Original Article

Bacterial and fungal keratitis in Upper Egypt: *In vitro* screening of enzymes, toxins and antifungal activity

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Purpose: This work was conducted to study the ability of bacterial and fungal isolates from keratitis cases in Upper Egypt to produce enzymes, toxins, and to test the isolated fungal species sensitivity to some therapeutic agents. **Materials and Methods:** One hundred and fifteen patients clinically diagnosed to have microbial keratitis were investigated. From these cases, 37 bacterial isolates and 25 fungal isolates were screened for their ability to produce extra-cellular enzymes in solid media. In addition, the ability of fungal isolates to produce mycotoxins and their sensitivity to 4 antifungal agents were tested. **Results:** Protease, lipase, hemolysins, urease, phosphatase, and catalase were detected respectively in 48.65%, 37.84%, 59.46%, 43.24%, 67.57%, and 100% out of 37 bacterial isolates tested. Out of 25 fungal isolates tested during the present study, 80% were positive for protease, 84% for lipase and urease, 28% for blood hemolysis, and 100% for phosphatase and catalase enzymes. Thirteen fungal isolates were able to produce detectable amounts of 7 mycotoxins in culture medium (aflatoxins (B1, B2, G1, and G2), sterigmatocystin, fumagillin, diacetoxyscirpenol, zearalenone, T-2 toxin, and trichodermin). Among the antifungal agents tested in this study, terbinafine showed the highest effect against most isolates *in vitro*. **Conclusion:** In conclusion, the ability of bacterial and fungal isolates to produce extracellular enzymes and toxins may be aid in the invasion and destruction of eye tissues, which, in turn, lead to vision loss.

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Infectious keratitis (Corneal ulcer) is a common disease of the cornea, resulting in significant vision loss. It is caused by various organisms, such as bacteria, fungi, virus, or protozoa. The severity of corneal infections usually depends on the underlying condition of the cornea and the virulence of the infecting microbes.[1] In the developing world, corneal opacity accounts for approximately 15% of treatable blindness, second only to cataract.[2] A significant proportion of these opacities is secondary to infectious keratitis.[3] Unfortunately, in the developing world, treatment of these visually disabling infections is often delayed for several weeks or more, and patients commonly present with very advanced keratitis. This type of ulceration is commonly associated with ocular trauma, mainly plant trauma, followed by post-operative infections.[3,4]

Ocular infections probably occur due to an interaction between various agents (bacteria and/or fungi), host (tissue and immunological mechanisms), and other factors.[4] For example, protease enzymes have been identified as a major pathogenic factor in *Serratia* keratitis.[5] Invasiveness of a fungal strain is aided by certain properties such as the capacity to adhere to the cells, to produce enzymes that destroy anatomical defenses and anti-microbial proteins, to survive and evade host defense mechanisms.[6] The secretion of enzymes such as phospholipase, protease, pseudocollagenase, and exotoxins causes coagulative necrosis with the loss of keratocytes and disruption of collagen lamellae.[7] Treatment of ocular fungal infections remains problematic, in part because of the expanding list of fungal pathogens and in other part because of the relatively short list of available therapeutic agents.[8] In treating ophthalmic mycoses, the ultimate aim is to preserve vision, and this depends on rapid diagnosis and efficient administration of appropriate antifungal therapy.[9] Due to the lack of information about keratitis in Upper Egypt and how the infectious agents can invade the eye, this work was designed to investigate the ability of bacterial and fungal species associated with keratitis to produce enzymes, toxins, and to test the activity of some antifungal agents.

Materials and Methods

A total of 115 patients clinically diagnosed to have microbial keratitis (microbial corneal ulcers) were investigated. All patients were admitted and treated in the department of ophthalmology of Assiut University Hospital, and their informed consent for corneal scrapings was obtained. Patients were from different areas of Upper Egypt. The patients were thoroughly examined using slit-lamp biomicroscope by an ophthalmologist. From each keatitis case, corneal scrapings were sent for direct microscopic examination and cultures. Bacterial and fungal isolates were identified by standard microbiological methods.[10,11]

Thirty-seven bacterial isolates and 25 fungal isolates obtained were screened for their ability to produce extracellular enzymes in solid media. Two-day old bacterial cultures grown on nutrient agar plates while seven-day old cultures of fungi grown on Sabouraud dextrose agar plates were used for inoculation. All of these isolates were assigned numbers. The ability of these organisms to produce enzymes was measured by the clear zone around the colony/mm (protease), the depth of visible precipitate/mm (lipase), the color intensity (urease), the color zone around the colony/mm (phosphatase), and O^2 bubbles evolution (catalase).[12] The following substrates were used:

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Casein for protease, Tween 80 for lipase, 2% urea solution for urease, diphosphophenolphthalein for phosphatase, and 10% hydrogen peroxide solution for catalase. For blood hemolysis, the appearance of a hemolytic zone around the colony was considered as a positive result. The tested fungi were incubated 7 days at 37°C. Hemolysis was recorded as α-hemolysis (partial blood hemolysis), β-hemolysis (complete blood hemolysis), and γ-hemolysis (no hemolysis).[13] Three replicates were used for each isolates in each enzyme test. Enzyme index was calculated according to method described by Ismail,[14] when possible.

An inoculum of each of 25 fungal isolates was transferred to 250 ml flasks, containing 50 ml potato dextrose broth. Three replicates were used, and the flasks were incubated at 28°C for 10 days as static culture. After the incubation period, the content of each flask (medium + mycelium) was transferred into a blender jar and homogenized for 2 minutes at low speed and 5 minutes in a high-speed blender (16000 rpm) with 50 ml chloroform. The extraction procedure was repeated again with the same volume of chloroform. The combined chloroform extracts were washed with equal volume of distilled water, filtered, dried over anhydrous sodium sulfate, and then evaporated to near dryness by flash evaporator.[15]

The dry crude extract was suspended in 50 ml chloroform and applied to a silica gel column (200 mesh, Merck) according to the method of AOAC.[16] The column was washed with 150 ml n-hexane followed by diethyl ether, and toxins were eluted with 200 ml of chloroform-acetone (9:1 v/v) solvents system. The elute was collected and evaporated to near dryness on a steam bath. The residue was diluted with chloroform to 1 ml.

The thin-layer chromatographic (TLC) technique was applied for semi-quantitative analysis of mycotoxins.[16] A Silica gel plate type 60 F_{254} of about 0.3 mm thickness was used. The samples to be analyzed were applied as 50 µl solution in chloroform. The spots were dried during application with a flow of cold air. The plates were developed in developing tanks of 15 × 30 × 30 x cm diameter (Zeiss, Jena, Germany) saturated with solvent vapor. Each sample was chromatographed in two series in selective solvent system. When the front of the system reaches a height of about 15 cm above the origin, the development was interrupted; the chromatogram was dried in air and then examined for the presence of mycotoxins. The developed plates were detected before and after spraying with the different reagents using short wave (254 nm) and long wave UV light (356 nm).

The disc susceptibility test[17] was employed using filter paper discs fully saturated with the antifungal agent (~10 µl). The antifungal activity of 4 types of anti-fungal therapeutic agents (amphotericin B (50 mg), cetrimide (100 mg), ketoconazole (200 mg), and terbinafine (250 mg)) against 25 fungal isolates of keratitis origin were tested using 3 concentrations (0.1%, 0.5%, and 1%), which are similar or nearby the used concentrations in treatment. Additional concentration of amphotericin B (0.005) was used because it is the applicable concentration that is used for severe eye infection. Discs were placed on the surface of Sabouraud dextrose agar seeded with the test organism. Cultures were incubated at 28°C for 48 hours, after which the zone of inhibition of fungal growth around discs was measured in mm, and the data were recorded as the mean of 3 replicates. Dimethyl sulfoxide (DMSO) was used to dissolve the antifungal agents and also as a control in this test because it showed no effect on the organisms tested.

Results

The ability of 37 bacterial isolates to produce some extracellular enzymes and blood hemolysis were shown in Table 1 and Fig. 1.

Protease was detected only in 18 out of 37 isolates tested. Two of these had enzyme index (PI) exceeding 2.4, and these were *Micrococcus luteus* and *Staphylococcus* sp. The other bacterial isolates had enzyme index ranging from 1.32 to 1.89. For lipase, only 14 showed positive capabilities of producing lipase, of which 7 isolates had at least a mean depth of precipitate of 6 mm. When testing the 37 bacterial isolates for hemolysis on human blood agar, 22 showed complete blood hemolysis (β- hemolysis). Regarding urease enzyme, out of 16 positive isolates, 2 were strong, 6 were moderate, and 8 were weak enzyme producers. For phosphatase enzyme, 25 isolates showed positive capabilities of producing phosphatase enzyme. Of these, 10 were strong, 5 were moderate, and 9 were weak producers. All 37 isolates tested could produce catalase enzyme. Of these, 30 were strong and 7 were moderate producers.

The ability of 25 fungal isolates from keratitis cases to produce some extracellular enzymes and blood hemolysis were shown in Table 2 and Fig. 2.

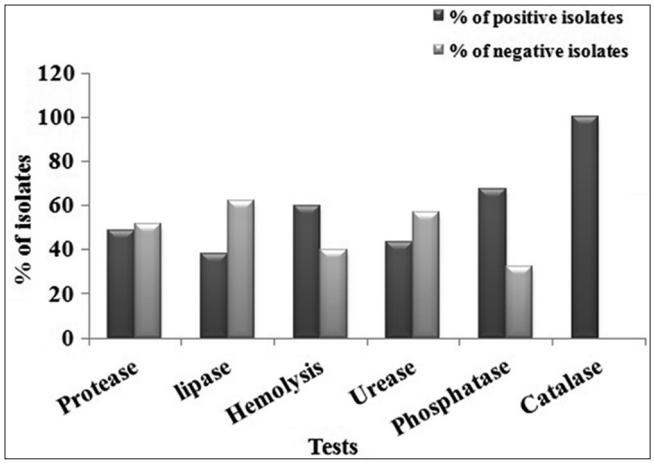

Figure 1: Extracellular enzymes and blood hemolysis produced by 37 bacterial isolates of keratitis origin

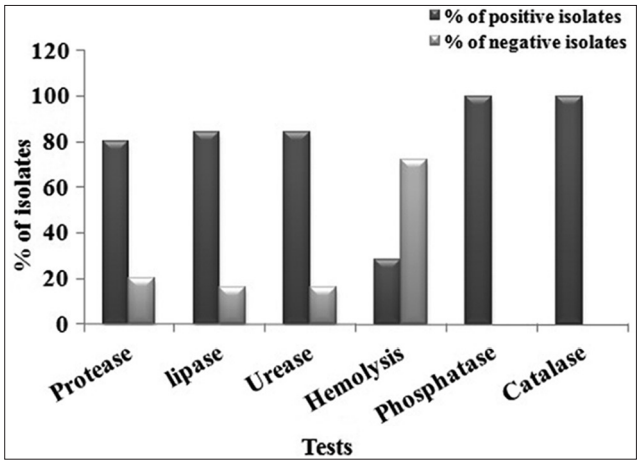

Figure 2: Extracellular enzymes and blood hemolysis produced by 25 fungal isolates of keratitis origin

Table 1: Extracellular enzymes and blood hemolysis produced by 37 bacterial isolates of keratitis origin*

Bacteria tested	BI No.	Protease			Lipase	Hemolysis	Urease	Phosphatase	Catalase
		MCD±SD	MDCZ±SD	PI	MDP± SD				
Escherichia coli	B3	NR	-	-	-	γ	-	w	m
Klebsiella pneumoniae	B7	6±0	9.67±1.37	1.61	5.33±0.85	γ	w	s	s
K.pneumoniae	B8	NR	-	-	-	γ	s	m	s
M. luteus	B10	NR	-	-	7±0	γ	-	w	m
M. luteus	B11	7±1	22.67±1.86	3.24	-	β	m	m	s
Pseudomonas aeruginosa	B12	13.67±3.9	25.67±2.34	1.88	12.33±0.85	β	s	s	s
Staphylococcus aureus	B13	8±1	14.67±1.75	1.83	4.66±0.85	β	-	-	s
S. aureus	B14	5±1	8.83±1.72	1.77	-	β	-	-	s
S. aureus	B15	4.33±0.85	6.5±0.55	1.5	4.33±0.85	β	m	s	s
S. aureus	B16	4.67±0.85	8.83±1.6	1.89	-	β	w	-	s
S. aureus	B17	5.67±085	9.5±1.22	1.68	5±1	β	-	w	m
S. aureus	B18	4.67±1.5	6.67±0.81	1.43	6.33±1.15	β	w	m	s
S. aureus	B19	6±1	9.67±0.51	1.61	-	β	-	m	s
S. aureus	B20	5.67±1.5	8.67±1.37	1.53	5.33±0.85	β	w	-	s
S. aureus	B21	5.67±2.1	8.67±1.5	1.53	6.33±0.85	β	m	m	s
S. aureus	B22	5.67±1.53	10.17±0.75	1.79	-	β	m	-	m
S. aureus	B23	NR	-	-	6±1	β	m	-	s
S. aureus	B24	NR	-	-	-	β	-	w	s
S. aureus	B25	NR	-	-	-	β	-	w	s
S. aureus	B26	NR	-	-	-	β	-	-	s
S. aureus	B27	NR	-	-	4.33±0.85	β	m	s	s
S. aureus	B28	NR	-	-	-	β	-	-	m
S. aureus	B29	NR	-	-	-	β	-	s	s
S. aureus	B30	NR	-	-	-	β	-	s	s
S. aureus	B31	NR	-	-	-	β	-	-	s
S. aureus	B32	NR	-	-	-	β	-	-	s
Staphylococcus sp.	B33	5.33±0.85	8.5±1.04	1.59	-	γ	w	s	s
Staphylococcus sp.	B34	5.33±0.85	7.5±1.38	1.41	4.66±0.85	γ	w	-	s
Staphylococcus sp.	B35	6.33±1.2	8.33±1.03	1.32	-	γ	w	w	s
Staphylococcus sp.	B36	5.67±0.85	14.33±1.21	2.53	13.33±1.15	γ	-	w	m
Staphylococcus sp.	B37	6.33±1.2	8.67±1.21	1.37	6±0	γ	-	w	m
Staphylococcus sp.	B38	NR	-	-	-	γ	w	s	s
Staphylococcus sp.	B39	NR	-	-	-	γ	-	w	s
Staphylococcus sp.	B40	NR	-	-	-	γ	-	w	s
Staphylococcus sp.	B42	NR	-	-	-	γ	-	s	s
Staphylococcus sp.	B43	NR	-	-	-	γ	-	s	s
Staphylococcus sp.	B44	NR	-	-	-	γ	-	-	s

*BI No.: Bacterial isolate number, MCD: Mean colony diameter (mm), SD: Standard deviation MDCZ: Mean diameter of clear zone (mm), PI: Protease index, NR: Not recorded, MDP: Mean depth of precipitate (mm), –: Means negative result, β: Complete hemolysis, α: Partial hemolysis, γ: No hemolysis, s: Strong enzyme producer, m: Moderate enzyme producer, w: Weak enzyme producer, MCD of isolates negative for protease was not recorded (NR)

Protease was detected in 20 out of 25 isolates tested. Three of these had enzyme index (PI) more than 1.75, and these were *Cladosporium cladosporioides* (Fungal isolate (FI) No. 3955) and *Candida* sp. (FI No. 3958, 3959). The other fungal isolates had enzyme index ranging from 1.01 to 1. 14. For lipase, 21 out of 25 isolates showed positive capabilities of producing the enzyme, of which 6 isolates had a mean depth of precipitate ≥20 mm, and these were *Aspergillus flavus* (3 isolates), *Cochliobolus spicifer*, *Fusarium solani,* and *F. verticillioides* (1 isolate each). The other fungal isolates had a mean depth of precipitate less than 20 mm. Regarding urease enzyme, out of 21 positive isolates, 8 were strong producer and had enzyme index (UI) of more than 2. The others fungal isolates had enzyme index of less than 2. When testing hemolysis of human blood on agar

Table 2: Extracellular enzymes and blood hemolysis produced by 25 fungal isolates of kiratitis origin*

Fungi tested	FI No.	Protease			Lipase	Urease			Hemolysis			Phosphatase	Catalase
		MCD±SD	MDCZ±SD	PI	MDP±SD	MCD±SD	MDCZ±SD	UI	MCD±SD	MDBH±SD	BHI		
Aspergillus flavus	3939	33.66±1.03	38.5±0.84	1.14	19.66±1.15	34.5±1.2	29.5±2.1	0.86	50.83±0.98	64.8±0.75	1.27	s	m
A. flavus	3940	34.16±0.75	37.16±2.71	1.09	20±0	41.3±1.03	31.8±1.5	0.77	42.3±1.5	56.3±2.3	1.33	s	m
A. flavus	3941	37.16±6.76	41±4.82	1.01	21±0	35.8±0.75	28.2±0.4	0.79	42.8±2.13	54.5±3.02	1.27	s	s
A. flavus	3942	34.33±0.52	37.16±1.47	1.08	17.33±0.58	32±1.4	26.7±1.2	0.83	44.8±4.5	60.5±1.2	1.35	s	s
A. flavus	3943	NR	-	-	20.66±1.15	38.8±3.3	35.7±3.01	0.92	54.5±4.2	68±2.1	1.25	w	m
A. flavus	3944	35.33±0.82	38±1.41	1.08	19.66±1.53	39.5±0.83	31.2±0.4	0.79	NR	γ	-	s	w
A. flavus	3945	41.16±1.17	42.83±0.75	1.04	19.66±0.58	36.8±1.5	31.7±1.6	0.86	NR	γ	-	s	s
A. flavus	3946	35±0.63	37.66±0.82	1.08	-	35.8±1.5	30.5±0.8	0.85	NR	γ	-	s	s
A. fumigatus	3947	34.33±2.88	37±2.37	1.08	19.33±0.58	22.5±2.7	68.3±2.6	3.04	NR	γ	-	s	m
A. niger	3950	45.66±2.5	49.33±1.51	1.08	4.66±0.58	29±1.3	20±0.63	0.69	NR	γ	-	s	m
A. terreus	3951	25.16±0.75	27.66±0.52	1.09	17.33±1.53	13.8±1.8	26.5±2.2	1.92	NR	γ	-	s	s
A. terreus	3886	28.66±2.07	34.33±3.78	1.19	18±1	17±4.8	28.3±3.5	1.66	NR	γ	-	w	s
A. terreus	3952	28.5±2.26	31.16±3.6	1.09	16.66±1.15	20±0	50±0	2.5	NR	γ	-	s	s
Cladosporium cladosporioides	3955	16.5±1.04	29.33±1.86	1.78	19.66±0.58	6.5±0.54	90±0	13.9	NR	γ	-	s	m
C. cladosporioides	3956	NR	-	-	8.33±0.58	3.3±0.52	19±0.9	5.76	NR	γ	-	s	s
Cochliobolus spicifer	3957	39.83±2.56	43.5±1.52	1.09	20.33±0.58	24.8±9.8	55.3±1.03	2.23	59.5±1.05	65±1.1	1.09	s	w
Candida sp.	3958	6.33±1.03	12.83±1.94	2.03	-	NR	-	-	NR	γ	-	w	s
Candida sp.	3959	6.16±0.75	13.16±1.17	2.14	11±1	NR	-	-	NR	γ	-	w	s
Fusarium oxysporum	3961	33±0.89	35.16±0.98	1.07	19.33±0.58	28.3±1.6	90±0	3.18	NR	γ	-	w	s
F. solani	3962	35.83±3.97	37.33±3.78	1.04	22.66±0.58	30.5±0.54	90±0	2.95	NR	γ	-	s	m
F. verticillioides	3963	32.66±0.82	35.16±0.98	1.08	20.66±0.58	18.7±1.4	8.6±1.4	0.46	41.3±1.5	48.8±3.1	1.18	s	w
Penicillium chrysogenum	3965	NR	-	-	-	NR	-	-	NR	γ	-	s	s
Stemphylium botryosum	3966	NR	-	-	-	NR	-	-	NR	γ	-	w	s
Sterile mycelia	3954	30.66±2.16	33.33±2.58	1.09	15.66±0.58	16±1.3	44.3±1.2	2.77	NR	γ	-	w	m
Trichderma hamatum	3967	NR	-	-	10±0	72±4.7	90±0	1.25	NR	γ	-	w	s

* FI No. Fungal isolate number, MCD: Mean colony diameter (mm), SD: Standard deviation, MDCZ: Mean diameter of clear zone/mm (protease), PI: Protease index, NR: Not recorded, MDP: Mean depth of precipitate (mm), MDCZ: Mean diameter of colour zone/mm (urease), UI: Urease index, MDBH: Mean diameter of blood hemolysis, BHI: Blood hemolysis index, -: Means negative result, γ: No hemolysis, s: Strong enzyme producer, m: Moderate enzyme producer, w: Weak enzyme producer, MCD of isolates negative for protease, Urease and blood haemolysis was not recorded (NR)

medium, only 7 out of 25 isolates were positive and showed complete hemolysis (β-hemolysis). These were related to *A. flavus* (5 isolates), *C. spicifer*, and *F. verticillioides* (1 isolates each). With respect to phosphatase enzyme, the 25 fungal isolates tested were positive. Of these, 17 isolates were strong producers and 8 were weak. For catalase, the 25 fungal isolates tested could produce catalase, of which 14 were strong, 8 were moderate, and 3 were weak producers.

Of 25 fungal isolates tested for toxin production, only 13 were able to produce detectable amounts of toxins in culture medium [Table 3].

Seven mycotoxin types were detected, and these were aflatoxins (B1, B2, G1, and G2), sterigmatocystin, fumagillin, diacetoxyscirpenol, zearalenone, T-2 toxin, and trichodermin. Aflatoxins (B1, B2, G1, and/or G2) were produced by 5 isolates of *Aspergillus flavus* and 1 isolate of *A. terreus*. Two isolates of *A. flavus* produced high aflatoxin B1 Level (>500 mg/50 ml medium) while the other isolates produced either moderate or low levels of aflatoxins. Sterigmatocystin was produced by 2 isolates of *A. fumigatus* and 1 isolate of *Penicillium chrysogenum*. Fumagillin was produced only by one isolate of *A. terreus*. Diacetoxyscirpenol was produced in moderate amount by *Fusarium oxysporum* and *F. solani* (one isolates each). Zearalenone was produced at high level by only 1 isolate of *F. solani*. T-2 toxin was produced in low amount by only 1 isolate of *F. verticillioides*. Trichodermin was produced in low amount by 1 isolate of *Trichoderma hamatum*.

Table 3: Mycotoxins produced by fungi isolated from keratitis cases*

Fungi tested	FI No.	Mycotoxin detected	Toxin Level
Aspergillus flavus	3939	-	-
A. flavus	3940	-	-
A. flavus	3941	Aflatoxin B1	L
A. flavus	3942	-	-
A. flavus	3943	Aflatoxin B1	H
A. flavus	3944	Aflatoxins B1, G1	M
A. flavus	3945	Aflatoxin B1	H
A. flavus	3946	Aflatoxin B1	L
A. fumigatus	3947	Sterigmatocystin	M
A. niger	3950	-	-
A. terreus	3951	-	-
A. terreus	3886	Aflatoxins B1, B2, G1, G2	M
A. terreus	3952	Fumagillin	L
Cladosporium cladosporioides	3955	-	-
C.cladosporioides	3956	-	-
Cochliobolus spicifer	3957	-	-
Candida sp.	3958	-	-
Candida sp.	3959	-	-
Fusarium oxysporum	3961	Diacetoxyscirpenol	M
F. solani	3962	Zearalenone	H
		Diacetoxyscirpenol	M
F. verticillioides	3963	T-2	L
Penicillium chrysogenum	3965	Sterigmatocystin	H
Stemphylium botryosum	3966	-	-
Sterile mycelia	3954	-	-
Trichderma hamatum	3967	Trichodermin	L
Total	25		
No. (%) of positive isolates		13 (52)	
No. (%) of negative isolates		12 (48)	

*FI No.: Fungal isolate number, L: Low (less than 100 mg/50 ml medium), M: Moderate (from 100-500 mg/50 ml medium), H: High (more than 500 mg/50 ml medium), -: Means negative for mycotoxins

Twenty-five fungal isolates of keratitis origin were tested *in vitro* against 4 antifungal therapeutic agents. [Table 4].

The minimal inhibitory concentration (MIC) of amphotericin B for *Aspergillus flavus* (FI No. 3939, 3940, 3941, 3942, 3943), *A. niger* (3950), *Cladosporium cladosporioides* (3955, 3956), *Candida* sp. (3959), *Penicillium chrysogeum* (3965), and sterile mycelia (3954) was at 0.1% concentration, while MIC for *A. flavus* (3945) and *A. fumigatus* (3947) was at 0.5% concentration. However, amphotercin B has no antifungal effect at all concentrations used on *A. flavus* (3944, 3946), the 3 *A. terreus* isolates tested, *Candida* sp. (3958), the 3 *Fusarium* species, and *Trichoderma hamatum* tested. Amphotericin B at 0.005% concentration was only active against *Cochliobolus spicifer*.

Cetyltrimethyl-ammonium bromide (citrimide) at 1% concentration was effective against all isolates except one (*F. verticillioides* no. 3963) while 0.5% was effective against 17 out of 25 isolates tested, and the latter concentration could be considered as the minimal inhibitory concentration for these 17 isolates where all of them showed no capabilities of growth at the lower (0.1%) concentration. Cetyltrimethyl-ammonium bromide at 1% concentration showed high activity against *A.terreus* (the 3 isolates tested) and *Cochliobolus spicifer*. On the other hand, cetyltrimethyl-ammonium bromide at 0.1% concentration has no effect on all isolates tested.

Ketoconazole at 1% and 0.5% concentrations was effective against all fungal isolates, except the 3 *Fusarium* species tested. However, 0.1% concentration was effective against only 9 isolates. The MIC of ketoconazole for *A. flavus* (FI No. 3941, 3942, 3945), *A. fumigatus*, the 3 *A. terreus* isolates, *A. niger*, *C. cladsporioides* (3955), *P. chrysogenum*, sterile mycelia, and *T. hamatum* was at 0.5% concentration.

Terbinafine at 1% and 0.5% concentrations was effective against all isolates except *Candida* sp. (3959) and *Fusarium solani*. On the other hand, terbinafine at 0.1% concentration was effective against 20 out of the 25 isolates tested. From the results shown in Table 4, it could conclude that the MIC of terbinafine could only be determined for two isolates; *Candida* sp. (3958) and sterile mycelia at 0.5% concentration where it had high effect against most isolates at the 3 concentrations used.

Discussion

Since enzymes are important chemical weapons aiding the pathogen to invade host tissue, it was essential to shed some lights on the ability of some bacterial and fungal species isolated from keratitis cases to produce these compounds.

Protease, lipase, hemolysins, urease, phosphatase, and catalase were detected respectively in 48.65%, 37.84%, 59.46%, 43.24%, 67.57%, and 100% out of 37 bacterial isolates tested. The ocular inflammatory response to bacterial infection was suggested to be multifactorial and involved many different

Table 4: *In vitro* antifungal activity of some antifungal theraputic agents*

Fungi tested	FI No.	Control (solvent)	Amphotericin B				Cetrimide			Ketoconazole			Terbinafine		
			1	0.5	0.1	0.005	1	0.5	0.1	1	0.5	0.1	1	0.5	0.1
Aspergillus flavus	3939	0	10.3	8.6	7.3	0	9	7	0	22	19.3	10	33	30	23
A. flavus	3940	0	9.7	8.3	6.3	0	8	6	0	23	20	9	38	34	23
A. flavus	3941	0	10	8	6	0	8	0	0	21	16	0	38	33	27
A. flavus	3942	0	13	9	8	0	7	0	0	19.3	16	0	35	30	22
A. flavus	3943	0	11	9	6	0	10	8	0	21	16.7	9	40	31	25
A. flavus	3944	0	0	0	0	0	7	0	0	18.7	11	8	36	32	25
A. flavus	3945	0	9	7	0	0	8	0	0	22	17	0	38	33	27
A. flavus	3946	0	0	0	0	0	7	0	0	20	15	10	39	30	25
A. fumigatus	3947	0	11	7	0	0	9	8	0	12	10	0	26	19	13
A. niger	3950	0	9	8	6	0	9	7	0	13	7	0	35	30	25
A. terreus	3951	0	0	0	0	0	20	15	0	17.3	12.3	0	37	32	25
A. terreus	3886	0	0	0	0	0	20	14	0	14	10	0	40	33	23
A. terreus	3952	0	0	0	0	0	17	11	0	17	11	0	34	29	21
Cladosporium cladosporioides	3955	0	12	11	10	0	14	9	0	11	8	0	25	20	10
C.cladosporioides	3956	0	12	10	9	0	15	8	0	35	23	10	42	37	30
Cochliobolus spicifer	3957	0	29.3	26	20	14	24	15	0	17	13	9	31	27	17
Candida sp.	3958	0	0	0	0	0	12	9	0	28.3	22	15	10	8	0
Candida sp.	3959	0	13	12	11	0	8	6	0	33	30	24	0	0	0
Fusarium oxysporum	3961	0	0	0	0	0	10	0	0	0	0	0	20	16	10
F. solani	3962	0	0	0	0	0	7	0	0	0	0	0	0	0	0
F. verticillioides	3963	0	0	0	0	0	0	0	0	0	0	0	21	15	10
P. chrysogenum	3965	0	12	9	7	0	12	9	0	11	9	0	39	35	25
Stemphylium botryosum	3966	0	21.3	19.3	17.3	9	13	8	0	19	15	0	10	8	0
Sterile mycelia	3954	0	12	9	7	0	12	9	0	11	9	0	10	8	0
Trichderma hamatum	3967	0	0	0	0	0	12	10	0	10	8	0	15	13	10

*FI No.: Fungal isolate number, Solvent: Dimethyl sulphoxide (DMSO) Figures are means of three replicates in mm, Concenterations of the antifungal agents were expressed as percentages

bacterial and host-derived factors.[18] Their study demonstrated that alpha, beta hemolysin, and lipase play a role in the ocular response and in the induction of clinical inflammation and infiltration of inflammatory cells. Moreover, Liesegang[5] reported that bacterial proteases attack peptide bonds, dissolve elastin, and acts as non-specific collagenases *in vitro*. He added that *Staphylococcus aureus* and other coagulase-positive strains are the most pathogenic and elaborate extracellular enzymes such as lipase, coagulase, staphylokinase, hyaluronidase, DNase, and lysozyme. Isolates of *S. aureus* showed different capabilities of producing different enzymes tested in this work; however, all produced β-hemolysin and catalase. *S. aureus* products such as lipoteichoic acid, peptidoglycan and alpha toxin have shown in cell culture to induce the expression of pro-inflammatory cytokines and adhesion molecules, which lead to the infiltration of inflammatory cells.[19] *Pseudomonas aeruginosa* which produced protease, lipase, β-hemolysin, urease, phosphatase, and catalase was also reported by Hobden,[20] to produce a multitude of secreted proteases and toxins that can be considered virulence factors for the eye. Of the 37 bacterial isolates *in vitro* tested during this study, some isolates were strong enzyme producers of urease (2 isolates) and catalase (30). In this respect, Cejkova *et al*.[21] studied catalase activity in the corneas of normal rabbit eyes of various age and found that the corneas of young and particularly aged rabbits might be more susceptible to oxidation injury as compared to the corneas of young adult animals.

Out of 25 fungal isolates tested during the present study, 80% were positive for protease, 84% for lipase and urease, 28% for blood hemolysis, and 100% for phosphatase and catalase enzymes. According to Gopinathan *et al*.,[22] the capability of fungal corneal pathogens to utilize collagen *in vitro* supports the assumption that the ability of a fungus to elicit corneal tissue damage is dependent, at least in part, on the utilization of corneal collagen *in vivo* by the infecting agent. Zhu *et al*.[23] had also identified extracellular proteases in cultures with collagen (*in vitro*) of a corneal isolate of *A. flavus* and concluded that the collagenase activity is a mediator of the severe corneal destruction caused by this isolate. Moreover, Dorner[7] reported that the secretion of enzymes and exotoxins causes coagulative necrosis with the loss of keratocytes and disruption of collagen lamellae.

Mycotoxins are low molecular weight fungal products that may be toxic to higher organisms. Only a few studies have investigated the role of fungal toxins as contributing factors in eye infections. In harmony with our results, Raza *et al*.[24] found that isolates of *Fusarium* species from 18 pateints with keratomycosis could produce mycotoxins *in vitro*. Of these isolates, 13 produced nivalenol, 6 produced deoxynivalenol, 9

gave T-2 toxin, and 2 showed the presence of diacetoxyscirpenol at different intervals. These authors were postulated that the virulence of *Fusarium* for the cornea might be partly related to its ability to produce mycotoxins. However, Thomas[6] found that some isolates of *Fusarium* from fungal keratitis produce various toxins, but it was difficult to correlate the production of toxins with the manifestation and progress of the disease.

The *in vitro* sensitivity test using different antifungal agents is greatly recommended to choose the most effective drug for therapy.

The minimal inhibitory concentration (MIC) of amphotericin B was at 0.1% or 0.5% concentrations for most fungal species tested while there was no effect on *Aspergillus terreus*, the 3 *Fusarium* species, and *Trichoderma hamatum*. Amphotericin B in solution or as an ointment has been used topically to treat keratitis; it is the treatment of choice for keratitis due to *Candida* species as reported by Tanure *et al*.[25] However, only 1 isolate of *Candida* (out of two tested in the present study) was affected when amphotericin B was used at 1%, 0.5%, and 0.1% concentrations. Amphotericin B was also used for treatment of 3 patients with keratitis and hypopyon due to *A. flavus* with minimal toxicity.[26] This is in harmony with our results *in vitro* on 6 out of 8 *A. flavus* isolates where they were inhibited mostly at 1%, 0.5%, and 0.1% concentrations of amphotericin B. Amphotericin B has a broad-spectrum of action including many *Aspergillus* species, *Candida* species, and *Penicillium marneffei* while *Aspergillus terreus* and *Fusarium* species are often resistant.[27]

Cetyltrimethyl ammonium bromide at 1% was more effective than 0.5% and 0.1% against all isolates tested except *F. verticillioides*. In agreement with our result *in vitro* on *F. salani* of keratitis origin, Mahmoud[28] reported that MIC of cetyltrimethyl ammonium bromide was 100 μg/ml, and he added that it had no pathological effect to eye corneal tissues and with no side effects during the treatment course of a rabbit model with keratitis.

Ketoconazole at 1% and 0.5% concentrations was effective against all fungal isolates, except the 3 *Fusarium* species tested. Marangon *et al*.[8] studied ketoconazole activity against species of *Aspergillus* (4 isolates), *Candida* (20), and *Fusarium* (9) and found that MIC values were 4, 0.032, and >16 μg/ml, respectively. In accordance with our findings, Al-Hussaini *et al*.[29] treated successfully 85% of mycotic keratitis cases in Upper Egypt with topical ketoconazole.

Terbinafine at the 3 concentrations used (1%, 0.5%, and 0.1%) showed high activity against most isolates tested, and this revealed that terbinafine was more effective *in vitro* than the other 3 antifungal therapeutic agents used in this study. In accordance with our results on all isolates of Aspergilli (16 of 4 species) and 1 out of 2 isolates of *Candida* sp., Richardson and Warnock[27] reported that terbinafine is active against *Aspergillus* species, *Candida albicans*, and some other *Candida* species. Terbinafine has a brood-spectrum and is considered a fungicidal *in vitro*, whereas the azoles (e.g., ketoconazole) are primarily fungistatic, and this may partially account for the greater efficacy of terbinafine.[30]

The present study showed the ability of bacterial and fungal isolates to produce extracellular enzymes and mycotoxins, which consider as contributing factors to invade the eye's tissues, and this can explain the severity of corneal infections. Thus, seeking for an efficient therapy is important in Upper Egypt where most of people are working in agriculture, which makes them more predisposing to keratitis through plant trauma.

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