In Vitro Antimicrobial, Antiviral and Cytotoxicity Activities of Aspergillus oryzae Isolated From El-Baida Marsh in Algeria

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ABSTRACT

This work covers the study of antimicrobial and antiviral activities of the Aspergillus oryzae strain isolated from saline soil (El-Baida marsh in Algeria). The crude extract obtained with ethyl acetate displayed antimicrobial activity against Gram-positive and Gram-negative bacteria and the yeast Candida albicans with a mean of 16.69 mm of inhibition zone and a minimal inhibitory concentrations MICs between 7.28 and 21.85 μgmL⁻¹. We also assessed the antiviral activity against Herpes simplex-2 Virus (HSV-2), in which no inhibitory effect was exhibited. In addition, cytotoxicity activity was tested in Caco-2 and RAW 264, a human epithelial and a murine macrophage cell line, respectively, revealing a no-toxic effect of the extract. The studied isolate extract possesses an antimicrobial property and its non-toxicity to the host cells becomes very important, and can be exploited for the production of new pharmacological and biotechnological agents.

Keywords: Aspergillus oryzae, antimicrobial activity, antiviral activity, cytotoxicity, fungal extraction.

1. INTRODUCTION

Filamentous fungi are extensive in the environment, and since a number of antibiotics have been isolated from different fungi such as Penicillium janczewskii, P. Canescens, the interest of the researchers towards bioactive compounds having different biological activities had increased. The discovery of penicillin was a real invention in medicine, and the research's interest for searching new antimicrobial metabolites from microorganisms is continued. Many intensive studies, mainly on terrestrial-derived and marine-derived fungi, displayed that fungi are a rich source of unique bioactive substances.

Fungi are important source of secondary metabolites with novel biological activities. Many fungal extracts substances have been found for their antimicrobial activity mainly from the fungus Penicillium, that they supply a rich source of compounds for therapeutic applications including antibacterial, antifungal and antiviral agents. Aspergillus oryzae is an important filamentous fungi in Japanese fermentation industry, as well as its capacity of the production of various enzymes makes it a perfect model for the research of protein secretion and gene expression.

A. oryzae has a great medicinal and pharmaceutical importance due to its ability to produce substances with antifungal activity such as Asperfurian and Oryzachlorin, in addition to antibiotics such as Penicillin. These countenances support the idea that A. oryzae is a suitable microorganism for fermentation and use in biotechnology.

Because of the increased interest of researchers in microorganisms and their applications in food as nutritional supplements, in health as therapeutic pharmaceuticals, and in industry as cosmetics, detergents. In addition, the interest of researchers is also attributed to the problem of salinity,
due to the transformation of vast agricultural areas annually into areas not suitable for agriculture. The question that arises is: If this soil is not suitable for agriculture, can it be exploited in other indirect ways in various fields? The aim of this study was to evaluate the antimicrobial and antiviral activities of the crude ethyl acetate extract of A. oryzae, isolated from saline soil, against some common pathogenic microorganisms, in addition to the study of its cytotoxicity.

2. MATERIALS AND METHODS

2.1. Study area

El-Baida marsh is one of the natural saline soils. It is located between latitudes 5° 53’ 20” E - 5° 53’ 30” E and latitudes 35° 57’ 80” N - 35° 54’ 20” N near Hammam El-Sukhna area (Setif region), an area of approximately 12,223 hectares. Soil surrounding the site is characterized by being less salty to alkaline clay with a gradient composition.7

2.2. Culture media

The synthetic (Yeast Extract Saccharose) YES medium consisting of sucrose 15% (w/v), yeast extract 2%, MgSO4 0.05%, metal traces 0.1% (ZnSO4.7H2O 1%, CuSO4.7H2O 0.5%) was used for secondary metabolites production. Muller Hinton agar MHA and broth MHB were used for bacterial isolates culture and sensitivity tests.

2.3. Fermentation and Antimicrobial assay

Seven days (potato dextrose agar) PDA plate fungal pure cultures were used to prepare discs. They were cut (6 mm diameter) and placed into 250 mL Erlenmeyer flask containing 100 mL of YES medium. The flasks were then incubated at 30 °C for 7 days. After fermentation, the mycelium with the medium was frozen at -10 °C for 24 hours, then crushed and centrifuged at 15000 rpm / 15 min. Seven samples were extracted twice with equal volumes (1:1 v/v) of ethyl acetate. The organic layers were evaporated to dryness in a rotary evaporator at 40 ± 1°C, the dry crude extracts were then dissolved in DMSO 50% and stored at 4°C until used.9,10

2.3.1. Screening assay for antimicrobial activity using disc diffusion method

Ethyl acetate extract of A. oryzae was tested against a panel of human pathogenic microorganisms including, five Gram positive bacteria, (Bacillus subtilis ATCC10876, Staphylococcus aureus ATCC25923, Staphylococcus aureus resistant to methicillin (SARM) ATCC43300, Listeria sp. and Enterococcus sp.), seven Gram negative bacteria (Salmonella typhi ATCC14028, Escherichia coli ATCC25922, Salmonella typhimurium ATCC13311, Pseudomonas aeruginosa ATCC27853, Klebsiella pneumonia ATCC700603, Escherichia coli resistant to a bactericide ATCC35218, and Salmonella enterica), and the yeast Candida albicans. Plates were prepared by spreading MHA and Sabouraud culture media by bacteria suspension 108 (Colony forming unit) CFU/mL and Calbicins 106 CFU/mL respectively, afterwards filter paper discs of 6 mm diameter were soaked with 20 μl of the fungal extracts and placed on the inoculated agar. The plates were then incubated at 37 °C, 24 h for bacterial isolates and 48-72 h for the yeast.8

Negative controls were prepared using the same solvents employed to dissolve the extracts. Antimicrobial activity was evaluated by measuring the zone of inhibition developed round the discs. Each assay in this experiment was performed in triplicate.

2.3.2. Determination of the minimum inhibitory concentration (MIC)

MIC is the lowest concentration that inhibits visible growth after time incubation period of 18-24 hours; here, its determination was made from the measurement of the turbidity induced by the growth of the studied germs.11 The MIC (μg/mL) of the crude extract of the fungus was determined using dilution method ELISA plate, where a series of two-fold dilutions (dissolved in DMSO up to 5% final DMSO concentration), were prepared in a 96 well-sterile microplate. 100 μl of corresponding media (Muller Hinton Broth) MHB for the bacteria and PDB (Potato Dextrose Broth) for the yeast were added, then 100 μl of the diluted extract was introduced in each well. These dilutions were inoculated with 25 μl of a solution containing 6 x 106 Colony Formating Unit colony- forming unite per mL (CFU/mL) of bacterial suspension (Bacillus aureus ATCC10876, S. aureus ATCC25923, SARM ATCC43300, E. coli ATCC25922, P. aeruginosa ATCC27853, K. pneumonia, ATCC700603) and 7 x 107 CFU/mL of yeast suspension Candida albicans.12,8 The well-which used as a negative control was prepared using the inoculum alone. The microplate was incubated at 37°C for 24 hour. The data was the mean of triplicates.13

2.4. Antiviral activity essay

Antiviral activity of A. oryzae extract was examined against Herpes simplex-2 (HSV-2), according to Anand et al.10 method. Briefly, 60μl of HEP-2 monolayer cells (a type of human epithelial cell) were grown in a plate of 96 wells and 40μl of viral suspension, obtained by seven successive ten-fold dilution in 2% FBS MEM (cow’s serum / medium Minimum Basic), was added into five wells of microtiter plates (Four for antiviral, one as positive control and one without virus as negative control). The 96 well microplates were incubated at 37°C in 5% CO2 atmosphere for 60 minutes to facilitate adsorption of virus to the cell line. The following concentrations of non-toxic fungal extracts of 40 μg, 30 μg, 20 μg and 10 μg were diluted with 2% FBS, MEM and 100 μl of each concentration with the medium were transferred into the virus inoculated monolayer cell lines and reincubated at 37 °C temperature for 3-4 days to allow multiplication of virus and subsequent development of cytopathic effect (CPE). Each well was observed under inverted microscope every day for presence or absence of cytopathic effect. The experiment was conducted under sterile conditions in duplicates.

2.5. In vitro cytotoxicity test of A. oryzae extract in Caco-2 cells and Raw 264 cells

Cytotoxic activity of A. oryzae extract in murine macrophage RAW 264 and human epithelial Caco-2 cells was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This test is indicative for mitochondrial NADH-dependent dehydrogenase activity, which is proportional to both cell viability and proliferation rates of treated cultures.12 Both cell lines used, RAW 264 and Caco-2, were provided by the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in RPMI Medium, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 units/mL) and streptomycin (1 mg/mL) in a humidified 5% CO2 atmosphere at 37 °C. Cells were seeded into 96 well plates at a density of 5 x 105 cells/well, grown until the formation of a monolayer, and thus incubated with different concentrations of the extract (50 and 100 μg/mL), not exceeding 1 % DMSO in content, for 24 h. Untreated and DMSO-treated cells were used as controls. The effect of the...
extract on cell viability was checked with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer’s protocol [3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution was added to each well and incubated for 1-4 h. The absorbance of the media was measured at 490 nm on a MRX Dynex microplate reader (Dynex Technologies, Chantilly, VA, USA). The cellular viability was calculated from the absorbance value and compared with that of the untreated control cells.

3. RESULTS

3.1. Antimicrobial assay

A. oryzae isolate was tested for its antimicrobial activity, by disc diffusion method, it showed strong effects against the species tested with an inhibition zone diameters ranging from 17.66 mm to 26 mm, the largest of which was against P. aeruginosa, followed by ERBC with 25 mm, then B. aureus by 24 mm, and SARM by 23 mm. Meanwhile, there was no effect on the bacteria Listeria sp., Enterococcus sp and Salmonella enterica. As for the yeast, Candida the isolate had a clear effect with 18 mm (Figure 1).

- The results showed that the MIC was 7.28 µg / ml for B. aureus and P. aeruginosa and 21.85 µg / ml for C. albicans and other tested bacteria (Table).

3.2. In vitro cytotoxicity assay

Incubation of Caco-2 and RAW 264 cells with different concentrations of A. oryzae extract (50 and 100 µg/ml) for 24 h, did not exhibit cytotoxicity as determined by MTT assay. In fact, the viability in treated cells reached similar values of those in untreated and DMSO-treated controls (Figure 2 and 3).

3.3. In vitro antiviral assay

The non-toxic concentrations of secondary metabolites of 40 µg, 30 µg, 20 µg and 10 µg were used for anti HSV-2 study. The secondary metabolites of A. oryzae did not show any inhibitory effect on HSV-2 and the complete cytopathic effect were observed (data not shown).

Table 1: Minimal inhibitory concentrations of A. oryzae ethyl acetate extract developed against a panel of bacteria and yeast

| Microorganisms       | E. coli | K. pneumoniae | S. aureus | SARM | C. albicans | B. aureus | P. aeruginosae |
|----------------------|---------|---------------|-----------|------|-------------|-----------|---------------|
| MIC (µg/mL)          |         |               |           |      |             |           |               |
|                      | 21.85±0.35 | 21.85±0.35 | 07.28±0.13 |      |             |           |               |

Figure 1: Antimicrobial activity of A. oryzae ethyl acetate extract expressed as diameter inhibition zone (mm) in disc diffusion method

Figure 2: Effects of A. oryzae extract on nitric oxide production in RAW 264 cells. The experiments were performed three times.
4. DISCUSSION

The marsh (very salty lakes) constitutes rare regions in the world that seem to be promoter environments to isolate fungi of new antimicrobial molecules. Fungal derived natural products have foremost served as lead structures for the development of antibacterial and antiviral agents.

In the present study, the fungal extract achieved the most activity against *P. aeruginosa*, *B. aureus*, *E. coli*-resistant and *S. aureus*-resistant bacteria. These findings are consistent with Bhattacharyya and Jha in their study of the antimicrobial activity of *A. niger* against *Bacillus subtilis* and *C. albicans* yeast. Same results obtained by Al-fakih and Almaqtri when testing the antibacterial activity of *A. oryzae* extract on seven bacterial species. However, the inhibition diameter ranged between 10-17.3 mm and the minimal inhibitory concentration (MIC) was between 176-285 µg/ml. Comparing with our results, this suggests that the isolated strain is better and more effective.

These results obtained are important since these bacteria are known to be pathogenic and resistant to many antibiotics, as they are responsible for hospital infections, such as *S. aureus* which is considered to cause skin infections, and *B. aureus* responsible of infections in many diseases, and causes food poisoning.

Both *Penicilium* and *Aspergillus* are known for their extensive production of the same metabolites with various biological activities, including antibacterial and antifungal activities. However initially, the antibiotics produced by *Aspergillus* were called aspirmiglin, but recent studies have found that *A. oryzae* is able to produce several secondary metabolites with antibiotic effect such as aspgirlormarmase, cyclopiazonic acid (CPA), kojic acid, 3-nitropropionic acid, maltorizine, violacatin, whose production has been investigated only on barley extract as an antibiotic, in addition to Asperfurans as an antifungal agent, as well as CJ-17.665A isolated from *A. ochraceus*.

*A. oryzae* fungus belongs to the family of *A. flavus*, but it doesn’t produce aflatoxin, however, some mycotoxins have been isolated from *A. oryzae*, including kojic acid, and β-nitropropionic acid (BNP), and cyclopiazonic acid (CPA), and some researchers have reported that kojic acid has strong inhibiting activity against tyrosinase and antibacterial activity, while recent studies have shown that kojic acid may be a possible carcinogen. Unlike Seshime, it is believed that the fungus contains the gene responsible for producing aflatoxin toxins but is inactive due to mutations in some regulatory genes. Regarding toxicity, our results demonstrate that the isolate *A. oryzae* has no toxic effect. In fact, incubation of both murine macrophage RAW 264 and human epithelial Caö-2 cell lines with different concentrations of the extract for 24 hours did not affect cell viability. These results are consistent with the study of Sosa et al., who found that the fungus did not produce aflatoxin B1, B2, G1, G2 as well as ochratoxin. As a result, *A. oryzae* is a safe fungus and was listed as “Generally Recognized as Safe” by the Food and Drug Administration (FDA), and its safety was supported by the World Health Organization (WHO). Fungal strains used in industrial biotechnology have a long and documented history of a safe use of food and feed applications. Strains of *A. niger* and *A. oryzae* species have been used to ferment food for more than two thousand years and to manufacture food enzymes for more than 50 years. Regulatory authorities consider hundreds of enzymes produced in these species safe.

The fact that the studied isolate extract possesses an antimicrobial property and its non-toxicity to the host cells becomes very necessary especially when it is used to prepare sterile or antiseptic substances or integrate it as one of the chemical components.

CONCLUSION

From the above study, it is concluded that *A. oryzae* extract showed an antimicrobial activity against sensitive and resistant pathogenic microorganisms. Thus, it is of great importance in the treatment of pathologies associated with them because these strains exhibit high resistance to the antibiotics used in current therapeutic applications. However, to value this work it is necessary to follow it with purification and application in vivo of these bioactive substances, especially for its non-toxicity, so this isolate can be exploited for production of new pharmacological and biotechnological agents.

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Conflict of Interest

The authors declare no conflict of interest.
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