Babol University Of Medical Sciences

IJMCM, Summer 2025, VOL 14, NO 3

International Journal of Molecular and Cellular Medicine

Journal homepage: www.ijmcmed.org

ORIGINAL ARTICLE

Evaluation of *Lactobacillus delbrueckii* Supernatant Effects on Cell Cycle Regulation, Apoptosis Induction, and Gene Expression in HT-29 Colorectal Cancer Cells

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ARTICLE INFO

Received: 2025/02/28
Revised: 2025/06/22
Accepted: 2025/07/8

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ABSTRACT

This study investigated the anti-tumor properties of *Lactobacillus delbrueckii*, a probiotic bacterium, against HT-29 human colorectal cancer cells.
The primary objective was to evaluate the effects of *Lactobacillus delbrueckii* supernatant on gene expression, cell viability, apoptosis, and cell cycle progression in HT-29 cells. In Gilan Province, Iran, a native strain of *Lactobacillus delbrueckii* was identified from the supernatant of local yogurt. After treating HT-29 cells with different doses of the supernatant, the MTT test was used to calculate the IC50 value 24 hours later. Annexin V/PI staining and flow cytometry were used to evaluate the induction of apoptosis, and real-time PCR was used to measure changes in gene expression. The results showed that the supernatant of *Lactobacillus delbrueckii* strongly and dose-dependently reduced the growth of HT-29 cells. In addition to causing apoptosis and stopping the cell cycle in the G0/G1 phase, the therapy also increased the number of Sub-G1 cells, which is a sign of cell death. According to gene expression study, anti-apoptotic genes (AKT, Bcl-2) were downregulated while pro-apoptotic genes (PTEN, p53, and Bax) were upregulated. Interestingly, the natural isolate outperformed a conventional strain in terms of anticancer efficacy. These results demonstrate *Lactobacillus delbrueckii*'s potential as a colorectal cancer treatment.

Keywords: *Lactobacillus delbrueckii*, Antineoplastic agents, Apoptosis, Colorectal neoplasms, Gene expression regulation, Cell cycle checkpoints.

Cite this article: Pahlaviani M, et al. Evaluation of *Lactobacillus delbrueckii* Supernatant Effects on Cell Cycle Regulation, Apoptosis Induction, and Gene Expression in HT-29 Colorectal Cancer Cells. International Journal of Molecular and Cellular Medicine. 2025; 14 (3):872-885. DOI: 10.22088/IJMCM.BUMS.14.3.872

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[DOI: 10.22088/IJMCM.BUMS.14.3.872]

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Introduction

Ranking as the third most often diagnosed disease and the second main cause of cancer-related death globally, colorectal cancer (CRC) is a significant global health concern. Startlingly, almost 1.9 million people were found to have CRC in 2020, highlighting its major influence on public health (1, 2). Driven by genetic predisposition and lifestyle choices including processed food consumption, physical inactivity, and disturbances in gut microbial balance, normal glandular cells in the colon or rectum undergo uncontrolled growth, resulting in this aggressive cancer (3, 4). Largely due to demographic changes toward an older population and the adoption of Western lifestyle choices, Iran is seeing a troubling increase in CRC diagnoses that mirrors worldwide trends (2, 5).

Advanced CRC's prognosis is still somewhat bleak; metastatic patients have under 15% five-year survival rates despite present therapy alternatives (6, 7). This dismal fact has driven the hunt for creative treatment strategies even further. Recent studies have focused on probiotics, particularly Lactobacillus strains, as possible supplementary cancer therapies. By disrupting the cell cycle, these good bacteria show extraordinary potential in stopping aberrant cell division (8, 9).

Moreover, they can control important cancer-related genes (6, 10-12) and trigger cellular suicide pathways in malignant cells. Their ability to control the immune system and prevent tumor development as well as their multifarious action make them especially interesting for CRC treatments (13). There are several good reasons for our study to concentrate particularly on Lactobacillus delbrueckii. Compared to conventional laboratory strains, native strains isolated from traditional yogurt in Iran's Gilan Province have shown remarkable capacity to inhibit cancer cell proliferation (14).

While many studies have shown its anti-cancer qualities, hence proving its possible therapeutic utility, this specific species works via different biological pathways setting it apart from other probiotics (12). Though cancerous cells sometimes develop resistance to this preventive biological process, the programmed cell death pathway known as apoptosis is a vital defensive mechanism against tumor formation (15). Colorectal cancer etiology comprises several molecular pathways that remain only partly defined, presenting prospects for alternative treatment approaches targeting disease symptoms and progression (7). Current studies show the therapeutic promise of probiotic formulations, from living bacteria to isolated cellular components like peptidoglycans and exopolysaccharides, which have quantifiable anticancer action (16). Recent research has indicated that alterations in gut microbial ecology and resulting changes in microbial metabolite profiles, especially short-chain fatty acids, phenolic compounds, and tryptophan derivatives, may significantly influence the onset and progression of colorectal cancer (4).

These microbial-derived biomolecules show concentration-dependent regulation of oncogenic pathways, able to either repress or accelerate neoplastic development under various physiological settings (13). Clinical studies indicate that a possible approach for restoring microbial ecosystems and lowering cancer risk is targeted dietary changes including prebiotic fibers, phytochemical-rich foods, and certain probiotic strains (8). Mounting studies show that changes in gut microbial homeostasis and following changes in microbial metabolic output, especially involving short-chain fatty acids, plant-derived polyphenols, and tryptophan catabolites, can both start and aggravate the growth of colorectal cancers, as shown in longitudinal cohort (4). Current research indicates that these microbiota-derived chemicals have concentration-dependent biphasic effects on oncogenic pathways; depending on microenvironmental circumstances, they may either inhibit or paradoxically promote tumorigenic processes (13). Clinical studies show that restoring microbial balance by means of strategic dietary changes including fermentable fibers, phytonutrient-rich foods, and clinically validated probiotic formulations is a realistic option that may also reduce cancer risk factors (8).

Probiotics have antitumor effects by means of a multifaceted mechanistic repertoire comprising: modulation of host physiological responses, suppression of aberrant cellular replication, activation of programmed cell death pathways in malignant cells, interference with pathogenic adhesion mechanisms, enzymatic detoxification of carcinogens, blockade of mutagenic processes, neutralization of procarcinogenic compounds, and enhancement of innate immune defenses via anti-inflammatory mediator secretion (15).Apart from being complementary treatment for confirmed colorectal cancer cases, growing clinical

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data places probiotic supplementation as a potential preventative tool that might lower illness frequency in high-risk groups (16).

Methods

Bacterial Strain and Culture Conditions

The *Lactobacillus delbrueckii* strain KHRS22 was isolated from traditional yogurt in Gilan Province, northern Iran (37.2809°N, 49.5831°E), and fully characterized in our prior genomic study, *which* confirmed its identity as *Lactobacillus delbrueckii* subsp. *bulgaricus* via biochemical profiling (Gram-positive, catalase-negative rods with homofermentative metabolism) and 16S rRNA sequencing (GenBank accession OQ970641) (17). Cryopreserved stocks (-80°C in MRS broth with 20% glycerol) were revived for the present study by two consecutive passages in fresh MRS media (Difco) at 37°C under anaerobic conditions (5% CO_2 , 10% H_2 , 85% N_2). Parallel studies used the reference strain *Lactobacillus delbrueckii* PTCC 1737 (Persian Type Culture Collection) grown under same conditions.

Preparation of bacterial supernatant

The supernatant was prepared by transferring colonies that were grown on MRS agar to MRS broth and incubating them at 37°C for 24 to 48 hours. Then, the pour plate method was employed to enumerate the viable cells on MRS agar plates via successive dilutions with two replicates. The subsequent equation was employed to ascertain the quantity of bacteria after the incubation period: N = CΣ / (d (n1 - n2)) N represents the total count of viable cells per milliliter. CΣ signifies the aggregate of colonies enumerated on all the plates; n1 indicates the quantity of plates retained in the first dilution; n2 suggests the quantity of plates retained in the second dilution; and d represents the dilution factor for the first dilution. The cellular sediment was isolated by centrifuging the active cell suspension at 4000 rpm for 10 minutes at 4°C after the bacterial growth media was transferred to a fresh tube. The detritus was rinsed multiple times with phosphate buffered saline (PBS) solution to guarantee the absence of cellular debris. Following the final centrifugation, the sediment obtained was lyophilized. At the time of use, different concentrations of 0.5, 0.75, 1, 1.5, and 2 milligrams/milliliter of supernatant were prepared using MRS broth and sterilized by a 0.22 µm filter.

Cell Culture and Treatment

HT-29 human colorectal adenocarcinoma cells were cultured in complete growth medium at 37°C in a humidified 5% CO_2 atmosphere. For viability assays, the cells were seeded in 96-well plates at an optimal density of 5×10^3 cells/well (200 µL final volume) to ensure proper monolayer formation without overcrowding. For apoptosis, cell cycle and gene expression studies, the cells were plated in 6-well plates at 2×10^5 cells/well (2 mL medium), allowing sufficient space for the cell proliferation during the treatment period. After 24 hours of attachment, cells were treated with various concentrations (0.5-2 mg/mL) of *Lactobacillus delbrueckii* supernatant for 24 hours.

Cell Viability Assessment (MTT Assay)

HT-29, the human colorectal cancer cell line, was supplied by the Iranian Biological Resource Center. The cells were then grown in 96-well plates at a density of 5×103 cells per well and allowed to incubate overnight at 37°C in a CO2 incubator. The cells were subsequently exposed for a full day to varying quantities of probiotic bacterial supernatant (0.5–2 mg/mL).The deleterious effects of the probiotic bacterial supernatant on HT-29 cells were evaluated using the MTT colorimetric assay. To achieve this, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and allowed to react for four hours at 37°C in an atmosphere with 5% CO2. The absorbance at 570 nm was quantified using an ELISA reader, and the formazan crystals that resulted were dissolved in DMSO. These data were employed to determine and publish cell viability percentages and IC50 values, which are the concentrations required to obstruct cell growth by 50% in comparison to untreated control cells.

Apoptosis Detection by Flow Cytometry

The Annexin V-FITC/propidium iodide (PI) double staining reagent (Roche, Germany) was employed to evaluate apoptosis induction. HT-29 cells were treated with IC_{50} concentrations of bacterial supernatant for 24 hours, and subsequently extracted and rinsed twice with phosphate-buffered saline (PBS). Annexin V-FITC and PI were used to stain the cells, which were resuspended in binding buffer in accordance with the manufacturer's protocol. Untreated cells were employed as a control. The BD

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FACScan flow cytometer (Becton Dickinson, USA) was employed to analyze the samples immediately. Cells were classified as viable (Annexin V^- /PI^-), early apoptotic (Annexin V^+ /PI^-), late apoptotic (Annexin V^+ /PI^+), or necrotic (Annexin V^- /PI^+) using FlowJo software for data acquisition and analysis.

Gene Expression Analysis Protocol

For gene expression studies, HT-29 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2 mL of complete growth medium. This plating density ensured optimal cell confluence (~70-80%) at the time of treatment. Following 24-hour exposure to bacterial supernatant at the established IC50 concentration (1.0 mg/mL), HT-29 cells were processed for the molecular analysis. The RNX-Plus reagent (SinaClon, Iran) was employed to extract total RNA from approximately 5×10^6 cells. An optimized protocol was implemented, which involved sequential lysis with 1 mL of RNX-Plus, phase separation through chloroform addition (200 µL, 15 sec vigorous mixing), and RNA precipitation with isopropanol during a 1-hour incubation at -20°C.

The RNA granules that were produced were rinsed with 75% ethanol, air-dried, and resuspended in nuclease-free water. The RNA integrity was verified through spectrophotometric assessment (NanoDrop 2000, A260/A280 ratio 1.8-2.0) and electrophoretic visualization of identifiable 28S/18S rRNA bands on 1% agarose gels. For cDNA synthesis, 1 µg of purified total RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) with oligo(dT)18 primers in a 20 µL reaction volume. The thermal profile included a 5-minute primer annealing step at 25°C, followed by 60-minute cDNA synthesis at 42°C and 5-minute enzyme inactivation at 70°C in a Bio-Rad thermal cycler.

The synthesized cDNA was diluted five-fold in nuclease-free water and stored at -20°C until qPCR analysis. β-actin (ACTB) was used as an internal reference gene for the quantitative assessment, BCL2-associated X protein(Bax), B-cell lymphoma 2 (Bcl2), tumor protein p53 (p53), protein kinase B (AKT), and phosphatase and tensin homolog (PTEN) gene expression was conducted using SYBR Green chemistry on a real-time PCR system. Each 20 µL reaction contained 2 µL cDNA template, 10 µL 2× SYBR Green Master Mix, and 0.5 µM of gene-specific primers. The amplification protocol comprised the initial denaturation (95°C, 10 min), 40 cycles of denaturation (95°C, 15 sec) and combined annealing/extension (60°C, 1 min), followed by melting curve analysis (60-95°C, 0.5°C increments) to confirm amplification specificity. All reactions were performed in triplicate, with cycle threshold (Ct) values normalized to β-actin and relative expression calculated using 2^(-ΔΔCt) method. The ΔΔCt (delta-delta Ct) method was implemented to ascertain the up-regulation or down-regulation of gene expression for PTEN/AKT, P53, and BAX/BCL-2.

This method normalizes target gene expression to the reference gene (β-actin) and compares treated samples to untreated controls. The formula $2^{-\Delta\Delta Ct}$ was employed to calculate the fold change in gene expression, where ΔΔCt is the difference between the control and the treated (Ct target gene−Ct β-actin). Up-regulation is indicated by a fold change >1, while down-regulation is indicated by a value <1. Accurate quantification of transcriptional alterations in response to probiotic supernatant treatment is guaranteed by this method, which has been validated by Livak and Schmittgen (2001).

The apoptotic activity of BAX/BCL-2 was evaluated by examining the ratio of their respective fold alterations. An elevated BAX/BCL-2 ratio indicates that apoptosis is facilitated. The regulatory effects of probiotic-derived compounds on key oncogenic and tumor-suppressive pathways in HT-29 and HUVEC cells were assessed using this standardized approach in the study (18).

Results

MTT test is used as a routine method to examine the viability and toxicity of cancer cell cultures compared to the control. The viability (IC_{50}) and/or toxicity (CC50) of supernatant bacterial strains of native and standard *Lactobacillus delbrueckii* were measured after 24 hours of treatment on HT-29 cancer cells and HUVEC. The results of MTT using different concentrations of *Lactobacillus delbrueckii* solutions are presented in graphs. The survival rate in the control group was considered 100%.

Figure 1 shows that supernatant from native and standard strains after cancer cell treatment had the maximum cytotoxicity relative to untreated cells (control) at the concentrations of 1.0 mg/mL and 2.0

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mg/mL, respectively (Figure 2). MTT assay showed that the supernatant from the native *Lactobacillus delbrueckii* strain exhibited significant cytotoxic effects on HT-29 colorectal cancer cells in a dose-dependent manner. The native strain exhibited an IC50 value of 1.0 mg/mL, whereas the standard strain exhibited a reduced activity with an IC50 of 2.0 mg/mL. Significantly, the supernatant demonstrated minimal toxicity toward normal HUVEC cells, suggesting selective anticancer activity.

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COMMENT     Sequences were screened for chimeras by the submitter using MEGA
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Figure 1. Registration of the 16SrRNA genomic sequence in the NCBI database

Cell cycle analysis

Following 24-hour treatment of HT-29 cells, it was found that the native supernatant strain was able to successfully block cell transit from G0/G1 phase to the following phases of interphase, as opposed to the control. Besides, the proportion of malignant HT-29 cells in Sub-G1 phase increased significantly when compared to the standard strain and control. Cancer cells increased by 34.82%, 5.85%, and 2.49% ($P<0.05$). After treatment, the percentages of cancer cells in G0/G1 phase were 76.29% for the control sample, 73.60% for the standard strain, and 35.01% for the native strain ($P < 0.05$). These findings clearly suggest that the native strain under investigation effectively terminated the cell cycle by inducing apoptosis, presumably commencing in the S phase (Figure 3).

The aforementioned figures are the average of two repetitions for each sample that was tested.Two-tailed t-test analysis was used via Prism software for calculation purposes. The pro-apoptotic genes of PTEN and P53 and were up-regulated after 24 hours. Bcl2 expression, an anti-apoptotic gene, decreased significantly after 24 hours ($P<0.05$). At the molecular

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level, real-time PCR analysis showed significant alterations in the expression of key regulatory genes. The native strain supernatant upregulated tumor suppressor genes *PTEN* (2.1-fold) and *p53* (2.4-fold), along with the pro-apoptotic *Bax* (1.8-fold). Conversely, it downregulated the survival gene *AKT* (0.6-fold) and anti-apoptotic Bcl-2 (0.7-fold). These changes resulted in a favorable Bax/Bcl-2 ratio shift, promoting apoptosis through the intrinsic mitochondrial pathway.

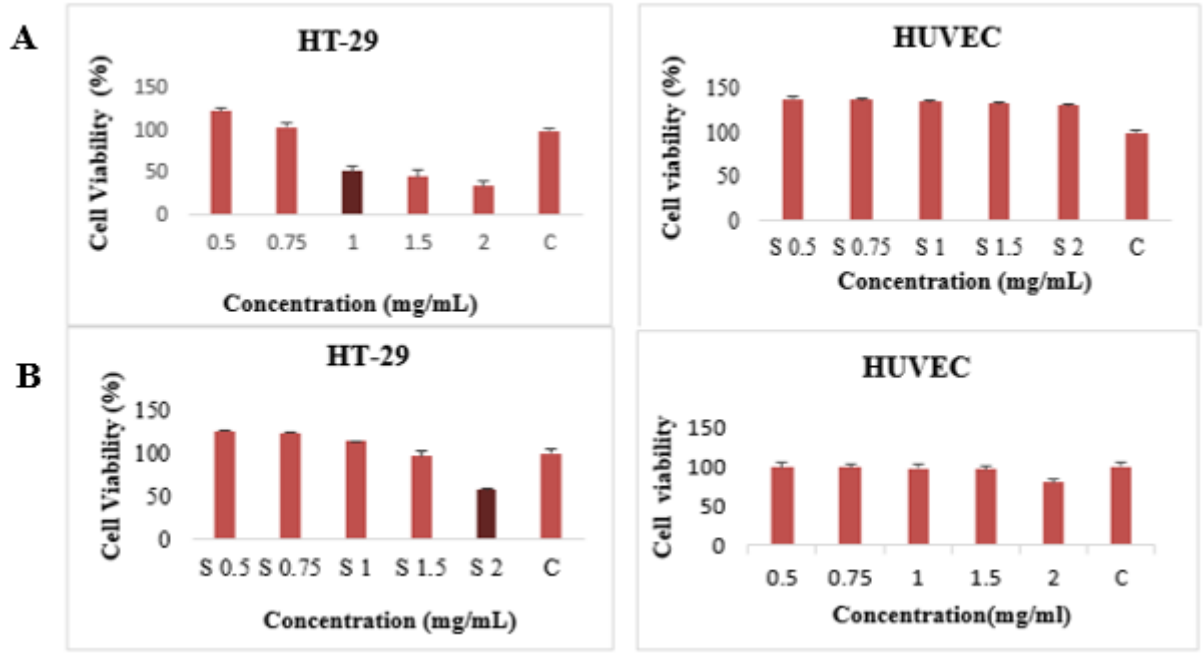


Figure 2. A) The survival percentage of HT-29 and HUVEC cells after treatment with different concentrations of the supernatant of the native strain of Lactobacillus delbrueckii. B) Survival percentage of HT-29 and HUVEC cells after treatment with different concentrations of the supernatant of standard strain Lactobacillus delbrueckii.

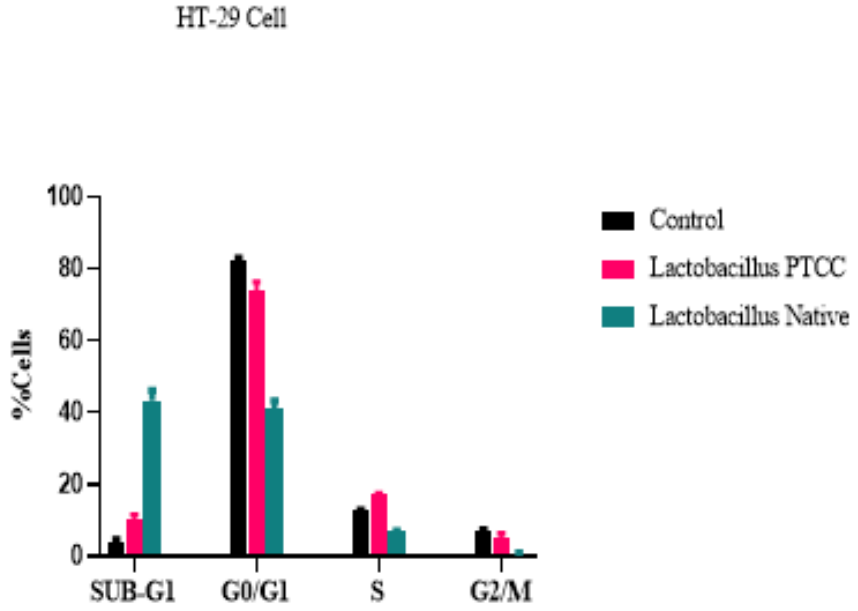


Figure 3. Statistical analysis of the inhibitory effects of native and standard Lactobacillus delbrueckii strains in different phases of the HT-29 cancer cell cycle compared to control.

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Moreover, gene expression data supported the results of apoptosis induction using the flow cytometry method (Figure 4). Examination of cell cycle distribution showed that treatment with the native strain supernatant produced a considerable increase of HT-29 cells in the G0/G1 phase (35.01%) relative to untreated controls (76.29%).Furthermore, a substantial increase in Sub-G1 population (34.82%) was observed, suggesting induction of apoptotic cell death. In contrast, standard strain showed only modest effects on cell cycle progression.

Apoptosis in HT-29 cells

The objective of this experiment was to ascertain the proportion of living and deceased cells, as well as to investigate cell proliferation and the cell cycle. The phenomena of necrosis and apoptosis were evaluated. The IC50 concentration of supernatant from both a native and a standard strain of *Lactobacillus delbrueckii* was administered to HT-29 cells for a 24-hour period. The flow cytometry results for treated, control, and standard cells are depicted in Figures 5A, 5B, and 5C, respectively. The figure shows that the percentage of necrotic cells (Q1), early and late apoptotic cells (Q2, Q3), and viable cells (Q4) in the treated and control cells differed significantly ($P<0.05$). The results showed that apoptosis caused the percentage of viable cells to drop from 74.6% (Q4) to 12.1% (Q3) and finally to 9.89% (Q2) after treatment with the native strain's supernatant.In contrast, the level of early and late apoptosis following the treatment of tumor cells with the supernatant of the standard strain was significantly lower compared to the level of apoptosis of the native strain, decreasing from 89.27% (Q4) to 3.85% (Q3), and then to 2.77% (Q2) ($P<0.05$).

However, the percentage of necrotic or secondary necrotic cells in cells treated with the supernatant of native and standard strains was approximately similar, and no significant difference was observed ($P>0.05$). As observed in Figure 6, morphological examination indicated apoptotic cell morphology resulting from tumor cell treatment with isolated *Lactobacillus delbrueckii* supernatant causing changes in the cellular and nuclear morphology in HT-29 tumor cells with the formation of apoptotic bodies (ApoBDs) and nuclear fragmentation compared to control HT-29 cells.

Flow cytometry using Annexin V/PI staining confirmed the pro-apoptotic effects of native strain supernatant. The treatment resulted in 9.89% early apoptotic (Annexin V+/PI-) and 12.1% late apoptotic (Annexin V+/PI+) cells, while untreated controls showed minimal apoptosis. Morphological examination via DAPI staining revealed characteristic apoptotic features including nuclear condensation and fragmentation in treated cells.

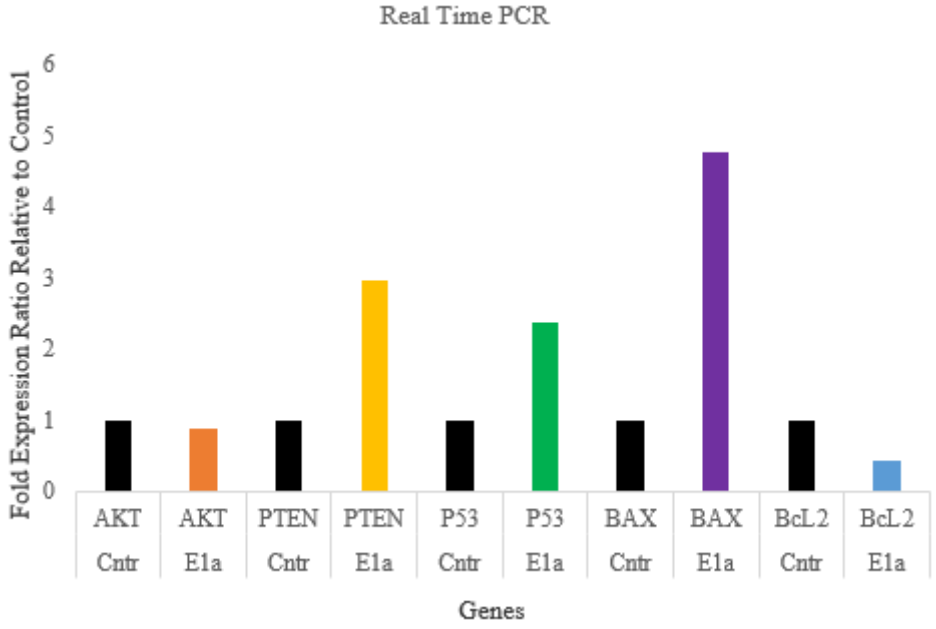


Figure 4. Comparison between PTEN, AKT, P53, BAX, and BCL2 gene expression in HT-29 cell culture treated with isolated native *Lactobacillus delbrueckii* supernatant at IC50 concentration of 1.0 mg/mL during a 24-hour incubation period

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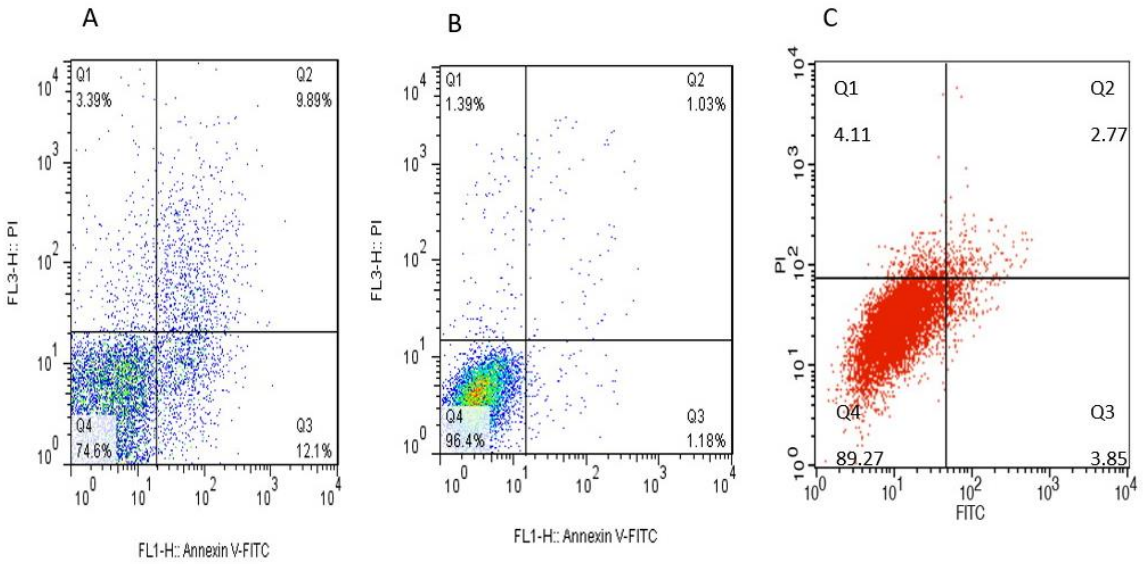


Figure 5. Flow cytometry assessment of apoptosis using (A) HT29 cells treated with the IC50 concentration of supernatant from the native strain of *Lactobacillus delbrueckii* isolated from local yogurt (B) Annexin V-PI - HT-29 cell control sample and (C) HT29 cells treated with the IC50 concentration of supernatant from the standard strain of *Lactobacillus delbrueckii*.

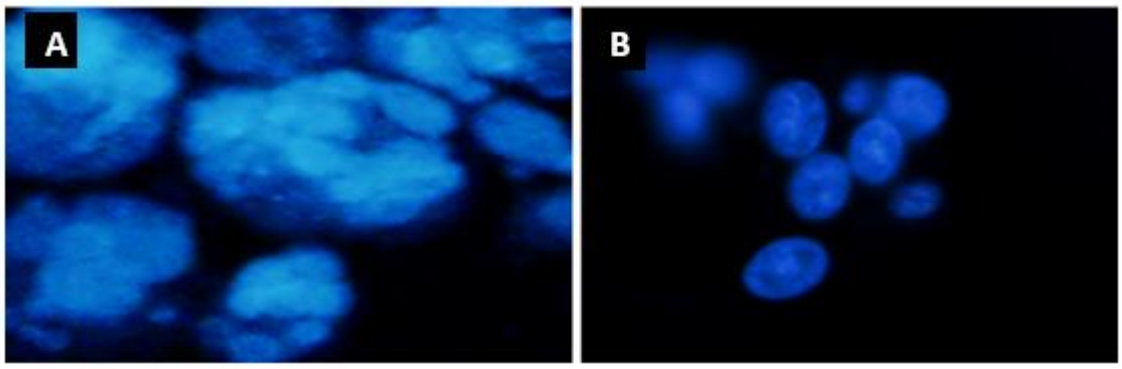


Figure 6. Normal and apoptotic HT-29 tumor cells in DAPI staining. A without treatment with supernatant of *Lactobacillus delbrueckii* isolate. B treatment with supernatant of *Lactobacillus delbrueckii* strain after 24 hours of incubation.

Data and Statistical Analysis

Quantitative gene expression data for PTEN, AKT, TP53, BAX, and BCL2 were normalized to β-actin as internal reference control. Relative expression levels were calculated using the ΔΔCt method, with results presented as mean ± standard deviation from three independent experiments. With a statistical significance threshold of $p < 0.05$, the experimental groups were compared statistically using either a one-way ANOVA with Tukey's post hoc test for multiple group analyses or Student's t-test for pairwise comparisons.

All statistical analyses were conducted using GraphPad Prism version 9.0, while flow cytometry data for apoptosis assays were processed using FlowJo software (version 10.8).

Discussion

Over the last several decades, a notable lot of study has been done to find novel probiotics that may satisfy market needs and are also good for health. Further study in this field is required because of the variety of species or strains among probiotics from various sources and the growing need for novel and potent probiotics in the worldwide market. Probiotics taken from the human or animal digestive system have certain qualities that set them apart from dairy

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products.For instance, they show more tolerance to bile salts and lower pH levels than dairy products (19). Sub-species of Lactobacillus bulgaricus isolated from local yogurt in Gilan province can be used as a biological preservative, this study found. Because of their strain durability in food and dairy products, they can be effective as a supplement or preferably as a co-culture with peptides such as lactoferrin during product expiration. This study looked at how Lactobacillus delbrueckii, a probiotic bacteria, affected HT-29 cells.

In particular, we looked at its capacity to cause cell death and stop cell proliferation. The results showed that Lactobacillus delbrueckii increased apoptosis and successfully stopped HT-29 cell growth. Apoptosis induction and anti-proliferative activities were dependent on the probiotic bacterial supernatant exposure dose. It is intriguing that the induction of apoptosis was more robust at higher supernatant concentrations. The probiotic strain under investigation showed the antagonistic and anti-tumor properties, as evidenced by the assessment of its probiotic properties, and anti-tumor activity in vaginal samples (16). The supernatant effects of strains on cancer cells such as HT-29 resulted in a 20-40% inhibition of their growth. The highest inhibitory concentration reported in this study was 50 µg/Ml (Figure 2). Furthermore, the cytotoxic effects of the supernatant solution on normal cells (HUVEC) were not significant (P>0.05).

Differences in effective concentration in studies may be in terms of the mass used after lyophilization of bacteria culture, differences in selected probiotic strains, or use of extract or supernatant or live cells in MTT method. These results were also consistent with the findings of Motevaseli et al., which demonstrated that supernatants from prevalent vaginal lactobacilli did not exhibit toxicity on normal cervical cells. In 2019, Chuah et al. conducted a study that revealed that the Lactobacillus plantarum metabolite exhibited limited cytotoxicity at a 26% (v/v) IC50 after 72 hours of incubation. Elsewhere, regarding the effect of growth environment on MTT assay in bacteria, Benov in 2019 showed using a glucose-containing buffer eliminates the effects of metabolites and restricts cell division. This study suggested that selecting an appropriate solvent for formazan decomposition should be considered an important factor. In MTT assay, unlike our research, the mentioned researchers did not use a standard probiotic strain for comparison (control group), which could be debatable in interpreting the results (20). Based on the current project, the results of the flow cytometry evaluation of cell cycle by Rabiee et al. in 2019 regarding the anti-proliferative effects of *Lactobacillus casei* isolated from traditional dairy products were able to show a significant cytotoxicity compared to the control, with cells in SubG1 phase growing by 0.326% compared to the control (4.6%) (21). The increase was shown to be 34.82% with the native strain and 5.85% with the standard strain of Lactobacillus. Comparable investigations conducted by this cohort of researchers may demonstrate inhibitory effects and cellular transition to the apoptotic phase.

The findings of Kaholi as well as Choui indicated a decline in tumor cell survival, respectively, through extract and supernatant of *Lactobacillus acidophilus* and fermentum (22, 23). However, Er et al. showed that the anti-tumor effects of many Lactobacillus strains on Caco-2 cells were not statistically significant (24). The difference in results among the studies can indicate strain characteristics, dosage, metabolite content used, differences in research methods, and appropriate time for metabolite release during analysis. In 2021, Dehghani et al. conducted a study on the probiotic performance of Lactobacillus rhamnosus isolated from traditional yogurt on HT-29 tumor cells.

The study revealed that the isolated supernatant was capable of showing inhibitory effects and anti-proliferative properties, with an increase in Sub-G1 peak and cell cycle arrest at G0/G1 phase at IC50 values of 1.95, 0.25, and 0.053 milligrams per milliliter during incubation times of 24, 48, and 72 hours, respectively. Ultimately, this prevented cellular proliferation (14). In agreement with our results, Borowicki et al. reported in 2011 that *Lactobacillus rhamnosus* GG supernatant was able to reduce the growth of HT-29 cancer cells and inhibit tumor cell proliferation at G0/G1 phase (8).

Building upon established cytotoxic effects of probiotic metabolites (IC50 0.1–0.5 mg/mL) reported in colorectal cancer models, our study provided novel mechanistic evidence that *Lactobacillus delbrueckii* supernatants coordinately regulate apoptotic pathways in HT-29 cells. The dose-dependent inhibitory effects observed in our study demonstrated significant agreement with the established cytotoxic profiles reported by Kumar et al. (2022) in their investigation of Lactobacillus-derived bioactive compounds against

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HT-29 colorectal cancer cells. The therapeutic potential of probiotic metabolites is further substantiated by the alignment of IC50 values between studies, which is notably evident in the comparable growth inhibition patterns observed at concentrations spanning from 0.1–0.5 mg/mL. Our extended analysis of apoptotic gene regulation offers novel insights that surpass previous characterizations of cytotoxic effects, while the consistency of these findings suggests conserved mechanisms of action across similar experimental models.

The observed modulation of PTEN/AKT signaling and TP53 activation, coupled with altered BAX/BCL2 ratios, extends beyond previous reports of ROS-mediated apoptosis by showing integrated genetic control of cell death processes. Particularly significant is the concurrent suppression of BCL2 with TP53 upregulation, which correlates with the oxidative stress cascade (ROS accumulation, ΔΨm reduction, caspase-3 activation) while establishing a new paradigm for probiotic-induced cancer cell elimination. These findings resolve previous dichotomous observations of concentration-dependent cytotoxicity versus metabolic stress effects by revealing their synergistic operation through defined transcriptional networks.

The gene expression profiles reveal that *Lactobacillus delbrueckii* supernatants coordinately modulate PTEN/AKT survival signaling and TP53-mediated apoptosis, while markedly altering the BAX/BCL2 ratio – effects that parallel the oxidative stress cascade described by Lin et al. (2019), where bacterial metabolites induce ROS accumulation, mitochondrial dysfunction (reduced ΔΨm), and caspase-3 activation. The first evidence of integrated genetic regulation bridging these phenomena is provided by our study, which establishes that probiotic cytotoxicity operates through synergistic mechanisms: direct concentration-dependent growth suppression coupled with ROS-mediated apoptotic induction. The observed concurrent BCL2 suppression and TP53 activation is particularly noteworthy, as it defines an optimized therapeutic window in which metabolic stress and transcriptional responses cooperatively facilitate the elimination of cancer cells.

This unified framework reconciles previous discrete observations of probiotic anticancer activity with our novel demonstration of coordinated pathway regulation in colorectal cancer models. The anti-tumor effects of *Lactobacillus delbrueckii* supernatant on HT-29 cells were further elucidated through gene expression analysis of key regulators in colorectal cancer pathogenesis. The selected genes—PTEN, p53, AKT, Bax, and Bcl-2—play critical roles in modulating apoptosis and cell cycle progression, making them ideal biomarkers for investigating the molecular mechanisms underlying the observed anti-proliferative effects. PTEN, a tumor suppressor, counteracts the PI3K/AKT pathway, which is frequently hyperactivated in colorectal cancer and promotes cell survival. In treated cells, the upregulation of PTEN and the subsequent downregulation of AKT indicate that the inhibitory pathway has been restored, resulting in the suppression of oncogenic signaling.

In the same vein, the elevated expression of p53, a master regulator of apoptosis and cell cycle arrest, suggests the reactivation of tumor-suppressive functions that are frequently compromised in cancer cells. The observed shift toward elevated Bax and reduced Bcl-2 expression favors programmed cell death, as the pro-apoptotic Bax and anti-apoptotic Bcl-2 serve as critical determinants of mitochondrial-mediated apoptosis.

These molecular changes align with our experimental findings of cell cycle arrest at the G0/G1 phase and increased Sub-G1 populations, showing the supernatant's ability to simultaneously target multiple oncogenic pathways. The coordinated modulation of these genes highlights the potential of *Lactobacillus delbrueckii* metabolites as multi-targeted therapeutic agents against colorectal cancer, warranting further investigation into their specific bioactive components and mechanisms of action. It was observed that P53 and PTEN genes increased while AKT gene decreased. AKT is a kinase involved in various biological processes, including apoptosis and cell proliferation. Its activation can inhibit proteins involved in apoptosis and increase the expression of anti-apoptotic proteins, promoting tumor cell survival. Nevertheless, the PTEN tumor suppressor protein, which is frequently mutated in cancer, regulates AKT activity.

Despite understanding the role of the PTEN/AKT signaling pathway in the induction of apoptosis in colorectal cancer, targeted interventions for this disease have not been entirely successful due to the complex feedback loops and interactions among pathway inhibitors. In cancer, PTEN is frequently

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targeted for inactivation and is recognized as a significant regulator of this pathway. Theoretically, PTEN inactivation should be accompanied by AKT activation to enhance the pathway in tumors (11). Thus, it can be concluded that PTEN acts as a negative regulator in the PTEN/AKT signaling pathway and can be targeted by regulatory proteins affected during cancer. Consequently, PTEN dysregulation may affect other oncogenic pathways alongside the PTEN/AKT pathway. Indeed, PTEN not only acts as an inhibitor of this pathway, but also inhibits other growth pathways through protein interactions, making it the main inhibitor of signal transduction (12, 25).

In fact, PTEN network is not independently functional; rather, it is intricately connected to other oncogenic signaling pathways and tumor-suppressive pathways, including those associated with Ras and p53. PTEN and p53 are not only involved in numerous similar cellular processes, but they also physically interact and modulate one another. The physical interaction between p53 and PTEN affects the acetylation status of p53 and its capacity to bind to DNA. The signaling pathway of phosphoinositide-3-kinase (PI3-K)/PTEN/AKT in colorectal cancer has been described by various research groups (26, 27). Since it has been shown that the expression of AKT and PTEN is disrupted in 60 to 70% of colorectal cancers, inhibiting this signaling pathway was proposed as a potential target in CRC (6, 7).

Bcl2 is an anti-apoptotic protein, while Bax can be involved in apoptosis through the intrinsic (mitochondrial) pathway. Bcl2 overexpression is frequently linked to chemotherapy resistance and aggressive malignancy, and it is negatively regulated by Bax. Furthermore, the Bax/Bcl2 ratio has been significantly altered by increased expression of PTEN and decreased expression of AKT, with overexpression of the Bcl-2 family being detected in approximately 94-30% of human CRC cases (20). Consequently, an additional objective of our investigation was to use real-time PCR to investigate modifications in the expression of BAX and BCL-2 genes to enhance the analysis of results.

HT-29 cancer cells were able to undergo apoptosis as a result of increased expression of PTEN (i), decreased AKT (ii), increased expression of BAX, and diminished expression of BCL-2 (iii), as well as potential variations in mitochondrial function, which were induced by our supernatant strain. Furthermore, increased expression and activity of P53 via cell cycle arrest and induction of apoptosis played an important role in limiting malignant cell growth, as shown by other studies (9). It was shown that mutations in this gene accelerate adaptive responses to stress conditions associated with cancer and can support tumor progression (28).

Since PTEN is one of the target factors for the P53 transcription factor, which inhibits PI3K activity, cells deficiency in PTEN exhibit persistently high levels of PIP3 and activate their downstream pathways. Both PTEN and p53 are tumor suppressors that work by preventing cell cycle progression and speeding up apoptosis. The qPCR method was used to conduct a simultaneous analysis of variations in P53 expression, PTEN/AKT, and BAX/BCL2 genes in this study. Mutations in P53 not only disrupt its tumor-suppressive activity, but also confer oncogenic properties to mutant p53 protein (29). Considering aforementioned reasons, changes in P53 gene expression were evaluated as its central role in tumor suppression using Benjamini and Hochberg method.

Our findings indicated a significant increase in P53 gene expression in tumor cells compared to control cells after treatment with native supernatant strain *Lactobacillus delbrueckii* during a 24-hour incubation period with HT-29 tumor cells (mRNA Fold change: 2.37, $P<0.0001$). It is possible to assert that PTEN functions as a negative regulator of the PI3K/AKT signaling pathway and primarily utilizes PIP3 as its lipid substrate. Akt activates mTORC1 and deactivates TSC1/2, whereas mTORC2 directly activates AKT. Nevertheless, the PI3K/AKT pathway is inhibited by PTEN. The final status of the cell is determined by Bcl-2 family factors, which influence the fate of the mitochondrial pathway. Inhibitors of the PI3K/AKT pathway can increase tumor cell sensitivity to chemotherapy-induced apoptosis and prevent drug resistance. PTEN enzyme prevents mdm2 activation by maintaining Akt in an inactive state.

When Akt is activated via the PI3 kinase pathway, it phosphorylates cytoplasmic Mdm2, leading to its movement into the nucleus. Once in the nucleus, Mdm2 tags p53 with ubiquitin, marking it for breakdown in the cytoplasm by proteasomes. Thus, the PI3K/AKT pathway may be a promising target for tumor treatment. There is abundant evidence showing the importance of P53 and Bcl-2 in cancer biology today (30). It can be inferred that precise qPCR

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analysis has the potential to enhance clinical diagnosis as well as the ability to predict and assess the prognosis of a disease. This implies that patients may benefit from enhanced therapeutic options, better outcomes, and expeditious treatment when they have confidence in the analytical results, which can assist in the identification and measurement of biomarkers in the early phases of disease.

In conclusion, PTEN/AKT pathway is the primary pathway for cell survival in cancer. PTEN functions as a negative regulator of the PTEN/AKT signaling pathway, which is susceptible to injury and can be affected by other oncogenic pathways if it is compromised. PTEN is regarded as the primary signaling pathway in colorectal cancer due to its ability to inhibit growth pathways. Consequently, the cancer apoptotic pathway is facilitated by the decrease in AKT expression and the increase in PTEN expression. Furthermore, PTEN regulates p53 either directly or indirectly. In this investigation, the expression of p53 was found to increase in HT-29 tumor cells that were treated with both native and standard solutions. However, tumor suppression with p53 can be achieved at multiple levels and through various mechanisms. Furthermore, p53 activates the expression of Bcl-2 family members and releases cytochrome C from mitochondria in the internal death pathway.

Therefore, this research verified that the induction of apoptosis in HT-29 cells is linked to increased Bax expression and decreased Bcl2 expression, resulting in accelerated apoptosis in response to variations in Bax/Bcl2 expression. Besides, the induction of apoptosis in cells treated with native isolates resulted in substantial alterations in gene expression when compared to cells treated with standard solutions, suggesting a greater potential for inducing apoptosis.The anti-tumor effects of *Lactobacillus delbrueckii* supernatant on HT-29 cells were further elucidated via gene expression analysis of key regulators in colorectal cancer pathogenesis.

The relative expression levels of target genes (PTEN, p53, AKT, Bax, and Bcl-2) were determined using 2-ΔΔCt method (18), where ΔΔCt = (Ct target - Ctβ-actin) treated - (Ct target - Ctβ-actin) control. Fold changes were calculated as 2-ΔΔCt, with values >1 indicating up-regulation and values <1 indicating down-regulation compared to untreated controls. The chosen genes are essential in regulating apoptosis and cell cycle progression, with PTEN opposing the often hyperactivated PI3K/AKT pathway in colorectal cancer. Our research indicated a notable up-regulation of PTEN (2.1-fold) and p53 (2.4-fold), accompanied by a down-regulation of AKT (0.6-fold), illustrating the reinstatement of tumor-suppressive signaling. The pro-apoptotic Bax showed 1.8-fold up-regulation while the anti-apoptotic Bcl-2 was down-regulated to 0.7-fold, shifting Bax/Bcl-2 ratio toward apoptosis. These molecular changes, quantified through rigorous qPCR analysis, provide mechanistic support for the observed cell cycle arrest and apoptotic effects, highlighting the potential of *Lactobacillus delbrueckii* metabolites as multi-targeted therapeutic agents against colorectal cancer.

While previous studies showed either cytotoxicity or oxidative damage in isolation, our work bridges these phenomena by showing their interdependence through defined molecular pathways. The study's limitations, such as the use of an in vitro model that is unable to completely replicate the complexities of the tumor microenvironment, necessitate contextualizing these insights. The multi-targeted mechanism is emphasized by the PTEN/AKT/p53 axis and the Bax/Bcl-2 ratio alterations. These findings align with prior studies (31) but highlight the native strain’s superior efficacy.

Nevertheless, the demonstration of synchronized pathway regulation supports the potential of probiotics as multi-target agents in cancer prevention or adjunct therapy. Future work should validate these mechanisms in vivo and identify the specific bioactive compounds responsible for these effects. This study reveals the unique anti-tumor activity of a native Iranian *Lactobacillus delbrueckii* strain, demonstrating its dual activation of PTEN (2.1-fold) and p53 (2.4-fold) to overcome PTEN-deficient resistance in HT-29 cells. The supernatant’s multi-target effects (BAX/BCL-2 modulation) and selective cytotoxicity (IC50 1.0 mg/mL) highlight its potential as a precision probiotic therapy, outperforming conventional strains. These findings establish regionally isolated probiotics as viable alternatives for CRC treatment.

Acknowledgements

We extend our sincere gratitude to the Research and Enterprise Committee of Islamic Azad University, Kazerun Branch, Iran, for their invaluable support throughout this study. The study was conducted in

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[DOI: 10.22088/IJMCM.BUMS.14.3.872]

accordance with the ethical principles of the Declaration of Helsinki and approved by the Ethics Committee of Islamic Azad University, Kazerun Branch (Approval Code: IR.IAU.KAU.REC.1404.019).

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