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Bioprocessed Black Rice Bran Potentiates the Growth Inhibitory Activity of an Immune Checkpoint Inhibitor against Murine Colon Carcinoma

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Abstract

This study determined the effect of orally fed polysaccharide-rich bioprocessed (fermented) black rice bran produced by culturing with shiitake (*Lentinus edodes*) mushroom mycelium on CT-26 colon cancer cells in vivo in an intracutaneously transplanted mouse tumor alone and in combination with intraperitoneally administered anti-PD-1 immune checkpoint inhibitor. Analysis of the isolated tumor weights at the end of the study shows that the average tumor size in control mice is 3.78 grams, and the average tumor size in mice treated with anti-PD-1 antibody is 2.16 grams. The average tumor size in mice treated with BRB-F alone is 2.25 grams, and the average tumor size in mice treated with anti-PD-1 antibody BRB-F combination is 1.38 grams. Thus, BRB-F or anti-PD-1 antibody alone each reduce tumor size by 40.5% or 42.9%, whereas the combination of BRB-F and anti-PD-1 antibody reduces tumor size by 63.5%, with their cooperative effect being statistically significant. The observed anti-tumor effects were accompanied by a series of biomarkers associated with cancer formation and inhibition. These results indicate that the reported potentiation of cancer therapy using drug-based medical chemotherapies with added checkpoint inhibitors in human patients are mechanistically similar with the functional food evaluated in the present study. These beneficial effects in mice challenge clinicians to investigate if the black rice bran food product can also protect against human cancer.

Keywords

Black Rice Bran, Mushroom Mycelia, Bioprocessing, Immune Checkpoint Inhibitor, Mice, Tumor Regression, Cancer Prevention, Biomarkers,

Mechanism, Research Needs

1. Introduction

Black rice bran contains bioactive phenolic and flavonoid compounds that may responsible for reported antioxidant and antiproliferative activities resulting in health-promoting effects including antiproliferative properties against breast cancer cells [1]. Indeed, Suttiaporn *et al.* [2] demonstrated the anti-leukemic cell activity of phytosterols and triterpenoids isolated from black rice bran. A review by Tan *et al.* [3] suggests that bioactive compounds from rice bran waste produced during the milling process, which removes the bran and germ and leaves the starchy endosperm, can benefit nutrition and health. Although thermal cooking decreases anthocyanin content and antioxidative activity, it does not affect the anti-inflammatory activity of black rice [4].

The antitumor effects of black rice bran have been reported previously. For example, Nam *et al.* [5] determined the anti-tumor promoting activity of 70% ethanol-water extracts of bran from the seeds of five pigmented rice cultivars. The extracts strongly inhibited phorbol-ester tumor promotion in lymphoblastoid B cells *in vitro*. In a related study, Nam *et al.* [6] also reported on antioxidative, antimutagenic, and anticarcinogenic activities of similar ethanol-water extracts in chemical and cell assays. Choi *et al.* [7] found that orally fed black rice bran protected mice against chemically induced inflammation (edema) of the skin. Kim *et al.* [8] discovered that mice fed a diet supplemented with the black rice bran compound γ-oryzanol significantly reduced tumor growth in CT-26 cancer mouse tumors, possibly resulting from the observed induction of splenic natural killer (NK) cells, activation of macrophages, and inhibition of angiogenesis, as well by activation of the immune system.

Choi *et al.* [9] observed that both black and brown rice brans exhibited antitumor effects in mice: a diet supplemented with black and brown rice inhibited the growth of transplanted tumors in mice by 35% and 19%, respectively. Tumor inhibition was associated with induction of NK activity and macrophages and inhibition of angiogenesis and other biomarkers. Additionally, a study by Tonchaiyaphum *et al.* [10] found that an ethanol extract of black rice bran could inhibit gastric ulcers; these authors also found that the 2000 mg/kg dose of oral black rice bran showed no acute toxicity in rats. A human oral intervention study by Jin-Min Kim *et al.* [11] reported that feeding cancer patients a cereal-based diet that contained 0.5% arabinoxylan-rich fermented rice bran powder and 5.5% black rice powder for up to 8 weeks improved chronic inflammation and health-related quality of life.

The bioprocessed black rice bran used in this study is a fermentation of black rice bran using *Lentinulus edodes* mycelium, which has been reported to exhibit various bioactive properties in previous studies. Bioprocessed black rice bran have been found to be able to prevent asthma [12], alcohol-induced hangovers

[13], LPS-induced endotoxemia [14] and infections [15]. Also worth noting is that bioprocessed black rice bran has been found to contain higher amounts of immune-active polysaccharides compared to black rice bran in its natural state.

Immune checkpoint inhibitors such as PD-1 that active exhausted antitumor T cells are used alone or with chemotherapy for the treatment of a wide range of cancers [16] [17] [18] [19]. To our knowledge, immune checkpoint inhibitors have not previously been evaluated in combination with anticancer functional foods such as the bioprocessed black rice bran. Also noteworthy is the report by Hwang *et al.* [20] that a polysaccharide isolated from the herbal medicine *Astragalus membranaceus* could be used as a topical mucosal adjuvant to enhance the anticancer effect of an immune check point inhibitor against pulmonary metastatic melanoma in mice. These considerations led us to determine the antitumor effects in mice of bioprocessed black rice bran alone and combination with an immune checkpoint inhibitor.

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, Hanks' balances salt solution (HBSS), and other cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phosphate-buffered saline (PBS), fetal bovine serum (FBS), and other cell culture reagents were purchased from Gibco BRL (Grand Island, NY). Calcein-AM was purchased from Calbiochem (San Diego, CA). Hematoxylin, eosin Y, lipopolysaccharide (LPS), recombinant interferon-γ (rIFN-γ), and other reagents were from Sigma Chemicals (St. Louis, MO).

2.2. Preparation of Black Rice Bran (BRB) and Its Isolated Fractions

The following samples were prepared for the evaluation of anti-tumor properties: BRB-F, bioprocessed (fermented) black rice bran/mushroom mycelia; BRB-F-S, supernatant fraction of BRB-F; BRB-F-W, water-soluble fraction of BRB-F; and BRB-F-P, polysaccharide fraction of BRB-F. BRB-F was produced according to the previously published method [12]. *Lentinus edodes* fungal mycelia were cultured on a potato dextrose agar (PDA) medium. The mycelium cultured in PDA media was inoculated into 50 mL of the liquid medium. Incubation experiments were performed in 250 mL Erlenmeyer flasks for 5 days at 28˚C on a rotary shaker (120 rpm) and used to seed the main liquid culture. The main liquid medium contained black rice bran (100 g/L). Subsequently, the medium was treated with amylase at 60˚C for 60 minutes for enzymatic digestion of particulate matter containing carbohydrates. The culture was then adjusted to pH 6.0 using HCl, and then sterilized in the autoclave. Experiments on the main liquid culture were initiated by inoculating the inoculum (10%) of pre-cultured mycelium using a 5 L fermenter (working volume of 3 L) at 28˚C and 150 rpm.

After 3 days, the culture mass was treated with amylase and an enzyme mixture for cell wall lysis containing cellulose, hemi-cellulase, pectinase, glucanase, mannose, and arabinase at 50˚C for 60 min. Subsequently, enzyme-treated cultures were extracted with hot water at 90˚C for 1 h and lyophilized with a solid material. BRB-F-S was obtained by removing insoluble residues from BRB-F, and BRB-F-W was further purified by removing formed submicro-sized lipid particles. BRB-F-P was sequentially purified by removing small-molecular impurities from BRB-F-W. Briefly, explaining each step, the bioprocessed black rice bran (BRB-F) was centrifuged (10,000 g, 10 min, 4˚C) and the supernatant was recovered to obtain BRB-F-S. Thereafter, nano-sized lipid particle impurities contained in BRB-F-S were extracted with methylene chloride, and an aqueous solution was obtained through liquid-liquid extraction to purify BRB-F-W. Low-molecular-weight impurities contained in BRB-F-W were removed by ultrafiltration (300 kDa cut-off), and a high-molecular fraction (UF retentate) was recovered to obtain BRB-F-P.

2.3. Mammalian Cell Cultures

The CT-26 mouse colon carcinoma cell line and the Yac-1 splenic natural killer cells (NK)-sensitive mouse lymphoma cell line, murine RAW 264.7 macrophage cell line from the American Type Tissue Culture Collection (Manassas, VA) were cultured in a modified DMEM containing 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS. Penicillin (100 U/mL) and streptomycin (100 mg/mL) were also added to the medium. Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. All cell lines used in this study were tested using a mycoplasma detection kit (Intron Biotechnology, Seongnam, Republic of Korea) before use.

2.4. Conditioned Medium Preparation

The cytotoxic effect of the conditioned medium containing macrophage-derived secretome on cancer cells was determined as follows. On day 1, RAW264.7 cells were cultured in 96-well plates (2×10^5 cells/well). On day 2, RAW 264.7 cells in 96-well tissue culture plates were treated with BRB, BRB-F and its purified fractions at three different concentrations in DMEM for 16 h. The treatment concentration of BRB-F and its isolated fractions was adjusted to ensure that the amount of polysaccharide contained in each isolated fraction was constant, and the treatment concentration for each sample was as follows. BRB, black rice bran extracts (10, 100, 1000 ng/mL); BRB-F, bioprocessed black rice bran extracts (10, 100, 1000 ng/mL); BRB-F-S, solid-liquid separation fraction of bioprocessed black rice bran extracts (4.03, 40.3, 403 ng/mL); BRB-F-W, water-soluble fraction of bioprocessed black rice bran extracts (2.97, 29.7, 297 ng/mL); BRB-F-P, polysaccharide fraction of bioprocessed black rice bran extracts (0.94, 9.4, 94 ng/mL). Simultaneously, CT-26 cells were cultured in 96-well plates (1×10^4

cells/well). On day 3, the culture supernatants of RAW264.7 cells were recovered and treated with CT-26 cells. After 16 h incubation, the cytotoxicity and the amounts of nitric oxide (NO) and TNF-α were measured in the same supernatant.

2.5. Nitric Oxide (NO) Generation Assay

Nitric oxide (NO) formation was measured by determining the concentration of its stable metabolite nitrite using a microplate assay as described by Narumi *et al.* [21], a method previously used in this laboratory. RAW 264.7 cells (2×10^5 cells/well) and CT-26 (1×10^4 cells/well) in a 96-well tissue culture plate were treated with BRB, BRB-F, and its purified products at three concentrations for 16 h. The treatment concentration of BRB-F and its isolated fractions was adjusted as in 2.4. After incubation, the culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N- [1-naphthyl] ethylenediamine dihydrochloride in 5% phosphoric acid) at room temperature for 15 min. The absorbance was then measured at 570 nm using a microplate reader against a standard of sodium nitrite.

2.6. Cell Cytotoxicity Assay

Cell cytotoxicity was assayed using the Cell Viability Assay Kit (EZCytox, DOGEN, Daejeon, Republic of Korea) according to the manufacturer's instructions. CT-26 cells (1×10^4 cells/well) were seeded in 96-well plates and incubated in 5% CO_2 incubator at 37˚C for 24 h. Then, BRB, BRB-F, and its purified products at three concentrations were cultured with conditioned media or vehicle for 16 h. The treatment concentration of BRB-F and its isolated fractions was adjusted as in 2.4. To measure cell cytotoxicity, 10 μL of the kit reagent was added to each well for 1 h. The culture medium was collected and measured at a wavelength of 450 nm absorbance using a microplate reader. Cell cytotoxicity was expressed by the following formula: % cytotoxicity = 100 × (absorbance of sample treated cell/absorbance of growth media treated cell).

2.7. Mice

Pathogen-free female BALB/c mice (6 weeks old) were purchased from Koatech (Gyunggi-do, Korea). The mice were housed in a stainless-steel cage under a 12 h light/dark cycle with a temperature range of 20˚C - 22˚C and relative humidity of 50% ± 10%. Mice were fed the pelletized normal commercial chow diet (Cat. No. 5 L79, Orient Bio, USA) and tap water *ad libitum* for 1 week after arrival for acclimation.

2.8. Tumor Transplantation Mouse Model and Treatment

BALB/c mice were intracutaneously transplanted with 1×10^6 cells of CT-26 mouse colon cancer cells in 200 μL of phosphate-buffered saline (PBS) into the lateral side of the back. The mice were divided into six groups (n = 10) and then

treated groups were orally administered with either of the extracts for 3 weeks (day 8 to 36). Representation: vehicle (−), negative control not tumor transplanted; vehicle (+), tumor transplanted mice positive control; BRB, black rice bran water extracts (40 mg/kg body weight); BRB-F-1, bioprocessed black rice bran extract (40 mg/kg body weight); BRB-F-S, solid-liquid separation fraction of bioprocessed black rice bran (16 mg/kg body weight); BRB-F-W, water-soluble fraction of bioprocessed black rice bran (12 mg/kg body weight); BRB-F-P, polysaccharide fraction of bioprocessed black rice bran (3.8 mg/kg body weight), mouse groups dietary administered with all extracts respectively. Control group mice were administered the same volume of PBS only. Mice were sacrificed at the end of the treatments for the isolation of peritoneal macrophages and excision of tumor masses and spleens (**Figure 1**).

2.9. Combination Therapy Model and Treatment

For the subcutaneous tumor model, a total of 1×10^6 cells of CT-26 mouse colon cancer cells were resuspended in 100 μL of phosphate-buffered saline (PBS) into the lateral side of the right back of the BALB/c (day 1) therapy with the PD-1 antibody was started when the tumor size reached 50 - 70 mm^3 (around day 8 - 10). Tumors were palpable within 10 days [22]. The mice were randomly divided into the following groups: CT-26 only, the CT-29 cell transplanted + saline treated group (10 mL/kg); *α*PD-1 only, the CT-29 cell transplanted + 200 μg/mouse PD-1 antibody; 40 mg/kg BRB-F, the CT-29 cell transplanted + 40 mg/kg BRB-F treated group; *α*PD-1 + 40 mg/kg BRB-F, the CT-29 cell transplanted + 200 μg/mouse PD-1 antibody + 40 mg/kg BRB-F treated group; BRB-F was administrated p.o. every day for 12 days (day 21). Mice were given the PD-1 antibody treatment through intraperitoneal (i.p.) injections every 3 days, for a total of four injections. Mice were sacrificed at the end of the treatments and tumor, peritoneal macrophages, NK cells, and serum were prepared from the normal and experimental groups of mice (**Figure 2**).

2.10. Tumor Growth

To evaluate the effects of each treatment, tumor masses were excised from the control and experimental groups of mice and weighed in an analytical balance.

2.11. NK Cell Cytolysis Assay

Spleen cells were isolated, and blood cells were removed as described by Trop *et al.* [23]. Spleens were crushed through a stainless mesh (size 60) in complete medium (CM) consisting of RPMI 1640 medium and 10% FBS plus antibiotics. The NK activity was evaluated as follows. Briefly, splenic mononuclear cells were obtained by centrifuging the spleen cell suspension on 2 mL of histopaque-1077 (Sigma Diagnostics, St. Louis, MO) to recover the cells in the interface, which were then washed three times with CM. The cells were resuspended in CM at 1×10^6 cells/mL. Yac-1 cells, used as the target cell, were labeled with Calcein-AM

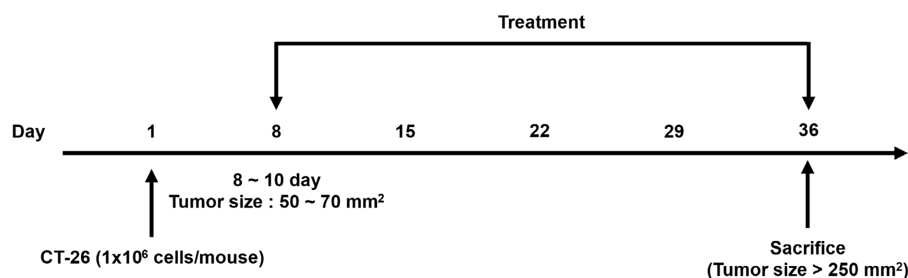

Figure 1. Scheme for tumor transplantation mouse model.

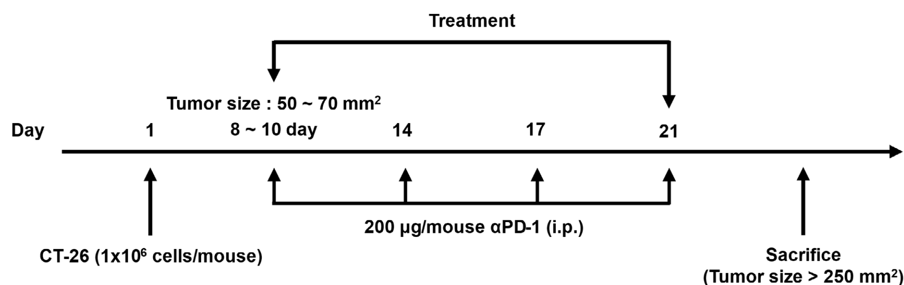

Figure 2. Scheme for combination therapy mouse model.

ester according to the method of Roden *et al.* [24]. Labeling of the cells (1×10^6 cells/mL) was in all cases performed at a final Calcein-AM concentration of 25 μM for 30 min. Purified mononuclear effector cells and labeled Yac-1 target cells were added to a 96-well plate and co-cultured for 3 h at 37˚C (20:1 effector: target ratio). Following centrifugation at 400 g, 100 μL of the supernatant from each well was harvested for measuring fluorescence released into medium using a spectrofluorometer (Molecular Devices, CA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Spontaneous fluorescence release was determined by culturing the labeled target cells in CM without effector cells. Maximum fluorescence was obtained from wells where target cells were incubated with a lysis buffer (50 mM sodium borate, 0.1% triton X-100, pH 9.0). Specific lysis was calculated according to the following formula:

% lysis = [1 − (experimental fluorescence—background fluorescence)/ (maximum fluorescence—background fluorescence)] × 100.

2.12. Nitrite Production Measurement of Peritoneal Macrophage

Isolation and purification of peritoneal macrophage cells from tumor-bearing mice treated with the bioprocessed black rice bran and its purified products were performed according to the method of Narumi *et al.* [21]. Peritoneal cells exudated through lavaging with HBSS reagent were plated onto 60 mm tissue culture dishes ($1 - 5 \times 10^6$ cells/dish) to produce macrophage cells firmly adhered onto the dishes. NO was measured by determining the concentration of its stable oxidative metabolite nitrite, using the microplate method described by Xie *et al.* [25] with slight modification. Briefly, isolated peritoneal macrophages were cultured in a 96-well plate (1×10^5 cells/well) with rIFN-γ (10 U/mL) and LPS (100

ng/mL) for 48 h. To measure nitrite concentrations, culture medium (100 μL) was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% N-(naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid] at room temperature for 15 min. The absorbance at 570 nm was determined with a microplate reader using a standard calibration curve for sodium nitrite.

2.13. Phagocytic Uptake Assay

The phagocytotic activity of peritoneal macrophage cells was measured following the method of Duperrier *et al.* [26] with slight modification. Briefly, isolated peritoneal macrophages were cultured in a 60 mm culture dish (1×10^5 cells) with rIFN-γ (10 U/mL) and LPS (100 ng/mL) for 48 h. After stimulation, cells (1×10^4 cells) were resuspended in PBS (1 mL) containing 5% FBS and cultured at 37˚C for 15 min. They were then incubated with Dextran-FITC (1 mg/mL) at 37˚C for 1 h. The reaction was stopped with cold PBS contacting 5% FBS and 1% sodium azide. The cells were then washed three times with cold PBS and analyzed on a FACSvantage instrument (Becton-Dickinson, Frankin Lakes, NJ).

2.14. Histology of Tumors and Assessment of Tumor Vascularity

For histologica1 analysis, the tumor tissue of the mice was fixed with 4% paraformaldehyde in 0.5 M phosphate buffer (pH 7.4). The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted onto glass slides. The sections were then dewaxed using xylene and ethanol and stained with hematoxylin and eosin Y (H&E). Blood vessels were counted in six blindly chosen random fields under the microscope at 200x magnification, and the microvessel density was recorded.

2.15. Enzyme-Linked Immunosorbent Assay (ELISA) of Cytokines and Eicosanoids

Extraction of eicosanoids prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) from tumor tissues was conducted by a described method. Briefly, tumor tissues from tumor-bearing mice treated with the bioprocessed black rice bran and its purified products were homogenized in a phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), and 10 mM ethylenediamine tetraacetic acid (EDTA). The homogenates were microcentrifuged at 14,000 g for 15 min at 4˚C to recover the supernatant. For quantitation of cytokines, peritoneal macrophages from each mice group were stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL) followed by recovery of the culture medium. Cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the culture medium and eicosanoids LTB_4 and PGE_2 in the supernatants were determined by ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The absorbance of the final solution at 420 nm was measured in a microplate reader.

2.16. Statistical Analysis

Results are expressed as the mean ± standard deviation (SD) of three independent experiments. Significant differences between means were determined using the Statistical Analysis Software package SAS (Cary, NC). $p < 0.05$ is regarded as significant.

3. Results

3.1. Effect of Bioprocessed Black Rice Bran on Tumor Cell Cytotoxicity through Macrophages

The direct or indirect cytotoxicity of BRB, BRB-F and sequentially purified fractions of BRB-F (BRB-F-S, BRB-F-W, BRB-F-P) against CT-26 cells was evaluated. The treatment of the developed material was BRB (10, 100, and 1000 ng/mL), BRB-F (10, 100, and 1000 ng/mL), and sequentially purified fractions of BRB-F, BRB-F-S (4.03, 40.3, and 403 ng/mL), BRB-F-W (2.97, 29.7, and 297 ng/mL), and BRB-F-P (0.94, 9.4, and 94 ng/mL), according to their respective extraction recovery rate. The results showed that all materials did not exhibit direct cytotoxicity in CT-26 cells. By contrast, treatment of CT-26 with the supernatant recovered after treatment of each material with RAW264.7 cells showed a concentration-dependent cytotoxic effect, and all purified fractions had more than a 2-fold higher cancer cell killing activity compared to BRB (**Figure 3**).

3.2. Effect of Bioprocessed Black Rice Bran on Macrophage Activation and TNF-α Release through Macrophages

Supernatants of RAW264.7 cells treated with each material were subjected to CT-26, and the amounts of NO and TNF-α were measured in the supernatants obtained. We measured NO production as an indicator of macrophage activation and found that BRB-F and its purified fractions treatment had a significant NO production effect. TNF-α production was measured in the same supernatant. BRB-F and its purified fractions treatment induced TNF-α production approximately 5-fold higher than BRB treatment (**Figure 4**). When the supernatants of macrophages cultured after treatment with each material was exposed to CT-26, there was no loss of NO and TNF-α before and after treatment, and direct treatment of each material with CT-26 resulted in no NO and TNF-α production (data not shown). Taken together, the *in vitro* results suggest that BRB-F and its purified fractions induce macrophage activation and induce cancer cell death via the macrophage-derived secretome. Notably, these effects were maintained during purification, and seemed to be mediated mostly by the polysaccharides contained in BRB-F.

3.3. Effect of the Bioprocessed Black Rice Bran and Its Purified Products on Growth of Tumors

To evaluate the anti-cancer activity of BRB-F and its isolated fractions in CT-26 tumor-bearing mice, CT-26 cells were injected subcutaneously at a concentration

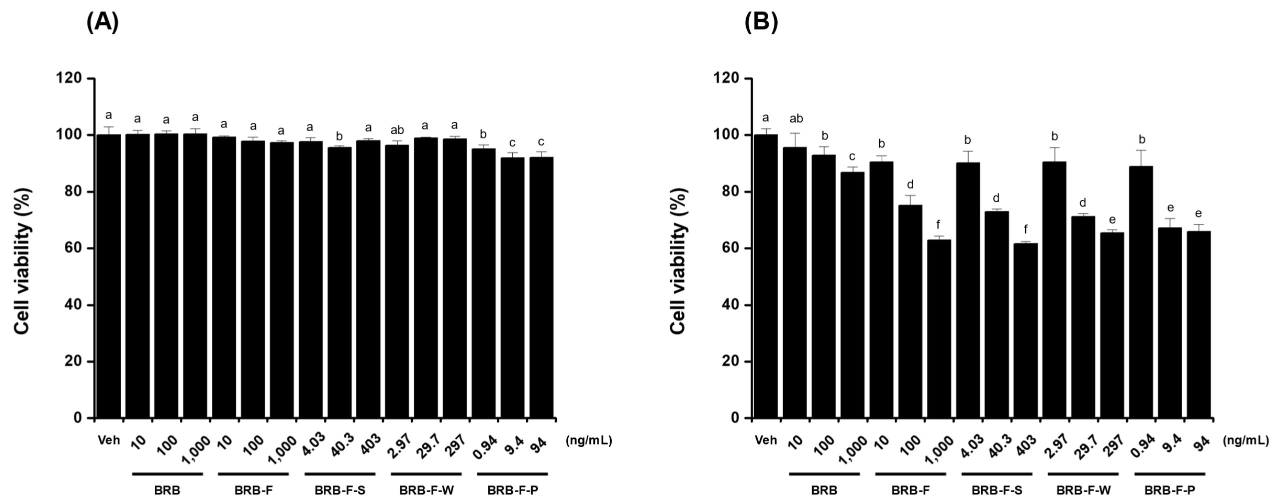

Figure 3. Direct and indirect cytotoxicity of bioprocessed black rice bran on CT-26 colon cancer cells. Direct cytotoxicity is assessed such that CT-26 cells were incubated with BRB (10, 100, 1000 ng/mL), BRB-F (10, 100, 1000 ng/mL), BRB-F-S (4.03, 40.3, 403 ng/mL), BRB-F-W (2.97, 29.7, 297 ng/mL), BRB-F-P (0.94, 9.4, 94 ng/mL). Indirect cytotoxiicity is assessed so that the conditioned medium contains the RAW264.7 cells-derived secretome in which then CT-26 cells are incubated. Data are expressed as the mean ± SD of triplicate experiments. Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

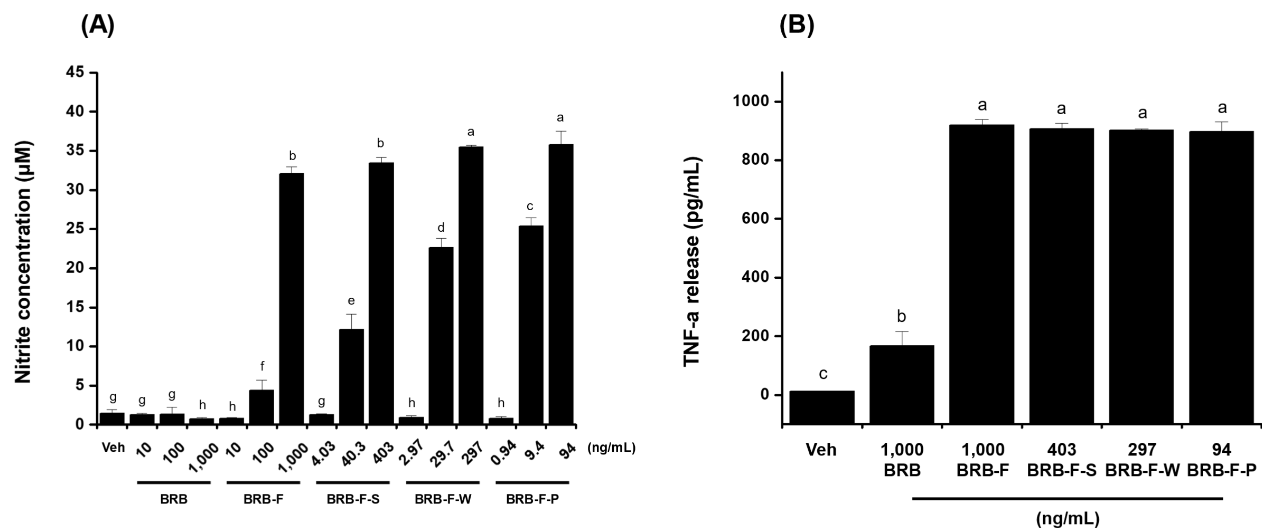

Figure 4. Effect of bioprocessed black rice bran on macrophage activation. RAW264.7 cells were incubated with or without serially diluted BRB (10, 100, 1000 ng/mL), BRB-F (10, 100, 1000 ng/mL), BRB-F-S (4.03, 40.3, 403 ng/mL), BRB-F-W (2.97, 29.7, 297 ng/mL), BRB-F-P (0.94, 9.4, 94 ng/mL) for 16 h. After, Conditioned medium is recovered and incubated with CT-26 (1×10^4 cells/well) for 16 h. The amount of NO and TNF-α in the medium is expressed as the mean ± SD of triplicate experiments. Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

of 1×10^6 cells/mouse to induce tumors. Tumors were weighed to evaluate tumor growth inhibition, and we observed tumor growth inhibition of 20%, 38%, 43%, 41%, and 41% in mice treated with BRB, BRB-F, and BRB-F-derived isolated fractions of BRB-F (BRB-F-S, BRB-F-W, BRB-F-P), respectively, compared to tumor-bearing mice (**Figure 5A**, **Figure 5C**). Notably, the tumor growth inhibitory effect of BRB-F showed about a 2-fold increase compared to BRB-treated mice. Histological examination of tumors harvested from tumor-bearing

mice confirmed the excellent effect of reducing neovascularization in the tissue (**Figure 5B**). Since the tumor growth inhibitory activity and angiogenesis inhibitory effect did not show any changes with purification, these effects were mostly attributed to the polysaccharides contained in BRB-F.

3.4. Inhibitory Effect of Eicosanoids in Tumor Microenvironment

The involvement of inflammation in cancer progression has been the subject of research for many years [27]. Therefore, to determine the effect of administration of the developed materials on the tumor microenvironment, the production of tumor growth-related eicosanoids (PGE_2 and LTB_4), which regulate angiogenesis and tumor immune evasion in tumor tissue, was evaluated. The results

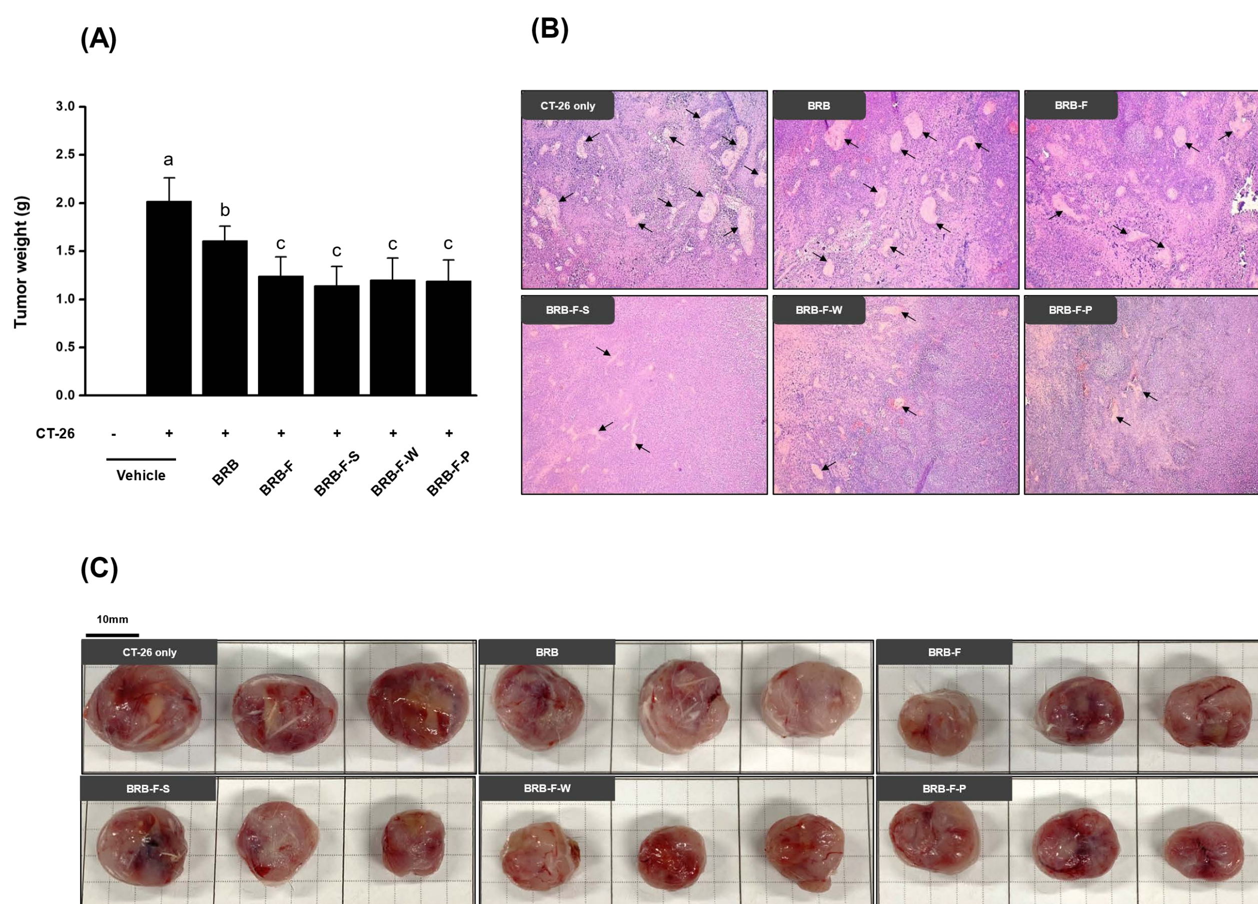

Figure 5. Effects of the bioprocessed black rice bran and its purified products on tumor growth in vivo. Mouse groups were dietarily administered with all extracts. BALB/c mice were intracutaneously transplanted with CT-26 mouse colon cancer cells (1x106 cells, 200 μL). After 1 week, each group was fed a standard diet supplemented with BRB, BRB-F and BRB-F purified products (BRB-F-S, BRB-F-W, BRB-F-P) and control without supplementation. (A) After 4 weeks, mice were sacrificed to measure tumor weight. (B) To observe blood vessel formation, paraformaldehyde-fixed and paraffin-embedded tumor sections were stained with hematoxylin and eosin Y (H&E). (C) Size distribution of the tumor tissues in six groups. Representation: vehicle (-), negative control not tumor-transplanted; vehicle (+), tumor-transplanted mice positive control; BRB, black rice bran water extracts (40 mg/kg body weight); BRB-F, bioprocessed black rice bran extract (40 mg/kg body weight); BRB-F-S, solid-liquid separation fraction of bioprocessed black rice bran (16 mg/kg body weight); BRB-F-W, water-soluble fraction of bioprocessed black rice bran (12 mg/kg body weight); BRB-F-P, polysaccharide fraction of bioprocessed black rice bran (3.8 mg/kg body weight), Results are expressed as means ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

showed that BRB, BRB-F, and BRB-F-derived isolated fractions (BRB-F-S, BRB-F-W, BRB-F-P) inhibited the production of PGE_2 by 4.9%, 40.9%, 43.2%, 41.8%, and 39.9%, respectively, and inhibited the production of LTB_4 by 10.2%, 39.8%, 42.1%, 36.5%, and 41%, respectively. In particular, the inhibitory activity of BRB-F and BRB-F-derived isolated fractions against eicosanoid production was found to be significantly higher than that of BRB (**Table 1**). Considering that the inhibitory activity did not change with purification, it seems that eicosanoid inhibitory activity was mostly originated from the polysaccharide fraction.

3.5. Effect of Macrophage Activation in Tumor-Transplanted Mice

Published studies indicate that physiological activities of macrophage cells are suppressed in tumor-transplanted animals [28]. Therefore, we isolated peritoneal macrophages from tumor-bearing mice and tested their activation. The isolated peritoneal macrophages were treated with IFN-γ and LPS to induce activation, and the production of nitrite, cytokines (TNF-α, IL-1β, IL-6), and activation of phagocytosis were measured as indicators of activation. The results showed that, compared to normal mice, peritoneal macrophages isolated from tumor-bearing mice were more than 50% less activated in the immune response. Administration with BRB slightly restored the immune response, but administration with BRB-F and its isolated fractions (BRB-F-S, BRB-F-W, BRB-F-P) induced macrophage activation similar to that of normal mice (**Table 2**). As there was no increase or decrease in activity due to purification, the macrophage activation effect was mostly attributed to the polysaccharide fraction.

3.6. Effect of NK Cell Activation in Tumor-Transplanted Mice

In addition to the macrophage activation effect of the developed material, we also confirmed the activation effect of NK cells, which play an important role in tumor immune response. The spleen was removed from tumor transplanted mice to isolate NK cells present in the spleen, and the killing activity of NK cells against YAC-1 cells was measured using fluorescently labeled YAC-1 cells as target cells. As a result, it was confirmed that tumor transplanted mice induced a slightly higher level of NK cell activation than normal mice. While the activation of NK cells was slightly increased in BRB-treated mice compared to tumor-bearing mice, the activation of NK cells in mice treated with BRB-F and BRB-F-derived isolated fractions (BRB-F-S, BRB-F-W, and BRB-F-P) was all increased more than 4-fold, confirming that administration of BRB-F and BRB-F-derived isolated fractions can induce a superior tumor immune response (**Figure 6**). As there was no change in activity with purification, the NK cell activation effect was mostly attributed to the polysaccharide fraction, suggesting that administration of the BRB-F and its isolated fractions can induce tumor suppression through restoration and activation of innate immune responses suppressed by tumor proliferation.

Table 1. Inhibitory effect of bioprocessed black rice bran and its purified products on release of eicosanoids in tumor-transplanted mice.

Sample	Eicosanoids (pg/mL)	
	PGE_2	LTB_4
CT-26 only	850.9 ± 59.8[a]	1873.7 ± 202.7[a]
BRB	809.4 ± 92.4[a]	1682.9 ± 185.4[a]
BRB-F	502.7 ± 62.8[b]	1128.4 ± 109.5[b]
BRB-F-S	483.3 ± 51.7[b]	1085.4 ± 112.7[b]
BRB-F-W	494.8 ± 42.8[b]	1189.7 ± 134.9[b]
BRB-F-P	511.7 ± 50.9[b]	1105.6 ± 128.5[b]

Values are expressed as means ± SDs (n = 10) in each column with the same letters are not significantly different at $p < 0.05$.

Table 2. Bioprocessed black rice bran and its purified products stimulate release of pro-inflammatory cytokines in peritoneal macrophages from tumor-transplanted mice.

Sample	Macrophage activity				
	Nitrite (μM)	Phagocytosis (%)	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
Normal	27.25 ± 3.13[b]	72.73 ± 8.83[b]	4285.3 ± 451.7[a]	251.7 ± 30.7[b]	403.8 ± 38.4[a]
CT-26 only	5.49 ± 0.47[d]	33.27 ± 5.17[c]	1938.4 ± 208.5[b]	108.5 ± 15.8[d]	121.6 ± 19.4[c]
BRB	11.28 ± 1.58[c]	41.56 ± 6.92[c]	2217.2 ± 209.4[b]	131.8 ± 14.4[c]	157.9 ± 16.8[b]
BRB-F	31.79 ± 2.54[a]	80.93 ± 7.24[a]	4048.2 ± 492.7[a]	285.4 ± 32.7[a]	411.9 ± 37.2[a]
BRB-F-S	34.26 ± 4.14[a]	83.42 ± 8.89[a]	4169.2 ± 438.2[a]	301.7 ± 28.9[a]	420.8 ± 48.2[a]
BRB-F-W	33.52 ± 3.29[a]	81.19 ± 9.54[a]	4282.5 ± 441.9[a]	288.9 ± 33.6[a]	404.4 ± 44.9[a]
BRB-F-P	34.17 ± 4.08[a]	84.43 ± 10.72[a]	4172.7 ± 382.5[a]	308.4 ± 19.8[a]	412.5 ± 40.5[a]

Values expressed as means ± SDs (n = 10) in each column with the same letters are not significantly different at $p < 0.05$. Normal mice macrophages are stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL). Tumor-transplanted mice macrophages are stimulated with rIFN-γ and LPS.

3.7. Effects of the Combination Therapy with Bioprocessed Black Rice Bran and PD-1 Antibody on Growth of Transplanted Tumor

Previous studies have shown that when the CT-26 colon cancer cell line is transplanted into mice and treated with the bioprocessed black rice bran, cancer cell proliferation is effectively suppressed through activation of the tumor-related immune response. We evaluated the synergistic effect of the combination treatment of immune checkpoint inhibitor and BRB-F in the tumor transplantation mouse model.

As a result of confirming the cancer cell proliferation inhibitory effect of single and combined treatment of the PD-1 antibody and BRB-F in tumor-transplanted mice, we found that the PD-1 antibody and BRB-F alone inhibited tumor weight growth by 42.9% and 40.5%, respectively, and the PD-1 antibody and BRB-F combined inhibited tumor weight growth by 63.5% (**Figure 7**). Compared to the single treatment with either PD-1 antibody or BRB-F, the

combined treatment resulted in an increased tumor growth inhibitory effect of approximately 1.5 times, so it was confirmed there was a synergistic effect of the combined treatment of the two materials.

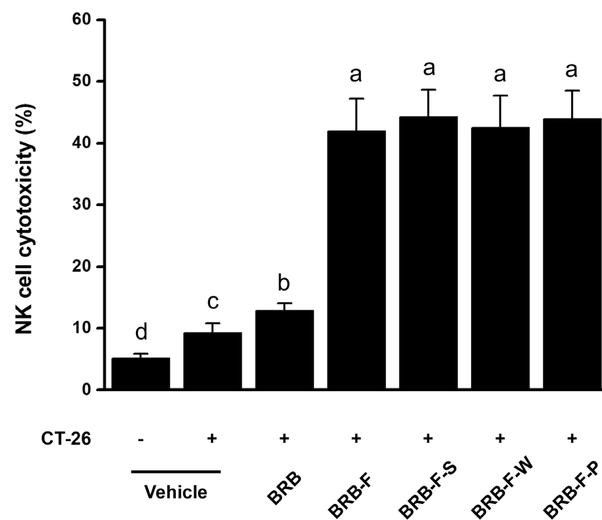

Figure 6. Effect of the bioprocessed black rice bran and its purified products on NK cytotoxicity activities. NK cells from tumor-transplanted mice subjected to orally administered with black rice bran extracts were incubated with Yac-1 target cells labeled with Calcein-AM for 3h (20:1 effector:target ratio). The NK cytolyic activity was evaluated by measuring Calcein-AM release from Yac-1 target cells using a fluorometer. Results are expressed as means ± SDs (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

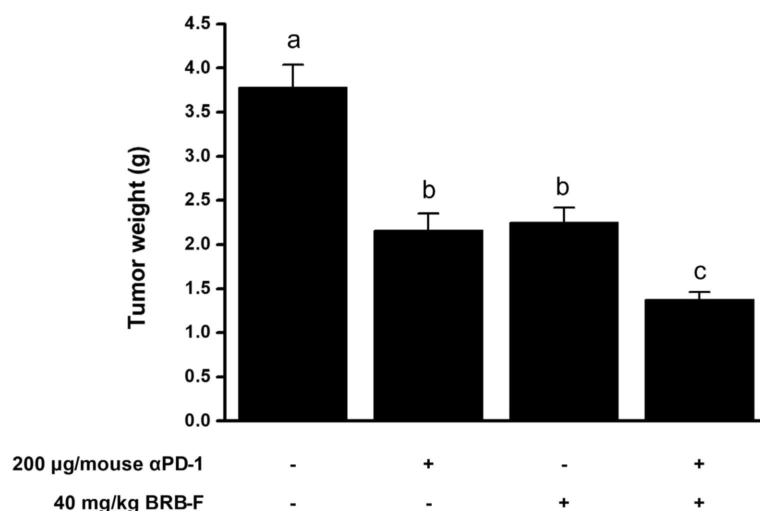

Figure 7. Effects of the combination therapy with bioprocessed black rice bran and the PD-1 antibody on tumor growth in vivo. BALB/c mice were intracutaneously transplanted with CT-26 mouse colon cancer cells (1×10^6 cells, 200 μL). Each group was fed a standard diet supplemented with BRB-F at 40 mg/kg and intraperitoneally administrated *α*PD-1 at 200 μg/mouse four times at 3-day intervals for 12 days after tumor induction. After 21 days, mice were scarified to measure tumor weight. Results are expressed as means ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

3.8. Inhibitory Effect of Eicosanoids in Combination Therapy Model

To evaluate the effect of the PD-1 antibody and BRB-F treatment on the tumor microenvironment, we determined the production of tumor growth-related eicosanoids in tumors. The results showed that the PD-1 antibody alone, BRB-F alone, and the combination of the PD-1 antibody and BRB-F inhibited the production of PGE_2 by 40.7%, 43.9%, and 56.9%, respectively, and inhibited the production of LTB_4 by 28.1%, 34.5%, and 56.9%, respectively (**Table 3**). In the case of the combined administration of the PD-1 antibody and BRB-F, it was confirmed that the activity of inhibiting the production of PGE_2 and LTB_4 was higher than that of the single treatment, and it is believed that the anticancer effects can be exhibited through more effective tumor growth factor inhibition.

3.9. Effect of Macrophage Activation in Combination Therapy Model

To evaluate the effect of the PD-1 antibody and BRB-F treatment on the tumor-related immune response, peritoneal macrophages were isolated from tumor-bearing mice and their activation was measured. Isolated peritoneal macrophages were treated with rIFN-γ and LPS to induce activation, and the production of nitrite, TNF-α, IL-1β, and IL-6 and the phagocytic capacity of macrophages were measured as indicators of activation (**Table 4**). The results demonstrated that the PD-1 antibody-treated mice showed little change in macrophage immune response, but mice treated with BRB-F alone and in combination with the PD-1 antibody showed more than double the macrophage immune response. These results suggest that the combination of PD-1 antibody and BRB-F may induce a stronger tumor immune response than the PD-1 antibody alone.

3.10. Effects of NK Cell Activation in Combination Therapy Model

In addition to the macrophage activation effect, we also confirmed the activation effect of NK cells, which plays an important role in the tumor-related immune response. The spleen was removed from tumor transplanted mice to isolate NK cells present in the spleen, and the killing activity of NK cells against YAC-1 cells was measured by targeting fluorescently labeled YAC-1 cells. The results demonstrated that the PD-1 antibody-treated mice showed a slight increase in NK cell activation compared to tumor-bearing mice, while BRB-F-treated mice showed an approximately 3-fold increase in NK cell activation compared to tumor-bearing mice (**Figure 8**). In particular, the activation of NK cells in mice treated with BRB-F in combination with the PD-1 antibody was increased by more than 4-fold, suggesting that BRB-F can induce an excellent tumor-related immune response even in combination with the PD-1 antibody. Taken together, these results suggest that the combination of the PD-1 antibody and BRB-F, compared to the PD-1 antibody alone, can induce effective tumor growth inhibi-

tion by inducing an additional tumor-related immune response through activation of the innate immune response, including activation of macrophages and NK cells by BRB-F, in addition to the anticancer effect of the PD-1 antibody.

4. Discussion

Cancer is a proliferation of cells resulting in unregulated tumor growth and metastasis, associated with changes in so-called biomarkers (antigens) and signaling pathways. Treatment includes the use drugs (chemotherapy) that destroy, preferably selectively, the cancer cells [29]. Colorectal cancer is the third leading cause of cancer-related fatalities with about 200,000 annual deaths in the United States despite early diagnosis and treatment progress [30].

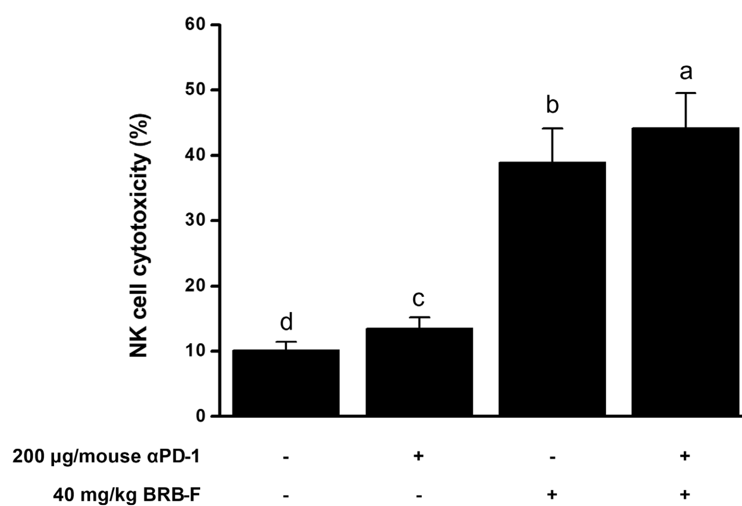

Figure 8. Effect of the combination therapy with bioprocessed black rice bran and the PD-1 antibody on NK cytotoxicity activities. NK cells from tumor-transplanted mice were incubated with Yac-1 target cells labeled with Calcein-AM for 3 h (20:1 effector:target ratio). The NK cytolyic activity was evaluated by measuring Calcein-AM release from Yac-1 target cells using a fluorometer. Results are expressed as means ± SD (n = 10). Bars sharing a common letter are not significantly different between groups at $p < 0.05$.

Table 3. Inhibitory effect of the combination therapy with bioprocessed black rice bran and PD-1 antibody on release of eicosanoids in tumor-transplanted mice.

Sample	Eicosanoids (pg/mL)	
	PGE_2	LTB_4
CT-26 only	816.3 ± 93.7[a]	1954.7 ± 214.9[a]
*α*PD-1 only	483.9 ± 54.1[b]	1405.5 ± 133.7[b]
40 mg/kg BRB-F	458.2 ± 55.8[b]	1280.5 ± 152.8[b]
*α*PD-1 + 40 mg/kg BRB-F	351.7 ± 31.4[d]	889.4 ± 72.5[c]

Values are expressed as means ± SDs (n = 10) in each column with the same letters are not significantly different at $p < 0.05$.

Table 4. The combination therapy with bioprocessed black rice bran and PD-1 antibody stimulate release of pro-inflammatory cytokines in peritoneal macrophages from tumor-transplanted mice.

Sample	Macrophage activity				
	Nitrite (μM)	Phagocytosis (%)	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
CT-26 only	8.29 ± 0.54^d	41.19 ± 5.27^b	1528.4 ± 132.5^c	95.7 ± 10.7c	126.8 ± 10.5^c
αPD-1 only	11.73 ± 1.52^c	45.58 ± 5.11^b	1944.5 ± 152.5^b	125.9 ± 16.7^b	162.8 ± 19.9^b
40 mg/kg BRB-F	31.81 ± 2.79^b	80.54 ± 7.29^a	3529.8 ± 407.9^a	257.1 ± 18.9^a	385.9 ± 42.5^a
αPD-1 + 40 mg/kg BRB-F	35.29 ± 2.69^a	83.76 ± 10.89^a	3382.5 ± 284.3^a	238.6 ± 30.8^a	401.7 ± 34.8^a

Values are expressed as means ± SDs (n = 10) in each column with the same letters are not significantly different at $p < 0.05$. Tumor-transplanted mice macrophages stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL).

In a previous study [9], we reported that, compared to the control diet without rice brans, tumor weights of mice fed diets with added black and brown rice brans that were intracutaneously inoculated with CT-26 colon cancer cells decreased by 35% and 19%, respectively, by the end of the two-week trial. The inhibition of tumor growth was associated with increased cytolytic activity of splenic NK cells, partial restoration of nitric oxide production and phagocytosis in peritoneal macrophages, released tumor necrosis factors from macrophages, reduction of angiogenesis (blood flow) inside the tumor, reduction in pro-angiogenic biomarker in mRNA and protein expression and other-cancer related biomarkers. Our related observations were that bioprocessed (fermented) black rice bran showed other beneficial effects, including inhibition of alcohol-induced hangovers [13] and allergic asthma in mice [12]. An edible alga bioprocessed with mushroom mycelia also protected mice against allergic asthma [31]. The multiple reports on the potentiating effects of the combination of medical chemotherapies and immune checkpoint inhibitors in human trials, as mentioned in the Introduction section induced us to determine if an immune inhibitor would also potentiate the anti-tumor effect of bioprocessed black rice bran and fractions isolated from the culture described in our anti-asthma study [12].

Figure 5 shows the size distribution of tumors as well as tumor weights of six isolated bioprocessed fractions without a checkpoint inhibitor. **Figure 7** shows the effects on tumor weights of one bioprocessed black rice bran diet (BRB-F with a high polysaccharide content) in combination with the administered PD-1 antibody inhibitor. The data show that the average tumor size in control mice is 3.78 grams. The average tumor size in mice treated with the PD-1 antibody is 2.16 grams. The average tumor size in mice treated with BRB-F is 2.25 grams. The average tumor size in mice treated with combined BRB-F and PD-1 antibody is 1.13 grams. We can summarize the data as follows. BRB-F or anti-PD-1 antibody alone each reduce tumor size by 40.5% or 42.9%, whereas the combina-

tion of BRB-F and PD-1 antibody reduces tumor size by 63.5%, with their cooperative effect being statistically significant. We used the BRB-F diet for the combination study because we also previously reported in a related study that mushroom polysaccharides showed an anti-tumor effect in mice like that of black rice bran [1] [9].

The following mechanism seems to govern the potentiation of tumor growth inhibitory activity of the checkpoint inhibitor PD-1 [18]. The PD-1 antibody binding to PD-1 on T-cells prevents T-cells from receiving an inhibitory signal from PD-1 ligands (PDL1 and PDK2) expressed on cancer and on myeloid cells such as macrophages present in the tumor. As a result of PD-1 antibody binding, the immune T cells become activated (less exhausted) so can better attack and destroy the cancer cells.

Additional experimental data on associated biomarkers shown in the Tables and Figures seem to be generally consistent with the anti-tumor effects. **Table 1** shows the relative potencies of the release of two eicosanoids (PGE_2 and LTB_4) in the control diet (CT-26 only), the not-bioprocessed black rice bran (BRB) and the four bioprocessed fractions (BRB-F, BRB-F-S, BRB-F-W, and BRB-F-P). The first two diets showed high values and the values for the other four isolates are much lower. **Table 2** shows trends in the release of pro-inflammatory cytokines in peritoneal microphages from tumor-transplanted mice by seven diets, **Table 3** shows the inhibitory effect of the combination treatment of BRB-F and anti-PD-1 on eicosanoid content. The value for PGE_2 and LTB_4 significantly decreased for both PD-1 and the two BRB-F diets. **Table 4** shows the increases in macrophage active biomarkers (nitrite, phagocytosis, TNF-α, IL-1β, and IL-6) elicited by the four diets, reinforcing the results in **Table 3**. Except for **Figure 4**, the graphical presentation of the data in **Figures 5-8** seem to confirm the trends shown in the Tables.

These observations indicate that inhibition of tumor growth is accompanied by a variety of biomarkers and signaling pathways associated with tumor formation and inhibition. That an immune checkpoint inhibitor potentiates the anti-tumor effect of a food product suggests it might also beneficially impact other food-related anti-carcinogens we studied, including the black rice bran component γ-oryzanol [8], mushroom polysaccharides [14] [28], the tomato glycoalkaloid tomatine [32] [33] [34], as well as potato and eggplant glycoalkaloids [35]. Also, what is particularly noteworthy is that the anticancer activity of BRB-F was not lost during to the purification and isolation steps. These results are consistent with our previous asthma suppression studies [12] and suggest that most of the anticancer activity seems to be due to the immunoactive polysaccharides contained in BRB-F, and that small-sized molecules other than the immunoactive polysaccharides do not contribute to the anticancer effect. Moreover, the results in **Figure 3** show that the polysaccharides contained in BRB-F and purified fractions do not have direct cancer-cell-killing activity, but are believed to inhibit the proliferation of cancer cells through the activation of immune cells.

Considering these results, it is likely that BRB-F and its purified fractions have the potential to be developed as immune checkpoint inhibitor combination therapy drugs with their own anticancer activity. Clinicians are challenged to demonstrate this possibility with human patients.

5. Conclusion

In conclusion, the described results show that administering the polysaccharide-rich functional food obtained from the culture of bioprocessed black rice bran and mushroom mycelia to mice with transplanted colon tumors significantly reduced tumor size by approximately 48%, as compared to the control diet without the added new food formulation. Several additional fractions isolated from the culture showed similar or lower reductions in tumor size. The administration of an immune checkpoint inhibitor potentiated (increased) the reduction from about 48% to 63%, which was significant. Studies associated with the possible mechanism of the inhibition of tumor growth showed that it was accompanied by the following *in vivo* biomarkers: eicosanoids, pro-inflammatory cytokines (nitrite, phagocytosis, TNF-α, IL-1β, IL-6), and NK cells, as well as histology of tumor tissues. It seems that the mechanism that governs the anti-tumor effects is like that reported for medical human chemotherapies. Also noteworthy is the finding of a reported human trial that found short-term consumption by elderly cancer patients of a cereal diet supplemented with 5% black rice bran and 0.5% arabinoxylan powders induced the formation of some the mentioned biomarkers as well as an apparent improvement in their quality of life, suggesting that there is a need for future clinical studies to investigate if dietary bioprocessed black rice bran food products supplemented to widely consumed foods such as breads, flatbreads [36], corn-based tortillas [37] [38], soy-based tofu [39] [40], cooked white rice [41] as well as other human foods and animal feeds can help ameliorate and/or prevent human carcinomas. It would also be of interest to determine if black rice bran and the black rice bran bioprocessed functional food would inhibit the heat-induced formation of the potentially carcinogenic compound acrylamide [42] in plant-based foods and heterocyclic amine compounds in animal-based foods [43]. Finally, we believe that characterization of the polysaccharide will be necessary through follow-up research, and that the isolated polysaccharide is expected to have potential to be developed as a pharmaceutical medicine that could be used in human and animal therapies of cancer and other diseases.

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Authors' Contributions

SPK conceived the idea and with KHL, KSK, WSH, WYL, JK, and SJL conducted

the experimental studies; MF and SPK interpreted the results and prepared a draft of the paper. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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