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ORIGINAL ARTICLE

Isolation and identification of the bacterium producing antitumor and antimicrobial compounds derived from Iranian swamp frog *(Rana ridibunda)* skin

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ABSTRACT

Background and Objectives: Cancer incidence and recurrence, antibiotic resistance, and overuse of antibiotics have become a global concern. The purpose of this study was to identify and isolate bacteria from the skin of the *Rana ridibunda*, Iranian swamp frog, which has produced antimicrobial compounds, and investigate its cytotoxic activity on the breast (MCF7) and glioblastoma (U87) cancer cell line.

Materials and Methods: An antibiotic-producing bacterium was isolated from the frog skin. The bacterium was identified based on 16S rDNA sequencing and biochemical and morphological characteristics. Antimicrobial activity of the culture supernatant was examined by disc diffusion and MIC methods. Cytoplasmic and cell wall extracts of bacteria were prepared by sonication. SDS-PAGE was then used to examine protein contents of them. The cancer cell lines were treated with cytoplasmic and cell wall extracts at different concentrations. The effects of cytotoxicity were assessed by MTT assay at 24 and 48 h intervals. Finally, the results were analyzed by SPSS.

Results: The isolated bacterium was identified as a new strain of *Bacillus atrophaeus*. MIC and disc diffusion methods showed that the *Bacillus atrophaeus* antimicrobial activity was broad spectrum. MTT assay showed IC50 values 30 µg/ml and 20 µg/ml for U87 and MCF7 cells after 24-48 h exposure, respectively.

Conclusion: The cytoplasmic extracts of *Bacillus atrophaeus* has anticancer potential and can be used as an alternative or complementary candidate in the treatment of cancer. Further *in vivo* and *in vitro* mechanistic studies are suggested to confirm the biological activities.

Keywords: Bacterial extract; *Bacillus atrophaeus*; Anticancer effect; Anti-microbial

INTRODUCTION

In the recent decade, clinical researches on the isolation of microbial products from microorganisms and the mechanisms of their actions have been in progress (1). Approximately 60% of prescribed drugs in cancer treatment were naturally attempted. Nowadays, many studies on environmental bacteria have been conducted, and these bacteria are con-

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sidered as important sources of new anticancer and antimicrobial compounds (2, 3). Excessive use of antibiotics, causing extensive side-effects and antibiotic resistance, has increased the attentions to the new antimicrobial compounds, which finally leads to introduce novel antibiotics (4, 5). Antimicrobial peptides are one of the necessary defense components against invasive pathogens. Organisms defend themselves against pathogenic microorganisms using producing and secreting antimicrobial compounds (6). Amphibians are rich sources of antimicrobial compounds with dramatic effects more similar to mammalian neuropeptides and hormones due to their skin glands. They are one of the primary constitutions of their defense systems. Frog's wet skin is an appropriate region for bacterial and fungal growth. However, secreting compounds from their skin highly prevent such microorganisms (7, 8). So far, there is no report about the isolation of bacteria with antimicrobial effects from swamp frog's skin. Therefore, the purpose of this study was isolation and identification of antimicrobial fraction- producing bacterium derived from Iranian swamp frog's *(Rana ridibumda)* skin and evaluation of its anticancer cell lines.

MATERIALS AND METHODS

Bacterial sampling and isolation. In this experimental study, the antimicrobial compound-producing bacterium was isolated from the swamp frog skin of Ahvaz. Sampling from different regions of frog skin was done under sterile conditions by swab and was inoculated into the Nutrient broth (Oxoid, England) and then incubated in a shaker incubator at 35°C (115 rpm) for five days. Collectives four bacteria isolated on nutrient agar by serial dilution (1-10 to 10-10). Based on morphology, the antimicrobial test has been carried out by well diffusion against other bacteria, and the best response has been selected for future studies on inhibition zone.

Bacterial identification. Bacterial isolates were identified based on morphological, biochemical, and phylogenetic methods (9, 10).

PCR for identification. PCR was performed using 5'-AGAGTTTGATCCTGGCTC -'3 (forward) and 3'-GCCTAAGGAGGT-5' (reverse) primers. DNA amplification condition was as follows: (A) denaturation at 94°C for 1 min, (B) annealing; 56°C for 40 seconds, (C) elongation; 72°C for 1 min (D) final elongation; 72°C for 5 min.

Sequencing and phylogenetic analysis. High pure PCR product kit (Roche, Germany) was used for Genomic DNA extraction. DNA absorbance was measured at both 260 nm and 280 nm to determine the DNA concentration and evaluating its purity. After amplification and gel electrophoresis, PCR products was recovered from the gel and amplified, and purified fragment were sequenced by automatic DNA Sequencer (SEQLAB, Germany) Dideoxy Chain Termination Methods according to Sanger (10, 11). The sequences were compared with similar sequences of the reference organisms by the BLAST algorithm (https://www.ncbi.nlm.nih.gov/).

Investigation of antimicrobial activity. Antimicrobial activity of the culture supernatant was examined against the indicator bacteria by disc diffusion and minimum inhibitory concentration (MIC) methods.

Disk diffusion method. The isolated bacterium strain was grown in a synthetic medium at 35°C and 140 rpm for 72-144 hours and in nutrient broth for 24-48 hours. The synthetic medium contained the following ingredients per liter: glucose, 10; K_2HPO_4, 0.5; NH_4Cl, 1; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.01; it also contained 1 mL of trace elements ($ZnCl_2$, 70mg; $MnCl_2.4H_2O$, 100 mg; $CoCl_2.6H_2O$, 200 mg; $NiCl_2.6H_2O$, 100 mg; $CuCl_2.2H_2O$, 20 mg; $NaMoO_4.2H_2O$, 50 mg; $Na_2SeO_3.5H_2O$, 26 mg; $NaVO_3.H_2O$, 10 mg; $Na_2WO_4.2H_2O$, 30 mg; in 1000 mL distilled water) (12). The culture medium was centrifuged at 3400 × g for 20 minutes and pH of the supernatant was adjustment to 7.0. Afterwards, 2 mL of the supernatant was dried on sterile antibiotic as-say disks and placed on Muller Hinton agar plates which were previously spread with indicator strains; then, the plates were incubated at 37°C for 18-24 hours and the clear zones were investigated. All the assays were conducted in triplicates. Standard tested microorganisms for Gram-Positive bacteria were used *Bacillus subtilis* ATCC 465, *Staphylococcus aureus* ATCC 25923 and the yeast *Candida albicans* ATCC 10231 and for Gram-Negative bacteria used *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 85327, and *Klebsiella pneumoni-*

ae. In additional *Candida albicans* ATCC 10231, and *Aspergillus niger* (isolated in our lab) were used as a standard fungus.

Minimum inhibitory concentration (MIC) method. The MIC was also used for antimicrobial assay. The culture supernatant was first washed with chloroform and the raffinate was extracted with methanol. The methanol extract was dried by a vacuum pump at 30°C. The resulted powder was dissolved in distilled water. Then, 250 µL of sterile Muller Hinton broth was poured in each well except for the first well, of a 96-well microtiter plate (Greiner, Nurtingen, Germany). Five mg of dried powder was dissolved in the first well and mixed well. Then, 100 µL of this well content was added to the second well and this manner was continued to the penultimate well; the last well was used as the negative control. The inoculum of the microorganisms was prepared from the 12-hour broth cultures and finally 100 µL of the standard bacteria suspension, adjusted to the 0.5 McFarland standard turbidity, was added to each well. The plate was incubated for 18-24 hours at 37°C. The lowest concentration that completely inhibited the growth of the bacterial cells was defined as the MIC (13).

Extraction of bacterial cytoplasmic and cell wall fractions. Initially, isolated bacterium cultured in nutrient broth for an overnight to make bacterial preparation. Then, the optical absorbance of the bacteria continuing culture was read at 600 nm. Under the condition, bacteria count was raised to 1.8×10^8 Colony Forming Unit (CFU) according to MacFarland Standard (14, 15). Continuing culture of bacteria centrifuged at 6000 rpm for 20 min and the pelt weighted and suspended in sterile PBS. The sonication method (VibraCell-USA, serial NO 50456) was used for preparing the cytoplasmic extract that in this study named as lysate. The bacterial suspension was lysed via a sonicator for 30 min and again centrifuged in 10000 rpm for 10 min (16). The prepared lysate was filtered by 0.22 µ sterile filter, and a 13-line culture was streaked from the plate, pre-filtered and filtered supernatant on nutrient agar and examined after 24 h incubation for confirmation of the Bacterial death. Samples were verified and selected to treat the cells if there was no bacterial growth on the plate. Then, supernatant and pelt, as bacterial lysate and cell wall extract, respectively, were isolated and stored at -20°C (17, 18).

SDS-PAGE gel. To examine lysate and pelt protein contents, SDS-PAGE gel (BIORAD) was used. According to BIORAD protocols, upper and down gels were prepared, and the tank was provided for placing the gel. Afterward, 10 ml volumes were pipetted into the wells, and the gel was run with 80 mA voltage.

Preparation and culturing cancer cells. Cell line MCF7 and U87 were used as human breast and glioblastoma cancer cells, respectively, provided by Tarbiat Modarres University Cell Bank. MCF7 and U87 cells were cultured in RPMI and DMEM-F12 medium, respectively, which reached by 10% FBS-supplemented and 100 µl of streptomycin-penicillin antibiotics and incubated at 37°C under CO_2 5% (19, 20).

Cytotoxicity test (MTT). MTT test was performed to assess the cell growth and mortality, according to protocol. The cells were trypsinized, collected from flasks, and counted using Neobar lam. 5000 cells were transferred into each well of 96-well plates containing 200 µl of culture medium. Next, various doses of bacterial lysate and cell wall, mentioned as pelt, were treated, and some remained treatment-free were considered as control. 24 h plates from MCF7 and U87 cell lines containing 40, 30, 20, 10 and 5 µg/ml concentrations of cytoplasmic lysate of the isolated bacteria and 20, 15, 10, 5 and 2.5 µg/ml concentration of its cell wall extract, and six treatment-free wells as control were considered. Also, three wells were considered for a triple repeat for each concentration. 48 h plates were prepared with concentration as well. All plates were incubated under CO_2 5%. At the end of incubation time, 10 µl of MTT solution was added to each well additionally incubated for 1 h at 37°C, forming formazan crystals. Then, 100 µl of DMSO (solvent) was added to each well, and their optical absorbance at 570 nm was read after a few minutes' incubation under room temperature by ELISA reader (ELx800™ BioTek, USA) (21-23).

Statistical analysis. Statistical analysis of data was performed using SPSS software, version 21. Results were reported by Repeated Measurement Test, and ANOVA one-way analysis of variance and all data were repeated as Mean ± SD. The level of significance (P-value) was $p< 0.05$. All tests repeated three times.

RESULTS

Identification of isolated bacterium. In this study, anaerobic, motile, Gram-positive, oxidase-negative, catalase-positive, endospore-containing coccobacillus bacterium was isolated from frog skin. The bacterium forms circular mucoid creamy colonies with irregular margins, the dry, wrinkled surface on nutrient agar (Fig. 1). Table 1 lists the morphological and biochemical features of the bacterium compared with *Bacillus atrophaeus*, with maximum similarity (95%).

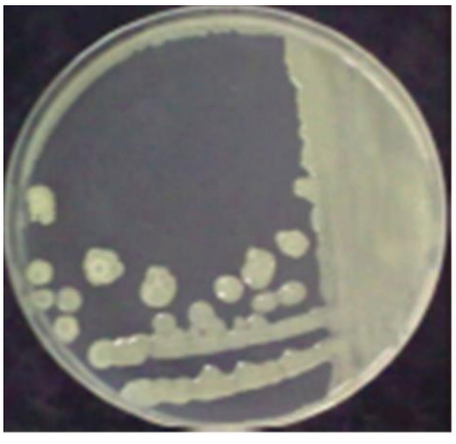

Fig. 1. Colonies of isolated bacterium derived from Frogs skin

Sequences results and phylogenetic analysis. After editing the nucleotide sequence, 16S rDNA fragment produced by PCR (1242 bp), which was explored in NCBI using BLAST software (Fig. 2). Results of the phylogenetic analysis showed the identity of the studied bacterium to several bacteria from the *Bacillus* genus. According to biochemical tests, it was suggested that its identity has more similarity to *Bacillus atrophaeus* (Table 1).

Evaluation of antimicrobial activity. Based on microbial sensitivity determination tests in two methods, disk diffusion and minimum inhibitory concentration (MIC), results showed a wide range of isolated antibacterial activity. The results of the disc diffusion method depicted in Table 2. It shows that the isolated bacterium in addition to Gram-positive bacteria, *Bacillus subtilis* ATCC 465, *Staphylococcus aureus* ATCC 25923, and the yeast *Candida albicans* ATCC 10231 had antimicrobial activity against Gram-negative bacteria such as *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae*, although in less extent.

SDS-PAGE gel. As shown in Fig. 3, the cell wall extract of the isolated bacterium has more bands, showing more rich protein contents compared to the

Table 1. Morphological and biochemical features of isolated bacterium bacterium and *Bacillus atrophaeus*

Features	GA	*Bacillus athrophaeus*
Colony morphology on Muller-Hinton agar	Large-Smile-Wet-Sticky-Smooth-Creamy	Opaque and transparent
Morphology	Bacilli	Bacilli
Staining	+	+
Growth under anaerobic condition	_	_
Motility	+	+
Catalase	+	+
Oxidase	-	-
H2S producing in TSI medium	-	-
Producing Indole	+	-
Spore formation	+	+
Capsule formation	+	+
Starch hydrolysis	+	+
Methyl-red (MR)	+	_
Voges-Proskauer (VR)	_	+
Nitrification	-	-
Denitrification	-	+
Glucose fermentation	+	+
Lactose fermentation	_	_
Sucrose fermentation	_	+

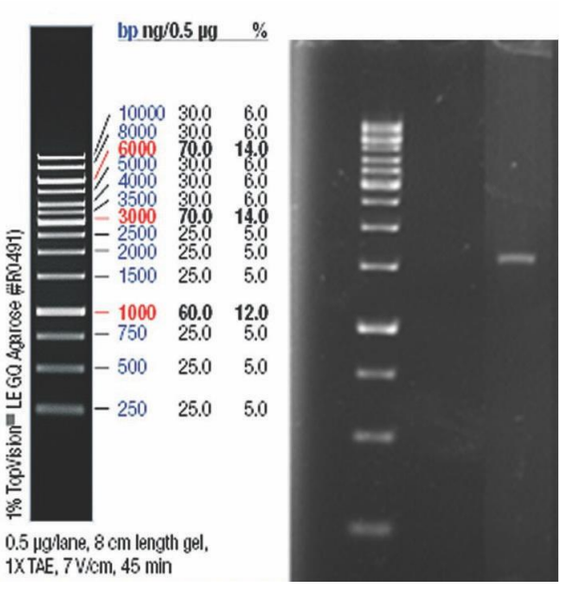

Fig. 2. gel electrophoreses of 16s rRNA fragment from PCR

Table 2. Evaluation of antimicrobial activity test against standard microorganisms by disc diffusion method

Indicator microbial strains	Growth inhibition zone (mm)
Bacillus subtilis ATCC 465	12
Staphylococcus aureus ATCC 25923	13
Escherichia coli ATCC 25922	11
Pseudomonas aeruginosa ATCC 85327	12
Aspergillus niger	15
Candida albicans ATCC 10231	16

bacterial cytoplasmic lysate.

Cytotoxicity test (MTT). The results showed that cytoplasmic extract had a significant ($p < 0.05$) cytotoxicity effect on cells. Fig. 4 shows the MCF7 cell's viability under treatment with different concentrations of isolated bacterium cytoplasmic lysate. The concentration of 20 µg/ml in 48 h and 10 µg/ml in 24 h were determined as IC50, and it could be concluded from comparison results from 24 and 48 h that bacterial cytoplasmic lysate has time and concentration-dependent cytotoxicity on MCF7 cell line.

Investigation of the effect of cytoplasmic lysate from isolated bacterium on U87 cells, has shown a significant cytotoxic effect. Fig. 5 depicts U87 cell viability treated via different concentrations of isolated bacterium cytoplasmic lysate. The concentration of 30 µg/ml in 24 h was determined as IC50. Furthermore, it could be concluded from comparison

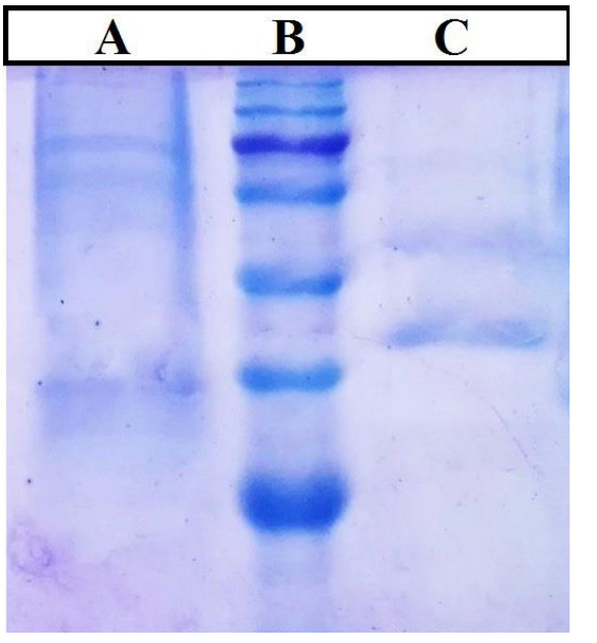

Fig. 3. Vertical SDS-PAGE gel electrophoresis. A) Cell wall extract of the isolated bacterium, B) ladder and C) cytoplasmic lysate of the isolated bacterium

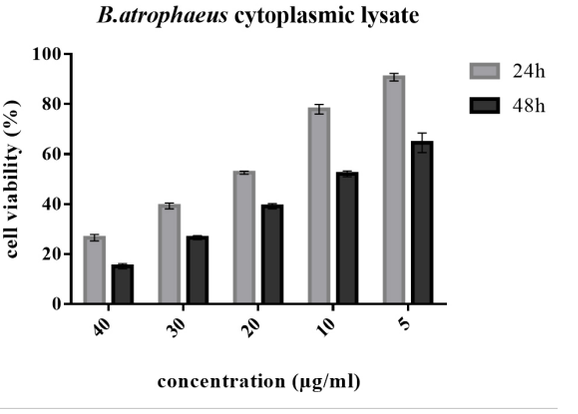

Fig. 4. Cytotoxicity effect of the cytoplasmic lysate of isolated bacterium on MCF7 cell line over 24-48h

results from 24 and 48 h that bacterial lysate, there is no time-dependent cytotoxicity the time. However, it can be conducted the cytoplasmic lysate of isolated bacterium has concentration-dependent cytotoxicity on the U87 cell line.

Fig. 6 demonstrates the stimulatory index of various concentrations cell wall extract has produced from isolated bacterium against MCF7 cells. There was a maximum stimulatory index over 24 h at 20 µg/ml concentration vs. control and similarly, but less, it was promoted cell proliferation over 48 h. So

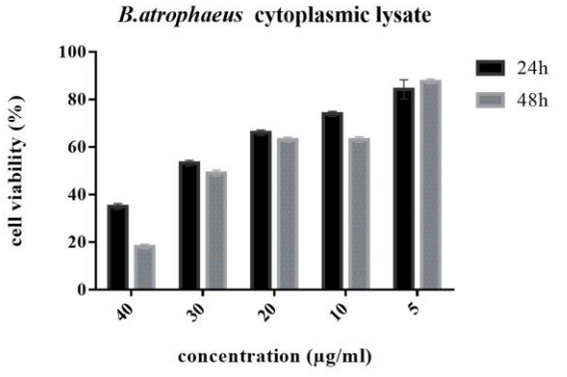

Fig. 5. Cytotoxicity effect of the cytoplasmic lysate of isolated bacterium on U87 cell line over 24-48h

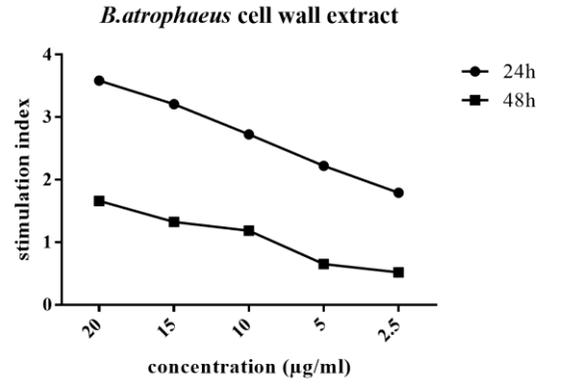

Fig. 6. Proliferation effect isolated bacterium cell wall extract on MCF7 cell line over 24-48h

it could be conducted that growth stimulation and cell proliferation were promoted with increasing cell wall extract concentration. This promotion was observed in both 24 and 48 h time ranges.

Fig. 7 represents the stimulatory index of isolated bacterium cell wall extract via various concentrations against U87 cells. The concentration 20 µg/ml has determined as a maximum stimulatory index over 24 h vs. control, whereas over 48 h, no promotion in cell proliferation was observed. So it could be conducted that growth stimulation and cell proliferation were promoted with increasing of wall concentration, still up to 24 h and more than it, the proliferation effects have stopped.

DISCUSSION

Currently, new treatment methods for a variety of cancers are under-investigation. Taking advan-

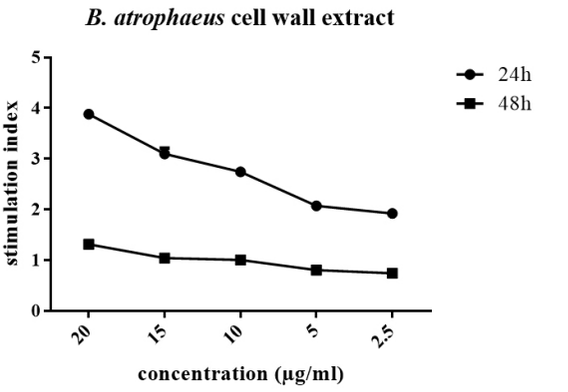

Fig. 7. Proliferation effect isolated bacterium cell wall extract on U87 cell line over 24-48h

tage of epigenetic processes against tumors such as gene silencing of MGMT (O6-methylguanine-DNA methyl transferase) by its promoter methylation and using this method besides of radiotherapy and chemotherapy had improved survival chances of patients developed non-removable tumor (24). The role of microbial metabolites in treating the disease, included cancer, is undeniable. The use of antibiotics is nowadays recognized as one of the new strategies for treating cancer and tumor cells. According to Scatena et al. patients with breast cancer received doxycycline for 14 days prior to antibiotic surgery. Almost in all cases it was observed that the number of cancer stem cells significantly decreased. It was a notable result because the cancer stem cells are responsible for cancer, metastasis, therapeutic resistance, and recurrence of cancers. Immunohistochemical analyzes of the effects of the FDA-approved drug show that the drug reduces expression of the underlying biomarkers of CD44 and ALDH1 by 67%. Reduction of cancer stem cells means reduced therapeutic resistance and recurrence of cancer. The study, conducted on a small group of patients (n = 15) at the University of Pisa Hospital in Italy, has raised hopes that using an inexpensive, yet effective, drug will prevent the growth and inhibition of cancer (25). We also know that antibiotics affect mitochondria, which are thought to be derived from bacteria that integrate with cells in the early stages of evolution. This is why some antibiotics that are used to destroy bacteria also affect the mitochondria. Because mitochondria plays an important role in the energy supply in cancer stem cells, any factor that causes them to deviate can lead to cancer. The growth and recurrence of many cancers are due to these cancer stem cells, which are

also one of the causes of cancer resistance. Lamb et al. (26) used five antibiotics on eight cell lines and found that four of them resulted in the eradication of cancer stem cells in each test. *In vitro*, antibiotics have no effect on normal cells, and since they have been approved for use in humans, new therapies can be easier than using new drugs. Hence, these studies provide a strong case for new human trials of using antibiotic components to fight cancer. In the present study, after examining the antibiotic properties of *Bacillus atrophaeus* bacterial fractions, we studied their effect on cancer cell lines. Recently, many studies have been done on microbial products. Senthilraja and Kathiresan (2015) reported the effect of extracts of three yeast species (*Candida albicans, Kuraishia capsulate* and *Saccharomyces cerevisiae*) against normal human hepatic cells (Vero), and two cancer cell lines- MCF7 and HEP G2. They observed that a low-dose of the extract caused cell proliferation and growth stimulation on, and a high-dose of yeast extract resulted in cancer cell death and had time-dependent toxicity (21). In our study, the cytotoxic effect of the cytoplasmic extract and the proliferation effect caused by the cell wall extract of *Bacillus atrophaeus* on the MCF7 cell lines was demonstrated. In another study, treating cell-free extract of Kefir with blood cancer cell line (acute erythroleukemia KG-1) caused decreasing in Erythroleokemia cancer cell line proliferation (27). Treatment of intestinal cancer cell line, Caco-2 and HT-29 with Kefir extract indicated that their proliferation was inhibited as dose, and time-dependent manner (28). The growth effect of kefir extract in this study is similar to the effect of *Bacillus atrophaeus* cell wall extract in the present study. While according to Fattahi et al. (2018), Kefir extract had a concentration-dependent effect in 24, and 48 h, and mortality was fallen over time (16). These results have shown that different microbial fractions could have different effects on cell lines. Vidhyalakshmi and Vallinachiyar (2013) have reported that several bacteria polysaccharides produced by some *Bacillus* and *Pseudomonas* strain had anticancer properties and were apoptosis-inducer in human breast cancer cell lines MCF7 (29). Since the main constituent of the bacterial cell wall is polysaccharide, this study also confirms the results shown in the present study on the proliferation effect of *Bacillus athrophaeus* cell wall extract. Assaeedil had investigated and confirmed *Bacillus* anticancer effects against cervical and hepatic cell lines. These results are in line with the results of the present study and revealed that the compounds derived from the bacterium have anticancer effects (30). St. Jean et al (2014) demonstrated that direct injection of the bacterium into tumor cells resulted in the death of up to 93% of them, which strengthens the hypothesis of using bacteria and their metabolites to treat cancer (31). In this study, *Bacillus athrophaeus* cytoplasmic and cell wall extracts were investigated upon both glioblastoma, and breast cancer cells over 24 and 48 h. Our results on breast cancer cells indicate that 20 mg/ml and 40 mg/ml of *Bacillus atrophaeus* cytoplasmic extract had maximum cytotoxic effects for 24 h and 48 h, respectively. Our results for breast cancer cells show that 20 µg/ml and 40 µg/ml cytoplasmic extracts of *Bacillus atrophaeus* had the maximum cytotoxic effects, respectively, which were confirmed by other studies. For U87 cell lines, cytoplasmic extracts were also shown to have a time-dependent and concentration-dependent lethal effect on cells. The toxic effect of the studied cytoplasmic extract on cell lines regarding the study of Vijaya Kumar et al. (2014) the toxic effect of the studied cytoplasmic extract can be attributed to the destructive effect of DNA in cancer cell lines and have minimum effects on a normal cell (32). Furthermore, Xiaohony Cao et al. (2009) study on human breast cancer cells has shown that surfactin produced by *Bacillus subtilis* had dose and the time-dependent lethality and inhibited the cell proliferation (33). Since one of the secondary metabolites produced by *Bacillus atrophaeus* is also surfactin, the toxic effect on cancer cells can also be attributed to this substance. On the other hand, examining parasporins and BT toxins produced by *Bacillus turginsis* against cervical cancer cell, by Aldeewan et al. (2014) proved the anticancer effects of these proteins (34). Therefore, the presence of these proteins and toxins in the cytoplasmic extract can also be considered as the source of their toxic effect on cancer cell lines. Another part of the results of this study has shown, 20 µg/ml of *Bacillus atrophaeus* cell wall extract for 24 and 48h could promote cell growth. This promotion can be a result of inflammatory and growth stimulatory compounds (i.e., bacterial peptidoglycans).

CONCLUSION

The results of the present study have shown that

some bacterial fractions of *Bacillus atrophaeus* have noticeable antimicrobial properties that can be considered as an alternative or supplement in the treatment of antibiotic-resistant bacterial infections in future studies. On the other hand, some cytoplasmic fractions of this bacterium due to its ability to induce death in cells can be used as a complementary component to the treatment of tumors and could be an interesting candidate for future studies to find new microbial products to treat cancer. Also, the cell wall extract of this bacterium can be used as a low-cost method for most stem cells due to its ability to stimulate cells to grow more.

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