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

Asmaa S. Yassein1*, Mohamed M. Hassan2 & Rokaia B. Elamary1

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 compounds1. They protect the mankind from the risks of obesity, cardiovascular illnesses, type 2 diabetes and hypertension2–3. *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 patients4–5.

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 diseases6,7. 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 animals8. OTA is the causative agent of Balkan endemic nephropathy, urothelial tumors, and testicular cancer in humans9–10.

Lipase, an enzyme belonging to the serine hydrolase class, catalyzes the hydrolysis of fats and oils to glycerol and fatty acids without requiring cofactors11. 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 States12. 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 increasing13.

Enzymes including isomerases, lyases, oxidoreductases, transferases, esterases and hydrolyases have been reported to induce antibacterial efficacy14. Lipase is a hydrolytic enzyme, has antimicrobial and antifouling properties15. However, its mode of action and its effects in most of the cases have not been clarified fully14.

---

1Faculty of Science, Botany and Microbiology Department, South Valley University, Qena 83523, Egypt. 2Faculty of Science, Department of Biology, Taif University, P.O.Box 11099, Taif 21944, Kingdom of Saudi Arabia. *email: asmaa.mohamed11@sci.svu.edu.eg
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. Several steps were involved in biofilm development beginning with surface adherence, microcolony formation, maturation and finally detachment stages. Biofilms protect pathogenic bacteria from human immune system, antibiotics and severe environmental conditions. Several bio-active and chemically synthesized compounds have been performed to suppress biofilm formation by pathogenic bacteria. Revitalize aminoglycosides also have been used to inhibit biofilm and pathogenic bacterial infections. Attenuating motility properties can be considered as highly potential for controlling biofilm formation since attachment was one of the main steps in biofilm formation. Using enzymes is also a good policy for biofilm elimination because enzymes are rabidly eco-friendly and degradable.

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 × 10^7 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. 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 fluorometric 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). They 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).

**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.
### 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.

| Fungal genera and species | Peanut | Almond | Coconut | Raisin |
|---------------------------|--------|--------|---------|--------|
|                           | Fungal count (CFU/g × 10^3) | %C | F% | NCI | Fungal count (CFU/g × 10^3) | %C | F% | NCI | Fungal count (CFU/g × 10^3) | %C | F% | NCI | Fungal count (CFU/g × 10^3) | %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. sydowi                 | –      | –      | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| A. terreus                | 0.51   | 2.56%  | 10 2  | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| A. ustus                  | –      | –      | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| 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   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| Echrysogenum              | 0.69   | 3.47%  | 25 5  | 0.36 | 2.14%| 35 7 | 0.57 | 3.77%| 30 6 | 0.54 | 5.79% | 35 7 |
| Edeilocoxii               | 0.12   | 0.6%   | 5 1   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| P. junicolusum            | 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. purpureogenum          | 0.12   | 0.6%   | 5 1   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| P. variabile              | 0.06   | 0.3%   | 5 1   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| Biscopus 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 atrus        | 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% | 9.33 | 100% |
| Number of genera = 12     | 11     | 5      | 5     | 5     | 5     | 5     |
| Number of species = 25    | 21     | 9      | 11    | 9     | 9     | 9     |
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,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.
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 Ismail28, 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 Ismail28,30–33. In contrast, Aspergillus section Flavi was the highest recorded in peanuts seeds followed by Aspergillus section Nigri and Aspergillus section Circumdati was the least34. Past study by Tournas et al5 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 Abdulla35, 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 al36,37. All the tested A. niger isolates were ochratoxin producers with variable readings by using fluorometric method (Table 2). In past investigation by Al-Sheikh38 confirmed that 57% and 60% of A. niger and A. carbonarius, respectively deteriorated peanut were ochratoxin producers. Magonli et al39, demonstrated that 32% of Aspergillus section Nigri isolated from peanut seeds in Argentinean had ability to produce ochratoxin A. Alhussaini40, 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 production40. Our obtained results were in-disagreement with past study by Palumbo & O’Keeffe41, reported that all the tested 171 isolates of Aspergillus section Nigri isolated from almonds showed negative results for ochratoxin A production. 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 al42, who reported that pks gene was detected in all Aspergillus niger positive ochratoxigenic 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 ochratoxin. All tested A. niger and A. tubingensis isolated from beef showed positive results for the presence of pks genes43. 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 was well-recognized to be the best producer of lipase enzyme and is favored in many industrial processes46, 47. A. niger, Fusarium oxysporum and Nectria haematococca isolated

Table 4. Antibacterial efficacy of crude lipase against some human pathogens.
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).
from beef luncheon were the highest lipase producers. Rai et al. isolated lipase producer A. niger from some oil contaminated soil samples. Earlier studies also, confirmed that A. niger was the highest lipase producing strain. Putri et al. 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. 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. 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. 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. 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. Bacterial biofilms are common populations of bacterial cells surrounded by a self-produced matrix of extracellular polymeric substances (EPS). EPS surrounding mixture include various exopolysaccharides, lipids, secreted proteins some of which can form amyloid fibers and extracellular DNA. 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. Plants contain various anti-biofilm compounds, as they have to prevent bacterial growth on their surfaces. Since lipase, an esterase, is a hydrolyzing enzyme, it is having the ability to act on the EPS produced by the organisms, by degrading protein components and the high molecular weight lipid of the biofilm. 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. Results of SEM confirmed antibacterial 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. was employed for isolation of mycobacteria 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, K<sub>2</sub>HPO<sub>4</sub>; 1, MgSO<sub>4</sub>. 7H<sub>2</sub>O; 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). The modified method described by Hermosa et al. was amplified as designed by El-Dawy et al. 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 5.1. 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. Sequences were deposited in GenBank under accession numbers MW029470.

**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 ml. Incubation the flasks at 28 °C for fifteen days. Filtration through a fluted filter paper (Whatman 2 V, Whatman plc, Middlesex, UK). Total ochratoxins were determined according to the method mentioned by El-Dawy et al. 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 micriliters 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 VICAM Series-4 fluorometer set at 360 nm excitation and 450 nm emissions.

**Molecular detection of ochratoxin-producing genes.** DNA extraction and purification were performed using DNA Promega Kit DNaseasy 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'-CAGACCATGCACACTTACGC-3', *Aopks*-R’5'-CTGGCAGTTCCAGTACCATGAG-3'. The 630 bp fragments were amplified, PCR was performed in a reaction volume of 25 µl according to Hussein et al. The reactions were done in a C1000. Thermocycler 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, 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<sub>2</sub>, 1; tween 80, 10 and agar, 15) and pH of the media was adjusted to 7. 250 µl of fungal spore suspension (8 × 10<sup>7</sup> 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.

**Quantitative estimation of lipase.** Trimetric method was applied for assay lipase with some modifications. Two disks (8 mm) of tested isolates were inoculated to minimal medium containing (g/l 1 yeast extract, 1 KCl, 1 MgSO<sub>4</sub>. 7H<sub>2</sub>O), pH 6 and supplemented with (1% v/v tween 80) and incubated at 30 °C on shaker incubation.
PCR products were checked on 1.5% agarose gel in 1 × TBE buffer. A gelpilot 100 bp plus DNA Ladder was used as annealing temperature at 59 °C and 1 s at 72 °C. Ten minutes at 72 °C was used as the final extension. The performing the reaction with initial denaturizing at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 50 s.

To study the virulence properties of target human pathogens in presence of crude lipase obtained from A. niger.  

**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’-CTCAAGATCCTGACTG-3’) and P2 (5’-CTGAACTCTCCTGGGATAG-3’). Twenty-five µl of 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.

**Detection 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.  

**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.  

**Antibiofilm efficacious 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 OD₅₉₅ 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.  

**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.
References

1. Carughn, A. et al. Pairing nuts and dried fruit for cardiometabolic health. *Nutr. J.* 5, 15–23. https://doi.org/10.1186/s12937-016-0142-4 (2015).

2. Li, M., Fan, Y., Zhang, X., Hou, W. & Tang, Z. Fruit and vegetable intake and risk of type 2 diabetes mellitus: Meta-analysis of prospective cohort studies. *BMJ Open* 4, e005497. https://doi.org/10.1136/bmjopen-2014-005497 (2014).

3. Hernandez-Alonso, P., Salas-Salvado, J., Baldrich-Mora, M., Juanola-Falgarona, M. & Bullo, M. Beneficial effect of pistachio consumption on glucose metabolism, insulin resistance, inflammation, and related metabolic risk markers: A randomized clinical trial. *Diabetes Care* 37, 3098–3105. https://doi.org/10.2337/dc14-1431 (2014).

4. Weidenborner, M. Pine nuts: The mycobiota and potential mycotoxins. *Can. J. Microbiol.* 47, 460–463 (2001).

5. Tournas, V. H., Niazis, N. S. & Kohn, J. S. Fungal presence in selected tree nuts and dried fruits. *Microbiol. Insights.* 8, 1–6. https://doi.org/10.4137/MBI.S24308 (2015).

6. Agripouloou, S., Kohiadima, A., Karaaiskakis, G. & Kapolos, J. Kinetic study of aflatoxins degradation in the presence of ozone. Food Control 61, 221–226. https://doi.org/10.1016/j.foodcont.2015.09.013 (2016).

7. Benedict, K., Chiller, T. M. & Mody, R. K. Invasive fungal infections acquired from contaminated food or nutritional supplements. *Foodborne Path. Dis.* 13(7), 343–349. https://doi.org/10.1089/fpd.2015.1208 (2016).

8. Pöhl-Lesiakowicz, A. Ochratoxin A and aristolochic acid involvement in nephropathies and associated urothelial tract tumours. *Arb. Hig. Rada.* Toksikol. 60(4), 465–483. https://doi.org/10.2478/thfud-2014-0020 (2009).

9. Wafa, E. W., Yahiya, R. S., Sobh, M. A., Eraky, I., El Baz, H., El Gayar, H. A. M., Bededer, A. M., Creppy, E. E. Human ochratoxi- cosis and nephropathy in Egypt: A preliminary study. *Hum. Exp. Toxicol.* 17, 124–129 (1998).

10. Schwartz, G. G. Hypothesis: Does ochratoxin A cause testicular cancer? *Cancer Causes Control.* 13, 91–100. https://doi.org/10.1007/ S10552-002-0123-8 (2002).

11. Singh, A. K. & Mukhopadhyay, M. Overview of fungal lipase: A review. *Appl. Biochem. Biotechnol.* 166(2), 486–520. https://doi.org/10.1007/s12010-011-9443-3 (2012).

12. Putri, D. N., Khootama, A., Perdani, M. S., Utami, T. S. & Hermansyah, H. Optimization of Aspergillus niger lipase production by solid state fermentation of agro-industrial waste. *Energy Rep.* 6, 331–335 https://doi.org/10.1016/j.egyr.2019.08.064 (2020).

13. Hasan, F., Shah, A. A. & Hameed, A. Industrial applications of microbial lipases. *Enzyme Microb. Technol.* 39(2), 235–251 (2006).

14. Kristensen, J.B., Meyer, R.L., Laursen, B.S., Shipovskov, S., Besenbacher, F. & et al.Antifouling enzymes and the biochemistry of marine settlement. *Biotechnol. Adv.* 26, 471–488 (2008).

15. Carvajal, J.C., McDaniel, C.S. & Wales, M.E. Enzymatic antimicrobial and antifouling coating and polymeric materials. In US Patent 2009/023311 (2009).

16. Prabhawatwi, V., Boobalan, T., Sivakumar, P. M. & Doble, M. Antifouling properties of intercellular active lipase immobilized porous polycaprolactam prepared by LB technique. PLoS ONE 9(5), e96152. https://doi.org/10.1371/journal.pone.0096152 (2014).

17. Achhi, N. B., Khan, F. & Kim, Y. M. Inhibition of virulence in Acinetobacterbaumannii by naturally-derived and synthetic drugs. *Curr. Drug Targets.* https://doi.org/10.2174/138945012166612013123335 (2020).

18. Khan, F., Tabassum, N., Pham, D. T. N., Olotokuyi, S. F. & Kim, Y. M. Diversity of bacteria and bacterial products as antibiofilm and pathogenic sensing drugs against pathogenic bacteria. *Curr. Drug Targets* 21(11), 1156–1179. https://doi.org/10.2174/138945012166619043161249 (2019).

19. Khan, F. et al. Chitosan and their derivatives: Antibiofilm drugs against pathogenic bacteria. *Colloids Surf. B Biointerfaces* 185, 1108–1127. https://doi.org/10.1016/j.colsurfb.2019.110627 (2020).

20. Mulat, M., Pandita, A. & Khan, F. Medicinal plant compounds for combating the multi-drug resistant pathogenic bacteria: A review. *Curr. Pharm. Biotechnol.* 20(3), 189–196. https://doi.org/10.2174/1872210513666190313513429 (2019).

21. Khan, F., Lee, J. W., Jiaavd, A., Park, S. K. & Kim, Y. M. Inhibition of biofilm and virulence properties of Pseudomonas aeruginosa by sub-inhibitory concentrations of aminglosides. *Microb. Pathog.* 146, 104249. https://doi.org/10.1016/j.micpath.2020.104249 (2020) (Epub 2020 May 11).

22. Khan, F., Pham, D. T. N., Olotokuyi, S. F. & Kim, Y. M. Regulation and controlling the pathogenic properties of Pseudomonasaerugi- nosa. Appl. Microbiol. Biotechnol. 104(1), 33–49. https://doi.org/10.1007/s00253-019-10201-w (2020).

23. Khan, F., Tabassum, N., Anand, R. & Kim, Y. M. Mycobiota and mycotoxins of nut products (pistachio, peanut, hazelnut and almond) in Tehran, Iran. *Pak. J. Nutr.* 6(5), 460–462 (2007).

24. Khosravi, A. R., Shokri, H. & Ziglari, T. Evaluation of fungal flora in some important nut products (pistachio, peanut, hazelnut and almond) from grapes. *Int. J. Food Microbiol.* 111(Suppl 1), S22–S27. https://doi.org/10.1016/j.ijfoodmicro.2006.03.009 (2006).

25. Botton, A. et al. A cDNA-AFLP approach to study ochratoxin A production in Aspergillus carbonarius. *Int. J. Food Microbiol.* 127, 105–115. https://doi.org/10.1016/j.jfoodmicro.2008.06.037 (2008).

26. Xavier, J. B., Picioreanu, C., Rani, S. A., Van Loosdrecht, M. C. M. & Stewart, P. S. Biofilm control strategies based on enzymatic marine settlement. *Microbiology* 151, 3817–3832 (2005).

27. Elmary, K. & Salem, W.M. Optimizing and purifying extracellular amylase from soil bacteria to inhibit clinical biofilm-forming bacteria. *PeerJ* 8, e10288. https://doi.org/10.7717/peerj.10288 (2020).

28. Ismail, M. A. Deterioration and spoilage of peanuts and desiccated coconuts from two Saharan tropical east african countries due to the associated mycobiota and their degradative enzymes. *Mycopathologia* 150(2), 67–84. https://doi.org/10.1007/s11036-008-0756-2 (2001).

29. Abdualla, N. Q. F. Evaluation of fungal flora and mycotoxin in some important nut products in Erbil local markets. *Res. J. Environ. Earth Sci.* 5(3), 330–336 (2013).

30. Perez, G., Susca, A., Epifani, E. & Mule, G. AFLP characterization of Southern Europe population of Aspergillus section Nigri from grapes. *Int. J. Food Microbiol.* 111(Suppl 1), S22–S27. https://doi.org/10.1016/j.jfoodmicro.2006.03.009 (2006).

31. Botton, A. et al. A cDNA-AFLP approach to study ochratoxin A production in Aspergillus carbonarius. *Int. J. Food Microbiol.* 127, 105–115. https://doi.org/10.1016/j.jfoodmicro.2008.06.037 (2008).
38. Al-Sheikh, H. M. LAMP-PCR detection of ochratoxigenic Aspergillus species collected from peanut kernel. Genet. Mol. Res. (GMR) 14(1), 634–644. https://doi.org/10.4238/2015.January.30.5 (2015).

39. Magonl, C., Astoraca, A., Posone, M.L., Fernandez-Juri, M.G., Barberis, C. & Dalcero, A.M. Ochratoxin A and Aspergillus section Nigri in peanut seeds at different months of storage in Cordoba, Argentina. J. Food Microbiol. 1, 119(3), 213–8, https://doi.org/10.1016/j.jfoodmicro.2007.07.056 (2007).

40. Yassein, A. S., El-Said, A. H. M. & El-Dawy, E. G. A. Biocontrol of toxigenic Aspergillus strains isolated from baby foods by essential oils. Flavour Frag. J. 35, 182–189. https://doi.org/10.1002/jf.3551 (2020).

41. Palumbo, J. D. & O’Keeffe, T. L. Distribution and mycotoxicogenic potential of Aspergillus section Nigri species in naturally contaminated almonds. J. Food Prot. 76(4), 702–706. https://doi.org/10.4315/0362-028X-JFP-12-431 (2013).

42. Massi, F. P., Sartori, D., Ferranti, L. de S., Imanaka, B. T., Taniwak, M. H. Vieira, M. L. C. & Fungaro, M. H. P. Prospecting for the lipolytic activity of the lipase produced by Aspergillus niger MTCC 2594. J. Gen. Appl. Microbiol. 53(4), 247–253. https://doi.org/10.2322/jgam.53.247 (2007).

43. Al-Sheikh, H. M. LAMP-PCR detection of ochratoxigenic Aspergillus niger. Biomed. Biotechnol. 21(3), 54–59. 10.12691/bb-2-3-3 (2014). https://pubs.scientific.net/bb/2-3-3.

44. Falony, G., Arnas, J. C., Mendoza, J. C. D. & Hernandez, J. L. M. Production of extracellular lipase from Aspergillus niger by solid state fermentation. Food Technol. Biotechnol. 44(2), 235–240 (2006).

45. Mahdik, N. D., Puntambekar, U. S., Bastawde, K. B., Khire, J. M. & Gokhale, D. V. Production of acidic lipase by Aspergillus niger in solid state fermentation. Proc. Biochem. 38(5), 715–721 (2002).

46. Ola, Z. A. & El-Sabaeny, A. H. Lipase production by Aspergillus niger under various growth conditions using a solid state fermentation. Microbiologia. (Madrid) 9, 134–141 (1993).

47. Kamin, N. R., Mala, J. G. S. & Puvanakrishnan, P. Lipase production from Aspergillus niger by solid state fermentation using jaggery oil cake. Process. Biochem. 35, 501–511 (1999).

48. Saleem, A. Effect of some food preservatives on lipolytic activity of beef luncheon fungi. Mycobiology. 36, 167–172 (2008).

49. Rai, B., Shreshtha, A., Sharma, S. & Joshi, J. Screening, optimization and process scale up for pilot scale production of lipase by Aspergillus niger. Biomed. Biotechnol. 21(3), 54–59. 10.12691/bb-2-3-3 (2014). https://pubs.scientific.net/bb/2-3-3.

50. Lequerte, Y., Boeb, G., Clarisse, M. & Fauill, C. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling 26, 421–431. https://doi.org/10.1080/09270964.2010.512330 (2010).

51. Davies, D. Understanding biofilm resistance to antibacterial agents. Nat. Rev. Drug Discov. 2, 114–122 (2003).

52. Pruteanu, M., Hernández Lobato, J. I., Stach, T. & Hengge, R. Common plant flavonoids prevent the assembly of amyloid curli. FEMS Microbiol. Lett. 9, 29–33 (1980).

53. Reifsteck, F., Ridgway, H. & Olson, B. H. Evaluation of cleaning strategies for removal of biofilms from reverse-osmosis membranes. Appl. Environ. Microbiol. 48, 395–403 (1984).

54. Wickert, K., Papkov, A. & Cossart, Y. Removal of biofilm from endoscopes: Evaluation of detergent efficiency. Am. J. Infect. Control 32, 170–176. https://doi.org/10.1016/j.ajic.2003.10.009 (2004).

55. Vyas, N., Sammons, R. L., Addison, O., Dehghani, H. & Walmsley, A. D. A quantitative method to measure biofilm removal efficiency from complex biomaterial surfaces using SEM and image analysis. Sci. Rep. 6, 32694. https://doi.org/10.1038/srep32694 (2016).

56. Domsh, K. H., Gams, W. & Anderson, T. H. Compendium of Soil Fungi, Taxonomically Revised by W. Gams. 672 (IHW , 2007). https://doi.org/10.1017/9780511612541.

57. Hermosa, M. R. et al. Genetic diversity shown in Trichoderma biocontrol isolates. Mycol. Res. 108, 897–906. https://doi.org/10.1017/S002703710300130X (2004).

58. Gubal, M. A., Hegazy, S. M. & Naga, Y. H. Aflatoxin production by Aspergillusflavus field isolates. Vet. Hum. Toxicol. 39, 519–521 (1994).

59. Lewis, L. et al. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environ. Health Perspect. 113(12), 1763–1767. https://doi.org/10.1289/ehp.7998 (2005).

60. Reddy, K. V., Naveen, K. & Reddy, B. Incidence and molecular detection of ochratoxigenic fungi from Indian cereal grains. Int. J. Pharm. Biol. Sci. 4(3), 31–40 (2013).

61. Hussein, M. A., El-Said, A. H. M. & Yassein, A. S. Mycobacteria associated with strawberry fruits, their mycotoxin potential and pectinase activity. Mycologia 11(2), 158–166. https://doi.org/10.1080/21501203.2017.1597919 (2020).

62. Elegado, F., Legaspi, C. L., Paet, J. M., Quelubin, F., Tolentino, J. E., Vilela, J., Jr, A. P., Maloles, J. & Zarate, J. Screening, identification and optimization of extracellular lipase production of yeast (Cryptococcus flavescens) isolated from a tree canopy fern in the mount Makiling forest reserve, Philippines. In AIP Conference Proceedings Vol. 2155 (1), 020029, https://doi.org/10.1063/5125333 (AIP Publishing LLC, 2019).

63. Ellof, J. N. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. 46, 711–713 (1998).
77. Lall, N., Henley-Smith, C.J., De Canha, M.N., Oosthuizen, C.B. & Berrington, D. Viability reagent, presto blue, in comparison with other available reagents, utilized in cytotoxicity and antimicrobial assays. *Int. J. Microbiol.* (2013).

78. Salem, W.M., El-Hamed, D.S., Sayed, W. & Elamary, R. Alterations in virulence and antibiotic resistant genes of multidrug-resistant Salmonella serovars isolated from poultry: The bactericidal efficacy of *Allium sativum*. *Microb. Pathog.* 108, 91–100. https://doi.org/10.1016/j.micpath.2017.05.008 (2017).

79. Sirelkhatim, A. et al. Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism. *Nano-Micro. Lett.* 7(3), 219–242 (2015).

80. Khan, E. et al. Streptomycin mediated biofilm inhibition and suppression of virulence properties in *Pseudomonas aeruginosa* PAO1. *Appl. Microbiol. Biotechnol.* 104(2), 799–816. https://doi.org/10.1007/s00253-019-10190-w (2020) (epub 2019 Dec 9).

81. Merritt, D.J., Turner, S.R., Commander, L.E. & Dixon, K.W. (eds). *Proceedings of the Fifth Australian Workshop on Native Seed Biology Brisbane, Australia* (2005).

82. Wang, J., Ma, M., Yang, J., Chen, L., Yu, P., Wang, J. & Zeng, Z. In vitro antibacterial activity and mechanism of monocaprylin against *Escherichia coli* and *Staphylococcus aureus*. *J. Food Prot.* 81(12), 1988–1996 (2018).

83. Kong, C. et al. Suppression of *Staphylococcus aureus* biofilm formation and virulence by a benzimidazole derivative, UM-C162. *Sci. Rep.* 8(1), 1–16 (2018).

84. Chin, C. Y., Hara, Y., Ghazali, A. K., Yap, S. J., Kong, C., Wong, Y. C., & Nathan, S. Global transcriptional analysis of *Burkholderia pseudomallei* high and low biofilm producers reveals insights into biofilm production and virulence. *BMC Genomics* 16(1), 471(2015).

Acknowledgements
Special thanks to South Valley University, Qena, Egypt. The authors also, extended their appreciation to Taif University, Saudi Arabia for funding this work through the program of Taif University Researchers Supporting Project number (TURSP-2020/59), Taif University, Taif, Saudi Arabia.

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
Correspondence and requests for materials should be addressed to A.S.Y.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021