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ORIGINAL ARTICLE

Enzymatic activity of clinical and environmental isolates of *Aspergillus flavus* section *Flavi*

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ABSTRACT

Background and Objectives: The ability of *Aspergillus* species to produce various extracellular enzymes has a pathogenic role in humans. These enzymes not only play a role in metabolism but also act as virulence factors. The secretion of hydrolytic enzymes facilitates tissue invasion and is considered a virulence factor for several fungi. The current study aimed to compare the enzymatic activity of *A. flavus* section *Flavi* isolated from otomycosis and environmental samples.
Materials and Methods: In the present study, eighty-one isolates of *A. flavus* including 41 clinical strains from otomycosis and 40 environmental isolates were identified using the calmodulin gene. The enzymatic activity of the isolates was assessed using standard culture media.
Results: All isolates exhibited phospholipase and urease activity. Esterase and hemolysin activity in otomycosis isolates were higher than in environmental isolates (88% > 62%), (98% > 72%). High levels of both extracellular and intracellular catalase activity were detected among otomycosis isolates.
Conclusion: All isolates were phenotypically identified as *Aspergillus* section *Flavi* and then characterized using the calmodulin gene. The mean activity of intracellular catalase enzymes (1.29) in both groups was higher than the mean activity of extracellular catalase enzymes (0.85). Additionally, the esterase (88%) and hemolysin (98%) activity of the clinical isolates was higher than that of the environmental isolates (esterase 62%, hemolysin 72%). All isolates exhibited phospholipase and urease activity.

Keywords: *Aspergillus flavus*; Otomycosis; Extracellular enzymes; Pathogenicity

INTRODUCTION

Host and organism factors play a crucial role in the development of aspergillosis in humans. *Aspergillus* species have the ability to produce various extracellular enzymes, which has both industrial and pathogenic (1). These enzymes, such as phospholipases, lipases, acid proteinases, catalase, and esterases, not only play a role in metabolism but also act as virulence factors (2). The secretion of these hydrolytic enzymes aids in tissue invasion, making them important virulence factors for fungi like the *A. flavus*

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complex (1, 3, 4). Research by Rouein et al. suggests that the mycelial forms of *A. flavus* exhibit higher catalase activity compared to the conidial form (5). Additionally, a study has shown significant differences in catalase activity between clinical and environmental isolates of *Aspergillus* (6).

Aspergillosis is a common fungal infection caused by *Aspergillus* species, which are common soil-dwelling, heterotrophic, anamorphic filamentous fungi (7). About 95% of aspergillosis cases are attributed to three main *Aspergillus* species: *A. fumigatus*, *A. niger*, and *A. flavus* (7). Among these, *Aspergillus* section *Flavi*, following *Aspergillus* section *Nigri*, is the second most common opportunistic pathogen responsible for aspergillosis in humans (3, 8). *A. flavus* is known to cause a variety of invasive and colonizing infections in humans, serving as a human pathogen, allergen, and mycotoxin producer (8). Specifically, *A. flavus* is often associated with fungal sinusitis and cutaneous infections, while chronic cavitary pulmonary aspergillosis is typically linked to *A. fumigatus* due to the smaller size of its spores, which are more likely to be deposited in the lower respiratory tract (9).

Comparing the enzymatic profiles of clinical and environmental *A. flavus* isolates provides a direct way to determine whether certain enzymes are markers of pathogenicity or just features of saprophytic growth in the environment. This comparison allows researchers to identify specific virulence-associated enzymes such as phospholipase, esterase and hemolysin that are significantly overexpressed in otomycosis. Furthermore, establishing a distinct enzymatic baseline for environmental saprophytes versus clinical pathogens strengthens the study by clarifying whether enhanced enzymatic activity is a result of host adaptation or an innate feature of the species. Therefore, the purpose of the current study is to compare the intra and extracellular enzymatic activity (including catalase, urease, phospholipase, esterase, and hemolysin) of *A. flavus* strains isolated from cases of otomycosis and environmental samples.

MATERIALS AND METHODS

Isolates and identification. A total of eighty-one isolates of *A. flavus* were analyzed, including 41 clinical strains obtained from patients with otomycosis and 40 environmental isolates. The clinical isolates were collected using sterile swabs from patients in Ahvaz, a province in southeast Iran, in 2022. These samples were cultured on Sabouraud dextrose agar (SDA) from Liofilchem, Italy. Additionally, 40 environmental strains (soil and air samples) of *A. flavus* were isolated from various sources in Ahvaz. The *A. flavus* isolates were initially screened based on their macroscopic morphological characteristics on SDA plates and microscopic slides. Pure cultures were then prepared from each isolate on SDA slants and stored at room temperature for further analysis.

DNA extraction and molecular identification of the isolates. Genomic DNA was extracted and purified following method presented by Hamzehee et al. (10). The isolates were cultured in glass flasks with 150 ml of Sabouraud dextrose broth (SDB) and incubated at 30-35ºC in a shaker incubator (100 rpm) for 24 hours under aerobic conditions. Mycelia were separated from the culture media using paper filters in sterile conditions. The mycelia were then homogenized in cryotubes with 500 µL of lysis buffer and glass beads using a SpeedMill PLUS Homogenizer (Analytica, Germany) for 6 minutes. Supernatants were separated, and DNA was purified using phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, USA). The DNAs were stored at -20ºC until use. The amplification of samples was carried out using calmodulin primers, Cmd5 (5'-GTCTCCGAGTACAAGGAGGC-3') and Cmd6 (5'-TCGCCGATRGAGGTCATRACGTG-3') (8). PCR products were sequenced using the forward primer (Cmd5), and all sequences were edited and aligned using MEGA11 software. Additionally, sequences were blasted using the NCBI Blast Database (https://blast.ncbi.nlm.nih.gov). Finally, all sequence data were deposited in the NCBI gene bank, and accession numbers were obtained.

Phospholipase activity. Phospholipase activity was assessed in *A. flavus* isolates following the method described by Jafarian et al. (11). SDA was supplemented with 5.8g NaCl (Merck, Germany), 0.055g CaCl2 (Merck, Germany), and 8% egg yolk as a phospholipid source. A 5 µL of 0.5 McFarland standard suspension of strains was inoculated on the medium and incubated at 25ºC for 7 days. Phospholipase-positive isolates produced a dense white zone of precipitation around the colonies. The phospholipase index (P index) was calculated as described by Price et al. as follows:

$$\text{P index} = \frac{\text{Colony diameter}}{\text{Colony diameter} + \text{Percipitation zone diameter}}$$

Esterase activity. The esterase activity of isolates was measured using a specific medium containing 10.0 g of Bacto Peptone (Difco, USA), 5.0 g of NaCl (Merck, Germany), 0.1 g of CaCl2 (Merck, Germany), 15.0 g of agar (Mir media, Iran), and 1000 mL of distilled water supplemented with 5 mL of sterile Tween 80 (Merck, Germany) (12). The standard suspension of each isolate was then inoculated into the media and incubated at 25ºC for 10 days. The presence of a clear halo around each colony indicates positive esterase activity. Finally, the esterase activity index (E index) for each isolate was calculated as described above for phospholipase activity.

Urease activity. The urease activity of tested isolates was assessed using slants of urea agar base medium (Merck, Germany) supplemented with 50 ml/L sterile 40% urea (Merck, Germany). After inoculating the medium with 5 µl of 0.5 McFarland standard spore suspension, it was incubated at 25ºC for 72 hours. A change in the culture medium color from orange to red indicates a urease-positive test and is recorded from 1+ to 3+ (13, 14).

Hemolytic activity. SDA supplemented with 7% sheep blood (Bahar Afshan, Iran) and 3% glucose (Merck, Germany) was used for hemolytic activity (3). 5 µL of standard spore suspension was inoculated on the culture medium and incubated at 25ºC for 5 days. The presence of clear hemolysis around the colony on the medium indicated positive results and was calculated as described above.

Catalase activity. All tested isolates were grown in SDA and incubated at 35ºC for 4-5 days. 1 ml of standard suspension was prepared from each isolate (equal to 0.5 McFarland) and inoculated into flasks containing 80 mL of 1% yeast extract medium (Merck, Germany). Flasks were incubated at 30ºC in a shaker incubator at 100 rpm for 3 days. Mycelia as fungal balls were collected by filtration using Whatman filter paper, washed twice with 0.05 M Tris-HCl buffer (pH = 7.5), and dried using 200 µl acetone. Approximately, one gram of dried mycelia was transferred to a microtube containing 500 µl of 10 mM Tris-HCl (pH=7.8) and acid-washed glass beads. A homogenized suspension was prepared using a SpeedMill PLUS Homogenizer. After centrifugation at 100g for 15 min, the supernatant was used for the extracellular catalase activity assay. Moreover, the liquid medium was used for the intracellular catalase activity assay. The Catalase Assay Kit (Navand Salamat, Iran) was used according to the manufacturer's instructions and read by Elisa Reader (BioTek, USA) at 540 nm. Finally, a standard curve was drawn and used to determine the concentration of catalase in samples (6).

Statistical analysis. In this study, a non-parametric t-test (online version of Social Science Statistics, https://www.socscistatistics.com/) was used to define the correlation between clinical and environmental data, and a P-value <0.05 was considered statistically significant.

Ethical statement. The ethics committee of Ahvaz Jundishapur University of Medical Sciences approved this project under reference no, IR.AJUMS.MEDICINE.REC.1400.034. The study was carried out as per the Helsinki Rules.

RESULTS

Isolates identification results. Isolate identification results in this study revealed 81 isolates of *Aspergillus* section *Flavi*, including *A. flavus* (97.5%), *A. tamarii* (1.2%), and *A. pipericola* (1.2%) from clinical and environmental specimens. Molecular analysis of the calmodulin gene confirmed 81 isolates of *A. flavus*, with 41 clinical and 40 environmental isolates. Accession numbers for A. flavus are LC706757 – 801, LC757827 – 31, LC830413, LC830414, LC820455, LC820456, LC820458 – 75, LC858709 – 13, LC858715, LC858716, *A. piericola* LC820454, *A. tamarii* LC858714.

Catalase activity. Regarding catalase activity, the study examined the catalase activity of 81 *Aspergillus* isolates (41 clinical and 40 environmental). The standard curve of catalase activity was linear with a regression coefficient of R2= 0.96 (Fig. 1). All isolates exhibited both intracellular and extracellular catalase activity. However, the ratio of enzymatic secretion varied among isolates. Clinical *Aspergillus* species demonstrated higher intracellular and extracellular catalase activity (4.47, 4.03 mU/ml) compared to environmental isolates (3.38, 3.33 mU/ml) as shown in Table 1.

Statistical analysis revealed a difference in catalase activity between extracellular and intracellular catalase among both clinical (P=0.0147) and environmental (P=0.0042) isolates. In other words, intracellular catalase activity in clinical and environmental isolates

of *A. flavus* is greater than extracellular catalase activity. Analysis of extracellular catalases in clinical and environmental isolates showed no difference in extracellular catalase secretion between them (P=0.289). Similarly, there was no significant difference in intracellular catalase activity among clinical and environmental isolates (P=0.264) (Table 1).

Table 2 displays the details of phospholipase, esterase, and hemolysin activities of clinical and environmental isolates of *A. flavus*. In this study, all isolates of *A. flavus* produced extracellular phospholipase. Specifically, 68% of clinical isolates and 53% of environmental isolates exhibited mild phospholipase activity. There was no significant difference in phospholipase activity between environmental and clinical isolates (P = 0.4782). Among our clinical isolates, 86% showed mild or strong hemolysin activities, while only 65% of environmental isolates displayed the same (P = 0.0001). Additionally, there was a significant difference in esterase activity between clinical and environmental isolates (P = 0.0084).

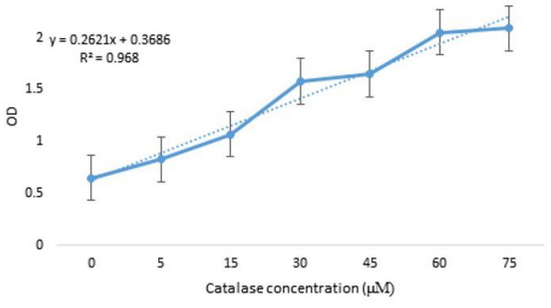

Fig. 1. The standard curve of catalase activity based on manufacturers' guidelines as the regression coefficient (R2=968) curve was linear

All isolates tested positive for urease activity however 72% had 2+ activity followed by 16%, 3+, and the remaining isolates were 1+. High urease activity (3+) was detected in 27% of clinical *Aspergillus* isolates while 88% of environmental isolates exhibited mild urease activity (2+) (Table 3).

In this study, a neighbor-joining phylogenetic tree was constructed based on calmodulin gene sequences from 41 clinical isolates (Fig. 2) and 40 environmental isolates (Fig. 3). As shown in Fig. 2 all clinical isolates were clustered in the *A. flavus*-clade. Meanwhile, all environmental isolates of *A. flavus* and one isolate

Table 1. The catalase activity of the clinical and environmental isolates of *Aspergillus flavus.*

Isolates	No	Enzyme type	Lowest	Highest	Mean	P value
Otomycosis	41	Extracellular	0.001	4.034	0.891	P=0.0147
		Intracellular	0.120	4.465	1.357	
Environment	40	Extracellular	0.120	3.329	0.798	P=0.0042
		Intracellular	0.120	3.378	1.227	
Otomycosis	41	Extracellular	0.001	4.034	0.891	P=0.289
Environment	40		0.120	3.329	0.799	
Otomycosis	41	Intracellular	0.120	4.465	1.357	P=0.264
Environment	40		0.120	3.378	1.227	

Table 2. The enzymatic activity of *Aspergillus flavus* isolates.

Origin of isolate (No)	Enzymes	1 (Neg)	0.9-0.99 (Weak)	0.8- 0.89 (Mild)	0.7-0.79 (Strong)	>0.69 (Very strong)
Clinical (41)	Phospholipase	0.0%	22%	68%	8%	2%
	Hemolysin	2%	0.0%	59%	27%	12%
	Esterase	12.2%	56%	17.1%	7.3%	7.3%
Environmental (40)	Phospholipase	0.0%	27%	53%	15%	5%
	Hemolysin	28%	2%	38%	27%	5%
	Esterase	38%	45%	15%	2%	0.0%
All strains (81)	Phospholipase	0.0%	26%	59%	11%	4%
	Hemolysin	15%	1%	48%	27%	9%
	Esterase	25%	49%	17%	5%	4%

of *A. pipericola* were clustered in the *A. flavus*-clade with only one isolate of *A. tamarii* clustered separately in the *A. tamarii*-clade. Additionally, the enzymatic patterns of both clinical and environmental isolates were also illustrated in these Figs.

DISCUSSION

Aspergillus section *Flavi* is one of the most important species in the *Aspergillus* genus due to its role in biotechnology, foods, and medicine. The section *Flavi* is phylogenetically divided into eight clades and 33 species including, *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae*, *A. tamarii*, *A. pseudotamarii*, and *A. pipericola* (15). Several studies have shown that *A. flavus* is not only one of the most common saprophytic fungi in tropical and subtropical regions but also an important etiologic agent of otomycosis (8, 13, 16, 17). In the present study, identification using the calmodulin gene confirmed that *A. flavus* from section *Flavi* was the only causative agent of otomycosis identified. *Aspergillus flavus* was the main soil isolate followed by *A. tamarii* and *A. pipericola* as rare species. Generally, *A. flavus* and *A. pipericola* have been clustered into the *A. flavus* clade whereas *A. tamarii* has a separate clade (15).

Urease enzymes play a crucial role in fungal pathogens that initiate infection via the respiratory system (16). Xiong et al., indicated that *A. fumigatus* clini-

Table 3. The urease activity of *Aspergillus flavus* isolates.

Urease	1+	2+	3+	Total
Clinical	7 (17%)	23 (56%)	11 (27%)	41 (100%)
Environmental	3 (7%)	35 (88%)	2 (5%)	40 (100%)
Total	10 (12%)	58 (72%)	13 (16%)	81 (100%)

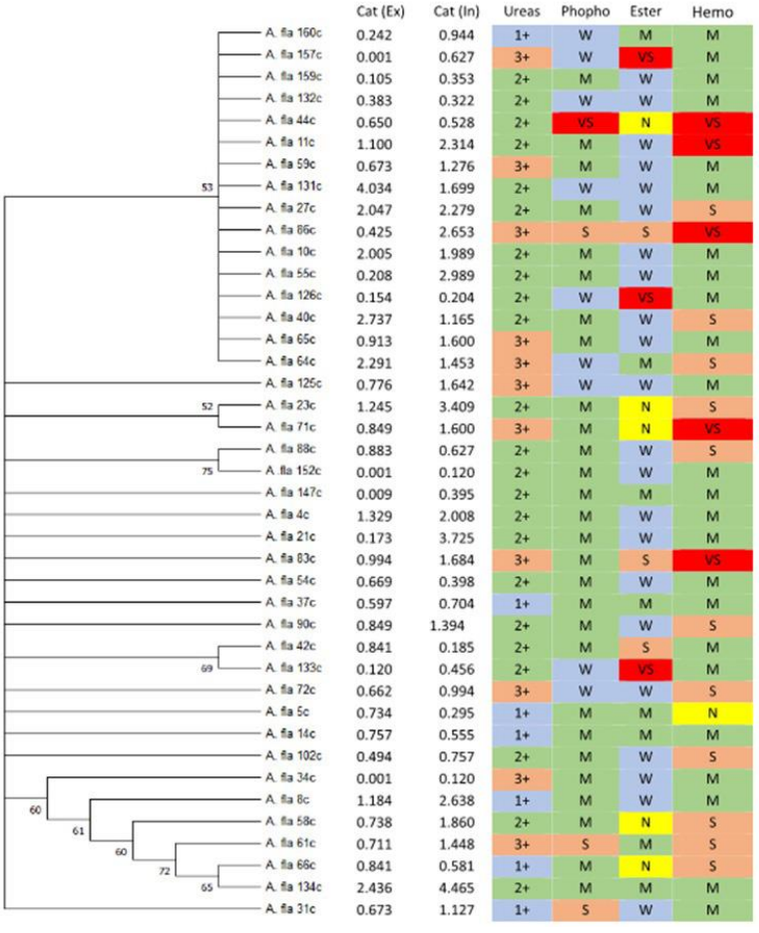

Fig. 2. Neighbor-joining phylogenetic tree based on calmodulin gene sequence data for clinical *Aspergillus flavus* isolates combined with enzyme activities. Cat (EX): Catalase (Extracellular); Cat (In): Catalase (Intracellular); Phospho: Phospholipase; Ester: Esterase; Hemo: Hemolysin; N: Negative; W: Weak; M: Mild; S: Strong; VS: Very strong

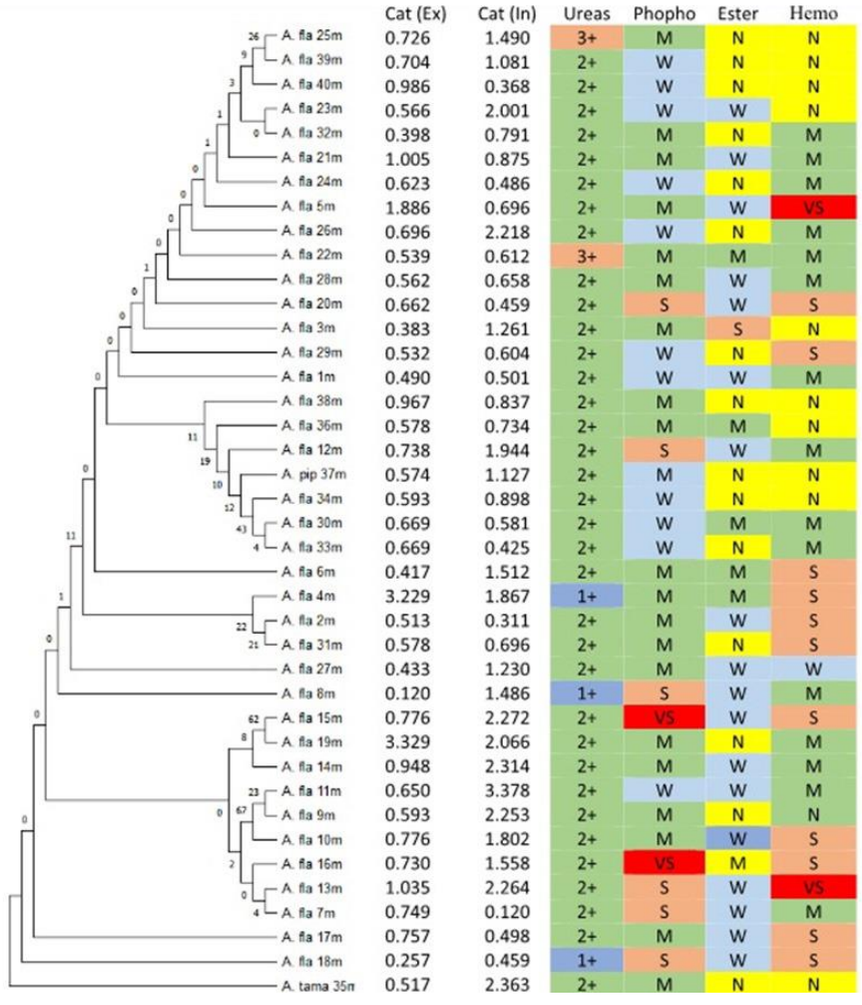

Fig. 3. Neighbor-joining phylogenetic tree based on calmodulin gene sequence data for environmental *Aspergillus flavus* isolates combined with enzyme activities. Cat (EX): Catalase (Extracellular); Cat (In): Catalase (Intracellular); Phospho: Phospholipase; Ester: Esterase; Hemo: Hemolysin; N: Negative; W: Weak; M: Mild; S: Strong; VS: Very strong

cal isolates have a high proportion of urease activity which, might contribute to the virulence of *A. fumigatus* (18). In our study, 100% of clinical and environmental isolates were positive for urease activity similar to findings of Frisvad et al. (15). In contrast to our study, only 37.6% of clinical isolates belonging to *Aspergillus* section *Flavi* produced urease enzyme in the study of Moharram et al. (13). A positive hemolytic activity was seen in 52.63% of human and avian *A. flavus* isolates studied by Ghorbel et al. (3). Raksha et al. report shows that 57.14% and 60% of clinical and environmental isolates of *A. flavus* positive for hemolysin (7). In our study, 98% of the clinical isolates and 72% of the environmental isolates had hemolytic activity. Moreover, statistical analysis has shown that clinical strains have more enzymatic activity than environmental isolates (P = 0.0001).

Phospholipase activity as an important virulence factor was described in several pathogenic fungi such as *Candida* species (19), *Cryptococcus* species (14), and *Aspergillus* species (7). In a study by Moharram et al., all Egyptian isolates of *Aspergillus* section *Flavi* originating from otomycosis showed phospholipase activity (13). Similarly, Ghorbel et al. reported phospholipase activity in all of the human and avian *A. flavus* isolates with high enzymatic activity in 35.5% of isolates (3). On the other hand, only 47.4% of *A. flavus* isolates in the Bavadharani et al. study had phospholipase activity (20). In our study, all tested isolates (clinical and environmental) were capable of producing phospholipase, however, the percentage in each grade for two sources was different.

The correlation between esterase activity and pathogenicity of *Candida* species is well described

by several researchers (19, 21, 22). Moreover, some studies indicated that *Aspergillus* species can produce this enzyme (7, 20). The production of esterase activity in clinical and environmental isolates of *Aspergillus* species was studied by Itor et al. They have reported that clinical strains had more esterase activity than environmental strains at a significant level (23). No more data about esterase activity in *A. flavus* with different sources for comparing our data. In the present study, 75% of isolates had esterase activity and we found that the difference in esterase secretion in the clinical and environmental isolates was significant (P = 0.016).

Several roles were described for catalase activity in fungal species, including association with aflatoxin B1 production, resistance to host phagocyte cells, and antifungal antibiotics (24-26). Moreover, Rouein et al. indicated catalase activity of the mycelium of *A. flavus* and *A. fumigatous* is more than that in conidia (5). Also, they found that catalase activity in the clinical isolates of *A. flavus* is higher than that in environmental isolates.

Although some data on intracellular catalase activity is available, rare information about extracellular catalase is available (26). In the present study, we evaluated the intracellular and extracellular catalase activity in the mycelium form of *Aspergillus* section *Flavi*. A significant difference was found in intracellular and extracellular catalase activities in clinical (P = 0.0147) and environmental (P = 0.0.0042) *Aspergillus* isolates. However, we cannot find a significant difference between catalase activity among clinical and environmental isolates while Gharaghani et al. reported high catalase activity among environmental *Aspergillus* species (6).

CONCLUSION

All 81 clinical and environmental isolates collected in Ahvaz were phenotypically identified as belonging to section *Flavi*, and further characterized as *A. flavus* strains (97.5%) through calmodulin gene analysis. Additionally, *A. tamari* (1.2%) and *A. pipericola* (1.2%) were identified as other strains within section *Flavi*. Our study revealed that the mean activity of intracellular catalase enzymes (1.29) in both groups was higher than the mean activity of extracellular catalase enzymes (0.85). Furthermore, the clinical isolates exhibited higher esterase (88%) and hemolysin (98%) activity compared to the environmental isolates, which showed esterase (62%) and hemolysin (72%) activity. All isolates, both clinical and environmental, displayed phospholipase and urease activity.

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