Anticancer Effects of *Bifidobacteria* on Colon Cancer Cell Lines

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Abstract

Background: Colorectal cancer (CRC), with a growing incidence trend worldwide, is resistant to apoptosis and have the uncontrolled proliferation. It is recently reported that probiotic microorganisms exert anticancer effects. The genus *Bifidobacterium*, one of the dominant bacterial populations in the gastrointestinal tract, has received increasing attention because of widespread interest in using as health-promoting microorganisms. Therefore, the present study aimed to assess the apoptotic effects of some *bifidobacteria* species on colon cancer cell lines.

Methods: The cytotoxicity evaluations performed using MTT assay and FACS-flow cytometry tests. Also, the effects of five species of *bifidobacteria* secretion metabolites on the expression level of anti- or pro-apoptotic genes including BAD, Bcl-2, Caspase-3, Caspase-8, Caspase-9, and Fas-R studied by real-time polymerase chain reaction (RT-PCR) method.

Results: The cell-free supernatant of all studied *bifidobacteria* significantly decreased the survival rates of colon cancer cells compared with control groups. Flow cytometric and RT-PCR results indicated that apoptosis is induced by *bifidobacteria* secretion metabolites and the mechanism for the action of *bifidobacteria* species in CRC prevention could be down-regulation and up-regulation of anti-apoptotic and, pro-apoptotic genes.

Conclusions: In the present study, different *bifidobacteria* species showed anticancer activity on colorectal cancer cells through down-regulation and up-regulation of anti-apoptotic and pro-apoptotic genes. However, further studies are required to clarify the exact mechanism of apoptosis induction by *bifidobacteria* species.

Introduction

Cancer as a major public health problem has a growing incidence trend worldwide. It is estimated that the number of global cancer deaths rises 45% from 2007 to 2030 (1, 2). CRC, the most common malignancy of the gastrointestinal tract, is the third and second most common cancer in males and the in females, respectively, and is the fourth leading cause of cancer death worldwide (3, 4). The number of new cases is increasing quickly because of the rising of different risk factors. The risk factors for CRC are including modifiable such as smoking, alcohol, physical inactivity and obesity, and nonmodifiable factors like familial risk, male sex, older age, and race/ethnicity (5). It has been indicated that the general population of intestinal bacteria and microbial dysbiosis may contribute to the initiation and the etiology of CRC (6) thus, any factor that could modify the gut microbiota may prevent the disease. Some studies proposed that the ingestion of certain microorganisms decreases both the risk of developing certain types of cancer and tumor growth and attention has been focused on probiotic yeasts and bacteria like *lactobacilli* and *bifidobacteria* (7–11).

The genus *Bifidobacterium* contains approximately 57 (sub) species. They are among the dominant bacterial populations in the gastrointestinal tract and the normal inhabitants of a healthy human gut. To

date, almost ten species/subspecies of *Bifidobacterium* has been detected in the human intestine. Among the bifidobacterial species, *B. catenulatum*, *B. pseudocatenulatum*, *B. adolescentis*, *B. longum*, *B. breve*, *B. bifidum*, *B. animalis*, and *B. dentium* are commonly found in the feces of healthy subjects (12, 13). Changes in the number or/and composition of their populations are one of the most frequent situations that present in some diseases like inflammatory bowel disease, colorectal cancer, or irritable bowel syndrome (14). During infancy, almost 80% of the gut microbiota consisted of bifidobacteria, and dysbiosis during infancy increases the risk of childhood diseases and could also affect the health of the host in the future (15).

In the last 15 years, the genus *Bifidobacterium* has received increasing attention due to widespread interest in using as health-promoting microorganisms, i.e., known as probiotics, in the food industry, and many products containing bifidobacteria such as fermented milk, yogurt, and healthy foods that produced for the microbiota modification (16, 17).

Some species of bifidobacteria are able to decrease carcinogen-induced DNA damage, pre-neoplastic lesions, and tumors in the colon of rats (18, 19). Administration of 3 bacteria including *Lactobacillus acidophilus*, *B. bifidum*, and *B. infantum* altered the gut microbiota and decreased colon cancer development by decreasing tumor incidence, multiplicity/count, and volume (7). The exact mechanisms responsible for the anti-cancer activity of these organisms are unknown yet, but some proposed mechanisms may influence metabolic, immunological, and protective functions within the colon, and also they may stimulate tumor cell apoptosis (20). Apoptosis is an active cellular process in which damaged or mutant cells undergo self-destruction and help the organism to control normal development. Each step of apoptosis requires many proteins such as caspases that block tumor progression (21). However, the exact molecular pathways by which *bifidobacteria* affect tumor cells remains unclear. Therefore, the present study aimed to assess the anticancer effects of some *bifidobacteria* species on HT-29 and Caco-2 cell lines in comparison to normal epithelial cells (KDR/293) by focusing on the main apoptosis pathways.

Materials And Methods

Cell-free supernatant preparation

Five species of *bifidobacteria* including *B. adolescentis* (ATCC 15703, PTCC1536), *B. animalis subsp. lactis* (PTCC1736), *B. animalis subsp. animalis* (ATCC 25527, PTCC 1631), *B. bifidum* (ATCC 29521, PTCC 1644), and *B. angulatum* (ATCC 27535, PTCC 1366) were obtained from the Persian Type Culture Collection (PTCC) from the Iranian Research Organization for Science and Technology (IROST). *Bifidobacterial* strains were cultured in de Man–Rogosa agar (MRS) (Merck, Darmstadt, Germany) at 37 °C for 72 hours in an anaerobic incubator. Then, the cultures were centrifuged at 4500 rpm for 15 min at 4 °C. Fifty ml of each supernatant mixed with 75 ml volume of methanol and gently agitated for 24 h. The methanolic extracts were dried by lyophilization and obtained dried materials were dissolved in different amounts (5–30 mg/ml) in each used cell culture including Dulbecco Modified Eagle medium

(DMEM) (Gibco, Grand Island, NY, USA), and Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma, St Louis, MO, USA). Finally, the pH of supernatants was adjusted to 7.2 points and before using, filtered through a 0.22 mm Millipore filter (Milli-Q, Millipore, Germany).

Cell Culture

Two human colon cancer cell lines (Caco-2, ATCC, HTB-37 and HT-29, ATCC, HTB-38) and one human epithelial normal cell line with the same embryonic origin (KDR/293) were purchased from Pasteur Institute (national cell bank of Iran). The purchased cells were cultured in 25 cm² plastic cell culture flasks and were incubated under standard conditions at 37°C in a humidified atmosphere with 5% CO₂ with medium renewal every 1–3 days. The cells were maintained in an RPMI-1640 (HT-29 and Caco-2) or high glucose concentration (4.5 g/l) DMEM (KDR/293) cell culture medium, respectively. Both media were supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 8 mM L-glutamine and 1% of mixture penicillin (100 IU/ml) and streptomycin (100 g/ml) (Sigma, St Louis, MO, USA).

Mtt Assay

Cell viability in treated and untreated cell lines was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St Louis, MO, USA) based on the capacity of viable cells to reduce a tetrazolium colorless salt to purple formazan in mitochondria. At first, the half-maximal inhibitory concentration (IC₅₀) for HT-29 and Caco-2 cells was determined using prescreening MTT tests (in the range of 10 to 100 µg/mL) at 24 and 48 hours. Briefly, the cells were washed twice with phosphate-buffered saline (PBS) (Sigma, St Louis, MO, USA) and trypsinized by adding 1 ml of trypsin/EDTA (Sigma, St Louis, MO, USA) solution. The cells were plated into 96well plates at 1.2×10^4 cells per well and added 200 µL of the growth medium, incubated for 24-h. After cell attachment, the medium was carefully removed from each well and the cells were treated with an effective dose (IC₅₀) of cell-free bacterial supernatant and growth medium. After 24 h or 48 h (determined time point) incubation, 50 µl of MTT reagent and 150 µL of fresh growth medium were added to each well and plates returned to the incubator for 4 hours. Then, the medium of each well was carefully removed and 200 µL of dimethyl sulfoxide (Merck, Germany) and 25 µL of Sorenson buffer (0.1 mol/L glycine, 0.1 mol/L NaCl, pH 10.5) were added to each well and kept for 15 minutes in the dark condition at room temperature. The absorbance was determined using enzyme-linked immune-sorbent assay plate reader (ELx 800; Biotek, Winooski, VT, USA) at 570 nm. The growth inhibitory effects of supernatant were calculated according to the following formula: the growth inhibition ratio = [(the absorbance of blank control group – the absorbance of experimental group)/the absorbance of blank control group] × 100% (22, 23). In the present study, 5-fluorouracil (5-FU) as approved anticancer drug (7 µL/well of 96-well plate) was used as the positive control.

Flow Cytometry

Three mL of growth medium including 1.2×10^5 cells was cultured in 6-well culture plates and incubated at growth condition. After 24 h, the cells were treated with 3 ml of the sterile growth medium containing determined dried materials of supernatant or 5-FU, as the positive control group, and incubated in the growth condition based on the determined time point. The treated/untreated control cells were detached by trypsin-EDTA, and supernatants were discarded by centrifugation at 900 rpm for 10 minutes. Finally, for detection of apoptosis, the cells were stained with Annexin V-FITC/propidium iodide (PI) apoptosis kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions, and data analysis was conducted using CELL Quest Pro software (BD Biosciences, San Jose, CA, USA). Each experiment was repeated 2 times with triplicate samples. All of the analyses were performed using 150000 cells at a rate of 900 cell/sec.

Quantitative Real-time Pcr Analysis

All untreated/treated cells were washed three times with PBS (pH 7.2) and total RNA was extracted from cells by direct lysis using 1 ml ice cold RNX-plus solution (Sina Clone, Iran), according to manufacturer's instruments. The obtained total RNA was solved in 50 μ L DEPC-treated water (Merck, Germany), and the quantity and quality of total RNA were evaluated by UV spectrophotometry and agarose gel electrophoresis, respectively. Complementary DNA (cDNA) was synthesized using one microgram of isolated RNA by Prime Script RT Reagent kit (Takara Bio Inc, Tokyo, Japan) according to the manufacturer's instructions. The specific primers for every gene including Bcl-2, BAD, Fas-R, caspase-3, caspase-8, caspase-9, and GAPDH as housekeeping gene were designed (10). All of the amplification reactions were carried out in triplicate for each sample, and every experiment mixture (20 μ L), containing 10 μ L SYBR Green PCR master mix (Takara Bio Inc, Tokyo, Japan), 1 μ L cDNA (1 μ g/ μ L), 1 μ L primer (forward and reverse), and 0.8 μ L 6-carboxy-X-rhodamine (ROX as reference dye), was subjected to ABI-step I plus (Applied Biosystems, Foster City, CA, USA) instrument. One cycle at 95 °C for 5 minutes followed by 40 cycles at 95 °C for 20 seconds, 60 °C for 35 seconds, and 72 °C for 10 seconds were selected as thermal cycling condition. Pfaffle method was used for interpretation of the results and the threshold cycle values were normalized to the expression rate of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (24).

Statistical analysis

The statistical package for the social sciences (SPSS Inc. Chicago, IL, USA version 16.0) was used for the statistical analysis. One-way ANOVA and Tukey's post hoc test were performed for analyzing differences between all treatments and multiple mean comparisons, respectively. Statistical significance was considered to be $P \leq 0.05$.

Results

The IC50s after treatment by prepared cell-free supernatants of *bifidobacteria* were determined a range between 65 µg/mL to 80 µg/mL for HT-29 and Caco-2 cells at 48 h and the control group (KDR/293 cells) were treated with the highest determined concentration (80 µg/mL).

After treatment by cell-free supernatant of all studied *bifidobacteria* species and 5-FU, the survival rates of colon cancer cells were significantly decreased compared with control groups. (Fig. 1). The survival rates of HT-29 cells after treatment by bifidobacteria were between 15.88 to 70.43 percent and in the caco-2 cell line were 28.19 to 55.45 percent. Also, the positive control group (5-FU) showed 65.07% and 51.77% survival rates in HT-29 and Caco-2 cells, respectively.

Flow cytometry was used for the detection of apoptotic rates quantitatively with Annexin V-FITC/PI staining. According to the results of the present study, treatment of cancer cell lines with determined IC-50 of methanolic extracts of *bifidobacteria* secretion metabolites increased the percentage of cells in early and late apoptosis phases compared with the control and normal KDR/293 cells. The highest percentage of induced apoptosis (early and late apoptosis) in the HT-29 cancer cell lines belonged to *B. bifidum* (53.32%) and the lowest was in *B. angulatum* (24.83%) (Fig. 2). Moreover, in Caco-2 cells, the percent of apoptosis was 79.78, 68.36, 61.07, 37.79 and 12.98 in *B. bifidum*, *B. animalis subsp. lactis*, *B. animalis subsp. animalis*, *B. adolescentis* and *B. angulatum*, respectively (Fig. 3). Also, in normal cells, the highest and lowest percentages of apoptosis were in *B. adolescentis* and *B. angulatum* groups, respectively. As well, 5-FU induced 52.77% (HT-29 cells), 34.36% (Caco-2 cells) and 28.64% (KDR/293 cells) apoptosis (Fig. 4).

Table 1 indicates the expression level of pro-apoptotic (caspase-3, caspase-8, caspase-9, Fas-R, and BAD) and anti-apoptotic (Bcl-2) key genes in the colon and normal cell lines after treatment with methanolic extract of *Bifidobacterial* supernatants and 5-FU compared with untreated control cells. 5-FU significantly increased the expression level of pro-apoptotic genes except for caspase-9 and decreased Bcl-2 as an anti-apoptotic gene. The expression level of the BAD, caspase-3, caspase-8, caspase-9, and Fas-R genes was increased by bacterial supernatants, but only in some species was statistically significant. Treatment with *B. adolescentis* significantly up-regulated the expression level of caspase-8, Fas-R and BAD genes in HT-29 and Caco-2 cancer cell lines. Also, up-regulation of the caspase-9 gene expression was significant in the HT-29 cells and down-regulation of the Bcl-2 gene expression was significant in the Caco-2 cell line. Moreover, *B. angulatum* was not able to up-regulate the expression level of genes significantly, and only its effect on Fas-R was significant.

Table 1

Important intrinsic and extrinsic apoptosis pathway gene expression ratio to control gene in the HT-29, Caco-2 cancer cells and KDR/293 normal cells.

Target genes *	Caspase-3	Caspase-8	Caspase-9	Bcl-2	Fas R	BAD
Control	1	1	1	1	1	1
HT-29	1	1	1	1	1	1
Caco-2	1	1	1	1	1	1
KDR/293						
5-FU **						
HT-29	1.60 [†]	1.89 [†]	1.30	0.66 [†]	2.20 [†]	1.22
Caco-2	1.65 [†]	1.80 [†]	1.27	0.47 [†]	2.05 [†]	1.41 [†]
KDR/293	1.25	1.30	1.15	0.95	1.10	1.16
B. adolescentis						
HT-29	1.20	1.60 [†]	1.42 [†]	0.79	1.85 [†]	1.52 [†]
Caco-2	1.36	1.48 [†]	1.33	0.67 [†]	1.92 [†]	1.71 [†]
KDR/293	1.35	1.24	1.12	0.92	1.25	1.22
B. animalis subsp. lactis						
HT-29	1.82 [†]	1.70 [†]	1.41 [†]	0.94	1.94 [†]	1.80 [†]
Caco-2	1.78 [†]	1.60 [†]	1.20	0.85	1.87 [†]	1.78 [†]
KDR/293	1.30	1.10	1.18	0.93	1.05	1.18
B. animalis subsp. animalis						
* Target genes were normalized to GAPDH as housekeeping control gene. All cell lines were treated with secretion metabolites of <i>bifidobacteria</i> containing indicated concentrations of dried materials from the methanolic extract for 48 h.						
** 5-FU was considered as positive control group versus untreated control groups.						
[†] All experiments were performed in triplicate (n = 3) and statistic significances were accepted at p < 0.05.						

Target genes *		Caspase-3	Caspase-8	Caspase-9	Bcl-2	Fas R	BAD
	HT-29	1.53 [†]	1.60 [†]	1.28	0.85	1.89 [†]	1.67 [†]
	Caco-2	1.65 [†]	1.75 [†]	1.39	0.74	1.75 [†]	1.72 [†]
	KDR/293	1.20	1.29	1.31	0.87	1.21	1.19
B. bifidum							
	HT-29	1.70 [†]	2.00 [†]	1.62 [†]	0.65 [†]	2.02 [†]	1.80 [†]
	Caco-2	1.80 [†]	1.86 [†]	1.49 [†]	0.70	2.00 [†]	1.65 [†]
	KDR/293	1.15	1.30	1.15	0.90	1.14	1.17
B. angulatum							
	HT-29	1.26	1.37	1.23	0.90	1.60 [†]	1.30
	Caco-2	1.15	1.24	1.15	0.87	1.74 [†]	1.25
	KDR/293	1.18	1.29	1.12	0.95	1.09	1.10
* Target genes were normalized to GAPDH as housekeeping control gene. All cell lines were treated with secretion metabolites of <i>bifidobacteria</i> containing indicated concentrations of dried materials from the methanolic extract for 48 h.							
** 5-FU was considered as positive control group versus untreated control groups.							
[†] All experiments were performed in triplicate (n = 3) and statistic significances were accepted at p < 0.05.							

Discussion

Probiotics are being investigated for their different health beneficial effects. Anticancer property is one of the most important preventive as well as therapeutic benefits, and the consumption of probiotics or prebiotics is suggested as a promising approach for the prevention of CRC (25). It is recently reported that probiotic microorganisms, specially *bifidobacterium* species exert anticancer effects (13).

Apoptosis is a controlled process in which cells are selectively eliminated and plays an important role in controlling cell numbers. Many cancer cell types including CRC are resistant to apoptosis and have uncontrolled proliferation. Therefore, the regulation of cell proliferation and death can be one of the

cancer treatment strategies, and probiotics are reported to be involved in regulating cell apoptosis and proliferation (11, 26).

To the best of our knowledge, this is the first study that reports the effects of *bifidobacteria* species on colorectal cell lines. We investigated the potential anticancer activities of five species of *bifidobacteria* on HT-29 and Caco-2 cell lines. In the present study, secretion metabolites of *bifidobacteria* species induced intrinsic and extrinsic apoptosis pathways in human colorectal cancer cells. Based on our findings, in HT-29 cancer cell lines, the highest and lowest levels of apoptosis were induced by *B. bifidum* and *B. angulatum*, respectively. The highest percentages of induced apoptosis in Caco-2 cells belonged to *B. bifidum*, followed by *B. animalis* subsp. *Lactis* and *B. animalis* subsp. *animalis*. Similarly, You et al. and Ku et al. suggested that *B. bifidum* strains inhibited the growth of several cancer cell lines including HT-29 (27, 28). *B. bifidum* has been previously reported to be anti-proliferative and protective effects against preneoplastic lesions in animal models of colorectal carcinogenesis (8). The anti-proliferative properties of *B. bifidum* on cancer cell lines are considered as an example of the interaction between *Bifidobacterium* spp. and host cells (29). The interactions between *B. bifidum* and colon cancerous cells leads to the suppression of cancer cell growth that indicates anticancer or antitumor effects of it. It is reported that whole peptidoglycan, a metabolite produced by *B. bifidum*, was capable to activate macrophages to produce large amounts of cytotoxic molecules including TNF- α , IL-6, and IL-12 (30). By considering the antitumor activity of these mediators, the cytotoxic molecules secreted by activated macrophages mediate the antitumor effects of the whole peptidoglycan. However, in this study, the main effective metabolites of *B. bifidum* was not characterized, and warrants further study. Further studies are necessary to elucidate the role of *B. bifidum* as a preventive or therapeutic agent.

The results of the present study indicated that *B. adolescentis* inhibited the proliferation of human colon cancer cell lines including HT-29, and Caco-2, which are similar to the results of Kim et al (31). The present study investigated the mechanisms of the effects of *B. adolescentis* on colon cancer cells. The observations revealed that the *B. adolescentis* was able to trigger apoptosis by upregulating the expression of caspase-8, Fas-R, and BAD in HT-29 and, Caco-2 cancer cell lines; our results indicated that up-regulation of caspase-8 subsequently stimulates the apoptosis pathway. Surface receptors for extrinsic apoptosis, such as TNF- α , are produced by immune system cells and Fas, which are able to activate the cytosolic protease, caspase-8. Caspase-8 activates caspase-3, caspase-6, and caspase-7 that leading to apoptosis induction (32). In this way, Kim et al revealed that *B. adolescentis* potentiated the production of TNF- α , as a cytokine that induces apoptosis, and their results supported our results (31). Furthermore, *B. adolescentis* has been used as a vehicle for systemic delivery of the antiangiogenic protein endostatin, and systemic administration of its spores that strongly inhibited angiogenesis and reduced tumor growth (33). Likewise, *B. adolescentis* effectively increased the expression level of caspase-9 in HT-29 cells, thus induced apoptosis through caspase-9 signaling. Caspase-9 activation is a consequence of mitochondrial outer membrane permeabilization and activation of the mitochondrial pathway of apoptosis (34); which we observed in *B. adolescentis*-treated HT-29 cells.

Additionally, our observations revealed that *B. adolescentis* significantly reduced the expression level of, a potent anti-apoptotic protein, Bcl-2, in caco-2 cell lines. It should be noted that apoptosis occurs via two major pathways: the intrinsic (mitochondria-mediated apoptosis) and the extrinsic pathways. BCL-2 family proteins are involved in the intrinsic apoptosis pathway and composed of two groups of proteins; one group includes proteins with pro-apoptotic traits such as BAX and BAK, and the other group proteins with anti-apoptotic properties such as BCL-2 and BCL-XL. Thus, the lower expression of BCL-2 which has anti-apoptotic activity can trigger the apoptosis pathway (34).

In the present study, the highest percentages of induced apoptosis in Caco-2 cells were induced by *B. bifidum*, followed by two subspecies of *B. animalis*: *animalis* and *lactis*. Numerous studies have focused on the potential effects of *B. animalis* strain on cancer cell lines, but the precise mechanism whereby probiotic bacteria exert their antitumorigenic effects remains undetermined yet (35, 36). Additionally, it is reported that *B. animalis* subspecies *lactis* exert preventive effects on colitis-associated colon cancer by inhibiting NF- κ B activity (37). Since NF- κ B is involved in cell proliferation and plays a critical role in the inflammatory processes, it provides a possible mechanistic link between inflammation and cancer (38). Based on different studies, NF- κ B has a pivotal role in inflammation, as well it can up-regulate several genes that suppress apoptosis, which indicates its critical role in inflammation-related carcinogenesis (39). However, in the present study, we did not evaluate the activity of NF- κ B, and further studies are required to investigate the effects of Bifidobacterium strains on NF- κ B signaling. Moreover, *B. animalis* has demonstrated anti-mutagenic activity during growth in the MRS broth which antagonizing the action of the carcinogen 2-amino-3-methylimidazo [4, 5-f] quinolone (40).

There are some limitations to the present study. Firstly, the anticancer effects of *bifidobacteria* species on other types of cancer cell lines were not investigated, and additionally, the specific compound(s) of the secretion metabolites of *bifidobacteria* species, which is involved in the antitumor activity, was not determined.

In conclusion, the present study showed that the possible mechanism for the CRC preventive effects of *bifidobacteria* species could be down-regulation and up-regulation of anti-apoptotic and pro-apoptotic factors. In addition to significant anti-cancer effects on cancer cell lines, fewer harmful side effects were observed on normal epithelial cells. However, performing more studies are recommended to determine the exact mechanisms of probiotics in human colon cancer.

Abbreviations

cDNA

Complementary DNA

CRC

Colorectal cancer

DMEM

Dulbecco's Modified Eagle's Medium

ELISA
Enzyme-linked immunosorbent assay
GAPDH
glyceraldehyde 3-phosphate dehydrogenase
IC50
half-maximal inhibitory concentration
IROST
Iranian Research Organization for Science and Technology
MRS
Man-Rogosa agar
MTT
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS
phosphate-buffered saline
PI
Propidium iodide
PTCC
Persian Type Culture Collection
RPMI
Roswell Park Memorial Institute medium
RT-PCR
real-time polymerase chain reaction
5-FU
5-fluorouracil

Declarations

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Authors' contributions

ZF, MHF, AS conceived and designed the experiments; MHF, SI performed the experiments and analyzed the data; AS, AHK reviewed the paper and provided comments. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Effects of secretion metabolites of different bifidobacteria species containing indicated concentrations of dried materials from the methanolic extract on the viability of HT-29, Caco-2 cancer cells, and KDR/293 normal cells for 48 h incubation by MTT assay test. Untreated cells were used as negative control, and 5-FU was used as positive control. MRS was used to determine its effect on cell viability.

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Figure 2

Flow cytometric analysis of treated/untreated HT-29 cells. The upper left quadrant: necrotic cells; the upper right quadrant: late apoptotic cells; the lower right quadrant: early apoptotic cells; the lower left quadrant: intact cells.

Figure 2

Flow cytometric analysis of treated/untreated HT-29 cells. The upper left quadrant: necrotic cells; the upper right quadrant: late apoptotic cells; the lower right quadrant: early apoptotic cells; the lower left quadrant: intact cells.

Figure 3

Flow cytometric analysis of treated/untreated Caco-2 cells. The upper left quadrant: necrotic cells; the upper right quadrant: late apoptotic cells; the lower right quadrant: early apoptotic cells; the lower left quadrant: intact cells.

Figure 3

Flow cytometric analysis of treated/untreated Caco-2 cells. The upper left quadrant: necrotic cells; the upper right quadrant: late apoptotic cells; the lower right quadrant: early apoptotic cells; the lower left quadrant: intact cells.

Figure 4

Flow cytometric analysis of treated/untreated KDR/293 cells. The upper left quadrant: necrotic cells; the upper right quadrant: late apoptotic cells; the lower right quadrant: early apoptotic cells; the lower left quadrant: intact cells.

Figure 4

Flow cytometric analysis of treated/untreated KDR/293 cells. The upper left quadrant: necrotic cells; the upper right quadrant: late apoptotic cells; the lower right quadrant: early apoptotic cells; the lower left quadrant: intact cells.