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Chemical Composition of *Aspergillus creber* Extract and Evaluation of its Antimicrobial and Antioxidant Activities

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Abstract

Among the species belonging to the *Aspergillus* section *Versicolores*, *Aspergillus creber* has been poorly studied and still unexplored for its biological activities. The present study was undertaken to analyze *A. creber* extract and to evaluate its *in vitro* antimicrobial and antioxidant activities. UHPLC-MS/MS analysis of *A. creber* extract allowed the characterization of five known fungal metabolites including: asperlactone, emodin, sterigmatocystin, deoxybrevianamide E, and norsolorinic acid. The highest antimicrobial activity was displayed against *Candida albicans*, with a mean strongest inhibition zone of 20.6 ± 0.8 mm, followed by Gram-positive drug-resistant bacteria. The MIC values of *A. creber* extract varied from 0.325 mg/ml to 5 mg/ml. *A. creber* extract was shown a potent antioxidant activity and a high level of phenolic compounds by recording 89.28% scavenging effect for DPPH free radical, 92.93% in ABTS assay, and 85.76 mg gallic acid equivalents/g extract in Folin-Ciocalteu assay. To our knowledge, this is the first study concerning biological and chemical activities of *A. creber* species. Based on the obtained results, *A. creber* could be a promising source of natural antimicrobial and antioxidant compounds.

Key words: Antimicrobial activity, antioxidant activity, *Aspergillus creber*, UHPLC-MS/MS, *Versicolores*

Introduction

Fungal secondary metabolites represent a diverse group of bioactive natural products often produced at a restricted part of the life cycle (Keller et al. 2005). *Aspergillus* species are among the major contributors to the secondary metabolites of fungal origin (Dewi et al. 2012; Bai et al. 2014). Although the functions of these compounds for the producing fungi are obscure or unknown, they confer important benefits to humankind as many of them have antibiotic and pharmaceutical activities (Yu and Keller 2005; Siddiquee et al. 2015). Isolation of a new fungal strain is often conducted to the identification of new natural products (Brakhage and Schroeckh 2011).

Aspergillus species assigned to section *Versicolores* are among the most ubiquitous fungi, frequently isolated from environmental samples (Gautier et al. 2016). The important characteristics that render these species of interest to the scientific community are their prevalent in indoor environments, their capacity to produce sterigmatocystin (STC), a carcinogenic mycotoxin, and their diverse biotechnological applications (Siqueira et al. 2016). Before using molecular methods for species identification, isolates belonging to section *Versicolores* were commonly reported as *A. versicolor* (Despot et al. 2016). In 2012, Jurjevic et al. revised the section *Versicolores* and accepted 13 species; among them, *Aspergillus creber* was described for the first time as a new species. Currently, the section *Versicolores* comprises 17 distinct species (Despot et al. 2017); among them, *A. versicolor* and *A. sydowii* are still the most commonly reported and studied species (Siqueira et al. 2016). However, very few reports are available concerning the species *A. creber* and those reports were only concerned with contamination by *A. creber* and its ability to

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produce STC. *A. creber* was reported as the most prevalent species in indoor air environments in USA (Jurjevic et al. 2012) and in Italian libraries (Micheluz et al. 2015). *A. creber* was also reported as among the lower producers of STC (Jurjevic et al. 2013).

Therefore, the lack of knowledge of this species recently assigned to section *Versicolores* has attracted our attention to more explore and study this species. In our research *A. creber* was assayed for its secondary metabolites diversity using UHPLC-MS/MS technique. Then, the antimicrobial activity of *A. creber* against some human pathogens and its ability to scavenge different free radicals, and to produce antioxidant compounds were also evaluated to improve the knowledge of this species, which has never been tested before for its biological activities.

Experimental

Materials and Methods

Fungal strain. The strain of *A. creber* employed in this study was isolated from maize grains collected from Batna region, Northeast Algeria. Morphological identification of the selected strain was made following the recommended method and media (Samson et al. 2014). Then, the identity was confirmed by the German Collection of Microorganisms and Cell Cultures (DSMZ) using the analysis of the Internal Transcribed Spacer rDNA region (ITS) and the calmodulin gene (CaM) sequences. The strain was kept on Potato Dextrose Agar (PDA) slants at 4°C in the fungal collection of the Laboratory de Mycologie, de Biotechnologie et de l'Activité Microbienne (LaMyBAM), University des Frères Mentouri Constantine-1.

Production and extraction of *A. creber* secondary metabolites. Under sterile conditions, a small amount of *A. creber* was transferred onto new PDA plates and incubated at 25°C until sporulation. The fungal spores were harvested by pouring 9 ml of sterile distilled water, containing 0.1% Tween 80, on the agar plate surfaces and transferred into sterile Falcon tubes. The prepared fungal suspension was inoculated aseptically in five 1000 ml Erlenmeyer flasks [5% (v/v)], each containing 250 ml of Czapek-Dox broth supplemented with yeast extract (Slack et al. 2009). The Erlenmeyer flasks were covered with the aluminum foil and incubated at 28 ± 2°C with shaking at 125 rpm for two weeks.

The culture broth was filtered, using Whatman filter paper number 1, to remove the mycelia. The filtered broth was extracted three times with equal volumes of ethyl acetate (EtOAc) and evaporated until dryness using a rotary evaporator at 45°C. The resulted ethyl acetate extract of the filtrate (EAF) was reconstituted in methanol to get a concentrated stock solution of 200 mg/ml, which was used for further analysis.

Ultrahigh-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) analysis of *A. creber* extract. A Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, USA) coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, USA) via a HESI-II electrospray ionization (ESI) source was used for the analysis of EAF. A volume of 1 µl of EAF was injected on a Luna Omega C18 column (50 × 2.1 mm, 1.6 µm particle size). The column thermostat was maintained at 25°C. A mobile phase consisting of eluent A (0.1% formic acid in water, 5 mM ammonium formate) and eluent B (0.1% formic acid in methanol, 5 mM ammonium formate) was used at a flow rate of 0.4 ml/min. The gradient elution was performed as follows: 0 min 20% eluent B; 0.5 min 40% eluent B; 6 min 100% eluent B; 8 min 20% eluent B; 10 min 20 % eluent B.

The Orbitrap mass spectrometer was operated in both positive and negative mode with the use of the following parameter settings: spray voltage, 4 kV; sheath gas (N2 > 95%), 35 arbitrary units; auxiliary gas (N2 > 95%), 10 arbitrary units; capillary temperature, 290°C; S lens RF level, 50; heater temperature, 305°C. Two scan events were carried out, the LC-MS was used in full scan mode at a resolution of 70 000 fwhm (full width at half maximum) in the range m/z 90–1000 without the use of any lock masses. The maximum injection time (MIT) was 100 ms with one micro scan, and the automatic gain control (AGC) target was set to 1e6. The MS/MS was performed in parallel reaction monitoring (PRM) mode, in order to obtain two product ions for each target compound, at a resolution of 35 000 fwhm with collision energy (CE) of 30, the AGC and MIT were set at 2e5 and 200 ms, respectively. The instrument control and data analysis were performed by Thermo Fisher Xcalibur v. 3.0.63 software.

Antimicrobial activity assay

Test microorganisms. Eight clinically isolated microorganisms, obtained from the laboratory of Clinical Microbiology, University of Federico II Napoli-Italy, were used to evaluate the antimicrobial activity of EAF. Four Gram-negative pathogenic bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Salmonella* Typhi) and two Gram-positive drug-resistant bacteria (DRB, *Staphylococcus aureus* producing beta-lactamase (SABL) and methicillin-resistant *Staphylococcus epidermidis* (MRSE)) were used for the antibacterial tests. Two pathogenic yeasts (*Candida albicans* and *Candida glabrata*) were used for anticandidal tests. Pathogenic bacteria were inoculated into the nutrient broth and incubated at 37°C for 24 h. *Candida* species were inoculated into Sabouraud Dextrose broth at 28°C for 48 h.

Antimicrobial activity determination. Antimicrobial activity of EAF against the test organisms was examined using disc diffusion method according to Turkoglu et al. (2007) with slight modification. Suspension of the organisms tested (0.5 McFarland standards) was spread on the sterile growth media plates containing Nutrient agar and Sabouraud Dextrose agar for bacteria and yeasts, respectively. Sterilized paper disks (6 mm) were impregnated with 20 µl of EAF and dried aseptically. The impregnated discs were placed on the surface of plates seeded with the test organisms and incubated at 37°C for 24 h for bacteria and at 28°C for 48 h for yeasts. Both chloramphenicol (10 µg/disk) and ketoconazole (30 µg/disk) were employed as positive controls for pathogenic bacteria and *Candida* species, respectively. Similarly, 20 µl of methanol and clean disks (without solvent) were used as negative controls. All experiments were carried out in triplicate. After incubation, antimicrobial activity was determined by measuring the diameter of inhibition zones around the discs.

Determination of minimum inhibitory concentration (MIC). The minimum inhibitory concentration (MIC) of EAF was carried out against the sensitive test microorganisms by using broth microdilution method following the recommendations of Clinical and Laboratory Standards Institute (CLSI) protocols for bacteria (CLSI 2012) and for yeasts (NCCLS 2002). Chloramphenicol and ketoconazole were used as a positive control. The MIC was taken as the lowest concentration at which no visible growth was observed.

Antioxidant analysis

2,2-diphenyl-1-picryl hydrazyl (DPPH) assay. The DPPH radical scavenging activity was determined according to Jakovljević et al. (2014). Different concentrations of EAF were prepared: 12.5, 25, 50, 100, 200, 400 µg/ml. To 1 ml of DPPH solution, 1 ml of each concentration was added. After incubation for 30 min at 37°C, the absorbance was measured at 517 nm against the blank. Ascorbic acid was used as positive control. The percentage of the DPPH scavenging activity was calculated using the formula:

$$\% \text{ inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 represents the absorbance of the blank sample, and A_1 represents the absorbance of the mixture.

2,2'-azino-bis-3- ethylbenzthiazoline-6-sulphonic acid (ABTS) assay. Using the same concentrations, ABTS assay was used as reported by Dudonné et al. (2009). The ABTS radical cation ($ABTS^{\bullet+}$) stock solution was prepared by mixing 7 mM of ABTS with 2, 45 mM of potassium persulfate. The mixture was left in the dark at room temperature for 12–16 h. For the study of EAF, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. To 3 ml of $ABTS^{\bullet+}$ diluted solution, 100 µl of each concentration was added, and the absorbance reading at 734 nm was taken at the end of the tenth minute. Percent inhibition of $ABTS^{\bullet+}$ was calculated using the previous formula.

Total phenolic content (TPC) assay. TPC of *A. creber* was determined using the Folin-Ciocalteu method. To 0.5 ml of EAF, 2.5 ml of Folin-Ciocalteu reagent was added. After 4 min, 2 ml of sodium carbonate (7.5%, w/v) were added, and the absorbance was measured at 760 nm after incubation for 2 h at room temperature (Gan et al. 2010). The TPC of the extract was expressed as mg Gallic acid equivalent per g of extract (mg GAE/g extract).

Results

Fungal strain. *Aspergillus* isolate showed the typical macro- and micro-morphological features described for section *Versicolores*. For species designation, sequencing of the ITS rDNA and CaM gene followed by a BLAST search revealed that the isolated strain had a high identity (99%) with *A. creber*. Thus, according to these results, the strain was identified as *A. creber* and its sequences data were submitted at GenBank with accession numbers: MH796366 and MH796367.

UHPLC-MS/MS analysis. After two weeks of growth in the fermentation broth, the secondary metabolites of *A. creber* were extracted, through EtOAc, filtered and concentrated *in vacuo* to give EAF, which was subjected to UHPLC-MS/MS analysis. On the basis of the fungal metabolites list established by Lehner et al. (2011), molecular mass data, MS/MS analysis and references, five known fungal metabolites were tentatively identified as asperlactone **(1)**, emodin **(5)**, sterigmatocystin **(7),** deoxybrevianamide E **(9)** and norsolorinic acid **(10)** (Table I) whereas five metabolites remained unidentifiable. The Total Ion Current (TIC) chromatograms of the ten peaks of detected compounds are shown in Fig. 1.

Antimicrobial activity. Results obtained for the antimicrobial activity of EAF are shown in Table II. In the case of bacteria, the highest zone of inhibition was displayed against Gram-positive DRB, including MRSE and SABL, followed by Gram-negative bacteria *K. pneumoniae* and *S. typhi* with an inhibition zone ranged from 8.5 ± 0.6 to 14.0 ± 0.2 mm. No activity was observed against *E. coli* and *P. aeruginosa* at the concentration used. In the case of yeasts, the EAF inhibited the growth of all tested yeasts (*C. albicans* and *C. glabrata*) with maximum inhibitory activity against *C. albicans*, which displayed an important zone of inhibition (20.6 ± 0.8 mm).

Table II showed also the MIC values of *A. creber* extract against the tested organisms, which showed an inhibition zone in the disc diffusion assay. The EAF of *A. creber* showed MIC values ranging from

Table I
Metabolites of *Aspergillus creber* as determined by UHPLC-MS/MS. The metabolites were tentatively identified using molecular mass data, MS/MS analysis and references.

Peak No.	Putative compound name	Adduction	Measured mass (m/z)	Productions (m/z)	t_R[a] (min)	Ref[b]
1	Asperlactone	[M+H]+	185.08081	141.05444, 113.05948	1.73	Vishwanath et al. 2009
2	NI	[M+Na]+	211.06121	195.03455, 133.02816	1.78	–
3	NI	[M+H]+	308.10992	280.95587, 145.10104	3.38	–
4	NI	[M+H]+	327.04734	309.18594, 191.15396	4.28	–
5	Emodin	[M+H]–	269.04590	ND	5.42	Sulyok et al. 2007; Lehner et al. 2011; Micheluz et al. 2016
6	NI	[M+H]+	251.09145	233.09526, 204.09319	5.49	–
7	Sterigmatocystin	[M+H]+	325.07053	ND	6.62	Lehner et al. 2011; Micheluz et al. 2016
8	NI	[M+Na]+	423.25041	405.27911, 239.14821	7.27	–
9	Deoxybrevianamide E	[M+H]+	352.20352	ND	8.04	Lehner et al. 2011; Micheluz et al. 2016
10	Norsolorinic acid	[M+H]–	369.09892	ND	8.24	Micheluz et al. 2016

[a] – Retention time; [b] – Reference; ND – not detected; NI – not identified

Table II
Antimicrobial activity and minimum inhibitory concentration (MIC) of ethyl acetate extract of *Aspergillus creber* against human pathogens.

	Zone of inhibition (mm)[a]		MIC of ethyl acetate extract (mg/ml)
	Ethyl acetate extract	Positive control[b]	
Bacteria			
Escherichia coli	0	23.4 ± 0.3	–
Klebsiella pneumoniae	10.0 ± 0.3	25.0 ± 0.4	2.5
Pseudomonas aeruginosa	0	20.8 ± 0.4	–
Salmonella Typhi	8.5 ± 0.6	21.5 ± 0.3	5
SABL[c]	12.8 ± 0.3	19.8 ± 0.2	0.625
MRSE[d]	14.0 ± 0.2	19.2 ± 0.2	0.625
Yeasts			
Candida albicans	20.6 ± 0.8	22.8 ± 0.4	0.325
Candida glabrata	13.0 ± 0.3	25.7 ± 0.5	1.25

[a] – Mean of three replicates (±) SD
[b] – Chloramphenicol and ketoconazole were used as the positive control for bacteria and yeasts respectively
[c] – *Staphylococcus aureus* producing beta lactamase
[d] – Methicillin resistant *Staphylococcus epidermidis*
0 – no zone of inhibition

0.325 to 5 mg/ml. The higher MIC values were recorded against *C. albicans* (0.325 mg/ml) and Gram-positive DRB (0.625 mg/ml) whereas the lower MIC values were recorded against *C. glabrata* (1.25 mg/ml) and Gram-negative bacteria *K. pneumoniae* (2.5 mg/ml) and *S. typhi* (5 mg/ml).

Antioxidant analysis. In the DPPH assay, the EAF of *A. creber* extract demonstrated a dose-dependent scavenging activity and the highest decolorization was recorded at 400 µg/ml (Fig. 2a). In the ABTS assay, like the DPPH assay, the EAF exhibited a rich scavenging effect and the highest decolorization was also recorded at a concentration of 400 µg/ml (Fig. 2b). In both assays, the scavenging activity of EAF was slightly lower than that of ascorbic acid. In the Folin-Ciocalateu assay, the EAF showed a high level of total phenols (Table III).

Table III
Antioxidant activities of *Aspergillus creber* ethyl acetate extract by DPPH and ABTS assays and its total phenolic content.

Activity	Extract	Ascorbic acid[a]
DPPH[a]	89.28 ± 0.32	91.39 ± 0.39
ABTS[a]	92.93 ± 0.30	93.03 ± 0.45
Total phenolic content (mg GAE/g)	85.76 ± 0.96	–

[a] – Percentage of inhibition at a concentration of 400 µg/ml. Values are mean of three replicates (±) SD

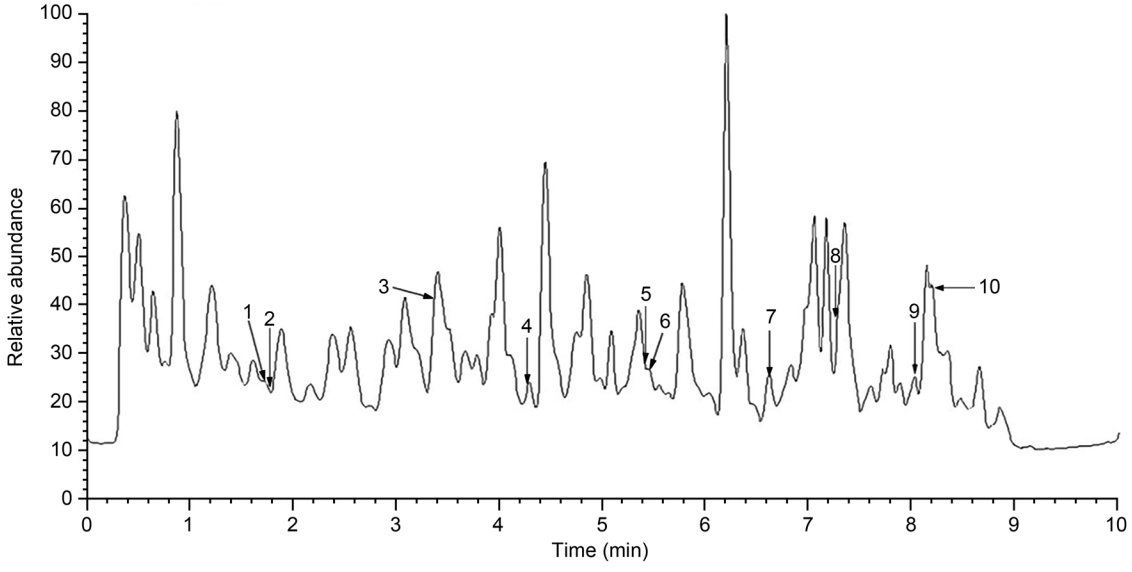

Fig. 1. Total Ion Current chromatogram of *Aspergillus creber* extract obtained with UHPLC-MS/MS. The retention times of the peaks (1–10) and their corresponding molecules names are presented in Table I.

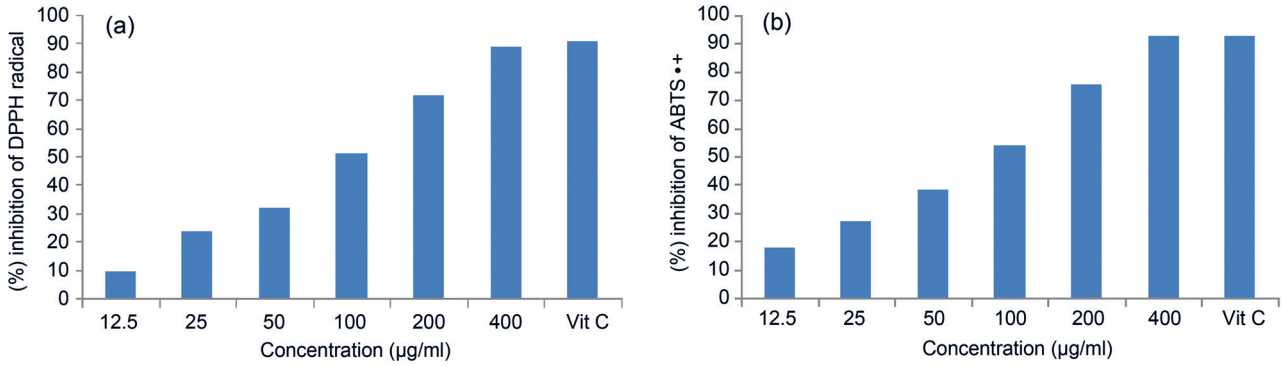

Fig. 2. Free radical-scavenging activities of *Aspergillus creber* extract and ascorbic acid (400 µg/ml) measured (a), in DPPH assay and (b), in ABTS assay.

Discussion

Fungi are major producers of secondary metabolites with different biological activities and various chemical structures (Abo-Elmagd 2014). Until today, except for *A. versicolor* and *A. sydowii*, there is no thorough study on metabolic profiles for the *Aspergillus* species belonging to section *Versicolores* (Despot et al. 2017). Moreover, to our knowledge, there are no published reports recorded the antimicrobial and the antioxidant activities of *A. creber*.

Our study demonstrated that *A. creber* is able to produce a variety of secondary metabolites previously reported as potent biologically active compounds. The biological activity of asperlactone, a polyketide metabolite, had been established for the first time by Balcells et al. (1995). It was the first fungal compound that exhibited insect growth regulating activity in *in vivo* tests. Asperlactone has also been reported as a potent antibacterial and antifungal metabolite (Chen et al. 2014). Emodin is the most studied anthraquinone derivative for its diverse biological activities, including antibacterial (Hatano et al. 1999), antifungal (Kim et al. 2004), antioxidant (Izhaki 2002), and anticancer properties (Cheshmi et al. 2017; Zhao et al. 2017). STC is a polyketide mycotoxin and a precursor of Aflatoxin B1 (AFB1), however, its toxicity is lower than that of AFB1 (Piontek et al. 2016). Deoxybrevianamide E is a prenylated indole alkaloid that belongs to the family of brevianamides. Although a variety of biological activities were exhibited by many brevianamides including antibacterial, anti-insect pests and antitubercular properties (Xu et al. 2017) there are no reports in the literature concerning the bioactivities of deoxybrevianamide E. Norsolorinic acid is an anthraquinone derivative fungal metabolite. Wang et al. (2008) reported that norsolorinic acid has antiproliferative activity on T24 human bladder cancer cells.

The ability of *A. creber* to produce STC is in accordance with previous studies demonstrated that most of *Aspergillus* section *Versicolores* are STC producers (Jurjević et al. 2013, Despot et al. 2016). Moreover, our findings are in agreement with the study of Micheluz et al. (2016) reported that *A. creber* is able to produce emodin, STC, deoxybrevianamide E and norsolorinic acid. However, to our knowledge, this is the first report concerning the production of asperlactone from the species *A. creber*.

In the antimicrobial assays, our study indicated that *A. creber* exhibited more potent antibacterial activity against Gram-positive than Gram-negative bacteria. This variation of susceptibility could be attributed to the morphologic difference in the composition of their cellular membranes, which influences their reaction to antibacterial compounds (Valle Jr et al. 2015). In addition to its antibacterial activity, the EAF of *A. creber* exhibited higher anticandidal activity against *Candida* species. In the case of *Candida* species, ergosterol is the main target of antifungal drugs (Martins et al. 2015). These results are in contrast to the previous studies reported that the metabolites produced by *A. versicolor,* the most studied species of *Aspergillus* section *Versicolores*, exhibited lower or no antibacterial and anticandidal activities (Zhuang et al. 2011; Song et al. 2012; Ebada et al. 2018). Another attractive finding on *A. creber* was the higher MIC values of its ethyl acetate extract against *C. albicans* (0.312 mg/ml) and Gram-positive DRB (0.625 mg/ml) since these species are the most common opportunistic pathogens (Martins et al. 2015; Knafl et al. 2017). Moreover, our findings can be considered very promising since the extract was crude and obtained by using non-optimized fermentation, which usually produces a poor yield of active compounds (Noor Ifatul et al. 2016).

Our study also brings additional data on *A. creber*, since the antioxidant activity and the TPC of this species were evaluated for the first time. The antioxidant ability of *A. creber* was tested using two different procedures, DPPH and ABTS assays. The DPPH assay is a widely used method that based on the reduction of the purple DPPH free radical to a yellow colored compound 1,1-diphenyl-2-picryl hydrazine (Floegel et al. 2011) whereas the ABTS assay is based on the reduction of the blue-green pre-formed $ABTS^{\bullet+}$ to the colorless ABTS form (Re et al. 1999). Thus, the higher scavenging activities of the EAF indicated its bioactive potential to neutralize the DPPH free radicals and $ABTS^{\bullet+}$. These results are in agreement with many other studies reported the antioxidant potency of filamentous fungi from different sources (Abo-Elmagd 2014; Kumaresan et al. 2015; Sharma 2015; Smith et al. 2015; Sugiharto et al. 2016). The slight difference between the antioxidant capacities of EAF and ascorbic acid may be explained either by the purity of ascorbic acid, whereas the extract of *A. creber* is a mixture of multiple compounds or by the capacity of some compounds in the fungal extract to neutralize or to inhibit the effect of active compounds (Dhankhar et al. 2012). Moreover, our data revealed that *A. creber* is able to produce much higher content of phenols than the other filamentous fungi in the literature (Jakovljević et al. 2014; Smith et al. 2015; Sugiharto et al. 2016). Many previous studies attributed the antioxidant activity of filamentous fungi to their TPC (Abdel-Monem et al. 2013; Smith et al. 2015, Nwobodo et al. 2017). In accordance with this, the higher percentages of inhibition of DPPH radical and $ABTS^{\bullet+}$ in our fungal extract might contribute to the high level of phenolic compounds.

In conclusion, UHPLC-MS/MS analysis revealed that *A. creber* could produce a variety of secondary metabolites including polyketides, anthraquinones, and alkaloids. Our findings suggest that *A. creber* might represent a novel source of natural bioactive products since we have demonstrated its ability to inhibit the growth of many pathogen microorganisms, mainly *C. albicans* and Gram-positive DRB, and its high antioxidant activity. Our work will increase the knowledge about the species A. creber newly described and serve as a prelude to a better understanding of the biology and the chemistry of its metabolites.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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