1734651

of some species of this genus to synthesize natural products with important applications and playing a crucial role in many biological activities including antioxidant, anti-inflammatory, etc. makes them useful for biotechnological applications.

Within *Aspergillus*, species such as *A. niger*, *A. oryzae*, and *A. terreus* are considered workhorses in biotechnology [6]. However, other lesser-known species, such as *A. quadrilineatus*, *A. niveus* and *A. wentii* have great biotechnological potential, which has already been described. Therefore, this work aims to highlight the secondary metabolites produced by these underexplored species and to investigate their biological activities.

2. RESULTS & DISCUSSION

2.1. Antioxidant activity

The antioxidant activity of the three fungal extracts was measured by four methods: DPPH free radical scavenging, ABTS radical scavenging, the reduction by the formation of Fe^{2+}-phenanthhroline complex and the FRAP test. Table 1 gives an overview of the obtained results.

Table 1. IC_{50} and ABS 0.5 values of fungal extracts for various antioxidant methods.

Extracts	IC_{50} (µg/mL)		$ABS_{0.5}$ (µg/mL)	
	DPPH	ABTS	Phenanthroline	FRAP
A. quadrilineatus	> 800 [a]	503.49 ± 1.28 [a]	> 200 [a]	> 200
A. niveus	583.58 ± 1.45 [b]	78.15 ± 1.41 [c]	60.51 ± 1.94 [b]	> 200
A. wentii	131.85 ± 0.72 [c]	87.71 ± 1.25 [b]	55.58 ± 1.08 [c]	> 200
Trolox	5.12 ± 0.21 [d]	3.01 ± 0.06 [d]	5.21 ± 0.27 [d]	5.25 ± 0.20
ascorbic acid	4.39 ± 0.01 [d]	3.04 ± 0.05 [d]	3.12 ± 0.02 [d]	3.62 ± 0.29

IC_{50}: 50 % inhibition concentration ; ABS 0.5: concentration indicating 0.50 of absorbance. Values are expressed as mean of three replications ± SD. Different letters indicate significant differences at $p \leq 0.05$ according to one way ANOVA followed by Tukey's HSD test.

Significant differences were detected among extracts ($p \leq 0.05$). *A. wentii* extract exhibited the strongest scavenging capacity, followed by *A. niveus* extract. The observed antioxidant activity in these species could be due to their high content of phenolic compounds, commonly known for their antioxidant properties [3].

2.1.1. DPPH scavenging assay

According to the results, *A. wentii* extract had a moderate activity of scavenging free radicals DPPH (IC_{50} = 131.85 ± 0.72 µg/mL), comparing it with that of Trolox (IC_{50} = 5.12 ± 0.21 µg/mL) and ascorbic acid (IC_{50} = 4.39 ± 0.01 µg/mL), and compared to extracts; *A. niveus* (IC_{50} = 583.58 ± 1.45 µg/mL), and *A. quadrilineatus* which recorded the lowest activity (IC_{50} > 800 µg/mL). Our results align with those reported by Hamed *et al.* [7], who observed a 46% DPPH free radical scavenging activity by the *A. unguis* strain at a concentration of 200 µg/mL. Additionally, the findings of Nuraini *et al.* [8] indicated that *A.minisclerotigens* and *A. oryzae* exhibited a strong antioxidant capacity, with IC_{50} values of 142.96 µg/mL and 145.01 µg/mL, respectively.

2.1.2. ABTS cation radical assay

The scavenging activity of the ABTS radical reflects an antioxidant ability to convert the blue/green-colored ABTS radical into its non-radical, colorless ABTSH form. As shown in Table 1, the *A. niveus* extract showed the highest activity (IC_{50} = 78.15 ± 1.41 µg/mL), followed by the *A. wentii* extract (IC_{50} = 87.71 ± 1.25 µg/mL). However, the *A. quadrilineatus* extract recorded the lowest activity among the extracts; IC_{50} = 503.49 ± 1.28 µg/mL, which is six times lower compared to that of *A. niveus*, Notably, *A. niveus* exhibited significant ABTS radical cation-scavenging activity in comparison to standard antioxidants Trolox (IC_{50} = 3.01 ± 0.06 µg/mL) and ascorbic acid (IC_{50} = 3.04 ± 0.05 µg/mL).

The extracts demonstrated a greater ability to scavenge free radicals in this activity compared to the DPPH free radical scavenging activity. This finding is supported by the ability of the ABTS radical cation to be more versatile than DPPH, since it is soluble in water and organic solvents, which makes it possible to assess the free radical scavenging capacity for lipophilic and hydrophilic substances [9]. Indeed, the potential of free radical scavenging largely depends on the specific free radical used as a reagent, which explains the differences in results from one method to another [10]. These results are consistent with those reported by Ton That Huu [11], in a similar study of five secondary metabolites of *A. tamarii*, who found a scavenging activity of the ABTS radical cation, with an inhibition percentage of about 18-32%.

2.1.3. Phenanthroline activity test

With varying degrees of potential, the extracts are able to reduce iron and form the Fe+2-phenanthroline complex. According to the results, the maximum reduction potential was observed with the *A. wentii* extract (ABS 0.5 = 55.58 ± 1.08 µg/mL), followed by *A. niveus* extract (ABS 0.5 = 60.51 ± 1.94 µg/mL). In addition, a relatively average activity was recorded by the *A. quadrilineatus* extract. Indeed, these results are noteworthy when compared to the reduction potentials of ascorbic acid (ABS 0.5 = 3.12 ± 0.02 µg/mL) and Trolox (ABS 0.5 = 5.21 ± 0.27 µg/mL).

The presence of reducing agents in these extracts allows to reduce Fe^{+3} to Fe^{+2}, which then forms a stable compound with phenanthrolin (red-orange). The spectrophotometric approach reveals this combination Fe^{+2}-phenanthrolin, enabling the measurement of ferrous ions and thereby determining the reducing power of the antioxidant. Moreover, these fungal species are well known for their antioxidant effect, for example, Wu *et al.* [12], proved that *A. niger* presented an absorbance of 0.3 at a concentration of 200 µg/mL.

2.1.4. Ferric Reducing Antioxidant Power (FRAP)

The obtained results indicate that the reduction capacity of the three studied extracts, as well as that of the standards, increases proportionally with the used concentrations. However, the extracts seem to have a slightly lower iron reduction potential compared to the two standards. The decline of the extracts' activity in this test may be justified by the degradation of secondary metabolites [3]. Interestingly, a species of the same genus *A. creber* has shown an absorbance between 0.2 and 0.3 at a concentration of 200 µg/mL [13]. indicating a reductive power similar to our results.

2.2. Anti-inflammatory activity

Table 2 reports the anti-inflammatory activity of fungal extracts at different concentrations as well as the results of one-way ANOVA. The *A. quadrilineatus* extract demonstrated the highest levels of BSA (Bovine Serum Albumin) denaturation inhibition activity, with inhibition percentages of 87.02% and 80.51 % observed at concentrations of 1000 µg/mL and 500 µg/mL respectively. The inhibition activity reached saturation at a concentration of 1000 µg/mL for the diclofenac standard.

Table 2. Anti-inflammatory activity (as % of inhibition) of different fungal extracts**.**

	% of inhibition				
	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	IC_{50} (µg/mL)
A. quadrilineatus	29.93 ± 1.41 ^{b}	46.94 ± 1.22 ^{b}	80.51 ± 0.71 ^{b}	87.02 ± 1.53	280.00 ± 0.43 ^{a}
A. niveus	-	-	-	-	-
A. wentii	-	-	-	-	-
Diclophenac	49.88 ± 1.96 ^{a}	88.36 ± 1.59 ^{a}	98.32 ± 1.50 ^{a}	S	128.83 ± 0.08 ^{b}

(-): No activity ; S: Saturation. Values are expressed as mean of three replications ± SD. Different letters indicate significant differences at $p \leq 0.05$ according to one way ANOVA followed by Tukey's HSD test.

The obtained results reveal that the inhibition of heat-induced denaturation of BSA depends on the concentration of extracts as well as the reference drug (diclofenac). Indeed, protein denaturation is linked to inflammation, it has been demonstrated that several anti-inflammatory drugs, such as salicylic acid, exhibit a dose-dependent reduction in heat-induced protein denaturation [14].The *A. quadrilineatus* extract showed significant anti-inflammatory activity, with an IC_{50} value of 280 ± 0.43 µg/mL. This result is relatively close to the IC_{50} value recorded by the standard anti-inflammatory; diclofenac (IC_{50} = 128.83 ± 0.08 µg/mL). While the IC_{50} values of extracts *A. niveus* and *A. wentii* extracts have not been determined in this study. This suggests that the fungal extract of *A. quadrilineatus* may contain significant amounts of anti-inflammatory compounds such as ethyl linoleate, oleic acid, n-hexadecanoic acid and ethyl palmitate. The findings recorded by Skanda and Vijayakumar [15] in their study on the *A. arcoverdensis* strain confirm the presence of an anti-inflammatory activity of the *Aspergillus* genus isolates. Specifically, they demonstrated that at a concentration of 1000 µg/mL, the fungal extract exhibited an inhibition of 31.34 %.

2.3. Antidiabetic activity

The inhibitory capacity of the different fungal extracts on alpha-amylase enzyme was evaluated by testing different concentrations of the extracts against acarbose. The obtained results are expressed as a percentage of inhibition of alpha amylase (Table 3).

Table 3. Antidiabetic activity (as % of inhibition) of different fungal extracts.

Extracts	% of inhibition						
	6.25 µg	12.5 µg	25 µg	50 µg	100 µg	200 µg	400 µg
A. quadrilineatus	-	4.52 ± 0,71[a]	7.69 ± 0.86 [a]	15.96 ± 1.81[a]	23.89 ± 0.59 [a]	28.10 ± 0.11 [a]	46.54 ± 0.86 [a]
A. niveus	-	-	1.57 ± 0.40 [b]	3.08 ± 0.92 [b]	8.86 ± 0.18 [b]	10.51 ± 1.51 [b]	22.82 ± 1.47 [b]
A. wentii	-	-	-	1.79 ± 0.26 [b]	2.81 ± 0.54 [c]	4.85 ± 0.89 [c]	13.50 ± 0.62 [c]
Acarbose	0.77 ± 0.17	0.81 ± 0.06 [b]	0.95 ± 0.05 [b]	0.11 ± 0.01 [c]	3.20 ± 0.09 [c]	3.67 ± 0.19 [c]	5.41 ± 0.09 [d]

(-): No activity. Values are expressed as mean of three replications ± SD. Different letters indicate significant differences at $p \leq 0.05$ according to one way ANOVA followed by Tukey's HSD test.

According to Table 3, it is apparent that the inhibition rate of extracts as well as that of acarbose increases proportionally with their concentrations, indicating a dose-dependent relationship. The *A. quadrilineatus* extract has a significantly higher inhibitory capacity compared to the other two extracts. Indeed, this inhibitory capacity was detectable even at a low concentration of 12.5 µg/mL. Furthermore, the inhibitory capacity of the three fungal extracts distinguishes from that of acarbose, especially for the *A. quadrilineatus* extract, which gave a rate of 46.54 % at a concentration of 400 µg/mL, compared to the reference (acarbose), which recorded a percentage of 5.41 % at the same concentration. This heightened inhibitory capacity may be attributed to the presence of some bioactive secondary metabolites including polyphenols that carry functional groupings similar to those found in starch. These groups can bind to the active site of alpha amylase by forming hydrogen bonds with residues present in this active site, thereby preventing its binding and inhibiting its activity [16]. To our knowledge, no inhibitory capacity of α- amylase has been previously evaluated on the studied fungal extracts, which reveals a promoter treatment for diabetes.

2.4. Antifungal activity

The organic extracts were tested for their antifungal activity against three different species by the well technique. The presence of an inhibitory zone around the well reflects the sensitivity of the target fungus. The results are presented in Table 4.

Table 4. Antifungal activity of the three fungal extracts.

	C. albicans	*F. Oxysporum*	*Alternaria* sp.
A. quadrilineatus	-	-	-
A. niveus	24.6 ± 0.84 mm	-	-
A. wentii	-	-	-

(-) : No activity

The results indicate that only the yeast *C. albicans* was sensitive, and this sensitivity was observed solely with the *A. niveus* extract. The latter showed significant activity, with an inhibition zone of 24.6 mm in diameter. Moreover, no activity was observed against the two fungi *F. oxysporum* and *Alternaria* sp. For the concentration effect, the results show that the diameter of the inhibition zone depends on the concentration of the tested extract. Therefore, the MIC (minimum inhibitory concentration) of *A. niveus* extract against *C. albicans* is determined to be 1.56 mg/mL with an inhibition zone diameter of 6.85 mm (Table 5).

Table 5. The effect of different concentrations of *A. niveus* extract on *C. albicans*.

Concentration (mg/mL)	25	12.5	6.25	3.125	1.56	0.78	0.39
Diameter (mm)	20.5 ± 0.07	17.9 ± 0.14	16.05 ± 0.21	12.85 ± 0.21	6.85 ± 0.35	-	-

(-) : No activity

The inhibition of yeast growth reflects the ability of *A. niveus* to synthesize bioactive molecules against *C. albicans*, that can target either its cell wall, its protein synthesis, or the inhibition of its metabolic pathways by causing its dysfunction [17]. This result aligns with those of Furtado *et al.* [18], who demonstrated the activity of *A. fumigatus* against *C. albicans*, and the results of Alkhulaifi *et al.* [19], who found significant antifungal activity of intra- and extracellular secondary metabolites from species including *A. athecius*, *A. terreus var. africans*, *A. flavus*, *A. terreus*, and *A. foetidus* against *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*.

2.5. Antiproliferative activity

After 3 days of incubation with fungal extracts, growth inhibition was measured using the MTS test. The results were expressed as the mean percentage of growth inhibition relative to a negative control. As shown in Figures 1 and 2.

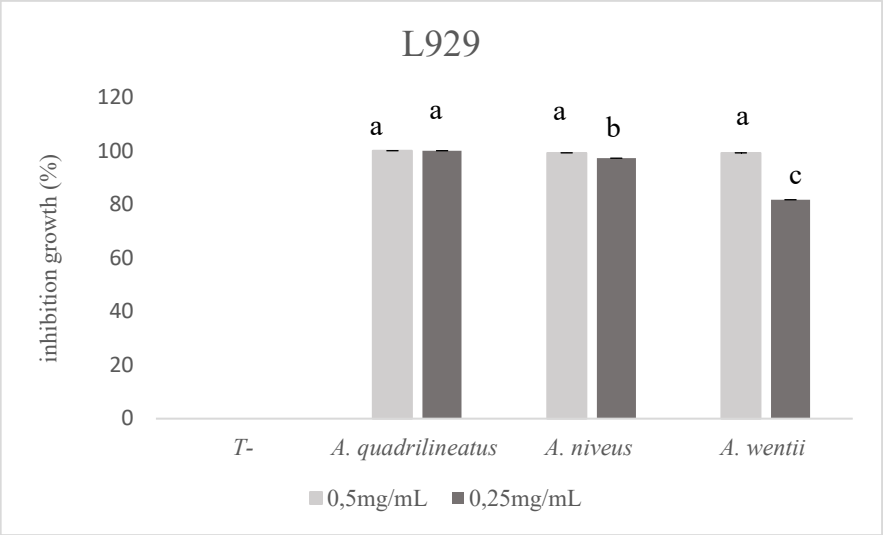

Figure 1. Growth inhibition percentage of L929 cells treated by fungal extracts.
Different letters indicate significant differences at $p \leq 0.05$ according to one way ANOVA followed by Tukey's HSD test.

The results showed that the *Aspergillus* genus had a remarkable potential cytotoxic effect against tumor cells. Indeed, the extracts prepared from the three fungal species had a cytotoxic effect against tumor cell lines used in this study (MCF-7), with a growth inhibition percentage of 100 % registered with *A. quadrilineatus* at the concentrations of 0.5 mg/mL and 0.25 mg/mL, *A. niveus* and *A. wentii* with the concentration of 0.5 mg/mL. According to other works, it has proven the cytotoxic power of the *Aspergillus* genus against several tumor lines [20]. Indeed, it was reported in previous works that the *A. wentii* strain was capable of inducing cell death of tumor cells by apoptosis and inhibiting metastases. It could inhibit the receptor of epidermal growth factor involved in the malignant transformation of cells in liver tumor cells [21]. The involvement of different compounds isolated from different species of the *Aspergillus* fungus has been proven. In fact, molecules such as wentilactone B from *A. wentii*, gliotoxin from *A. fumigatus*, austocystin D from *A. puniceus*, *A. turkensis*, *A. pseudoustus* and *A. ustus* had inhibited the growth of tumor lines *in vitro* (including MCF-7) [22, 23, 20]. In the other hand, our extracts had the same effect on normal cell line used in this study (L929). It should be noted, that in previous studies, the *A. wentii* was not toxic against non-cancerous cell lines such as HUVEC, Normal Human Liver L02 cell line, and Chang Liver (HeLA derivative) cell line [21].

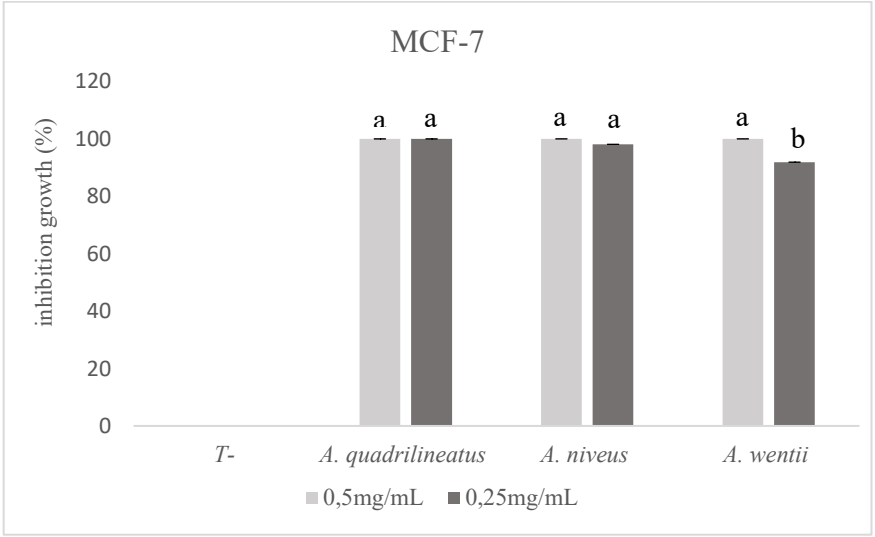

Figure 2. Growth inhibition percentage of MCF-7 cells treated by fungal extracts.

Different letters indicate significant differences at $p \leq 0.05$ according to one way ANOVA followed by Tukey's HSD test.

3. CONCLUSION

The main objective of this work was to evaluate the different biological activities of three fungal species; *A. quadrilineatus* (MH109538), *A. niveus* (MH109544) and *A. wentii* (MH109545). The findings revealed that the *A. wentii* extract exhibited significant antioxidant activity, with an IC_{50} value of 131.27 ± 0.35 µg/mL using the DPPH method. Moreover, both ABTS and phenanthrolin methods indicated remarkable antioxidant activity in the *A. wentii* and *A. niveus* extracts compared to the positive controls. All three extracts demonstrated a distinguished alpha amylase inhibitory activity compared to the reference molecule (acarbose). On the other hand, the *A. quadrilineatus* extract showed potent anti-inflammatory activity, with an IC_{50} value of 280.00 ± 0.43 µg/mL. Regarding antifungal activity, the well diffusion method indicated that *A. Niveus* extract displayed a notable antifungal effect against the yeast *C. albicans*. Lastly, the MTS assay results demonstrated that the *Aspergillus* genus had a considerable cytotoxic potential effect against the MCF-7 tumor cell line, as well as cytotoxicity against fibroblast immortalized cell line L-929.

4. MATERIALS AND METHODS

4.1. Biological material

Three fungal species belonging to *Aspergillus* genus were used in this study: *Aspergillus quadrilineatus* (MH109538), *Aspergillus niveus* (MH109544) and *Aspergillus wentii* (MH109545). These fungi were isolated from different Algerian soils [24].

4.2. Fermentation and extraction of bioactive metabolites

Fermentation was carried out on the liquid Czapek-dox culture medium (20 g of sucrose, 2 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 g ferrous sulfate and 1000 mL distilled water, pH = 7) added with 2.5 g/L of yeast extract, due to its favorable composition for the production of bioactive molecules. Vials of 500 mL containing 100 mL of culture medium were inoculated by eight discs of each seven-day fungal culture and incubated at 28 °C for 14 days (static fermentation). Afterwards, the secondary metabolites were extracted using 100 mL of chloroform. The contents of the vials were crushed using an ultra-turrax before being filtered on Whatman paper N°1 (11 µm). To separate the aqueous phase from the organic phase, the filtrate was passed through a decanting ampoule. This procedure was repeated several times, by successively adding decreasing volumes of chloroform to recover as many metabolites as possible. The obtained organic phase was dried using the rotary evaporator to obtain a raw extract [25, 26].

4.3. Antioxidant activity

The antioxidant activity of the three fungal extracts was evaluated according to four methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging, the reduction by the formation of phenanthroline Fe^{2+} ferrous complex, and the Ferric Reducing Antioxidant Power (FRAP) assay. Trolox and ascorbic acid were used as standards.

4.3.1. DPPH scavenging assay

The test was carried out on 96-well microplates, a volume of 40 µL of each extract at different concentrations (12.5, 25, 50, 100, 200, 400 and 800 µg/mL diluted in methanol), was mixed with 160 µL of the freshly prepared DPPH solution (0.1 mM). The mixture was incubated at room temperature in the dark for 15 min. Subsequently, the absorbance was measured at 517 nm against both a blank and a negative control (40 µL methanol, 160 µL DPPH) prepared under the same operating conditions [27, 28]. The inhibition percentage was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = (A_{Control} - A_{sample})/A_{Control} \times 100$$

where:

$A_{Control}$: negative control absorbance

A_{Sample} : extract (or standard) absorbance

4.3.2. ABTS cation radical assay

The test was conducted on a 96-well microplate, using the Re *et al.* Method [29]. The cation radical ABTS was generated by mixing 7 mM of an aqueous solution of ABTS with 2.45 mM of potassium persulfate, the whole was then stored protected from light and at room temperature for 16 h before use. The solution obtained was subsequently diluted with ethanol to obtain an absorbance of 0.7 ± 0.02 at 734 nm. After this, 160 µL of ABTS solution was added to 40 µL of methanol extract solution at different concentrations (12.5, 25, 50, 100, 200, 400 and 800 µg/mL). The mixture was kept at room temperature in the dark for 10 min. The absorbance was read at a wavelength of 734 nm, methanol was used as a negative control. The inhibition percentage was calculated as follows:

$$ABTS^{\circ+} \text{ scavenging effect } (\%) = (A_{Control} - A_{Sample})/A_{Control} \times 100$$

where:

$A_{Control}$: negative control absorbance

A_{Sample} : extract (or standard) absorbance

4.3.3. Phenanthroline assay

For this test, a volume of 10 µL of each extract at different concentrations (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) was mixed with 50 µL of anhydrous iron chloride (0.2 %), 30 µL of phenanthroline (0.5 %) and 110 µL of methanol. The mixture was incubated at 30 °C for 20 min and the absorbance was measured at 510 nm. A blank was used replacing the extract with methanol [30].

4.3.4. Ferric Reducing Antioxidant Power (FRAP)

This approach was determined by the Oyaizu method [31]. On a 96-well microplate, 10 µL of each extract at different concentrations (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) were added with 40 µL phosphate buffer (pH 6.6) and 50 µL of ferricyanide potassium solution (K_3 [Fe (CN) $_6$] (1 %), the mixture was incubated at 50 °C for 20 min. The reaction was then stopped by the addition of 50 µL of trichloroacetic acid (TCA) (10 %). Finally, 40 µL of distilled water and 10 µL of anhydrous iron chloride solution (0.1 %) were added. The absorbance of the reaction medium was read at 700 nm against a blank, replacing the extract with methanol.

The results were expressed as an IC_{50} values (µg/mL), corresponding to the 50 % inhibition concentration, in both DPPH and ABTS tests [32], and as ABS 0.50 (µg/mL), corresponding to the concentration indicating 0.50 of absorbance, in Phenanthroline and FRAP assays [30].

4.4. Anti-inflammatory activity

The anti-denaturation test of BSA was determined as described by Kar [33]. Briefly, 100 µL of each extract at different concentrations (diluted in ultra-pure water) were added to 100 µL of BSA. The mixture was incubated at 72 °C for 20 min, and the absorbance was measured at 660 nm against a blank prepared from 100 µL of Tris-HCl buffer (0.05 M, pH = 6.6) and 100 µL of extract, as well as to a negative control prepared from 100 µL BSA and 100 µL of ultra-pure water. Diclofenac (known as a nonsteroidal anti-inflammatory drugs) served as the standard for comparison. The inhibition percentage was calculated as follows:

$$\% \text{ Inhibition} = (A_{Control} - A_{Sample}) / A_{Control} \times 100$$

where:

$A_{Control}$: negative control absorbance

A_{Sample} : extract (or standard) absorbance

4.5. Antidiabetic activity

The antidiabetic activity of the three fungal extracts were determined by the alpha-amylase enzyme inhibition method as described by Zengin *et al.* [34]. Each extract (25 µL) at different concentrations was deposited in the wells, followed by adding 50 µL of the alpha amylase solution. Incubation was carried out for 10 min at 37 °C. Subsequently, 50 µL of starch (0.1 %) was added, and another 10 min incubation was performed. Thereafter, 25 µL of hypochloric acid (1M) and 100 µL of iodine/potassium iodide (IKI) were added, and the results were read at 680 nm. Acarbose was employed as standard, and the inhibition percentage of alpha-amylase was determined using the following formula:

$$\% \text{ Inhibition} = 1 - |(Ac - Ae) - (As - Ab) / (Ac - Ae)|$$

where:

Ac = Absorbance [25 µL Solvent + 50 µL buffer solution + starch + HCl + IKI]

Ae = Absorbance [25 µL Solvent + enzyme + starch + HCl + IKI]

As = Absorbance [Extract + enzyme + starch + HCl +IKI]

Ab = Absorbance [Extract + 125 µL buffer solution + IKI].

4.6. Antifungal activity

4.6.1. Preparation of fungal inocula

The antifungal activity was evaluated against *Candida albicans* yeast and filamentous fungi, including *Fusarium oxysporum* and *Alternaria* sp., *C. albicans* was cultured on Sabouraud medium and incubated for 24 to 48 hours at 37°C, while the filamentous fungi were seeded on Potato Dextrose Agar (PDA) and incubated at 28°C for a period of 14 days.

Spore suspensions were obtained by scraping cultures after adding physiological water. These suspensions were then diluted to an absorbance of 0.2 to 650 nm. followed by an additional dilution 1/10th. On the other hand, the yeast suspension was adjusted with sterile physiological water to a density of 0.5 Mc Farland [35 , 36].

4.6.2. The well technique

To assess the antifungal properties, an agar well diffusion assay was followed. Each indicator strain previously prepared inoculated onto Petri dishes filled with Sabouraud medium. Wells (3 mm) were sterilely dug on the agar surface and then filled with 20 µL of each fungal extract (50 mg/mL in Dimethylsulfoxide). The dishes were initially incubated at 4 °C for 30 min, then at 28 °C for 48 to 72 hours for molds and 24 to 48 at 37 °C for *C. albicans*. The inhibition activity was identified by measuring the diameter of the inhibition zone around the well [37, 38]. Subsequently, a dilution range of 25 to 0.19 mg/mL of the extracts exhibiting antifungal activity was prepared to determine the MIC, using the same method mentioned above [39].

4.7. Antiproliferative activity

4.7.1. Cell lines

Cell lines used in this study belong to laboratory of Histology-Cytology, University of Liège. The L-929 cells are a type of adherent mouse fibroblast immortalized cell line often used as an alternate test system

for toxicity assessment and the human breast cancer cell line MCF-7/Bos (Michigan Cancer Foundation Clone 7).

4.7.2. Culture cell

200 µL of cell suspension (5 x 103 cells) in DMEM culture medium (Dulbecco's Modified Eagle Medium, GibcoTM, US) were cultured 24 h at 37 °C, with 5 % CO_2 and saturated with water, in 96-well culture plates in a cell incubator. The culture medium was enriched with 10 % of Fetal Bovine Serum (FBS) previously decomplemented 1 h at 56 °C, and 1 % antibiotics (Penicillin and streptomycin). After that, culture medium was sucked with a vacuum and 200 µL of each fungal extract diluted in DMSO 0.5 % (0.5 mg/mL and 0.25 mg/mL) were deposed in each well. Cells were incubated for 72 h at 37 °C. Untreated cells serve as a negative control.

4.7.3. The MTS assay

Cytotoxic effect of fungal extracts was assessed in vitro using the MTS assay. This test involves the conversion of the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium) to a formazan in living cells. Wells were rinsed with 100 µL of modified DMEM (F12), 20 µL of MTS (10X) were added to each well and the plate was incubated for 45 min in dark at 37 °C. Finally, the absorbance was read at 492 nm with a microplate reader PowerWave x (Bio-tec INC, USA). Data was presented by relative growth inhibition to untreated cells.The growth inhibition percentage was calculated as follows:

$$\% \text{ Growth inhibition} = 100 - [(100/A_{Control}) \times A_{Sample}]$$

where:

$A_{Control}$: negative control absorbance

A_{Sample}: extract absorbance

4.8. Statistical analysis

All analyses were carried out in triplicate and the experimental data were reported as means ± standard deviation. Statistical analysis was carried out using XLSTAT software (Addinsoft SARL, New York, NY, USA). Significant differences were determined at $p \leq 0.05$ by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test.

Acknowledgements: The authors wish to thank the support of the Algerian Ministry of Higher Education and Scientific Research (MESRS, DGRSDT)
Author contributions: Concept - A.B., Design – A.B., V.B., R.L., A.S., Supervision –A.B., Resource – C.B., M.C.D.P.G., Materials – A.B., C.B., M.C.D.P.G., Data Collection &/or Processing – A.B., V.B., R.L., A.S., Analysis &/or Interpretation - F.Z.M., Literature Search – A.B., R.L., A.S., Writing - A.B., R.M., R.L., A.S., Critical Reviews - A.B, F.Z.M., V.B., M.C.D.P.G.
Conflict of interest statement: The authors declare that there are no conflicts of interest related to this article.

REFERENCES

[1] Rani A, Saini KC, Bast F, Mehariya S, Bhatia SK, Lavecchia R, Zuorro A. Microorganisms: A potential source of bioactive molecules for antioxidant applications. Molecules. 2021; 26(4):1142. https://doi.org/10.3390/molecules26041142.

[2] Brakhage AA. Regulation of fungal secondary metabolism. Nat Rev Microbiol. 2013; 11: 21–32. https://doi.org/10.1038/nrmicro2916

[3] Arora DS, Chandra P. Antioxidant Activity of *Aspergillus fumigatus*. ISRN Pharmacol. 2011; 2011:619395. https://doi.org/10.5402/2011%2F619395

[4] Berdy J. Thoughts and facts about antibiotics: Where we are now and where we are heading. J Antibiot. 2012; 65: 385–395. https://doi.org/10.1038/ja.2012.27

[5] Sadorn K, Saepua S, Boonyuen N, Laksanacharoen P, Rachtawee P, Prabpai S, Kongsaeree P, Pittayakhajonwut P. Allahabadolactones A and B from the endophytic fungus, *Aspergillus allahabadii* BCC45335. Tetrahedron. 2016; 72(4): 489 495. https://doi.org/10.1016/j.tet.2015.11.056

[6] Frisvad JC, Larsen TO. Chemodiversity in the genus *Aspergillus*. Appl Microbio. Biotechnol. 2015; 99: 7859–7877. https://doi.org/10.1007/s00253-015-6839-z

[7] Hamed AA, Abdel-Aziz MS, Abd El Hady FK. Antimicrobial and antioxidant activities of different extracts from *Aspergillus unguis* SPMD-EGY grown on different media. Bull Natl Res Cent. 2018; 42(1): 29. https://doi.org/10.1186/s42269-018-0027-0

[8] Nuraini FR, Setyaningsih R, Susilowati A. Antioxidant activity of bioactive compound produced by endophytic fungi isolated from endemic plant of South Kalimantan *Mangifera casturi Kosterm*. AIP Conf Proc. 2019; 2120(1) https://doi.org/10.1063/1.5115751.

[9] Gülçin I. Antioxidant properties of resveratrol: A structure–activity insight. Innov Food Sci Emerg Technol. 2010; 11(1): 210-218. https://doi.org/10.1016/j.ifset.2009.07.002

[10] Salar RK, Purewal SS, Sandhu KS. Bioactive profile, free-radical scavenging potential, DNA damage protection activity, and mycochemicals in *Aspergillus awamori* (MTCC 548) extracts: A novel report on filamentous fungi. 3 Biotech. 2017; 7(3): 164. https://doi.org/10.1007/s13205-017-0834-2

[11] Ton That Huu D, Phuong HT, Diem Tran PT, Souvannalath B, Trung HL, Ho DV, Viet CLC. Secondary metabolites from the grasshopper-derived entomopathogenic fungus *Aspergillus tamarii* NL3 and their biological activities. Nat Prod Commun. 2022; 17:12. https://doi.org/10.1177/1934578X221141548

[12] Wu P, Tan H, Zhan J, Wang W, Hu T, Li S. Optimization of bioprocess extraction of *Poria cocos* polysaccharide (PCP) with *Aspergillus niger* β-glucanase and the evaluation of PCP antioxidant property. Molecules. 2020; 25(24): 5930. https://doi.org/10.3390/molecules25245930

[13] Sakhri A. PhD Thesis. Exploitation et caractérisation des substances bioactives sécrétées par une souche d'*Aspergillus* sp. Department of microbiology, Faculty of Natural and Life Sciences, University of Mentouri Brothers Constantine, Algeria, 2020.

[14] Moharram AM, Zohri AA, Omar HM, Abd El-Ghani OA. *In vitro* assessment of antimicrobial and anti-inflammatory potential of endophytic fungal metabolites extracts. Eur J Biol Res. 2017; 7 (3): 234-244. http://dx.doi.org/10.5281/zenodo.839696

[15] Skanda S, Vijayakumar BS. Antioxidant and anti-inflammatory metabolites of a soil-derived fungus *Aspergillus arcoverdensis* SSSIHL-01. Curr Microbiol. 2021; 78(4): 1317-1323. https://doi.org/10.1007/s00284-021-02401-3

[16] Yan JK, Wu LX, Qiao ZR, Cai WD, Ma H. Effect of different drying methods on the product quality and bioactive polysaccharides of bitter gourd (*Momordica charantia L.*) slices. Food Chem. 2019; 271: 588-596. https://doi.org/10.1016/j.foodchem.2018.08.012

[17] Zida A, Bamba S, Yacouba A, Ouedraogo-Traore R, Guiguemdé RT. Anti- *Candida albicans* natural products, sources of new antifungal drugs: A review. J Mycol Méd Suppl. 2017; 27(1): 1-19. https://doi.org/10.1016/j.mycmed.2016.10.002

[18] Furtado NAJC, Said S, Ito IY, Bastos JK. The antimicrobial activity of *Aspergillus fumigatus* is enhanced by a pool of bacteria. Microbiol Res. 2002; 157(3): 207-211. https://doi.org/10.1078/0944-5013-00150

[19] Alkhulaifi MM, Awaad AS, AL-Mudhayyif HA, Alothman MR, Alqasoumi SI, Zain SM. Evaluation of antimicrobial activity of secondary metabolites of fungi isolated from Sultanate Oman soil. Saudi Pharm J. 2019; 27(3): 401-405. https://doi.org/10.1016/j.jsps.2018.12.009

[20] Bladt TT, Frisvad JC, Knudsen PB, Ostenfeld LT. Anticancer and antifungal compounds from *Aspergillus, Penicillium* and other filamentous fungi. Molecules. 2013; 18: 11338-11376. https://doi.org/10.3390/molecules180911338

[21] Zhang Z, Miao L, Sun W, Jiao B, Wang B, Yao L, Huang C. Wentilactone B from *Aspergillus wentii* induces apoptosis and inhibits proliferation and migration of human hepatoma SMMC-7721 cells. Bio Pharm Bull. 2012; 35: 1964–1971. https://doi.org/10.1248/bpb.b12-00368

[22] Kanoh K, Kohno S, Asari T, Harada T, Katada J, Muramatsu M, Kawashima H, Sekiya H, Uno I. (−)-phenylahistin: A new mammalian cell cycle inhibitor produced by *Aspergillus ustus*. Bioorg Med Chem Lett. 1997; 7 (22): 2847–2852. https://doi.org/10.1016/S0960-894X(97)10104-4

[23] Wang CCC, Chiang YM, Kuo PL, Chang JK, Hsu YLP. Norsolorinic acid inhibits proliferation of T24 human bladder cancer cells by arresting the cell cycle at the G0/G1 phase and inducing a Fas/membrane-bound Fas ligand-mediated apoptotic pathway. Clin Exp Pharmacol. 2008; 35: 1301–1308. https://doi.org/10.1111/j.1440-1681.2008.05007.x

[24] Bramki A, Ghorri S, Jaouani A, Dehimat L, Chaouche NK. Antibacterial activity of *Aspergillus* isolated from different Algerian ecosystems. Afr J Biotechnol. 2017; 16 (32): 1699-1704. https://doi.org/10.5897/AJB2017.16086

[25] Gengan RM, Chuturgoon AA, Mulholland DA, Dutton MF. Synthesis of sterigmatocystin derivatives and their biotransformation to aflatoxins by a blocked mutant of *Aspergillus parasiticus*. Mycopathologia. 1998; 144 (2): 115-122. https://doi.org/10.1023/A:1007064304292

[26] Bramki A, De Pauw E, Frahtia M, McCann A, Mazzucchelli G, Dehimat L. Chromatographic separations and characterization of bioactive secondary metabolites of *Apergillus quadrilineatus* (MH109538) fungal strain. AsPac J Mol Biol Biotechnol. 2020; 28(3): 13-21. https://doi.org/10.35118/apjmbb.2020.028.3.02

[27] Blois MS. Antioxydant determinations by the use of a stable free radical. Nature. 1958; 4617(181): 1119-1200. https://doi.org/10.1038/1811199a0

[28] Tel G, Apaydın M, Duru ME. Antioxidant and cholinesterase ınhibition activities of three *Tricholoma* species with total phenolic and flavonoid contents: The edible mushrooms from Anatolia. Food Anal Methods. 2012; 5: 495–504. https://doi.org/10.1007/s12161-011-9275-4.

[29] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999; 26 (9-10): 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

[30] Szydlowska-Czerniak A, Dianoczki C, Recseg K, Karlovits G, Szlyk E. Determination of antioxidant capacities of vegetable oils by ferric-ion spectrophotometric methods. Talanta. 2008; 76(4): 899-905. https://doi.org/10.1016/j.talanta.2008.04.055

[31] Oyaizu M. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet. 1986; 44(6): 307-315. http://dx.doi.org/10.5264/eiyogakuzashi.44.307

[32] Carmona-Jiménez Y, García-Moreno MV, Igartuburu JM, Garcia Barroso C. Simplification of the DPPH assay for estimating the antioxidant activity of wine and wine by-products. Food Chem. 2014; 165: 198 204. https://doi.org/10.1016/j.foodchem.2014.05.106

[33] Kar B, Kumar RS, Karmakar I, Dola N, Bala A, Mazumder UK, Hadar PK. Antioxidant and *in vitro* anti-inflammatory activities of *Mimusops elengi* leaves. Asian Pac J Trop Biomed. 2012; 2(2): S976-S980. https://doi.org/10.1016/S2221-1691(12)60346-3

[34] Zengin G, Sarikurkcu C, Aktumsek A, Ceylan R, Ceylan O. A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. Endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. Ind Crops Prod. 2014; 53: 244-251. https://doi.org/10.1016/j.indcrop.2013.12.043

[35] Barboucha G, Rahim N, Boulebd H, Bramki A, Andolfi A, Salvatore MM, Masi M. Chemical Composition, In Silico Investigations and Evaluation of Antifungal, Antibacterial, Insecticidal and Repellent Activities of Eucalyptus camaldulensis Dehn. Leaf Essential Oil from ALGERIA. Plants. 2024; 13: 3229. https://doi.org/10.3390/plants13223229

[36] Hossain MA, Biva IJ, Kidd SE, Whittle JD, Griesser HJ, Coad BR. Antifungal Activity in Compounds from the Australian Desert Plant *Eremophila alternifolia* with Potency Against *Cryptococcus* spp. J Antibiot. 2019; 8: 1-12. https://doi.org/10.3390/antibiotics8020034

[37] Yamaç M, Bilgili F. Antimicrobial activities of fruit bodies and/or mycelial cultures of some mushroom isolates. Pharm Biol. 2006; 44: 660- 667. https://doi.org/10.1080/13880200601006897

[38] Prabavathy D, Valli Nachiyar C. Study on the antimicrobial activity of *Aspergillus* sp. isolated from *Justicia adathoda*. Indian J Sci Technol. 2012; 5(9): 3317-3320. http://dx.doi.org/10.17485/ijst/2012/v5i9/30679

[39] Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J Biol Sci. 2018; 25 (2): 361–366. https://doi.org/10.1016/j.sjbs.2017.02.004.