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A new report on gene expression of three killer toxin genes with antimicrobial activity of two killer toxins in Iraq

Najwa Mohammed Jameel Ali Abu-Mejdad[1], Adnan I. Al-Badran[1], Abdullah H. Al-Saadoon[2] and Mohammed Hussein Minati[3*]

Abstract

Background: The K1, K2, and K28 toxins are usually encoded by several cytoplasmically genetic satellite dsRNAs (M1, M2, and M28), which are encapsulated with virus-like particles (VLPs) and reliant on an additional assembly of assistant yeast viruses (L-A) for their reproduction and encapsidation. Ascomycetous yeast species that have these VLPs are especially attractive targets for finding killer toxins like proteins. This is because the organisms are known in producing a large variety of secondary metabolites and extracellular enzymes, which have medical importance as alternative drugs for resistance bacterial strains, particularly multi-resistance drugs (MRD).

Results: For the first time, 31 type strains of yeasts were tested for killer toxin production in Iraq via the measurement of gene expression of three killer toxin genes (M1, M2, and M28) within the mycovirus in yeasts. All the type strains gave an expression for the three killer toxins with variable levels. The highest expression was recorded for the killer toxin genes in *Torulaspora delbrueckii* followed by *Wickerhamomyces anomalus*. Determined antibacterial activity of two killer toxins appeared with high inhibition zone against pathogenic strains of bacteria. Cytotoxicity against human blood cells was not found. These results considered the first record of killer toxins isolated from type strains in Iraq.

Conclusion: The two typical strains *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* showed the highest level of gene expression for the three killer toxins.

Keywords: Antibacterial activity, Cytotoxicity, Gene expression, Mycovirus, Killer toxins, RT-PCR, Yeasts

Background

Gene expression of killer toxins

The K1, K2, and K28 toxins are usually encoded by several cytoplasmically genetic satellite dsRNAs (M1, M2, and M28), which are encapsulated with virus-like particles (VLPs) and reliant on an additional assembly of assistant yeast viruses (L-A) for their reproduction and encapsidation. The responsibility of M dsRNAs is confined to both killer activity and self-immunity, especially in phenotypic yeast strains that are characteristic in their producing killer toxins (Vepštaitė-Monstavičė et al., 2018). Moreover, these toxins are capable of killing yeasts with non-killer activity in addition to yeasts of the reverse killer group, whereas the generating yeasts stay immune to their private toxins and to that formed by other strains of identical killer class. Along with the statements above, the system of killer strains provides a distinctive model for studying as long as infection by L-A virus is accompanying with the existence of a satellite M dsRNA, enveloped in a capsid that is encoded by a helper virus. L-As are independently reproducing viruses that do not need M RNA for their replication (Becker & Schmitt, 2017).

As any virus, the yeast L-A and M viruses exploit the host's translation machinery to produce proteins for virus capsid assembly, dsRNA replication, and toxin

* Correspondence: abo_azher70@yahoo.com
[3]Biology Department, College of Education-Qurna, University of Basra, Basra, Iraq
Full list of author information is available at the end of the article

synthesis. However, dissimilar to other viruses, the yeast virus presence in the cell is symptomless (causes neither growth disadvantage nor death of the cell) (Kyrychenko et al., 2018). The killer phenotype in *Saccharomyces cerevisiae* is strictly associated with the presence of dsRNA viruses belonging to the *Tortiviridae* family, the best characterized class of mycoviruses (Marquina et al., 2002).

Killer toxins of *Candida kruseii*, isolated from fermented vegetables, exhibited growth inhibition against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Bacillus cereus (Waema et al.,* 2009*)*. The killer activity of *Saccaromyces cerevisiae* against bacterial strains was reported by Valzano et al. (Valzano et al., 2016).

However, the ability of killer toxin from *D. hansenii* DSMZ70238 to kill *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes* was recently demonstrated by Al-Qaysi et al. (Al-Qaysi et al., 2017). Ascomycetous yeast species are especially attractive targets for finding a killer toxin like protein. This is because the organisms are known in producing a large variety of secondary metabolites and extracellular enzymes, which have medical importance as alternative drugs for resistance bacterial strains, especially multi-resistance drugs (MRD) (Liu et al., 2015). However, based on this background, we searched for killer toxins produced by ascomycetous yeasts in soil by using two type strains of yeasts (*Torulaspora delbrueckii* and *Wickerhamomyces anomalus*) as test strains. Therefore, the aim of this study was to identify and measure the level of gene expressions that are responsible for coding to killer toxins in 31 different type strains with antibacterial activity and cytotoxicity of two killer toxins.

Methods

Extraction of total RNA

Total RNA were extracted from each yeast isolate according to the procedure of SV total RNA isolation system (Promega, USA).

Reverse transcription of RNA

Total RNA of 300 ng from each sample was used generated cDNA by GoTaq 2 step RT-qPCR system (Promega, USA). Thermal cycler condition for cDNA synthesis was represented annealing temperature 25 °C for 5 min, incubation 42 °C for 1 h, and inactivate the reverse transcriptase at 70 °C for 15 min with 1 cycle.

Quantitative real-time PCR

The cDNA served as templates for quantitative real-time PCR (qRT-PCR) that was performed by using SYBER-Green PCR core reagents. A total of 30 ng of cDNA of each sample was used to measure the gene expression of M1, M2, M28, and ACT1 as a housekeeping gene and primers for relative gene expression as shown in Table 1.

The volume for each single reaction was GoTaq qPCR Master 10 μl, forward primer 1 μl, reverse primer 1 μl, and cDNA template 8 μl. DNA amplification was performed under the following reaction conditions: an initial heating cycle of 95 °C for 2 min, 40 cycles alternating between denaturation at 95 °C for 15 s, and primer annealing and extension at 60 °C for 15 min. The data was analyzed by calculating the expression level of the genes of interest using cycle threshold number (CT) value. Relative quantification of the expression of M1, M2, and M28 was obtained using ACT1 as housekeeping gene, and the expression level of each gene was calculated according to a Livak method (Livak & Schmittgen, 2001) as follows:

$$\Delta CT = CT_{(\text{target gene})} - CT_{(\text{reference gene})}$$

Yeast strains

The killer yeast strains *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* were isolated from soil, Basra, Iraq.

Pathogenic bacteria type strains

E. coli and *Staphylococcus aureus* were obtained as two type strains, and their identification was based on 16

Table 1 Candidate housekeeping gene and their primer sequence for qRT-PCR

Primer's name	*Primer's sequence 5′ to 3′*	*Size*	*Primer's orientation*
M1	5′-TCAAAAGATCAGACACAGCCGA-3′	900 bp	Forward
	5′-GATACCATGCTCGCACATAGGA-3′		Reverse
M2	5′-AGTGGGTGTCATACTCGGTCTA-3′	581 bp	Forward
	5′-CAGCTGCGCATACTTTCTGATC-3′		Reverse
M28	5′-CGCAACAGAAAGAAGCAATGGA-3′	865 bp	Forward
	5′-AGCCGCCATCCTGATAATAAGG-3′		Reverse
Housekeeping gene ACT1	5′-CTTCCGGTAGAACTACTGGT-3′	520 bp (Frohloff et al., 2001)	Forward
	5′-CCTTACGGACATCGACATCA-3′		Reverse

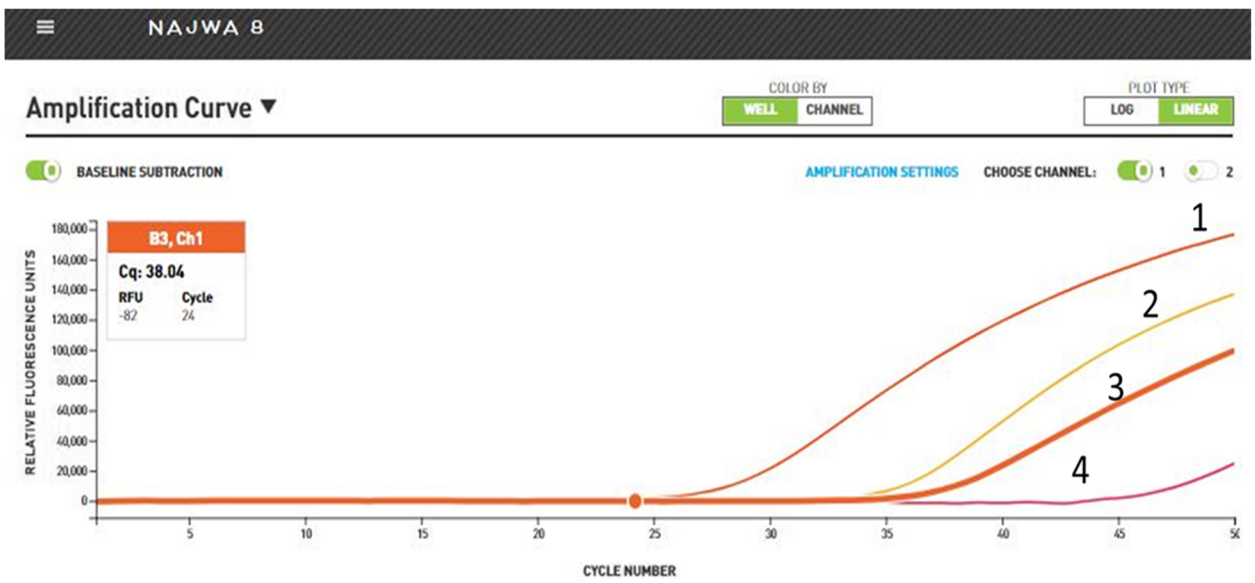

Fig. 1 The fluorescent Ct curve for *Kt* (1 = M28, 2 = M1, 3 = M2, and 4 = ACT) genes for *Torulaspora delbrueckii*

rDNA in bacteriology Lab, Biology Department, College of Science, University of Basra, from burn infections.

Culture media

Yeast malt extract broth (YMB) with 1% SDS: yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g, SDS 2 gm, and distilled water 1000 ml (Jorgensen et al., 2015). Nutrient agar (Himedia, India) was used for antibacterial activity and activation pathogenic bacteria. Potato dextrose Agar (Oxoid, UK) was used for activating type strains of yeasts.

Isolation and purification of two killer toxins (TK, WK)

During this study, isolation and purification of the two killer toxins (TK and WK) were done by Abu-Mejdad et al. (Abu-Mejdad, 2019).

Antibacterial activity

Susceptibility test of killer toxins as antibacterial

Twenty-five microliters from the suspension of bacteria (*Staphylococcus aureus* and *E. coli*) that was prepared previously was added to the nutrient agar for bacteria and then spread by L-shaped glass spreader. The plates

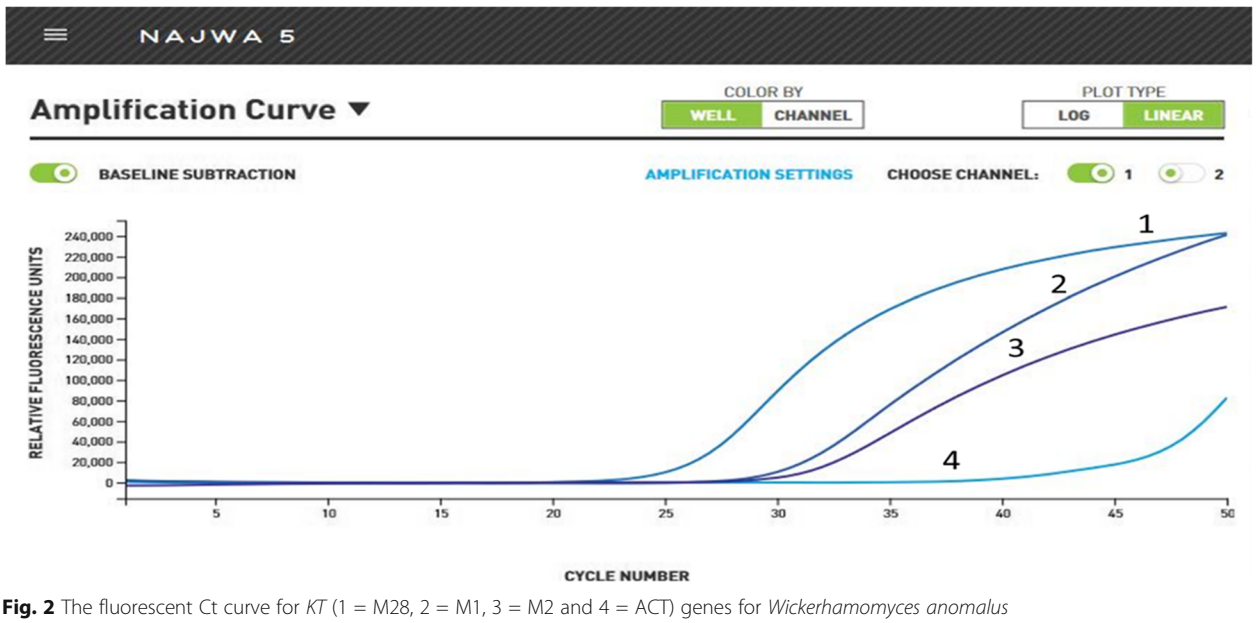

Fig. 2 The fluorescent Ct curve for *KT* (1 = M28, 2 = M1, 3 = M2 and 4 = ACT) genes for *Wickerhamomyces anomalus*

Table 2 Relative gene expression of M28 gene for 31 strains

No	Type strains	Gene expression
1	*Wickerhamomyces anomalus*	2141218
2	*Torulaspora delbrueckii*	260333.2
3	*Meyerozyma caribbica*	205674
4	*Cnadida tropicalis*	193235.4
5	*Lodderomyces elongisporus*	145433.5
6	*Debaryomyces hansenii*	93326.55
7	*Geotrichum candidum*	72214.45
8	*Galactomyces pseudocandidum*	48308.85
9	*Symmetrospora folicola*	42642.37
10	*Hanseniaspora uvarum*	31651.8
11	*Rhodotorula diobovata*	30786.28
12	*Naganishia albida*	28329.16
13	*Rhodotorula mucilaginosa*	24322.43
14	*Nagishiana vishniacil*	21469.49
15	*Vishniacozyma carnescens*	15716.58
16	*Yarrowia lipolytica*	15076.35
17	*Candida glabrata*	13682.08
18	*Cystobasidium minutum*	11425.74
19	*Galactomyces reessii*	9345.136
20	*Naganishia uzbekistanensis*	7486.107
21	*Naganishia albidosimilis*	5220.6
22	*Cutaneotrichosporon dermatis*	4837.346
23	*Naganishia liquefaciens*	4011.706
24	*Cystobasidium benthicum*	2091.033
25	*Filobasidium oeirense*	2033.853
26	*Candida membranifaciens*	1710.26
27	*Wickerhamomyces onychis*	1530.726
28	*Naganishia adeliensis*	321.7954
29	*Naganishia diffluens*	143.0128
30	*Aureobasidium melanogenum*	97.00586
31	*Pichia fermemtans*	32

Table 3 Relative gene expression of M1 gene for 31 strains

No.	Type strains	Gene expression
1	*Wickerhamomyces anomalus*	58251.19395
2	*Torulaspora delbrueckii*	27175.14288
3	*Pichia fermemtans*	4240.445128
4	*Debaryomyces hansenii*	1389.162831
5	*Symmetrospora folicola*	1323.369299
6	*Candida membranifaciens*	837.531708
7	*Lodderomyces elongisporus*	749.6118763
8	*Candida glabrata*	588.1335578
9	*Naganishia vishniacil*	467.8817025
10	*Geotrichum candidum*	410.1477773
11	*Cnadida tropicalis*	308.6868039
12	*Hanseniaspora uvarum*	247.2797002
13	*Galactomyces pseudocandidum*	240.5178238
14	*Naganishia adeliensis*	230.7201184
15	*Vishniacozyma carnescens*	157.5864849
16	*Yarrowia lipolytica*	151.1670607
17	*Wickerhamomyces onychis*	123.6398501
18	*Naganishia albidosimilis*	114.5632091
19	*Rhodotorula diobovata*	107.6347412
20	*Naganishia liquefaciens*	105.4196502
21	*Rhodotorula mucilaginosa*	97.68058937
22	*Galactomyces reessii*	63.11889309
23	*Filobasidium oeirense*	40.22442798
24	*Cystobasidium benthicum*	36.50443891
25	*Naganishia uzbekistanensis*	31.55944654
26	*Meyerozyma caribbica*	27.28431654
27	*Cutaneotrichosporon dermatis*	6.408559021
28	*Candida membranifaciens*	3.226567037
29	*Naganishia adeliensis*	0.301451957
30	*Naganishia diffluens*	0.141610486
31	*Aureobasidium melanogenum*	0.028955877

were left to dry for 15 min at room temperature and then wells with 6-mm diameter were made using cork-borer. After that, 100 μl of killer toxins (TK and WK) at concentration 200 mg/ml DW was added individually to each well and incubated at 37 C° for 24 h for bacteria. The results were read by measuring the inhibition zone diameter in mm (Fakruddin et al., 2017). The positive control used in this experiment was tetracycline for bacteria.

Cytotoxicity test

Cytotoxicity of killer toxins against human red blood corpuscles RBCs was tested according to (Hsiao et al., 2014) in a concentration 0.2 g dissolved with phosphate-buffered saline. The phosphate-buffered saline was used as a control sample.

Results

Gene expression detection of three killer toxins (M1, M2, and M28)

The gene expression for killer toxins (KT) were detected by real-time PCR. Extracting total RNA of 31 strains, then cDNA was synthesized for 31 strains from the total RNA for real-time PCR reaction. The results from real-time PCR were illustrated by Ct for killer toxins (M1, M2, and M28 with reference gene ACT) genes for *Wickerhamomyces anomalus* and *Torulaspora delbrueckii.* The results showed that the gene expression of M28 for

Table 4 Relative gene expression of M2 gene for 31 strains

No.	Type strains	Gene expression
1	*Wickerhamomyces anomalus*	35610.13
2	*Torulaspora delbrueckii*	2665.148
3	*Cnadida tropicalis*	1209.336
4	*Meyerozyma caribbica*	522.7582
5	*Hanseniaspora uvarum*	146.0178
6	*Nagishiana vishniacil*	73.51669
7	*Symmetrospora folicola*	70.52193
8	*Lodderomyces elongisporus*	53.81737
9	*Galactomyces pseudocandidum*	44.01734
10	*Cystobasidium minutum*	43.71329
11	*Geotrichum candidum*	35.75319
12	*Candida membranifaciens*	33.59093
13	*Candida glabrata*	26.90869
14	*Vishniacozyma carnescens*	25.99208
15	*Debaryomyces hansenii*	22.00867
16	*Rhodotorula mucilaginosa*	21.85664
17	*Naganishia albidosimilis*	19.83532
18	*Rhodotorula diobovata*	17.5087
19	*Naganishia uzbekistanensis*	15.34823
20	*Yarrowia lipolytica*	13.08643
21	*Galactomyces reessii*	12.295
22	*Cystobasidium benthicum*	9.781122
23	*Filobasidium oeirense*	7.361501
24	*Wickerhamomyces onychis*	5.278032
25	*Naganishia adeliensis*	5.133704
26	*Naganishia albida*	5.063026
27	*Pichia fermemtans*	3.182146
28	*Naganishia liquefaciens*	2.496661
29	*Naganishia diffluens*	1.717131
30	*Aureobasidium melanogenum*	1.214195
31	*Cutaneotrichosporon dermatis*	0.752623

two tested strains was highest compared with M1 and M2, which showed less gene expression, respectively, as shown in Figs. 1 and 2.

Meanwhile, the results of M28, M1, and M2 gene expression showed that *Wickerhamomyces anomalus* and *Torulaspora delbrueckii* had the highest expression compared to the remaining 29 isolates that appeared in less gene expression, as shown in Tables 2, 3, and 4.

The high value of gene expression was compared with the lowest value to determine the fold change for *Wickerhamomyces anomalus.* The fold change values for the genes M28, M1, and M2 were 66.913, 2011.714, and 47314.69, respectively, while the fold change of *Torulaspora delbrueckii* for the genes M28, M1, and M2 were 8.135, 938.497, and 3541,146, respectively. These results clarified the fold change for all genes. The highest fold change value occurred in *Wickerhamomyces anomalus* compared to *Torulaspora delbrueckii.* This means that the *Wickerhamomyces anomalus* has a high ability in production amount compared with *Torulaspora delbrueckii*, as shown in Table 5.

Table 5 The fold change of three genes for *Wickerhamomyces anomalus* and *Torulaspora delbrueckii*

Isolates	M28 gene	M1 gene	M2 gene
Wickerhamomyces anomalus	66.913	2011.714	47314.69
Torulaspora delbrueckii	8.135	938.497	3541.146

Antibacterial activity of TK and WK killer toxins

The antimicrobial activity of TK and WK compounds at 200 mg/ml was tested against *E. coli* and *Staphylococcus aureus*. The results showed that the WK compound had the higher inhibitory effect against all tested isolates in comparison with TK. The results were also compared with positive control compounds (tetracycline) as antibacterial compound, which was used at 200 mg/ml concentration, as shown in Table 6 and Figs. 3 and 4.

The cytotoxicity test of killer toxin

Table 7 and Fig. 5 show the results of killer toxins that had no toxicity against the human red blood cells with the concentration 0.2 g/ml by using positive control tap water and negative control phosphate-buffered saline (PBS).

Our results showed that WK and TK were non-hemolytic for blood and consequently are considered nontoxic to human blood.

Discussion

Relative gene expression

Previous studies such as Rodríguez-Cousiño et al. (Rodríguez-Cousiño et al., 2011) have indicated the role of genes (M1, M2, and M28) in encoding proteins called K1, K2, and K3, which are produced from non-yeast species but are similar in their production of proteins. These genes are important for encoding proteins responsible for lethal activity. In the recent study, two species

Table 6 Rates of inhibition zone diameters of WK and TK against bacterial clinical isolates.

No.	Bacterial species	Inhibition zone rates (mm)	
		WK	TK
1	*Staphylococcus aureus*	34	28
2	*E. coli*	23	20
3	Tetracycline	Zero	Zero

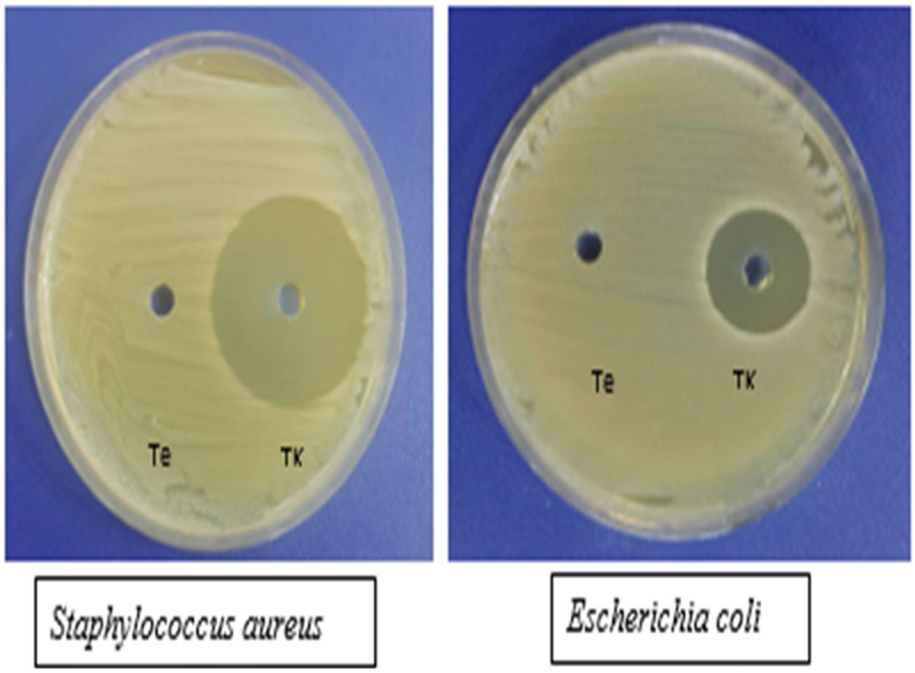

Fig. 3 Antimicrobial activity of TK for *Torulaspora delbrueckii* against *Staphylococcus aureus* and *E.coli* at a concentration of 200 mg/ml

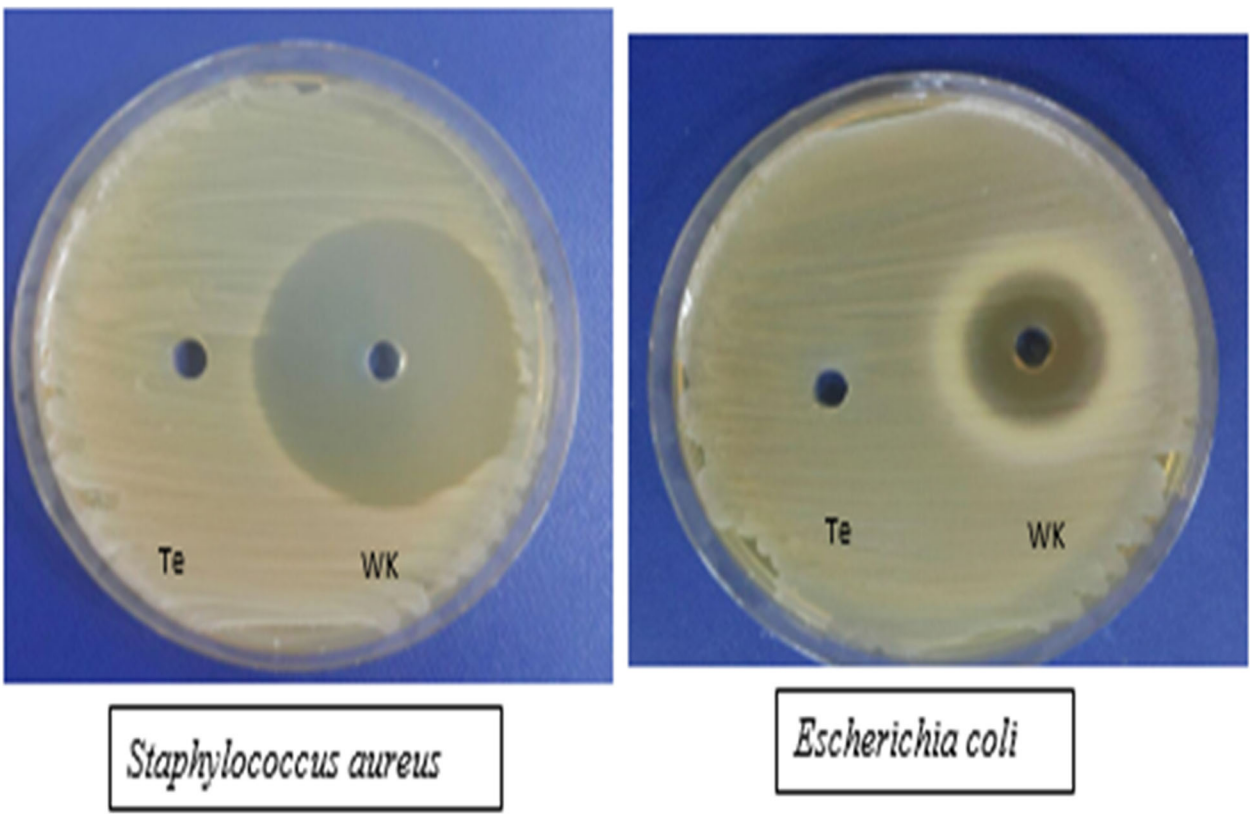

Fig. 4 Antimicrobial activity of WK for *Wickerhamomyces anomalus* against *Staphylococcus aureus* and *E.coli* at a concentration of 200 mg/ml

Table 7 Cytotoxicity test of killer toxins in concentration 0.2 g/ml

Compound	Toxicity against RBC
TK	−ve
WK	−ve
Tap water	+ve
PBS	−ve

−ve 0% lysis, *+ve* lysis 100%

showed a high expression of these genes compared to the remaining 29 isolates, which showed a less expression.

In general, gene expression (inhibition or stimulation) among different strains may be caused by abiotic factors, such as temperature, salinity, and acidity (Chen & Chou, 2017). This is consistent with the results of the current study, as the isolates were selected from high salinity and acidic soils as well as testing their ability in the lab on salt tolerance using YPGA + NaCl medium. Another explanation is attributed to the influence of competition mechanisms where the yeasts found in an extreme environment, the higher genetic expression of these toxins appeared as a defense mechanism and vice versa (Wisniewski et al., 2007).

Or maybe due to genetic factors as these toxins are coded from different genetic sources. Yeast may express such genes themselves by using their genome, mitochondrial genome, and plasmid or sometimes depend on the coexistent virus, which is found in all members of Eumycota Kingdom (King et al., 2011). However, the predominance of expressed genetic sources differed from strain to another or sometimes the yeasts use more than one source, which affects the level of gene expression negatively or positively. Previous studies have indicated the role of dsRNA in gene expression for three toxins (Ramírez Fernández et al., 2015; Schaffrath et al., 2018) in particular for the two species of fungi (*Wickerhamomyces anomalus* and *Torulaspora delbrueckii*). This result is concordant to the present study, which demonstrated the role of symbiotic virus.

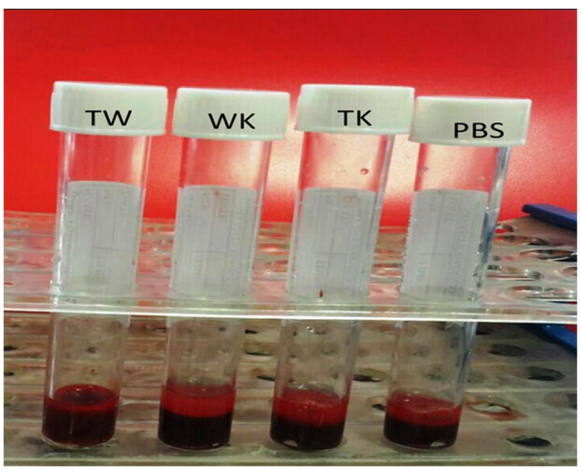

Fig. 5 The cytotoxicity test of two killer toxins. TW tap water (positive control), TK *Torulaspora delbrueckii* killer toxin, WK *Wickerhamomyces anomalus* killer toxin, PBS phosphate-buffered saline (negative control)

Antibacterial activity of TK and WK killer toxins

In this study, the TK and WK toxins with a concentration of 200 mg/ml were tested against 2 clinical isolates. In general, the results showed that the effect of WK was higher than that of TK and the reason may be attributed to the differences in the chemical composition of their compounds and in mechanism action (Becker & Schmitt, 2017). In terms of K28, early studies in the nineties have demonstrated that a specific primary receptor in the cell wall allows rapid K28 absorption via an energy-independent binding mechanism. In contrast, Vadasz et al. (Vadasz et al., 2000) stated that the ionophoric killer toxin (K2) has the ability of killing cells by causing shrinkage as a characteristic of cytosol efflux, which can be seen clearly by an electron microscope. The cytosolic efflux and cell death are the results of initial interaction between yeast toxin and specific cell wall receptors, followed by the formation or activation of endogenous ion channels in the plasma membrane of target cells (Ahmed et al., 1999).

After different purification methods, the standard antibodies were not showing any efficacy against the tested isolates, which were 100% resistant. Our results also showed that the activity of killer toxins against Gram-positive bacteria was higher than those with Gram negative. This might be attributed to the nature of their wall. Sasaki et al. (Sasaki et al., 1984) studied 150 strains of *Saccharomyces* for inhibitive activity against Gram-positive and Gram-negative bacteria and found only eleven yeasts inhibiting all tested bacteria. The active mechanism of killer toxins may change the cell cycle arrest in G1 phase, increase the membrane permeability of ions, generate ion channel, damage the plasma membrane, and inhibit the glucan synthesis (Buzzini et al., 2007).

The cytotoxicity of WK and TK killer toxins

The cytotoxicity test is known as one of the most biological evaluation and screening tests that use blood cells in vitro to observe the cell morphological effects by medical materials, such as killer toxins. During this study, the two toxins were not displaying any hemolysis on blood cells. This is in accordance with Rodríguez et al. (Rodríguez et al., 2014), who

reported that the peptide had no hemolytic effect on the blood cells. Another reason is that the high specificity of killer toxins generated from the producer killer cell may secret toxins binding only with a specific receptor in the sensitive cell resulted from two cells of the same species but they differ genetically (Chang et al., 2015).

Conclusion

The two typical strains *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* showed the highest level of gene expression for the three killer toxins. All isolates of the non-*Saccharomyces cerevisiae* that tested for gene expression of the three toxins gave uneven positive results indicating the presence of mycovirus in all the examined isolates. Also, two purification killer toxins exhibited antibacterial activity with no toxicity against blood cells.

Abbreviations

ACT1: Actin 1; cDNA: Complementary deoxynucleic acid; CT: Cycle thresholder; dsRNA: Double strand ribonucleic acid; DW: Distilled water; *E. coli*: *Escherichia coli*; K1: Killer toxin 1; K2: Killer toxin 2; K28: Killer toxin 28; MRD: Multi-resistance drugs; PBS: Phosphate-buffered saline; qRT-PCR: Quantitative real-time PCR; RNA: Ribonucleic acid; RT-PCR: Reverse transcript polymerase chain reaction; SDS: Sodium dodecyl sulfate; Te: Tetracycline; TK: *Torulaspora delbrueckii* killer toxin; TW: Tap water; UK: United Kingdom; USA: United State America; VLPs: Virus-like particles; WK: *Wickerhamomyces anomalus* killer toxin; YPGA: Yeast extract peptone glucose Agar medium

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Authors' contributions

All work was done by NA and MM under the supervision and advice of AA and AA. NA has drafted the work and MM was a major contributor in substantively editing, revising, and writing the manuscript. The authors read and approved the final manuscript.

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Author details

[1]Biology Department, College of Science, University of Basra, Basra, Iraq. [2]Pathological Analyses Department, College of Science, University of Basra, Basra, Iraq. [3]Biology Department, College of Education-Qurna, University of Basra, Basra, Iraq.

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