Extraction and preliminary study of antibacterial compounds of three species of *Aspergillus* genus

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Abstract. In the interest of discovering new antibiotic molecules, the antibacterial activity of three fungal strains namely: *Aspergillus quadrilineatus*, *Aspergillus niveus*, and *Aspergillus wentii* isolated from particular ecosystems was sought against six bacterial strains including three with Gram-positive staining (*Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*) and three with Gram-negative staining (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*). The results of the agar cylinder technique highlighted that the three fungal strains showed a considerable antibacterial activity. In order to optimize the extraction conditions of the bioactive molecules, five solvents in different polarities were tested, of which chloroform turned out to be the best one. After the selection of this solvent, four culture media of different compositions were used in order to determine the most adequate medium for the production of antibacterial substances. The results revealed that Czapek-dox medium supplemented with yeast extract turned out to be the most favorable one for the production of bioactive molecules from both strains: *A. quadrilineatus* and *A. niveus*, while the most suitable medium for the *A. wentii* strain was Sabouraud. In addition, a study of the antibacterial effect of organic extracts by the Biolog micro-culture system was performed using a range of concentrations. The obtained results revealed that the extracts of the three fungal strains presented a remarkable activity with different concentrations and this, against all the tested bacterial strains. It was recorded only for the three used fungal species, the antibacterial activity was studied for the first time by the Biolog system.

Keywords: antibacterial activity, *Aspergillus*, bioactive molecules, Biolog

INTRODUCTION

The bacterial resistance to antibiotics is one of the most serious threats to global health today. Every day, new mechanisms of resistance emerge and spread on a global scale, compromising our ability to treat the most common infectious diseases (Azoun, 2016).

In front of this alarming situation, the appropriate use of antibiotics is absolutely necessary to extend the clinical validity of these molecules. However, the long-term solution to counteract microbial resistance is to develop or

search for new molecules (Madigan and Martinko, 2007; Saisivam *et al.*, 2008; Berger Savin, 2014). In the 1990s, the discovery of bioactive metabolites of microbial origin grew exponentially thanks to technological advances. Unfortunately, among the discovered metabolites, many are analogues of already known molecules, compounds with no antibiotic activity or even minor compounds. The invention of new innovative chemical families becomes scarce (Berdy, 2005). Faced with these emerging needs and multiresistant germs, the

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search of new molecules represents a need (Sharma *et al.*, 2005) which cannot be filled either by: the extraction of new derivatives in mutants of listed strains, the realization of new semi-synthetic molecules from known structure, the synthesis of new derivatives or the obtaining of new substances produced by new bacterial or fungal species isolated from explored or less explored ecosystems (Kitouni, 2007). This last path that was adopted during our study.

In addition, fungi are among the best candidates for the production of biologically active secondary metabolites. This kind of microorganisms is responsible for the production of about 22% active molecules with antibacterial effect on an industrial scale (Atoui, 2006). In fact, the genera *Aspergillus* and *Penicillium* as well as the species of the order Monilliales constitute the most important reservoirs (Botton *et al.*, 1990).

For this reason, we sought to study the antibacterial activity of *Aspergillus* genus fungal strains isolated from different ecosystems as producers of active metabolites against resistant bacteria for the purpose of using their bioactive molecules.

MATERIALS AND METHODS

Producing strains. Three strains of *Aspergillus* genus were used to evaluate their antibacterial activity: *Aspergillus quadrilineatus* (MH109538) isolated from the soil taken from an arid zone; Laghouat (located at 400 km south of Algiers), *Aspergillus niveus* (MH109544), and *Aspergillus wentii* (MH109545) isolated from a thermal soil in the region of Teleghma (located in north-eastern Algeria). It should be noted that isolation and identification steps of fungal strains have been detailed in another article (Bramki *et al.*, 2017).

Demonstration of the antibacterial activity of fungal strains by the agar cylinder technique. 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), in the exponential growth phase. The opacity of the bacterial suspensions in sterile physiological water was equivalent to 0.5 McFarland (a bacterial concentration estimated at 10^6 CFU/ml) (Cavalla and Eberlin, 1994).

The fungal strains were seeded on PDA medium (Potato Dextrose Agar). After 14 days of incubation at 28°C, agar cylinders of 6 mm in diameter were removed and deposited on the surface of Mueller-Hinton medium previously seeded following the NCCLS technique (National Committee for Clinical Laboratory Standard) by tested bacteria. The Petri dishes carrying the agar cylinders were placed at 4°C for 4 h, to allow the diffusion of the bioactive substances elaborated by the fungal strains, then incubated at 37°C for 18 to 24 h (Tortorano *et al.*, 1979; Gungi *et al.*, 1983).

Study of the antibacterial activity

Choice of the optimum solvent for the extraction of bioactive molecules

Preparation of extracts. Five solvents of different polarities were tested separately; chloroform, hexane, methanol, butanol and ethyl acetate to extract bioactive substances (Loucif, 2011; Boughachiche, 2012). 500 ml flasks, containing 100 ml of PDB (Potato Dextrose Broth) culture medium, were inoculated with eight disks of a seven days aged culture for each strain. After 14 days of incubation at 28°C (static fermentation), an identical volume of the tested solvent (100 ml) was added to each flask containing the fungal culture. The mixture was crushed using ultra turrax, then filtered on Whatman paper No. 1. The filtrate was transferred to a separatory funnel, then, the organic phase was concentrated by vacuum evaporation using a Rotavapor. (Gengan *et al.*, 1999; Ghorri, 2015). Each fungal extract was dissolved in dimethylsulfoxide (DMSO) in order to obtain a concentration of 100 mg/ml.

Discs technique. To evaluate the antibacterial activity of organic extracts, sterile discs of 6 mm

in diameter prepared from Wattman paper No. 1, containing 10 µl of organic extracts. After drying, the discs were carefully placed on Petri dishes containing Mueller-Hinton medium previously seeded by the tested bacteria. Before incubation at 37°C, the dishes were left for 2 h at 4°C to allow the diffusion of the bioactive substances (Yamaç and Bilgili, 2006 ; Hazalin *et al.*, 2009). Results' reading was done after 18 to 24 h. Any growth of inhibition zone around discs, even of a small diameter, was considered a positive result.

Choice of the optimum medium for the production of bioactive substances. Four liquid culture media of different composition: PDB, Sabouraud, Czapek dox supplemented with 2.5 g/l of yeast extract and MEB (Malt Extract Broth) recommended for the production of secondary metabolites were tested in order to determine the most adequate medium for the production of bioactive molecules. After a static fermentation of 14 days, an extraction of secondary metabolites with chloroform was performed. The preparation and the antibacterial activity test of the obtained extracts have been done as described previously (choice of the solvent) (Mathan *et al.*, 2013). It should be noted that three repetitions were performed for each test, and the diameters of the inhibition zones were measured in millimeter.

Study of the antibacterial activity of organic extracts by the micro-culture system (Biolog). From a concentration of 100 mg/ml (100 mg of extract in 1 ml of DMSO solvent), a successive one-half dilutions have allowed to prepare a range of concentrations ranging from 100 to 1.56 mg/ml (Harrar, 2012). The bacterial suspensions were prepared from cultures aged from 18 to 24 h, and were calibrated using a Biolog turbidimeter

to reach a final turbidity of 85% T, then, a volume of 220 µl of Mueller Hinton Broth (supplemented with 1% of « Biolog Redox Dye Mix F » for Gram-positive bacteria and « Biolog Redox Dye Mix A » for Gram-negative bacteria), 15 µl of the diluting extract and the same volume of the bacterial suspension were placed into each well of the microplate.

Both DMSO solvent and bacteria only were performed as negative and positive controls respectively were performed. The microplates were placed in an Omnilog incubator-reader for 24 h at 37°C, and the phenotypic behavior was recorded by a CCD camera (Al-Bayati, 2008; Benslama, 2014; Ghorri, 2015).

Indeed, this test was confirmed by the disc technique on Mueller Hinton Agar, and the discs were impregnated with 15 µl of each extract.

RESULTS AND DISCUSSION

The demonstration of the antibacterial activity of fungal strains by the agar cylinder technique. The antagonism test by the agar cylinder technique showed the ability of the three fungal strains to produce molecules with antibacterial effect. In fact, the strongest inhibition zones were observed against *S. aureus* with diameters of 33.67 mm, 33 mm, and 31.33 mm for the three fungal strains *A. quadrilineatus*, *A. niveus*, and *A. wentii* respectively. In contrast, *E. faecalis* showed a sensitivity only with *A. quadrilineatus* with a diameter of 20 mm. On the other hand, no inhibition zone was observed with the four tested strains *B. subtilis*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae* (Table 1).

Table 1. Demonstration of the antibacterial activity of fungal strains by the agar cylinder technique.

Strain	Diameter of inhibition zones (mm)		
	<i>A. quadrilineatus</i>	<i>A. niveus</i>	<i>A. wentii</i>
<i>S. aureus</i>	33.67±6.03	33.00±2.65	31.33±3.21
<i>B. subtilis</i>	-	-	-
<i>E. faecalis</i>	20.00±2.00	-	-
<i>E. coli</i>	-	-	-
<i>P. aeruginosa</i>	-	-	-
<i>K. pneumoniae</i>	-	-	-

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

Our results agreed with those of Irobi *et al.* in 2000, whereby the *A. quadrilineatus* fungal strain has a considerable antibacterial effect against Gram-positive bacteria. 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 (Maria *et al.*, 2005, Madki *et al.*, 2010). These biologically active secondary metabolites are synthesized at the end of growth (Attalah and Kacem-chaouche, 1992).

Study of antibacterial activity

Choice of the optimum solvent for the extraction of bioactive molecules. The obtained results from antibacterial test showed that all the organic extracts have an antibacterial activity on at least one of the tested bacteria, with the exception of hexane extract of *A. quadrilineatus* strain, and that the most significant effect was especially obtained from the chloroform extracts, followed by ethyl acetate and butanol (Figures 1, 2, and 3).

Figure 1. Antibacterial activity of organic extracts of the fungal strain *A. quadrilineatus*.

Figure 2. Antibacterial activity of organic extracts of the fungal strain *A. niveus*.

Figure 3. Antibacterial activity of organic extracts of the fungal strain *A. wentii*.

Regarding the chloroform extracts, considerable inhibition zones were observed by *A. niveus* strain extract against both *B. subtilis* and *S. aureus* with diameters of 25 mm and 18.33 mm, in that order. As well as, *A. quadrilineatus* strain extract exhibited a remarkable effect on *S. aureus* bacterium with an inhibition zone of 16.67 mm.

The same results were detected by ethyl acetate and butanol extracts of *A. niveus* fungal strain, where the average of inhibition zones' diameters ranged from 15 mm to 20 mm against the same bacterial strains: *B. subtilis* and *S. aureus*.

These results revealed that the chloroform was the best solvent for the extraction of fungal metabolites, especially that have an antibacterial effect, is in agreement with the results of Kitouni's and Loucif's results in 2007 and 2011, successively, who found that chloroform is among the best solvents used for the extraction of bioactive molecules. However, this solvent is often used for the extraction of secondary metabolites (Kosalec *et al.*, 2004; Abdelaziz, 2006, Praveena and Padmini, 2011).

The appearance of the antibacterial activity by polar solvents (methanol and butanol), solvents of intermediate polarity (chloroform and ethyl acetate) and another non-polar (hexane), assumes that it is either several molecules of different polarity (each soluble in a different solvent) or one or more molecules soluble in various solvents. This last hypothesis is more probable since, according to Guernet and Hamon (1981), antibiotics' structure is generally complex, containing polar and non-polar characters, resulting in an affinity for very diverse solvents.

Choice of the optimum medium for the production of bioactive substances. The antibacterial effect test of each fungal strain showed that the diameters of the inhibition zones vary from one culture medium to another and from one tested bacterium to another.

For the *A. quadrilineatus* fungal strain, the represented results in Figure 4 showed that the most important zones of inhibition were obtained by the disks impregnated with molecules from Czapek-dox medium, with diameters of 17.67 mm against *S. aureus* bacterium and 15 mm against *E. faecalis* bacterium, followed by the PDB medium with diameters of 16.67 mm and 13.67 mm against the bacteria *S. aureus* and *E. faecalis*, respectively.

Regarding the *A. niveus* fungal strain, the results in Figure 5 proved Czapek-dox medium as the most suitable medium for the production of antibacterial metabolites, followed by the PDB medium, in which the highest diameters against the two bacterial strains *B. subtilis* and *S. aureus* were shown.

In effect, Czapek-dox liquid medium supplemented with yeast extract was chosen as a specific medium for optimal production of secondary metabolites (Pamel *et al.*, 2010). In addition, this medium contains yeast extract and sodium nitrate, which are good sources of nitrogen, adequate for the production of antibiotics; it also contains sucrose as a carbon source as well as mineral salts (Pandey *et al.*, 2005).

However, the extract of the *A. wentii* fungal strain, coming from Sabouraud medium, reacted differently compared to other media, giving more distinct results. Moreover, considerable zones of inhibition: 24.33 mm, 20.33 mm, and 18.67 mm in diameter were observed against the three bacterial strains: *S. aureus*, *B. subtilis*, and *E. faecalis*, respectively (Figure 6).

The best performance of Sabouraud's medium compared to other media can be explained by the presence of peptone, which is a source difficultly metabolized and therefore favors the production of antibiotics (Voelker and Altaba, 2001).

Several studies have shown that the nature of carbon, nitrogen, and the mineral sources of culture media influences enormously the production capacity of antibiotics in fungi. Furthermore, Boussaber *et al.* in 2012, showed that the antibacterial activity of the actinomycete

strains depends mainly on the tested bacteria and the composition of the culture media.

Figure 4. Effect of different culture media on antibacterial activity of the fungal strain *A. quadrilineatus*.

Figure 5. Effect of different culture media on antibacterial activity of the fungal strain *A. niveus*.

Figure 6. Effect of different culture media on antibacterial activity of the fungal strain *A. wentii*.

Study of the antibacterial activity of organic extracts by the micro-culture system (Biolog).

The study of the antibacterial effect of organic extracts by the Biolog micro-culture system revealed that the extracts of the three fungal

strains presented a remarkable activity with different concentrations, against all the tested bacteria.

The results in Figures 7, 9, and 11 showed that the chloroformic solubilized extracts in DMSO with the concentrations 100, 50, and 25 mg/ml could almost inhibit the total development of the tested bacterial cells, unlike positive control (bacteria only) and negative control (DMSO + bacteria) that demonstrated a good development in wells.

Otherwise, the results mentioned in Figures 8, 10, and 12 corresponding to the concentrations of the extracts ranged from 12.5 to 1.56 mg/ml, indicated a diminution in the antibacterial effect during the decrease of the extracts' concentrations with all the tested bacteria.

However, the antagonism test performed by the discs imbibed with the fungal extracts confirmed the Biolog test (Tables 2, 3, and 4).

In regards to the tested germs, Gram-positive bacteria appear to be more sensitive to the bioactive molecules of the three fungal strains in comparison with Gram-negative bacteria. This is related to the results obtained by Hasavada *et al.* (2006), Atta *et al.* (2009), and Prabavathy and Nachiyar (2012). These results can be explained by the fact that these two groups of microorganisms differ morphologically (Kumara *et al.*, 2010).

Figure 7. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. quadrilineatus* strain with different concentrations. 1-2: test bacteria (2 repetitions); 3: the bacterium with DMSO; 4-12: the different concentrations (from 100 to 25 mg/ml); A-F: the different bacterial strains.

Figure 8. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. quadrilineatus* strain. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.

Figure 9. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. niger* strain with different concentrations. 1-2: test bacteria (2 repetitions); 3: the bacterium with DMSO; 4-12: the different concentrations (from 100 to 25 mg/ml); A-F: the different bacterial strains.

Figure 10. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. niger* strain. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.

Figure 11. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. wentii* strain with different concentrations. 1-2: test bacteria (2 repetitions); 3: the bacterium with DMSO; 4-12: the different concentrations (from 100 to 25 mg/ml); A-F: the different bacterial strains.

Figure 12. Kinetics of bacterial cell development. Antibacterial effect of chloroformic extract of *A. wentii* strain. 1-12: the different concentrations (from 12.5 to 1.56 mg/ml); A-F: the different bacterial strains.

Table 2. Effect of different concentrations of chloroformic extract of *A. quadrilineatus* fungal strain on the test bacteria.

Bacteria	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.56 mg/ml
<i>S. aureus</i>	17.00±1.41	14.00±0.00	12.50±0.71	10.50±0.71	9.00±0.00	8.00±0.00	7.00±0.00
<i>B. subtilis</i>	14.00±1.41	12.50±0.71	11.33±0.58	11.00±0.00	10.00±0.00	9.50±0.71	8.00±0.00
<i>E. faecalis</i>	14.50±2.12	12.50±0.71	12.00±0.00	11.50±0.71	11.00±0.00	10.00±0.00	9.33±0.58
<i>E. coli</i>	15.50±0.71	15.00±0.00	13.50±0.71	13.00±1.41	10.00±0.00	9.33±0.58	8.50±0.71
<i>P. aeruginosa</i>	9.50±0.71	8.33±0.58	7.00±0.00	-	-	-	-
<i>K. pneumoniae</i>	10.00±1.41	9.50±0.71	9.33±0.58	8.00±0.00	8.00±0.00	8.00±0.00	7.00±0.00

* (-): Diameter of inhibition zone ≤ 6 mm

Table 3. Effect of different concentrations of chloroformic extract of *A. niveus* fungal strain on the test bacteria.

Bacteria	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.56 mg/ml
<i>S. aureus</i>	19.00±1.41	14.00±1.41	12.50±0.71	8.50±0.71	8.00±0.00	7.50±0.71	7.00±0.00
<i>B. subtilis</i>	26.50±2.12	17.50±0.71	14.00±1.41	10.50±0.71	9.50±0.71	8.00±0.00	7.50±0.71
<i>E. faecalis</i>	16.50±0.71	13.50±0.71	12.00±0.00	11.00±0.00	9.33±0.58	9.00±0.00	8.00±0.00
<i>E. coli</i>	13.00±1.41	12.50±0.71	11.00±0.00	10.00±0.00	9.00±0.00	8.50±0.71	7.00±0.00
<i>P. aeruginosa</i>	10.00±0.00	9.50±0.71	8.00±0.00	7.00±0.00	7.00±0.00	-	-
<i>K. pneumoniae</i>	11.33±0.58	10.00±0.00	9.50±0.71	9.00±0.00	7.50±0.71	7.00±0.00	7.00±0.00

* (-): Diameter of inhibition zone ≤ 6 mm

Table 4. Effect of different concentrations of chloroformic extract of *A. wentii* fungal strain on the test bacteria.

Bacteria	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.56 mg/ml
<i>S. aureus</i>	14.00±1.41	12.50±0.71	12.33±0.58	11.00±0.00	11.00±0.00	9.50±0.71	7.50±0.71
<i>B. subtilis</i>	14.50±2.12	13.00±1.41	11.50±0.71	11.00±1.41	10.0±0.00	8.50±0.71	7.00±0.00
<i>E. faecalis</i>	16.50±0.71	14.00±0.00	13.33±0.58	11.50±0.71	8.00±0.00	7.00±0.00	7.00±0.00
<i>E. coli</i>	13.00±1.41	11.50±0.71	11.00±0.00	11.00±0.00	10.00±0.00	8.33±0.58	8.00±0.00
<i>P. aeruginosa</i>	10.00±0.00	9.33±0.58	8.00±1.41	8.00±0.00	7.00±0.00	-	-
<i>K. pneumoniae</i>	13.00±1.41	10.00±0.00	9.33±0.58	9.00±0.00	9.00±0.00	8.00±0.00	7.50±0.71

* (-): Diameter of inhibition zone ≤ 6 mm

CONCLUSION

In the context of research for new antibiotic molecules, three fungal strains of *Aspergillus* genus were tested against six bacterial strains. The highlighting test revealed that the three fungal strains have the ability to produce antibacterial molecules. In order to optimize the extraction conditions of these bioactive molecules, five solvents with different polarities were tested, in which chloroform turned out to be the best solvent. Fermentation on different media has shown that the nature of carbon, nitrogen, and mineral sources of the culture media influences enormously the antibiotics' production capacity in fungi. Therefore, the research perspectives would consist in completing the characterization, the purification and the identification of these molecules, as well as the optimization of their biosynthesis for medical purposes.

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