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Evaluation of Anti-bacterial, Anti-adenoviral, and Apoptosis-inducing Activity of *Bacillus clausii* Supernatant

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Abstract

Background: *Bacillus clausii* is being studied as a probiotic candidate. There is insufficient information on the antimicrobial and anticancer effects of *B. clausii*.

Objectives: The present investigation was designed to evaluate the anti-bacterial, anti-adenoviral, and apoptosis-inducing activity of *B. clausii* cell-free supernatant (CFS).

Methods: First, the supernatant of *B. clausii* was collected after culture for 24 h. Then, its anti-bacterial impact on several genera of bacteria was assessed through the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Adenovirus 5 (Ad5) was exposed to the CFS under four conditions, including pre-treatment: First infecting cells with CFS and then with the virus; pre-incubation: Incubation of the supernatant and virus for 1.5 hours and then adding to the cells; competition: Infection of cells with the simultaneous mixture of the supernatant and virus, and post-treatment: First infecting cells with the virus and then with CFS. The median tissue culture infectious dose ($TCID_{50}$) technique determined the virus titer. Real-time PCR was performed to assess the E1A expression. After exposure to the CFS, real-time PCR was utilized to measure the expression of *MicroRNA-145*, *BCL-2*, and *BAX* in HeLa cancer cells.

Results: *Bacillus clausii* supernatant showed an inhibitory effect on Methicillin-resistant *Staphylococcus aureus (MRSA)*, *Enterococcus faecalis, S. aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Acinetobacter baumannii*. The Ad5 titers were reduced by about 4.61, 4, 3.9, and 3.1 Log_{10} $TCID_{50}$/mL in pre-treatment, pre-incubation, competition, and post-treatment tests (CFS dilution: 1/4), respectively. Similar results of the viral titration were seen when experimental and control E1A expression levels were compared. Also, *B. clausii* supernatant during 48 h exposure to HeLa cells increased the transcript of the *BAX, BCL-2*, and *miR-145* genes to 9.1, 2.3, and 55 folds, respectively, compared to the untreated condition.

Conclusions: *Bacillus clausii* can be a potent antimicrobial and anticancer agent. Further research is required to learn about the spectrum of anti-bacterial, antiviral, and anti-cancerous activities of *B. clausii*.

Keywords: Anti-bacterial, Antiviral, Adenovirus, *Bacillus*

1. Background

Bacillus clausii is known as a probiotic bacterium. Previous research has shown that *B. clausii* has anti-bacterial properties, but the current data are insufficient. Recent studies have reported that the antimicrobials produced by *B. clausii* have activities against *Clostridium difficile* and *Staphylococcus aureus in vitro* (1). *Bacillus clausii* produces the class I antibiotic clausin, which works against Gram-positive bacteria (2). According to the current research, *B. clausii* has no anti-bacterial action on Gram-negative germs but has an inhibitory effect on some Gram-positive germs. More research is required to determine the range of its antimicrobial properties. Several bacilli have shown antiviral action against human viruses, such as *B. subtilis* to inhibit the HSV and influenza virus (3), *B. horneckiae* against herpes simplex virus 1 (4), and *B. licheniformis* against herpes simplex virus 2 (5). Additionally, the ribonuclease (binase) of *B. pumilus* has been developed as an inhibitor of rhinovirus serotype 1A and influenza A in cell culture (6). The direct activity of *B. clausii* and its metabolites against human viruses is unknown and needs more focus.

Probiotics have been shown to have anticancer properties. Also, *B. licheniformis, B. subtilis, B. coagulans,* and *B. cereus* have been studied for their ability to suppress cancer development or trigger apoptosis (7). However, there is

little knowledge of *B. clausii*. *Bacillus* strains have been assessed for their effects on the *BAX* and *BCL-2* genes in cancer cells (8-10), but no data have been published on *B. clausii* and cervical cancer HeLa cells. In addition, the possible role of *Bacilli* has not been assessed in upregulating the expression level of miR-145 involved in apoptosis.

2. Objectives

This work aimed to get further information on the antibacterial activity of *B. clausii* supernatant. Additionally, the possible inhibitory effect of *B. clausii* supernatant against adenovirus type 5 (Ad5) is investigated. Adenoviruses are one of the most common causes of respiratory diseases in humans. However, no study has published data on the inhibitory effect of probiotics on adenoviruses. We also aimed to evaluate the impact of the bacterial supernatant on the expression of genes associated with apoptosis in the HeLa cell line, such as *BAX*, *BCL-2*, and *miR-145*.

3. Methods

3.1. Bacteria, Cell Line, and Virus

Bacillus clausii (ATCC 700160) was purchased as lyophilized from the Iranian Biological Resource Center (IBRC). Fifty milliliters of *B. clausii* culture (10^6 CFU/mL) was cultured in a tryptic soy broth medium for 24 h. The culture suspensions were centrifuged (700 g, 25 minutes) to extract the cell-free supernatants. The IBRC provided *S. aureus (ATCC 25923), Escherichia coli (ATCC 25922), Acinetobacter baumannii (ATCC 1960), Methicillin-resistant S. aureus (MRSA) (ATCC 43300), Pseudomonas aeruginosa (ATCC 27853),* and *Enterococcus faecalis (ATCC 25912)*. The virology department at Tarbiat Modares University (Tehran, Iran) provided HeLa cells, HEK-293 cells, and Ad5 as gifts.

3.2. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Anti-bacterial activity of *B. clausii* was assessed on *S. aureus*, *MRSA*, *E. faecalis, E. coli, and P. aeruginosa*. Several colonies of isolates were put into the Brain Heart Infusion (BHI) medium (HiMedia, India) and cultivated for 24 h at 37°C. One hundred microliters of bacterial suspensions (1.5×10^5 CFU/mL) were added into 96-well plates and treated with 100 μL of different concentrations of each compound (dilutions 1/2 to 1/28). After 24 h storing at 37°C, the Minimum Inhibitory Concentration (MIC) was determined as the lowest dose at which the bacteria could not grow visibly. To find the minimum bactericidal concentration (MBC), TSB tubes with the lowest *B. clausii* supernatant were used to inoculate Trypticase soy agar using the pour plate technique (11).

3.3. Adenovirus Titration

According to a previously described procedure, we carried out a median tissue culture infectious dose ($TCID_{50}$) experiment to evaluate the titer of adenovirus type 5 (12).

3.4. Cytotoxicity Assay of Bacillus clausii Supernatant

The HeLa cells were grown in cell culture microplates (20,000 cells per well) in triplicate to test the cytotoxicity of *B. clausii* supernatant. Dimethylthiazolyl-diphenyl tetrazolium bromide (MTT) test was used to determine cytotoxicity using a previously described procedure (13).

3.5. Antiviral Activity Assay

We cultured HEK-293 cells in six-well plates. Bacterial supernatants (dilutions 1/4 and 1/8) and Ad5 (MOI = 0.1) were added to cells in different conditions, including pre-treatment, pre-incubation, competition, and post-treatment, as previously described (14). The Ad5-infected cells were considered a control (without exposure to the bacterial supernatant).

3.6. Relative Expression of E1A Adenovirus and BAX, BCL-2, and microRNA-145 Genes in HeLa Cancer Cells

The *E1A* expression levels in different experimental assays were measured to approve $TCID_{50}$ results. A real-time PCR test determined the level of the BCL-2, BAX, and microRNA-145 transcripts to evaluate the influence of *B. clausii* supernatant on the apoptosis activation in the HeLa cell line. First, 1×10^5 HeLa cells were grown in each well of six-well plates. The cells were treated with dilutions of bacterial supernatant (1/4 and 1/8) for 24 h and 48 h at 37°C. The RNX kit (SinaClon, Iran) isolated total RNA from the cultivated cells. A commercial cDNA Synthesis Kit (AddBio, South Korea) was used to transcribe one microgram of total RNA.

The cDNA for the *miR-145* and *U6* genes (as a control gene) was synthesized using the following RT primers: RT-U6–5′-ATATGGAACGCTTCACGAATTTGC-3′, RT-miR-145-5p-5′-TCGTATCCAGTGCAGGGTC CGAGGTATTCGCACTGGATACGACAG. The real-time PCR was done in the ABI StepOnePlusTM equipment and specific primers (Table 1). The PCR mixture included 12.5 μL of Ampliqon master mix (Denmark), 0.5 μL (10 μmol) of each primer, 2 μL of cDNA, and 9.5 μL of Distilled Water (DW). Amplifications were carried out using the following cycling profile: 94°C for 10 minutes and then 40 cycles, including 20 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The results were normalized against

the expression of human beta-globin (U6 for miR-145), and the 2^{-ddCT} technique was utilized to estimate the relative expression of genes.

3.7. Statistical Analysis

GraphPad Prism 6.0 software was used to compare $TCID_{50}$ and real-time PCR data between the control and treated groups by a student's *t*-test.

4. Results

4.1. Minimum Inhibitory Concentration/Minimum Bactericidal Concentration

By determining the MIC and MBC, the anti-bacterial activity of *B. clausii* supernatant was examined against several bacterial genera (Table 2). The cell-free supernatant of *B. calusii* had an inhibitory effect on *E. faecalis, S. aureus, MRSA, E. coli, P. aeruginosa*, and *A. baumannii*. The anti-bacterial effect of *E. faecalis* occurred at a lower concentration of *B. calusii* supernatant than that of other bacteria. The bacterial supernatant had no bactericidal activity against *MRSA, P. aeruginosa*, and *A. baumannii*.

4.2. Cell Viability Assay

The results of HeLa cell viability are shown in Figure 1. The MTT findings revealed that incubating HeLa cells for 24 and 48 hours with bacterial supernatant at a concentration of 1/2 reduced viable cells proportion to 65% and 40%, respectively. Additionally, 80% of cells were still alive after exposure to CFS at concentrations above 1/4 for 24 h and above 1/8 for 48 h.

4.3. Anti-adenoviral Effect of Bacillus clausii Supernatant

The human Ad5 titer was 8.91 ± 0.14 Log_{10} $TCID_{50}$/mL when *B. clausii* supernatant was absent (control). The Ad5 titer was calculated after incubation of CFS (dilution: 1/4 and 1/8) and HEK-293 cells in different conditions. Data are summarized in Table 3 and Figure 2. The Ad5 titer in pre-treatment, pre-incubation, competition, and post-treatment conditions with a concentration of 1/4 supernatant dropped by 4.61, 4, 3.9, and 3.1 Log_{10} $TCID_{50}$/mL, respectively. As demonstrated in Figure 2, the Ad5 titer was significantly lower (P-value 0.05) in all experiments than in the control group. The mean Ad5 titer in the pre-incubation and competition tests was similar. Similar results of the viral titration were shown when the E1A expression in the experimental and control tests was compared (Figure 2B). The most significant reduction in E1A-adenovirus expression was seen in the pre-treatment assay.

4.4. Effect of Bacillus clausii Supernatant on the Expression of BAX, BCL-2, and miR-145 Genes

When *B. clausii* supernatant was added to HeLa cells for 24 h at a concentration of 1/8, the levels of *BAX, BCL-2*, and *miR-145* genes increased to 2.8, 1.16, and 6.3 folds, respectively, compared with untreated cells. In addition, 1/4 dilution of the bacterial supernatant during 48 h increased *BAX, BCL-2*, and *miR-145* genes by 5.5, 1.5, and 9.1 folds (Figure 3). A concentration of 1/8 *B. clausii* supernatant during 48 h could elevate the level of *BAX, BCL-2*, and *miR-145* genes by 5.4, 1.7, and 24 folds, respectively, compared to the untreated condition.

5. Discussion

Several *Bacillus* species are investigated in humans as potential probiotic candidates. The current study evaluated the anti-bacterial, anti-adenoviral, and apoptotic effects of *B. clausii* supernatant. Data showed that the *B. clausii* supernatant inhibits some bacterial strains and human adenovirus type 5. Furthermore, it remarkably induced the level of the apoptosis-linked genes *BAX* and *miR-145*. Several studies have demonstrated that *B. clausii* strains produce metabolites with bacterial inhibitory and anti-fungal effects. In the current work, *B. clausii* supernatant after 24 h of culture had anti-bacterial effects on *E. faecalis, S. aureus, MRSA, E. coli, P. aeruginosa*, and *A. baumannii*. Clausin is a lantibiotic recently isolated from *B. clausii* and is active against some gram-positive microbes (3). In the previous studies, it inhibited *M. luteus* at MIC = 16 mg/L and *MRSA* at MIC = 128 mg/L (4). Similarly, in the present study, *B. clausii* had an inhibitory effect on *MRSA* at a MIC equal to 1/4 dilution of the bacterial supernatant. Ripert et al. showed that compounds secreted by *B. clausii* inhibit toxins of two pathogens, *C. difficile* and *B. cereus* (16). Using a colony overlay test, Ripert et al. examined the inhibitory effect of the strains O/C, N/R, SIN, and T of *B. clausii*, which are probiotics.

The *B. clausii* CFS had inhibitory effects on *S. aureus, E. faecium, Lactobacillus lactis*, and *C. difficile* but had no inhibitory effect on gram-negative germs (*E. coli, Salmonella typhimurium, S. flexneri, Vibrio cholerae, V. parahaemolyticus,* and *P. fluorescens*) (1). In the present work, contrary to the findings of Urdaci et al., *B. clausii* supernatant had an antimicrobial effect on *E. coli, P. aeruginosa,* and *A. baumannii*. This disagreement in results may be due to variations in the research techniques or strains utilized in the two investigations. One of the aims of the present study was the in vitro assessment of the anti-adenoviral activity of *B. clausii*. The *B. clausii* CFS showed an antiviral impact on all

Table 1. Primers Used in Real-time PCR Assay for Determination of Expression Levels of Apoptosis-related Genes *BAX*, *BCL-2*, and *microRNA-145*

Primers	Sequence (5-3)	Product Size (bp)	References
miR145-forward	TCCAGTTTTCCCAGGAATCCC	85	(12)
miR145-reverse	ATCCAGTGCAGGGTCCGA		
U6-forward	GAGAAGATTAGCATGGCCCCT	90	(12)
U6-reverse	ATATGGAACGCTTCACGAATTTGC		
Bax-forward	CCTGTGCACCAAGGTGCCGGAACT	99	(15)
Bax-reverse	CCACCCTGGTCTTGGATCCAGCCC		
Bcl-2-forward	TTGTGGCCTTCTTTGAGTTCGGTG	114	(15)
Bcl-2-reverse	GGTGCCGGTTCAGGTACTCAGTCA		
B-Actin-forward	GGCGGCACCACCATGTACCCT	202	(15)
B-Actin-reverse	AGGGGCCGGACTCGTCATACT		
E1A-forward	ACCTCCTAGCCATTTTGAACCAC	122	(12)
E1A-reverse	GTCAATCCCTTCCTGCACCG		

Table 2. Determination of Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) of *Bacillus clausii* Supernatant Against Bacteria

Test	*Enterococcus faecalis*	*Staphylococcus aureus*	*MRSA*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Acinetobacter baumannii*
MIC	1/32	1/16	1/4	1/16	1/4	1/4
MBC	1/16	1/8	-	1/8	-	-

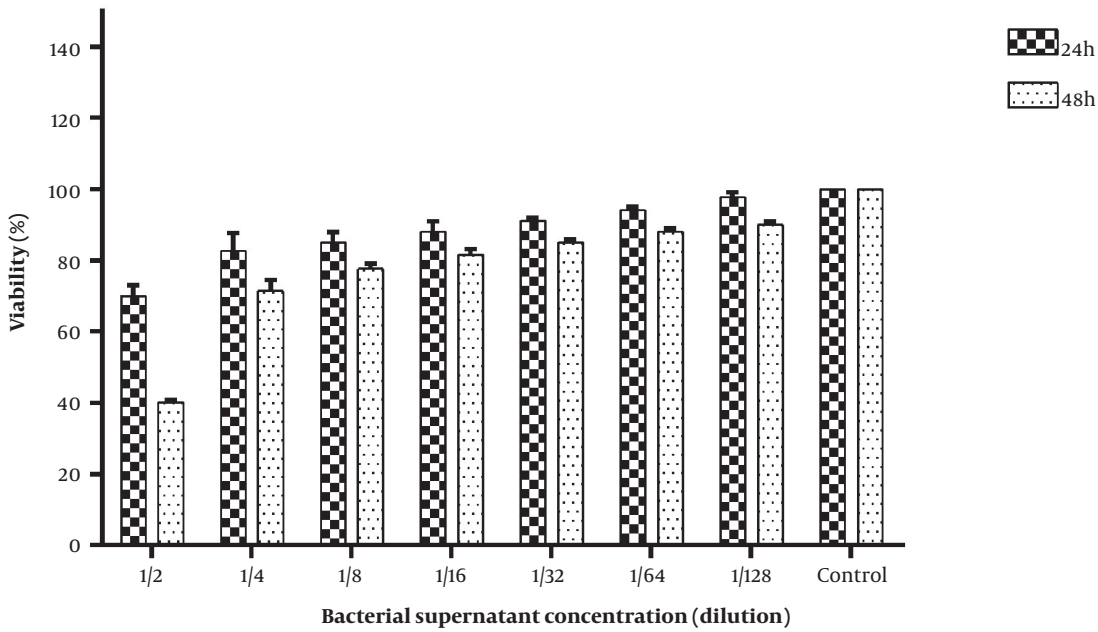

Figure 1. MTT assay for the viability of HeLa cells after treatment with different concentrations of *Bacillus clausii* supernatant

tests conducted in different conditions. However, the pre-treatment condition showed the highest drop in Ad5 titer. The virus's titer was 4.61 Log10 $TCID_{50}$/mL lower than the control. It is conceivable that the supernatant of *B. clausii* can cause interference in adenovirus adhesion to the target cell. As a result, it keeps the virus from entering the

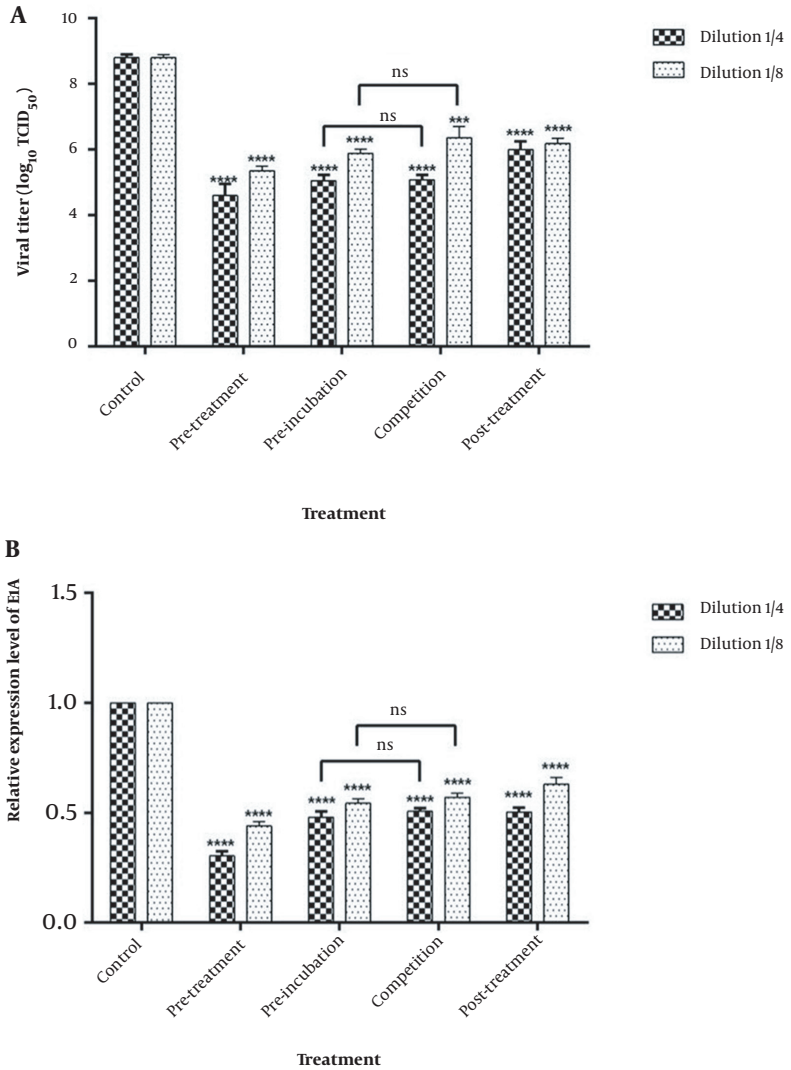

Figure 2. Human Ad5 titer (A) and E1A expression level (B) in different experimental conditions of *Bacillus clausii* inoculation. Statistical differences between the mean virus titer (or E1A expression) at each stage of bacterial inoculation and the mean virus titer in the absence of bacteria (control) were analyzed by the student test (ns: Not significant; **** P-value < 0.0001, *** P-value < 0.001).

cell. This finding was obtained in a few investigations on other probiotics. The culture supernatant of vaginal lactobacilli displayed a strong neutralizing effect on herpes simplex virus type 2 before viral entrance (17). Contrary to the present study, Mousavi et al. found that the CFS of *L. crispatus* did not show a significant antiviral activity on HSV (18).

MicroRNA-145 is a tumor suppressor often expressed in healthy, normal cells but drastically downregulated in malignant cells (12, 19). Recent research has suggested that miR-145 may be essential for controlling tumor cell growth, migration, invasion, and death in many malignancies (20). According to Pan et al., raising miR-145 levels in A549 cells resulted in a notable rise in the expression of *BAX* and a decline in the expression of MMP2, MMP9, and BCL-2. The A549 cells' apoptosis is accelerated by increasing the proportion of BAX to BCL-2 (21). The molecular mechanisms by which miR-145 promotes apoptosis in HeLa cells are unknown. However, Li et al. suggested that *miR-145* can increase cell death in the HeLa cell line by modulating Wnt/β-catenin (20). In the current study, 24 and 48-hour incubations of *B. clausii* supernatant with HeLa cell line increased the level of miR-145 by 24 and 55 folds, respectively. Therefore, *B. clausii* supernatant can have an antitumor effect on HeLa cells through increasing tumor sup-

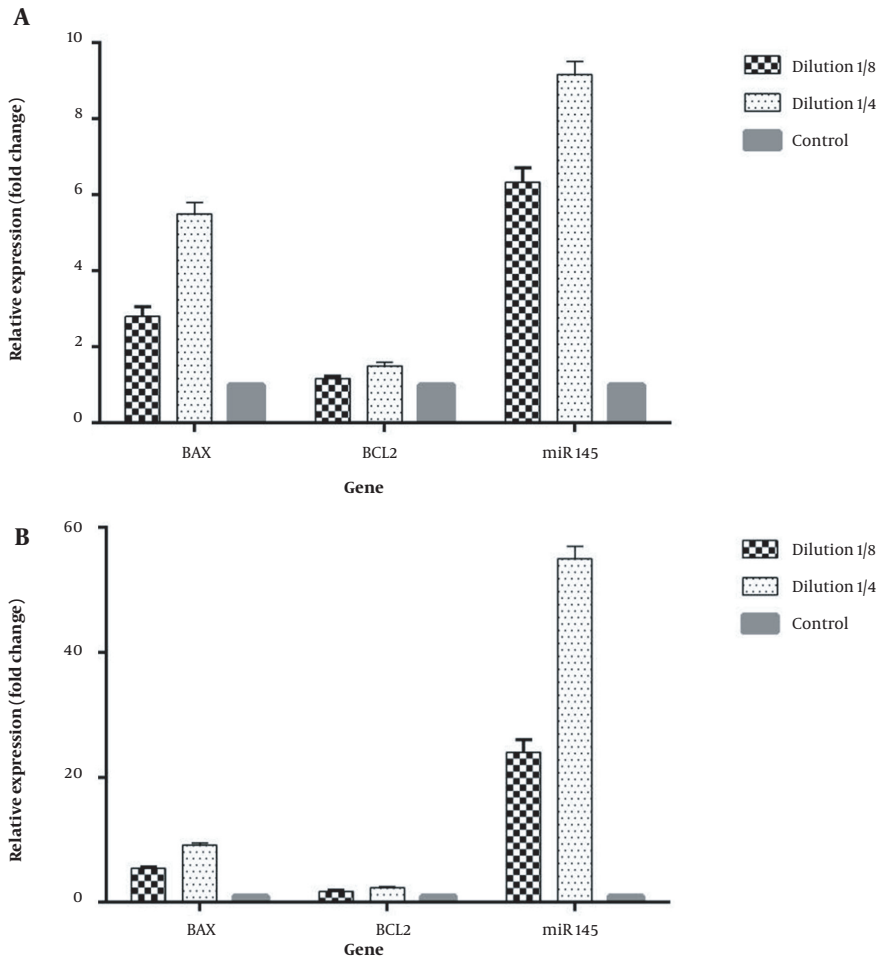

Figure 3. The expression of apoptosis-related genes BAX, BCL-2, and miR-145 in HeLa cancer cells after exposure to *Bacillus clausii* supernatant for 24 (A) and 48 (B) hours.

Table 3. Results of Ad5 Titer in Different Experimental Assays of *Bacillus clausii* Supernatant Inoculation

Different Experimental Assays	Mean Ad5 Titer (Log_{10} $TCID_{50}$/mL ± SD)	
	Supernatant Dilution: 1/4	Supernatant Dilution: 1/8
Pre-treatment	4.30 ± 0.28	5.33 ± 0.14
Pre-incubation	4.91 ± 0.16	5.91 ± 0.28
Competition	5.00 ± 0.14	6.08 ± 0.16
Post-treatment	5.75 ± 0.16	6.33 ± 0.28
Control	8.91 ± 0.14	8.91 ± 0.14

pressor miR-145. This finding has been reported in a few previous studies. Fahmy et al. found that *B. longum* can induce the production of microRNA-145 in murine colorectal tumors (22). In the study of Anton et al., the expression of miRNA145 in cervical cells was upregulated after contact with bacteria-free supernatants of *Gardnella vaginalis* (23).

5.1. Conclusions

Bacillus clausii can be a potent antimicrobial and anticancer agent. However, further studies can determine its anti-bacterial, antiviral, and anticancer activity spectrum.

Footnotes

Authors' Contribution: M.SH. conceived and designed the evaluation and drafted the manuscript. M.Z. participated in designing the evaluation, performed parts of the statistical analysis, and helped to draft the manuscript. Z. F. re-evaluated the data, performed the statistical analysis, and revised the manuscript.

Conflict of Interests: None of the authors have any competing interests.

Ethical Approval: This study is approved under the ethical approval code of IR.IAU.QOM.REC.1401.012 (Link: ethics.research.ac.ir/CommitteeCertificate.php?id=605).

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