Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 5, 2023, 476
https://doi.org/10.33263/BRIAC135.476

Cytotoxic Potentials of Cell-Free Supernatant Derived from *Lactobacillus casei* CRL431 on HCT-116 and HT-29 Human Colon Cancer Cell Lines

Amin Abbasi [1], Aziz Homayouni Rad [1,*], Leili Aghebati Maleki [2,*], Hossein Samadi Kafil [3], Amir Baghbanzadeh [2]

[1] Department of Food Science and Technology, Faculty of Nutrition & Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran; aminabasi.tbz.med.ac@gmail.com (A.A.); homayounia@tbzmed.ac.ir (A.H.R.);
[2] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; aghebatil@tbzmed.ac.ir (L.A.M.); amirbaghbanzadeh@gmail.com (A.B.);
[3] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; Kafilhs@tbzmed.ac.ir (H.S.K.);
* Correspondence: homayounia@tbzmed.ac.ir (A.H.R.); and aghebatil@tbzmed.ac.ir (L.A.M.);
Scopus Author ID 57197853612

Received: 4.07.2022; Accepted: 15.08.2022; Published: 28.12.2022

Abstract: The increasing incidence of colorectal cancer (CRC) is one of the major concerns of today's society. The scientific community has always sought to reduce the side effects of CRC treatment methods. Today, in line with this issue, gut microbial-derived biomolecules (postbiotics), as novel bioactive constituents, have received special attention. This study aimed to investigate postbiotics' potential antigenotoxic and cytotoxic effects derived from *L. casei* CRL431. The antigenotoxic and cytotoxic effects of postbiotics derived from *L. casei* CRL431 were assessed by applying SOS Chromo Test and MTT assay on HCT-116 and HT-29 cell lines. Also, further supportive cellular analyses such as cell cycle assay, DAPI staining, and scratch wound-healing assay were performed to investigate the effect of postbiotics on some cellular pathways. Based on the outcomes, the postbiotics of *L. casei* CRL431 showed significant antigenotoxic and cytotoxic effects, 10–50%, and 70–80%, respectively. Besides, the treatment of cancerous cells with the postbiotics of *L. casei* CRL431 affected the cell cycle and suppressed the initial (G0/G1) phase of the cell's division, influenced the nucleus of the treated cells, triggered significant fragmentation reactions, and significantly inhibited the cell migration as outcomes for the cell cycle assay, DAPI staining, and scratch wound-healing assay, respectively. Postbiotic metabolites derived from *L. casei* CRL431 exhibited significant antigenotoxic and cytotoxic potential as well as reducing-metastasis properties against colon cancer.

Keywords: colorectal cancer; postbiotic; anticancer therapy; *L. casei* cell-free supernatant; HT-29; HCT-116.

Abbreviations: CRC: Colorectal Cancer; SCFAs: Short-Chain Fatty Acids; RPMI: Roswell Park Memorial Institute Medium; DAPI: 4′,6-diamidino-2-phenylindole; FBS: Fetal Bovine Serum; MRS: De Man, Rogosa and Sharpe; CFS: Cell-Free Supernatant; DMH: 1,2-dimethylhydrazine; IF: Induction Factor; 5FU: 5-Fluorouracil; PBS: Phosphate Buffered Saline; SD: Standard Deviation; LAB: Lactic Acid Bacteria; GIT: Gastrointestinal Tract; 2D: Two-Dimensional; 3D: Three-Dimensional; PIF: Plantain Inflorescence.

1. Introduction

According to the results of epidemiological studies, colorectal cancer (CRC) is the second leading cause of cancer mortality along with other types of cancer, and the World Health Organization predicts that by 2030 it will cause approximately 17 million deaths [1-3]. The high frequency of colon cancer has incited researchers to look for novel and natural components as adjuncts to prevent its expansion. Hippocrates was right a long time ago (400 BC) that "Death sits in the bowel" because currently, pieces of evidence confirm the significant physiological role of the intestinal microbiome in the genesis and/or progression of autoimmune disorders, metabolic disturbances, alcoholic liver disease, irritable bowel syndrome, and colorectal cancer [4-6]. Therefore, the expansion of various functional food formulations applying natural biological interventions such as probiotics, prebiotics, and synbiotics has received attention as preventive measures for numerous gastrointestinal disorders [7-10]. Although several studies have shown the positive effects of probiotics in the treatment of CRC, some potential adverse effects such as sepsis, bloating, the spread of antibiotic resistance properties, overstimulation of the immune system, and systemic infections have been reported that limit their use in some at-risk individuals (specifically includes the population of children, the elderly and patients with underlying diseases). Therefore, as safe biomolecules mainly derived from probiotics, postbiotics are getting attention as an alternative biological approach in medicine, particularly CRC therapies [11-13].

The term "postbiotic" refers to the ruptured microbial cell, cell elements, and/or cell metabolites that are primarily produced by live probiotic cells during the fermentation process or as a result of environmental factors such as the predominance of the dysbiotic gut microbiome. In this regard, postbiotic compounds are created using laboratory techniques in their pure form, and when taken in appropriate amounts, they offer the host many biological health advantages. Postbiotics can modulate the function of the host's commensal microbiota with certain chemical structures by altering specific biological processes, regulatory reactions, metabolism, and/or behavioral feedback [14-16]. Several postbiotic metabolites, including linoleic acid, short-chain fatty acids (SCFA), lactic acid, exopolysaccharides, peptides, glycoproteins, proteins, and peptidoglycans have been shown to have strong anticancer potential [17,18]. In addition, investigators also showed interest due to the unique function of postbiotics, such as immunomodulatory action, intestinal barrier maintenance, genotoxins deactivation, and cytotoxicity, even in the progressive phases of cancer [19-22]. Raman *et al*. established that the potential binding capability of some probiotics (*Lactobacillus*, *Bifidobacterium*) to mutagenic constituents can be traced back to postbiotic metabolites such as polysaccharides, secreted glycoproteins, and peptidoglycans [8]. In particular, irreversible mutagen coupling and antimutagenicity are attributed to SCFA [23]. It has been found that butyrate-based short-chain fatty acids have significant properties of inducing cancer cell differentiation, apoptosis, inhibiting angiogenesis, and regulating normal cell proliferation [24,25]. Interestingly, investigators also demonstrated that cell-free supernatant-based postbiotics from *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus* GG, and *Escherichia coli* Nissle, are involved in inducing apoptosis and therefore play an important role in the growth-preventing of colon cancer cells at an early stage [26,27]. It is assumed that colon carcinogenesis as a multi-stage process will occur due to genetic mutations, leading to uncontrolled cell division and suppression of apoptosis [28-30]. Hence, the ideal anticancer compound can exert its functional properties through various mechanisms, such as disrupting

the genotoxic characteristics of common carcinogenic compounds in the intestinal environment or stimulating the cytotoxic response in cancer cells, which inhibits and controls the progression of cancer. In this regard, the present study investigated the potential function of postbiotic metabolites derived from *L. casei* CRL431 in generating antigenotoxic and cytotoxic responses in HCT-116 and HT-29 colon cancer cells.

2. Materials and Methods

2.1. Bacterial strain.

To evaluate the potential antigenotoxicity activity, the *E. coli* PQ37 strain was kindly provided by the Microbiology Laboratory of Drug Applied Research Center, Tabriz University of Medical Sciences, Iran. The investigated bacterial strain was maintained by inoculation in a nutrient agar medium containing ampicillin (10%) and incubated for 24 h at 37 °C, and glycerol stocks were kept at −70 °C.

2.2. Preparation of bacterial strains and cell-free supernatant.

Cinnagen Co. (Iran), provided the *L. casei* CRL431 strain for this study. The fresh microbial suspension was created by sub-culturing *L. casei* CRL431 in MRS broth (containing 8 g/L meet extract, 4 g/L yeast extract, 2 g/L triammonium citrate, 20 g/L dextrose, 1 g/L polysorbate 80, 10 g/L peptones, 5 g/L sodium acetate trihydrate, 0.1 g/L magnesium sulfate, 0.05 g/L manganese sulfate, and 2 g/L dipotassium phosphate). It was then standardized using a visible-ultraviolet spectrophotometer (Beckman, MO, USA; model DU-530) in the 600 nm range.

The growth of *L. casei* CRL431 in MRS broth is the first stage in the synthesis of cell-free supernatant (CFS) type of postbiotics, which should be carried out at a temperature of 37 ± 1 °C for 48 hours in a CO_2 incubator (BINDER INC., Germany). Due to the considerable impact that various microbial strain growth conditions and extraction techniques have on the postbiotic function, this approach was repeated in 2000 mL Erlenmeyer flasks to collect adequate and suitable volumes of CFSs. In order to obtain CFS, bacterial cells were first removed using centrifugation at 6000 × g for 10 minutes at 4 °C. Next, the culture supernatant was collected, lyophilized (Biobase, China) (pump pressure: 100 mTorr, freezing temperature: −40 °C, and shelf temperature: −60 °C), and utilized as CFS in future studies [27]. The prepared CFSs were filtered through a 0.22 um millipore filter before use in each *in vitro* assessment.

2.3. Cell culture.

HT-29 and HCT-116 colon cancer cell lines were purchased from Pasteur Institute, National Cell Bank of Iran, Tehran, Iran, and were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, penicillin 10 U/mL, and streptomycin 100 mg/mL by incubating in 5% CO_2 incubator at 37 °C. Simultaneously achieving cell culture confluency to 80-90%, the detachment of cells was performed via trypsin. While seeding in the relevant culture plates, the culture media were replaced regularly over 2-3 days.

2.4. Antigenotoxicity assay.

The SOS Chromo test, as a colorimetric bacterial assay for genotoxins, was used to investigate the potential antigenotoxic activity of CFS derived from *L. casei* CRL431, in this

assessment, the 1,2-dimethylhydrazine (DMH) was applied as the genotoxic factor [31]. Briefly, *E. coli* PQ37 was added to different concentrations of *L.casei* CFS (5, 10, 15, 20, 25, 30, 35, 40 µL) and incubated (37 °C for 2 h) with DMH (80 µL). Then alkaline phosphatase and ß-galactosidase activities were evaluated. Antigenotoxicity result was expressed in terms of % induction factor (IF=Ratio of ß-galactosidase to Alkaline phosphatase of test/Ratio of ß-galactosidase to Alkaline phosphatase of control) reduction, which is equal to IF of Control−IF of test×100/IF of control. All treatments were performed three times in three replications.

2.5. Cytotoxicity assay.

In this study, the cytotoxicity assay, according to [32], was utilized to evaluate the cytotoxic potential of CFS of *L. casei* CRL431. Briefly, the investigated cell lines (180 µL) were seeded in 96-well microtiter plates with a density of 2×10^4 cells per mL, and their incubation was performed under 37 °C for 24 h conditions. Subsequently, various concentrations (0.1, 1, 10, 100, 1000 µg/mL) of *L. casei* CFS and 7 µL/well 5-Fluorouracil (5FU) (50 mg/mL) as a positive control were added and incubated at 37 °C for 48 h. After the determined culture period, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated similarly to the growth condition for 4 h. Afterward, culture media was removed, and the solubilization of formed blue formazan crystals was performed by addition of 150 µL of dimethyl sulfoxide. Absorbance was measured at 570 nm using an ELISA reader (ELx 800; Biotek, Winooski, VT, USA). A comparison of intensity levels of treated cells with untreated control cells was applied to calculate the relative growth inhibitory effect of *L. casei* CRL431 CFS. It is noteworthy that all tests were performed three times in three replications.

2.6. Cell cycle analysis.

Cell cycle assay is one of the complementary and confirmatory tests in studies focusing on anticancer functions of gut microbial-derived biomolecules. In the present study, in addition to cytotoxicity, the capability of the investigated postbiotic metabolites to affect the cell cycle and induce its arrest responses in HCT-116 cells after 48 h of treatment by flow cytometry was investigated [18]. Briefly, a density of 3×10^6 cells per mL of HCT-116 cells was seeded in each well of a six-well cell culture plate and incubated in a 5% CO_2 incubator at 37 °C for 5 h. Subsequently, the seeded cells were treated with 100 µL postbiotic metabolites and incubated similarly to the growth condition for 48 h. After that, cells were collected from the wells, centrifuged at 850 g for 10 min, and washed twice. Finally, the cell's fixation was performed with cold ethanol (70%), and then 1 mL of PI master mix solution containing 950 µL of phosphate-buffered saline (PBS),10 µL of RNase, and 40 µL of the PI solution was added, and the incubation was performed at 37 °C for 30 min. Following this, the cell cycle analysis was performed by flow cytometry (Becton Dickinson, San Jose, CA).

2.7. DAPI staining.

DAPI (4′,6-diamidino-2-phenylindole) staining was performed to evaluate morphological alterations in the nucleus of cancer cells. Here, 3×10^5 cells/cm^2 were seeded in 6-well plates and, after overnight incubation, treated with the CFS of *L.casei* CRL431 and 5FU for 48 h before being fixed with paraformaldehyde 4% for 10 min. The immobilized cells were then washed with PBS (three times), and 0.1% Triton X-100 was used to permeate for 10 min. Eventually, the cells were washed with PBS (three times) afore being stained with DAPI

(250 ng/mL) at room temperature for 5 min. An Olympus BX64 inverted fluorescence microscope equipped with a U-MWU2 fluorescence filter was used to evaluate the stained cells.

2.8. Scratch assay.

Evaluation of cell migration potential *in vitro* conditions was performed by scratch assay in a two-dimensional space. The investigated cells were seeded at a density of 2×10^5 cells/cm^2 in six-well plates and stored for confluent monolayer formation. The HT-29 and HCT-116 cells were exposed to CFS of *L.casei* CRL431 (100 µg/mL) for 24 and 48 h. The cells have then scratched with a scraper throughout the layer, followed by washing steps with PBS to eliminate the floating cells and cellular remnant. Then, to prevent cell proliferation, the cell culture medium was replaced with 1% FBS, and subsequently, the relevant images of the same field were taken regularly at 24 and 48 h intervals [33].

2.9. Statistical analysis.

Numerical data were presented as the mean ± standard deviation (SD). Each experiment was repeated at least three times. The Excel spreadsheet software (Microsoft, Redmond, WA) was applied for data analysis. Statistical analyses were performed using the Graphpad Prism (version 5; Graphpad Software, San Diego, CA) and the one-way ANOVA test, followed by Tukey's multiple comparison test. A P value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Antigenotoxicity assay.

It was demonstrated that CFS derived from *L.casei* CRL431 at the concentrations of 5, 10, 15, 20, 25, 30, 35, and 40 µL exhibited antigenotoxicity and caused a significant reduction of 10-50% in the induction factor of genotoxicity.

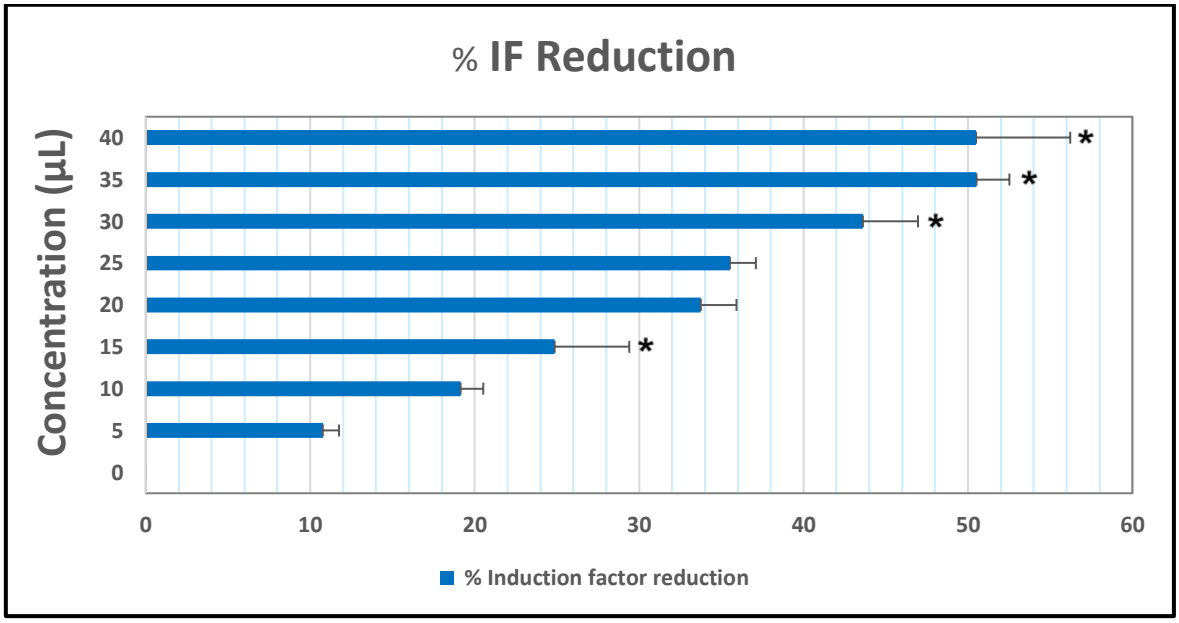

Figure 1. Antigenotoxic activity (%IF reduction) of different concentrations of *L.casei* CRL431 cell-free supernatants (CFS) against *E. coli* PQ37. Values are mean ± SD, **P* < 0.05 v/s DMH-treated.

More specifically, at the investigated concentrations of CFS, the minimum/maximum concentration that induces significant ($P \leq 0.05$) genotoxicity inhibition in PQ37 cells was found to be related to the 5 and 35 µL of CFS, respectively (Figure 1).

3.2. Cytotoxicity assay.

The growth inhibitory effect of CFS derived from *L.casei* CRL431 on the viability of colon cancer cell lines (HT-29, HCT-116) was assessed by MTT assay. The IC_{50} value was characterized to be about 100 µg/mL at 48 h (Figure 2). As shown in Figure 2, there is a direct relationship between cell viability and the dose of postbiotics, so after exposure to cancer cell lines with *L. casei* CFS, cell viability is dependent on the dose being reduced ($P \leq 0.05$).

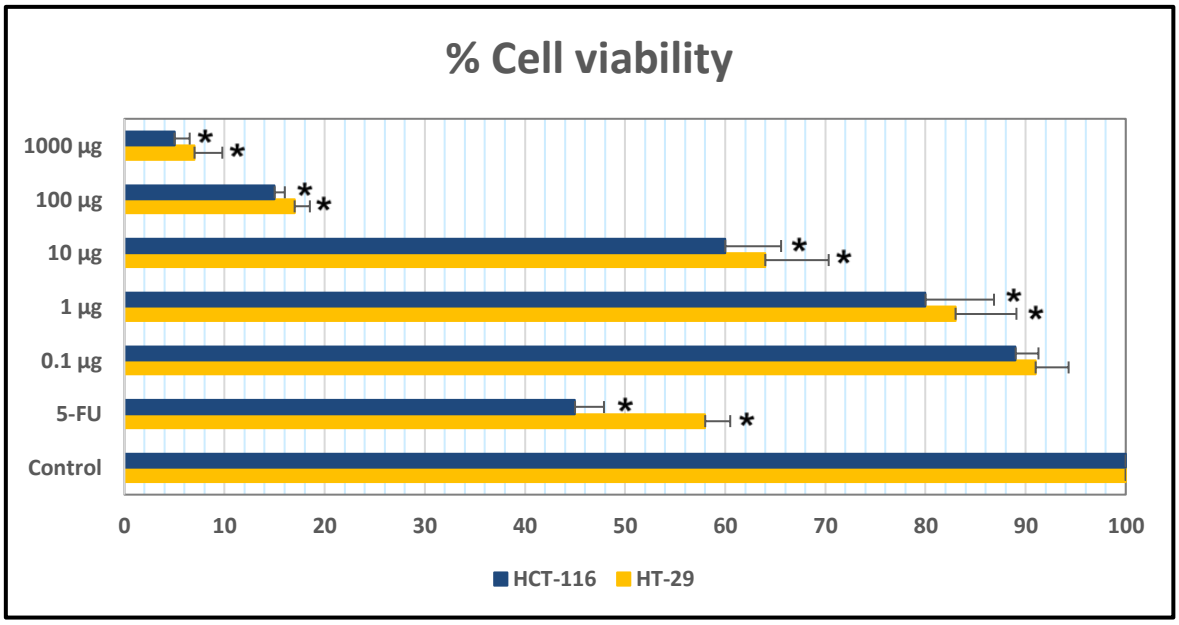

Figure 2. MTT assay of different concentrations of *L.casei* CRL431 cell-free supernatants (CFS) on human colon cancer cell lines after 48 h treatment, 5-FU was used as a positive control. Values are mean ± SD, **P* < 0.05 v/s untreated cells.

3.3. Cell cycle analysis.

The inhibitory capability of CFS derived from *L.casei* CRL431 was also detected in the cell cycle of HCT-116 cells. It was found that 48 h of postbiotic treatment arrested the cell cycle of the HCT-116 cell population in the sub-G1 (G0/G1) (80.56 ± 6.2%) and G2M (12.6 ± 3.7%) phase, respectively. These findings indicate that exposure of cancer cells to postbiotic metabolites arrested cell growth and division in the early stages (sub-G1) of their cell cycle (Figure 3).

3.4. DAPI staining.

For evaluating the morphological alterations as a result of stimulation of apoptosis responses on treated/untreated cell lines, DAPI staining was performed. As shown in Figure 4, a 48-h treatment of 100 µg/mL of *L. casei* CFS affected the nucleus of the investigated cells and caused noteworthy fragmentation reactions in the cells' nucleus compared to the untreated control cells. The cells treated with the CFS of *L. casei* exhibited fundamental fragmentation in the chromatin within the nucleus of investigated colon cancer cells, but their morphology did not alter in the untreated control cell line (Figure 4). The symptoms of natural apoptotic

responses in these cells include separated nuclei and the formation of apoptotic bodies in cancer cells.

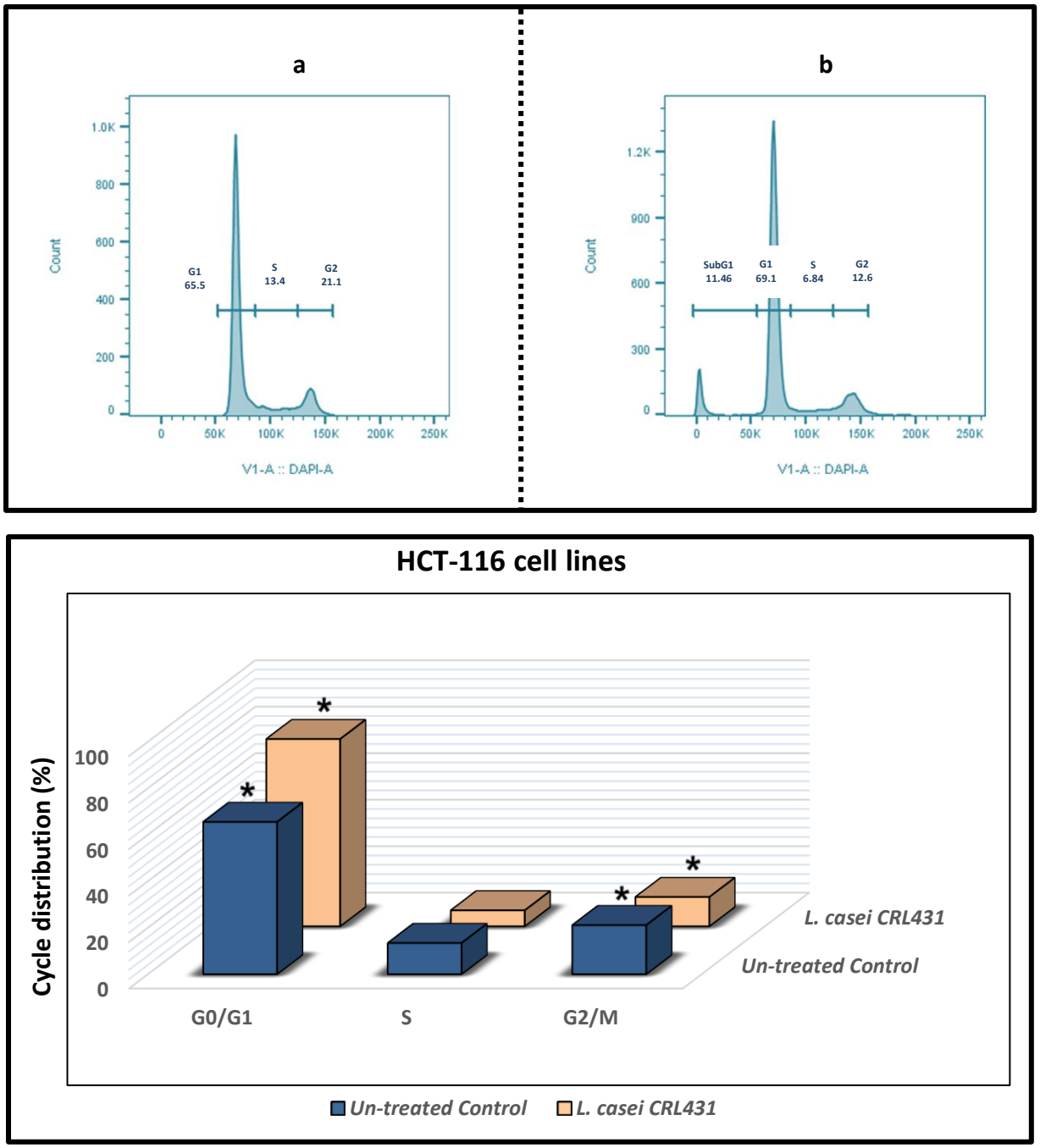

Figure 3. Effect of *L.casei* CRL431 postbiotics treatment on the cell cycle of HCT-116 cells by flow cytometry. a: untreated and b: postbiotic treated.

3.5. Scratch assay.

To investigate the effect of *L.casei* CRL431 CFS on the motility of HT-29 and HCT-116 colon cancer cell lines, the scratch assay was accomplished by determining the expanse of cell migration into the scratched zone. The cells were treated with the conditioned media for 24 and 48 h. Cell migration was meaningfully prevented in reply to the *L. casei* CRL431 CFS treatment, as shown in Figure 5.

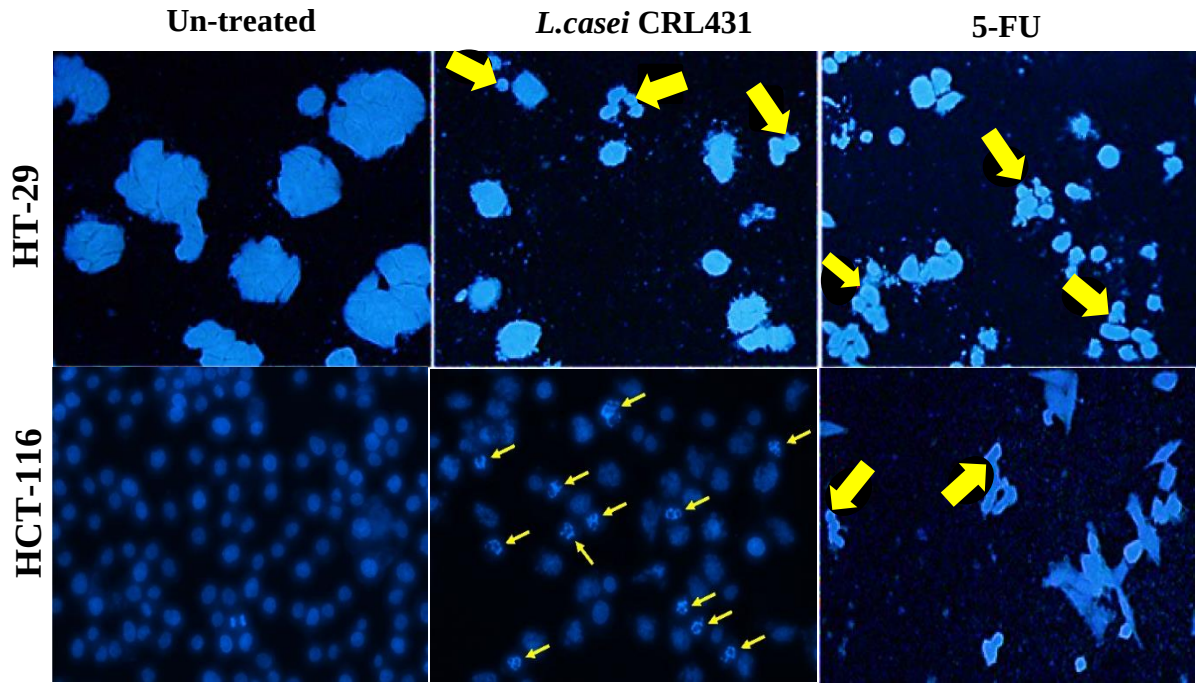

Figure 4. Fluorescent microscopy of DAPI staining of *L.casei* CRL431 cell-free supernatants (CFS) (100 µg/mL) and 5-FU (50 mg/mL) on treated and untreated control colon cancer cell lines (magnification×100). Arrows demonstrate apoptotic nuclei.

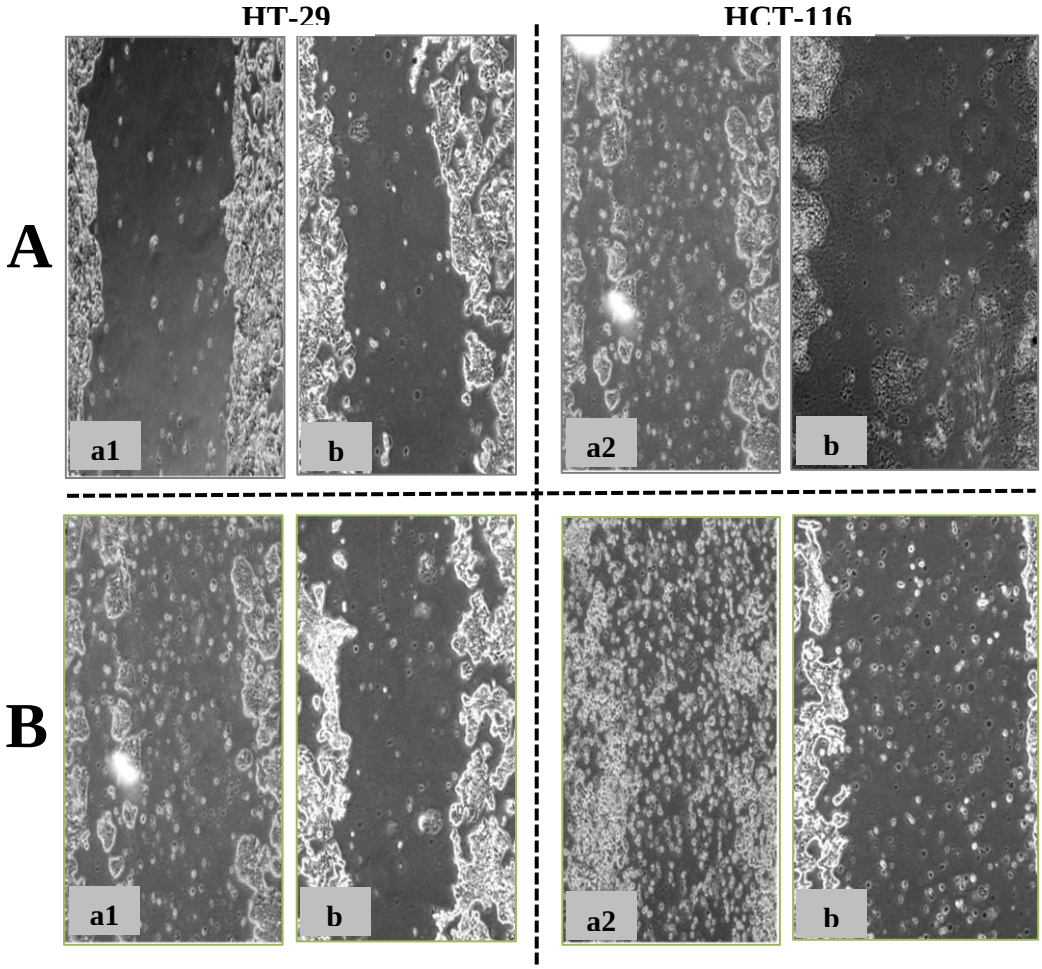

Figure 5. Cell migration was assessed using the scratch assay. The confluent cells were treated with the cell-free supernatants (CFS) (100 µg/mL) obtained from *L. casei* CRL431 for 24 **(A)** and 48 **(B)** h. a1 and a2 demonstrate untreated cells. Also, the treated cells are presented in b1 and b2.

Colon cancer is one of the most common virulent tumors of the gastrointestinal tract and the principal cause of cancer-associated death worldwide. Its prevalence is high in Western countries, so in Iran, it is ranked fourth among women and third among men [34,35]. Radiation therapy, chemotherapy, and surgery are some of the most important therapeutic approaches in the treatment of colon cancer. Still, each of these methods, due to their nature and adaptability in the host, can be associated with some unavoidable side effects, which usually significantly overshadows the patient's quality of life [36,37]. In many cases, cancer cells are resistant to conventional therapies [38,39], so targeting pathways involved in angiogenesis, invasion, intracellular signaling cascades, and programmed cell death (apoptosis) as key signaling pathways can be a new approach to generating specific cytotoxic responses against cancer cells [40]. Currently, cancer treatment based on intestinal microbiota is considered one of the new methods compatible with the host body [41]. In this regard, the presence of lactic acid bacteria (LAB) as integral, safe, and functional components in fermented/functional foods has usually been associated with improving the health status of consumers [42,43]. LABs, due to the presence of prebiotic compounds in the matrix of these foods and their inherent properties, synthesize an extensive range of biologically active molecules such as biopeptides and organic acids that have multiple crucial physiological functions [44,45]. The available scientific evidence confirms the dramatic growth and proliferation-inhibitory effect of LAB and their bio-metabolites on the stomach, bladder, colon, and breast cancer cells [1,46,47]. The main action anticancer mechanisms of LAB include modulation of immune system responses, modification of intestinal microbiome composition, and possessing significant anti-proliferative and antioxidant properties [48]. In addition, LABs and their derived biometabolites can exhibit their clinical health effects by contributing to the recovery (after cancer surgery) and reducing the length of hospital stay [49], eliminating local infection of the surgical incision [50], hindering/diminishing some undesirable effects of conventional cancer treatments (antibiotic- and chemotherapy-stimulated diarrhea) [51], and establish and maintain microbial eubiosis in the gastrointestinal tract [52]. On the other hand, there are some reports of some side effects of LABs (e.g., the transmission of antibiotic resistance genes and virulence factors [1], stimulation of acute inflammatory responses [53], and the production of biogenic amines [54, 55]), which has led scientists to find the main mechanisms of their health-promoting effects and now attribute most of their effects to their metabolites (postbiotics) that is considered a response to environmental conditions [56]. The use of postbiotics as an adjunct to the prevention and treatment of cancer has been strongly linked to the regulation of response/function of both adaptive and innate immune systems, which usually act as a bridge between the two sections and provide effective feedback. The results of laboratory-scale studies and animal models confirm the significant effect of postbiotics in reducing proliferation, invasion of cancer cells, and inflammatory pathways. This is especially true in the case of gastrointestinal cancer [57]. Therefore, the present study investigated some of the cellular mechanisms involved in causing the anticancer effects of postbiotics produced by *L.casei* CRL431. In the present study, we tried to move from fermented foods to the development of functional foods by selecting key microbial strains involved in the fermentation process as probiotics (*L.casei* CRL431) and also by producing safe, functional, and effective postbiotics in creating biological effects.

It was demonstrated that CFS derived from *L.casei* CRL43 exhibited antigenotoxicity autonomous of live strain, signifying that postbiotic metabolites generated by this strain do contain antigenotoxic constituents that may either straightly bind to genotoxins or deactivate

genotoxic ingredients due to modification of milieu such as either scavenging reactive oxygen metabolites or lowering pH. Researchers have also found that CFS of LAB, e.g., *L. plantarum*, *L. acidophilus*, *Bifidobacteria*, and *Streptococcus cremoris* exhibited significant antigenotoxic effects that were mostly due to short-chain fatty acids, peptidoglycans, exopolysaccharides, and glycoproteins [8,23,58].

Additional cellular/molecular mechanisms being researched for the anticancer effects of postbiotics include inducing anti-proliferative action against cancer cells, reducing metalloproteinase-9 levels, pro-apoptotic cell death pathways, increasing tumor cell death through autophagy, increasing apoptosis and necrosis, inhibiting cancer invasion, encouraging immune feedbacks, coupling to mutagenic and carcinogenic elements, and reducing microbial translocation [59,60]. We demonstrated that postbiotic metabolites derived from *L.casei* CRL43 revealed a significant cytotoxic effect versus investigated cancer cells, further signifying the attendance of antitumor constituents, which is in concordance with previous investigations where CFS of *Bifidobacterium adolescentis* SPM0212 also suppressed the growth of Caco-2, SW-480, and HT-29 colon cancer cell lines to a greater extent compared with live/heat-inactivated probiotic cells [61,62]. In addition to the mentioned cytotoxic effects, the results showed that these effects were specific and time- and dose-dependent, so the best responses in a period of 48 h and at a concentration of 100 μg/mL for *L.casei* CRL43 CFS were obtained. Numerous studies have reported the cytotoxic and anti-proliferative effects of biological macromolecules isolated from probiotics from various sources in different cell lines of human cancers. Tan *et al*. investigated the inherent cytotoxic potential of proteinaceous constituents generated via *L. plantarum* I-UL4 cultivated in various specific media (Modified de Man, Rogosa, and Sharpe broth containing Tween 80 and yeast extract) on MCF-7 breast cancer cell line. In this study, the percentage of apoptotic MCF-7 cells treated with *L. plantarum* I-UL4 postbiotics cultivated in the modified culture medium increased significantly ($P < 0.05$) from 24 to 48 h incubation. The results obtained in this study have shown the potential of producing postbiotics as a supplement to human health and as a preventive agent against cancer [63]. The previous study of Karaçam *et al*. demonstrated that the biofilm formation, growth, and antibiotic resistance characteristics of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were significantly hindered by the CFSs derived from *Streptococcus salivarius* M18 as the oral probiotic. Also, they stated that these antimicrobial properties of postbiotics were more impressive under acidic circumstances. The researchers then performed a study to assess the growth-inhibition response of colon cancer cells when exposed to the *S. salivarius* M18 postbiotics, considering the biological approach and naturally acidic characteristics of the tumor microenvironment. In this study, in both two-dimensional (2D) and three-dimensional (3D) cell culture models, postbiotics derived from *S. salivarius* M18 exhibited significant anticancer activity in the pH circumstances imitating the tumor's acidity in the body. The growth inhibition effect was more prominent when colon cancer cells were treated with cell-free supernatant obtained from an inulin-enriched medium. The results of this study indicate the potential of functional products (food/pharmaceutical) containing postbiotic compounds to target low-pH environments, including the acidic microenvironment of tumors [64]. Sharma *et al*. designed a study to isolate effective LAB and monitor the anticancer potential of their postbiotic metabolites. In this study, probiotics were isolated from different sources (fresh fruits (apples, oranges, orange peel, tomato)), vegetables (cucumbers, radishes, carrots, radish leaves, turmeric, cabbage), and the feces of healthy infants) and their CFSs were assessed for cytotoxic and antigenotoxic potentials by the MTT assay and SOS chromo test on

HT-29 and Caco-2 cells. Among 60 samples of isolated LAB, only 10 isolates had more than 30% antigenotoxic properties, and four isolates had 70-80% cytotoxicity. Besides, organic extracts of these four CFS dissolved in carboxymethyl cellulose exhibited 80-90% cytotoxicity. In this study, the most effective isolate had probiotic properties, and its phylogenetic properties were attributed to *L. rhamnosus* MD 14. Also, the evaluation of the physicochemical properties of the derived postbiotic extract showed the presence of organic acids and heat-sensitive proteins [32]. In a study by KB *et al*., the inherent potential of soluble dietary fiber plantain inflorescence (PIF) was investigated to introduce it as a prebiotic agent. PIF showed its prebiotic potential by increasing the selective growth of the studied probiotics and subsequently preventing the progression of colorectal cancer. *Bifidobacterium bifidum* and *L. casei* use PIF fiber as the main substrate during fermentation. PIF fiber, as a potential prebiotic, and probiotic during the fermentation process, leads to the production of noteworthy amounts of SCFAs in the derived CFS that can initiate apoptotic signaling in HT-29 cancer cells and lead to cell death. The postbiotics also caused DNA damage and increased production of reactive oxygen species in HT-29 cells, leading to an apoptotic response [65].

It is noteworthy that the type of parent microbial strain, cell phenotype, the presence of stimulants (prebiotics) in the environment, the presence of bioactive micro-and macro-molecules, and methods used in the preparation of postbiotics are among the factors affecting the function and biological effects of postbiotics [66]. In this regard, based on the result of related studies to the investigated probiotic strain (*L.casei* CRL43), the SFC of *L. casei* can contain a wide variety of biological compounds (organic acids, peptides, short-chain fatty acids, carbohydrates, enzymes, etc.). Some of the biological compounds in the chemical profile of postbiotics derived from *L. casei* CRL431 include cis-1,3-Dimethylcyclopentane, Heptane, 4H-Pyran-4-one, 2,3-dihydro-3,5-di, L-Pipecolinic acid, 1-Butanamin, Propanoic acid, Pyrrolo [1,2-a] pyrazine-1,4-dione, Benzoic acid, phenol, 3,5-dimethoxy, trans-2-Cyclohexene-1,4-diol, 5-Fluoroveratraldehyde, N, 3-Diethyl-3-nonanamine, Cyclopentane, 2-Pyrrolidin-2-yl-4-amino-6-dimethylamino-s-triazine, Purin-8-ol, 6-amino-8,9-dihydro, Cyclohexane carboxylic acid, vaccenic acid and octadecynoic acid [32,67]. Hence, this study found that postbiotic metabolites of *L.casei* CRL43 diminished the division of investigated cancer cells by influencing the cell cycle and arresting it in the initial cell division (G0/G1) phase. This result may be due to the attendance of short-chain fatty acids in the postbiotic metabolites described to stimulate cell cycle arrest and variation in colon cancer cell lines mostly by the hindrance of histone deacetylase that results in hyperacetylation of histone residues and imperfect transcription with silencing of cell cycle-regulated genes [68]. Correspondingly, Chen *et al*. have also shown that short-chain fatty acids could increase the expression of p21 via selectively controlling histone acetylation, thus stimulating cell cycle arrest in cancer cell lines (SW1116 and Colo-320) at the G1 stage. Besides, in this study, the biological compounds in the CFSs of *L. rhamnosus* GG and *L. plantarum* A7 effectively hindered the expansion of cancer cells related to the organic acids [69]. According to the outcomes of studies, peptide/protein-based postbiotics can also significantly inhibit the growth and progression of cancer cells by various molecular pathways [70]. In this regard, Yan *et al*. have also exhibited that two protein-based postbiotics (p75 and p40) with the origin of *L. rhamnosus* GG have a significant function in establishing gut homeostasis [71]. The significant reduction in cytotoxicity after physicochemical treatment, such as thermal treatment, proposed the heat-unstable nature of active biomolecules sensitive to hydrolysis, denaturation, and evaporation and is in concordance with the study of Nepelska *et al*. [72]. Based on this study, it can be stated that

CFS-based postbiotics derived from *L.casei* CRL431 suppressed the genotoxicity of chemical carcinogen, stimulated cytotoxicity, arrested cell cycle in G0/G1 phase, and reduced migration rates in the investigated (HT-29, HCT-116) colon cancer cell lines. Nevertheless, the precise anticancer molecular mechanism of these postbiotics is underway.

4. Conclusions

According to available scientific literature, the incidence and prevalence of chronic diseases such as colorectal cancer is high in most developed and developing countries and leads to high mortality rates. On the other hand, conventional therapies are sometimes associated with some side effects that reduce the quality of life of patients. Therefore, researchers are trying to find adjunctive therapies with low side effects and costs [73]. According to the results, the postbiotic metabolites produced in this study have the potential to cause significant antigenotoxic and cytotoxic effects, arrest the cell cycle in cancer cells and also prevent metastasis. Due to their safe origin, they can be applied in the development of functional food formulations and as novel biotherapeutic approach for both prevent and adjuvant treatment policies in CRC patients with high compatibility and without any drastic unwanted secondary effects. However, additional metabolomic investigations are required to identify novel postbiotics and monitor their safety profile, anticancer activity, and stability during the food/pharmaceutical manufacturing processes, marketing, and gut circumstances. Furthermore, randomized, double-blind clinical trials are required to determine the precise dose and optimum administration frequency of postbiotic supplements for colon cancer patients.

Funding

This research was funded by the Research Vice-Chancellor of Tabriz University of Medical Sciences, grant number 64644.

Acknowledgments

This study was supported by the Vice-Chancellor for Research of Tabriz University of Medical Sciences, Tabriz, Iran, with reference number 64644. The authors would like to express their thanks to the Research Vice-Chancellor of Tabriz University of Medical Sciences for the financial support of this study.

Conflicts of Interest

The authors declare no conflict of interest.

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