

Research Paper

In Vitro Enzymatic Virulence Factors of Dermatophytes Species Isolated From Clinical Specimens



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ABSTRACT

Background: Dermatophytes are keratinophilic fungi that affect the stratum corneum of the skin and keratinous structures. Virulent factors play a vital role in the pathogenesis and antifungal resistance of dermatophytes.

Objective: This study aims to evaluate the activity of extracellular enzymatic and biofilm formation as virulence factors of dermatophyte isolates.

Methods: Fifty-eight dermatophyte isolates belonged to 27 *Trichophyton rubrum* (46.6%), 19 *T. mentagrophytes* (32.8%), and 12 *Microsporum canis* (20.7%) for evaluating the activity of phospholipase, hemolysin, proteinase, and biofilm formation were examined. The biofilm formed was analyzed by scanning electron microscopy (SEM).

Findings: Evaluation of extracellular enzymes production revealed that 86.2%, 77.6%, and 57% of dermatophyte strains were shown to be phospholipase, hemolysin, and proteinase producers, respectively. Furthermore, all isolates of *T. rubrum* and *M. canis* can produce phospholipase and hemolysin, respectively. There was a statistically significant difference between phospholipase activity and dermatophyte strains ($P < 0.05$). In addition, biofilm formation ability was observed in 41.5% of dermatophyte isolates. The highest level of biofilm production was found in 93% of dermatophytes isolated from nail chips. A significant difference between biofilm formation with dermatophyte isolates and different body sites was observed ($P < 0.05$).

Conclusion: The activity of hydrolytic enzymes and biofilm formation as important pathogenic factors may play a role in the persistence of dermatophytosis infections. Our results showed that dermatophyte isolates have enzymatic activity and biofilm production at different levels. Therefore, understanding the function of these factors is essential to controlling the spread of dermatophytosis infection.

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1. Introduction

Dermatophytosis is one of the contagious fungal infections. This disease is the most common fungal infection in humans and animals [1] and can be transmitted through contact with hair, patient skin, animal wool, birds, and skin lesions [2]. Dermatophytes are a group of filamentous fungi which infect keratinous tissue of the human body, like skin, hair, and nails. This group of organisms is classified into three genera: Trichophyton, Microsporum, and Epidermophyton [3, 4]. *T. rubrum* is the predominant etiologic agent in dermatophytosis, followed by *T. mentagrophytes*, *E. floccosum*, *M. canis*, or *M. gypseum* as etiologic agents isolated from patients with dermatophytosis [5].

Dermatophytosis is an important health issue, and its estimated prevalence is about 20% to 25% of the world's population [6, 7]. Knowledge of the ecology and epidemiology of dermatophytes and the factors affecting their transmission are of particular importance in prevention and treatment [8]. Sometimes some dermatophytosis infections are associated with treatment failure and high rates of re-infection because the mechanism of pathogenesis and inflammatory responses are poorly understood [9].

Hydrolytic enzymes, including secretory proteinases, phospholipases, and hemolysin, are virulence factors of fungi that are important in pathogenesis [9, 10]. More than 20 proteases can be produced by dermatophytes involved in adhesion and invasion of the keratin structure [11, 12]. In addition, the enzyme phospholipase helps to invade dermatophytes by hydrolysis of phospholipids and hemolysin through the absorption of iron [13, 14]. Biofilm formation is another fungal pathogen that is one of the important causes of antifungal resistance and frequent recurrence of infections by forming a dense cell network [15, 16]. Studies on the hydrolytic enzymes and the ability to form biofilm structures of dermatophytes are fewer than studies on the ability to produce these factors in *Candida*, *Cryptococcus*, *Aspergillus*, *Malassezia*, and *Histoplasma*. Therefore, we aimed to evaluate and compare the activities of proteinase, phospholipase, hemolysin, and biofilm formation in dermatophytes isolated from hair, skin, and nail lesions.

2. Materials and Methods

Fungal strains

A total of 58 dermatophytes isolated *T. rubrum* (n=27), *T. mentagrophytes* (n=19), and *M. canis* (n=12) were examined. All isolates were collected from skin scrapings (55%), nail chips (24%), and hair (21%) of patients with dermatophytosis referred to the dermatology clinic of Razi Skin Hospital in Tehran, Iran. All isolates were inoculated onto Mycobiotic agar containing chloramphenicol (0.5 g/L) and cycloheximide (0.5 g/L) (Merck Co, Darmstadt, Germany). These cultures were sent to the Department of Mycology, Qazvin University of Medical Science. The dermatophyte isolates were identified based on macroscopic and microscopic characteristics with lactophenol catechin blue (LCB), the ability to hydrolyze urea, and hair perforation tests. All isolates were stored at -80°C until use.

Production of hydrolytic enzymes

The enzymatic activity was assessed as previously reported [17, 18]. In brief, dermatophyte isolates were cultured on potato dextrose agar (Difco) and incubated at 28°C for two weeks. Conidia suspension was prepared by covering the cultures with sterile saline, and the final concentration was adjusted to 0.5 McFarland (1×10^4 conidia per inoculum).

The proteinase activity was evaluated by adding fresh cell suspension to BSA (bovine serum albumin) medium (2% dextrose, 0.1% KH_2PO_4 , 0.05% magnesium phosphate, 2% agar, and 1% BSA). All plates were incubated at 37°C for 7 days. Phospholipase activity was assessed by adding 10 μL of conidia suspension to the surface of each SDA medium plate containing 1 M NaCl, 5 mM CaCl_2 , and sterile egg yolk emulsion incubated at 37°C for 7 days. In addition, the hemolytic activity of isolates was evaluated in an SDA medium supplemented with 5% sheep blood. Similarly, aliquots of 10 μL of suspension of dermatophytes conidia were inoculated on the culture medium of hemolysis. The cultures were incubated for 7 days at 37°C. The enzymatic activity values for each isolate were calculated by the ratio of colony diameter (a) to colony diameter plus the diameter of the clear zone (mm) around the colony (b). Enzyme activities were expressed as Pz (a/b) value. The enzyme activity assay was repeated three times for each strain [19].

Biofilm formation

Biofilm evaluation was performed based on the method described by Toukabri et al. with some modifications [15]. Briefly, the conidia suspension (1×10^6 conidia per inoculum) in RPMI-1640 medium (Sigma, St Louis, MO, USA) was prepared, then 200 μ L aliquots of suspension were transferred to 96-well polystyrene plates. After incubation at 37°C for 3 h, the wells were washed twice with sterile PBS to remove non-adherent cells. Then, 200 μ L of RPMI was added for biofilm maturation in the wells, and the plates were incubated at 37°C for 72 h. After this period, the wells were washed twice with sterile PBS and stained with crystal violet for 15 min. Finally, fixation was performed using ethanol-acetone (80:20, v/v). The absorbance values were determined using an ELISA reader at 630 nm.

Biofilm microscopic analyses

The features of the *T. rubrum* filamentous structure were analyzed by scanning electron microscopy (SEM) [20]. In brief, the biofilm was prepared as described above. Then, biofilm cells were fixed with glutaraldehyde solution and incubated at 4°C overnight. After this time, the biofilms were dehydrated with various dilutions of ethanol (20%, 40%, 60%, 80%, and 100%).

Statistical analysis

The Pz values were analyzed by the Student t test in SPSS, version 22 (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to compare the activity of hydrolytic enzymes between different isolates. P less than 0.05 were considered significant.

3. Results

The results of virulence activity showed that 86.2% of the dermatophyte isolates had phospholipase activity. Maximum phospholipase production was seen in all *T. rubrum* strains (100%) (Table 1). Among positive phospholipase isolates, 14 strains (24%) had high activity, 19 strains (32.8%) as moderate producers, and 17 isolates (29.3%) as weak producers (Figure 1a). There was a statistically significant difference between phospholipase activity and dermatophyte strains ($P < 0.05$). In contrast, no significant relationship was observed between enzyme activity and different body sites ($P > 0.05$).

The hemolytic enzyme activity was observed in 45 dermatophyte isolates (77.6%). All isolates of *M. canis* (100%) showed the ability to produce hemolysin, while 77.8% of *T. rubrum* and 63.2% of *T. mentagrophytes* could produce hemolysin (Table 1). Low hemolysin activities were detected in 25 strains (43%) tested, whereas 20 isolates (34.5%) presented moderate activity (Fig. 1b). There was no statistically significant difference between hemolysin activity with dermatophyte strains and different body sites ($P > 0.05$).

Among the dermatophyte isolates tested, proteinase activity was observed in 57% of the isolates. The results showed that the ability to produce proteinase of *T. rubrum* species (70.4%) is higher than *T. mentagrophytes* and *M. canis* (Table 1). According to the enzymatic activity classification, 9 cases (15.5%) had strong activity, 20 (34.5%) were classified as moderate producers, and 4 (7%) had low activity (Figure 1). Statistical results showed no significant difference between proteinase activity with dermatophyte strains and different body sites ($P > 0.05$).

Table 1. Virulence factors (phospholipase, hemolysin, and proteinase) for different species of dermatophyte

Phospholipase Activity (n)	Virulence Factors		
	Hemolysin	Proteinase Activity	Species
<i>T. rubrum</i> (n=27)	27(100)	21(77.8)	19(70.4)
<i>T. mentagrophytes</i> (n=19)	14(73.7)	12(63.2)	9(47.4)
<i>M. canis</i> (n=12)	9(75)	12(100)	5(41.7)
Total (n=58)	50(86.2)	45(77.6)	33(57)
P	0.000*	0.058	0.147*

* Significant.

Table 2. Biofilm formation of dermatophyte species isolated from clinical samples

Variables	Species			Anatomical Site		
	<i>T. rubrum</i> (n=27)	<i>T. mentagrophytes</i> (n=19)	<i>M. canis</i> (n=12)	Skin (n=32)	Nail (n=14)	Hair (n=12)
Biofilm formation	15 (55.6%)	9 (47.4%)	-	11 (34.4%)	13 (93%)	-
P	0.000*				0.000*	-

* Significant.

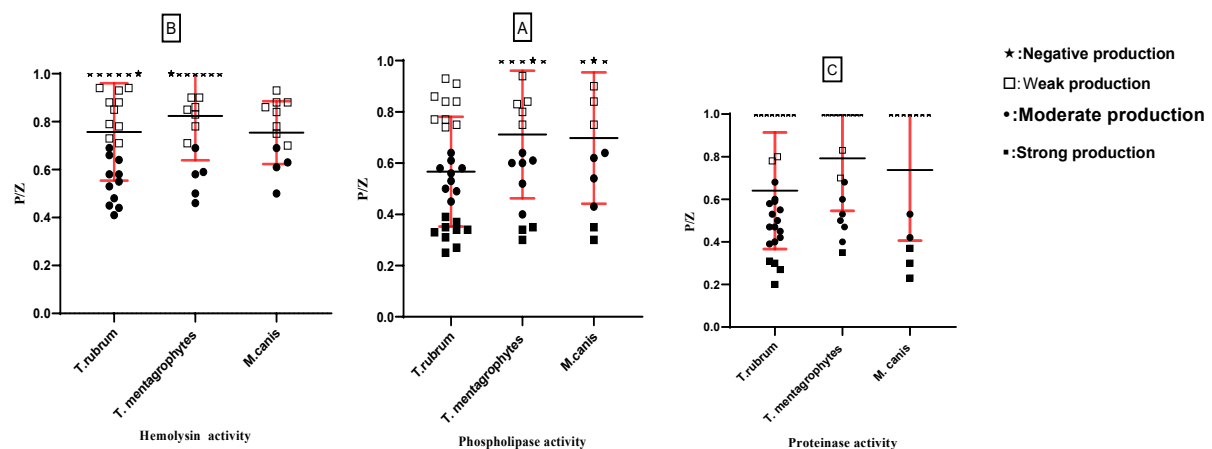
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The rate of biofilm production was detected in 41.5% of dermatophyte isolates (Table 2). Among positive biofilm isolates, 5 strains (8.6%) of dermatophytes were classified as strong biofilm producers, 12 strains (20.8%) as intermediate producers, and 7 isolates (12%) as weak biofilm producers. Biofilm production was not noted in *M. canis* isolates, while the maximum biofilm production capacity was reported in 55.6% of *T. rubrum*. A significant difference between biofilm formation with dermatophyte isolates was observed ($P < 0.05$). On the other hand, biofilm production at the highest level was found in 93% of dermatophytes isolated from nail chips (Table 2). There was a statistically significant difference between biofilm production and different body sites ($P < 0.05$). The result of SEM of the network of dense hyphal by *T. rubrum* isolated from a nail chip is shown in Figure 2.

4. Discussion

The pathogenesis of dermatophytosis infections is due to their virulence factors and host resistance [21]. Despite studies on the keratinolytic activity of derma-

tophytes, the association between other enzymes produced by the fungus and the clinical manifestations of dermatophytosis is not well understood [22, 23]. According to our results, 86.2% of dermatophyte isolates could produce phospholipase, of which 100% of *T. rubrum* strains revealed higher phospholipase activity than *T. mentagrophytes* and *M. canis*. Phospholipases, as one of the virulence markers, facilitate the colonization of host cells by attaching the fungus to the target tissue and destroying the cell membrane following the hydrolysis of phospholipids [24]. These results are consistent with previous results and confirm the importance of this enzyme in the first stage of dermatophytosis infection and fungal colonization. Gnat et al. reported that 96% of dermatophyte isolates from clinical specimens were phospholipase producers [25]. Muhsin et al. showed that all dermatophyte species produced phospholipase [17]. Iron uptake is an essential requirement for the invasion of fungi, which is caused by the enzyme hemolysin with a cytotoxic effect on erythrocytes cells. In our study, *M. canis* isolates rendered the highest hemolysin activities, while *T. mentagrophytes* isolates showed the lowest activity of this enzyme. The enzyme hemolysin

**Figure 1.** Median Pz values of phospholipase

(A), hemolysin (B), and proteinase (C) activities in dermatophyte isolates recovered from skin, nail, and hair samples.

Three replicates were used for each isolate, with the mean of each represented.

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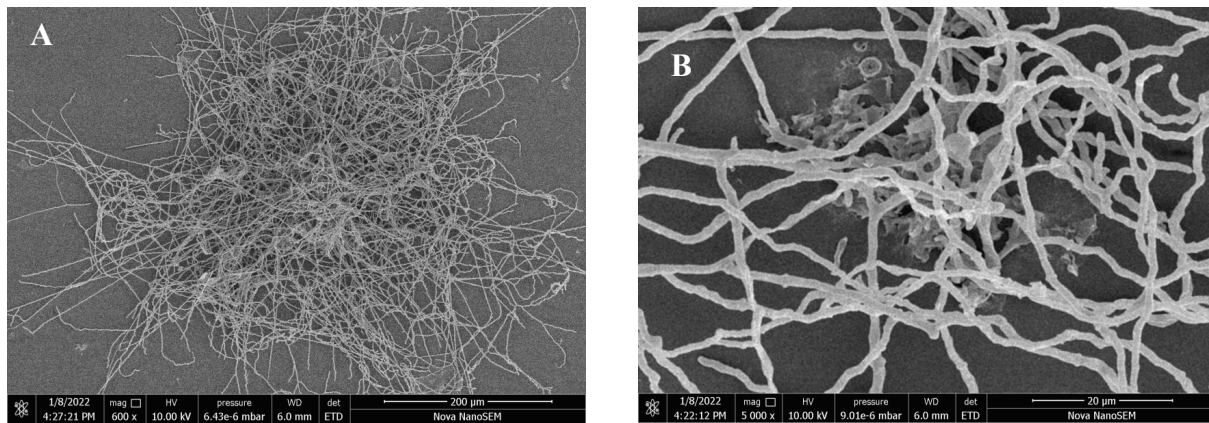


Figure 2. Demonstration of dense biofilm networks of *trichophyton rubrum* with scanning electron micrographs

600× magnification in A and 5000× magnification in B (FEI scanning electron microscope with 200 µm and 20 µm scale, respectively).

is produced for dermatophyte survival strategies during infection [13]. These enzymes may play an important role in balancing host immunity and the ability of dermatophytes to reduce the immune response. Similarly, Aneke et al. reported that 100% of *M. canis* isolated from human clinical samples produced hemolysin [26]. Ramos et al. showed that all strains of *M. canis* could produce hemolysin. In this study, low and moderate levels of enzyme production were observed in 84.6% and 15.4% of the strains, respectively [19]. In our study, most dermatophyte isolates (43%) presented low hemolysin production, and none of the strains showed high activity. The low level of hemolysin production may be due to the negligible contact of fungal elements with the host's blood during infection [19].

Protease enzyme with proteolytic activity and protein breakdown plays a major role in the penetration of dermatophytes during infection [27]. Studies have shown that the secretion of proteases by dermatophytes is essential for their pathogenicity [11]. Our study showed that protease activity in *T. rubrum* (70.4%) is higher than in *T. mentagrophytes* (47.5%) and *M. canis* (41.7%). The study by Gnat et al. showed that 100% of *T. mentagrophytes* produce protease. However, 80% and 67% of *T. rubrum* and *M. canis*, respectively, produce protease [25]. Ramos et al. reported that 100% of *M. canis* isolates were capable of aspartic-protease activity at different levels [19]. These different results may be due to the type of host (human or animal) and the anatomical region. Biofilms are usually involved in the resistance of fungal agents to antifungals [28, 29]. Studies show that the ability to form biofilms may be related to the unresponsiveness of dermatophytes to antifungals [30, 31]. In the present study, biofilm production was evaluated by dermatophytes, and it was shown that the highest

biofilm formation ability is related to *T. rubrum* (55.6%). The first study in Brazil, evaluating the ability to produce biofilms by *T. rubrum* and *T. mentagrophytes* isolates *in vitro*, showed that both species could form biofilm [16]. In addition, the highest level of biofilm production was found in 93% of dermatophytes isolated from nail chips. Studies show that the formation of fungal biofilms in nails contributes to chronic onychomycosis [32]. Burkhart et al. showed that biofilm cells attached to the nail plate provided the basis for the growth, survival, and chronicity of the infection [31]. Gupta et al. reported that the main cause of therapeutic resistance in onychomycosis is biofilm formation, and treatment should include biofilm destruction [33].

5. Conclusion

Generally, dermatophytosis infections are cutaneous, and the fungus inability to penetrate deep tissues. On the other hand, host immunological reactions lead to the production of mild to intense metabolic products. In addition, extracellular enzymes and biofilm formation as virulence factors are important in fungal pathogenesis. Therefore, it seems that standard methods for determining virulence factors are necessary. Also, the relationship between virulence factors and resistance to antifungals should be investigated.

Ethical Considerations

Compliance with ethical guidelines

The study was approved by the Ethics Committee of [Qazvin University of Medical Science](#) (Code: IR.QUMS.REC.1398.179).

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Authors' contributions

Responsible for design and Supervision: Faezeh Mohammadi; Collaborate on practical work: Amirhossein Gholamlou and Hadi Aliyari; Collaborate on sampling patients referred and participate in collecting samples: Zeinab Ghasemi; Data analysis: Monirsadat Mirzadeh; Contributed to the writing of the article: All authors

Conflict of interest

The authors declared no conflict of interest.

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References

- [1] AL-Janabi AA. Dermatophytosis: Causes, clinical features, signs and treatment. *J Symptoms Signs*. 2014; 3(3):200-3. [Link]
- [2] Hainer BL. Dermatophyte infections. *Am Fam Physician*. 2003; 67(1):101-9. [Link]
- [3] Moriello KA. Dermatophytosis. In: Noli C, Colombo S, editors. *Feline dermatology*. Cham: Springer; 2020. [DOI:10.1007/978-3-030-29836-4_13]
- [4] Mercer DK, Stewart CS. Keratin hydrolysis by dermatophytes. *Med Mycol*. 2019; 57(1):13-22. [PMID]
- [5] Kanbe T, Suzuki Y, Kamiya A, Mochizuki T, Kawasaki M, Fujihira M, et al. Species-identification of dermatophytes *Trichophyton*, *Microsporum* and *Epidermophyton* by PCR and PCR-RFLP targeting of the DNA topoisomerase II genes. *J Dermatol Sci*. 2003; 33(1):41-54. [DOI:10.1016/S0923-1811(03)00150-6]
- [6] Havlickova B, Czaika VA, Friedrich M. Epidemiological trends in skin mycoses worldwide. *Mycoses*. 2008; 51(Suppl 4):2-15. [PMID]
- [7] Lopes G, Pinto E, Salgueiro L. Natural products: An alternative to conventional therapy for dermatophytosis? *Mycopathologia*. 2017; 182(1-2):143-67. [PMID]
- [8] Aly R. Ecology and epidemiology of dermatophyte infections. *J Am Acad Dermatol*. 1994; 31(3 Pt 2):S21-5. [PMID]
- [9] Vermout S, Tabart J, Baldo A, Mathy A, Losson B, Mignon B. Pathogenesis of dermatophytosis. *Mycopathologia*. 2008; 166(5-6):267-75. [PMID]
- [10] Al-Khikani FH. Dermatophytosis and the role of enzymes in pathogenesis. *Matrix Sci Med*. 2021; 5(1):28. [DOI:10.4103/MTSM.MTSM_23_20]
- [11] Monod M. Secreted proteases from dermatophytes. *Mycopathologia*. 2008; 166(5-6):285-94. [PMID]
- [12] Achterman RR, White TC. Dermatophyte virulence factors: Identifying and analyzing genes that may contribute to chronic or acute skin infections. *Int J Microbiol*. 2012; 2012:358305. [PMID]
- [13] Aktas E, Yigit N. Hemolytic activity of dermatophytes species isolated from clinical specimens. *J Mycol Med*. 2015; 25(1):e25-30. [PMID]
- [14] Chinnapun D. Virulence factors involved in pathogenicity of dermatophytes. *Walailak J Sci Technol*. 2015; 12(7):573-80. [Link]
- [15] Toukabri N, Corpologno S, Bougnoux ME, El Euch D, Sadfi-Zouaoui N, Simonetti G. *In vitro* biofilms and antifungal susceptibility of dermatophyte and non-dermatophyte moulds involved in foot mycosis. *Mycoses*. 2018; 61(2):79-87. [PMID]
- [16] Costa-Orlandi CB, Sardi JC, Santos CT, Fusco-Almeida AM, Mendes-Giannini MJ. *In vitro* characterization of *Trichophyton rubrum* and *T. Mentagrophytes* biofilms. *Biofouling*. 2014; 30(6):719-27. [PMID]
- [17] Muhsin TM, Aubaid AH, al-Duboon AH. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. *Mycoses*. 1997; 40(11-12):465-9. [PMID]
- [18] Shanmuga PC, Prabha R, Rajesh M. Phenotypic Characterization of enzymatic and non-enzymatic virulence factors in etiopathogenesis and evolution of drug resistance among dermatophytes. *J Pure Appl Microbiol*. 2020; 14(4):2661-7. [DOI:10.22207/JPAM.14.4.43]
- [19] Ramos MLM, Coelho RA, Brito-Santos F, Guimarães D, Premazzi M, Zancopé-Oliveira RM, et al. Comparative analysis of putative virulence-associated factors of *Microsporum canis* isolates from human and animal patients. *Mycopathologia*. 2020; 185(4):665-73. [PMID]
- [20] Mohammadi F, Hemmat N, Bajalan Z, Javadi A. Analysis of biofilm-related genes and antifungal susceptibility pattern of vaginal *Candida albicans* and non-*Candida albicans* species. *Biomed Res Int*. 2021; 2021:5598907. [PMID]
- [21] Martinez-Rossi NM, Peres NT, Rossi A. Pathogenesis of dermatophytosis: Sensing the host tissue. *Mycopathologia*. 2017; 182(1-2):215-27. [PMID]
- [22] Elavarashi E, Kindo AJ, Rangarajan S. Enzymatic and non-enzymatic virulence activities of dermatophytes on solid media. *J Clin Diagn Res*. 2017; 11(2):DC23-5. [PMID]
- [23] Viani FC, Dos Santos JL, Paula CR, Larson CE, Gambale W. Production of extracellular enzymes by *Microsporum canis* and their role in its virulence. *Med Mycol*. 2001; 39(5):463-8. [PMID]
- [24] Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev*. 2000; 13(1):122-43. [PMID]

- [25] Gnat S, Łagowski D, Nowakiewicz A, Zięba P. Phenotypic characterization of enzymatic activity of clinical dermatophyte isolates from animals with and without skin lesions and humans. *J Appl Microbiol.* 2018; 125(3):700-9. [PMID]
- [26] Aneke CI, Rhimi W, Hubka V, Otranto D, Cafarchia C. Virulence and antifungal susceptibility of *Microsporum canis* strains from animals and humans. *Antibiotics.* 2021; 10(3):296. [PMID]
- [27] Mandujano-González V, Villa-Tanaca L, Anducho-Reyes MA, Mercado-Flores Y. Secreted fungal aspartic proteases: A review. *Rev Iberoam Micol.* 2016; 33(2):76-82. [PMID]
- [28] Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. *Int J Microbiol.* 2012; 2012:528521. [PMID] [PMCID]
- [29] Berman J, Krysan DJ. Drug resistance and tolerance in fungi. *Nat Rev Microbiol.* 2020; 18(6):319-31. [PMID]
- [30] Hryniewicz-Gwóźdź A, Kalinowska K, Plomer-Niezgoda E, Bielecki J, Jagielski T. Increase in resistance to fluconazole and itraconazole in *Trichophyton rubrum* clinical isolates by sequential passages *In vitro* under drug pressure. *Mycopathologia.* 2013; 176(1-2):49-55. [PMID]
- [31] Burkharta CN, Burkhart CG, Gupta AK. Dermatophytoma: Recalcitrance to treatment because of existence of fungal biofilm. *J Am Acad Dermatol.* 2002; 47(4):629-31. [PMID]
- [32] Gupta AK, Foley KA. Evidence for biofilms in onychomycosis. *G Ital Dermatol Venereol.* 2019; 154(1):50-5. [PMID]
- [33] Gupta AK, Daigle D, Carviel JL. The role of biofilms in onychomycosis. *J Am Acad Dermatol.* 2016; 74(6):1241-6. [PMID]

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