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

***Nacef Houda Sara[1], Belhattab Rachid[2], Galvez Julio[3], Rodriguez-Sojo María Jesus[3,4], Vezza Teresa[3,4]**

[1] Department of Microbiology and Laboratory of Applied Microbiology, Faculty of Nature and Life Sciences, Ferhat Abbas Setif 1 University, Algeria

[2] Department of Biochemistry and Laboratory of Applied Microbiology, Faculty of Nature and Life Sciences, Ferhat Abbas Setif1 University, Algeria

[3] Department of Pharmacology, Biomedical Research Center (CIBM), 18071-Granada University, Spain

[4] Institute of Biosanitary Research of Granada (ibs.GRANADA), 18012-Granada, Spain

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.

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***Address for Correspondence:**

Nacef Houda Sara, Department of Microbiology and Laboratory of Applied Microbiology, Faculty of Nature and Life Sciences, Ferhat Abbas Setif 1 University, Algeria

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* [1,2] 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.[3]

Fungi are important source of secondary metabolites with novel biological activities.[4] Many fungal extracts substances have been found for their antimicrobial activity mainly from the fungus *Penicillium*,[5] that they supply a rich source of compounds for therapeutic applications including antibacterial, antifungal and antiviral agents .[2] *Aspergillus oryzae* is an important filamentous fungi in Japanese fermentation industry, as well as its capacity of the production of various enzymes makes it an 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 Asperfuran 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.[6]

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 salty, 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%, $MgSO_4$ 0.05%, metal traces 0.1% ($ZnSO_4$,$7H_2O$ 1%, $CuSO_4$,$7H_2O$ 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 (6mm diameter) and placed into 250 mL Erlenmeyer flask containing 100mL 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 2-3 hours, then crushed and centrifuged at 15000 rpm / 15 min.[8] Finally; 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, (*Bcillus aureus ATCC*10876, *Staphylococcus aureus ATCC*25923, *Staphylococcus aureus resistant to methicillin (SARM) ATCC*43300, *Listeria sp.* and *Enterococcus sp.*), seven Gram negative bacteria (*Salmonella typhi ATCC*14028, *Escherichia coli ATCC*25922, *Salmonella typhimirium ATCC*13311, *Pseudomonas aeruginosa ATCC*27853, *Klebsiella pneumonia ATCC*700603, *Escherichia coli* resistant to a bactericide *ATCC*35218, and *Salmonella entarica*), and the yeast *Candida albicans.* Plates were prepared by spreading MHA and Sabouraud culture media by bacteria suspension 10^8 (Colony forming unit) CFU/mL and *C.albicans* 10^6 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 10^7 Colony Formatting Unit colony- forming unite per mL (CFU/mL) of bacterial suspension (*Bcillus aureus ATCC*10876, *S. aureus ATCC*25923, *SARM ATCC*43300, *E. coli ATCC*25922, *P. aeruginosa ATCC*27853, *K. pneumonia, ATCC*700603*)* and 7 x 10^7 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% CO_2 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-diphenyl-tetrazolium 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.[14] 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% CO_2 atmosphere at 37 °C. Cells were seeded into 96 well plates at a density of 5 x 10^5 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. aeroginosa*, 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 entarica*. As for the yeast, *C.allbicans* 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).

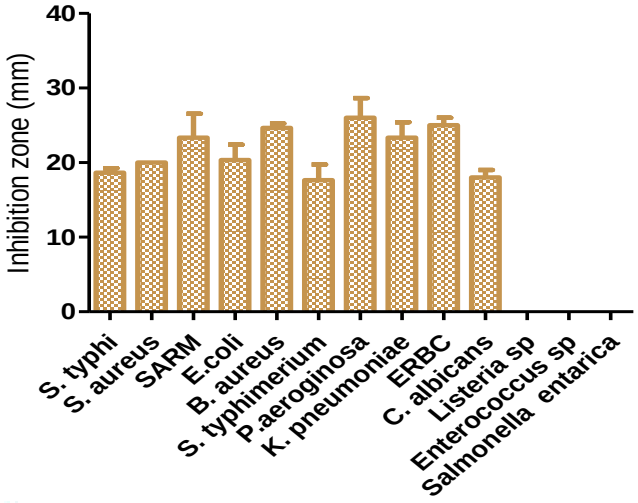

Figure1: Antimicrobial activity of *A. oryzae* ethyl acetate extract expressed as diameter inhibition zone (mm) in disc diffusion method

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			07.28±0.13	

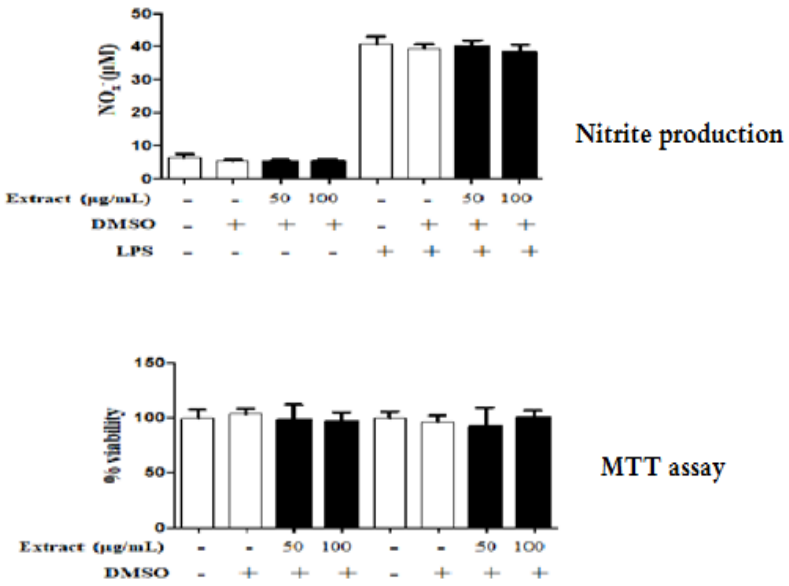

Figure 2: Effects of *A. oryzae* extract on nitric oxide production in RAW 264 cells. The experiments were performed three times.

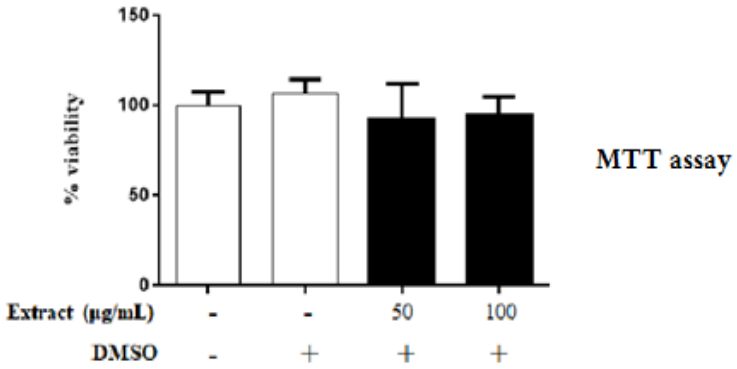

Figure 3: Cytotoxicity test of *A. oryzae* extract in CaCo-2 cells. The experiments were performed three times

4. DISCUSSION

The marsh (very salty lakes) constitutes rare regions in the world that seem to be promoter environments to isolate fungi source of new antimicrobial molecules.[15] Fungal derived natural products have foremost served as lead structures for the development of antibacterial and antiviral agents.[16]

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[17] 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[3] 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[18], such as *S. aureus* which is considered to cause skin infections[19], and *B. aureus* responsible of infections in many diseases, and causes food poisoning.[20]

Both *Penicilium* and *Aspergillus* are known for their extensive production of the same metabolites with various biological activities, including antibacterial and antifungal activities.[3] However initially, the antibiotics produced by *Aspergilli* were called aspirgillin, but recent studies have found that *A. oryzae* is able to produce several secondary metabolites with antibiotic effect such as aspergillormarasmine, cyclopiazonic acid (CPA), kojic acid, 3-nitropropionic acid, maltorizine, violacetin, whose production has been investigated only on barley extract as an antibiotic[21], in addition to Asperfuran as an antifungal agent[22], as well as CJ-17.665A isolated from *A. ochraceus*.[3]

A. oryzae fungus belongs to the family of *A. flavus*, but it doesn't produce aflatoxin[23], 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 anti-bacterial activity, while recent studies have shown that kojic acid may be a possible carcinogen.[24] Unlike Seshime[25], 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 non-toxic effect. In fact, incubation of both murine macrophage RAW 264 and human epithelial Caco-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.[26], 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).[24] 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.[27]

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.[28]

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.

REFERENCES

1. Takahashi JA, Castro MCM de, Souza GG, et al. Isolation and screening of fungal species isolated from Brazilian cerrado soil for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus, Salmonella typhimurium, Streptococcus pyogenes*and *Listeria monocytogenes*. JMM. 2008; 18:198–204. https://doi.org/10.1016/j.mycmed.2008.08.001
2. Kaur H, Onsare JG, Sharma V, Arora DS. Isolation, purification and characterization of novel antimicrobial compound 7-methoxy-2, 2-dimethyl-4-octa-4′, 6′-dienyl-2 H-napthalene-1-one from *Penicillium sp*. and its cytotoxicity studies. AMB Express. 2015; 5:40. DOI: 10.1186/s13568-015-0120-9.
3. Al-Fakih A A, and Almaqtri W Q A. Overview on antibacterial metabolites from terrestrial *Aspergillus* spp. Mycol. 2019; 10:191-209. DOI: 10.1080/21501203.2019.1604576.
4. Baker DD and Alvi KA. Small-molecule natural products: new structures, new activities. Curr Opin Biotech. 2004; 15:576–583. https://doi.org/10.1016/j.copbio.2004.09.003.
5. Petit P, Lucas EM, Abreu LM, et al. Novel antimicrobial secondary metabolites from a *Penicillium sp.* isolated from Brazilian cerrado soil. EJBiotechnology. 2009; 12:8–9 DOI: 10.2225.
6. Junichiro M, Sumiko O-K, Tomohiro A, Nishimura M, Koike H, Masayuki M. Penicillin biosynthesis in *Aspergillus oryzae* and its overproduction by genetic engineering. J. Biosc. Bioeng. 2010; 110: 8-11. doi: 10.1016/j.jbiosc.2010.01.001.
7. Aliat T and Kaabeche M. Phytoecological characterization of the wetland Chott El Beida (Setif, Algeria). Bulletin de l'Institut Scientifique Rabat. 2013; 35:35–41.
8. Chaudhary R and Tripathy A. Isolation and Identification of Bioactive Compounds from *IrpexLacteus* Wild Fleshy Fungi. J Pharm Sci. 2015; 7:11
9. Sadrati N, Daoud H, Zerroug A, et al. Screening of Antimicrobial and Antioxidant Secondary Metabolites from Endophytic Fungi Isolated from Wheat (*Triticum Durum*).J Plant Prot Res. 2013; 53:129-136. https://doi.org/10.2478/jppr-2013-0019.
10. Anand D, Marimuthu S, Saravanan P, Rajarajan S. *In Vitro* Antiviral Activity of Fungal Secondary Metabolites against *Herpes Simplex* Virus-2. Int. J. Pharm. Sci.2016; 38:73-77.
11. Moroh J L A, Bahi C, Dje K, Loukou Y, Guede-Guina F. Study of the antibacterial activity of *Morindamorindoides* (Baker) milne-redheat (*rubiaceae*) acetatique extract (ACE) *in-vitro* growth of *Escherichia coli* strains. Bulletin de la sociétéroyale des sciences de Liège. 2008; 77:44-61.
12. Qadri M, Johri S, Shah BA, et al. Identification and bioactive potential of endophytic fungi isolated from selected plants of the Western Himalayas. Springerplus. 2013; 2:8. https://doi.org/10.1186/2193-1801-2-8.
13. Rahmoun NM, Boucherit-Otmani Z, Boucherit K, et al. Antibacterial and antifungal activity of lawsone and novel naphthoquinone derivatives. Med Mal Infect. 2012; 42:270–275. https://doi.org/10.1016/j.medmal.2012.05.002.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65(1–2):55–63. doi: 10.1016/0022-1759(83)90303-4.
15. Boughachiche F, Reghioua S, Oulmi L, et al. Isolement d'actinomycétales productrices de substances antimicrobiennes à partir de la sebkha de AïnMLila. Sciences & Technologie C, Biotechnologies. 2005 ; 23:5–10.
16. Hussain H, Kliche-Spory C, Al-Harrasi A, et al. Antimicrobial constituents from three endophytic fungi. Asian Pac J Trop Med. 2014; 7:S224–S227. https://doi.org/10.1016/S1995-7645(14)60236-4.
17. Bhattacharyya P, Jha DK. Optimization of cultural conditions affecting growth and improved bioactive metabolite production by a subsurface *Aspergillus* strain. Int. j. appl. biol. pharm. 2011; 2:133-143.
18. Sahu M C, Debata N K, Padhy R N. Antibacterial activity of *Argemone mexicana* L. against multidrug resistant *Pseudomonas aeruginosa*, isolated from clinical samples. Asian Pac. J. Trop. Biomed. 2012; S800-S807. https://doi.org/10.1016/S2221-1691(12)60316-5.
19. Camporese A, Balick MJ, Arvigo R, Esposito RG, Morsellino N, De Simone F, Tubaro A. Screening of anti-bacterial activity of medicinal plants from Belize (Central America). J. Ethnopharmacol. 2003; 87:103–107. DOI: 10.1016/s0378-8741(03)00115-6.
20. Kumar VP, Chauhan N S, Padh H, Rajani M. Search for antibacterial and antifungal agents from selected Indian medicinal plants. J. Ethnopharmacol. 2006; 107:182– 188. DOI: 10.1016/j.jep.2006.03.013.
21. Blumenthal CZ. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichodermareesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul Toxicol Pharmacol. 2004; 39:214–228. DOI: 10.1016/j.yrtph.2003.09.002
22. Farid M A F, Hoda M, and Abdel Halim S. Amylase production from *Aspergillus oryzae* LS1 by solid-state fermentation and its use for the hydrolysis of wheat flour. Iran J Biotechnol. 2011; 9: 267-274.
23. Barbesgaard P, Heldt-Hansen HP, Diderichsen B. Mini-review On the safety of *Asperyillus oryzae*: a review. Appl Microbiol Biotechnol. 1992; 36: 569-572. DOI: 10.1007/BF00183230.
24. He B, Tu Y, Jiang C, Zhang Z, Li Y, Zeng B. Functional Genomics of *Aspergillus oryzae*. Strategies and Progress. Microorganisms. 2019; *7*(4):103. DOI: 10.3390/microorganisms7040103.
25. Seshime Y, Juvvadi PR, Fujii I, Kitamoto K. Discovery of a novel superfamily of type III polyketide synthases in *Aspergillus oryzae*. BiochemBiophys Res Commun. 2005; 331:253–260. DOI: 10.1016/j.bbrc.2005.03.160.
26. Sosa A, Kobashigawa E, Galindo J, et al. Mycotoxicological studies of an *Aspergillus oryzae* strain. J. Yeast Fungal Res. 2013; 4:26–32. DOI: 10.5897/JYFR13.0111.
27. Frisvad J C, Møller L L, Larsen T O, Kumar R, Arnau J. Safety of the fungalworkhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger, Aspergillus oryzae*, and *Trichoderma reesei.* Appl. Microbiol. Biotechnol. 2018; 102(22):9481-9515. doi: 10.1007/s00253-018-9354-1.
28. Muhammad Jamal Ali Abu Majdad, N. *In vitro* Antibacterial activity of bitter melon and turmeric extracts. Thi-Qar University Journal. 2009; 04:04.