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Prevalence of lipase producer *Aspergillus niger* in nuts and anti-biofilm efficacy of its crude lipase against some human pathogenic bacteria

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Nuts are the natural source of healthy lipids, proteins, and omega-3. They are susceptible to fungal and mycotoxins contamination because of their high nutritional value. Twenty-five species comprising 12 genera were isolated from 80 samples of dried fruits and nuts using the dilution plate method. Peanut recorded the highest level of contamination followed by coconut; almond and raisin were the lowest. *Aspergillus* was the most prevalent genus and *A. niger*, was the most dominant species. The morphological identification of the selected *A. niger* isolates as they were detected in high frequency of occurrence was confirmed by using 18SrRNA sequence. Ochratoxin biosynthesis gene *Aopks* was detected in the tested isolates. Lipase production by the selected *A. niger* isolates was determined with enzyme activity index (EAI) ranging from 2.02 to 3.28. *A. niger*-26 was the highest lipase producer with enzyme activity of 0.6 ± 0.1 U/ml by the trimetric method. *Lip2* gene was also detected in the tested isolates. Finally, the antibacterial and antibiofilm efficiency of crude lipase against some human pathogens was monitored. Results exhibited great antibacterial efficacy with minimum bactericidal concentration (MBC) of 20 to 40 µl/100 µl against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and Methicillin-resistant *Staphylococcus aureus* (MRSA). Interestingly, significant anti-biofilm efficacy with inhibition percentages of 95.3, 74.9, 77.1 and 93.6% was observed against the tested pathogens, respectively.

Dried fruits and nuts are enriched source of healthy fatty acids, protein, potassium, dietary fibers and bioactive compounds[1]. They protect the mankind from the risks of obesity, cardiovascular illnesses, type 2 diabete and hypertension[2,3]. *Alternaria*, *Aspergillus, Candida, Fusarium, Mucor*, *Rhizopus, Penicillium*, *Trichoderma*, and *Cladosporium* are the most common genera causing nuts spoilage, and their ingestion may cause mycoses especially in immunocompromised patients[4,5].

Many species of fungi produce mycotoxins, secondary metabolites of small molecular sizes (MW < 300) that are toxic to humans and animals cause mycotoxicoses when ingested, leading to cancer and liver diseases[6,7]. A total of 145 secondary metabolites were discovered in *Aspergillus* section Nigri; among them are ochratoxin A (OTA), which are the most toxic to humans and animals[5]. OTA is the causative agent of Balkan endemic nephropathy, urothelial tumors, and testicular cancer in humans[8–10].

Lipase, an enzyme belonging to the serine hydrolase class, catalyzes the hydrolysis of fats and oils to glycerol and fatty acids without requiring cofactors[11]. Fungi are considered the best producers of lipase among all microorganisms, especially, *Aspergillus niger* which was generally recognized as safe (GRAS) by Food and Drug Administration (FDA) in the United States[12]. Lipase has a wide range of industrial applications, such as in the food industry, as detergent additives, pharmaceutical industry, and biofuel production; therefore, the universal demand for the lipase enzyme is increasing[13].

Enzymes including isomerases, lyases, oxidoreductases, transferases, esterases and hydrolases have been reported to induce antibacterial efficacy[14]. Lipase is a hydrolytic enzyme, has antimicrobial and antifouling properties[15]. However, its mode of action and its effects in most of the cases have not been clarified fully[14].

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Biofilm is a complex medium involving live and dead bacterial cells, exopolysaccharides, proteins and carbohydrates on a material surface with a serious problem in biomedical applications[16]. Several steps were involved in biofilms development beginning with surface adherence, microcolony formation, maturation and finally detachment stages[17]. Biofilms protect pathogenic bacteria from human immune system, antibiotics and severe environmental conditions[18]. Several bio-active and chemically synthesized compounds have been performed to suppress biofilm formation by pathogenic bacteria[19–22]. Revitalize aminoglycosides also have been used to inhibit biofilm and pathogenic bacterial infections[23]. Attenuating motility properties can be considered as highly potential for controlling biofilm formation since attachment was one of the main steps in biofilm formation[24,25]. Using enzymes is also a good policy for biofilm elimination because enzymes are rabidly eco-friendly and degradable[26,27].

This study was established for the isolation and identification of mycobiota associated with four kinds of nuts, determination the ochratoxigenic potential of some *A. niger* isolates, their lipolytic activity, and finally studying the ability of crude lipase from *A. niger* to inhibit the growth and biofilm formation of some human pathogens.

Results

Mycobiota contaminating nuts. Twenty-five fungal species comprising 12 genera were obtained from the 80 tested samples of nuts by using dilution plate method. *Aspergillus* was the most prevalent genus as it was isolated from 100% of the samples. *Penicillium* was the second genus in frequency as it was isolated from 62.5% of total samples. From the above genera *A. niger*, *A. flavus*, *P. chrysogenum* and *P. oxalicum* were the most frequent species (Table 1). *Rhizopus stolonifer* was isolated from 60% of peanut, 45% of almond and 35% of raisin but not detected in coconut. The remaining genera and species were isolated in rare frequency accounting collectively 1.59×10^3 CFU/g as illustrated in Table 1.

Multiple alignment of different *A. niger* isolates. The 5.8S gene in rDNA sequences were subjected to multiple alignments using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/page2.html). Among the five isolates of *A. niger*, 5.8S gene nucleotide sequences showed 98% similarity all strains. When the sequences were aligned with the database sequences, they showed 95% similarity with *A. niger* strain AHBR5, except the *A. niger*-27 similarity sequence, which shared 98% similarity with *A. niger* strain AHBR5 (Fig. 1). Phylogenetic tree was drawn with MEGA 7.1 program show that more similarity among *Aspergillus niger* and low similarity with *A. niger* AHBR5 (Fig. 2).

Ochratoxigenic potential of *A. niger*. Our results indicated that all the tested *A. niger* isolates had ability to produce ochratoxins by using flourometric method with variable levels (2.6–3.2 ppb) with the highest reading recorded by *A. niger*-27 recovered from coconuts as shown in Table 2.

Detection of ochratoxins biosynthesis genes. Polymerase chain reaction (PCR) was applied using two sets of primer for gene involved in ochratoxin biosynthetic pathway. Bands of the fragments of *Aopks* gene can be visualized in all tested *A. niger* isolates at 549 bp (Fig. 3).

Preliminary screening of *A. niger* isolates for lipase production. The tested *A. niger* isolates had ability to produce lipase enzyme in solid medium containing tween 80 with enzyme activity index (EAI) ranging from 2.02 to 3.28 as summarized in Table (3). White precipitate diameter was between 11.5 ± 0.5 and 21.8 ± 7.42 mm. *A. niger*-29 showed the highest diameter 21.8 ± 7.42 mm and the lowest was observed in *A. niger*-27 with 11.5 ± 0.5 mm.

Assay of lipase enzyme. Lipase activity was determined in liquid medium by using trimetric method showed that *A. niger*-26 obtained from almond recorded the highest lipase activity (0.6 ± 0.1 U/ml-min) followed by *A. niger*-30 (0.3 ± 0.1 U/ml-min), *A. niger*-28 and *A. niger*-29 with the same reading (0.233 ± 0.11547 U/ml-min) and *A. niger*-27 was the least (0.2 ± 0.1 U/ml-min) (Table 3).

Detection of *A. niger lip2* gene. PCR was performed for *Lip2* gene detection in the tested *A. niger* isolates using two sets of primers. *Lip2* gene was detected at 1276 bp in all the *A. niger* isolates (Fig. 4).

Studying the virulence properties of target human pathogens in presence of crude lipase obtained from *A. niger*. In the current study, we extended the utility of using crude lipase from *A. niger* to explore its potential as antibacterial against some human pathogens. This was performed using INT reduction assay. Results exhibited an excellent effect of crude lipase against both Gram negative and Gram positive tested strains. Where MIC ranged from 10 to 20 µl/100 µl and MBC from 20 to 40 for *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and Methicillin-resistant *Staphylococcus aureus* (MRSA) Table 4.

Antibiofilm activity lipase enzyme. In our study crude lipase from *A. niger* MW029470 was examined as antibiofilm agent against four human pathogens by spectrophotometric methods. The ability of the four tested human pathogens *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and Methicillin-resistant *Staphylococcus aureus* (MRSA) to form biofilm were confirmed before treatment with lipase as shown in Fig. 5A–D{C}, respectively. The results exhibited significant inhibition for biofilm formation in the four tested pathogens. The highest significant percentages of inhibition were 95.3, 74.9, 77.1 and 93.6 for *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and Methicillin-resistant *Staphylococcus aureus* (MRSA) Fig. 5A–D{50}, respectively.

Fungal genera and species	Peanut				Almond				Coconut				Raisin			
	Fungal count (CFU/g ×10³)	%C	F%	NCI	Fungal count CFU/g ×10³	%C	F%	NCI	Fungal count (CFU/g×10³)	%C	% F	NCI	Fungal count (CFU/g ×10³)	%C	F%	NCI
Acremonium hyalinulum	0.06	0.3%	5	1	–	–	–	–	-	-	-	-	-	-	-	-
Alternaria alternata	0.18	0.9%	5	1	0.03	0.18%	5	1	-	-	-	-	-	-	-	-
Aspergillus	15.68	78.75%	100	20	15.12	89.68%	100	20	11.67	77.18%	100	20	6.39	68.49%	100	20
A. fumigatus	0.65	3.26%	15	3	0.06	0.36%	10	2	0.42	2.78%	30	6	0.51	5.46%	45	9
A.flavus	1.53	7.68%	55	11	4.2	24.91%	65	13	5.85	38.69%	100	20	3.81	40.84%	95	19
A.niger	12.99	65.2%	100	20	10.83	64.23%	100	20	4.92	32.54%	75	15	2.07	22.19%	70	14
A.sydowii	–	–	–	–	0.03	0.18%	–	–	0.12	0.79%	10	2	–	–	–	–
A.terreus	0.51	2.56%	10	2	–	–	–	–	0.12	0.79%	10	2	–	–	–	–
A.ustus	–	–	–	–	–	–	–	–	0.24	1.58%	5	1	–	–	–	–
Emericella nidulans	0.24	1.2%	5	1	–	–	–	–	0.03	0.21%	5	1	0.12	1.29%	5	1
Eurotium amstelodami	–	–	–	–	0.03	0.18%	5	1	–	–	–	–	–	–	–	–
E. chevalieri	0.12	0.6%	5	1	–	–	–	–	–	–	–	–	–	–	–	–
Fusarium dimerum	0.12	0.6%	5	1	–	–	–	–	0.09	0.6%	10	2	–	–	–	–
F. solani	0.12	0.6%	10	2	–	–	–	–	–	–	–	–	–	–	–	–
Mucor circinelloides	–	–	–	–	–	–	–	–	–	–	–	–	0.24	2.57%	15	3
Paecilomyces variotii	0.12	0.6%	5	1	–	–	–	–	–	–	–	–	–	–	–	–
Penicillium	1.5	7.53	55	11	0.57	3.38%	40	8	2.01	13.29%	80	16	2.04	21.86%	75	15
P. aurantiogriseum	0.06	0.3%	5	1	–	–	–	–	–	–	–	–	–		–	–
P.chrysogenum	0.69	3.47%	25	5	0.36	2.14%	35	7	0.57	3.77%	30	6	0.54	5.79%	35	7
P.duclauxii	0.12	0.6%	5	1	–	–	–	–	–	–	–	–	–		–	–
P.funiculosum	0.06	0.3%	5	1	–	–	–	–	–	–	–	–	0.06	0.64%	5	1
P. oxalicum	0.39	1.96%	20	4	0.21	1.25%	20	4	1.44	9.52%	75	15	1.44	15.43%	70	14
P.purpurogenum	0.12	0.6%	5	1	–	–	–	–	–	–	–	–	–		–	–
P.variabile	0.06	0.3%	5	1	–	–	–	–	–	–	–	–	–		–	–
Rhizopus stolonifer	1.62	8.14%	60	12	1.11	6.58%	45	9	–	–	–	–	0.54	5.79%	35	7
Scytalidium lignicola	0.06	0.3%	5	1	–	–	–	–	1.32	8.73%	75	15	–	–	–	–
Stachybotrys atra	0.06	0.3%	5	1	–	–	–	–	–	–	–	–	–	–	–	–
Sterile mycelia	0.03	0.15%	5	1	–	–	–	–	–	–	–	–	–	–	–	–
Total	19.91	100%			16.86	100%			15.12	100%			9.33	100%		
Number of genera = 12	11				5				5				5			
Number of species = 25	21				9				11				9			

Table 1. Colony forming units (CFU/g), percentage (%C), frequency (F%) and number of cases of isolation (NCI) of mycobiota contaminating nuts. CFU/g: Colony forming unit per gm of 80 samples of peanut, almond, coconut and raisin (20 of each) on rose Bengal chloramphenicol agar medium (RBCA). %C: Percentage of each isolate to the total isolates for each type of nuts. F%: Frequency of each isolate. NCI: number of cases of isolation of each isolate out of 20 sample of peanut, almond, coconut and raisin.

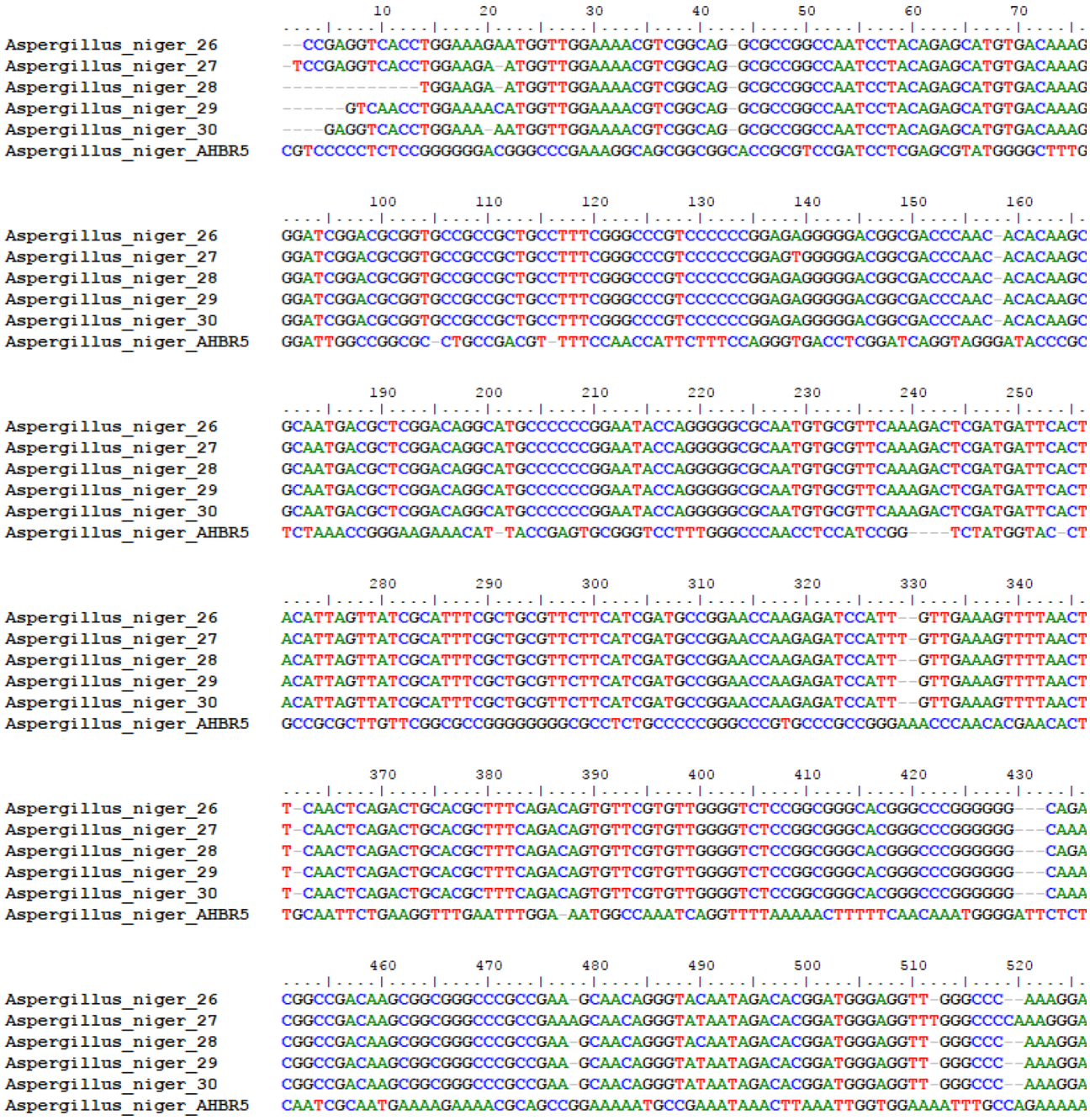

Figure 1. Comparison alignment of nucleotide sequences of ITS region in five *A. niger* isolates with different relation nucleotide sequences of some strains at NCBI database using BioEdit program.

Scanning electron microscopy (SEM). Results of SEM were revealed in Figs. 6 and 7. For antibacterial efficacy of crude lipase for the tested bacteria, the micrographs showing that some cells shorten and getting smaller such as *Escherichia coli* (Fig. 6a,b). Other cells were curved and divided like *Proteus mirabilis* (Fig. 6c,d). Cells of *Pseudomonas aeruginosa* (Fig. 6e,f) distortion occur in cell shape to spherical instead of bacillus. Finally, cells of Methicillin-resistant *Staphylococcus aureus* (MRSA) that begin to swell up with irregular spherical shape Fig. 6g,h.

SEM micrographs for biofilm structure revealed that, in control, there are typically heterogeneous distributions of biofilm with higher number of adhered cells also cells arranged in the form of aggregates or simply as individualized cells without slimy material in their vicinity (Fig. 7a,c,e,g). In contrast, to treatment with crude lipase where micrographs showing a uniform layer of cells with negligible clumping (Fig. 7b,d,f,h).

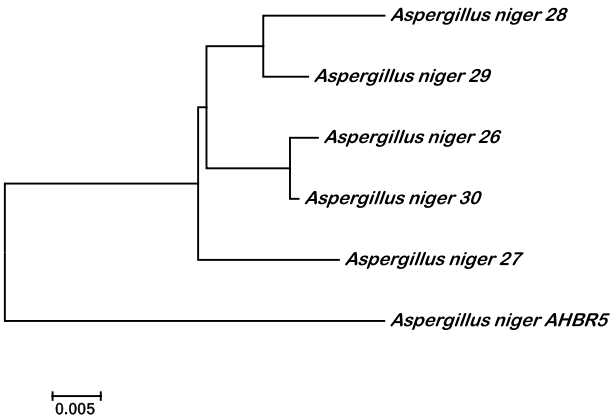

Figure 2. Phylogenetic tree and relationship among five strains of *A. niger* compared with some *A. niger* strains at NCBI.

Fungal isolates	Source	Accession number	Ochratoxins level
A. niger-26	Almond-5	MW029470	2.7
A. niger-27	Coconut-9	MW029471	3.2
A niger-28	Almond-16	MW029472	2.6
A. niger-29	Peanut-2	MW029473	3.1
A. niger-30	Peanut-13	MW029474	2.8

Table 2. Ochratoxigenic potential of *A. niger* isolates.

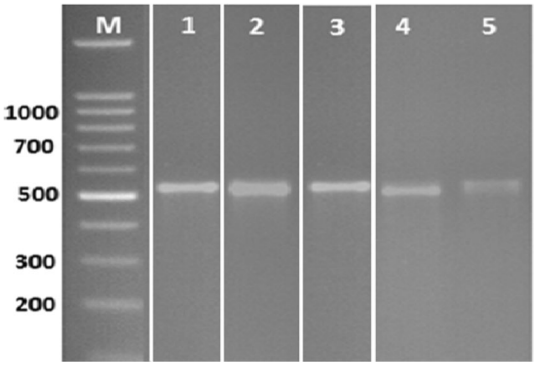

Figure 3. PCR amplification of *Aopks* genes (549 bp) for *A. niger* isolates. Whereas, (1) *A. niger*-26 and (2) *A. niger*-27, (3) *A. niger*-28, (4) *A. niger*-29 and (5) *A. niger*-30. Sample lanes from different gels have been juxtaposed together in this figure.

Fungal isolates	Clear zone diameter (mm)	Enzyme activity index (EAI)	Lipase activity (U/ml-min)
A. niger-26	14 ± 2.08	3.07	0.6 ± 0.1*
A. niger-27	11.5 ± 0.5	2.78	0.2 ± 0.1*
A. niger-28	13.8 ± 1.04	3.11	0.233 ± 0.11547*
A. niger-29	12.5 ± 0.87	3.28	0.233 ± 0.11547*
A. niger-30	21.8 ± 7.42	2.02	0.3 ± 0*

Table 3. Lipolytic activity of *A. niger* isolates. *Means significant value in comparison with control with LSD at 0.05 was 0.16 for *A. niger*-26 and *A.niger*-29, 0 for *A. niger*-27 and *A. niger*-30 and 2.44 for *A. niger*-28. Values expressed as mean ± Standard deviation.

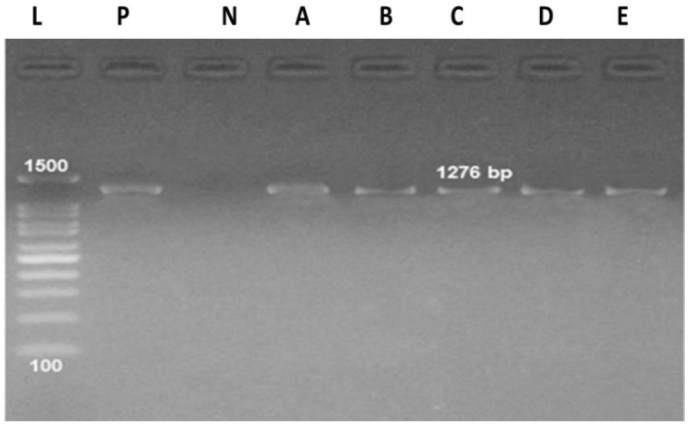

Figure 4. PCR amplification of *Lip2* genes (1276 bp) for *A. niger* isolates. whereas, **(A)** *A. niger*-29, **(B)** *A. niger*-26, **(C)** *A. niger*-28, **(D)** *A. niger*-27 and **(E)** *A. niger*-30.

Tested pathogens	Antibacterial efficacy/100 µl	
	MIC	MBC
Escherichia coli	10 µl	20 µl
Pseudomonas aeruginosa	20 µl	20 µl
Proteus mirabilis	10 µl	20 µl
Staphylococcus aureus (MRSA)	30 µl	40 µl

Table 4. Antibacterial efficacy of crude lipase against some human pathogens.

Discussion

Nuts and dried fruits are healthful foods that protect human body from many chronic diseases. Their high nutritional value makes them a suitable medium for fungal contamination. In the current study, peanut were the highest contaminated samples this may be due to the high moisture content of peanut samples in harmony with Ismail[28], who reported that peanut samples were highly deteriorated with fungi than coconut. *Aspergillus* was the most prevalent genus followed by *Penicillium*. This were previously confirmed by Khosravi et al.[29], who showed that *Aspergillus* followed by *Penicillium* were the most frequent genera deteriorated 60 samples of nuts. From the above genera *A. niger*, *A. flavus*, *P. chrysogenum* and *P. oxalicum* were the most frequent species (Table 1) and these results were previously obtained by Ismail[28,30–33]. In contrast, *Aspergillus* section Flavi was the highest recorded in peanuts seeds followed by *Aspergillus* section Nigri and *Aspergillus* section Circumdati was the least[34]. Past study by Tournas et al[5] found the same results that *A. niger* followed by *Penicillium* were the most common mold in nuts and dried fruits. *Rhizopus stolonifer* was isolated from peanuts, almonds and raisins in high and moderate frequency of occurrence (Table 1). In a study by Abdulla[35], reported that *Aspergillus*, *Rhizopus* and *Penicillium* genera were more frequently detected than other genera of fungi in nuts.

The molecular identification of the tested *A. niger* confirmed the morphological identity and more similarity among *A. niger* isolates was observed and low similarity with *A. niger* AHBR5 except *A. niger*-27 (Figs. 1, 2) and the obtained results in agreement with Perrone et al[36,37]. All the tested *A. niger* isolates were ochratoxin producers with variable readings by using fluorometric method (Table 2). In past investigation by Al-Sheikh[38] confirmed that 57% and 60% of *A. niger* and *A. carbonarius*, respectively deteriorated peanut were ochratoxin producers. Magonli et al[39], demonstrated that 32% of *Aspergillus* section Nigri obtained from peanut seeds in Argentinean had ability to produce ochratoxin A. Alhussaini[30], found that 33.3% of *Aspergillus* section Nigri biserriate and one isolate of uniserriate isolated from nuts were ochratoxin A producers. The tested *A. niger* isolates recovered from baby foods recorded positive results for ochratoxins production[40]. Our obtained results were in-disagreement with past study by Palumbo & O'Keeffe[41], reported that all the tested 171 isolates of *Aspergillus* section Nigri isolated from almonds showed negative results for ochratoxin A production. In this study, ochratoxin biosynthesis gene *Aopks* was detected in all the tested isolates at 549 bp (Fig. 3). The obtained results in harmony with Massi et al.[42], who reported that *pks* gene was detected in all *Aspergillus niger* positive ochratoxgenic strains isolated from Brazilian foods amongst, nuts and dried fruits. *Aopks* genes were detected at 549 bp in *A. niger* isolates that had ability to produce ochratoxins[43,44]. All tested *A. niger* and *A. tubingensis* isolated from beef showed positive results for the presence of *pks* genes[45]. The selected *A. niger* isolates were lipase producers qualitatively on Tween 80 solid medium and quantitatively by using trimetric titration method with the highest activity recorded by *A. niger*-26 isolated from almond (Table 3). *A. niger* is well-recognized to be the best producer of lipase enzyme and is favored in many industrial processes[46,47]. *A. niger*, *Fusarium oxysporum* and *Nectria haematococca* isolated

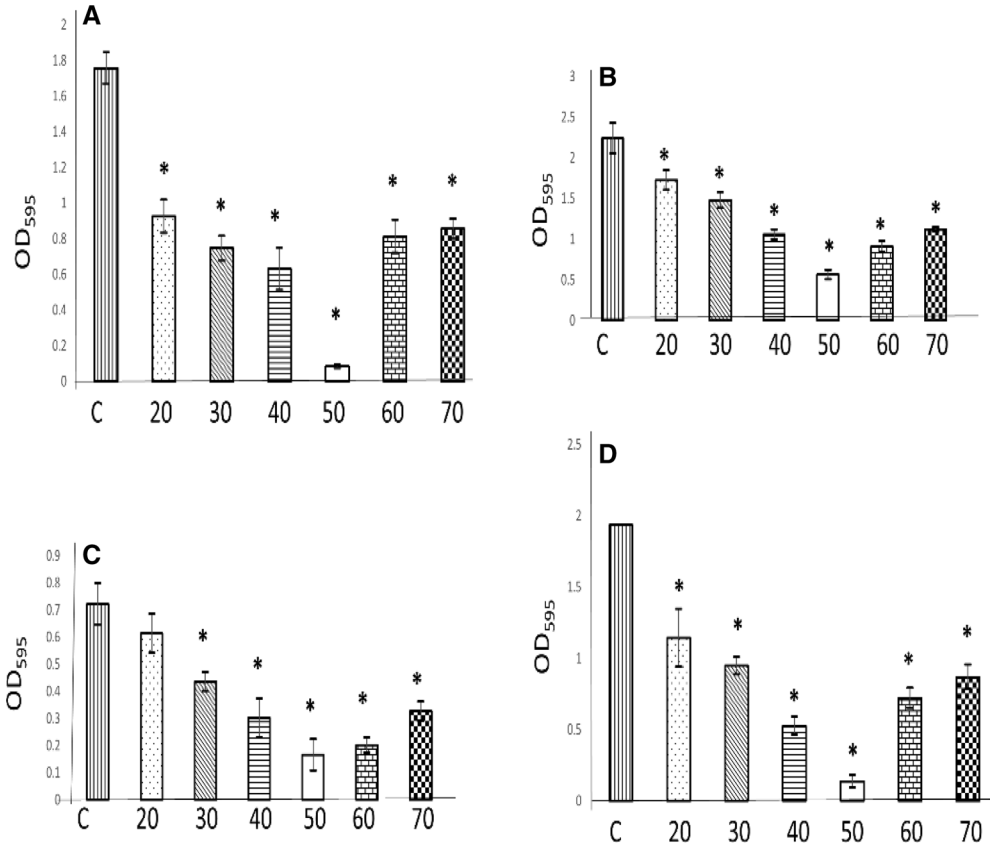

Figure 5. Antibiofilm activity of crude lipase produced by *A. niger* isolated from nuts against some human pathogenic bacteria. **(A)** *Escherichia coli*; **(B)** *Pseudomonas aeruginosa*; **(C)** *Proteus mirabilis*; **(D)** methicillin-resistant *Staphylococcus aureus* (MRSA). C: control (amount of biofilm of the tested strains). 20, 30, 40, 50, 60, and 70 µl: added volumes of crude lipase for determination the optimum volume in inhibiting biofilm. Shown are the averages from at least three independent measurements. The error bars indicate the standard deviations. Asterisk: means values are highly significant compared with control.

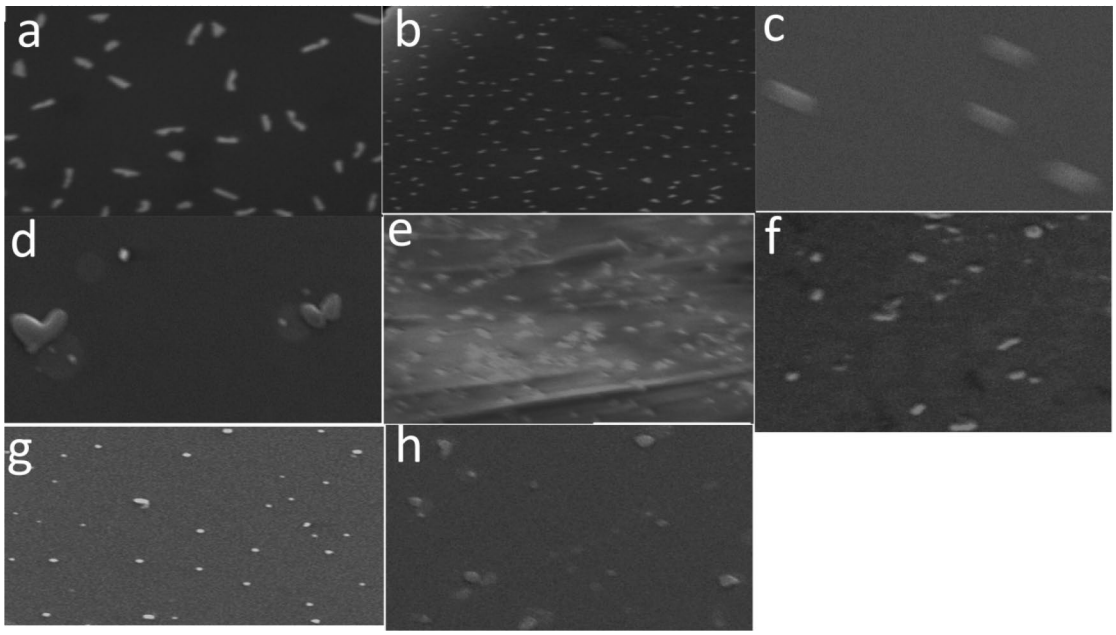

Figure 6. Scanning electron microscopy micrographs of treated bacteria with crude lipase. **(a,c,e,g)** Untreated *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA), respectively (control). **(b,d,f,h)** Treated *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) with crude lipase, respectively (treatments).

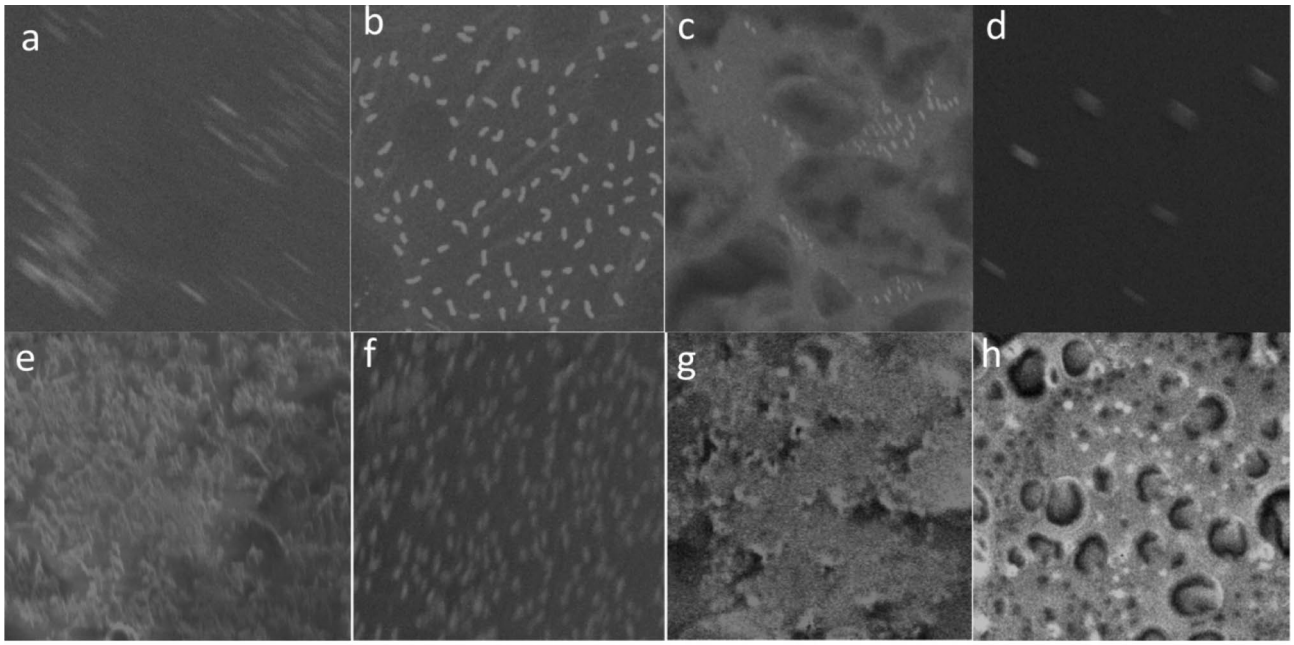

Figure 7. Scanning electron microscopy micrographs of biofilm structure. **(a,c,e,g)** Biofilm formation by untreated *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA), respectively (control). **(b,d,f,h)** Biofilm formation by treated *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) with crude lipase, respectively (treatments).

from beef luncheon were the highest lipase producers[48]. Rai et al.[49], isolated lipase producer *A. niger* from some oil contaminated soil samples. Earlier studies also, confirmed that *A. niger* was the highest lipase producing strain[50–54]. Putri et al.[12], optimized the production of lipase by *A. niger* by using agro-waste and revealed that 1% olive oil was the highest inducer, yielding dry lipase extract with highest activity unit (176 U/ml enzyme). *Lip2* gene was visualized at 1276 bp in all the tested *A. niger* isolates (Fig. 4). Yang et al.[55], reported *Lip2* gene a novel lipase gene cloned from *A. niger*. Lipase exhibits antibacterial activity against *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* with MBC of 20 µl/100 µl (Table 4). This may be due to that lipase acting on the lipopolysaccharide of Gram negative cell wall as well as the esters of exopolysaccharide present in the biofilm. Furthermore, lipolytic enzyme acts on a lipid substrate Such as phospholipids and other hydrophobic molecules, to hydrolyze or esterify a bond[16]. Lipases are esterases capable of hydrolyzing any ester bond. They act on the lipoprotein, lipopolysaccharide and phospholipids which surrounds the peptidoglycan layer leading to the hydrolysis of the lipid bilayer. The lipopolysaccharide complex is an endotoxin present on the outer membrane of the cell wall and this toxicity leads to a wide spectrum of nonspecific pathophysiological reactions including fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death. When lipase works on lipid A, the chances of infection are reduced[56]. In most of the Gram positive bacteria, lipoteichoic acids are present and the lipid tail present here plays a major role in the bacterial attachment. There is a possibility for the lipase to act on this lipid tail thereby preventing its adherence to a surface[16]. Our results confirmed antibacterial activity of lipase on Gram positive bacteria (MRSA) with MBC of 40 µl/100 µl (Table 4). Bacterial biofilms pose a great threat to human life not only because they involved in a lot of chronic infectious human diseases but also, they highly resistant to different antimicrobial agents. This generates a strong demand for finding suitable anti biofilm agents[57]. Bacterial biofilms are common populations of bacterial cells surrounded by a self-produced matrix of extracellular polymeric substances (EPS) [58]. EPS surrounding mixture include various exopolysaccharides, lipids, secreted proteins some of which can form amyloid fibers and extracellular DNA[59]. Most of the antimicrobial agents fail to penetrate the biofilm owing to the presence of EPS which acts as a barrier protecting the bacterial cells within the biofilm. So, the remedy will be the use of compounds that able to degrade the biofilm EPS. Enzymes have been recognized to be effective for the degradation of the biofilms EPS[60,61]. Plants contain various anti-biofilm compounds, as they have to prevent bacterial growth on their surfaces[62,63]. Since lipase, an esterase, is a hydrolyzing enzyme, it is having the ability to act on the EPS produced by the organisms[64], by degrading protein components and the high molecular weight lipid of the biofilm[65]. In the current investigation crude lipase, was examined as antibiofilm agent against four human pathogens by spectrophotometric methods. The highest significant percentages of inhibition were 95.3, 74.9, 77.1 and 93.6 for *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and Methicillin-resistant *Staphylococcus aureus* (MRSA), respectively. Although, all added volumes of lipase significantly inhibited biofilm formation, the suitable volume that gives highest inhibition percentage was 50 µl (Fig. 5). Scanning electron microscopy (SEM) has been used widely for qualitative observation of biofilm before and after treatments, biofilm disturbance due to its high resolution and

is usually applied in biological assays of biofilm removal effectiveness also antimicrobial treatments[66–68]. Results of SEM confirmed antbacterial and antibiofilm properties of crude lipase against the tested human pathogens.

In conclusion, Peanuts were the highest contaminated samples among the tested types. *A. niger* was the most isolated species from nuts. All the selected *A. niger* isolates were lipase producers with highest enzyme activity was recorded by *A. niger* MW029470 and showed positive results for the presence of *Lip 2* gene. Crude lipase from *A. niger* MW029470 showed highly inhibition of the tested pathogens growth with MBC of 20 to 40 µl/100 µl and significantly inhibited biofilm formation of 4 biofilm former human bacterial pathogens. The significant percentages of inhibition were 95.3, 74.9, 77.1, and 93.6 for *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and Methicillin-resistant *Staphylococcus aureus* (MRSA), respectively.

Materials and methods

Collection of nuts samples. Eighty samples of peanut, almond, coconut and raisin (20 samples of each type) were purchased from different supermarkets at Qena Governorate, Egypt. All samples were kept in a refrigerator until mycological analysis.

Isolation of fungi. The modified method described by Tournas et al.[5] was employed for isolation of mycobiota contaminating nuts. A known weight of each sample was blended with 90 ml of 0.1% peptone in blender jar under aseptic conditions for minute. Serial dilutions were made to obtain the suitable one. One ml of the suitable dilution was poured in sterilized petri plate followed by 20 ml of rose Bengal chloramphenicol agar (RBCA) medium containing g/l (peptone; 5, glucose; 10, kH_2PO_4; 1, $MgSO_4.7H_2O$; 0.5, rose Bengal; 0.05, chloramphenicol; 0.1, and agar 15.5). Triplicates of each sample were prepared. Plates were incubated for a week at 28 °C. The developed fungal colonies were counted, examined and identified (based on macro- and microscopic features)[69].

Sequence analysis of 5.8S-ITS region. ITS1 and ITS2 regions together with 5.8S gene in rDNA from *A. niger* strains were amplified as designed by Hermosa et al.[70]. The purified bands were determined using the sequencer Gene analyzer 3121 in Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, KSA. The realized sequence was aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.10. Then, a consensus sequence was generated from each alignment made. The sequencing data were compared against the Gene Bank database (http://www.ncbi.nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences on the Gene Bank database.

Accessions numbers. Sequences were deposited in GenBank under accession numbers MW029470-MW029474.

Ochratoxins production by *A. niger* isolates. Five isolates of *Aspergillus niger* with the highest number of colonies and high frequency of occurrence were tested for their ability to produce ochratoxins by cultivation in conical flasks containing 50 ml of yeast extract sucrose (YES) liquid medium with composition sucrose, 40 g, yeast extract 20 g, and distilled water, 1000 m1. Incubation the flasks at 28 °C for fifteen days[71]. Filtration through a fluted filter paper (Whatman 2 V, Whatmanplc, Middlesex,UK). Total ochratoxins were determined according to the method mentioned by El-Dawy et al.[44] in 10 ml fungal filtrate by adding 90 ml (methanol: water) (80:20 v/v) and the filtrate was diluted (1:4) with distilled water and re-filtered through a glass-fiber filter paper. Ten milliliters of the glass-fiber filtrate were placed on Ochra test WB SR Column (VICAM, Watertown, MA, USA) and allowed to elute at 1–2 drops/s. The columns were washed twice with 10 ml of distilled water, and ochratoxins were eluted from the column by adding 1 ml of methanol HPLC and delivered in clean cuvette. 1.5 ml ochratoxin eluting agent was added and the total ochratoxins concentration were measured after calibration VICAMSeries-4 fluorometer set at 360 nm excitation and 450 nm emissions[72].

Molecular detection of ochratoxin-producing genes. DNA extraction and purification were performed using DNA Promega Kit DNeasy Blood & Tissue (Valencia, CA, USA). Two published primers were used for the specific detection of ochratoxin biosynthesis genes. The sequence of primers was as following: *Aopks*-F '5-CAGACCATCGACACTGCATGC-'3, *Aopks*-R '5- CTGGCGTTCCAGTACCATGAG-'3[73]. The 630 bp fragments were amplified, PCR was performed in a reaction volume of 25 µl according to Hussein et al.[74] The reactions were done in a C1000.

Thermo Cycler BioRad, Germany with initial denaturizing at 94 °C for 5 min, followed by 30 cycles of 1 min. at 94 °C, 1 min. at 58 °C and extension at 72 °C for 1 min[43], then final step as extension at 72 °C for 10 min. PCR products were checked on a 1.3% agarose gel and stained with ethidium bromide.

Screening *A. niger* isolates for lipase production. Tween 80 agar plate was used for screening the tested isolates for lipase production containing (g/l peptone, 15; NaCl, 5; $CaCl_2$, 1; tween 80, 10 and agar, 15) and pH of the media was adjusted to 7. 250 µl of fungal spore suspension (8×10^7 spores/ ml) was inoculated to 8 mm cavity on the media and incubated at 28 °C for 4 days. Appearance of white precipitate around the fungal colony indicates the ability to produce lipase enzyme[49].

Quantitative estimation of lipase. Trimetric method was applied for assay lipase[75] with some modification. Two disks (8 mm) of tested isolates were inoculated to minimal medium containing (g/l 1 yeast extract, 1 KCl, 1 $MgSO_4.7H_2O$), pH 6 and supplemented with (1% v/v tween 80) and incubated at 30 °C on shaker incuba-

tor at 150 rpm for 3 days. 1.5 ml suspension from liquid medium was centrifuged at 3000 rpm for 10 min. Assay mixture containing 500 µl 0.1 M phosphate buffer (pH 6.8), 500 µl tween 80 and 250 µl crude enzyme was incubated at 37 °C for 20 min. Three ml of acetone: ethanol (1:1) was added to stop the reaction. The liberated fatty acids were titrated with 1 N NaOH solution with bromothymol blue indicator. In control 250 µl distilled water was added instead of crude enzyme. Lipase activity (U/ml-min) was calculated from the following equation:

$$\text{Lipase activity} = (T - C) \times N \times df/(t \times v).$$

T is the titration volume, C is the control, N is the normality of NaOH, df is the volume of assay/volume of enzyme, t is the incubation time, v is the sample.

Detection of *lip2* gene in *Aspergillus niger* isolates. *Aspergillus niger lip2* gene was detected by using 2 documented primers. The sequences of primers were as following: *P1* (5'-CTCAAGAGTATCCTGCACTG-3') and *P2* (5'-CTGAACCTTCCTTGGGATAG-3')[55]. Twenty-five µl as volume was used for PCR reaction by mixing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer, 6 µl of DNA template and 4.5 µl of water was added to make the volume up to 25 µl. Applied biosystem 2720 thermal cycler was used for performing the reaction with initial denaturizing at 94 °C for 5 min, followed by 35 cycles of 30 s. at 94 °C, 50 s. as annealing temperature at 59 °C and 1 s. at 72 °C. Ten minutes at 72 °C was used as the final extension. The PCR products were checked on 1.5% agarose gel in 1 × TBE buffer. A gelpilot 100 bp plus DNA Ladder was used to determine the fragment sizes. The gel was photographed by a gel documentation system. Data was analyzed through computer software.

Studying the virulence properties of target human pathogens in presence of crude lipase obtained from *A. niger*. *Determination of minimum inhibitory concentration (MIC).* MIC was evaluated by *p*-iodonitrotetrazolium violet choloride (INT) formazon assay (0.2 mg/ml, SIGMA-ALDRICH). Overnight cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and Methicillin-resistant *Staphylococcus aureus* (MRSA) were adjusted to OD_{595} of 0.01 into tryptic soy broth (TSB). 100 µl of each freshly prepared bacterial culture were placed into 96-well plates plus different volumes of crude lipase (10–100 µl, 8 replicates were made for each volume in complete raw). After 24 h incubation at 37 °C, to confirm bacterial growth suppression and deficiency of metabolic activity, 40 µl INT was added to the microplate wells and re-incubated at 37 °C for 30 min. The MIC in the INT assay was defined as the lowest concentration that suppressed bacterial growth and prevented color change[62,76–78].

Determination of minimum bactericidal concentration (MBC). The bactericidal efficacy was defined as a 99.9% decrease in CFU (3 logs) in the initial inoculum during 24 h of incubation. The MBC was determined by inoculating sterilized tryptic soy agar (TSA) fresh plates with 50 µl from each well of overnight MIC plates. Viable colonies were counted after 24 h at 37 °C. The limit of detection for this assay was 10 cfu ml^{-1}[62,79].

Static biofilm assay. The tested bacterial strains {*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and Methicillin-resistant *Staphylococcus aureus* (MRSA)} were obtained kindly from international Luxor hospital. The ability of the verified pathogens for biofilm formation was determined using 96-well polystyrene plates[80]. Bacterial strains were subcultured on tryptic soy agar for 24 h at 37 °C, suspended in tryptic soy broth and adjusted to an OD_{595} of 0.02. 130 µl of each adjusted isolate culture were put in the microtitre plate (U bottom, Sterilin) at 37 °C for 24 h. After incubation the wells were washed six times with distilled water, Furthermore, the wells were stained with 0.1% crystal violet for 10 min. the wells were again washed with distilled water (4 times) to remove excess stain[81]. Finally, the wells were destained by 210 µl of ethanol 96% and the OD_{595} was read using infiniteF50 Robotic (Ostrich) microplate plate to quantify the amount of biofilm.

Antibiofilm efficacy of crude lipase. The effect of crude lipase enzyme with the highest activity from *A. niger* MW029470 free of ochratoxin after 3 days of incubation as antibiofilm against four human pathogenic biofilm former bacteria was done by spectrophotometric methods. Different volumes (20, 30, 40, 50, 60, and 70 µl) were added to 130 µl of the tested pathogens at OD_{595} of 0.02 after 24 h incubation at 37 °C for allowing biofilm formation. The plates then incubated for further 24 h and then stained with crystal violet as described previously[27].

Scanning electron microscopy (SEM) analysis of antibacterial and antibiofilm efficacy of crude lipase. Preparation of samples for antibacterial was performed as described by Wang et al.[82]. While for biofilm was done as described by Kong et al.[83] and Chin et al.[84] with little modifications. Biofilms were allowed to form on the slides at 37 °C for 24 h alone (control) and after treatment with crude lipase following which, the samples were fixed in 4% (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) at 4 °C for 12 h. Subsequently, the samples were washed three times in phosphate buffer, dehydrated through a graded ethanol series, dried in a critical-point drying apparatus with liquid carbon dioxide; slides coated with gold and viewed using (JEOL JSM-5500LV, Japan).

Statistical analysis. The variability degree of results was expressed in form of means ± standard deviation (mean ± SD) based on triplicates determinations (n = 3 for replicate plates). The data were statistically analyzed by one-way ANOVA analysis and compared using the least significant difference (LSD) test at 0.05 (*) levels. It was done to compare between control and treatments.

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Author contributions

A.Y. conceived, designed the manuscript, performed the practical work and wrote some parts of the article. R.E. shared in the manuscript design, performed practical work, wrote some parts of the article, analyzed the data. M.H. assisted in the molecular identification part and gave some feedbacks about the research. All the authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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