**In Vitro** Evaluation of Anti-Lung Cancer and Anti-COVID-19 Effects using Fermented Black Color Ginseng Extract

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Abstract

Ginseng is known as the “king” of herbal plants and has been used widely in Asia for centuries. Ginseng contains active saponins, including protopanaxadiols, protopanaxatriols, and other compounds. There are many methods for processing ginseng, such as steaming, fermentation, expansion, and conversion of active compounds, which can improve its biological activity. In this study, we investigated the cytotoxic and oxidative effects of fermented black color ginseng (FBCG), black ginseng (BG), and white ginseng (WG) on a human lung carcinoma cell line (A549). Moreover, we found that treatment with FBCG induced oxidative stress in the A549 cell line and increases the apoptosis percentage; these effects were linked to the stimulation of the caspase 3/mitogen-activated protein kinase (caspase 3/MAPK) pathway. We also evaluated the anti-coronavirus disease-2019 (COVID-19) effect of FBCG on a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected Vero E6 cell line. Our results suggest that FBCG not only inhibits the replication of this strain of virus in the cell but also reduces the number of viral RNA (vRNA) copies in the extracellular environment. Taken together, these data show that FBCG has both potential anti-lung cancer and anti-COVID-19 effects.

Keywords

fermented black color ginseng, *Aspergillus niger*, antilung cancer, caspase 3/MAPK, COVID-19

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Introduction

Ginseng (Panax ginseng Meyer) is one of the oldest and most widely known traditional medicinal herbs and has been used to promote longevity and regulate bodily functions for centuries.¹ In East Asian countries, especially in China, Korea, and Japan, ginseng is used widely as an over-the-counter drug and as an adjuvant for cancer therapy.² The main commercially valuable part is the ginseng main root, which usually is harvested after 4 to 6 years of growth,³ and ginsenosides, which are extracted from P. ginseng Meyer, are the main active compounds with a wide range of pharmacological activities. More than 170 types of ginsenosides have been isolated and determined from *P. ginseng*, and these include both naturally occurring and transformed compounds; in addition, more than 100 saponins have been identified from raw or processed ginseng.⁴ Some of the saponin ginsenosides are compounds with a dammarane structure of two main types: panaxadiol (PPD) and panaxatriol (PPT). Ginseng and its saponins have been reported to have a variety of anti-cancer efficacies.¹

The Global Cancer Observatory (GCO) has appraised that the prevalence and mortality of cancers are increasing annually, and that cancer has become a primary cause of death worldwide.⁵ Among the reported cases of cancer-associated death, approximately 40% of patients died of lung cancer.⁶ Besides, over the past decade, novel synthetic chemotherapeutic agents have been applied to treat cancer patients, although the effects have not been as good as expected. Therefore, it is imperative to develop new, affordable, anti-cancer drugs that have fewer negative interactions for patients.

*Aspergillus niger (A. niger)* is recognized as a safe (generally regarded as safe [GRAS]) microorganism that has been used in the food industry for a long time, and has become a novel part of the ginseng fermentation process. During fermentation by *A. niger*, both PPD- and PPT-type ginsenosides, compounds K, Rg5, Rg3, Rk1, and F2 could be converted from ginsenosides

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Rb1, Rb2, and Re. Fermented ginseng has been effective with the following properties reported: anti-proliferative, anti-obesity, intestine protective, and neuroprotective.

Chinese clinical practice has proven that integrated Chinese and Western therapy approaches are effective for controlling the COVID-19 epidemic. Among them, proprietary herbal medicines containing ginseng and ginseng preparations have accounted for 56.41% of all applied treatments. COVID-19, which is caused by SARS-CoV-2, was declared as a pandemic by the World Health Organization. Currently, several treatment protocols have been proposed based on symptomatic treatment, supportive care, anti-virus therapy, and a combination of Chinese and Western medicine.

In the present study, we investigated the apoptotic effects and mechanisms of fermented black color ginseng (FBCG), black ginseng (BG), and white ginseng (WG) on A549 cells. We also detected the suppressive function of FBCG on SARS-CoV-2 vRNA replication.

Results

In Vitro Cell Cytotoxicity

To evaluate the apoptosis induction and the potential anti-SARS-CoV-2 effect of this FBCG, A549 cells were treated with FBCG, BG, and WG for 24 h; Vero E6 cells were treated with FBCG (Figure 1a and b). A significant reduction in cell viability was observed at 200 µg/mL in the A549 cell line for FBCG (Figure 1a). Compared with BG and WG, FBCG showed the highest cytotoxic effect in the A549 cell line. However, FBCG up to 25 µg/mL did not show cytotoxicity in Vero E6 cells (Figure 1b).

Intracellular Reactive Oxygen Species (ROS) Generation

To detect the intracellular ROS, A549 cells were treated with FBCG, BG, and WG for 24 h. The increase of ROS was evaluated by a dichlorodihydro-fluorescein diacetate (DCFH-DA) probe (Figure 2a). To quantify the intracellular ROS levels in the A549 cancer cell line, we examined the ROS production with a spectrofluorometer, and the results are shown in Figure 2b; analysis indicated that ROS induction was related to the 100 µg/mL FBCG treatment.

FBCG Treatment-Induced Apoptosis of A549

A549 cancer cells were monitored after different sample treatment by performing Hoechst-33258 staining. The cell membrane remained intact in untreated cells and prohibited Hoechst dye intake, showing low staining of the cells. Whereas, when the cells were treated with FBCG, the cell membrane of the cancer cells was damaged due to the penetration of the dye into the cells and these cells showed positive apoptotic results (Figure 3a). The BCL2 associated X gene (BAX)/Bcl2 ratio significantly increased in A549 cancer cells (Figure 3b and c).

The expressions of caspase 3 (CASP3) and p53 in the A549 cell line were analyzed by western blotting and quantitative polymerase chain reaction (qPCR) (Figure 4a-c). However, the western blot analysis did not indicate increased p53 accumulation at the protein level; it showed that the cells underwent apoptosis by activating CASP3 rather than p53 (Figure 4a). Phosphorylation of the p38/MAPK protein slightly increased in the A549 cancer cells (Figure 4d).

Anti-SARS-CoV-2 Efficacy

To investigate whether FBCG influenced viral replication of SARS-CoV-2, Vero E6 cells were infected with SARS-CoV-2 in the absence or presence of FBCG or positive controls (Remdesivir [Rem] and chloroquine phosphate [C.P]). As a result, this assay indicated that FBCG potently inhibited the

![Figure 1.](image_url) Cytotoxicity of FBCG, BG, and WG on the A549 cell line (a) and toxicity evaluation of FBCG on Vero E6 cell line (b). Values shown are mean ± SD of 3 independent experiments. ***P < .001 versus control cells. Abbreviations: FBCG, fermented black color ginseng; BG, black ginseng; WG, white ginseng.
replication of SARS-CoV-2. In contrast, Rem did not show significant antiviral activity (Figure 5a). On the other hand, after treatment with FBCG, the vRNA copies showed a dosage-dependent reduction in the culture medium (Figure 5b). When treated with a 25 µg/mL sample, the cells showed the same significant reduction as the positive control Rem.

Figure 2. Oxidative stress potential of WG, BG, and FBCG on A549, the ROS was detected by DCFH-DA probe (a), ROS generation was measured after 24 h of treatment (b). Values shown are mean ± SD of 3 independent experiments. Abbreviations: WG, white ginseng; BG, black ginseng; FBCG, fermented black color ginseng; DCFH-DA, dichlorodihydro-fluorescein diacetate; ROS, reactive oxygen species. *P < .05, **P < .01, and ***P < .001 versus control cells.

Figure 3. Evaluation of apoptosis in the A549 cell line. Cells were treated with WG, BG, and FBCG for 24 h. Hoechst staining positive apoptotic cells are pointed with arrows (a), relative protein level was evaluated by western blotting (b), and relative gene expression (c) was detected by qPCR. Values shown are mean ± SD of 3 independent experiments. *P < .05, **P < .01, and ***P < .001 versus control cells. Abbreviations: WG, white ginseng; BG, black ginseng; FBCG, fermented black color ginseng; qPCR, quantitative polymerase chain reaction.
Figure 4. The potential apoptotic mechanism of FBCG on A549 cell line. After 24 h treatment, the relative protein level was evaluated by western blotting (a), caspase 3 (b), and p53 (c) gene expression was detected by qPCR. Phosphorylated expression of p38 protein level was presented by western blot (d). Values shown are mean ± SD of 3 independent experiments. *P < .05, **P < .01, and ***P < .001 versus control cells. Abbreviation: qPCR, quantitative polymerase chain reaction; WG, white ginseng; BG, black ginseng; FBCG, fermented black color ginseng.

Figure 5. The anti-SARS-CoV-2 potential of FBCG. The effects of FBCG on SARS-CoV-2 replication (a) and viral RNA copy numbers in cell culture medium of the SARS-CoV-2 infected Vero E6 cells (b). Values shown are mean ± SD of 3 independent experiments. *P < .05, **P < .01, and ***P < .001 versus control cells. Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WG, white ginseng; BG, black ginseng; FBCG, fermented black color ginseng.
Discussion

In this study, we found that FBCG demonstrated high toxicity on a human lung carcinoma cell line (A549) compared with BG and WG. To avoid the toxic side effects, Nature Bio Pharma Co., Ltd tested the amount of benzopyrene in FBCG. Benzopyrene is a type of the polycyclic aromatic hydrocarbon that is, a primary carcinogen in grilled and broiled foods, tobacco, and many industrial processes. The level of benzopyrene is used to indicate the risk of carcinogenicity. Supplemental Figure S1 shows the specific peak for benzopyrene; it was not observed in the high-performance liquid chromatography (HPLC) spectrogram of FBCG, which indicates the level of benzopyrene in FBCG was not detected. The Korea Food and Drug Administration (KFDA) limited the maximum level for benzopyrene in food products to 5.0 μg/kg. The data for this study indicate a level far below the KFDA standard for benzopyrene. Similarly, our previous study confirmed that the benzopyrene contents of BG and WG were less than 5.0 μg/kg. Therefore, these materials can safely be used as oral ginseng products.

In addition, FBCG treatment induced the ROS levels related to cellular apoptosis. P. ginseng extract has been reported to induce cell death in various cancer cells through DNA damage by generating ROS and activating multiple pro-apoptotic markers. ROS are generated and eliminated in the biological system and play important roles in a variety of normal biochemical functions and abnormal pathological processes. In addition, mitochondria damage can cause ROS to be released from mitochondria, the major source of ROS. Therefore, the antiproliferation effect of FBCG was determined by evaluating the ROS levels. This assessment was important because intracellular oxidative stress is a known mechanism of cell death in various types of cell lines.

Many studies have reported that a balance between cell proliferation and death is crucial for tissue homeostasis in multicellular organisms. Apoptosis or programmed cell death occurs through an evolutionarily conserved cellular program in various physiological and pathological situations. Many diseases are associated with apoptosis, such as cancer, autoimmune disease, and neurodegenerative disease. Thus, the induction of apoptosis in cancer cells is one of the molecular bases for anti-cancer therapeutic interventions. The results of our study showed an increase in morphological alteration when cells were treated with high concentrations of FBCG. Furthermore, cell number and size were significantly reduced, which was likely attributable to FBCG involvement in cell detachment and altered morphologies.

Human lung cancer (A549) cells were treated with FBCG, BG, and WG to induce nuclear cell death by involving an apoptotic mechanism that upregulated the caspase 3/activation of stress-responsive MAPKs gene expression such as p38 phosphorylation. The antiapoptotic member Bcl2, and the proapoptotic member BAX have been proven to be critical players in the intrinsic apoptosis pathway, and the ratio of BAX/Bcl2 is a key factor for regulating apoptosis. Figure 3b and c confirm that the BAX/Bcl2 ratio significantly increased in A549 cancer cells.

The caspase (cysteine-aspartic acid proteases) family plays an important role in the apoptosis process. A previous study reported that application of P. ginseng extract could induce apoptosis in A549 cells by activating p53 and caspase 3. Changes in the expression of p53 correlated with changes in 2 downstream Bcl2 family proteins; BAX and Bcl2. This observation is in agreement with the transcriptional expression change of p53 in the A549 cell line. Our data suggest that the expression of the p53 gene tends to increase in A549 cells that are gradually treated with increasing concentrations of WG, BG, and FBCG; the CASP3 gene also showed increased expression after treating these cells with the three different samples and concentrations. Moreover, cells treated with FBCG further upregulated both CASP3 and p53 transcripts. However, protein level analysis did not indicate an increase in p53 accumulation. Phosphorylation of the p38/MAPK protein expression slightly increased in the A549 cell line. The increasing ratio of BAX/Bcl2 results in apoptosome formation and activation of its effector, CASP3, which was indicated by the increased transcriptional expression of CASP3 in the A549 cancer cell line (Figure 4b). Our data are the first to show that FBCG inhibits cancer cells proliferation by inducing apoptotic-mediated cell death. This result suggests that FBCG might serve as a potential antitumor agent against various cancer cells, although additional in vivo experimental analysis is needed.

Moreover, recent studies reported that ginseng and ginseng products demonstrated high toxicity on a human lung carcinoma cell line (A549) compared with BG and WG. To avoid the toxic side effects, Nature Bio Pharma Co., Ltd tested the amount of benzopyrene in FBCG. Benzopyrene is a type of the polycyclic aromatic hydrocarbon that is, a primary carcinogen in grilled and broiled foods, tobacco, and many industrial processes. The level of benzopyrene is used to indicate the risk of carcinogenicity. Supplemental Figure S1 shows the specific peak for benzopyrene; it was not observed in the high-performance liquid chromatography (HPLC) spectrogram of FBCG, which indicates the level of benzopyrene in FBCG was not detected. The Korea Food and Drug Administration (KFDA) limited the maximum level for benzopyrene in food products to 5.0 μg/kg. The data for this study indicate a level far below the KFDA standard for benzopyrene. Similarly, our previous study confirmed that the benzopyrene contents of BG and WG were less than 5.0 μg/kg. Therefore, these materials can safely be used as oral ginseng products.

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Moreover, recent studies reported that ginseng and its production provided a potential defense against respiratory infections, even COVID-19. COVID-19 and influenza can present with similar symptoms, and co-infections with both illnesses can cause more acute and complicated or fatal outcomes. Additionally, COVID-19 and influenza have similar high-risk groups and both can prove detrimental for older patients and those with chronic diseases, and previous studies have reported that ginseng and ginseng products could effectively prevent and treat influenza. Thus, ginseng extracts against COVID-19 have been proposed as part of anti-SARS-CoV-2 measures.

To investigate whether FBCG impacts SARS-CoV-2 viral replication, Vero E6 cells were infected with SARS-CoV-2 in the absence of FBCG, presence of FBCG, or presence of positive controls (Rem and C.P). Remdesivir (Rem) is an anti-virus drug that targets a viral RNA-dependent RNA (Ribonucleic acid) polymerase and was identified early as a promising therapeutic candidate for COVID-19. Chloroquine phosphate (C.P) is an antimalarial drug that has been used worldwide for more than 70 years and recent reports have confirmed that C.P has an impact against COVID-19. Our results indicated that FBCG efficiently inhibits intracellular and extracellular viral replication. Accordingly, FBCG should be considered as a potential therapeutic candidate to prevent new vRNA synthesis in asymptomatic and symptomatic patients to relieve the course of COVID-19.
Conclusions
In our study, we determined the potential of FBCG extract in inducing apoptosis and DNA damage in lung cancer (A549). It has been identified that, among different caspase pathways, activation of the caspase 3/MAPK pathway is required for apoptosis from FBCG. Furthermore, FBCG extract inhibited the replication of SARS-CoV-2.

Experimental
Reagents and Materials
Bacterial culture medium was supplied by Difco, MB Cell (Gangnam-go, Seoul, South Korea). All cell lines were purchased from the Korean Cell Line Bank. Fresh ginseng was collected from the ginseng resource of Kyung Hee University in South Korea. The strain A. niger KHNT-1 (NCBI Accession number: MT804610) was isolated from fermented soybean (Meju), a fermented traditional food collected in the Gyeonggi province in South Korea.

Preparation of FBCG Extract
Briefly, fresh white ginseng roots were steamed three times before the fermentation process. Then the steamed samples were dipped in the KHNT-1 (A. niger) cell suspension for 1 min and fermented at room temperature for 3 days; this process was repeated seven times to complete the fermentation process. The samples gradually changed to a black color during the process. Finally, the dried fermented samples were extracted by 80% methanol, evaporated under a vacuum, and used for the following experiments.

Cell Culture
Human lung carcinoma cell line (A549) was grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S), in a 5% CO2 incubator at 37 °C. African green monkey kidney cells (Vero E6) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% P/S and 10% FBS.

Cytotoxicity Assay
The cell cytotoxicity of black fermented ginseng toward the 2 cell lines (A549 and Vero E6) was evaluated using an (3, 4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT stock solution (10×) (5 mg/mL) was dissolved in phosphate-buffered saline (PBS) (pH 7.4), filter sterilized, and stored at 4 °C. We used 5×10^4 cells per well (96-well plates) containing 100 μL of the complete culture medium. After incubating overnight, test samples at different concentrations were added into 96-well plates. The final concentration of dimethyl sulfoxide (DMSO) Hybri-Max in all assays was less than 0.1%. Based on the preliminary test results, the cytotoxic activities of FBCG, BG, and WG were determined at various concentrations ranging from 0 to 200 μg/mL. The culture plates were incubated in 37 °C supplied with a humidified atmosphere of 5% CO2. Then, after 24 h, 0.05% of the 100 μL MTT reagent was added to each well, and incubated for 3 h. The MTT solution was removed, and 200 μL DMSO was added to each well. Finally, the plate was shaken in a microplate shaker for 10 min under dark conditions to dissolve the purple formazan crystals. The DMSO solution was used as a negative control. The optical density values were recorded using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Instruments, Inc.) at 570 and 670 nm, respectively. Blank values were subtracted from experimental values.

ROS Generation
Intracellular ROS values were measured by 2′,7′-DCFH-DA. In brief, A549 cells (2×10^4/well) were treated with different extracts of 100 μg/mL for 24 h. Then, 100 μL of DCFH-DA was added at 20 μM and incubated for 30 min. Next, media were discarded and cells were washed twice with 1x PBS. The supernatant was kept in a 96-well plate in the dark and the fluorescence intensity was determined with a spectrofluorometer with excitation at 485 nm and emission at 520 nm.

Apoptosis Detection
Hoechst-33258 staining was performed to capture the apoptotic induction of FBCG in the A549 cell line. Cells were seeded into a 6-well plate at a density of 1×10^5 cells/well in 2 mL complete medium and incubated overnight. The cells were treated for 24 h and then stained with Hoechst-33258 solution at 2 μg/mL for 20 min following our previous protocol.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis
Treated and non-treated A549 cell monolayers grown in 100 mm cell culture dish (Corning Costar) were treated with 100 μg/mL of FBCG, BG, and WG. After 24 h of treatment, the total RNA was extracted from non-treated and treated cultures using Qiagen lysis reagent. For qPCR, 500 ng of total RNA was reverse transcribed using oligo (dT) 15 primer (0.2 mM), and Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (10 units/μL) and cDNA were synthesized using a Superscript First-Strand Synthesis Kit (Invitrogen), according to the manufacturer’s instructions. The qPCR was performed to quantify the messenger RNA (mRNA) expression level with a real-time rotary analyzer. The mRNA levels were quantified using SYBER® Green SensiMix plus Master Mix. The fluorescent product was detected at the last step of each cycle and measured in the real-time reverse transcriptase PCR.
The total proteins (20 μg) were mixed with 5× sodium dodecyl sulfate (SDS) buffer as per the manufacturer’s instructions and then boiled for 10 min and electrophoresed on a 12% SDS-PAGE gel. After electrophoresis, the proteins from the gels were transferred onto a polyvinylidene fluoride (PVDF) membrane using an electroblotting apparatus. The membrane was then blocked with 5% skim milk in TBS-T (tris-buffered saline with 0.1% tween 20) at room temperature for 1 h and further incubated overnight at 4 °C with the specific monoclonal or polyclonal antibody stated previously; each of the solutions was at a dilution of 1:1500. After washing with 1× TBS-T 5 times at 5-min intervals, the membranes were incubated with a goat anti-rabbit IgG (H&L) horseradish peroxidase conjugated secondary antibody at a 1:5000 dilution for 2 h at RT. Finally, after washing with 1× TBS-T 5 times, the blots were developed with an enhanced chemiluminescence (ECL) reagent and immediately exposed to an X-ray film.

Western Blotting
A549 cells (1 × 10⁶ cells/well) were treated with three extracts at 100 μg/mL for about 24 h. The cells were then harvested and washed twice with cold PBS. The cell pellets were lysed using Pro-prep™ protein extraction solution. The lysates were centrifuged at 13 500 rpm for 10 min at 4 °C. The protein content of the supernatant was determined using a Bio-Rad Protein Assay kit, and bovine serum albumin (BSA) was used as the standard. The total proteins (20 μg) were mixed with 5× sodium dodecyl sulfate (SDS) buffer as per the manufacturer’s instructions and then boiled for 10 min, and electrophoresis was conducted in a thermocycler, and the genomic increase of the fluorescence corresponding to the exponential increase of the product was used to determine the threshold cycle (Ct) in each reaction using the formula 

\[ 2^{-\Delta\Delta CT} \] . The housekeeping gene that encodes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a standard for all samples. The primer sequences are shown in Table 1.

Detection of vRNA Copies
Vero E6 cells were seeded in 12 well plates at 3 × 10⁵ cells/well and incubated overnight. After cells were infected with SARS-CoV-2, they were treated with different concentrations of FBCG and incubated. After 18 h of treatment, the virus was collected from the culture medium. The vRNA copies were detecting by qPCR performed using the primers in Table 2. The titration of SARS-CoV-2 were performed on Vero E6 cells, as described previously. Cells were incubated for 1 h with SARS-CoV-2 in the presence of DMEM or Eagle’s minimum essential medium with 0.3% BSA to infect the cells with SARS-CoV-2.

Plaque Reduction Assay
Vero E6 cells were infected with 50 or 100 plaque-forming units (PFUs) of SARS-CoV-2 and treated with the samples diluted from 1/40 to 1/640 with serum-free medium at 37 °C in 5% CO₂ over 1 h. After rinsing two times with PBS to remove attached viruses, the cells were overlaid with 0.6% agarose diluted in DMEM with 0.3% BSA. The plates were incubated at 37 °C under 5% CO₂ conditions over 3 days. The cells were stained with 0.4% crystal violet dye solution (Sigma) for 10 min and washed with PBS three times. The plaque reduced ratio was calculated compared with normal cells.

Statistical Analysis
All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software). Error bars indicate the standard error (SD) of the mean and mean values were compared using analysis of variance with Duncan’s test. All experiments were performed independently at least three times.

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Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
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Supplemental Material
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