Original Article

The Study of Extracellular Protein Fractions of Probiotic Candidate Bacteria on Cancerous Cell Line

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Abstract

Introduction: Probiotics are live microorganisms, habituated in the human intestine, which have a beneficial effect on our health. In spite of many reports about the anticancer effect of these bacteria in in–vivo and in–vitro, their mechanisms of action are not completely understood. The goal of this study was to compare the extracellular fractions of Lactobac*illus casei* and *L. paracasei* on the anti proliferation and apoptosis induction in K562 cell line.

Materials and Methods: *L. casei* and *L. paracasei* were cultured in MRS broth medium. Then extracellular secretions were collected and after enrichment, analyzed by electrophoresis. Fractionation were determined by gel filtration chromatography using sephadex G100 column, and the anticancer properties were evaluated.

Results: The results of SDS–PAGE showed various molecular weight of fractionated proteins of *L. casei* and *L. paracasei*. Bioactivity assessment illustrated that anti proliferative effects on K562 cells is dose and time dependent and the cytotoxic effects was parallel with protein concentration and the increase of time from 36 to 72 hours.

Conclusion: Regarding the cell cytotoxicity results, the fractionated extracellular proteins of *L. casei* and *L. paracasei* have significant effects in inhibition of cancer cell proliferation. However, more study is needed to better elucidate the mechanisms of extracted proteins, and its effect on other human cancer cell lines.

Keywords: Anticancer, blood cancer, extracellular fraction, gel filtration chromatography, K562

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Introduction

Cancer is a disease that is caused by the accumulation of genetic defects, chronic oxidative stress and inflammation, which increases cancer cell proliferation.[1] Today, a variety of cancer treatments including surgery, chemotherapy, radiation and targeted therapy are being used to cure patients.[2]

The probiotics refer to the bacteria which are associated with beneficial effects on human health.[3] The probiotics are substances produced by microorganisms which promote the growth of other microorganisms. They act as a live microbial nutrition supplement which beneficially affects human health. These bacteria are able to resist acid and bile conditions and have beneficial health effects such as adherence to the intestinal mucosa, and production of the antimicrobial substances.[4] Probiotic bacteria confront cancer by reducing mutagenic and genotoxic effects. Recently, studies showed that lactobacillus bacteria reduces the occurrence and the number of tumors in colon, liver, small intestinal and mammary tissue.[5] Probiotics also play a beneficial role in several medical conditions such as diarrhea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, cancer, depressed immune function, hepatic diseases, genitourinary tract infections, etc.[6] Moreover, Lactic acid bacteria (LAB) help promoting health and reducing the risk of various diseases such as cancer by producing variable biological products.[7] Important roles of lactobacilli are the enhancement of immunity, maintenance of intestinal microbial balance and prevention of gastrointestinal infection.[8–9] Anticancer activities were found in peptidoglycans isolated from *Bifidobacterium infantis* and *L. casei*, as well as glycoproteins found in the supernatants of *L. plantarum*.[10] Probiotic bacteria suppress intestinal inflammation and down regulate immune responses in the gut inhibiting the proliferation of mononuclear cells and some cell lines.[11]

Also, probiotics can be used for treating dysfunctions of the gut mucosal barrier, such as gastroenteritis, food allergy, colon cancer and inflammatory disease.[9] In addition, *Lactobacillus* have an anti–cancer activity and prevent metastasis.[12] However, previous studies have shown that some LAB such as *L. acidophilus*, *L.casei*, *L. rhamnosus* and *Bifido-bacterium longum* are able to inhibit the growth of tumor cells induced in rodents. In this study, we evaluated the effects of gel filtration fractionated extracellular proteins of *L.casei* and *L.paracasei* onto the K562 cancer cell line.

Materials and Methods

Reagents

Sodium deoxycholate (DOC), MTT [3–(4, 5–dimethylthiazolyl)–2,

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5–diphenyl–tetrazolium bromide, were purchased from Sigma Chemical (St. Louis, MO). Polyethylene Glycol (PEG) and MRS broth were obtained from ScharlauChemie (ScharlauChemie S. A, Barcelona, Spain). Sephadex G–100, Tris, acrylamide and bis–acrylamide, Sodium dodecyl sulfate (SDS), Glycine (Amersham Biosciences, Piscataway, NJ, USA), Fetal bovine serum (FBS), RPMI 1640 from Gibco (Gibco, Paisley, England), Trichloroacetic acid (TCA) from Merck (Merck, Darmstadt, Germany), V–FITC kit from (eBiosciences; Vienna, Austria), as well as other chemicals were purchased from Sigma–Aldrich.

Bacteria strain, Medium, and Growth Condition

Lactobacillus species were inoculated in MRS broth and grown under aerobic conditions at 37ºC for 24 – 48 h. Bacterial cultures were harvested by centrifugation (20 min, 3500 g, 4ºC), and the supernatant was collected in sterile containers.

Extracellular protein preparation

Extracellular proteins were concentrated through three methods: DOC and TCA precipitation techniques, lyophilisation and dialysis using PEG.[13] Briefly, the supernatant was filtered through syringe filter (0.45µm) for removing bacterial cells. For DOC and TCA method, the DOC detergent (at a final concentration of 0.015%) and TCA were added and the contents mixed by vortexing. Proteins were allowed to precipitate overnight at 4ºC. Proteins were recovered by centrifugation (30 min, 1000 g, 4ºC), followed by dialysis. For lyophilisation Christ alpha 1–4 lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterodeam Harz, Germany) was used. As a third protein enrichment method, the protein solution was dialyzed using PEG. Protein concentration was measured using the Bradford technique.[14]

Gel filtration chromatography (GFC):

The Sephadex G–100 was prepared according to the manufacturer's instruction; briefly the powder was swollen with distilled water and stored overnight at 4 ºC. After three washing processes, the gel was slowly packed into a column (1.5 × 75cm) and equilibrated by phosphate buffer, pH 7.00. The sample was loaded into the column, washed with phosphate-buffered solution at a flow rate of 0.3 ml/min and the fractions were collected.

For evaluation of chromatography fractionation, SDS–PAGE was performed according to the Laemmli with 5% stacking gel and 16% separating gel. Samples were boiled for 5 min in a sample buffer (4% SDS, 25% glycerol, 5% 2–mercaptoethanol, 125 mM Tris–HCl, pH 6.8, and 0.005% bromophenol blue). The gels were electrophoresed at constant voltage, initially 50 V, followed by 150V. [15] After migration, the gel was stained with silver staining.[16–17]

Cell culture

Human K562 leukemia cell line was purchased from the Pasteur Institute of Iran Bank cell and cultured in RPMI 1640 supplemented with 10% heat–inactivated FBS. Then the human K562 leukemia cell line was incubated in 5% CO2 and 86% humidity incubator at 37°C. Cell viability was assessed by the trypan blue exclusion test.[17]

Measurement of cell proliferation

To evaluate the cytotoxicity effects of chromatography fractions on cells, MTT test was done. Briefly, after centrifugation and counting using trypan blue, K562 cells were seeded in 96–well tissue culture plates, with a density of 10,000 cells. Then, 100 µL of culture medium containing various concentrations of extracellular extraction (100, 200, 400 and 800 µg/ml) and the relevant fractions were added to the wells and were incubated. Three wells as a negative control and three wells (which were treated with taxol) as a positive control was considered.[18]

After 36 and 72 h post incubation, the supernatants were removed and incubated with 0.5 mg/ml MTT (100 µL) for 4 h in a humidified incubator at 37ºC. The cell cultures were centrifuged at 1000 g for 5 min and the supernatants were discarded. Subsequently, 200 µL of dimethyl sulfoxide (DMSO, Sigma) and 25 µL Sorenson buffer were added. The optical density (OD) was measured at 570 nm by an enzyme linked immune absorbent assay plate reader (BIO–RAD). Growth inhibition percent of cells was calculated as follows:

% Inhibition =100 – (Test OD/Non–treated OD) × 100).

Apoptosis detection by Flow Cytometry

K562 cells were seeded in 6–well tissue culture plates at a density of 0.5×10^6 cells/well and incubated at 37 ºC, 5% CO_2 in an incubator for 12 h. The cells were treated with the *L. casei* F11 and *L.paracasei* F21 at a concentration of 100 µg/ml and then incubated for 36 and 72 h. Apoptosis was measured by Annexin V–FITC kit, according to the manufacturer's instructions. Annexin V–positive cells were measured at a fluorescence intensity of 1×10^4 cells using the FACS Calibur system (Becton & Dickinson, San Jose, CA, USA) within 1 hour.

Statistical Analysis

All experiments were performed at least three times, each time in triplicate and the data was shown as a mean ± standard deviation (SD). The data were analyzed using IBM SPSS Statistics 21 software. Statistical comparisons of the groups were performed by two–way analysis of variance (ANOVA) followed the Tukey's test post–hoc test at ($P \leq 0.05$).

Results

Protein Enrichment

We used three methods for concentration of extracellular protein from *L. casei* and *L. paracasei*: TCA with DOC detergent, lyophilisation and dialysis using PEG, followed by SDS–PAGE analysis.

Figure1 represents that the quality and number of protein bands enriched with DOC and TCA method is better than other methods. SDS–PAGE profiles of crude extracellular from *L. casei* and *L. paracasei* show that the weight range of protein bands was 20 to 120 kDa and little difference was found in the diversity of bands.

GFC of proteins on Sephadex G100

Fractionation of *L. casei* and *L. paracasei* was done using the GFC method. The chromatograms show 4 peaks from *L. casei* and 3 peaks from *L.paracasei*. Figures 2 and 3 illustrate the SDS–PAGE profile of extracellular protein from *L. casei* and *L. paracasei* isolated using the GFC.

The results of MTT assay from extracellular proteins of *L.casei* and *L. paracasei* showed antiproliferative effects on K562 cell line. This antiproliferative activity is dose and time dependent.

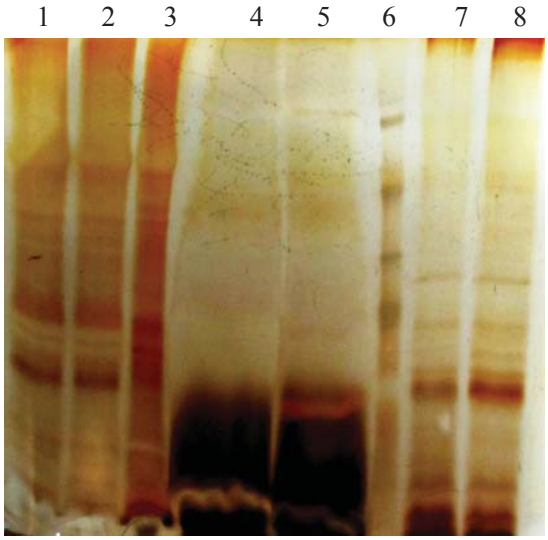

Figure 1. Comparative electrophoresis profiles of secreted proteins using three concentrated methods; **Lane 1,2:** proteins secreted by *L. casei* precipitated with DOC and TCA; **Lane 3:** proteins secreted by *L. paracasei* precipitated with DOC and TCA; **Lane 4:** *L. casei* secreted proteins were concentrated with PEG; **Lane 5:** *L. paracasei* secreted proteins were concentrated with PEG; **Lane 6:** Molecular weight standard (120,85,50,35,25,20kDa); **Lane 7:** proteins secreted by *L. casei* concentrated with lyophilisation, Lane 8: proteins secreted by *L. paracasei* concentrated with lyophilization.

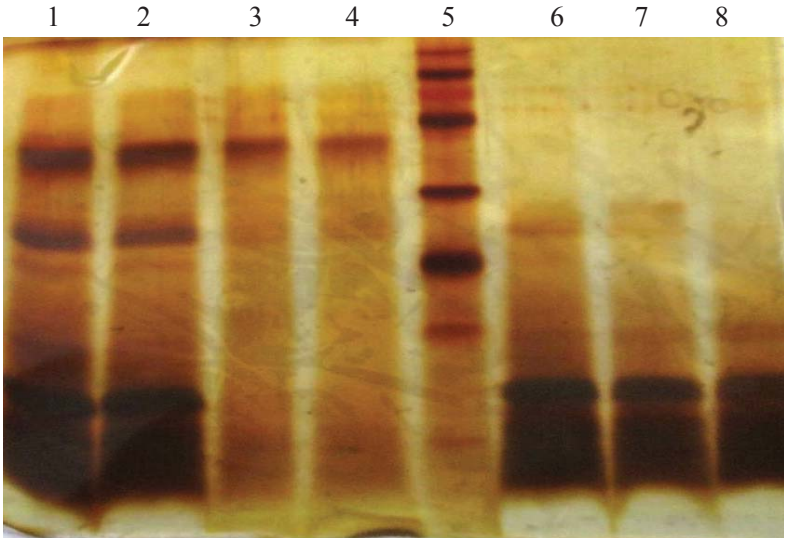

Figure 2. SDS–PAGE pattern of *L.casei* proteins fractionated by GFC; **Lanes 1, 2:** *L. casei* secreted crude proteins were precipitated with DOC and TCA; **Lane 3:** fraction 11; **Lane 4:** fraction 16; **Lane 5:** Molecular weight standard (120,85,50,35,25,20kDa); **Lane 6:** fraction 22; **Lane 7:** fraction 33; **Lane 8:** fraction 50.

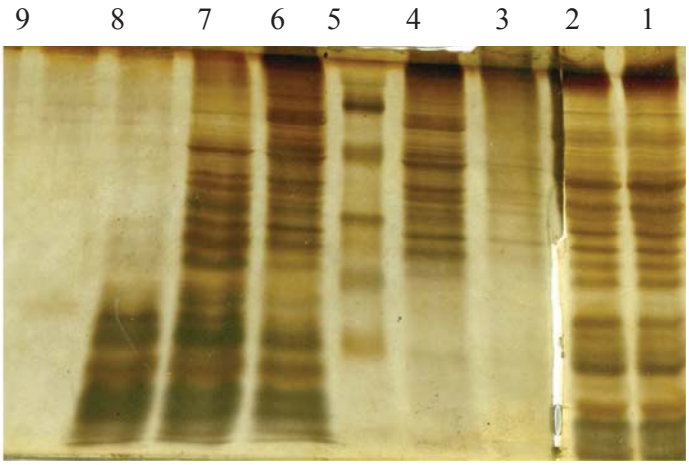

Figure 3. SDS–PAGE pattern of *L. paracasei* proteins fractionated by GFC; **Lanes 1, 2:** crude proteins secreted by *L.paracasei* precipitate with DOC and TCA; **Lane 3:** fraction 4; **Lane 4:** fraction 16; **Lane 5:** Molecular weight standard (120,85,50,35,25,20kDa); **Lane 6:** fraction 21; **Lane 7:** fraction 35; **Lane 8:** fraction 47; **Lane 9:** fraction 52.

So, as the concentration reduced, a reduction in the cytotoxic effects was observed. Differences between treatments of variable concentrations containing the same determined letter(s) at the top of each column are not significant. Data are illustrated as mean ± S.D (Figures 4 and 5).

In *L.casei*, the highest cell cytotoxicity percent is related to the fraction 11 and cytotoxicity increased with the elevation of time and concentration. Butin *L. Paracasei* the fraction 21 had the highest cell cytotoxicity percent and cytotoxicity increased with the elevation in time and concentration (Table 1).

Flow cytometric analysis of apoptosis

Annexin V–FITC/PI flow cytometry analysis was performed to confirm the apoptosis effect of extracellular proteins from *L.casei*and *L. paracasei* on K562 cell line.

Cells were treated with 100 µg/mL *L.casei* and *L. paracasei* fractions of 36 and 72 h. The early stage apoptosis for *L. casei* (F 11) and *L. paracasei* (F 21) were found 18.38% and 20.04% (lower, right), and late stage apoptosis found 2.51% and 10.06%, respectively (upper right) after 36 hours treating time (Figure 6).

The early stage apoptosis was increased to 21.87% and 48.36% and the late stage apoptosis was increased to 24.05%, but slightly decreased to 9.33% after 72 hours treating time for *L.casei* (F 11) and *L. paracasei* (F 21), respectively (Figure 6).

Discussion

Some strains of probiotic *lactobacilli* are shown to have an

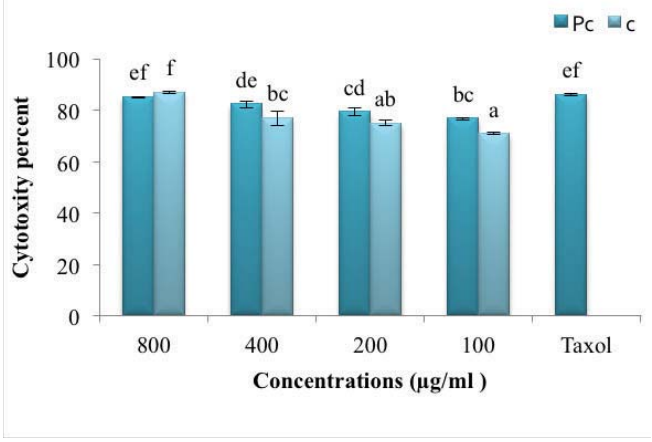

Figure 4. Effect of different concentrations of *L.casei* and *L. paracasei* secreted proteins on K562 cell line in 36 h. Data are the mean ±SD. $P < 0.05$ as determined by two–way analysis of variance followed by the Tukey's test. Differences between treatments of different concentrations containing the same determined letter(s) are not significant, c: *L. casei*, pc: *L. paracasei*.

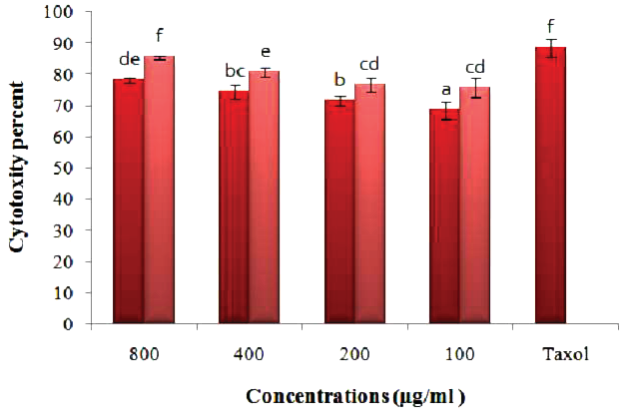

Figure 5. Effect of different concentrations of *L.casei* and *L. paracasei* secreted proteins on K562 cell line in 72 h. Data are the mean ±SD. $P < 0.05$ as determined by two–way analysis of variance followed by theTukey's test. Differences between treatments of different concentrations containing the same determined letter(s) are not significant, c: *L. casei*, pc: *L.paracasei*.

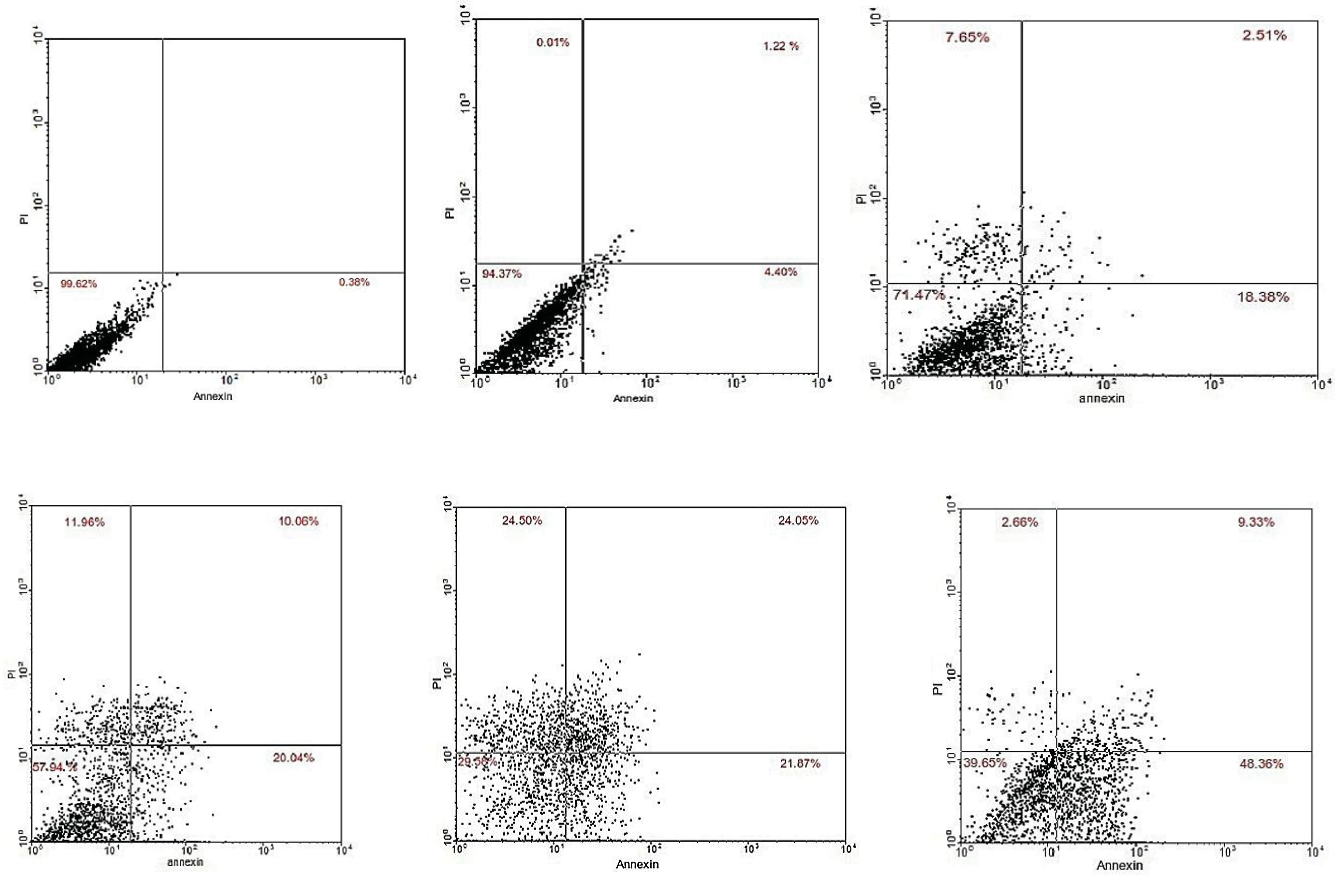

Figure 6. Flow cytometric analysis of K562 cells: after incubation without treating for 36 h **(a)** and 72 h **(b)** with treating by 100 µg/mL of *L. casei* (F 11) for 36 h **(c)** and 72 h **(e)** with treating by 100 µg/mL of *L. paracasei* (F 21) for 36 h **(d)** and 72h **(f)** Dots with Annexin V/PI (lower left), Annexin V+/PI– (lower right), and Annexin V+/PI+(upper right) Annexin V–/PI+(upper left) features represent viable intact, early apoptotic, late apoptotic, and necrotic cells, respectively.

Table 1. Comparison of cytotoxic activity of different concentrations of extracellular fractions from *L.casei* and *L. paracasei* on K562 cell line at 36 and 72 h. The data are expressed as mean ± standard deviation (SD) for at least three independent determinations in triplicate for each experimental point.

Bacteria	Fractions	Concentrations (µg/ml)							
		36 h				72 h			
		100	250	500	1000	100	250	500	1000
L. casei*	F11 (IC50 = 97.68)	40.15 ± 1.13^{a}	75.39 ± 1.05^{c}	78.59 ± 1.0^{d}	79.39 ± .893^{d}	66.70 ± 0.90^{b}	86.73 ± 0.97^{e}	89.40 ± 0.84^{ef}	91.52 ± .1.13^{f}
	F22 (IC50 = 142.6)	26.37 ± 1.02^{a}	71.50 ± 1.19^{e}	72.62 ± 0.92^{cde}	74.62 ± 1.07^{de}	38.48 ± 0.94^{b}	72.38 ± 1.13^{cd}	75.57 ± 0.90^{e}	79.73 ± 1.14^{f}
	F50 (IC50 = 843.7)	1.88 ± 0.98^{a}	14.73 ± 1.08^{b}	41.82 ± 1.08^{d}	53.57 ± 1.07^{e}	4.07 ± 0.99^{a}	27.06 ± 1.02^{c}	42.04 ± 1.0^{d}	57.11 ± 0.98^{f}
L.paracasei**	F4 (IC50 = 175)	24.70 ± 1.01^{a}	59.64 ± .93^{b}	67.40 ± 1.06^{d}	71.36 ± 1.06^{e}	63.77 ± 0.91^{c}	82.46 ± 0.91^{f}	84.63 ± 1.18^{f}	88.51 ± 1.11^{g}
	F21 (IC50 = 101.7)	41.77 ± 1.13^{a}	68.79 ± 1.05^{c}	76.33 ± 1.01^{d}	87.24 ± 0.86^{e}	64.54 ± 1.29^{b}	84.40 ± 0.88^{e}	86.77 ± 1.22^{e}	91.87 ± 1.05^{f}
	F47 (IC50 = 2360)	4.41 ± 1.21^{a}	6.50 ± 1.07^{ab}	15.47 ± 1.11^{d}	25.48 ± 0.87^{e}	7.49 ± 1.23^{b}	12.41 ± 0.98^{c}	44.59 ± 0.79^{f}	53.60 ± 1.24^{g}

*: Means ± SD followed by the difference letter(S) in each row show significantly differ ($P < 0.05$) in *L.casei* fractionated proteins on K562 cell line at 36 and 72 h; **: Means ± SD followed by the difference letter(S) in each row show significantly differ ($P < 0.05$) in *L. paracasei* fractionated proteins on K562 cell line at 36 and 72 h.

effect on reducing cancer occurrence and infectious diseases in experimental animal models and in humans.[19] Fichera and Giese have shown that tumor cell lines loss the viability during the incubation with *L. casei* or its peptidoglycan.[20] *L.casei* and *L. paracasei* are probiotic organisms in humans which trigger immune function stimulation and exhibitanti–tumor effects. Some other LAB whole bacteria, bacterial cell wall peptidoglycans or other cellular components and their metabolites could inhibit the proliferation of tumor cells.[21] Also, the cytoplasm, cell wall extracts and peptidoglycan of heat–killed whole cell LAB have significant antiproliferative effect on several cancer cells.[22] In vitro studies indicated a profound inhibitory activity of *Bifidobacterium* on HT–29, HepG2, and Vero cell lines.[23] Recently, Nami, et al. demonstrated that the secreted metabolites of *L. acidophilus* decreased the proliferation and viability of HeLa, HT–29, AGS, and MCF–7 cell lines, but did not show a significant inhibitory or cytotoxic effects on HUVEC cells.[24] *L.gasseri* and *L. Acidophilus* showed a cytotoxic effect on the three types of tumor cell lines, including liver carcinoma (HepG2), *Rhabdomyosarcoma* (RD) and Mice mammary gland carcinoma cell lines (AMN).[25] LAB strains possess antioxidant properties and inactivate ROS via enzymatic mechanisms such as coupled NADH oxidase/peroxidase system and catalase.[26] Han, et al. demonstrated that the whole cellular extract of *L. casei* have anti–tumor activity on Huh7 human hepatoma cells by the trypan blue dye exclusion method using Annexin–V–FITC and propidium iodide.[27]

Also, Verdenell, et al. studies revealed that daily consumption of food products enriched with the two potential probiotic strains (*L. Rhamnosus* and *L. paracasei*) improve beneficial intestinal microbiota.[28]

In this study, extracellular protein from *L. casei* and *L. paracasei* were concentrated in different ways: TCA/DOC, lyophilization and using PEG. The quality and number of protein bands showed that the TCA/DOC technique was the proper method for protein enrichment among other mentioned methods. It seems that our work is the first to compare the three previously mentioned methods of protein concentration. In this study, for the first time, GFC using G100 was used for *L. casei and L. paracasei* proteins fractionation. In addition, the MTT assay was performed to assess the cytotoxic activity of secreted protein fractions. The results of MTT assay showed that *L.casei* and *L. Paracase*i extra cellular proteins have antiproliferative activity on K562 cell line compared to the negative control. Since this activity is dose and time dependent, by reducing the concentration of proteins, the cytotoxic property was decreased. Moreover, by increasing the time from 36 to 72 hours the cell cytotoxicity was increased. This antiproliferative effects property for *L. Casei* was for fraction 11 (with high molecular weight). But for *L. paracasei*, the most cytotoxic effect was related to the fraction 21 (Medium molecular weight). Also, flow cytometric analysis confirmed the potential role of *L.casei* and *L. paracasei* fractions on inhibition of growth and induction of apoptosis onto K562 cell line, so by increasing the time from 36 to 72 hours the apoptosis was increased.

The main limitation of our study was the lack of information to clarify the exact mechanism of extracted protein against the cancer cell proliferation (in this study, the effects on k562 cell line was evaluated) by the focus on characterizing the mean proteins through isoelectric focusing approach or other proteomics methods. Also, lack of the evaluation of molecular pathways which are associated with cell apoptosis and might be responsible for anti–cancer effect of extracted protein is one of the main limitation of the present study. With more *in vivo* experiments in animals, as well as protein identification in the mentioned fractions, more effective procedures for inhibition of cancer or other related diseases will be designed in the future.

In conclusion, regarding our results, the fractionated supernatants of *L. casei* and *L. paracasei* have potential anticancer effects. However, more investigations are needed to determine the mechanisms of their cytotoxic and apoptotic effects on human cancer cells.

Conflict of Interest: *None.*

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