Chromatographic separations and characterization of bioactive secondary metabolites of *Aspergillus quadrilineatus* (MH109538) fungal strain

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Abstract. In the interest of searching new antibiotic substances, the antibacterial effect of *Aspergillus quadrilineatus* fungal strain (MH109538) has been evaluated against sensitive bacteria (*S. aureus*, *E. faecalis*, *B. subtilis* and *E. coli*) and resistant bacteria (*P. aeruginosa* and *K. pneumonia*), following the disks technique. The obtained result confirms the capacity of this strain to produce antibacterial molecules. In order to analyze and characterize these bioactive metabolites present in the crude extract, which was fractionated by column chromatography. The fractions were then separated by thin-layer chromatography. The detected spots were sampled and tested against the most sensitive bacteria (*S. aureus*). The results have revealed that only one fraction showed an antibacterial activity. This active fraction was analyzed by liquid chromatography coupled with mass spectrometry (LC/MS) in order to obtain the elemental composition of the present compounds. The molecular ions fragmentation (LC/ MS/MS) were used to determine the deployed formula of these compounds. The analysis of the results by Xcalibur 2.2 software reveals the presence of 24 molecules, and this, based on the retention times of the peaks obtained in the chromatogram, where, the m/z ratios, the formulas, as well as the intensities of the various compounds detected are determined.

Keywords: *A. quadrilineatus* (MH109538), bioactive metabolites, chromatographic techniques

INTRODUCTION

Faced with the loss of antibiotic therapy effectiveness, threaten by the emergence of multi-resistant germs, the discovery of new molecules has become an absolute necessity (Berger Savin, 2014). The fields of investigation are vast. However, natural resources are exploited again to lead to the discovery of new bioactive molecules reservoirs, consisting in exploring these molecules especially from microorganisms. Fungi are among the best candidates for the production of

biologically active secondary metabolites. This group of microorganisms is responsible for around 22% of active molecules with antibacterial effect produced on an industrial scale (Atoui, 2006). Indeed, the species of *Aspergillus* genus prove to be a true metabolic factories responsible for the synthesis of chemical compounds plethora (Youcef Ali, 2014), including substances with antibacterial effect (Madki *et al.*, 2010 ; Barakat & Gohar, 2012 ; Al-Shaibani *et al.*, 2013 ; Liu *et al.*,

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2017). For this reason, the fungal specie *A. quadrilineatus* (MH109538), isolated from soil taken from an arid zone (Laghout; located at 400 km south of Algiers) has been screened to see its activity towards sensitive and resistant bacteria (primary screening). In order to analyze and characterize these bioactive metabolites, usually present as traces in the crude extract, a whole range of methods was used essentially based on the principle of molecules chromatographic separation, then their detection with spectrometry.

MATERIALS AND METHODS

Preparation of the crude extract

The used fermentation medium was Czapek dox supplemented with 2.5 g/L of yeast extract. 500 mL flasks, containing 100 mL of liquid culture medium, were inoculated with eight disks of seven days aged culture. After 14 days of incubation at 28°C, an identical volume of chloroform (100 mL) was added to each flask containing the fungal culture, the mixture was crushed using ultra turrax, and then filtered on Whatman paper N°01. After decantation, the chloroform phase was concentrated by vacuum evaporation using a Rotavapor (Gengan *et al.*, 1999 ; Zerroug, 2011 ; Ghorri, 2015).

Demonstration of the antibacterial activity

Preparation of bacterial strains

The used microbial support was composed of six bacterial strains, four ATCC strains (American Type Culture Collection), which were: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC, 25922), *Pseudomonas aeruginosa* (ATCC, 27853), and two clinical strains namely: *Enterococcus faecalis* and *Klebsiella pneumoniae*. Antibacterial tests must be performed from young cultures (18 to 24 h). The opacity of the bacterial suspensions in sterile physiological water was equivalent to 0.5 McFarland (Gonelimali *et al.*, 2018 ; Manandhar *et al.*, 2019).

Highlight test by disks technique

A quantity of crude extract was dissolved in dimethylsulfoxide (DMSO) in order to obtain a

concentration of 100 mg/mL. Then, sterile disks of 6 mm in diameter, prepared from Whatman paper No. 1, containing 10 µL of the tested extract. After drying, the disks were carefully placed on Petri dishes containing Mueller-Hinton medium previously seeded by the tested bacteria. After that, the Petri dishes are incubated at 4°C in a refrigerator for 2 h to allow the diffusion of bioactive substances, thereafter placed in an oven at 37°C for 18 to 24 hours (Yamaç and Bilgili, 2008 ; Razmavar *et al.*, 2014). It should be noted that three repetitions were performed, and the diameters of the inhibition zones were measured in millimeter.

Separation of metabolites by column chromatography

The used column measured 22 X 400 mm prepared according to the following process: the tube was provided at the bottom end with a grid and cotton, then filled at two-thirds with chloroform and next with 5 g of sodium sulfate (granular anhydrous). After checking that the top surface of the sodium sulfate layer was flooded, a quantity of 10 g of silica gel 60 was added, stirring carefully after each addition to remove air bubbles. The preparation was left to stand for 1 hour, and then 15 g of sodium sulfate were added carefully, thus forming the upper layer of the column. The chloroform excess was eliminated through the lower tube track placed for this purpose. 50 mL of chloroform extract were added to 100 mL of hexane, then, the mixture was quantitatively transferred to the column with the elimination of the elapsed liquid. Afterwards, a volume of 100 mL of diethyl ether was added and allowed to descend again to the upper surface of the sodium sulfate layer. Then, the elution was carried out with 150 mL of mixture (chloroform - methanol: 97/3 V/V) (Zinedine, 2004; Abdelaziz, 2006).

Thin layer chromatography (TLC)

After column fractionation, the bioactive molecules were separated again by TLC, which was carried out on silica gel plates << 60F254 >>. A volume of almost 200 µL of each fraction was gradually deposited, using capillary in the form of spots. The atmosphere of the tank was saturated previously by a mobile phase consisting of three solvents (Toluene / Ethyl acetate / Formic acid;

50/40/10) (Siddiqui, 2004; Awad, 2005). After migration, the plates were dried then visualized under UV light ($\lambda = 254$ and 365 nm) (Móricza *et al.*, 2016). The spots that appeared were scraped and deposited on the surface of the Mueller Hinton medium seeded with the most sensitive test bacteria (*S. aureus*) to demonstrate their antibacterial activity. Afterwards, the Petri dishes were placed at 4°C for 4 h to allow the diffusion of bioactive substances, and then incubated at 37°C for 18 to 24h (Boughachich, 2012).

Analysis by (LC/MS) and (LC/MS/MS)

The active fraction was scraped, suspended in 3 mL of chloroform and subjected to a rotary mixer for 1 h. After centrifugation at 15000 g for 5 min, the supernatant was collected and evaporated to dryness using a vacuum ramp. Then the fraction was resuspended in 500 μL of water/methanol mixture (97.5%; 2.5%), then filtered on a 0.45 μm millipore membrane so that it can be analyzed.

The used chromatographic system was composed of a binary pump system in order to generate a gradient, two columns (a pre-concentration column and an analytical column) and an injector. The different elements were connected to a computer, allowing the different components of the chromatographic system to be controlled automatically.

4 μL of filtered extract was automatically injected into the device; Acquity M-Class UPLC class (Waters, Milford, MA, USA) connected to a Q Exactive (Thermo Scientific, Bremen, Germany). The sample was loaded on a trap column (Symmetry C18 5 μm , 180 μm x 20 mm, Waters) in 100% of solvent A (Water with 0.1% formic acid) for 3 min, and then separated on the analysis column (HSS T3 C18 1,8 μm . 75 μm x 250 mm, Waters) with a flow rate of 600 nL/min. A solvent gradient system was applied in order to separate the secondary metabolites from the used fraction. The mobile phase was composed of two solvents: solvent A (as previously described) and solvent B (Acetonitrile with 0.1% formic acid). Table 1 represents the used linear gradient program.

Operating conditions of ionization parameters (LC-ESI-MS and LC/MS/MS)

The UPLC device was coupled to a Q-Exactive (Thermo Scientific, Bremen, Germany), in

positive ion mode to a nano-electrospray – MS with an Orbitrap type analyzer. The system was controlled by Xcalibur 2.2 software (Thermo Fisher Scientific). The recorded data with an m/z ratio of 80 to 1200 were then analyzed using the full MS scan mode.

Table 1. Gradient program used for the analysis and separation of metabolites.

Time (min) / Solvent	A	B
0 min	98%	2%
5 min	93%	7%
135 min	70%	30%
150 min	60%	40%
154 min	10%	90%
162 min	98%	2%
180 min	98%	2%

The parameter values to obtain optimal signal-to-noise ratio, in positive mode, were as follows: A capillary voltage of ± 3.5 kV, a capillary temperature of 320°C , a cone gas flow rate of 6 L/min, a target value of automatic gain control (AGC) equal to 3×10^6 charges, a maximum injection time of 200 ms, with a resolution equal to 70000.

The data were acquired in profile mode. In order to allow the metabolites identification, additional MS acquisitions were carried out using collision-induced dissociation. On the Exactive Orbitrap, the data was acquired using the DDMS2 (Data Dependent Acquisition) method (TOP 10), and the parameter values were set as follows: A collision energy “HCD” equal to 27, a resolution equal to 17500, a target value of the automatic gain control (AGC) equal to 1×10^5 charges, a maximum injection time of 200 ms, an isolation window equal to 2m/z. The number of MS/MS acquisition has been set to 10 for an MS spectrum (Top 10), Dynamic exclusion was set at 10.0 S., and the Lock mass at 445.12003, with mass accuracy equal to 5 ppm.

Figure 1 summarizes the procedure of characterizing bioactive secondary metabolites of the fungal strain *A. quadrilineatus* (MH109538); from fermentation and extraction of fungal metabolites to LC/MS analysis of the active fraction and the comparison of results with the ChemSpider database.

Figure 1. Process of characterizing bioactive secondary metabolites.

RESULTS AND DISCUSSION

Demonstration of the antibacterial activity

The highlight test by disks technique revealed the capacity of the fungal strain *A. quadrilineatus* (MH109538) to produce antibacterial molecules, where the most significant effects are observed against the two bacteria *S. aureus* and *E. faecalis* with inhibition zones diameters of 17.67 and 15.0

mm respectively. In contrast, the bacteria *B. subtilis* and *E. coli* were less sensitive to bioactive metabolites, where the recorded diameters are equal to 13.0 mm, followed by *K. pneumoniae* with a diameter of 11.67 mm. Otherwise, no inhibition zone was observed against the *P. aeruginosa* strain (Table 2). Our results were agree with those of Irobi *et al.* in 2000 (in different working conditions), whereby the *A. quadrilineatus* fungal strain has a considerable antibacterial effect

against *B. subtilis* (with inhibition zones from 0 to 42 mm in diameter) and *S. aureus* (with inhibition zones from 0 to 38 mm in diameter). Abdelaziz in 2006, also showed that *Aspergillus* fungal strains had a considerable antibacterial effect against the tested bacteria. However, *Aspergillus* species are known by their production of antibacterial substances (Madki *et al.*, 2010 ; Ola *et al.*, 2014 ; Xu *et al.*, 2015 ; Pillai *et al.*, 2016 ; Phainuphong *et al.*, 2017).

Chromatographic separation

In order to study the natural substances of fungal origin, different methods of analytical chemistry, aimed at detecting molecules in complex mixtures are used depending on the type of compounds and the studied matrices, often involving separated or coupled chromatographic techniques to different detection systems (Cigic and Prosen, 2009).

Within this framework, the crude extract of the fungal strain was fractionated using column chromatography. Afterwards, the molecules are separated again using the thin layer

chromatographic technique. Generally, to achieve pure biomolecules, it is essential to use all these different chromatographic techniques, and at each step it is necessary to test the biological activity of the fractions. Therefore, the spots that appeared are scraped and tested against the most sensitive bacteria (*S. aureus*) (Boughachiche, 2012). The results reveal that only one fraction showed an antibacterial activity with inhibition zone of 14 mm in diameter. This active fraction has an Rf equal to 0.526, and a yellow color in visible light as well as under the two wavelengths 254nm and 365nm. In addition, this minority fraction represents almost all of the crude extract activity, which confirms our results on the other non-active fractions. This proves that the steps applied for the separation of bioactive molecules allowed to obtain a suitably separated fraction and therefore, concentrate the active ingredient of the *A. quadrilineatus* (MH109538) fungal strain. In fact, several consulted studies have mentioned that thin layer chromatography is applied in most cases of secondary metabolites purification (Neilson, 2000 ; Bajpai *et al.*, 2016).

Table 2. Demonstration of the antibacterial activity of fungal strain by the disks technique.

	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. faecalis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>
<i>A. quadrilineatus</i>	17.67±1.53	13.0±1.0	15.0±2.0	13.0±2.64	-	11.67±1.53

*(-): Diameter of the inhibition zone <6 mm.

Analysis by (LC/MS) and (LC/MS/MS)

In order to identify and characterize the bioactive molecules present in the fraction, considered as pure, we used the liquid chromatography technique coupled with simple spectrometry (LC/MS) and in tandem mode (LC/MS/MS). Indeed these techniques are widely used because not only they are a detection methods often encountered for the analysis of secondary metabolites, but above all, they provide structural information on the analyzes studied (Bains and Tewani, 1987 ; Gupta *et al.*, 1989 ; Benserradj, 2014).

In order to obtain a satisfying separation of secondary metabolites. The UPLC system was carried out in a linear elution gradient of 180 min. Figure 2 shows the chromatographic profile obtained by UPLC analysis of the strain *A. quadrilineatus* (MH109538) active fraction. It has been observed that the secondary metabolites sought in the active fraction cover a wide range of

polarity and that 180 minutes are necessary to separate all of these molecules.

In mass spectrometry, the molecule is analyzed in an ionized form and to quantify it, the detector records the intensity of the signal associated with this specie (m/z ratio) as a function of time (Figure 3).

The analysis by Xcalibur 2.2 software (Thermo Fisher Scientific) of chromatographic peaks and MS spectra reveal the presence of 24 different peaks correspond to 24 molecules, of which the majority (very intense peaks) are those with elution times 46.05, 63.30, 76.93, 86.61, 137.90, 157.18, 157.20, 157.77, and 158.01 min.

The different compounds detected with their m/z ratios, their formulas (the formulas proposed are compatible with the requirements imposed on the spectrometer but these are the candidates to be checked), their retention times and their intensities are represented in Table 3. In order to identify bioactive metabolites, additional MS

acquisitions were performed using collision induced dissociation. The masses of the different obtained fragments are represented in Table 4.

The obtained results are compared with those described in the literature (the ChemSpider database; <http://www.chemspider.com>), in order to achieve the determination of bioactive metabolites nature and to arrive at their

identification. This comparison was based on the mass/charge ratios, the crude formulas and the pseudo-molecular ions of each compound. Indeed, ChemSpider, proposed to us for each compound, a variety of metabolites, which have the same formulas and the same masses to those obtained in our fraction.

Figure 2. UPLC chromatogram of the *A. quadrilineatus* strain active fraction.

Figure 3. Sum of mass spectra obtained by ESI-MS in positive mode of the *A. quadrilineatus* strain active fraction.

Table 3. The different compounds detected in the active fraction by UPLC-MS analysis.

Compound	Retention time	m/z ratio	Ion type	Formula	Intensity
1	46.05	453.3429	$[M+H]^+$	$C_{24}H_{45}O_4N_4$	1.59×10^8
2	63.30	566.4278	$[M+H]^+$	$C_{30}H_{56}O_5N_5$	2.73×10^8
3	76.93	679.5119	$[M+H]^+$	$C_{28}H_{59}O_9N_{10}$	1.14×10^9
4	86.61	792.5946	$[M+H]^+$	$C_{38}H_{74}O_5N_{13}$	6.39×10^8
5	137.90	177.546	$[M+H]^+$	$C_{10}H_9O_3$	4.87×10^8
6	154.21	191.1430	$[M+H]^+$	$C_{13}H_{19}O$	1.20×10^7
7	155.53	161.091	$[M+H]^+$	$C_{11}H_{13}O$	2.41×10^7
8	155.79	221.1172	$[M+H]^+$	$C_{13}H_{17}O_3$	1.61×10^7
9	156.16	242.2841	$[M+H]^+$	$C_{16}H_{36}N$	1.62×10^7
10	156.26	312.2532	$[M+NH_4]^+$	$C_{18}H_{34}O_3N$	1.52×10^7
11	156.56	197.1536	$[M+H]^+$	$C_{12}H_{21}O_2$	3.44×10^7
12	157.18	195.1015	$[M+H]^+$	$C_{11}H_{15}O_3$	1.67×10^8
13	157.20	167.0703	$[M+H]^+$	$C_9H_{11}O_3$	1.09×10^8
14	157.25	227.1639	$[M+H]^+$	$C_{13}H_{23}O_3$	7.50×10^7
15	157.36	385.0920	$[M+H]^+$	$C_{20}H_{17}O_8$	3.21×10^7
16	157.39	402.1179	$[M+NH_4]^+$	$C_4H_{20}O_{13}N_9$	3.25×10^7
17	157.41	231.1590	$[M+H]^+$	$C_{12}H_{23}O_4$	2.26×10^7
18	157.77	199.1692	$[M+H]^+$	$C_{12}H_{23}O_2$	1.06×10^8
19	157.97	253.1798	$[M+H]^+$	$C_{15}H_{25}O_3$	6.83×10^7
20	158.01	415.2114	$[M+H]^+$	$C_9H_{27}O_7N_{12}$	1.51×10^8
21	158.23	251.1492	$[M+H]^+$	$C_{11}H_{23}O_6$	1.21×10^7
22	158.69	215.1277	$[M+H]^+$	$C_{11}H_{19}O_4$	1.01×10^7
23	159.18	294.2062	$[M+NH_4]^+$	$C_{17}H_{28}O_3N$	7.53×10^7
24	159.22	221.1172	$[M+H]^+$	$C_{13}H_{17}O_3$	4.54×10^7

For example; the database proposed five different metabolites (cyclic peptides) for the second compound (Tr: 63.30). Therefore, the identification confirmation of these metabolites requires: either the use of standards (pure molecules), by comparison of the chromatograms and mass spectra of these standards with those obtained in our fraction, which must have a similarity in the retention times, the molecular weights and the molecular weights of the fragments, with our obtained compounds, or the use of other spectrometric identification techniques such as nuclear magnetic resonance (NMR of proton and carbon 13) which studies the

evolution of a spin system specific to each nucleus subjected to a magnetic field (Silverstein *et al.*, 2007).

This technique will be complementary to the results already obtained and can help in structure determination. Indeed, the secondary metabolites of natural origin (vegetable, marine, fungal or bacterial), have characteristics making their study a little difficult such as; the very small amount of the active ingredient, often with mixtures of molecules with close structures making the extraction and purification steps long and which can also lead to antagonistic or synergistic effects between the molecules (Garneau *et al.*, 2002).

Table 4. The different compounds detected with their MS/MS fragments.

Compound	MS/MS Fragment Masses
1	435.3324, 417.0467, 340.2583, 322.2482, 284.1721, 209.1645, 114.0914, 96.0809, 69.0699
2	548.4172, 453.3443, 435.3332, 341.2424, 322.2491, 228.1596, 209.1650, 114.0906, 96.0813
3	661.5021, 566.4319, 548.4182, 452.3597, 435.3335, 341.2438, 322.2492, 228.1594, 209.1652, 114.0919, 96.0813
4	774.5840, 679.5123, 661.4995, 548.4176, 452.3607, 435.3329, 341.2442, 322.2490, 228.1590, 209.1650, 114.0915, 96.0810
5	149.0233, 121.0285
6	149.0961, 135.0805, 121.0651, 107.0495
7	146.0727, 133.1012, 105.0703
8	179.1067, 161.0961
9	181.1222, 155.0702, 130.1590, 87.0810, 71.0862, 57.0706
10	295.2267, 277.2162, 259.2054, 165.1274, 113.0964, 85.1017
11	179.1432, 151.1483, 141.0911, 123.0807, 95.0860, 57.0707
12	167.0703, 149.0598, 123.0807, 95.0496
13	149.0598, 139.0390, 121.0286, 95.0496
14	209.1533, 177.1273, 149.1325, 135.1169, 107.0859, 97.0651, 93.0703, 79.0548
15	359.0761, 341.0658, 237.0760, 193.0497, 149.0234
16	385.0914, 359.0753, 341.0649, 237.0760, 193.0493
17	171.1017, 147.0653, 129.0548, 111.0444, 101.0601
18	181.1590, 163.1482, 153.1640, 111.1172, 107.0859, 97.1017, 83.0861, 69.0706, 55.0550
19	197.1174, 179.1066, 141.0547, 123.0442, 97.0290, 57.0707
20	135.0805, 119.0858
21	211.0816, 193.0710, 171.0139, 153.0033
22	71.0499
23	221.1172, 203.1066, 175.1117, 165.0546, 161.0960, 57.0706
24	203.1079, 193.1233, 179.1075, 175.1125, 165.0554, 161.0968, 137.0603, 57.0709

CONCLUSION

To analyze the metabolites with antibacterial effect present in the crude extract of the *A. quadrilineatus* fungal strain (MH109538), a molecules separation was used by chromatographic techniques. The antibacterial effect test of the different spots has proven the activity of only one fraction. After the analysis of this fraction by UPLC-MS, 24 peaks corresponding to 24 molecules were revealed with determination of their chemical characteristics: m/z ratios, formulas, retention times and intensities.

In perspective, this work is a preliminary step for broader studies, more in-depth and more accomplished including; the purification and

identification of bioactive metabolites produced by this fungal strain, and in cases where these molecules turned out to be new, additional studies should be considered, such as: *In vivo* toxicity study, the determination of the action mechanism of these substances, as well as optimizing the biosynthesis of these bioactive molecules for medical purposes.

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