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ORIGINAL ARTICLE

Anti-proliferative effects of cell wall, cytoplasmic extract of *Lactococcus lactis* and nisin through down-regulation of *cyclin D1* on SW480 colorectal cancer cell line

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ABSTRACT

Background and Objectives: Colorectal cancer is one of the most types of cancer. Researchers have shown that lactic acid bacteria have antitumor activity. The cell wall of *Lactococcus lactis*, as the bacterial cytoplasmic extract and nisin can affect the proliferation of cancer cells. Since cyclin D1 plays an important role in the progression of the cell cycle, its regulation can also be a therapeutic approach. We investigated the antiproliferative effect of cell wall, cytoplasmic extract and nisin on SW480 cancer cell line and the expression level of cyclin D1 gene in treated cancer cells.

Materials and Methods: SW480 cell lines were treated with different concentrations of bacterial cell wall, cytoplasmic extract and nisin. MTT test was also performed. The expression level of cyclin D1 gene was determined using Real time PCR. Data were analyzed using Graph Pad Prism software.

Results: The growth rate of cancer cells treated with nisin has significantly decreased compared to the cancer cells treated by other two substances ($p< 0.05$). Survival rates of the cancer cells treated by nisin at a concentration of 2000 µg, cytoplasmic extract, and cell wall were 34%, 47% and 49%, respectively. Real-time PCR results showed that cyclin D1 mRNA expression has significantly decreased in nisin treated sw480 cells ($P<0.05$).

Conclusion: The results of this study show that nisin, bacterial cytoplasmic extract, and bacterial cell wall have antiproliferative effects, which are associated with the decreased expression of cyclin D1 in SW480 cell line.

Keywords: Cell wall; Cytoplasmic extract; Nisin; Cyclic redundancy check; Cyclin D1

INTRODUCTION

One of the leading causes of death worldwide is colorectal cancer (CRC) (1). Despite the advances in anticancer therapies, the incidence of cancer is globally increasing (2). Recent studies showed that probiotics are living microorganisms playing important roles in fighting against cancer (2, 3).

Lactococcus lactis is a probiotic bacterium that is found in fermenting foods, especially in yogurt and cheese (4, 5). *Lactococcus lactis* is one of the lactic acid bacteria (LAB) that seems to be useful due to its non-pathogenic properties, including lactose fermentation and tumor suppressor. Nisin is a low molecular weight antibacterial peptide that is produced by *Lactococcus lactis* subsp. Lactis (6, 7). LAB inhibits ammonia production through the mechanism

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of altering the physical and chemical conditions of the colon. As a result, these bacteria can help in preventing cancers, especially colon cancer (8). Recently, the apoptotic and anticancer effects of nisin on the SW480 cell line have been investigated (9).

Components of lactic acid bacteria including cell wall, cytoplasmic fraction and the other cellular components have shown different inhibitory activities against tumor growth (10, 11). It was shown that, the cell wall of *Bifidobacterium infantis* plays an important role in reducing tumor proliferation in mice (11). Also, the cytoplasmic extract of *Lactococcus lactis ssp lactis* has disrupted the cell cycle of SNUC2 cancer cells (12).

Moreover, Type D cyclins play a key role in the cell cycle, with overexpression of cyclin D associated with tumor proliferation in various tumors such as esophageal, colorectal and prostate cancers (13, 14). Also, the *ccnd1* gene encodes the CCND1 protein, which is overexpressed in many types of tumors. Therefore, the regulation of cyclin D1 levels could be considered as a therapeutic approach for human colorectal cancer (15-17).

The cyclin D type family includes cyclin D1, cyclin D2 and cyclin D3. Notably, few reports have been published on the role of CCND2 and CCND3 carcinogenesis in contrast to CCND1. Cyclin D1, by activating cyclin kinase (CDK) 4/6 and inactivating retinoblastoma protein (pRb), can promote the cell cycle from the stage G1 to S (18).

In the present study, we investigated the effects of nisin, cell wall, and cytoplasmic extract of nisin producing *Lactococcus lactis* on SW480 colorectal cancer cell line, as well as the expression level of cyclin D1 gene in these cells after performing the treatment.

MATERIALS AND METHODS

Preparation of cell wall and cytoplasmic extract of *L. lactis ssp lactis*. Nisin producer microorganism, *L. lactis ssp lactis* (PTCC 1336) was obtained from the Persian Type Culture Collection, Iran, Tehran. Lyophilized bacteria (*L. lactis ssp lactis* PTCC1336) were cultured on MRS (Man, Rogosa, Sharpe) broth medium (liofilchem, Italy) for 72 hours and then incubated at room temperature (30°C).

Subsequently, the cells were harvested using a refrigerated centrifuge (3500 rpm, 15 min, at 4°C). The resulting precipitate was then washed twice with 0.1 M phosphate buffer (pH 6.9). Afterward, the bacteria were lysed using the Freeze-Thaw method and then sonicated in ice for 30 minutes. The cell walls were precipitated from the supernatant by centrifugation at 15000 rpm for 30 minutes at 4°C. The supernatant was finally used as a cytoplasmic extract. The cell walls were then placed for one day in the Standard Freeze Dryer (ZB111, UK) and the quantities needed were then weighted to obtain different concentrations of cell wall. After determining protein content in cytoplasmic extract using Bradford method, the desired dilutions of cytoplasmic extract were prepared, sterilized with 0.2 micron syringe filter, and then stored at -80°C until use (12-19).

Nisin. Nisin was purchased from Sigma-Aldrich, Germany (N5764). To prepare different concentrations of nisin, RPMI1640 (Bioidea, IRAN) medium was used.

Cell culture. SW480 colon cancer cell line and NIH3T3 (mouse embryo fibroblast cells) were purchased from Pasteur Institute of Iran. Cells were grown in RPMI medium that contained 1% fetal bovine serum (Gibson USA) and 1% penicillin - streptomycin antibiotic (Gibson USA), which were then incubated at 37°C and 5% CO_2. After 3 days, the cells were passaged using trypsin (Gibson USA).

MTT assay test. MTT test was used to evaluate the cell viability. Accordingly, this test is performed based on the reduction of dimethyl thiazole diphenyl tetrazolium bromide into formazan insoluble and purple crystals by the mitochondrial dehydrogenase enzymes of living cells. Notably, the intensity of the purple color produced is related to the amount of cells that are metabolically active.

Approximately 10^3 SW480 cells were cultured in each well of 96-well plates. Then, the first plate of 96 wells was treated by different concentrations (0, 50, 100, 200, 400, 600, 800, 1000, 1200, 1500 and 2000 µg/ml) of cell wall of *L. lactis ssp lactis.*

The second plate of 96 wells was treated by different concentrations (0, 50, 100, 200, 400, 600, 800, 1000, 1200, 1500 and 2000 µg/ml) of cytoplasmic extract of *L. lactis ssp lactis.* Moreover, the final plate of 96-well plates was treated by different concentrations (0, 50, 100, 200, 400, 600, 800, 1000, 1200, 1500 and 2000 µg/ml) of nisin. The plates were then incubated for 24 hours at 37°C. In this study,

zero concentration was considered as control.

Afterward, the cells were washed with PBS and 20 µL of MTT solution was then added to each well. The microplates were further incubated for 3 h at 37°C.

Subsequently, 100 µL of dimethyl sulfoxide (DMSO sigma) was added to each well, and all these wells were gently shaken. In addition, the optical absorption was measured at 570 nm using an ELISA reader (Bio-Rad, USA). Simultaneously, the anti-proliferative activity of the three substances was performed on a noncancerous NIH 3T3 (mouse embryo fibroblast) as normal cell.

RNA extraction and quantitative real time PCR. To extract RNA from SW480 cells, approximately 500,000 cells/well were seeded in 6-well plates for 24 hours. With respect to the MTT test results and IC50 obtained, we tested the Real Time PCR at 4 concentrations. Cells were separately treated by different concentrations (0, 600, 800 and 1500 µg/ml) of nisin, bacterial cell wall, and bacterial cytoplasmic extract for 24 hours. Next, RNA was extracted using a kit (Parstous, Iran). In addition, RNA quantification was performed using nanodrop spectroscopy (Eppendorf, Hamburg, Germany) at 260 nm.

One microgram of total RNA was then converted to cDNA using the cDNA synthesis kit (Takara, Japan). Also, real time PCR was performed using a Cyber Green Master Mix (Ampliqon, Denmark) PCR Mixture with primers (Bioneer, Korea) for cyclin D1 gene as follows: forward, 5'ATGGAACACCAGCTCCTGTGCTGC-3' and reverse, 5'TCAGATGTCCACGTCCCGCACGT-3' and GAPDH gene: forward, 5'GGGCTGCTTTTAACTCTGGT-3' and reverse, 5'TGGCAGGTTTTTCTAGACGG-3' (20). Quantitative real-time PCR was performed using Rotor-gene 6000 (Corbett life silences, Sydney, Australia). The expression level of cyclin D1 was normalized with housekeeping gene using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis. Data were analyzed by Graph Pad Prism software and $P<0.05$ was considered as an statistically significant level.

RESULTS

Antiproliferative effect of nisin, cell wall, and cytoplasmic fraction of *L. lactis ssp lactis*. MTT assay was performed to determine the inhibitory effects of *L. lactis ssp lactis* cell wall, cytoplasmic extract of *L. lactis ssp lactis*, and nisin on SW480 colorectal cancer cells.

The results of the present study show that the highest viability of SW480 colorectal cancer cells was obtained in the treatment with bacterial cell wall, bacterial cytoplasmic extract and nisin, respectively.

Statistical analysis of MTT assay showed that the growth rate of cancer cells treated with nisin has significantly reduced compared to the cancer cells treated by the bacterial cell wall and cytoplasmic extract. Treatment by 800, 1000, 1200, 1500 and 2000 µg/ml nisin had significantly decreased the cell viability compared to the control group ($P< 0.05$) (Fig. 1). Cell wall of *L. lactisssp lactis*, cytoplasmic extract of *L. lactis ssp lactis* and nisin had only a minor toxic activity on NIH 3T3 (mouse fetal fibroblasts) ($P> 0.05$).

Expression level of cyclin D1 mRNA following the treatment with nisin, cell wall and cytoplasmic extract of *L. lactis ssp lactis* using quantitative Real Time PCR. To determine the anti-proliferative effects of nisin, cytoplasmic extract of *L. lactis ssp lactis* and bacterium cell wall on SW480 cells for 24 hours, the mRNA levels of cyclin D1 were analyzed. The results showed that the mRNA levels of cyclin D1 have decreased in SW480 cells after the treatment with nisin for 24 hours. Moreover, a significant reduction was observed in the expression of cyclin D1 mRNA as compared to the control ($p<0.05$). Moreover, the level of cyclin D1 mRNA was not altered significantly in the presence of cell wall and cytoplasmic extract of *L. lactis ssp lactis* ($p> 0.05$) (Fig. 2).

DISCUSSION

L. lactis ssp lactis is one of the lactic acid bacteria that produce peptide and protein bacteriocins. In some studies, the growth inhibition of cancer cells by bacterial cell wall, cytoplasmic extract, and some natural products like nisin were confirmed (21-24). These researchers showed that the antibacterial effects of the cytoplasmic extract of the LAB are due to neutralizing the organic acids that are present in the cytoplasmic membrane of the bacteria (25, 26). One of the important bacteriocins produced by *L. lactis ssp lactis* is nisin, which has recently been examined for its effects on cell growth besides its antimicro-

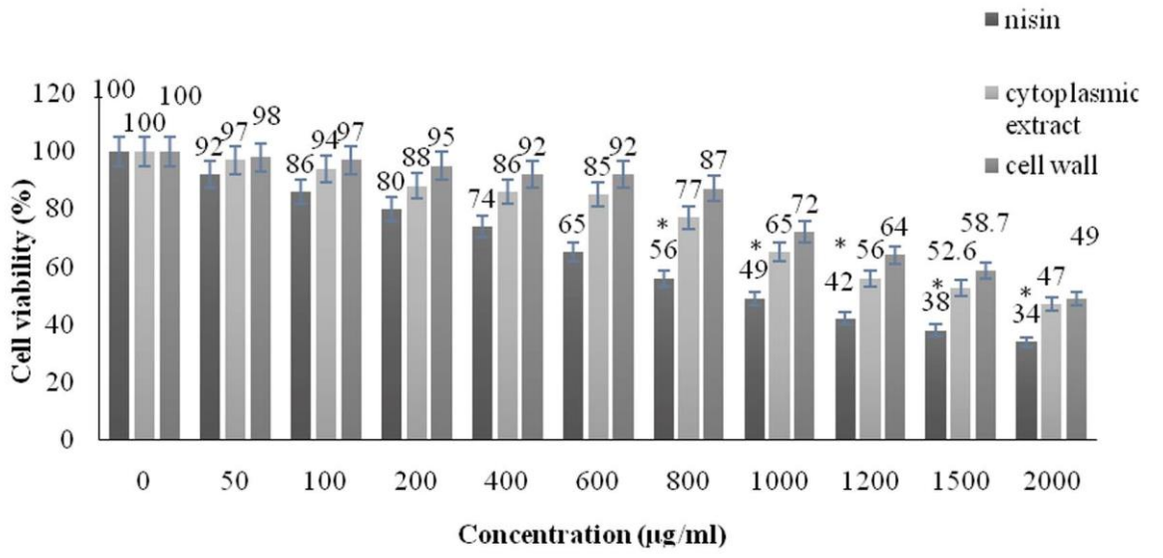

Fig. 1. SW480 cells were treated with, cell wall of *L. lactis ssp lactis*, bacterial cytoplasmic extract and nisin at doses of 0, 50, 100, 200, 400, 600, 800, 1000, 1200, 1500, 2000 µg/ml for 24 h. Cell viability was evaluated with the MTT assay and results are reported as relative cell viability (%). All data were normalized to the control group which was considered to be 100%. *P<0.05 versus control group (0 µg/ml).

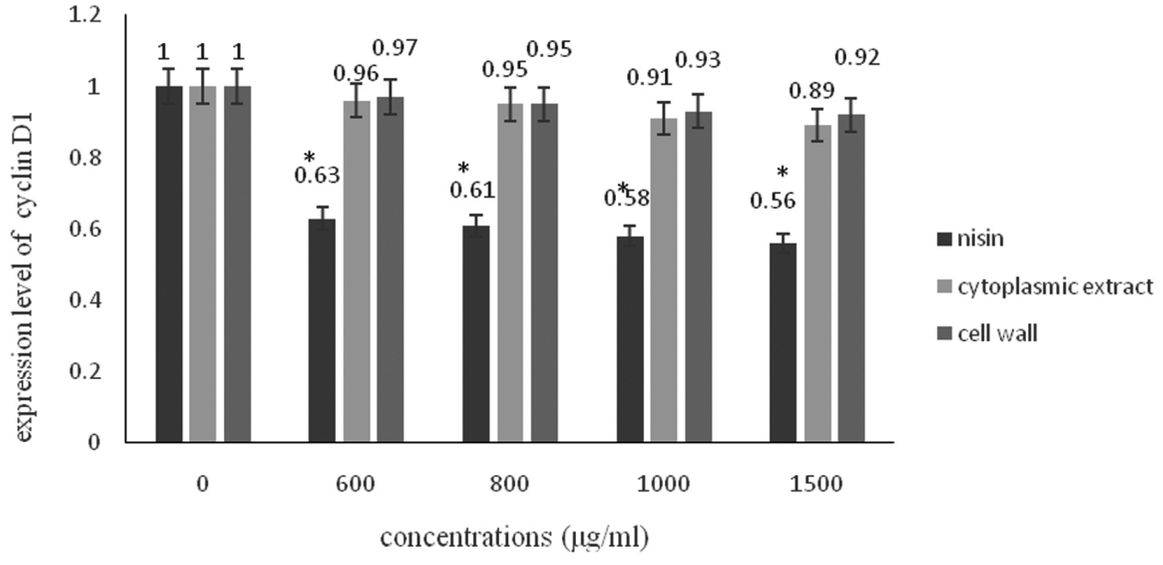

Fig. 2. Bacterial cell wall, bacterial cytoplasmic extract and nisin decreased genes expression level of cyclinD1 in SW480 cells in a dose-dependent manner. SW480 cells were treated with three substances respectively (0, 600, 800, 1000, 1500 µg/ml) for 24 h. The expression level of mRNAs was analyzed by real-time quantitative PCR and normalized by GAPDH expression. *P<0.05 versus control group (0 µg/ml).

bial activity (7, 9, 27). The level of cyclin D1 gene increases in different cancers, including colorectal cancer (28). Notably, cyclin D1 is involved in the activation of transcription of genes that causes genetic stability, cell growth and metastasis (29, 30). Consequently, the control of cyclin D1 levels is known as a therapeutic approach for human colon cancer. In this study, it was found that all three tested substances in a dose dependent manner reduced the proliferation of the SW480 cell line. The novelty of the present study

was that for the first time in the world, a comparison was separately performed among three substances related to a probiotic bacterium as well as the effects of each one of them on genes expression level of cyclin-D1in colorectal cancer cell line. Similar to our previous study, treatment with 800, 1000, 1200, 1500 and 2000 µg/ml of nisin had significantly decreased the cell viability compared to the control group ($p<0.05$) (9). In another study by Sam Maher et al. (31), nisin with concentrations of 115 µM and 89.9 µM had a toxic effect on Caco2 and HT29 colorectal cancer cells, respectively. Moreover, our results show that nisin concentration was directly correlated with cell viability. Notably, the decrease in cell proliferation of SW480 cell line treated with bacterial cell wall and cytoplasmic extract was not significant ($p>0.05$).

Contrary to our study, in a previous study conducted by Kim et al. the cytoplasmic extract of *L. lactis ssp lactis* bacteria had a significant anti-proliferative activity against SNUC2A colorectal cancer cell line. Furthermore, they showed that *L. lactis ssp lactis* cytoplasmic extract with a concentration of 100 µg/ml prevented the proliferation of more than 80% of snuc2 cancer cell line (12).

Kim et al. showed that the cytoplasmic extract of *L. lactis ssp lactis* reduced the Cyclin E protein levels, increased the Cyclin A protein expression and resulted in no change in the protein level of cyclin dependent kinase 1 (CDK1) in SNUC2A colorectal cancer cells (12).

However, in our study, at a concentration of 100 µg/ml of the cytoplasmic extract of the *L. lactis ssp lactis* bacteria, the viability of cancer cells was about 97%. This variation may be related to the difference among the tested cells.

Also, in a study by Wang et al. WPG (whole peptidoglycan) of *Lactobacillus paracasei ssp. paracasei M5* strain had anti-cancer effects on the HT-29 human colon cancer cell line (32). The highest concentration of cell wall of *Lactobacillus paracasei ssp. paracasei M5* strain tested in Wang et al.'s study was 160 µg/ml, which inhibited the growth of 20% of HT 29 cancer cell line. Accordingly, the results of their study show that the cytotoxic effects of bacterial cell wall on cancer cells are related to the activation of a number of genes in the apoptotic pathway.

The highest concentration of cell wall of *L. lactis ssp lactis* used in this study was about 2,000 µg/ml, which inhibited the growth 49% of SW480 cancer cell line. Similar to our study, in the research conducted by Wang et al. (32) in 2018, increasing the concentration of cell wall decreased the proliferation of cancer cell line; however, it was not significant ($p<0.05$).

The reason for the difference between the results of the present study and the other studies can be due to the type of cancer cell line in these studies. Researchers have shown that SW480 colon cancer cell line have a higher drug resistance compared to the other colon cancer cells line (27). However, nisin has been previously approved for cytotoxicity. This study examined the effect of nisin on the expression level of the cyclin D1 gene, which is one of the key genes in the cell proliferation cycle. Human colon cancer cell line (SW480) was used to investigate the effect of bacterial cytoplasmic extract, bacterial cell wall, and nisin on the level of the cyclin D1 gene expression. The results of this study show that in the colorectal cancer cell line treated by bacterial cytoplasmic extract and bacterial cell wall, the levels of cyclin D1mRNA decreased in a dose dependent manner, which was not significant ($p>0.05$). Moreover, in the colorectal cancer cell line treated by different concentrations of nisin, the levels of cyclin D1 significantly decreased in a dose dependent manner ($p>0.05$). An interesting finding in the present study is that nisin has a potent antiproliferative effect on SW480 cells compared to the bacterial cytoplasmic extract and the bacterial cell wall. Notably, 34%, 47% and 49% of the cancer cells survived in the treatment by 2000 µg/ml of nisin, cytoplasmic extract and bacterial cell wall, respectively. In this study, it was indicated that the cytoplasmic extract of *L. lactis ssp lactis* had a higher anticancer effect compared to the bacterial cell wall ($p<0.05$), which could be due to the presence of some proteins like nisin in the cytoplasmic extract. Previous studies have investigated the effect of nisin on caspase gene expression and apoptosis pathway in the SW480 cell line (9, 27). According to the results of this study, it is necessary to investigate the anti-proliferative and antitumor activities of nisin, bacterial cytoplasmic extract and bacterial cell wall associated with the decreased expression of cyclin D1 in different cancer cell lines.

CONCLUSION

The results of this study show that nisin, the cytoplasmic extract of *L. lactis ssp lactis*, and the cell

wall of this bacterium have antiproliferative and antitumor effects, which are associated with the decreased expression of cyclin D1 in SW480 cancer cell line. Further analysis on the types of proteins and amino acids present in the bacterial cytoplasmic extract and the cell wall of probiotic bacteria should be performed as well as on the effect of these proteins on the signaling pathways of cancer cells.

In future studies, it is suggested that other cancer cells, as well as the expression of different genes other than cyclin D1, be examined. Also, further research is needed on proteins extracted from different probiotic bacteria other than *L. lactis ssp lactis* and their impact on other human cancer cell lines.

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