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Extract of the bioconverted fine root of ginseng induces apoptosis and cell cycle arrest in mouse colon cancer cells

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Abstract

Cancer is the major cause of death worldwide, and the anticancer effect of ginseng and its main root has been studied. However, study of fine root of ginseng (FRG) is still insufficient. The purpose of this study was to discover a new anticancer effect from FRG, which does not show an anticancer effect, through a bioconversion technique. We measured and compared cell viability in FRG- and bioconverted fine root of ginseng (BFRG)-stimulated CT26 cells to investigate differences caused by bioconversion. Cell viability of CT26 was suppressed upon treatment with BFRG, unlike FRG. The effect of BFRG on apoptosis and cell cycle arrest was investigated by flow cytometry. BFRG-stimulated CT26 cells showed an increased apoptotic cells and cell cycle arrest. Additionally, BFRG induced mitochondrial impairment by reducing the expression of anti-apoptosis protein Bcl-2. When confirming the signaling pathway, it was found that the p38 MAPK pathway was activated by BFRG. Collectively, our results reveal anticancer effects against colorectal cancer and represent potential targets for anticancer drug development.

Keywords Colon cancer, Fine root of ginseng, Apoptosis, Cell cycle arrest

Introduction

Cancer is the major cause of death worldwide, and colorectal cancer is the second most commonly diagnosed cancer among men in Korea and the third most commonly diagnosed cancer among women. Changed dietary patterns, lifestyle factors are increasing the incidence of cancer [1] and the incidence of colorectal cancer in Korea is increasing by ~ 6% every year. Moreover, the death rate ranks third globally [2].

Currently used drugs for treating colorectal cancer include 5-fluorouracil, capecitabine, oxaliplatin, irinotecan, and cetuximab. Metabolic inhibitors such as 5-fluorouracil and capecitabine cause dermatitis and palmar-plantar erythrodysesthesia in patients [3, 4], whereas oxaliplatin, a platinum complex, causes side effects such as neurotoxicity, gastrointestinal tract toxicity, anemia, and thrombocytopenia [5]. Irinotecan causes complications, such nausea, hair loss, and severe gastrointestinal toxicity [6]. Prolonging the patient's survival and increasing the survival rate are the main goals of chemotherapy, but the patient's quality of life is also an important consideration. Therefore, treatment using natural products with less cytotoxicity and side effects than chemoprevention agents is in the spotlight.

Fine root of ginseng (FRG) is the thin root of ginseng. Ginseng has been used for the treatment and prevention of diseases for ≥2,000 years and is associated with beneficial effects on blood pressure [7], oxidative stress reduction [8], anti-inflammatory activity [9], liver injury [10], and glucose action [11]. Additionally, various bioactive compounds occur in the leaves, stems, and roots of ginseng, which have been studied for metabolism,

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cardiovascular disease, and carcinogenesis [12]. Ginseng inhibits the growth of various cancers such as lung cancer [13], breast cancer [14], gastric cancer [15], and prostate cancer [16], but no research has been conducted on the pharmacological properties of FRG.

The major targets for anticancer therapy are apoptosis and proliferation inhibition. Apoptosis, "programmed cell death," triggers cell death by activating Bax by internal and external stimuli, followed by cleavage of hundreds of proteins using cleavage of caspases and PARP [17]. The most distinctive feature of cancer cells is their continuous and abnormal proliferation. The checkpoints that occur between cell cycle stages serve to stop incomplete cell cycles, but cancer cells continue to proliferate by avoiding them. Thus, stopping the cell cycle of cancer cells is one way to prevent their proliferation.

Bioconversion means a technology that converts existing materials using the functions of biocatalysts. Bioconversion techniques using enzymes such as *Trametes versicolor* have the advantage of efficiency and toxic substances-free. Accordingly, industrial added value is being created in metabolic engineering and synthetic biology branch, and the development of medicines such as therapeutic agents and physiologically active substances using microorganisms is developing.

This study aimed to investigate whether BFRG, using *Trametes versicolor*, affects the viability and proliferation of colorectal cancer cells. Here, we newly report a bioconversion technique to discover the anticancer effect of FRG, which itself does not show anticancer effect in colorectal cancer cells.

Material and methods

Extraction of FRG

FRG was purchased from the Korean herbal medicine market. 30% ethanol was added 10 times to 500 g of FRG, followed by reflux extraction twice for 3 h each. After extraction, FRG was concentrated at a temperature of 38 °C using a vacuum concentrator. After concentration, FRG was used for bioconversion.

For bioconversion, the concentrate was resuspended in distilled water, before the same amount of *Trametes versicolor* crude enzyme solution was added at 25 °C and shaking at 200 rpm for 24 h.

The *T. versicolor* coenzyme was prepared as follows. *T. versicolor* was inoculated into PDA medium (with 50 mg/mL ampicillin) and cultured for one week. Thereafter, 10 g of bran was mixed with 10 mL of DW before *T. versicolor* was inoculated into the mixture. The mixture was incubated at 25 °C for 13 days. The cultured *T. versicolor* was suspended in 1 mM citrate buffer (pH 4.5) and left at 4 °C for 24 h. The suspension was centrifuged, filtered, and used for bioconversion after measuring the laccase activity. For bioconversioned RG (BFRG) extracts, the diluted sample and the bioconversion crude liquid were mixed at a weight ratio of 1:1, and incubated at 25 °C for 24 h with shaking at 200 rpm. For FRG, non bioconversion solution was added instead of bioconversion solution under the same conditions. After the reaction product was fractionated with ethyl acetate, each fraction layer was concentrated under low pressure to obtain a stock having a concentration of 50 mg/mL, which was then used in this experiment.

Cell culture

CT26 cells (mouse colon cancer cell line) and NIH3T3 (mouse epithelial cell) cells were obtained from the Korea Cell Line Bank (Seoul, Korea). CT26 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. NIH-3T3 cells were cultured in DMEM supplemented with 10% newborn calf serum. All cells were cultured with penicillin (100 μg/mL)/streptomycin (100 μg/mL) in a humidified 5% CO_2 incubator at 37 °C.

Cell viability (MTT) assay

CT26 and NIH-3T3 cells were seeded in a 96-well plate (2×10^5cells/well) and maintained at 37 °C in a CO_2 incubator. After incubation for 24 h, the cells were exposed to 5, 10, 15 μg/mL of FRG and BRFG for 24 h. The control cells were treated with 15 μg/mL of DMSO alone in medium before the medium was removed and 500 μg/mL of MTT solution in phosphate-buffered saline (PBS) was added. After incubation for 3 h, iso-propan-2-ol was added to each well for 1 h. Absorbance at 595 nm was measured using ELISA reader (Infinite f50, TECAN, Männedorf, Switzerland).

Cell cycle phase analysis by flow cytometry

Cell cycle distribution was analyzed by flow cytometry (Thermo Fishier Scientific, Inc.) after staining with a propidium iodide (PI; Invitrogen) solution. CT26 cells (2×10^5 cells/mL) were cultured on 60-mm culture dish and treated with each concentration of BFRG (control, 5, 10, 15 μg/mL) for 24 h after attached. Subsequently, the cells were harvested, washed in cold PBS, and fixed with 400 μL of cold 95% ethanol at 4 °C for 1 h. The fixed cells were exposed to RNase A (100 μg/mL) for 30 min at 37 °C. Cells were washed with PBS and PI (100 μg/mL), which were added for 30 min at 37 °C incubation. The cell cycle was investigated using flow cytometry and the percentage of cell cycle cells was calculated.

Annexin V-FITC/PI staining assay

The CT26 cells were cultured on 60-mm culture dish (2×10^5 cells/mL) and exposed to BFRG at concentration

of 5, 10, and 15 μg/mL for 24 h. The cells were collected and washed with 2% FBS in PBS. Annexin V-Alexa Fluor 647/PI was added and mixed after adding 100 μL of binding buffer. After staining, the samples were protected from light for 15 min at room temperature. Apoptotic cells were detected by flow cytometry to determine the apoptotic cell number.

Protein isolation and western blot analysis

Proteins of interest, were analyzed using western blotting. Cells were seeded in 6-well plates (at a density of 2×10^5 cells per well) and incubated at 37 °C for 24 h and stimulated with the indicated concentrations of FRG and BFRG. After 24 h, cells were lysed in lysis buffer (RIPA buffer) containing a protease inhibitor (GenDepot, Katy, TX, USA) and phosphatase inhibitor cocktails (GenDepot, Katy, TX, USA). The total protein concentration in the supernatant was measured using Bradford solution (Bio-Rad Lab., Hercules, CA, USA) with bovine serum albumin as the standard. The samples were boiled for 10 min before they (30 μg of total protein) were separated by 7.5%, 10% or 15% SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis. Proteins were transferred to Amesrsham Protan 0.45 μM nitrocellulose membranes, blocked in 5% skim milk in TBST (1-M Tris at pH 8.0, 1.5-M NaCl, and 0.05% Tween 20) for 1 h, and incubated overnight with each primary antibody (Table 1) at 4 °C. After washing with TBST for 10 min for 3 times, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit or -mouse IgG antibodies at room temperature for 1 h. After washing with TBST for 10 min for 3 times again, proteins were detected using ECL prime or select western blotting detection reagent (GE Healthcare, Buckinghamshire, UK). The ImageJ software was used to quantify the reported protein expressions. All experiments were performed in triplicate.

Table 1 Primary antibodies used in the present study

Antibody	Origin	Company	Catalog no
Caspase 3	Rabbit monoclonal	Cell Signaling Technology, Inc	9662S
PARP	Rabbit monoclonal	Cell Signaling Technology, Inc	9542S
Bax	Rabbit monoclonal	Cell Signaling Technology, Inc	2772S
Bcl-2	Rabbit monoclonal	Santa Cruz Biotechnology, Inc	sc-492
p38	Rabbit monoclonal	Cell Signaling Technology, Inc	8690S
p-p38	Rabbit monoclonal	Cell Signaling Technology, Inc	4511S
Actin	Mouse monoclonal	Santa Cruz Biotechnology, Inc	sc-47778

Statistical analysis

Each experiment was performed at least in triplicate, and the results were presented as mean ± standard deviation (SD). Two-way analysis of variance was used to test for statistical significance and, where necessary, the *t* test and One-way ANOVA was used to compare each experiment with the control. A p-value < 0.05 was considered statistically significant.

Results

Bioconversion of FRG reduces viability of CRC cells

To study the ability of FRG and BFRG to inhibit proliferation of CRC cells, CT26 cells were exposed to FRG and BFRG for 24 h at each concentration (5, 10, 15 μg/mL). The results indicated that the number of CT26 cells decreased in a dose-dependent manner. To measure cell viability, an MTT assay was performed on normal epithelial NIH-3T3 cells and CT26 cells. At the 15 μg/mL concentration, FRG and BFRG did not cause any cytotoxicity in normal epithelial cells (Fig. 1B), so we selected the concentrations 5, 10, 15 μg/mL. As shown in Fig. 1A, CT26 cells exposed to FRG for 24 h showed no decrease in cell viability in the 10 μg/mL treatment group, but 65% cell viability in the BFRG 10 μg/mL treatment group. At the concentration of 15 μg/mL, 99% cell viability was observed when cells were exposed to FRG, but the BFRG-treated group showed a significantly lower cell viability of 39%.

BFRG arrests cell cycle of CRC cells

Unlike normal cells, cancer cells are characterized by endless and continuous cell proliferation. As abnormal cell proliferation can lead to metastasis or invasion, the suppression of cancer cell proliferation is vital for cancer treatment. To examine the ability of BFRG to inhibit cell proliferation, we measured the number of CT26 cells in each cell cycle phase by flow cytometry 24 h after exposure to BFRG. The percentage of cells in G0/G1 phase increased but that of the cells in the S phase of DNA replication decreased in a dose-dependent manner (Fig. 2A, B). At the highest concentration of 15 μg/mL, the number of cells in S phase was 2.6 times lower than those in the control. These findings indicate that BFRG inhibits cell proliferation through G0/G1 and S phase regulation.

BFRG induces apoptosis in CRC cells

After confirming the cell viability and cell cycle arrest effect in CT26, we performed flow cytometry to study the effect of BFRG on cell apoptosis. Exposure of CT26 cells to BFRG for 24 h resulted in 2.4% at the lowest

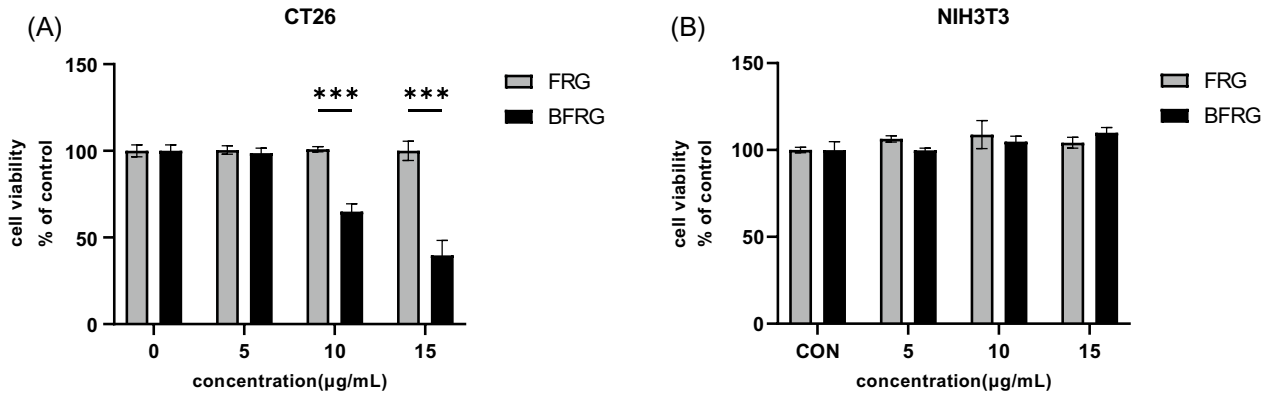

Fig. 1 Dose dependent inhibitory effects of FRG and BFRG on the viability of **A** CT26 and **B** NIH3T3 cells. Cells were treated with various concentrations (0–15 μg/mL) for 24 h. Data are presented as the mean of triplicate values ± SD. ***$p < 0.001$

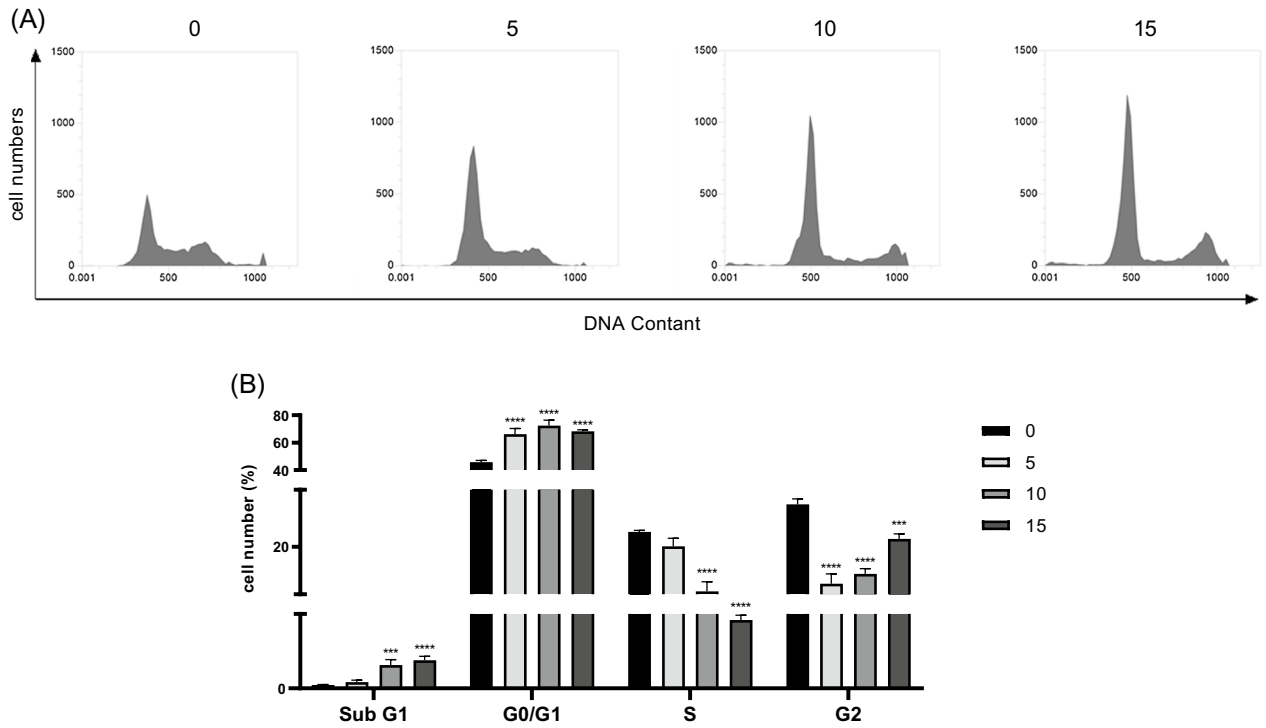

Fig. 2 Cell cycle analysis of CT26 cells treated with BFRG. The flow cytometry analyzed CT26 cells that were treated with BFRG at 0, 5, 10, 15 μg/mL. **A** Histogram demonstrate the number of cell (*y*-axis) versus the DNA content (*x*-axis). **B** The quantitative values of the phase population. Data are presented as the mean of triplicate values ± SD. ***$p < 0.001$; ****$p < 0.0001$

concentration of 5 μg/mL, 7.0% at 10 μg/mL, and 20.9% at the highest concentration of 15 μg/mL of the cells being apoptotic, indicating that BFRG significantly induces apoptosis in CT26 cells in a dose-dependent manner (Fig. 3A, B).

BFRG induces mitochondrial impairment in CRC cells

Apoptosis is activated by mitochondria, and caspase 3 is located at the end of the caspase cascade and is a key protein for apoptosis [18]. Additionally, activated caspase 3 induces the activation of PARP and apoptosis through

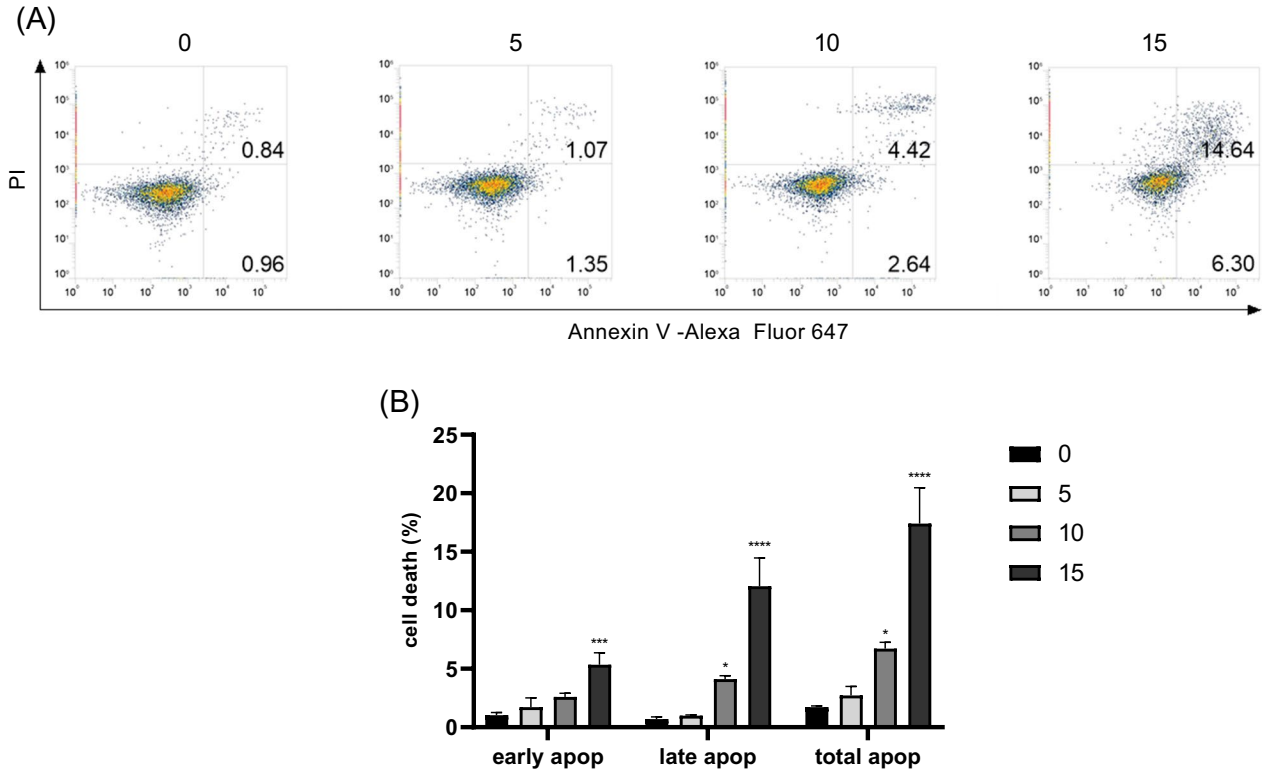

Fig. 3 Effects of BFRG on the apoptosis in CT26 cells. **A** Cells were treated with BFRG at 0, 5, 10, 15 μg/mL for 24 h. Next, all samples were double stained with Annexin V-Alexa 647/PI and analyzed by flow cytometry. The upper right quadrant indicates late apoptosis, and the lower right quadrant indicates early apoptosis. The lower left quadrant illustrates live cells. **B** Quantification of the early, late and total apoptotic cells were shown. Data are presented as the mean of triplicate values ± SD. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$

DNA fragmentation [19]. Our findings indicate that caspase 3, which is not expressed in FRG, was significantly expressed in BFRG (Fig. 4A, B). Particularly, at the highest concentration of BFRG, 15 μg/mL, the expression of cleaved caspase3 was ~ 100-fold and that of cleaved PARP was ~ 192-fold higher than that of the control group (Fig. 4A, B). In the intrinsic pathway of apoptosis, Bax, which is located on the outer mitochondrial membrane, induces apoptosis by impairing the mitochondrial membrane potential when it is activated [20], and Bcl-2 inhibits the activity of Bax. Our western blot data shows that the expression of Bcl-2, which has anti apoptotic activity, was reduced in BFRG-treated CRC cells (Fig. 4C, D). These results suggest that BFRG induces intrinsic apoptosis through damage to the mitochondrial membrane potential.

BRFG activates p38 MAPK in CRC cells

Many anticancer compounds induce apoptosis through the MAPK pathway. Therefore, we measured the effect of BFRG on the MAPK pathway. As shown in Fig. 5, BFRG significantly increased p38 phosphorylation. The BFRG 15 μg/mL treatment group increased the expression level of phospho-p38 to 14-fold higher than that of the control group. The protein p38 indicates the intrinsic apoptosis pathway by inactivating Bcl-2 or induces apoptosis by causing caspase 3 cleavage.

Discussion

The treatment of cancer using natural products rather than synthetic drugs is increasingly becoming popular, and ginseng, one of the most popular natural products, has been reported to have noteworthy pharmacological anticancer activity [21]. For example, Panax ginseng's ginsenoside Rp1 has anticancer activity against breast cancer cells [22], ginsenoside Rk3 inhibits the proliferation of lung cancer cells [23], and ginsenoside 20(S)-Rh2 exerts anticancer activity in human cervical cancer cells [24].

An important aspect of our study is that we discovered the anticancer effect of FRG on colorectal cancer for the first time through a bioconversion technique. The main ginsenoside content of the main root and fine root of ginseng is different, and the main root has a dominant content of ginsenoside Rg1, while the fine root has a high content of ginsenoside Rb1. Ginsenoside Rg1 is known to

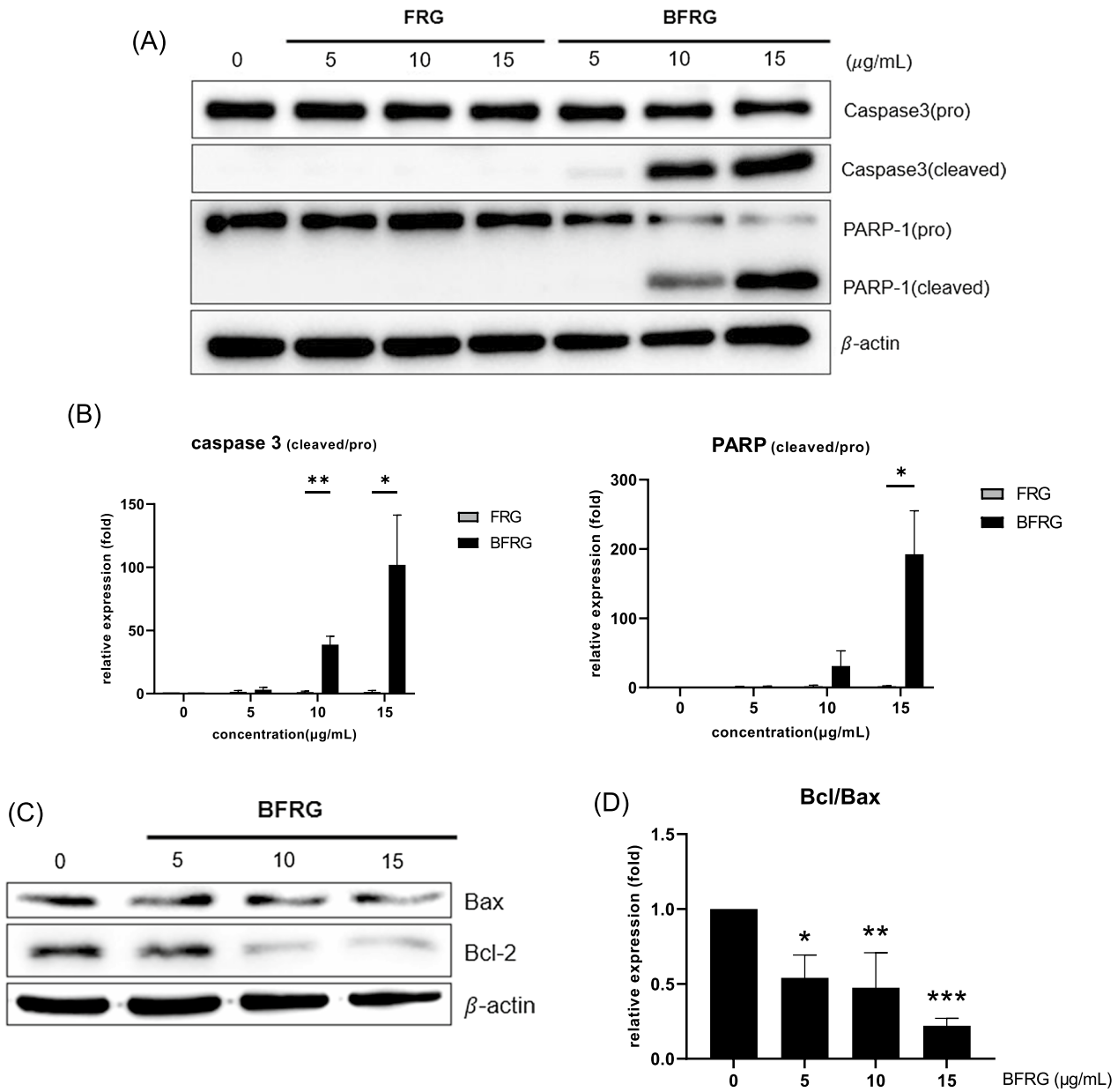

Fig. 4 Effects of FRG and BFRG on mitochondria of CT26 cells. **A** Apoptosis related proteins in CT26 cells were detected by Western blotting following treatment with 5, 10, 15 μg/mL FRG or BFRG for 24 h. **B** Quantitative analysis of Western blot of caspase3 or PARP. **C** Mitochondrial impairment related proteins in CT26 cells were detected by Western blotting following treatment with 5, 10, 15 μg/mL BFRG for 24 h. **D** Quantitative analysis of Western blot of Bcl/Bax. Data are presented as the mean of triplicate values ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

inhibit cell proliferation in various cancer cells [25], but the anticancer effect of ginsenoside Rb1 has not been identified. *Trametes versicolor* has high β-glucosidase activity [26]. β-glucosidase breaks the β-glucoside bond of glycosides, and we predict that due to the bioconversion technique *using Trametes versicolor*, the content of sugar derivatives bound to glucose of ginsenoside will decrease, and the content of ginsenoside, which has an anticancer effect, will increase by inducing the binding of new sugar derivates.

Apoptosis is a key pathway for regulating mammalian cell homeostasis and morphogenesis; its misregulation leads to cancer [27]. Many previous studies have focused on apoptosis in their study of the mechanism of action of many anticancer drugs. Therefore, we studied the effect of BFRG on apoptosis. We found that

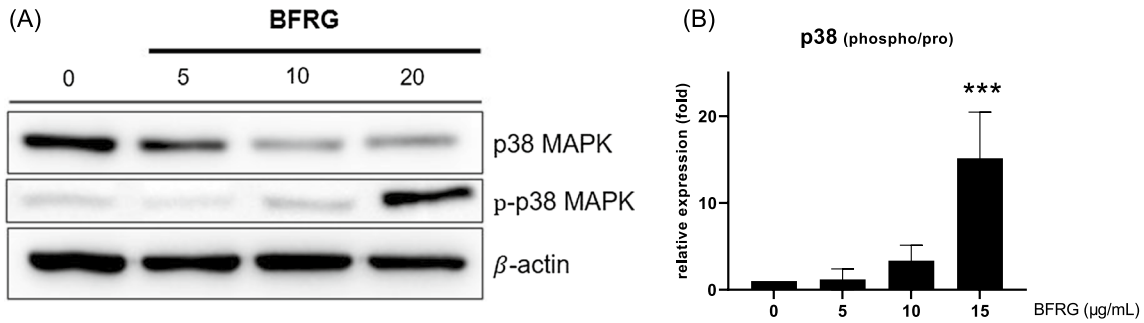

Fig. 5 **A** The protein levels of p-p38, p38 were detected after CT26 cells were exposed to 5, 10, 15 μg/mL BFRG for 24 h. **B** Quantitative analysis of Western blotting of p38. Data are presented as the mean of triplicate values ± SD. ***$p < 0.001$

BFRG significantly induced both early and late apoptosis and inhibited cell proliferation. Caspase 3 is an important mediator of apoptosis, catalyzing the cleavage of many cellular proteins when it is activated [28]. Bax occurs in the mitochondrial outer membrane and plays a role in pro apoptosis through involvement in the loss of mitochondrial membrane potential and release of cytochrome C. In contrast, Bcl-2 plays an anti-apoptotic activity by inhibiting the activity of Bax. Our findings indicate that BFRG induces apoptosis by causing damage to the mitochondrial membrane potential through a decrease in the Bcl/Bax ratio.

Persistent and excessive cell division is a hallmark of cancer cells [29]. The cell cycle, consisting of G0/G1/S/G2/M phase, is regulated through checkpoints occurring between the G1 and S phases and between the G2 and M phases [30]. These checkpoints are responsible for repairing damaged DNA or preventing excessive cell cycle progression. Additionally, the intervals between cell cycle phases are finely adjusted by cyclin-dependent kinases, which are activated when specific cyclins are involved [31]. Our findings indicate that exposure of colorectal cancer cells to BFRG inhibits cell proliferation by arresting the cell cycle at the G0/G1 phase.

We acknowledge the following limitations of our study. First, we did not perform in vivo experiments to confirm the in vitro dose–response relationships and we did not confirm if experimentally significant differences were also clinically significant. Second, the component analysis of FRG and BFRG was not performed. To verify this limitation, confirmation reproducibility using human-derived cell lines and in vivo test. Additionally, component comparison analysis of FRG and BFRG should be performed and future studies using random survival forest (RSF) and Cox proportional hazard regression will attempt to analyze the relationship between BFRG and patient survival in colorectal cancer [32].

In conclusion, bioconverted FRG (BFRG) induces apoptosis, which causes colorectal cancer cell death by impairing the mitochondrial membrane potential. BFRG also induces cell cycle arrest, resulting in suppression of colorectal cancer cell proliferation. This suppression of colon cancer cell proliferation occurs through the phosphorylation of p38. Therefore, we propose a bioconversion technique using *Trametes versicolor* as a way to use FRG for colorectal cancer treatment.

Abbreviations

CRC	Colorectal cancer
FRG	Fine root of ginseng
BFRG	Bioconverted fine root of ginseng
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
PARP	Poly(ADP-ribose) polymerase
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
p38 MAPK	P38 mitogen-activated protein kinases
p-p38 MAPK	Phospho-p38 mitogen-activated protein kinases

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Author contributions
YS designed, performed the experiment and wrote the manuscript; JC gave advice and supervised the experiment; JON, as corresponding authors, conceived the study and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data analyzed during this study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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