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Targeting Colorectal Cancer: *Limosilactobacillus Fermentum* Gl As A Promising Probiotic Approach

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Abstract

Colorectal cancer (CRC) remains a primary global health concern, necessitating innovative therapeutic strategies. Recent studies have brought attention to the promise of probiotics in the context of cancer therapy. This promise arises from their capability to influence various signaling pathways involved in the cell cycle, cellular proliferation, and programmed cell death (apoptosis). The present study examines the potential of Limosilactobacillus fermentum GL to trigger apoptosis and hinder cell proliferation in vitro within colorectal cancer (CRC) cells. To achieve this objective, we prepared supernatant and bacterial extract and co-cultured them with HT-29 cells. The cytotoxic impact of the bacterial cell extract on the HT-29 cell line was evaluated using the MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) method. Quantitative RT-PCR analysis was utilized to assess changes in the mRNA expression levels of p53, PTEN, and caspase-3. Additionally, apoptosis was analyzed through flow cytometry using the V-FITC kit. The results revealed that co-culturing with L. fermentum GL reduced HT-29 cell proliferation and increased apoptosis. These effects were accompanied by the concurrent induction of p53, PTEN, and caspase-3 expression. In summary, L. fermentum GL presents a promising probiotic approach for targeting colorectal cancer cells. The harnessing of probiotics' potential in cancer therapy has the potential to transform treatment approaches, providing a safer and more effective complement to exciting therapies.

Introduction

Colorectal cancer (CRC) ranks among the most common gastrointestinal (Gl) malignancies on a global scale. The pathogenesis of CRC is complex and its diagnosis is often difficult in the early stages of the disease [1]. Surgery, chemotherapy, and radiotherapy are the most common treatment methods with severe side effects [2,3]. The primary hallmark of cancer cells is unregulated cellular growth and resistance to apoptosis [4]. An anticancer agent is typically characterized by its ability to induce apoptosis in cancer cells [5]. Among various gastrointestinal cell lines, HT-29 stands out as a human colorectal adenocarcinoma cell line with epithelial characteristics, making it responsive to chemotherapeutic drugs. Additionally, the HT-29 cell lines serve as an in-vitro model for investigating the absorption, transport, and secretion processes in intestinal cells [6]. In tandem with the progress in developing novel diagnostic and therapeutic strategies for gastrointestinal cancers, numerous probiotic strains have found utility as nutritional supplements [7]. Probiotics, beneficial microorganisms that regulate digestive enzymes in both animals and humans, exhibit inhibitory effects on cancer-related factors in vitro and in vivo. They also play a vital role in attending cancer-inducing compounds and tumors in laboratory animals [8,9]. Probiotics are commonly incorporated into fermented dairy products like yogurt, with lactobacilli serving as significant sources of these beneficial microorganisms

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[10]. Yogurt is a dairy product prepared by fermenting milk with *lactobacillus* species. Today, the preventive and nutritional properties of yogurt are widely accepted. When administered in sufficient amounts, probiotics promote host health and microbial balance [11,12]. A study demonstrated that quantifying *lactobacillus fermentum* can be employed to assess the role of apoptosis in potential anti-cancer effects against in vitro colorectal cancer (CRC) cells [13]. It has also been proposed that probiotics impede tumorigenesis and cancer progression through apoptosis. However, only a limited number of studies have elucidated the precise mechanism underlying apoptosis induction, which holds promise for cancer therapy [14]. Phosphatase and tensin homolog (PTEN) is a tumor suppressor protein known for its phosphatase activity and negative regulatory function within the Phosphoinositide 3-kinases/ Protein kinase B (P13K/AKT) pathway. This pathway governs numerous processes linked to cell metabolism, proliferation, and survival [17]. PTEN plays a critical role in suppressing signal transduction initiated by several membrane growth factor receptors through the P13K/AKT signaling cascade [18]. PTEN also modulates the levels of the tumor suppressor protein P53 by its degradation mediated by Mouse-double-minute-2 homolog (Mdm2) [19]. Furthermore, PTEN interacts with p53 and alters its transcriptional activities in hypoxic conditions [20]. Conversely, p53 upregulates PTEN transcription, and the absence of p53 results in PTEN loss [21]. In addition to the mentioned genes, Caspases constitute a family of endoproteases crucial for programmed cell death and inflammation [22]. Among them, Caspase-3, an inhibitor cleaved by Caspase-8 or Caspase-9, is responsible for inhibiting apoptosis by cleaving essential intracellular proteins [23,24]. Numerous anticancer treatments, such as cytotoxic drugs, radiotherapy, or immunotherapy, induce tumor cell death by activating Caspase-3. However, the full extent of probiotics' impact on CRC remains incompletely understood. The current study seeks to investigate the role of *Limosilactobacillus fermentum GL* in HT-29 cells and explore its role in colorectal carcinogenesis. Therefore, this study aims to uncover the tumor-suppressing effects of *L. fermentum GL* in CRC.

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Materials & Methods

Sampling, isolation, and selection of bacteria

The current investigation took place in the microbiology laboratory of Lahijan University of Sciences in Iran in the year 2022. A total of 20 samples of traditional dairy products, including cow's milk, cheese, yogurt, buttermilk, and local curd, were gathered from various regions within Guilan province. These samples were subsequently stored at a temperature of 4^{o}C. To isolate *lactobacilli*, 1 ml or 1mg of each sample was combined with 99 ml of 0.1% peptone water. Subsequently, 50 µl of each dilution was cultured on two MRS Agar mediums and incubated under both aerobic and anaerobic conditions for 24 to 48 hours at a temperature of 37°C to cultivate an enriched culture. Nyastin at a concentration of 100 mg/ml was utilized to prepare the culture medium to inhibit yeast growth. Several individual colonies were selected at random and subsequently cultured in 10 ml MRS broth. Initial screening of the isolates involved morphological assessments, such as Gram staining and other morphological characteristics, of these individual colonies. To identify the type of bacteria catalase, oxidase, movement, temperature tolerance, phenol, NaCl, PH, Bile salt, and fermentation test of various sugars were used. All purified isolates were numbered and stored at -80 C^{o} in an MRS Broth medium containing equivalent amounts of 30% glycerol.

Amplification of 16S rRNA

Chromosomal DNA from the overnight broth cultures was isolated using a DNA extraction kit (Carmania Pars Gene (KPG-GPNB), Iran) according to the manufacturer's instructions. The quality of the extracted DNA was subsequently assessed using both a gel documentation system (UVTEC, UK) evaluated using a gel documentation system (UVITEC, UK) and spectrophotometric method (NanoDrop DeNovix- DS-11, USA). For amplification of the 16S rDNA, we employed the 16F primer (5′ AGAGTTTGATCCTGGCTCAG-3′) and 16R primer (5′-CTAGTACCAAGGCATTCACC-3′). Polymerase Chain Reaction (PCR) amplification was conducted in 20µl volume, which included 10µl of master mix (SINACLON, Iran), 2µl of 16S rRNA gene-specific primers, 3µl of DNA, and 5µl of deionized water. The cycling program comprised an initial pre-denaturation step at 94 °C for 5 minutes followed by 32 cycles of

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denaturation at 94°C for 45 seconds, annealing at 55 °C for 45 seconds, and a final extension at 72°C for 10 minutes.

PCR product electrophoresis

PCR product electrophoresis was conducted on a 1% agarose gel, stained with DNA-safe stain, and then observed using a gel documentation system (UVITEC, UK). The Ladder 100bp plus (Sinaclon, Iran) was used as a ladder.

Sequencing and analysis of 16S rRNA gene

The PCR products obtained from the isolates were subjected to purification using the QIAquick PCR purification (QIAGEN, Hilden, Germany), following the manufacturer's guidelines. Subsequently, the purified products underwent sequencing through Bioneer Korea Company (Bioneer, South Korea). The resulting sequences of the PCR products were then subjected to a molecular identification search within the GenBank database (http: //www.ncbi. nlm.nih.gov).

Cell culture

HT-29 and Human Umbilical Vein Endothelial Cells (HUVEC) were procured from the Pasteur Institute of Iran. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) and %1 penicillin-streptomycin (pen-strep) from Capricorn-Germany. The cell culture was maintained in a 37 °C incubator (Memmert, Schwabach, Germany) within a humidified atmosphere containing 5% CO2.

MTT survival assay

To assess cell viability in response to *Lactobacillus*, we employed the MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) assay. Approximately 1×10^5 cells per well were seeded into 96-well culture plates and allowed to incubate for 24 hours. Subsequently, various concentrations of bacterial cell extract (0.5, 0.75, 1, 1.5, and 2 mg/ml) were applied to HT-29 and HUVEC cell lines at 24-hour intervals. Following treatment, the controls of the 96-well plates were gently removed, and 5 mg/ml MTT solution (Sigma, Taufkirchen, Germany) was added, incubating for 4 hours in a 5% CO2 atmosphere at 37°C. Afterward, 200 µl of dimethyl sulfoxide (DMSO) was added and incubated for 30 minutes at 37 °C to dissolve the

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crystals. The absorbance was measured using a SunriseTM microplate reader (Tecan, Switzerland) at 570 nm. Each experiment was conducted in triplicate.

RNA extraction

RNA from all treated samples was extracted utilizing an RNA extraction kit (Parstous, Iran) following the manufacturer's guidelines. The concentration of RNA was assessed using a spectrophotometer method (NanoDrop DeNovix- DS-11, USA), and its quality was checked by electrophoresis.

Synthesis of cDNA

Complementary DNA synthesis was conducted using a kit from Kiagene Fanavar, Iran. The primer sequences were designed using oligo 7 software and then submitted to the Primer-BLAST online tool available on the NCBI website (www.ncbi.nlm.nih.gov) with details provided in Table 1. The primers were custom-synthesized by the Takapouzist Company. The cycling program consisted of initial incubation at 25 °C for 10 minutes, followed by extension at 47 °C for 60 minutes. The reaction was terminated by heating at 85°C for 5 minutes, and the samples were held at 4°C.

Real-time polymerase chain reaction

Real-time (RT)-PCR was conducted in triplicate using the Rotor-Gene 6000 cycler from Qiagen, Germany. The thermal cycling protocol involved an initial activation step at 95°C for minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The qPCR assay was performed in the final reaction volume of 16µl, comprising 7.5µl of SYBR Green qPCR master mix, 4.5µL of nuclease-free water, 1µL each of forward and reverse primers, and 2µl of cDNA. The Cycle Threshold (CT) value was determined for each sample. To quantify the expression levels of the target genes about GAPDH the $2^{-\Delta\Delta CT}$ formula was used.

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Flow cytometry assay

To assess apoptosis induction in the cells, we employed the Annexin V-FITC kit from eBioscience, Affymetrix, Santa Clara, California, USA, following the manufacturer's instructions. Approximately 2×10^5 cells per well were subjected to treatment with various concentrations corresponding to the IC50 values of the bacterial extract for 48 hours. Subsequently, the cells were harvested and washed with PBS. For apoptosis detection, the cells were re-suspended in a binding buffer, stained with Annexin V-FITC, and incubated at room temperature for 15 minutes. Finally, the cells were re-suspended in the PI binding buffer and prepared for apoptosis measurement. Cellular analysis was conducted using a flow cytometry device (FACSCalibur-BECTON DICKINSON) and the flow cytometric data were analyzed using FlowJo software.

Statistical analysis

All experiments were conducted in triplicate, and the mean values were calculated using Minitab version 16 software, Standard deviations were determined using GraphPad Prism version 9 software. Statistical analysis was carried out utilizing One-Way Unstacked ANOVA, with significance set at $p < 0.05$.

Results

Bacteria isolation

The dairy samples were subjected to culture on MRS agar for a period of 24 to 48 hours at a temperature of 37°C following appropriate dilution. Colonies that emerged on the agar surface were scrutinized, and pure cultures were subsequently isolated from the round white colonies. Gram staining and catalase tests were employed to examine these colonies. The bacterial colonies had biochemical properties of *Lactobacillus* including rod-shaped, gram-positive, catalase-negative, and oxidase-negative, movement-negative. Then the confirmed colonies revealed tolerance to 4%, 8%, and 10% NaCl, 0. 3%,0.4%, 0.5%, and 0.6% phenol, 2,3,4, and 5 PH, 0.3%, 0.5% and 0.8% bile salt and growth at 15°C,37°C and 45°C [39]. Finally, samples were used for molecular identification.

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Molecular characterization

PCR was conducted to amplify the 16S rRNA gene in the chosen isolates, and the resulting PCR products were subjected to analysis via agarose gel electrophoresis. The PCR bands observed in the samples were approximately 1700 bp in size when visualized on 1% agarose gel, indicating successful amplification of the 16S rRNA gene. to confirm the identity of the PCR products, the positive samples were sent to Bioneer in South Korea for sequencing. For species identification, the obtained sequences were compared with those present in the NCBI database using Blast. The Blast results revealed that one of the samples exhibited a sequence similarity of 88.61%, confirming its affiliation with *Lactobacillus fermentum* GL. Furthermore, the phylogenetic tree generated from the 16S rRNA sequence of the *L. fermentum* GL is depicted in Figure 1. The sequence of the Extracted *L. fermentum* GL has been deposited at https://www.ncbi.nlm.nih.gov/nuccore/OP555803.

MTT Assay

The HT-29 and HUVEC cells underwent treatment with varying concentrations (0.5, 0.75, 1, 1.5, and 2 mg/ml) of *L. fermentum* GL cytoplasmic extract for 24 hours. Cell viability was assessed utilizing the MTT assay. Based on the results, the cytoplasmic extract exhibited no significant impact on the cell viability of HT-29 cells at concentrations of 0.5 and 0.75 mg/ml. However, at concentrations of 1, 1.5, and 2 mg/ml, cell viability was reduced by approximately 50±4.87%, 40±8.61%, and 25±5.80%, respectively. The calculated IC_{50} value for the cytoplasmic extract in HT-29 cells was 1.43 mg/ml. Furthermore, the cytotoxicity study indicated that the concentrations of cytoplasmic extract employed had no inhibitory effects on the normal HUVEC cell line for 24-hour duration (P <0.05), as illustrated in Figure 2.

Real-Time PCR Assay

The qPCR investigation was conducted to assess the expression levels of p53, PTEN, and caspase-3 genes in HT-29 cells following exposure to IC_{50} concentration of *L. fermentum* GL cytoplasmic extract (1.43 mg/ml) for 24 hours. The results revealed a significant upregulation in the expression of these genes, with p53 showing a 2.9-fold increase (P <0.001), PTEN exhibiting

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a 1.9-fold increase (P <0.05), and Caspase-3 displaying a 2.7-fold increase (P <0.001), as depicted in Figure 3.

Flow cytometry assay

HT-29 cells were exposed to the IC_{50} concentration of *L. fermentum* GL cytoplasmic extract (1.43 mg/ml) for a duration of 24 hours, and the quantification of apoptotic and necrotic cells was determined through a flow cytometry assay. Figure 4 illustrates the flow cytometry results for both control and cytoplasmic extract-treated cells. Notably, the percentage of viable cells (Q4), and apoptotic cells (Q2, Q3) in the cytoplasmic extract-treated cells markedly differed from those in the untreated cells. The findings indicated a significant decrease (P *<0.02*) in the percentage of viable cells, decreasing from 96.4% in the control cells to 76.8% in the cytoplasmic extract-treated cells. Additionally, the percentage of apoptotic cells in the treated group significantly increased (P *<0.02*) compared to the control group. However, it's worth noting that the percentage of necrotic cells in both the control and treated cells remained relatively similar.

Discussion

Cancer remains a significant global health challenge, ranking as the second leading cause of death worldwide [25]. A hallmark feature of cancer cells is their uncontrolled proliferation and resistance to apoptosis, making any agent capable of inducing apoptosis in these cells a potential anticancer candidate. Probiotics have emerged as adjunctive therapies to enhance the efficacy of conventional anticancer treatments [26]. Our study demonstrated that the cytoplasmic extracts of *Lactobacillus fermentum* GL exhibited significant growth-inhibitory effects on HT-29 cells by suppressing their proliferation. Additionally, *L. fermentum* GL induced apoptosis, underscoring its potential as a tumor-inhibiting agent. Previous research has also shown that the ectopic expression of probiotics can inhibit proliferation and induce apoptosis in various cancer cells [26–28]. However, the precise mechanisms underlying the effects of probiotics on cancer cells remain incompletely understood [25]. It's noteworthy that the bacteria used in our study, *L. fermentum* GL, were isolated from traditional dairy samples in Iran, and this research marked the first investigation into their anti-proliferative, apoptosis-inducing, and anticancer properties. To delve deeper into the tumor-inhibiting effects of *L. fermentum* GL in colorectal cancer, our study

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examined its impact on key genes, including p53, Caspase 3, and PTEN. Studies have emphasized the prognostic significance of p53 mutations in colorectal cancer [29]. Furthermore, investigations have indicated that abnormalities in p53 may independently affect prognosis, particularly in patients with more favorable baseline prognoses [30]. Our study supports the potential of p53 as a target for *L. fermentum* GL function. Metabiotics from L. rhamnosus MD have been found to inhibit colorectal cancer by upregulating p53 expression [31]. Likewise, several other studies involving different probiotic strains have shown the induction of p53 expression in various cancer types [28,32–34]. PTEN, a well-known tumor suppressor gene, plays a vital role in inhibiting cancer by enhancing Tp53 stability and transcriptional activity, leading to cell cycle arrest [35]. Our findings indicated that *L. fermentum* GL administration upregulated PTEN expression in HT-29 cells. This aligns with previous research demonstrating that the probiotic *L. plantarum* induces apoptosis through PTEN pathways [25]. Our flow cytometry results provide strong evidence of *L. fermentum* GL supernatant-induced apoptosis in HT-29 cells, with negligible necrosis. Additionally, our study revealed that L. fermentum GL induced the expression of caspase-3, a key enzyme in the intrinsic apoptotic cascade. Other studies have shown similar effects of probiotics on apoptosis induction in CRC cells [13,27,28,36-39]. However, research on the role of *L. fermentum* GL in the later stages of CRC, such as metastasis, remains limited, and further investigations are warranted in this regard.

Conclusion

In summary, our study marks the first to demonstrate that *Lactobacillus fermentum* GL supernatants possess significant potential to inhibit proliferation and induce apoptosis via p53, PTEN, and caspase-3 dependent pathways in colorectal cancer (CRC) cells. Importantly, unlike traditional chemotherapy, the consumption of probiotics is associated with fewer side effects. Therefore, we propose that the utilization of *L. fermentum* GL, as outlined in this study, could emerge as an innovative and promising approach to probiotic-based therapy for colorectal cancer.

Conflicts of interest

No conflict of interest was reported

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Availability of data and materials

The study protocol and the datasets analyzed are available from the corresponding author upon request.

Consent for publication

Not applicable

Credit authorship contribution statement

Hossnieh Kafshdar Jalali: Writing – original draft, Investigation, Methodology, Formal Analysis, Funding acquisition

Khosro Issazadeh: Writing – review & editing, Conceptualization, Data curation, Project administration

Abbas Ali Rezaeian: Writing – review & editing, Data curation, Supervision

Competing interests

The authors declare that they have no competing interests in this work.

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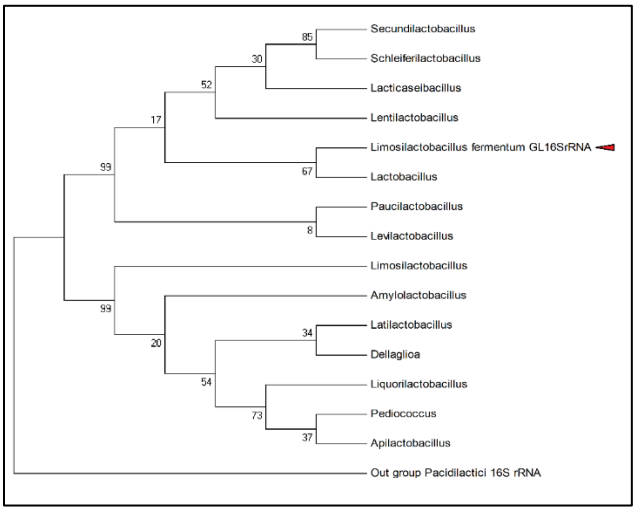

Figure 1. Phylogenetic tree resulting from 16S rRNA sequence analysis

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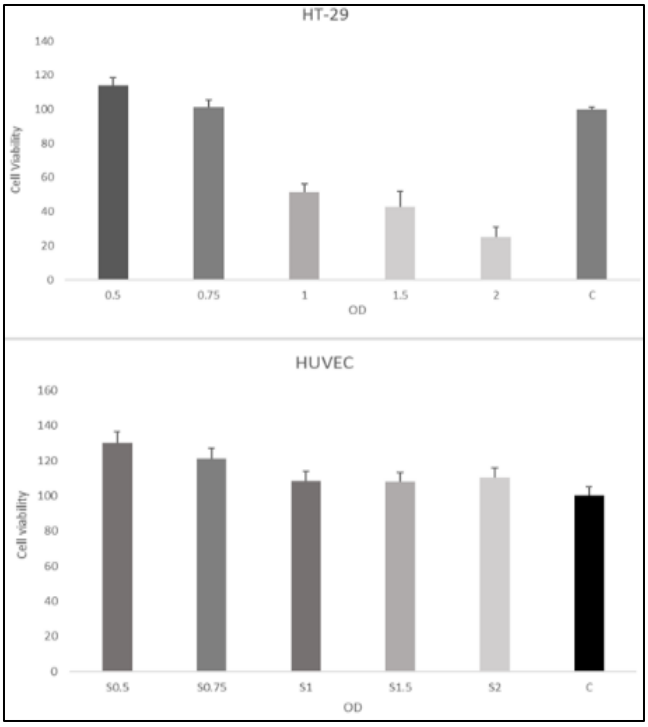

Figure 2. MTT results and determining the effect of the bacterial extract on normal HUVEC cells and HT-29 by a significant level less than 0.05.

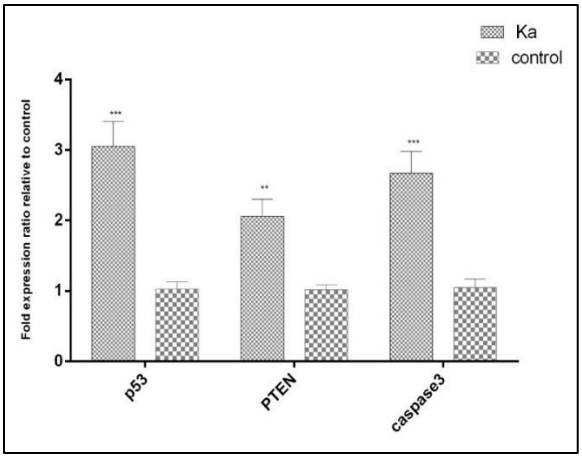

Figure 3. An increased expression of desired genes (Ka: control of Lactobacillus cells, GAPDH: Intra control).

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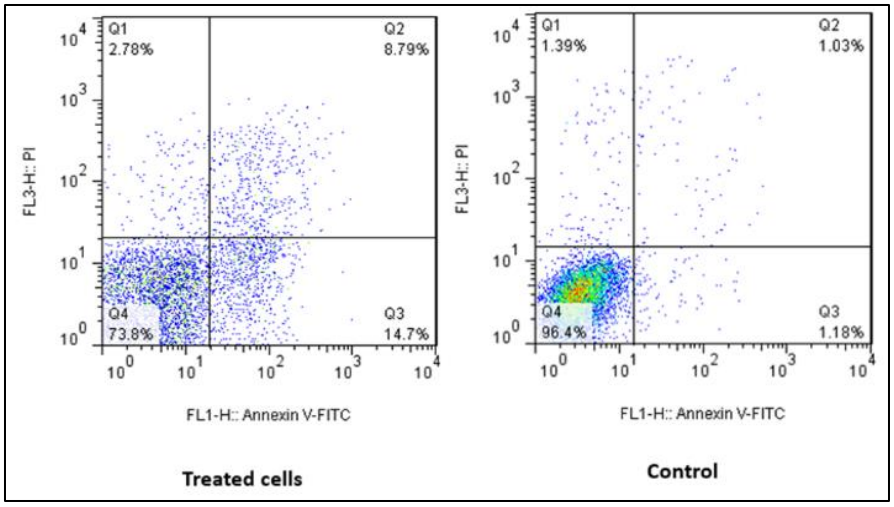

Figure 4. Flow cytometry outcomes of control and cytoplasmic extract-treated cells

Table 1. Properties of primers used for quantitative real-time PCR assay

Gene	Primer	Primers' Sequences (5-3)	Amplicon Size (bp)	TM
GAPDH	Forward	F: GAGCCAAAAGGGTCATCATC	6135	57.30
	Reverse	R: TAAGCAGTTGGTGGTGCAGG	6253.1	59.35
PTEN	Forward	F:5 AGGCACAAGAGGGCCCTAGATTTCT-3	7675	62.98
	Reverse	R: 5- ACTGAGGATTGCAAGTTCCGCCA-3	7048.6	62.43
p53	Forward	F: 5- AGTCTAGAGCCACCGTCCA-3	5757.8	58.83
	Reverse	R: 5- TCTGCGCACACCTATTGCAAGC-3	6968.5	62.43
Caspase-3	Forward	F:3-GTGGAACTGACGATGATATGGC-3	6839.5	60.25
	Reverse	R:3-CGCAAAGTGACTGGATGAACC-3	6464.2	59.82

(GAPDH**:** glyceraldehyde-3-phosphate dehydrogenase**;** PTEN**:** Phosphatase and tensin homolog; p53: A tumor protein; Caspase-3: Cysteine-aspartic acid protease-3)

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