

Research article

Anti-cancer potential of *Lactiplantibacillus plantarum* NG28 through the induction of intrinsic apoptosisSowmya Shankar¹, Shankar Jayarama², Shubha Gopal¹¹Department of Studies in Microbiology, University of Mysore, Manasagangotri, Mysuru, Karnataka, India²Department of Studies in Food Technology, Davangere University, Shivagangotri, Davangere, Karnataka, India

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Corresponding author: **Shubha Gopal**. Email: sguom@yahoo.com**ABSTRACT**

Introduction: Probiotics and fermented foods have become the focal point of research across different scientific disciplines, with recent findings indicating that *Lactiplantibacillus plantarum* (*L. plantarum*) isolated from various origins, holds significant promise in combating numerous diseases, including cancer. Hence, our current study seeks to explore how the *L. plantarum* NG28 strain might counteract the growth of HT-29 colorectal adenocarcinoma cells, potentially contributing to anti-cancer strategies.

Methods: The effect of the ethyl acetate extract of *L. plantarum* NG28 strain on HT-29 cells was evaluated in several ways. First, the cytotoxicity of HT-29 cells was determined by MTT assay. Additionally, the changes in cell morphology of HT-29 cells through DAPI staining and the induction of apoptosis using Annexin V-FITC/PI staining followed by flow cytometry were investigated. By using the RT-PCR and western blot techniques the expression of pro-apoptotic genes and proteins such as Bax, Caspase-9, and Caspase-3, as well as the anti-apoptotic gene Bcl-2 was examined.

Results: The findings revealed that the ethyl acetate extract of *L. plantarum* NG28 strain had a significant impact on the viability of HT-29 colon cancer cells in a dose-dependent manner, in contrast to its minimal effect on HUVEC cells. DAPI staining revealed the noticeable DNA damage and condensation of treated HT-29 cells and the flow cytometry strengthened the occurrence of apoptosis in the treated HT-29 cells. RT-PCR and western blot techniques investigated the intrinsic apoptosis of HT-29 cells through the increased expression levels of pro-apoptotic genes / proteins and decreased expression levels of anti-apoptotic gene.

Conclusion: The study demonstrated the anti-cancer activity of ethyl acetate extract of the *L. plantarum* NG28 strain against HT-29 colon cancer cells, primarily through the intrinsic apoptotic pathway. This suggests that *L. plantarum* NG28 strain could be used as a therapeutic tool in the inhibition and medication of colorectal cancer.

Keywords: Probiotics; *Lactiplantibacillus plantarum*; colon cancer; HT-29; apoptosis.

INTRODUCTION

The human intestines play host to intricate communities of microorganisms known as gut microbiota, which play a prominent function in maintaining wellness of the mucosal barrier (1). To ensure the body functions normally and to guard against diseases, it is essential to maintain a harmonious relationship between gut microbiota and host. Various factors such as dietary choices, antibiotic usage, environmental influences, age and stress can influence the composition of the gut microbiota (2,3). When this balance is disrupted, referred to as dysbiosis, has been related with promotion of numerous diseases, namely inflammatory bowel disease, asthma, rheumatoid arthritis, obesity, irritable bowel syndrome, and numerous classes of cancer (4).

Cancer is the result of cells dividing uncontrollably due to mutations that occur during DNA replication. Its primary risk factors include exposure to infectious agents, harmful substances and UV radiation, as well as an individual's lifestyle and genetic background (5,6). Among cancers like lung, prostate, and breast

cancer, colorectal cancer stands out as one of most frequent and extreme form of cancers (7). In fact, it ranks as the third most prevalent cancer globally, and its occurrence among young people is on the rise. Diet plays a crucial role in enhancing the body's immunity against various diseases including colorectal cancer. Therefore, maintaining a proper diet can help in protection against the development of colorectal cancer. Engaging in physical activity, adopting the right diet, and considering other associated factors can all contribute in the prevention of colorectal cancer.

Traditional fermented foods may have several health advantages, including anticancer effects (8,9). They are important components of healthy diet because of their probiotic content and bioactive molecules. Fermented foods and probiotic microbes have unique beneficial characteristics such as enzyme production, peptide formation, antioxidant and anti-inflammatory activities and antimicrobial compounds. As a result, probiotic-fermented foods have procured the interest of researchers from a variety of disciplines.

Probiotics are live, harmless microbes that when supplied in the proper proportions contribute significantly to the host's health advantages (10). Probiotics benefit the body through a number of methods, including decreasing pH, producing antimicrobial compounds, modifying the host immune response and competing with pathogens for colonization, nutrients and other growth factors in the gut (11). Probiotics offer a pivotal benefit by persisting in the body without causing any adverse reactions. They have shown significant promise in treating various disorders including, ulcerative colitis, irritable bowel syndrome, acute infectious diarrhea as well as dermatological and respiratory allergic conditions (12). Lactic acid bacteria widely recognized as safe and effective food additives due to their historical use in fermented foods, also take part in reduction of pro-carcinogens and this contributes to cancer prevention and treatment (13). Among these bacteria, *L. plantarum* a gram-positive lactic acid bacterium with potential probiotic and functional qualities, is actively being investigated for its anti-cancer effects across various types of cancer (14,15). Nevertheless, limited information is available regarding the precise mechanisms behind these effects. Therefore, the present study focused the mechanism responsible for the anti-cancer properties of *L. plantarum* NG28 in HT-29 cells.

Our previous study has demonstrated the probiotic potential of *L. plantarum* NG28 strain isolated from ngari, a fermented fish product produced in Manipur and also proved its antioxidant and anti-inflammatory activities (16). The aim of the current study was to analyze the effectiveness of *L. plantarum* NG28 species on the initiation of apoptosis in HT-29 cells.

MATERIALS AND METHODS

Penicillin/streptomycin (P/S), DMEM, MTT, and fetal bovine serum (FBS) were purchased from Hi-Media (India). Bax, Bcl-2 and GAPDH primers were purchased from Eurofins Scientific (India). The primary antibodies caspase-9, caspase-3, β -Actin and secondary antibodies were from Cell Signalling Technology (USA). Other excellent quality reagents and chemical products were purchased from Hi-media, SRL chemicals (India).

Cell culture and maintenance

The HT-29 colon cancer cells and HUVEC (human umbilical vein endothelial cells) of normal origin were carefully maintained under sterile conditions at a temperature of 37°C. They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The culture environment was kept in a humidified atmosphere with 5% CO₂ at 37°C. Passaging of the cells was performed every four days using trypsin-EDTA buffer and the culture medium was refreshed every two days.

Preparation of ethyl acetate extract of *L. plantarum* NG28

The *L. plantarum* NG28 strain, which had been previously isolated from ngari was employed in this study (15). To cultivate *L. plantarum* NG28, overnight culture was incubated in 1000 ml of de Man, Rogosa and Sharpe (MRS) broth at a temperature of 37°C for duration of 72 hours. Then the culture was subjected to centrifugation at 10,000 rpm for 15 minutes at 4°C. The pellet was washed three times with distilled water, to eliminate the residual MRS broth medium. Then the pellet was suspended in distilled water and sonicated for a period of 30 minutes. After centrifugation at 12,000 rpm for 15 minutes at 4°C, the resulting supernatant was filtered and mixed with an equal quantity of ethyl acetate. The mixture was vigorously agitated for two hours using an orbital shaker. After the agitation, the ethyl acetate portion was concentrated through vacuum evaporation, with the remaining material being dissolved in dimethyl sulfoxide (DMSO) to a desired concentration. The cell free extract was filtered by using a 0.45 μ m filter and stored at -20°C for further experiments (17).

Determination of cytotoxicity by MTT assay

The cytotoxic effect of ethyl acetate extract of *L. plantarum* NG28 on HT-29 colon cancer cells and HUVEC normal cells was assessed by MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] (18). Briefly, 1×10^4 cells per well were seeded to 96 well plates and incubated for 24 h. Then the cells were treated with the extract of *L. plantarum* NG28 in different concentrations (6.25 μ g/ml, 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, 100 μ g/ml, and 200 μ g/ml). After incubation of twenty-four hours, 0.5 mg/ml MTT solution was added to each well and left it in the dark for four hours. The MTT-containing liquid was carefully removed and 200 μ L of DMSO was added to each well to dissolve the formazan crystals. After another 30-minute of incubation, absorbance was measured at 570 nm and the percentage of cell viability was measured. Then the IC₅₀ value was calculated

DAPI staining

The induction of apoptosis in HT-29 cells by *L. plantarum* NG28 extract was assessed by microscopic examination using DAPI staining (19, 20). HT-29 cells were seeded at a density of 2×10^4 in confocal dishes and incubated overnight. The cells were treated with the extract of *L. plantarum* NG28 (IC₅₀ concentration of 30.5 μ g/ml) and incubated for 24 h. After incubation, the treated cells were washed with culture media and substituted it with a freshly prepared fixative solution, which consisted of DMEM containing 4% formaldehyde. After incubation of 5 minutes, the cells were washed twice with PBS. Followed by permeabilization using PBS containing 0.1% Triton X-100 for 5 minutes at a temperature of

37°C, the cells were stained with DAPI at a concentration of 1 µg/ml and incubated for 15 minutes. The slides were then examined using fluorescence microscopy after being cleaned with PBS.

Assessment of apoptosis by flow cytometry

Annexin V-FITC/PI staining followed by flow cytometry, was used for the determination of apoptosis or necrosis in treated HT-29 cells (21). The HT-29 cells were seeded at a density of 5 x 10⁵ cells per well in a six well plate and treated with 30.5 µg/ml of *L. plantarum* NG28 extract for 24h. After incubation, PBS was used to wash the untreated and treated cells and stained with Annexin V-FITC/PI as described in the commercial manual. Finally, the cells were analysed with flow cytometry. After performing the flow cytometry, the cell populations were defined using four quadrant gates, represented as, quadrant 1 (Q1): necrotic cells (Annexin V-/PI+); quadrant 2 (Q2): late apoptotic cells (Annexin V+/PI+); quadrant 3 (Q3): live cells (Annexin V-/PI-) and quadrant 4 (Q4): early apoptotic cells (Annexin V+/PI-).

Analysis of expression of genes and proteins related to apoptosis

RT-PCR analysis of gene expression

The expression levels of apoptosis related genes such as Bax and Bcl-2 were measured by RT-PCR (22). HT-29 cells were seeded at a density of 5 x 10⁵ cells per well in a six well plate and treated with *L. plantarum* NG28 extract for 24h. Total RNA was isolated from the untreated or treated HT-29 cells by using the RNA Xpress solution (RNA Trizol) according to the manufacturer's manual. Using superscript III One-step RT-PCR kit, 1 µg of RNA from the untreated and treated HT-29 cells was subjected to reverse transcription. The complementary DNAs were synthesized at 50°C for 30 min followed by incubation at 94°C for 2 min. Subsequently, 30 cycles of PCR were carried out with denaturation for 45 seconds at 94°C, annealing for 45 seconds at 53°C, extension for 1.5 min at 72°C and a final extension for 7 min at

72°C. Different sets of primers used for the amplification of Bcl-2, Bax, and GAPDH were shown in the Table 1. The PCR products were resolved on 1.5% agarose gel and documented using the UvpBio-Doc system.

Western blotting

Western blotting was employed for the determination of pro-apoptotic proteins in treated HT-29 cells (23). HT-29 cells were initially seeded in a 6-well plate at a density of 5 X 10⁴ cells per well and incubated to grow for a period of 24 hours. Following this incubation, the cells were exposed to *L. plantarum* NG28 extract at a concentration of 30.5 µg/ml (IC₅₀ value). To extract the total proteins and remove any insoluble debris, a lysis buffer containing a protease inhibitor was employed, and the resulting mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant obtained from this process was used for quantifying the proteins and for western blotting. A 20µg protein sample was loaded onto a 12.5% SDS polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. After 1-hour of blocking with 5% skim milk at room temperature, the proteins were subjected to an overnight incubation with primary antibodies at 4°C, followed by thorough washing with 0.1% Tween 20 solution in tris-buffer saline. The primary antibodies caspase-9, caspase-3 and β-actin was identified by employing goat anti-rabbit IgG secondary antibodies coupled with horse radish peroxidase, followed by 1h of incubation at room temperature. The detection was achieved through the application of enhanced chemiluminescence (ECL) Western Blotting Substrate.

RESULTS

Cytotoxic effect of *L. plantarum* NG28 extract on HT-29 cells

To examine the cytotoxic properties of the *L. plantarum* NG28 extract, we administered various concentrations of the extract (6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml) to HT-29 cells and HUVEC normal cells.

Table 1: List of primers used in RT-PCR

Gene	Product Number	Primer Sequence (5' – 3')	
Bcl-2	B9179	F	CCT GTG GAT GAC TGA GTA CC
		R	GAG ACA GCC AGG AGA AAT CA
Bax	B8304	F	GTT TCA TCC AGG ATC GAG CAG
		R	CAT CTT CTT CCA GAT GGT GA
GAPDH	P7732	F	TGC MTC CTG CAC CAC CAA CT (*M=A or C)
		R	YGC CTG CTT CAC CAC CTT C (*Y=T or C)

*where M = A or C, Y = T or C that was used as a loading control

After 24-hours of incubation period, our results as depicted in Fig. 1, clearly demonstrated a dose-dependent reduction in the viability of HT-29 cells. The IC₅₀ value was found to be 30.5 µg/ml and this concentration was used for the further assessments.

On the other hand, the *L. plantarum* NG28 extract did not show substantial reduction in the cell viability of HUVEC normal cells (data not shown), hence it could be considered as safe for normal cells.

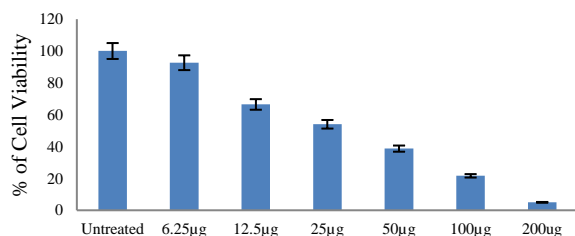


Fig. 1: Cytotoxic effect of *L. plantarum* NG28 extract on HT-29 cells in dose dependent manner after 24 h of incubation using MTT cell viability assay.

Microscopic assessment by DAPI staining

DAPI staining was used to examine the morphological changes of HT-29 cells. As depicted in Fig. 2, the untreated cells displayed their usual, healthy appearance, while the treated cells showed alterations in their morphology, such as DNA damage and cell condensation. The cell nuclei were stained in blue. The normal cells were marked with white arrows, whereas cells with condensed and damaged DNA, exhibited higher fluorescence were marked with yellow arrows.

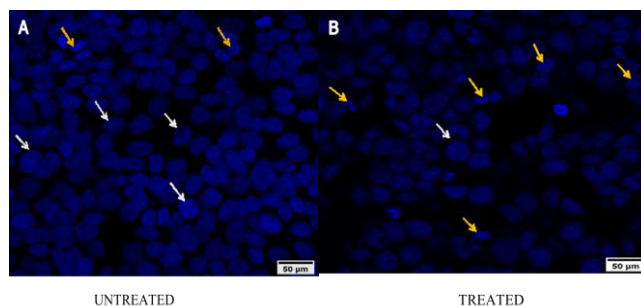


Fig. 2: The morphological changes in the HT-29 cells. (A) Untreated, (B) Treated with ethyl acetate extract of *L. plantarum* NG28 for 24 h of incubation.

Assessment of apoptosis by flow cytometry

Flow cytometry allows the study of aspects of apoptosis. The flow cytometric analyses revealed the induction of apoptosis in treated HT-29 cells with 15.74% of early apoptotic cells and 42.99% of late apoptotic cells as shown in Fig. 3B. Whereas in untreated cells the apoptosis was significantly low (Fig. 3A). The results indicated that the extract of *L. plantarum* NG28 elicited cytotoxic effects on HT-29 cells through apoptosis (Fig. 3C).

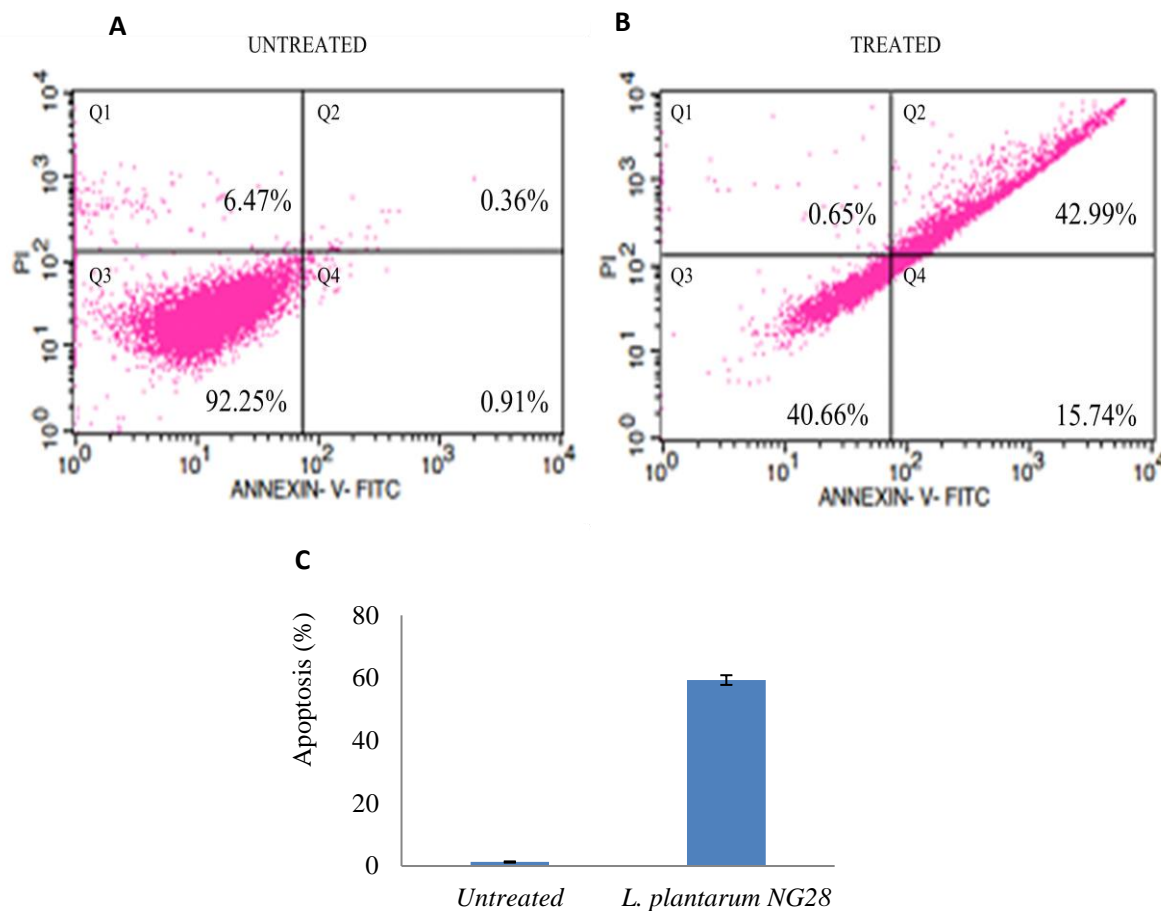


Fig. 3: Flow cytometry analyses to determine apoptosis of HT-29 cells after staining with Annexin V-FITC/PI. (A) Untreated, (B) Treated with *L. plantarum* NG28 extract for 24 hours, (C) Quantitative study of cell apoptosis by Flow cytometry after staining with Annexin V and PI.

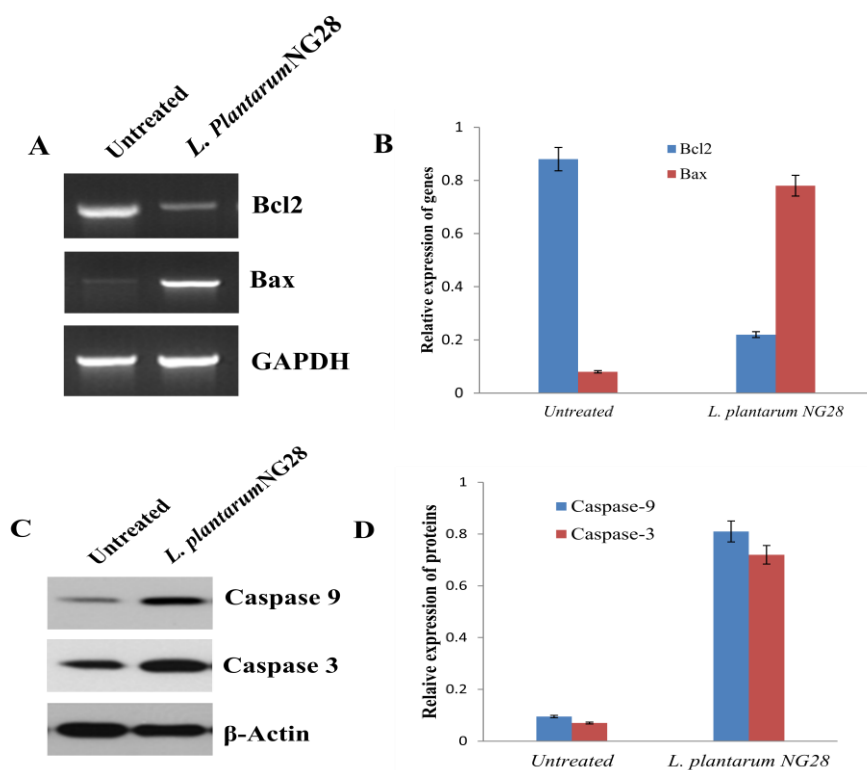


Fig. 4: The expression of genes / proteins associated with the intrinsic apoptotic pathway in HT-29 cells when treated with ethyl acetate extract of *L. plantarum* NG28 for 24 hours. (A) examining the expression of Bax and Bcl-2 genes using RT-PCR, (B) quantifying gene expression with GAPDH as a control gene, (C) analysing caspase 9 and caspase 3 expression through Western blotting, (D) quantifying protein expression with β -Actin as the internal loading control.

Analysis of expression of genes and proteins associated with apoptosis

To determine the apoptosis pathway, the apoptosis related genes and proteins were analyzed in HT-29 cells after the treatment of the *L. plantarum* NG28 extract. The RT-PCR analysis of gene expression indicated that the pro-apoptotic Bax gene showed a significant increase, while the anti-apoptotic Bcl-2 gene displayed a reduction, as depicted in Fig. 4A. Fig. 4B illustrates the relative gene expression of Bcl-2 and Bax, with GAPDH serving as the internal loading control. Additionally, western blot results in Fig. 4C clearly revealed the increased expression levels of caspase-9 and caspase-3 in treated HT-29 cells, compared to decreased expression levels in untreated cells. Fig. 4D presents the relative protein expression levels of caspase-9 and caspase-3, with β -actin as the internal loading control.

DISCUSSION

In recent times, probiotics have received formal recognition, since they have been shown to turn many health benefits to the human. The primary interest in the application of probiotics has been in the prevention and treatment of gastrointestinal infections and diseases. Studies have confirmed that probiotics have beneficial effects in regulating proliferation and apoptosis of cancer cells.

In our previous study, The *L. plantarum* NG28, isolated from ngari has proved its probiotic potential and also exhibited antioxidant and anti-inflammatory

activities (16). Thus, in the present study series of experiments have conducted to investigate the anticancer effect of ethyl acetate extract of *L. plantarum* NG28 in HT-29 colon cancer cells.

The MTT assay is a widely employed method, used to determine viable cell number in proliferation and cytotoxicity studies. The cytotoxic effect of *L. plantarum* NG28 extract was determined by treating HT-29 colon cancer cells and HUVEC normal cells with various concentrations of the *L. plantarum* NG28 extract. The results exhibited that; the viability of HT-29 cells gradually decreased with increasing of dose of the extracts. And the safety of the extract has proven by the unaffected HUVEC cells. Numerous research studies have documented how probiotic strains can impede the spread of cancerous cells. A previous study supports our results by determining the reduction of viability of Caco-2 cells treated with the *L. plantarum* metabolites in dose dependent manner (24). In addition, a examine by Wei *et al.*, the exopolysaccharide extract of *Lactobacillus fermentum* YL-11 proved the antiproliferative activity by inhibiting the Caco-2 and HT-29 cells up to 45.6 % (25).

In the microscopic examination by DAPI staining, the treated HT-29 cells exhibited damaged DNA and condensed cells, whereas the non-treated HT-29 cells showed no morphologic changes. The finding matches with the previous study by Nami *et al.*, showing the DNA damage and cell condensation in HeLa cells treated with *L. plantarum* 5BL (26). In our study,

DNA damage was clearly exhibited in treated cells in comparison with the untreated cells which showed intact nuclear architecture.

The probiotics have been proved in inhibiting the proliferation of cancer cells by inducing apoptosis. The induction of apoptosis was determined by flow cytometry after staining with Annexin V-FITC/PI. Annexin V serves as a particular ligand that tightly attaches to phosphatidylserine, making it a valuable tool for identifying cells in various phases of programmed cell death. On the other hand, PI exclusively marks cells with compromised cell membranes, enabling the identification of cells that are in the late apoptotic and dead stages. The flow cytometry displayed the induction of apoptosis in treated HT-29 cells with 15.74% and 42.99% of early and late apoptotic cells respectively. In a previous study, Pourramezan *et al.*, determined the 17.30% of apoptosis and 14.05% of necrosis in Caco-2 cells treated with fraction (F4) of *Lactobacillus hilgardii* strains compared to untreated Caco-2 cells with 3.33% apoptosis and 4.57% necrosis (27).

Apoptosis serves as a crucial role in maintaining the balance of cell populations within human organs. Apoptosis essentially a form of programmed cell death which is meticulously regulated. In contrast, necrosis, another type of cell demise occurs because of sudden cellular injury or damage. Apoptosis can be triggered via two pathways: intrinsic pathway and extrinsic pathway. The intrinsic pathway activated by internal signals when cells are under stress, hinges on the release of Cytochrome-C proteins from the mitochondria. The regulation of Cytochrome-C release is directed by the Bcl-2 family of proteins, where key pro-apoptotic members include Bax, Bak, Bad, and Bid, while anti-apoptotic proteins comprise Bcl-2 and Bcl-xL. The intrinsic pathway is characterized by the activation of caspase 9. The extrinsic pathway activated when external molecules latch onto death receptors on the cell surface and this process involves the initiation of caspase 8. In both pathways, the initiator caspases once activated set off a chain reaction that leads to the activation of caspase 3. The caspase 3 results the cell death as it breaks down the proteins within the cell. In our study, RT-PCR and western blotting results demonstrated the intrinsic apoptotic pathway in the treated HT-29 cells by exhibiting the up-regulation of pro-apoptotic genes/protein Bax, caspase 9 and caspase 3 and down-regulation of anti-apoptotic gene Bcl-2. In agreement with the results, Lee *et al.*, showed the induction of apoptosis through intrinsic pathway by up-regulation of Bax gene and caspase 3 and down-regulation of Bcl-2 genes in HCT-116 cells treated with cell free supernatant of *Lactobacillus fermentum* (28). Russo *et al.*, determined that, the extracts of *Lactobacillus rhamnosus* GG caused a decrease in the expression of anti-apoptotic Bcl-2 gene and increased expression of

the pro-apoptotic Bax gene (29). Based on our findings, the study clearly confirmed that the *L. plantarum* NG28 extract induced apoptosis in HT-29 cells through intrinsic pathway.

CONCLUSION

Researchers from various fields are focusing their attention on the healthy profit of probiotics. There is a raising interest in enhancing probiotic products to maximize their health advantages. In relation to the anti-cancer effects of probiotics, the current study sought to examine the effect of *L. plantarum* NG28 strain on HT-29 cells. The ethyl acetate extract of *L. plantarum* NG28 was found to exhibit a highly specific cytotoxic effect on tumor cells while being safe for normal cells. This was accompanied by a notable increase in the expression levels of pro-apoptotic genes and proteins like Bax, caspase-9, and caspase-3, along with a simultaneous decrease in the anti-apoptotic gene Bcl-2, indicating the activation of the intrinsic pathway of apoptosis. Consequently, the *L. plantarum* NG28 strain could be considered as a potent therapeutic agent for the prevention and treatment of colorectal cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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