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ORIGINAL ARTICLE

In vitro effects of purified lacticin from whey isolated *Lactococcus lactis* culture on *Staphylococcus aureus* and MCF-7 breast cancer cells

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ABSTRACT

Background and Objectives: Bacteriocins are interested as antibacterial and anticancer agents due to their high specificity and low side effects. This study aimed to isolate bacteria which produce bacteriocins of the lacticin family from whey and to investigate their antibacterial and anticancer effects.
Materials and Methods: Lactic acid bacteria were isolated from different whey samples. The presence of the lacticin gene in the isolates was checked using PCR and then the inhibitory effects of their bacteriocin was investigated on *Staphylococcus aureus* utilizing well plate method. The protein content was separated by dialysis. The presence of lacticin was checked with the help of SDS-PAGE. The lacticin producing bacterium was identified through the sequencing of the 16S rRNA gene. Finally, the cytotoxicity of the obtained protein was studied on the MCF-7 breast cancer cells using MTT and scratching tests.
Results: The isolated lacticin-producing *Lactococcus lactis* was able to grow in acidic conditions (pH = 2.5 for 3 h) and in bile salts (0.3% for 24 h). The bacterium produced 4.2 µg/µl bacteriocin with a molecular weight of 3.1 KD. The lacticin showed antibacterial effect against *S. aureus*. The cancerous cells treated with lacticin had slower growth than the control in Scratch test. Based on the MTT results, more than 80% of cancerous cells were inhibited at a concentration of 7 µg/ml lacticin with IC_{50} = 5.2 µg/ml.
Conclusion: The bacteriocin produced in this study is a promising antibacterial and anticancer agent.

Keywords: Probiotic; Lacticin; *Lactococcus lactis*; Anticancer; Antibacterial

INTRODUCTION

Probiotics are known as live microorganisms that, if used in sufficient amounts, improve the health of the host by improving the microbial flora of the body. Their resistance to acidic conditions, digestive enzymes, and bile salts leads to their viability in intestinal conditions. Various species of the genera *Bifidobacterium, Lactobacillus, Enterococcus, Saccharomyces, Lactococcus, Leuconostoc, Pediococcus*, and *Streptococcus* are considered as probiotics (1, 2). Probiotics have antimicrobial activity against many pathogenic bacteria and also have anticancer properties by producing bioactive compounds such as short-chain fatty acids, bacteriocins, ethanol, diacetyl, lactic acid, 2, 3-butanediol, acetaldehyde, benzoic acid, and glycoproteins (3). These metabolites also modulate insulin sensitivity and the body's immune system and represent anti-inflammatory effects. Dairy products are known as one of the main

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foods containing probiotic bacteria (4).

Bacteriocins are protein metabolites produced by a wide range of bacteria and archaea, typically with a molecular weight less than 10 KD (5). Bacteriocins are divided based on molecular weight, specificity of action mechanism, amino acid content, and structural features (6). The use of bacteriocins in foods as natural preservatives is of great interest, as bacteriocins can be added directly or indirectly to food with the desired bacteriocin-producing strain. Nisin and pediocin are two examples of commercial bacteriocins (7). Studies demonstrated that bacteriocins are active against a wide range of pathogens, including those caused by the antibiotic-resistant *S. aureus.* Nisin has been classified as a bacteriocin with the ability to replace antibiotics in treating skin infections caused by *S. aureus* (8). Moreover, a prior study suggested that the penicillin-like bacteriocin peptide from a *Paenibacillus* sp. significantly suppresses infection in mice due to *S. aureus* (9). It has been shown that lacticin consumption significantly reduces the amount of *S. aureus* accumulation in the spleen and liver of mouse models that were injected with this bacterium (10).

Lacticin is one of the bacteriocins produced by probiotic bacteria. This dietary bacteriocin which is produced by lactococci, is made up of two distinct peptides. Acting broadly against Gram-positive bacteria like *Staphylococcus, Clostridium, Listeria,* and *Streptococcus* spp., lacticin exhibits antimicrobial action. Despite its antimicrobial properties against Gram-positive bacteria, lacticin is not yet proven to exhibit significant inhibitory effects on Gram-negative bacteria (11, 12). *Lactococcus lactis* is among the bacteria that produce this bacteriocin (13).

L. lactis is a Gram-positive, non-motile, and spherical bacterium that produces lactic acid from sugars in a homofermentative manner. This bacterium has a special place in dairy industries and was previously known as *Streptococcus lactis.* This bacterium is used in the early stage of many cheeses production. This bacterium can create a variety of bacteriocins and is not harmful or invasive (14, 15).

Along with lung and colon cancers, breast cancer is among the most prevalent cancers worldwide that claims the lives of about 700,000 individuals annually. Due to the complexity and multifactorial nature of cancer and the side effects of chemical drugs, alternative methods have been used to treat this disease in recent years (16). It is highly promising that short-chain fatty acids and other probiotic metabolites such as pyridoxine and lacticin increase the activation of apoptosis in cancerous cells (17-19). Therefore, this study aimed to investigate the antibacterial and anticancer effects of bacteriocins of the lacticin family from whey isolated and to investigate their antibacterial and anticancer effects.

MATERIALS AND METHODS

Isolation of bacteria from whey. Several whey samples were prepared from different sources and 1 ml of each sample was transferred to separate falcons. After centrifugation (M12P, Neuation) for 5 min at 1000 rpm, 100 µl of the supernatant was removed and cultured on Man–Rogosa–Sharpe (MRS) agar medium (Ibresco, Iran). After incubating the samples for 24 h at 37°C in the presence of 5% CO_2 , the obtained colonies were Gram stained and observed using a microscope (Nikon) (20).

Biochemical tests. In order to initially identify the isolates, catalase (using 3% hydrogen peroxide) and oxidase (using oxidase disk) test results, and resistance to acid and bile salts were checked (21). For testing the resistance to bile salts. Bile Esculin Agar (BEA) is a selective-differential culture medium used for the isolation and identification of probiotics. Bile salts are the selective agent, while aesculin is the differential component. Bacteria that hydrolyze aesculin react with the products of the reaction with ferric citrate in the medium and produce insoluble ferric salts, which cause the medium to turn black. For this purpose, after overnight cultivation in MRS medium, the bacteria were centrifuged for 10 minutes at 5000 rpm. Then the supernatant was discarded and the bacteria were washed using phosphate buffer. Then they were centrifuged again for 10 minutes at 5000 rpm and after pouring the supernatant twice, the pellet was mixed with MRS broth with bile salts (0.3%). The number of viable bacteria was examined at different times using the pour plate technique. Growth was also studied by measuring optical absorbance at 600 nm (22).

Identification of lacticin-producing isolates. Initially, bacteria were inoculated into MRS broth and incubated at 37°C for 48 h. Then the culture supernatant was separated utilizing centrifugation at 5000 rpm for 10 min. The bacteriocin was purified by add-

ing 70% ammonium sulfate to the supernatant (1:1), and the obtained solution was kept at 4°C for 24 h. Next, the resulting solution was centrifuged at 10,000 x g at 4°C for 30 min. The precipitate was dissolved in 1ml 0.1 M phosphate buffer (pH = 7) and placed inside the dialysis bag. The protein was then dialyzed in the presence of 0.1 M phosphate buffer (pH = 7) (23). Total protein content was assessed utilizing Bradford test trough Coomassie blue staining and determination of the optical density (OD) of samples at the wave length of 595 nm. In the following, the protein concentration of the sample was determined using a standard curve. The molecular weight of the protein produced by them was determined on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In order to molecularly identify the lacticin-producing isolates, a 530 bp fragment in 16S rRNA gene was amplified using universal primers F8 and 518R, and the amplified product was sequenced. After verifying the generated sequence using Bioedit software, an alignment was conducted in NCBI using BLAST server (24).

PCR for the identification of isolates containing lacticin gene. In order to confirm the presence of the lacticin gene fragment in the isolated bacteria, first the DNA extraction was performed by boiling, and then PCR was performed using primer pairs (FMHLc48:5'-GTGACAGAAAGTGAATTGGACC-3' and RMHLc48: 5'-AGAGCAGCGAGTAAATACAAATTG-3') specific for lacticin gene amplification (25). For DNA extraction, a single colony of each bacterium was dissolved in 10 µl double distilled water. Then the microtubes were placed in a thermocycler (TC-SQ, manufactured by Boeco) for 10 min at a temperature of 94°C to lyse the bacteria. After that, the microtubes were immediately placed on ice (26). The PCR master mix containing PCR buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (200 µM), and each primer (0.5 µM) was added to the lysed bacteria in a total volume of 50 µl which was adjusted with double distilled water. Then, PCR was performed according to the temperature conditions listed in Table 1 (27). The PCR product with a 117 bp length was visualized utilizing agarose gel electrophoresis and confirmed on polyacrylamide gel electrophoresis and staining with silver nitrate method (28).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) test for cell survival assessment. First, 50,000 cells were added to each well of a 96-well plate, and then 180 µl of complete culture medium was added to each. Then 20 µl of concentrations of 10, 100, and 1000 µg/ml of purified lacticin containing extract was added to the wells. The plate was incubated for 48 h under CO_2 (5%) at 37°C. Then, 20 µl of MTT solution was added to each well, and the plate was incubated again for 2 h under CO_2 (5%) at 37°C. Ultimately, the content of wells was removed, and 100 µl dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Finally, the OD of each sample well and the control well was determined at the wave length of 492-630 nm. The survival percentage of cells exposed to each concentration of lacticin was calculated according to the following formula (29):

$$\text{Survival (\%)} = \frac{\text{Sample OD} - \text{Blank OD} \times 100}{\text{Blank OD} - \text{Control OD}}$$

Table 1. Optimal temperature conditions for performing the PCR colony reaction

Cycle	Time	Temperature steps (°C)	Steps
1	5 min	94	Initial annealing
35	sec 35	94	Denaturation
	sec 35	57	Annealing
	1 min	72	Amplification
1	5 min	72	Extention
1	1 min	10	Cooling

Scratch test for cell survival assessment. The cells were grown in 96-well plates as described above with different concentrations of purified lacticin containing extract. Then the cells were scraped with a scraper. In this test, a number of cells were cut in a certain line, and pictures were taken of that section at different hours. The greater the ability of cells to migrate and invade, the faster they fill the scratched area. Finally, the photos taken were compared (30).

Antibacterial activity assessment. First, 100 µl of microbial suspension (OD 600 = 0.08) of *S. aureus* was cultivated on Mueller Hinton agar. Then, wells with diameters of 8 mm were cut in the agar with a punch in sterile conditions. Finally, 100 µl of each concentration of purified lacticin containing extract was poured into each well and the plate was incubated at a temperature of 35°C for 24 h. After what, the growth inhibition zones around the well were determined (31).

Statistical analysis. The growth rate of cells in the scratch test was calculated with the help of Image J software, and all statistical analysis were performed using GraphPad Prism 9 software and the independent T test.

RESULTS

Isolation of *Lactococcus* from whey. Different whey samples were collected from dairy industries in Isfahan and were cultured on MRS agar for 24 h at 37°C in the presence of 5% CO_2. Then, the colonies were examined under a microscope. Finally, eight Gram-positive rods (the isolates 6G, 8G, 13G, 7L, 8L, 9L, 10L, and 12L) that were oxidase-negative, catalase-negative, and spore-free were obtained and kept for molecular identification. Among the isolates, 6G, 8G, 13G, and 12L were resistant to bile salts.

PCR to confirm the presence of lacticin gene in isolates. A PCR was conducted using specific primers for the lacticin gene in order to identify isolates containing this gene for further investigations. Two of the eight isolates, 8 G and 12 L, had the lacticin gene. Fig. 1 displays the agarose gel electrophoresis results which visualized an amplified 117 bp fragment.

PCR product on polyacrylamide gel. To validate the DNA fragments more precisely, electrophoresis of the products on a polyacrylamide gel was done and then they were stained with the silver nitrate method. The results of this test also confirmed the presence of the lacticin gene in two isolates (8 G and 12 L) (Fig. 2).

Antibacterial activity assessment of lacticine against *Staphylococcus aureus.* The results are shown in Fig. 3. Lacticin derived from two isolates (8 G and 12 L) was able to prevent the growth of *S. aureus*, using the qualitative test well diffusion in agar and create a 14-mm-diameter zone of inhibition around the well.

The tolerance of isolate 8G to acidic conditions (pH = 2.5 for 3 h) and bile salt (0.3% for 24 h) was examined. In both cases, *Escherichia coli* strain TOP10F and the culture of isolate 8G in MRS broth with neutral pH and salt-free condition were used as control. In acidic condition the growth of *E. coli* strain TOP10F was inhibited but no significant growth inhibition was seen by *L. lactis* isolate 8G (Fig. 4). In the presence

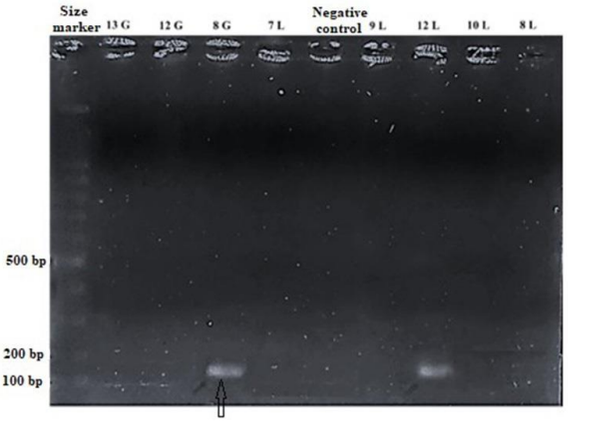

Fig. 1. PCR product showing the presence of lacticin gene with a size of 117 bp in two isolates 8 G and 12 L. Arrows indicate the target band locations compared to a 100 bp size marker

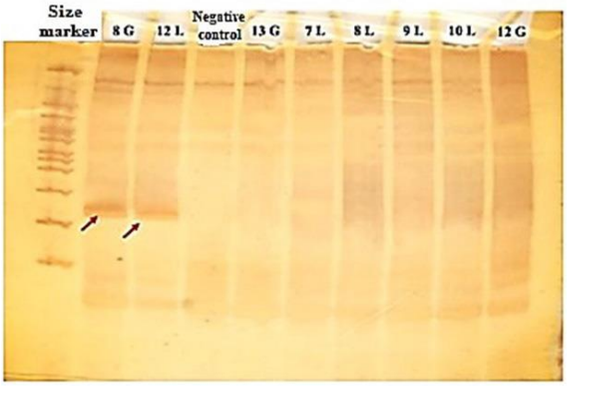

Fig. 2. Examination of PCR products of the Lacticin gene in 10% acrylamide gel to confirm the size of the product. Arrows indicate the locations of target bands compared to a 50 bp size marker

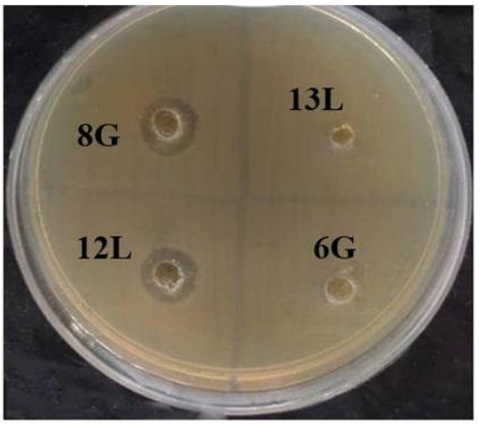

Fig. 3. Antibacterial activity assessment of the derived lacticine against *S. aureus* Resistance to acid and bile

of bile, a growth delay (< 15 min) min was seen by *L. lactis* isolate 8G which was lower than that by of *E. coli* strain TOP10F (> 30 min) (Fig. 5), compared to the control condition.

SDS-PAGE results. After identifying the presence of the lacticin gene in both 8 G and 12 L, lacticin protein with a molecular weight of 3.1 KD was observed in the two isolates utilizing SDS-PAGE (Fig. 6).

Quantifying the amount of lacticin production using Bradford's test. After computing the OD associated with the proteins produced by isolate 8G at a wavelength of 595 nm and entering it into the formula for the slope of the line obtained from the standard curve, i.e., y = 0.0256 x - 0.0535 (Fig. 7), the amount of lacticin produced was determined to be 4.2 µg/µl.

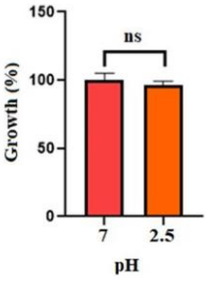

Fig. 4. Investigating tolerance to acidic conditions in *L. lactis* isolate 8G did not show a significant difference between acidic and neutral conditions ($p \leq 0.05$, Sig. = 0.327)

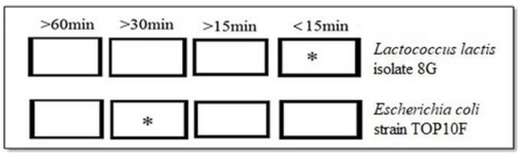

Fig. 5. There was a delay in the growth of *L. lactis* isolate 8G compared to the control bacterium (*E. coli* strain TOP10F) in the presence of bile (0.3%)

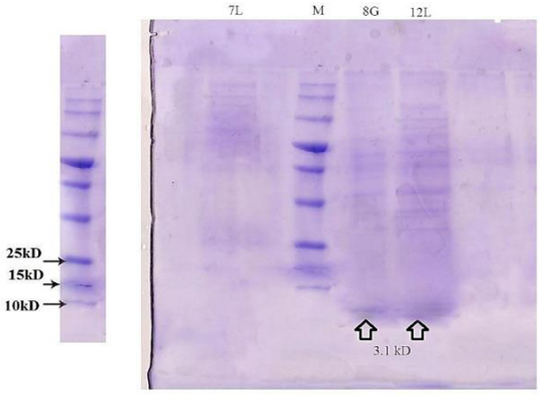

Fig. 6. The results of SDS-PAGE of the protein obtained from two studied isolates (8G and 12L)

Molecular identification of the selected lacticin producing isolate. Based on the blast analysis, the isolate was related to *L. lactis* and the sequence data was deposited as *L. lactis* HDA01 with the accession number O38929.1 in GeneBank. The phylogenic tree which obtained using neighbor-joining method is shown in Fig. 8.

Effect of lacticin on MCF-7 breast cancer cell viability. The comparative diagram of proliferation was drawn after 36 h in terms of the speed of growth of cells in the scratch area and calculation of the diameter of the scratch using Image J software. The toxic effect was increased by purified protein containing lacticin at the concentration 5 µg/ ml and escalated in higher

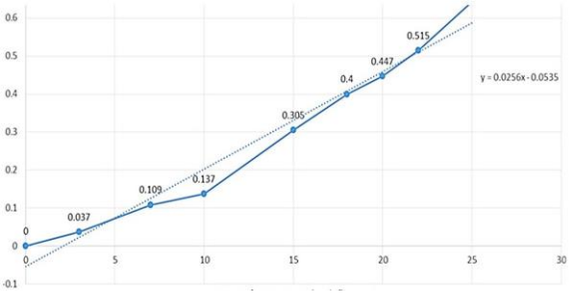

Fig. 7. Standard curve for protein concentration assessment by Bradford's test

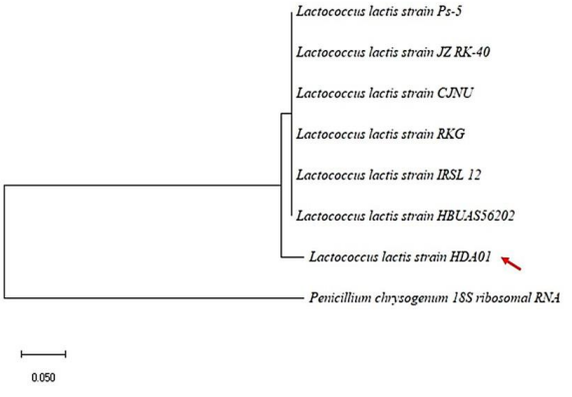

Fig. 8. Phylogenetic relationships of *L. lactis* HDA01 Based on 16S rRNA sequence obtained by using the neighbor-joining method and 2000 bootstrap replications

concentrations. The IC_{50} of lacticin was determined as 5.2 µg/ml. The filling speed was significantly lower (Sig. = 0.012) in the treatment group compared to the control. Fig. 9 shows the performance of scratch test on the examined cells. The cell viability assessment by MTT test on MCF-7 cancerous cells treated with different concentrations of purified protein containing lacticin after 24 and 36 h are shown in Fig. 10. The results showed that more than 80% of cells were killed at a concentration of 7 µg/ml lacticin.

DISCUSSION

Two out of 8 isolates obtained from whey samples in this study had the lacticin gene. The production of bacteriocin lacticin by the superior isolate was ascertained. Lastly, the pathogenic bacterium *S. aureus* and the MCF-7 cell line were used to test the anti-bacterial and anti-cancer properties of the purified bacteriocin. The enormous complexity of cancer means that developing innovative therapeutic approaches has always been a top focus. In the meantime, 5 million people worldwide are affected by breast cancer each year, making it one of the most common malignancies among women. However, because anticancer medications have a wide range of adverse effects, it is imperative to produce and develop natural anticancer substances (32). Antimicrobial peptides (AMP) have been employed for cancer treatment in a number of studies because of their significant anticancer capabilities, taking into account the negative effects of chemotherapy for cancer patients (33). Natural antimicrobial peptides have a major role in the efficiency of anticancer peptides, which are a successful treatment with few adverse effects (34). Antimicrobial peptides can target the negatively charged membranes of cancer cells because of their low molecular weight, amphipathic, and cationic nature (35). Bacteriocins are antimicrobial peptides derived from prokaryotic cells that are ribosomally produced by eubacteria microorganisms. By forming holes in the target cell's cytoplasmic membrane, these peptides kill the cell. Lacticin, an anticancer peptide with a molecular weight of 3100 Daltons has been isolated from *L. lactis* with the ability to inhibit 50% of cancer cells in a concentration of 5.2 µg/ml (36). Lacticins are mostly generated by the *Lactococcus* spp. This peptide has potent anticancer effects in addition to its antibacterial capabilities against *S. aureus* (37). Lacticin, at 7 µg/ml, inhibited over 80% of MCF-7 cancer cells in the current investigation and demonstrated beneficial effects against pathogenic *S. aureus.* Different lactic acid bacteria have also shown antibacterial activity due to the production of active peptides. For example, Fornitano et al. demonstrated the activity of *Lactobacillus rhamnosus* against *S. aureus.* The bacteria exhibited growth inhibition, interference with coagulase synthesis, and biofilm formation (38). A strain of *Lactobacillus gasseri* have also had the ability to inhibit the growth of *S. aureus*, *Klebsiella oxytoca*, and *E. coli*, according to research conducted Gao et al. In the current investigation, *S. aureus* was unable to grow in well plate test due to the bacteriocin generated by *L. lactis* (39). According to Layus et al.'s study, *Lactobacillus plantarum* was able to inhibit the growth of both methicillin-resistant *S. aureus* (MRSA) and multi-drug-resistant *Pseudomonas aeruginosa* strains that were isolated from diabetic foot ulcers (40). In this investigation, pathogenic *S. aureus* was subjected to a 14 mm di-

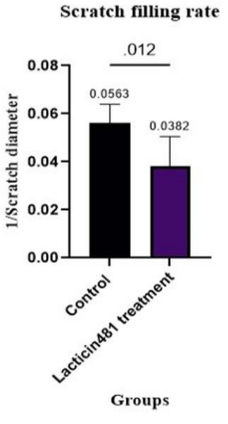

Fig. 9. The filling speed was significantly reduced (Sig. = 0.012) in the treatment group compared to the control

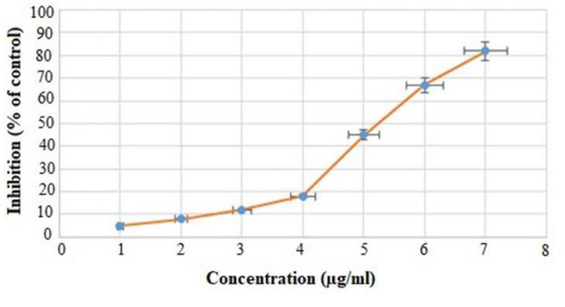

Fig. 10. MTT analysis of MCF-7 cell viability after 24 and 36 h exposure to purified protein containing lacticin

ameter zone of growth inhibition, indicating a relatively high antibacterial impact of the bacteriocin generated by *L. lactis.* The isolate which was studied for production of lacticin production, was detected as probiotic lactic acid bacterium because of biochemical measures including catalase and oxidase, and resistance to bile and acids (41). The bacterium which was detected as *L. lactis* based on 16S rRNA sequence (42) had been isolated previously with special carbohydrate activity from Swiss cheese (43). *L. lactis* HDA01 which was isolated in the present study, produced bacteriocin with a molecular weight of 3 KD and carried lacticin gene, showed cytotoxicity on MCF-7 cell line. Abdul Khaliq et al. also showed the inhibitory impact of bacteriocin derived from *L. lactis* on MCF-7 and CCL-119 lymphoid cancerous cells. Additionally, this bacteriocin demonstrated inhibitory effects on *Pseudomonas aeruginosa* and *E. coli* (44). Hamdem et al. also showed the lethal effects of bacteriocin bropsin 13 generated by *Lactococcus brevis* on MCF-7 breast, HepG2 liver, and Caco-2 clone cancer cells by over 91%, 95%, and 93%, effectiveness respectively (45). The lacticin produced in the present study, showed toxicity effect on MCF-7 breast cancer cells by the concentrations higher than 5 µg/ ml. Avand et al. demonstrated that nisin derived from *L. lactis* in conjunction with tryptone could inhibit 50% of MCF-7 cancer cells at a concentration of 5 µM and an incubation temperature of 37°C. Furthermore, their investigation demonstrated that the combination of doxorubicin and nisin enhances their anticancer effectiveness with synergistic effects (46). Physical damage can be effectively repaired by substances that can accelerate cell migration and increase cell mobility (47). This study included the scratch test in addition to the MTT test, and it found a substantial decrease in the proliferation of MCF-7 cells following treatment with proteins containing lacticin.

CONCLUSION

The current study's findings demonstrated that lacticin, extracted from *L. lactis* HDA01, significantly reduced the growth of *S. aureus.* Additionally, the results of examining this bacteriocin's cytotoxic effects on cancerous cells demonstrated that it can stop the growth and kill the cells. Simultaneous antibacterial and anticancer effects of the studied protein which is reported in the present study is promising and provide a reference for future research on its medicinal effects.

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