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Abstract: To investigate the bioactivities of fresh garlic and its processed product, black garlic, we conducted comparative analyses of antioxidant, anti-inflammatory, innate immune activation and anti-cancer activities, in addition to chemical composition (sugar, amino acid, polyphenol contents) of these materials. Simultaneous assay using neutrophil-like cells showed that fresh garlic exhibited antioxidant and innate immunostimulatory activities, whereas black garlic displayed a potent anti-inflammatory effect. The antioxidant activity index was correlated with phenol and flavonoid contents, while the innate immunostimulatory activity was correlated with fructan content. Furthermore, some black garlics with low fructose content were found to inhibit the proliferation of UM-UC-3 cancer cells, while other black garlics rich in fructose increased UM-UC-3 cell proliferation. It was shown that the thermal processing steps of fresh garlic could change the composition of sugars, antioxidants, and amino acids, which have different effects on neutrophil-like cells and UM-UC-3 cells, as well as on bioactivities.

Keywords: *Allium sativum*; black garlic; functional food; anticancer activity; processed food

1. Introduction

Garlic (*Allium sativum* L.) bulb is believed to contain various bioactive substances that contribute to health, such as organosulfur compounds, phenolic compounds, and polysaccharides [1], and is used worldwide as a spice and ingredient in other foods and pharmaceuticals. Garlic has been cultivated and used since ancient times; it was already being cultivated and eaten in ancient Egypt [2]. Garlic is also known to produce a variety of bioactive components and exhibit different bioactivities depending on the method of preparation or processing. Black garlic is a processed food produced by aging fresh garlic for 10 to 40 days at 60 to 90 °C under a humidity-controlled environment of 70 to 90% [3]. Black garlic is characterized by dark brown turned scales, which is due

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to the Maillard reaction [4]. Black garlic has no pungent smell peculiar to garlic and has a sweet taste like dried fruit. Furthermore, black garlic has been reported to exhibit a number of bioactivities, including antioxidant, anti-inflammatory, and anti-cancer effects [5-7]. Sulfides, cysteine derivatives, allicin, and other sulfur-containing compounds have been proposed as key factors in garlic's antioxidant and other bioactivities [8-10]. On the other hand, non-sulfur compounds commonly found in plants, such as polyphenols, are also known to have health-promoting effects [11, 12]. The biological properties of garlic may be due to the synergistic effects of various phytochemicals in garlic and their proportions. However, few studies have conducted comprehensive comparative analyses of the differences in bioactivities and content composition among the varieties of garlic used to produce black garlic. Therefore, there are still many unclear points regarding changes in the chemical compositions during the production process of black garlic and the relationship between the various biological activities and active compounds. As indices for evaluating the bioactivity of foods, many *in vitro* experimental systems, such as radical scavenging and chemiluminescence, are used in experiments to evaluate antioxidant effects, and many experimental systems using model experimental animals are used to evaluate anti-inflammatory effects. However, these systems do not accurately reflect *in vivo* effects and have cost and ethical problems. Therefore, the Simultaneous Evaluation Cell Assay for Antioxidant, Anti-inflammatory, and Innate Immune Activation was developed by Kazumura *et al*, which utilizes the biological defense response of innate immune cells to simultaneously measure not only the reactive oxygen species (ROS) scavenging capacity of foods, but also the effects of foods on innate immune function [13].In this study, the bioactivities of fresh and black garlic was evaluated by four methods: (1) evaluation of antioxidant, anti-inflammatory, and innate immune-activation effects using the Simultaneous Evaluation Cell Assay described above, (2) measurement of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, (3) measurement of the viability of cancer cells, (4) evaluation of total phenol, flavonoid compounds, carbohydrate, and amino acids contents.

2. Results and Discussion

2.1. Simultaneous Monitoring of Superoxide and Intracellular Calcium Ions in Neutrophils by Chemiluminescence and Fluorescence

Simultaneous monitoring of superoxide and calcium ion concentrations in neutrophil-like cells stimulated with chemotactic peptide f-MLP by chemiluminescence and fluorescence, respectively, was used to evaluate bioactivities [13]. The bioactivities evaluated in this experiment were antioxidant, anti-inflammatory, and innate immune-activation effects. Compared to the control, if calcium ions were taken up normally and superoxide (O_2^-) was scavenged by the sample, it was determined to be an antioxidant effect. If calcium ion uptake into the cells was inhibited and superoxide production was suppressed, it was determined to be anti-inflammatory. When calcium ion uptake was similar to or higher than that of the control and superoxide production was higher, it was determined to be an innate immune-activation effect. In fresh garlic, with the exception of 'White-roppen' (obtained in 2019), the calcium ion influx was kept stable, and the concentration of O_2^- was significantly reduced as compared to the control at extract concentrations of 0.3 to 3.0 × 10^{-2} mg mL^{-1}. Thus, these garlics were confirmed to exhibit antioxidant activity at 0.3 to 3.0 × 10^{-2} mg mL^{-1} (Figure 1AB; Supplementary Tables S1 and S2). Fresh garlic 'White-roppen' (obtained in 2019) showed antioxidant activity at concentrations ranging from 1.0 × 10^{-3} to 3.0 × 10^{-3} mg mL^{-1} (Figure 1AB; Supplementary Tables S1 and S2). In addition, fresh garlic clones 'Iki-ooninnniku', 'Chile-60', and 'Spain-225' showed no change in calcium ion influx at 1.0 mg mL^{-1}, and the intensity of O_2^- increased significantly. Therefore, these 1.0 mg mL^{-1} extracts of fresh garlic were suggested to have an innate immunostimulant effect (Figure 1A; Supplementary Table S1). A high positive correlation (r = 0.93) was observed between the fructan content and neutrophil-like cell O_2^- production (Figure 2A). The higher content of fructan in garlic tended to increase O_2^- production by neutrophil-like cells, suggesting that O_2^- production is related to innate immune responses and that fructan content is strongly associated with the innate immune-activation effects of neutrophil-like cells.

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Polysaccharides derived from plant materials have immunostimulatory properties [14] and also have stimulating effects on neutrophils, which are responsible for innate immunity. Fresh garlic showed antioxidant and innate immune-activation effects, while black garlic showed anti-inflammatory effects. In all black garlic extracts, the influx of calcium ions and the intensity of O_2^- were significantly reduced, and anti-inflammatory effects were observed when high concentration extracts of 0.1 to 1.0 mg mL^{-1} were added (Figure 1AB; Supplementary Tables S1 and S2). In particular, black garlic purchased in Indonesia showed a strong anti-inflammatory effect, with a significant decrease in reactive oxygen species concentration and intracellular calcium ion concentration at the high concentration. Furthermore, the same garlic showed different bioactivities before and after processing.

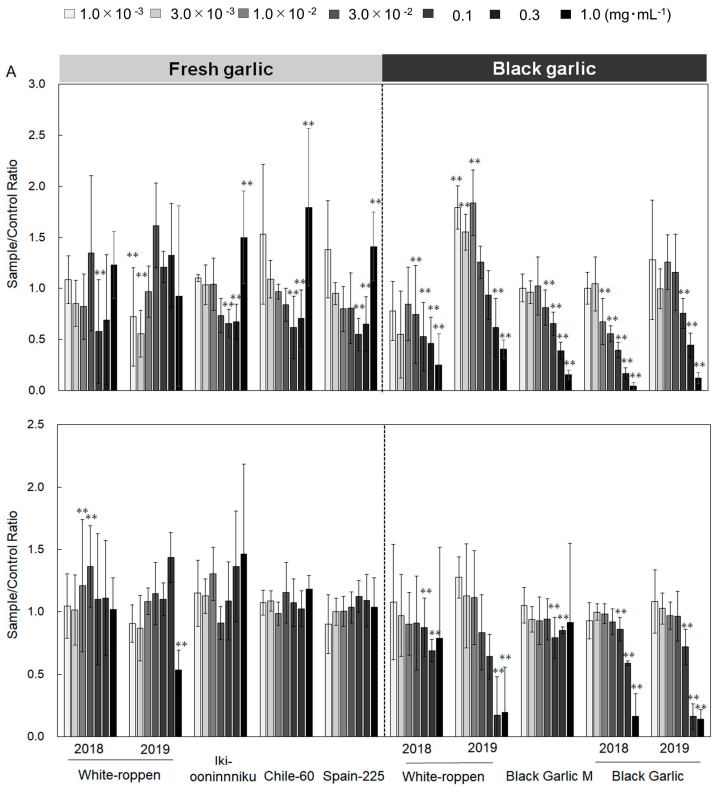

Figure 1. Superoxide production (A) and calcium ion uptake (B) by neutrophil-like cells induced by fresh and black garlic. * and ** symbols indicate statistically significant difference at 5% and 1% level, respectively comparing to control. Bar indicates ± SD (n=3).

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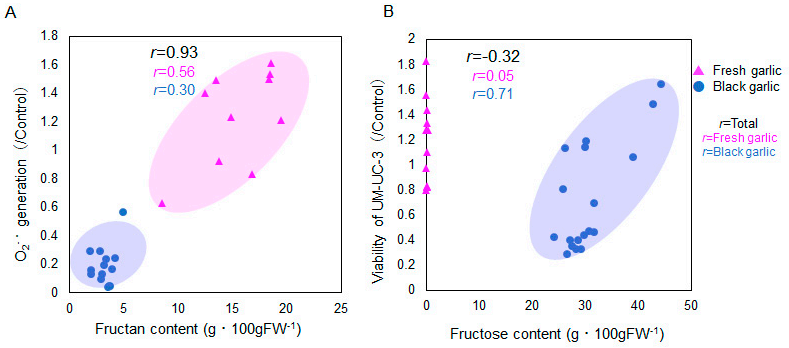

Figure 2. Relationship between fructan content and indicators of innate immunostimulatory activity (A), fructose content and cancer cell activity (B) in fresh and black garlic.

2.2. Evaluation of Antioxidant Activity

DPPH radical scavenging activity and total phenol and flavonoid content, which are antioxidant components, were significantly higher in black garlic than in fresh garlic. Total phenolic and flavonoid compound content showed a high negative correlation with O_2^- production and a high positive correlation with DPPH radical scavenging activity (Figure 3). In other words, the higher the total phenolic and flavonoid content, the stronger the antioxidant capacity to scavenge O_2^- and DPPH radicals. The results suggest that the increase in antioxidant capacity due to the processing of black garlic is due to the increase in total phenolic and total flavonoid compounds [15]. Many phenolic and flavonoid compounds in plants are found as glycosides [16]. In some reports, heating plants inhibits the activities of oxidative and hydrolytic enzymes that destroy antioxidant components, whereas tissue disruption raises the available antioxidant content and increases antioxidant capacity [17]. Furthermore, in an experiment to evaluate the bioactivities of anthocyanidin and anthocyanin using the innate immune response, anthocyanidins such as cyanidin and delphinidin showed antioxidant and anti-inflammatory effects, while anthocyanins, such as cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside, and delphinidin 3-rutinoside, showed innate immune activation [18]. Therefore, it is assumed that heat treatment during the processing of black garlic might increase available phenolic compounds, leading to high antioxidant activity in black garlic.

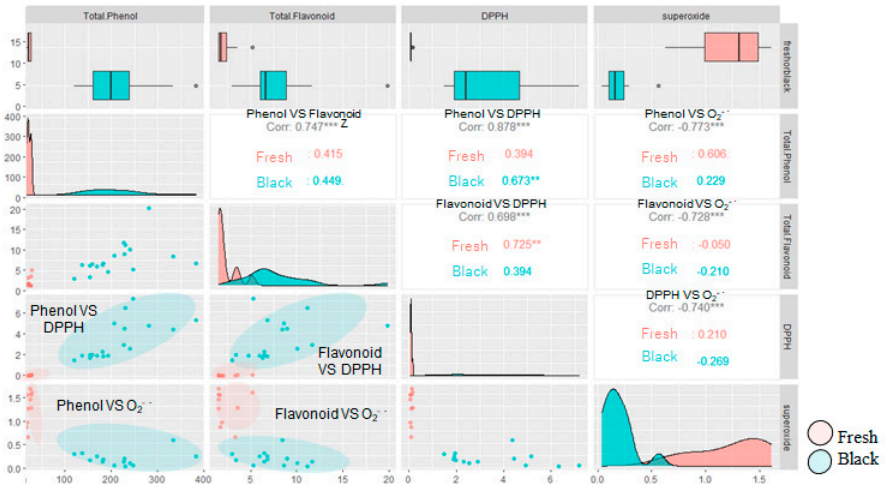

Figure 3. Relationship between total phenol, flavonoid contents in fresh and black garlic and each index. ^{z} symbol indicates Correlation coefficients (*r*).

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2.3. Evaluation of Anticancer Effect

Cancer cell viability was reduced in 'Black Garlic M' and 'Black Garlic' when the concentration of the sample extracts in the medium was as high as 0.3–1.0 mg mL^{-1}. On the other hand, 'White-roppen' and its processed black garlic increased the activity of cancer cells and stimulated their proliferation when the concentration of the extract was increased (Figure 4; Supplementary Table S3). Cancer is a disease caused by genetic errors. Most reactive oxygen species generated in cells are removed by scavenging enzymes and antioxidants, but when overproduction occurs or the scavenging system is unable to keep up, oxidative DNA damage increases, and the possibility of genetic problems that lead to carcinogenesis is high [19]. This suggests that black garlic, which exhibits radical scavenging and antioxidant activity, may contribute to the suppression of cancer development. The inhibitory effect on cancer cell proliferation was examined in terms of the induction of apoptosis. S-allylmercaptocysteine inhibits the proliferation of two leukemia-derived cell lines in a concentration-dependent manner [20]. It has been suggested that ajoene may induce apoptosis by increasing the production of peroxides and activating transcription factors [21]. The results indicate that the high radical scavenging and antioxidant effects of black garlic contributed to the inhibition of cancer cell viability, and the apoptosis-inducing substances inhibited the proliferation of cancer cells. There was a high positive correlation between the fructose content of black garlic and the growth rate of cancer cells (Fig. 2B; Supplementary Tables S3 and S5). When testing the addition of fructose to UM-UC-3 cells, the activity of UM-UC-3 cells increased with increasing concentrations of fructose in the medium and was significantly higher at 250 μg mL^{-1} as compared to the control (Figure 5; Supplementary Table S4). Cancer cells generally require large amounts of energy and are believed to use the glycolytic pathway, which provides energy quickly, as their main pathway of energy acquisition [22]. In this case, the large amount of fructose contained in black garlic extract may have provided the energy source for cell proliferation. It has also been reported that the addition of fructose to pancreatic cancer-derived cell lines stimulated the synthesis of nucleic acids and nucleotides and increased proliferation [22]. It is possible that the same activation of the nucleic acid synthesis pathway in UM-UC-3 cells also led to cell proliferation. These results suggest that fructose in the medium increased the activity of UM-UC-3 cells, and that the effect of fructose in black garlic on the proliferation of UM-UC-3 cells exceeded the effect of anti-cancer substances when the fructose content in black garlic was increased. Furthermore, the sugars in garlic and black garlic were suggested to influence the response of innate immune cells as well as cancer cells. For fresh and black garlic to have higher bioactivity, the composition of the sugars in garlic should be considered not only antioxidant contents.

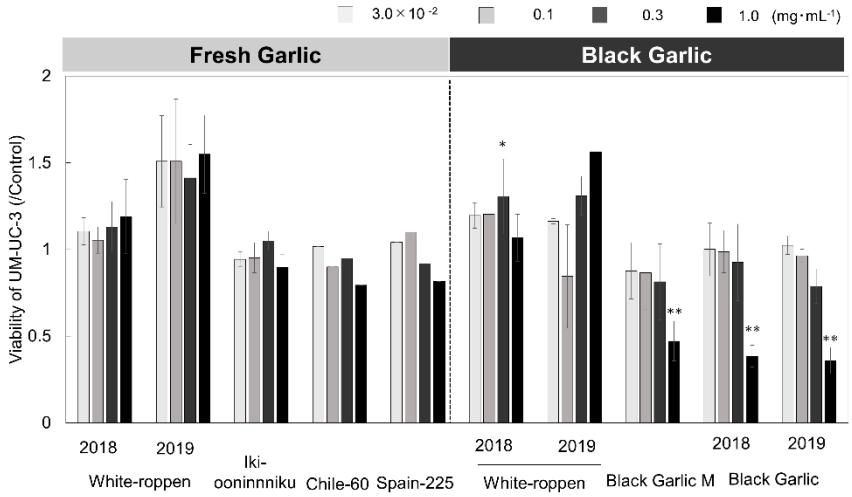

Figure 4. Effects of fresh and black garlic extracts on cell viability of UM-UC-3. * and ** symbols indicate statistically significant difference at 5 % and 1 % level, respectively comparing to control. Bar indicates ± SD (n=1-5).

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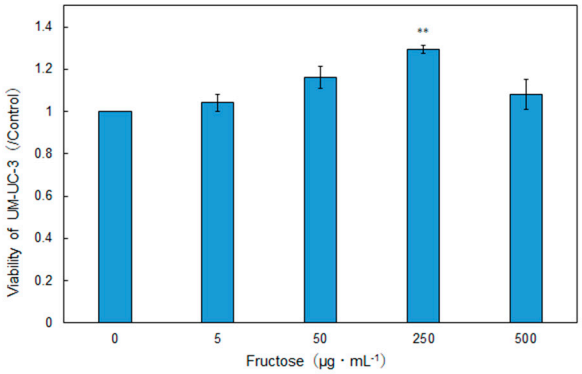

Figure 5. Effect of fructose on cell viability of UM-UC-3 (bladder cancer cell) . ** symbols indicate statistically significant difference at 1 % level, respectively comparing to control by Dunnett's test (n=3).

2.4. Amino Acid and Sugar Contents

Amino acid content was measured, and a total of 34 amino acids were found. The amino acids in fresh and black garlic are quantitatively and qualitatively different. Fresh garlic tended to have higher total amino acid content than black garlic, with arginine and asparagine accounting for most of the total amino acid content. On the other hand, black garlic tended to have lower total amino acid content than fresh garlic but higher contents of amino acids such as alanine, glycine, tyrosine, and phosphoserine than fresh garlic (Figure 6; Supplementary Table S5). The Maillard reaction occurs in the process of black garlic production. In their study of the antioxidant components of aged garlic extract, Ryu *et al.* isolated fructosyl arginine, a Maillard compound that exhibits strong hydrogen peroxide scavenging activity [23]. The formation of this compound correlates with the formation of glucose in garlic extract during ripening and is considered to be formed by the non-enzymatic reaction of fructose—produced by the breakdown of fructan—with arginine, which is present in large quantities in garlic [24]. It is possible that during the black garlic production process, amino acids were consumed in the same way as in the aged garlic extract, resulting in the formation of Maillard compounds, which have antioxidant properties, thus increasing the antioxidant activity of the garlic. Regarding carbohydrate content, fructan was the major sugar in fresh garlic and fructose in black garlic. Fructan content decreased after processing into black garlic, and conversely, the fructose and glucose contents increased (Figure 7; Supplementary Table S5). This supports previously reported results [25]. During the heat treatment process, fructan, the main storage sugar of garlic, was decomposed, and fructose and glucose accumulated. The results suggest that a dried fruit-like sweetness and stickiness characteristic of black garlic are produced in this process.

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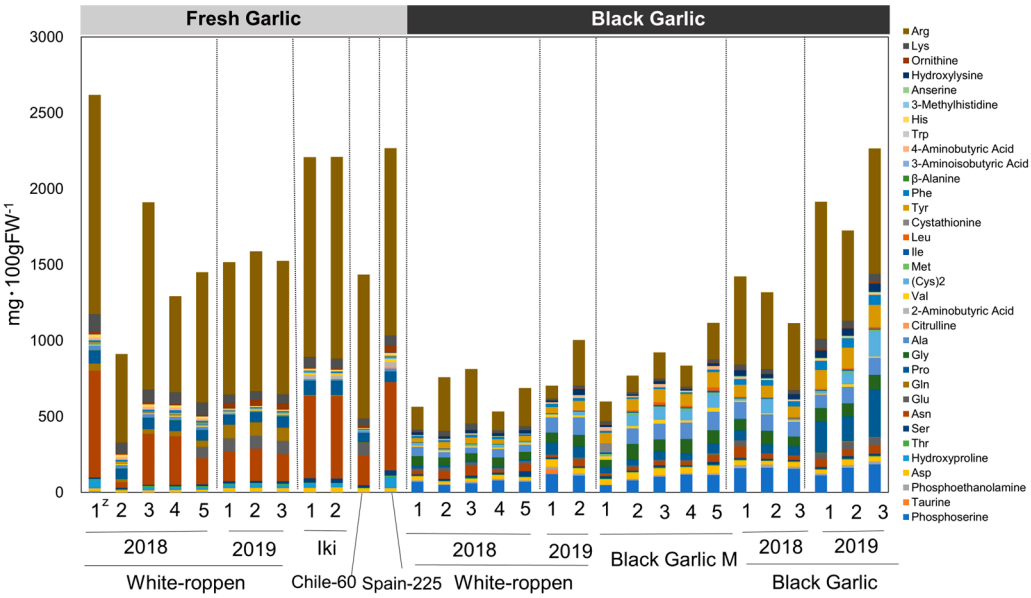

Figure 6. Amino acid contents of fresh and black garlic （mg 100gFW^{-1}）. ^{z} symbol indicates the number of bulbs. 1-5 bulbs were analyzed for each samples.

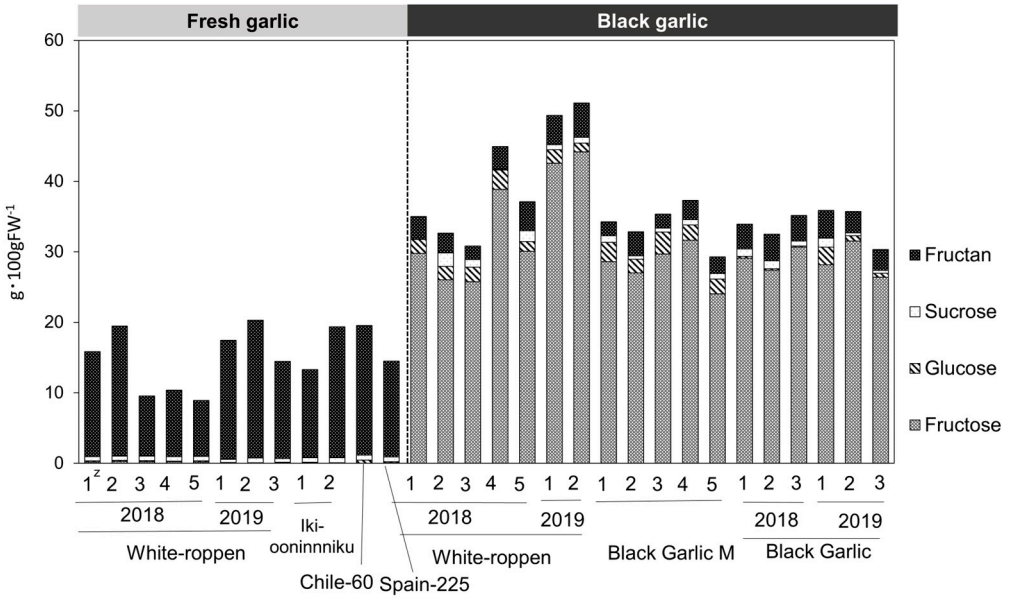

Figure 7. Carbohydrate content in fresh and black garlic (g 100gFW^{-1}). ^{z} symbol indicates the number of bulbs. 1-5 bulbs were analyzed for each samples.

3. Materials and Methods

3.1. Plant Materials

Fresh and black garlic were collected from Japan and abroad. In addition, two garlic accessions managed by Yamaguchi University were used in this study (Table 1). These accessions were obtained from local markets or national institutions in each country. Detailed information regarding these accessions was reported by Etoh **[26]** and Hirata *et al.* **[27]**. 'White-roppen' black garlic was processed in a rice cooker using warm mode for two weeks. Freeze-dried powder of fresh and black garlic bulbs is used as the experimental material. To prevent the loss of components, they are cut into thin slices on ice, immersed in liquid nitrogen for a few seconds, and then dried in a freeze-dryer (TAITEC vacuum freeze-drying equipment VD-250R) for 3 weeks. For each sample, 1 to 5 bulbs were prepared (Table 1).

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Table 1. Plant materials used in this study.

Materials		Number of bulbs	Collected site	Accession information
Fresh garlic	'White-roppen' 2018	5	Japanese local market	—
	'White-roppen' 2019	3	Japanese local market	—
	'Iki-ooninnniku'	2	Saga University, Japan	Hirata et al. (2016b)
	'Chile-60'	1	Chile	Etoh (1985)
	'Spain-225'	1	Spain	Hirata et al. (2016b)
Black garlic	'White-roppen' 2018	5	Processed White-roppen 2018	—
	'White-roppen' 2019	2	Processed White-roppen 2019	—
	Black Garlic M	5	Japanese local market	—
	Black Garlic 2018	3	Indonesian local market	—
	Black Garlic 2019	3	Indonesian local market	—

3.2. Sample Preparation

3.2.1. Hot Ethanol Extraction

The freeze-dried powder of the sample was extracted with 70% ethanol to obtain the extract. Twenty mg of the freeze-dried powder was weighed and placed in a 15 mL plastic tube. Then, 2.5 mL of 70 % ethanol was added to the tube, and it was mixed with a vortex mixer for 5 minutes. Next, the mixture was heated at 80 °C for 15 minutes and sonicated for 5 minutes. Finally, the supernatant was collected after centrifugation. The extracts were stored at -25 °C until measurement.

3.2.2. Dimethyl Sulfoxide (DMSO) Extraction

The experimental samples were extracted with dimethyl sulfoxide (DMSO), and the extracts were used for the measurement. Ten mL of DMSO was added to 2 g of freeze-dried sample. After mixing with a vortex mixer for 10 minutes, the samples were centrifuged (25 °C, 300×g, 5 min), and only the supernatant was used. The extracts were stored at -80 °C until measurement. The samples were diluted with DMSO before measurement.

3.3. Simultaneous Evaluation for Antioxidant, Anti-Inflammatory, and Innate Immune Activation

The bioactivities of plant materials were evaluated according to the method of Kazumura (2013), which utilizes the innate immune response of neutrophils. We utilized neutrophil-like cells obtained by inducing the differentiation of human acute promyelocytic leukemia cell line (HL-60) as surrogates for neutrophils. The HL-60 cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation, Japan) supplemented with 10% fetal bovine serum (FBS, Biosera, France) and 1% Penicillin–Streptomycin (P/S, FUJIFILM Wako Pure Chemical Corporation, Japan) at 37 °C in a humidified 5% CO_2 atmosphere. Differentiation was induced by 96-hour incubation at 4.0×10^5 cells mL^{-1} in medium containing 1.3 % sterile DMSO. Differentiated neutrophil-like cells were incubated for 45 minutes in medium with a calcium ion indicator, 3 μM fluo-3 AM. Neutrophil-like cells obtained following the method above were used to prepare the assay solution. The assay solution

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contains 1.0×10 ^{5}cells mL^{-1} of neutrophil-like cells in RH buffer; 0.5 μM MCLA, which is a superoxide-sensitive chemiluminescence reagent; 1 mM calcium chloride as a source of calcium ions; and DMSO extraction or DMSO. A simultaneous fluorescence and chemiluminescence measurement system (CFL-C2000, Hamamatsu Photonics, Japan) was used for the assay. Data analysis was performed using dedicated analysis software that can simultaneously analyze the three data obtained from the measurements. The stimulus enhancement of the fluorescence and chemiluminescence signals was determined by automatically calculating the baseline and peak area under the curve. The peak area ratio of each sample compared to the control was calculated. The t-test was used to determine the significant difference between the O_2^- production and calcium ion concentration of neutrophil-like cells. A p-value < 0.05 was considered statistically significant.

3.4. DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured based on the method of Kondo *et al.* [28]. Samples were extracted with 70 % ethanol and used for assay. Extracts were used for assay immediately after extraction. Seventy-two μL of DPPH dissolved in ethanol, pH 6.0 MES (Morpholinoethanesulfonic acid monohydrate) buffer, and 30 % ethanol were mixed in the 96 well plate. Then 72 μL of sample extract diluted with 70 % ethanol was added. The absorbance at 520 nm was measured with a microplate reader after incubation for 20 minutes at room temperature in the dark. A regression equation was calculated based on the dilution ratio of the samples, and the half-inhibition concentration (IC_{50} (mgFW)) of each sample was calculated. Results were expressed as the reciprocal of IC_{50} (mgFW^{-1}).

3.5. Measurement of Cancer Cell Viability with CCK-8 Kit

The UM-UC-3 cell line was obtained from the European Collection of Cell Cultures (ECACC). UM-UC-3 cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation, Japan) supplemented with 10 % FBS and 1 % P/S at 37 °C in a humidified 5 % CO_2 atmosphere for 3 days were used for the assay after confirming that they had reached 70 to 90 % confluence. The cells were dissociated from the flask and suspended, and the cell suspension was centrifuged (1,500 rpm × 5 min) to remove the old medium. The cells were seeded into 96-well plates (200 μL, 5,000 cells/well). Cells were incubated for another 24 hours after adding 2 μL of DMSO extract per well. The experiment was performed according to the protocol of the assay kit. CCK-8 reagents (DOJINDO, Japan) were added 10 μL to each well after removing 100 μL of supernatant. The treated cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere for 3.5 hours. The absorbance at 450 nm was measured with a microplate reader. The cell viability was expressed as the ratio of absorbance between sample groups and the control group.

3.6. Determination of Soluble Sugars (Glucose, Fructose, and Sucrose)

Soluble sugar content, including fructose, glucose, and sucrose, was determined by the HPLC method using hot 70 % ethanol extract. Before injection, the extract was filtered through a 0.45 μm filter. The measurements were performed under the following conditions. The identification of substances was done by retention time. The sugar content of the sample was determined from the peak area of the standard and that of the sample.

Column: NH2 (4.0 mm inner diameter × 250.00 mm long, Kanto Chemical, Japan); column temperature: 40 °C; mobile phase: 80% acetonitrile solution; flow rate: 1.0 mL min^{-1}; injection volume: 100 μL; detector: refractive index detector (L-2490, HITACHI); data collection time: 20 min

3.7. Determination of Fructan Content

The fructan in extracts was determined by the thiobarbituric acid method [29] with minor modification. The 70 % ethanol extract was diluted 5 times and placed in a test tube at 20 μL. Then, 10 μL of 25 mM ammonium acetate buffer was added. In addition, 10 μL of invertase solution was added and left at room temperature for 5 minutes to degrade the sucrose in the sample. Fifty μL of

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distilled water and 10 μL of 10 N sodium hydroxide solution were added and heated in boiling water for 10 minutes to decompose the fructose in the solution. After cooling rapidly in ice water, 1 mL of thiobarbituric acid solution and 1 mL of 12 N hydrochloric acid were added and heated in boiling water for 6 minutes. Finally, the absorbance at 432 nm was measured with a spectrophotometer. The concentration of fructan in the extract was determined using a calibration curve derived from 1-kestose (FUJIFILM Wako Pure Chemical Corporation, Japan), and the fructan content per 100 g fresh weight of plant sample was calculated.

3.8. Determination of Total Phenolic Compounds

The content of total phenolic compounds in the 70 % ethanol extract was determined by the Folin–Ciocalteu method [30]. The 70 % ethanol was diluted 5 times with distilled water. To 1 mL of the diluted solution in a test tube, 1 mL of Folin–Ciocalteu reagent was added. After 3 minutes, 1 mL of 10% sodium carbonate solution was added. The mixture was incubated for 1 hour at room temperature in the dark. The absorbance at 530 nm was measured with a spectrophotometer (U-2000, Hitachi High-Technologies Corporation, Tokyo, Japan). The total phenolic compound content were expressed as milligram catechol equivalents (mg catechol) per 100 g fresh weight.

3.9. Determination of Total Flavonoid Compounds

The total flavonoid content was determined by the colorimetric method using hot 70 % ethanol extract. The method was adopted from the approach of Vu *et al.* [31]. The 70 % ethanol extract of the sample and *n*-hexane were placed in a test tube at a ratio of 1:1 and separated into two layers after stirring. Chlorophyll, carotenoids, and other pigments dissolved in the hexane layer were removed. The 0.5 mL of 70 % ethanol layer was diluted with the same amount of 70 % ethanol in a test tube, and 2 mL of 2 % aluminum chloride solution was added. After allowing the reaction to proceed for 1 hour in the dark, the absorbance at 420 nm was measured. The total flavonoid compound content was calculated using a calibration curve prepared with quercetin. The results were expressed as milligram quercetin equivalents (mg quercetin) per 100 g fresh weight of plant sample.

3.10. Determination of Amino Acid Contents

The freeze-dried powder of the sample was extracted with pH 2.2 lithium citrate buffer solution (FUJIFILM Wako Pure Chemical Corporation, Japan). Briefly, 10 mL of lithium citrate buffer was added to 50 mg of sample powder. The solution was vigorously mixed with a vortex mixer for 5 minutes and ultrasonicated for 5 minutes. The extract was centrifuged (20 °C, 5,000 rpm, 30 min), and the supernatant was collected. Before injection, the extract was filtered through a 0.20 μm filter. Samples were measured immediately after extraction. The amino acid analysis system of SHIMADZU was used to measure amino acids by the post-column derivatization method. Amino acids were analyzed using the gradient from three mobile phase types (S228-21195-95, SHIMADZU, Japan) and were derivatized using two OPA reagents (S228-21195-93, SHIMADZU, Japan). Before use, sodium hypochlorite was added into the OPA reagents. Mobile phases and reagents were purchased from SHIMADZU. The HPLC conditions are as follows.

Column: Shim-Pack-Amino-Li (6.0 mm inner diameter × 100 mm long, SHIMADZU, Japan); injection volume: 50 μL; detector: fluorescence detector (RF-20A, SHIMADZU); excitation wavelength: 350 nm; fluorescence wavelength: 450 nm; flow of mobile phases: 0.6 mL min^{-1}; flow of OPA reagents: 0.2 mL min^{-1}

The time program was based on the Shimadzu manual. From the injection to 22 min, mobile phase A was flowed 100%, and from there the concentration of mobile phase B was increased step by step to reach 100% at 111 min. From 133 min, mobile phase C was flowed to wash the column. The analysis was carried out for 163 minutes.

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3.11. Statistical Analysis

Statistical analyses were performed with EZR [32], which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R Commander designed to add statistical functions frequently used in biostatistics. Each sample was measured three times (HPLC analysis was done once), and data were expressed as the mean ± SD. Except simultaneous evaluation for antioxidant, anti-inflammatory, and innate immune activation, one-way ANOVA followed Dunnett's test was used to determine the difference between groups. A p-value < 0.05 was considered statistically significant.

4. Conclusions

Both fresh and black garlic have a variety of benefits that contribute to human health and show different bioactivities depending on the process of preparing fresh garlic into black garlic and the type of fresh garlic used to make the black garlic. The thermal processing of garlic changes the composition of sugars, antioxidants, and amino acids and has different effects on neutrophil-like cells and cancer cells. This suggests that the process of thermal processing of garlic can also affect bioactivities. Furthermore, a black garlic strain was found to have both very potent antioxidant and anti-inflammatory effects and to exhibit anti-proliferative effects on bladder cancer cells.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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