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Green Pepper (*Piper nigrum* L.) Extract Suppresses Oxidative Stress and LPS-Induced Inflammation via Regulation of JNK Signaling Pathways

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Abstract: In this study, we compared the physicochemical properties and the antioxidant capacities of black and green pepper. Green pepper from India (GPI) and Sri Lanka (GPS) had higher Hunter L* and b* values and lower a* values than black pepper from India (BPI) and Sri Lanka (BPS). The contents of chlorophyll a and b, flavonoids, and phenolic compounds in GPI and GPS were higher than those in BPI and BPS. The peppercorns showed the following decreasing order of 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging capacities: GPI > GPS > BPI > BPS. High-performance liquid chromatography showed that the highest piperine content was present in GPI (8613.27 ± 45.86 mg/100 g). We further investigated the anti-inflammatory capacity of the green pepper. GPS and GPI significantly suppressed lipopolysaccharide (LPS)-induced nitrite production and inducible nitric oxide synthase expression without being cytotoxic to RAW 264.7 cells. GPS and GPI also suppressed the LPS-induced phosphorylation of mitogen-activated protein kinases, but not p65. GPS had a higher inhibitory effect on LPS-induced c-Jun phosphorylation and translocation from the cytosol to the nucleus than GPI. Thus, the findings of our study suggest that green pepper has the potential to be an effective nutraceutical against oxidative and inflammatory stress.

Keywords: green pepper; antioxidant; anti-inflammation; nutraceutical; piperine; mitogen-activated protein kinases (MAPKs)

1. Introduction

The "rate-of-living hypothesis" postulates that animals with high metabolic rates have short life spans [1]. This hypothesis suggests that reactive oxygen species (ROS) present at the cellular level induce damage in an organism, and that those ROS are intimately linked to aging [2]. Diverse evidence has shown that overproduction of ROS and impairment of enzymatic and nonenzymatic antioxidant systems disrupt homeostasis in living organisms [3]. Antioxidants are substances that have the capacity to prevent or reduce the damage caused by free radicals and unstable molecules that are generated during energy production or during reactions to environmental and other types of stresses in cells. Multiple studies have demonstrated that botanical extracts and their constituent compounds can act as superior antioxidants [4,5]. Therefore, administration of antioxidants derived

from natural materials such as plant sources may be an optimal strategy for preventing inflammatory diseases, thereby prolonging the longevity.

Immune cells such as macrophages and neutrophils recognize pathogens such as infectious organisms, viruses, and bacteria via pattern recognition and they produce nitric oxide (NO) in order to eliminate these pathogens [6]. However, the overproduction of NO causes damage to the host tissues, thereby resulting in inflammation and progression of various diseases [7,8]. Although NO has a critical role in the regulation of vascular homeostasis, neurotransmission, and inhibition of platelet aggregation, the abnormal expression of NO is generally considered to be a marker of inflammation [9]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcriptional factor, plays a critical role in inflammation by regulating the expression of genes involved in inflammation, including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), as well as inflammatory cytokines such as interleukin-1, tumor necrosis factor, and interleukin-6. Various compounds have been reported to exhibit anti-inflammatory effects by inhibiting NF-κB activity and the associated signaling pathways [10,11].

Plants are a rich source of antioxidants, and herbs and spices are a primary source of natural antioxidants [12,13]. Pepper (*Piper nigrum* L.) has long been cultivated in India, Indonesia, Brazil, Malaysia, and Sri Lanka, and it has a high commercial value owing to its rich flavor and spiciness [14]. The dried fruit of the pepper varies in color and can be black, green, or white. Black pepper fruits are harvested when mature and dried in the sun. Green pepper fruits are harvested when unripe, 10–15 days before maturation, and they are processed to maintain the green color [15]. Pepper has physiological effects owing to its antimicrobial, anti-inflammatory, detoxifying, and anti-allergic properties [16–19]. Piperine, the representative component of pepper, has various physiological activities including antitumor, antioxidant, and anti-epileptic effects [20–22]. Additionally, recent studies have shown that mitogen-activated protein kinases (MAPKs) and activator protein (AP)-1 are strongly involved in inflammation via up-regulation of iNOS expression and nitrite production in RAW 264.7 cells [23,24].

In this study, we aimed to investigate the protective properties of green pepper in oxidative and inflammatory stress. We found that green pepper has higher chlorophyll content, total flavonoids, total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity than black pepper. Although green pepper from India (GPI) and green pepper from Sri Lanka (GPS) have similar inhibitory effects on lipopolysaccharide (LPS)-induced nitrite production and iNOS expression, GPI showed higher inhibition effects on LPS-induced c-Jun N-terminal kinase (JNK) and c-Jun phosphorylation and translocation of c-Jun from the cytosol to the nucleus than GPS, while GPS showed higher inhibition activity on p38 MAPK phosphorylation than GPI.

2. Materials and Methods

2.1. Materials

The green and black pepper used here were commercially available and purchased online. Each sample was classified by color, passed through a 60-mesh sieve, and stored in the dark at room temperature.

2.2. Extraction

The pepper sample (5 g) was homogenized with 100 mL of 80% ethanol in a mixer (KWG-150, Sunway Electric Manufacture (He Shan) Co., Ltd, China), and the solution was incubated with shaking for 24 h (DH. WIS 02011, DAEHAN Scientific Co., Ltd. Korea). The homogenate was centrifuged at 15,000 rpm for 10 min (Mega21R, Hanil, Korea). The supernatant was collected and filtered through the Minisart syringe filter (pore size: 0.45 μm) and concentrated under reduced pressure in a rotary evaporator (N-1000, Eyela, Japan). The final volume was adjusted to 20 mL, divided into

aliquots and stored at −80 °C prior to quantitative analysis of the physicochemical characteristics and antioxidant activity.

2.3. Color

The color of pepper was expressed as L* value (brightness range of 0 to 100 where 0 is black and 100 is white), a* value (redness index), and b* value (yellowness index). For individual measurements, five replicates were made with each of the two colored peppercorns, yielding a mean value, standard deviation, and relative standard deviation (%).

2.4. High-Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) Analysis

Piperine profiling was performed by using the HPLC method with some modification [25]. For quantitative analysis, a 0.1-g homogenized sample was dissolved in 50 mL methanol. The mixture was ultrasonically extracted at 50 °C for 20 min, cooled at room temperature, and filtered through a 0.45-µm syringe filter (Minisart).HPLC analyses were performed using an Agilent 1100 HPLC system (Agilent, Santa Clara, CA, USA) with a diode array detector (DAD, 340 nm). Eclipse C18 plus (4.6 × 150 mm, 5 µm, Agilent, Santa Clara, CA, USA) was utilized at 25 °C for piperine quantification. The mobile phase was acetonitrile/1% citric acid (45:55, isocratic). The sample injection volume was 10 µL, and the flow rate was set to 1 mL per minute with a run time of 20 min.

2.5. Chlorophyll Contents

The chlorophyll content of each colored pepper was determined by the method described by Barman et al. [26], with some modifications. Four grams of each sample was pulverized in 50 mL of 80% acetone in a mortar, followed by shaking for 24 h. After centrifugation for 10 min at 15,000 rpm, the supernatant was collected and filtered through a 0.45-µm Minisart filter. The absorbance of the filtrate was measured at 645 and 663 nm (Optizen POP, Mecasys, Korea), and these values were used to calculate the chlorophyll concentration according to following equation.

$$\text{Chlorophyll a (mg/L)} = 12.72\ OD663 - 2.58OD645 \quad (1)$$

$$\text{Chlorophyll b (mg/L)} = 22.88\ OD_{645} - 5.50\ OD_{663} \quad (2)$$

$$\text{Total chlorophyll (mg/L)} = 7.22\ OD_{663} + 20.3\ OD_{645} \quad (3)$$

2.6. Total Flavonoids and Total Phenolics

Total flavonoid content was measured using a colorimetric assay [27]. One milliliter of the sample extract and 4 mL of distilled water were added to a 15 mL tube, along with 0.3 mL of 5% $NaNO_2$. The solution was allowed to react at room temperature for 5 min. Next, 0.3 mL of 10% $AlCl_3$ was added, and the solution was mixed thoroughly and allowed to react at room temperature for 6 min. Thereafter 2 mL of 1N NaOH was added along with 2.4 mL of distilled water to adjust the total volume to 10 mL. The absorbance of the solution was measured at a wavelength of 510 nm using a spectrophotometer (Optizen POP).Total phenolic content analysis was performed using the Folin–Ciocalteu colorimetric method [27]. A sample volume of 0.2 mL was added to a 15 mL tube with 2.6 mL of distilled water, to which 0.2 mL of the Folin-Ciocalteu reagent was added. After reacting for 6 min at room temperature, 2 mL of 7% Na_2CO_3 was added, followed by a 90 min incubation at room temperature under dark conditions. The absorbance of the sample was measured at 750 nm using a spectrophotometer (Optizen POP), and a standard curve was prepared using gallic acid. The total phenolic content was expressed in mg gallic acid equivalents (GAE)/100 g FW.

2.7. Radical Scavenging Effect

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured using a previously described method, with some modifications [28]. A DPPH solution was prepared at a concentration of 100 μM and diluted with 80% methanol to an absorbance of 0.65 ± 0.02 at a wavelength of 517 nm. The sample extract (50 μL) was mixed with 2950 μL of diluted 0.1 mM DPPH solution and allowed to react for 30 min. The absorbance of the sample solution was measured at a wavelength of 517 nm in the spectrophotometer (Optizen POP). The antioxidant activity of the sample was expressed as mg vitamin C equivalents (VCE)/100 g FW.

The 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging ability of the extract was determined using the ABTS radical scavenging assay [28]. The radical solution was prepared by dissolving 27.117 mg of 2,2''-azobis (2-amidinopropane) dihydrochloride (AAPH) and 137.175 mg of ABTS in 100 mL of phosphate buffer saline (PBS) with stirring for 5 min. After reacting for 40 min in a water bath (70 °C), the solution was cooled to room temperature, and the working ABTS solution was prepared by dilution with PBS to obtain an OD_{734} of 0.63 to 0.67. Thereafter, 20 μL of the sample was mixed with 980 μL of diluted ABTS solution and allowed to react at 37 °C for 10 min. The absorbance of the solution was measured at 734 nm using the spectrophotometer (Optizen POP). The antioxidant activity of the extract was expressed as mg VCE/100 g FW.

2.8. Cell Culture

The murine RAW 264.7 cell line (Korean Cell Line Research Foundation) was maintained in DMEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and the cells were cultured with 5% CO_2 at 37 °C. The medium was replaced and cells were passaged every two days.

2.9. Cell Viability

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, based on the manufacturer's instructions (Promega, Madison, WI, USA). RAW 264.7 cells at a concentration of 3×10^5 cells/mL were seeded in 96-well plates and incubated at 37 °C in a 5% CO_2 incubator overnight. Cells were then treated with green pepper extracts (GPEs) at increasing concentrations for 24 h and 20 μL of MTS reagent in each well for 1 h of incubation. The absorbances of each well were measured at 490 nm using a microplate reader (Bio-Rad Inc., Hercules, CA, USA).

2.10. Nitrite Assay

RAW 264.7 cells at a concentration of 3×10^5 cells/mL were seeded in a 96-well plate and incubated at 37 °C for 24 h in a 5% CO_2 incubator. Cells were then treated with green pepper (GPS and GPI) and LPS (1 μg/ml) for 1 h and 24 h. The NO concentration was determined using the Griess reagent (0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The cultured medium (100 μL) and Griess reagent (100 μL) were mixed and the solution was incubated at 37 °C for 10 min. The concentration of nitrite was determined using a standard curve generated with sodium nitrite. The absorbance of each well was measured at 550 nm using a microplate reader (Bio-Rad Inc.).

2.11. Western Blot Analysis

RAW 264.7 cells were seeded in a 60 mm cell culture dish for 24 h and treated with GPEs (15 and 30 μg/mL) in DMEM. Cells were pretreated with GPEs for 1 h before being stimulated with LPS (1 μg/mL) and incubated for different times (30 min or 24 h). Cells were collected after incubation and washed twice with cold PBS. Total cell lysates were extracted with a lysis buffer (Cell Signaling Technology, MA, USA) mixed with a protease and phosphatase inhibitor cocktail

(Thermo Scientific, MA, USA) and maintained on ice for 30 min with vortexing. Total protein contents were measured using a DC Protein Assay Kit reader (Bio-Rad Inc.) following the manufacturer's instructions. Each respective protein sample was boiled in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and separated electrophoretically on a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore, Immobilon®-P transfer membrane, MA, USA). The membrane was blocked in TBST (tris-buffered saline and polysorbate-20) buffer containing 5% skim milk for 1 h at room temperature. Then, specific primary antibodies were incubated with the membrane at 4 °C overnight. After hybridization with horseradish peroxidase-conjugated secondary antibody (Thermo Scientific) at room temperature for 1 h, the protein bands were visualized using a chemiluminescence detection kit (ATTO, Tokyo, Japan) and a GeneGnome XRQ NPC imaging system (Syngene, Cambridge, UK).

2.12. Immunofluorescence

RAW 264.7 cells were seeded at a concentration of 5×10^4 cells/mL on eight-well chamber slides (ibidi, Munich, Germany). After incubation for 24 h, the medium was exchanged for medium containing GPE followed by another 1 h of incubation. Cells were then treated with LPS and incubated for 5 min. After incubation, the cells were fixed with 4% formaldehyde, permeabilized with ice-cold methanol, and treated with specific anti-p65 primary antibodies overnight at 4 °C. After washing, Alexa Fluor®488 conjugated goat antirabbit IgG H&L conjugated to Alexa Fluor®488-conjugated labeled secondary antibodies (Abcam, Cambridge, UK) were used for visualization. For nuclear visualization, cells were stained with VECTASHIELD®(Vector Laboratories, Burlingame, CA, USA). The prepared cells were then observed under a fluorescence microscopy (Zeiss Axioscope, Germany), and images were recorded.

2.13. Statistical Analysis

For statistical analysis, one-way analysis of variance was performed using the SAS 9.4 statistical program (SAS Institute, Inc., Cary, NC, USA), and Duncan's multiple range test was used to determine the significance of each average value ($p < 0.05$). The data were expressed as mean ± standard deviation from triplicate determination.

3. Results

3.1. Color and Chlorophyll Content Analysis

The Hunter L*, a*, b* values and chlorophyll contents of green and black pepper from India and Sri Lanka are shown in Table 1. The chromaticity analysis results showed that GPI and GPS had high L* (48.21 ± 0.04 and 49.47 ± 0.06) and b* (19.16 ± 0.01 and 19.43 ± 0.02) values and low a* (−0.95 ± 0.02 and 0.27 ± 0.05) values, respectively. Compared to green pepper, black pepper from India (BPI) and Sri Lanka (BPS) had high a* values (3.44 ± 0.01 and 3.64 ± 0.02) and low L* (42.03 ± 0.01 and 36.66 ± 0.03) and b* (11.43 ± 0.01 and 12.35 ± 0.02) values, respectively. The color of green pepper is closely associated with the content of chlorophyll [29]. GPI had the highest chlorophyll a content (28.97 ± 0.09 mg/L), followed by GPS (21.92 ± 0.07 mg/L), BPI (13.68 ± 0.01 mg/L), and BPS (7.15 ± 0.02 mg/L). The mean chlorophyll b contents in GPI and GPS were 15.41 ± 0.06 mg/L and 8.62 ± 0.03 mg/L, respectively. The chlorophyll a and b contents of green pepper were higher than those of black pepper.

3.2. Total Flavonoids and Total Phenolic Contents and Radical Scavenging Effects

The antioxidant and anti-inflammatory effects of natural materials are dependent on the flavonoid and phenolic components in the material [30,31]. Total flavonoid contents in the pepper extracts were measured using $AlCl_3$ with a colorimetric assay. The highest flavonoid content was found in GPS (1083.43 ± 8.24 mg CE/100 g), followed by GPI (923.43 ± 11.43 mg CE/100 g), BPI (576.97 ± 14.70 mg

CE/100 g), and BPS (275.00 ±10.10 mg CE/100 g) (Table 2). The total phenolic contents were expressed in GAE/100 g dry extract weight. GPI and GPS had the highest phenolic contents (1414.63 ± 13.85 and 1414.63±10.56 mg GAE/100 g, respectively), BPI and BPS had low phenolic contents (985.69 ± 5.22 and 589.39 ± 9.19 mg GAE/100 g, respectively).

Table 1. Hunter L*, a*, and b* values, and chlorophyll contents of green and black pepper from India and Sri Lanka.

Color	Origin	L*	a*	b*	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)
Green	India	48.21 ± 0.04 ^{b}	−0.95 ± 0.02 ^{d}	19.16 ± 0.01 ^{b}	28.97 ± 0.09 ^{a}	15.41 ± 0.06 ^{a}
	Sri Lanka	49.47 ± 0.06 ^{a}	0.27 ± 0.05 ^{c}	19.43 ± 0.02 ^{a}	21.92 ± 0.07 ^{b}	8.62 ± 0.03 ^{b}
Black	India	42.03 ± 0.01 ^{c}	3.44 ± 0.01 ^{b}	11.43 ± 0.01 ^{d}	13.68 ± 0.01 ^{c}	4.08 ± 0.03 ^{c}
	Sri Lanka	36.66 ± 0.03 ^{d}	3.64 ± 0.02 ^{a}	12.35 ± 0.02 ^{c}	7.15 ± 0.02 ^{d}	3.12 ± 0.01 ^{d}

All data are expressed as mean ± SD (n = 3) on a dry weight basis; different letters in a column indicate values that are significantly different at $p < 0.05$.

DPPH radical scavenging capacity was measured to determine the antioxidant activity and was expressed by as mg VCE/100 g dry extract weight. The highest DPPH radical scavenging potency was shown by GPI (522.83 ± 26.00 mg VCE/100 g), followed by GPS (518.67 ± 7.21 mg VCE/100 g), BPI (362.59 ± 7.86 mg VCE/100 g), and BPS (194.42 ± 5.67 mg VCE/100 g).

ABTS radical scavenging activity was also measured and is expressed as mg VCE/100 g dry extract weight. The highest ABTS radical scavenging potency was found in GPI (1941.91 ± 67.44 mg VCE /100 g), followed by GPS (1902.77 ± 30.07 mg VCE/100 g), BPI (1269.66 ± 84.01 mg VCE/100 g), and BPS (526.45 ± 55.89 mg VCE/100 g).

Table 2. Antioxidant compounds and radical scavenging effect of green and black pepper.

Color	Region	Total Flavonoid (mg CE/100 g)	Total Phenolic (mg GAE/100 g)	DPPH (mg VCE/100 g)	ABTS (mg VCE/100 g)
Green	India	923.43 ± 11.43 ^{b}	1414.63 ± 13.85 ^{a}	522.83 ± 26.00 ^{a}	1941.91 ± 67.44 ^{a}
	Sri Lanka	1083.43 ± 8.24 ^{a}	1414.63 ± 10.56 ^{a}	518.67 ± 7.21 ^{a}	1902.77 ± 30.07 ^{a}
Black	India	576.97 ± 14.70 ^{c}	985.69 ± 5.22 ^{b}	362.59 ± 7.86 ^{b}	1269.66 ± 84.01 ^{b}
	Sri Lanka	275.00 ± 10.10 ^{d}	589.39 ± 9.19 ^{c}	194.42 ± 5.67 ^{c}	526.45 ± 55.89 ^{c}

All data are expressed as mean ± SD (n = 3) on a dry weight basis; different letters in a column indicate values that are significantly different at $p < 0.05$.

3.3. Quantification of the Major Compounds in Pepper by HPLC

Piperine is an alkaloid compound in pepper that has a significant influence on the taste and flavor of pepper [32]. Quantitative analyses shown that piperine concentrations of 8613.27 mg/100g and 5087.97 mg/100g, respectively (Table 3 and Figure 1). Moreover, piperine concentrations in BPI and BPS were determined to be 3291.65 mg/100 g and 7388.50 mg/100 g, respectively.

Table 3. Piperine contents of green and black pepper.

Color	Origin	Piperine (mg/100 g)
Green	India	8613.27 ± 45.86 ^{a}
	Sri Lanka	5087.97 ± 29.78 ^{c}
Black	India	3291.65 ± 3.53 ^{d}
	Sri Lanka	7388.50 ± 37.00 ^{b}

All data are expressed as mean ± SD (n = 3) on a dry-weight basis; different letters in a column indicate values that are significantly different at $p < 0.05$.

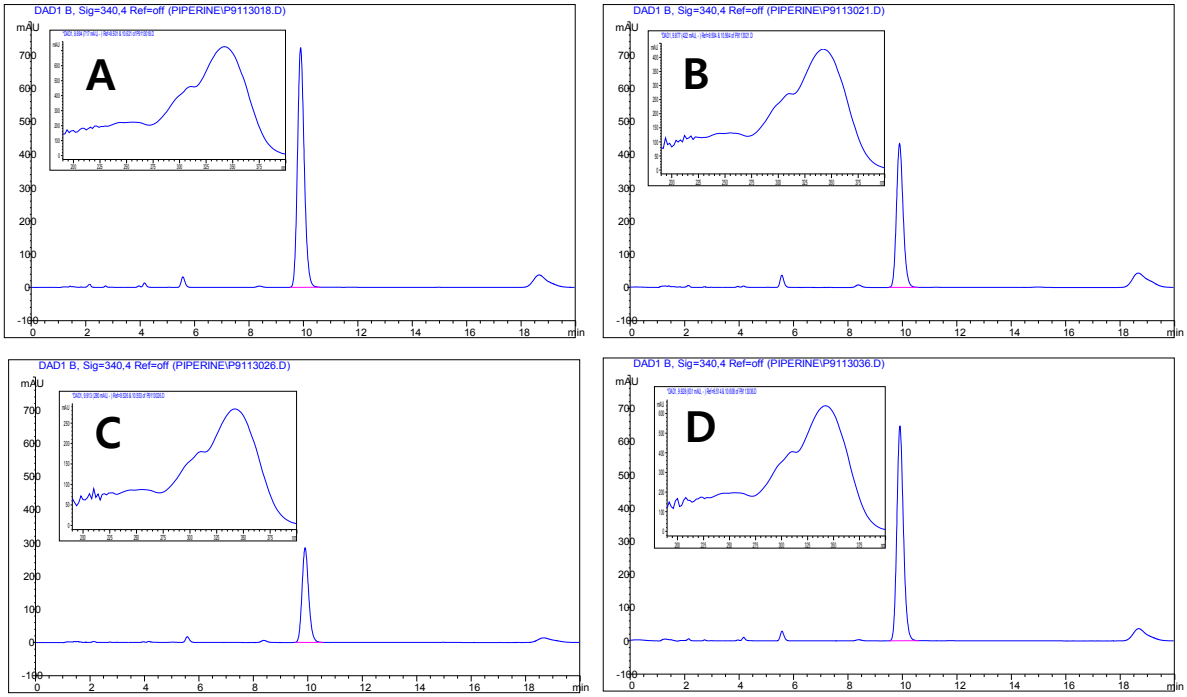

Figure 1. Chromatogram and absorption spectra of GPI (**A**), GPS (**B**), BPI (**C**), and BPS (**D**).

3.4. Effect of GPEs on LPS-Induced Nitrite Production, iNOS Expression, and Cell Viability of RAW 264.7 Cells

Abnormal production of NO is a critical marker in inflammation. RAW 264.7 cells are quite sensitive to LPS treatment, and they produce NO in response to this treatment [33]. Because the green pepper extract (GPE)s showed stronger antioxidant activities and higher concentrations of piperine, we further investigated the effect of GPI and GPS on LPS-induced NO production and the subsequent inflammatory signaling pathways in RAW264.7 cells. Using the Greiss assay, we found that GPEs significantly suppressed the LPS-induced nitrite production in a dose-dependent manner without affecting the cell viability of RAW 264.7 cells (Figure 2A,B). Because NO production is dependent on stress-mediated iNOS expression, we evaluated the effect of the GPEs on LPS-induced iNOS expression in RAW 264.7 cells. Pretreatment with GPE significantly suppressed the LPS-induced iNOS expression in RAW 264.7 cells (Figure 2C).

3.5. Effect of GPEs on LPS-Induced NF-κB and MAPK Signaling Pathways in RAW 264.7 Cells

We investigated the effects of GPE on LPS-induced NF-κB and MAPK signaling pathways in RAW 264.7 cells, because these are the primary signaling pathways regulating LPS-induced iNOS expression. GPEs suppressed the LPS-induced phosphorylation of p38 and JNK1/2 and slightly suppressed the phosphorylation of extracellular-signal-regulated kinase (ERK)1/2 in RAW 264.7 cells (Figure 3A). Interestingly, although NF-κB is a canonical signaling pathway involved in LPS-induced NO production and inflammation in immune cells including macrophages and neutrophils, GPEs did not affect the phosphorylation of p65 in RAW 264.7 cells (Figure 3B).

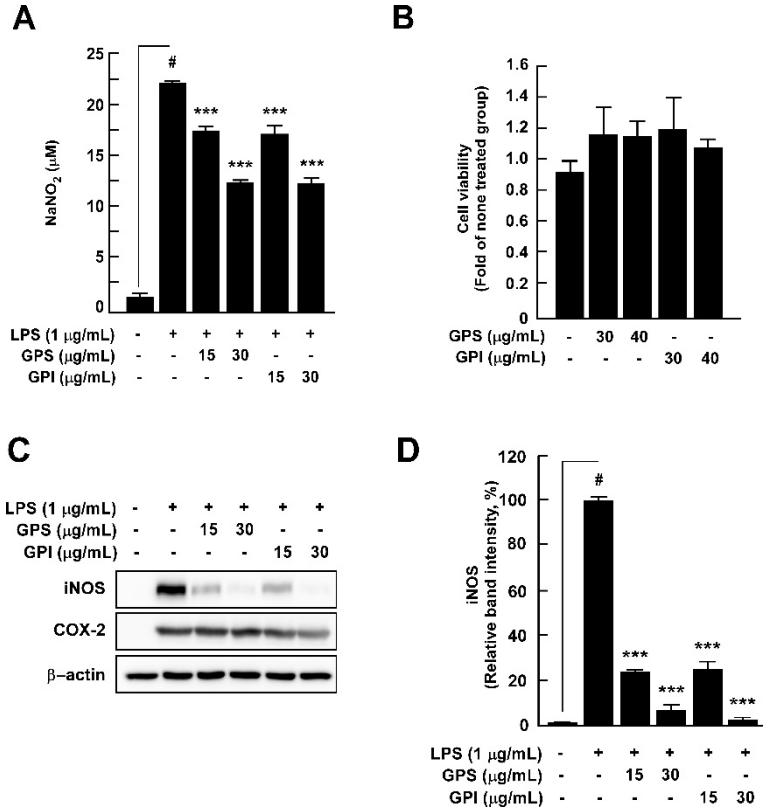

Figure 2. Effects of GPEs on LPS-induced nitrite production, cell viability, and phosphorylation of MAPKs and p65 in RAW 264.7 cells. (**A**) GPE suppressed the LPS-induced nitrite production in RAW 264.7 cells. Cells were pretreated with GPE in the presence or absence of LPS (1 μg/mL) for 24 h. (**B**) GPEs did not affect the cell viability at the tested concentrations. The cells were treated with increasing concentration of GPE for 24 h. (**C**) GPEs suppressed the LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. (**D**) Quantification of iNOS suppression by GPEs. Expression levels of iNOS, COX-2, and β-actin were determined by western blot. Data are presented as mean ± SD of three independent experiments. $^{\#}$ $p < 0.05$ between control and LPS-exposed cells (no GPE); * $p < 0.05$; ** $p < 0.01$.

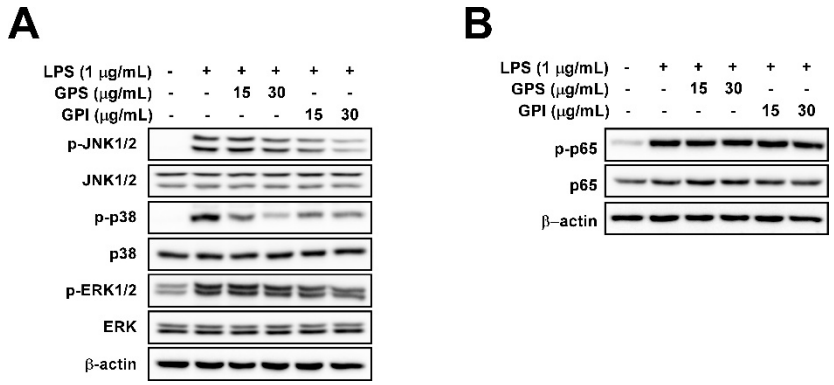

Figure 3. Effects of the GPEs on LPS-induced MAPK and NF-κB signaling pathways in RAW 264.7 cells. (**A**) GPEs suppressed the LPS-induced phosphorylation of JNK1/2, p38, and ERK1/2 in RAW 264.7 cells. (**B**) GPEs did not affect the LPS-induced phosphorylation of p65 in RAW 264.7 cells. Cells were treated with the indicated concentrations of GPEs and then stimulated with LPS (1 μg/mL). The levels of phosphorylation and expression were detected by western blot.

3.6. Effect of GPE on LPS-Induced Phosphorylation of c-Jun and c-Jun Translocation from the Cytosol to the Nucleus in RAW 264.7 Cells

Because GPS and GPI only affected the LPS-induced phosphorylation of MAPKs, but not p65, we next evaluated whether GPS and GPI affected the LPS-induced phosphorylation of c-Jun and c-Jun translocation from the cytosol to the nucleus. Western blot analysis and immunofluorescence analysis results showed that although GPI suppressed LPS-induced c-Jun phosphorylation and c-Jun translocation from the cytosol to the nucleus, GPS only suppressed the LPS-induced phosphorylation of c-Jun, but not c-Jun translocation in RAW 264.7 cells (Figure 4).

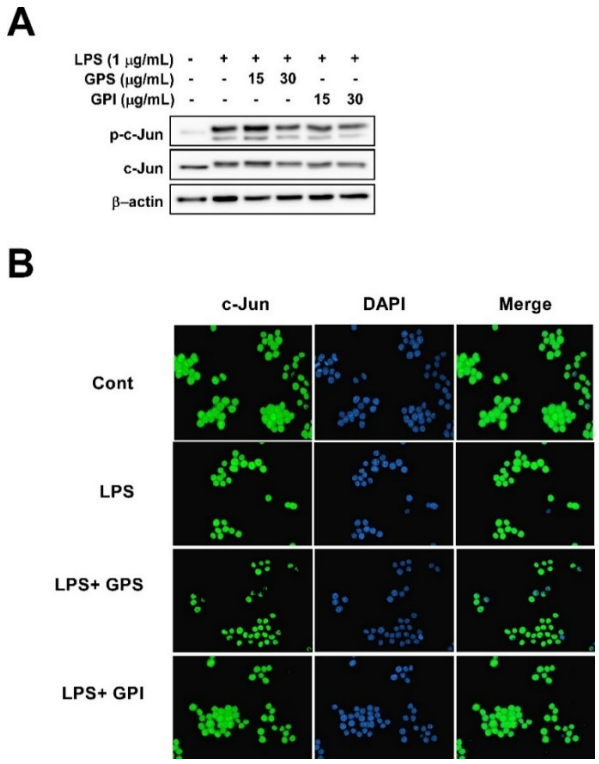

Figure 4. Effects of GPE on LPS-induced phosphorylation of c-Jun and nuclear translocation in RAW 264.7 cells. (**A**) GPEs suppressed the LPS-induced phosphorylation of c-Jun in RAW 264.7 cells. Cells were treated with the indicated concentration of GPE for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. The levels of phosphorylation and expression were detected by western blot. (**B**) GPE suppressed the LPS-induced c-Jun expression in the nucleus of RAW 264.7 cells. Cells were treated with the indicated concentration of GPE for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. The levels and locations of c-Jun were detected by immunofluorescence as described in the Materials and Methods section.

4. Discussion

ROS are the key metabolites generated during ATP production, and their overproduction can affect the redox homeostasis [34]. Under normal conditions, ROS are neutralized or eliminated by enzymatic and non-enzymatic antioxidant systems [1]. However, structural damages can occur in the cells when the cell's ROS influx level increases rapidly due to environmental or oxidative stress [1]. These oxidative stresses directly induce inflammation and they are further correlated with the occurrence of various diseases including diabetes, cardiovascular diseases, neurodegenerative diseases, and several types of cancers [35–37]. Therefore, ROS production needs to be regulated by the intake of antioxidants, which can prevent the inflammation caused by oxidative stress and limit the development of inflammatory diseases.

Pepper is one of the most widely used spices in the world, with a distinctive flavor and tangy taste, and it is considered as the king of spices [29,31]. Pepper can be black, green, or white depending on the degree of ripeness and the processing method used after harvest. The spiciness of green pepper is lesser than that of black pepper, and it is harvested in the unripe state [15]. The degree of maturation and the color of the pepper fruit have a strong effect on the biological activity of the pepper fruit [38]. Therefore, we evaluated the color, flavonoid contents, phenolic contents, and antioxidant activity of black and green pepper. The colors of the peppercorns are dependent on the chlorophyll content and various phytochemicals, which affect the flavonoid and phenolic contents and therefore, the antioxidant capacity. The contents of flavonoids and nonphenolic pigments are affected by the degree of the pepper fruit maturation and postharvest processing [39]. Our chromaticity analysis results showed that green pepper had high Hunter L* and b* values and low a* values, while black pepper had low Hunter L* and b* values and high a* values. As expected, the chlorophyll, flavonoid, and phenolic contents in GPI and GPS were higher than those in BPI and BPS.

The chlorophyll, flavonoid, and phenolic contents are closely associated with the antioxidant capacity of botanical extracts [30,31]. As expected, we observed a positive correlation between chlorophyll a and b, flavonoid, and phenolic contents and the DPPH and ABTS radical scavenging capacities of GPI and GPS (Tables 1 and 2). Similarly, a previous study reported that the fluorescence recovery after photobleaching (FRAP) and ABTS scavenging capacity of *Salvia coccinea* (Lamiacease) are dependent on the chlorophyll and phenolic contents [40]. Several studies have shown the antioxidant activity of pepper [41,42]. Supercritical fluid extract of *P. nigrum* L. were reported to have EC_{50} values of 103.28 and 316.27 μg/mL [43]. Klitschko and colleagues evaluated the antioxidant activity of 11 fruits in Brazil and among them, murici was determined to have the highest DPPH and ABTS radical scavenging capacities of 295.12 ± 26.87 and 235.94 ± 0.12 mg VCE/100 g, respectively. In this study, GPI and GPS showed relatively high DPPH radical scavenging capacities of 522.83 ± 26.00 mg VCE/100 g and 518.67 ± 7.21 mg VCE/100 g, respectively and the ABTS radical scavenging capacity of 1941.91 mg VCE /100 g and 1902.77 mg VCE /100 g, respectively.

Because piperine is a major component of pepper [44], we predicted that the higher antioxidant activity of green pepper, as compared to that of black pepper, may be correlated with the piperine content. Interestingly, although BPS had the lowest DPPH and ABTS scavenging capacities, its piperine content (7388.50 ± 37.00 mg/100 g) was similar to that of GPI (8613.27 ± 45.86 mg/100 g), which had the strongest DPPH and ABTS radical scavenging capacities (Table 2). A previous study using supercritical extraction with carbon dioxide and gas chromatography with flame ionization detection (GC-FID) and gas chromatography and mass spectrometry (GC-MS) analysis showed that *P. nigrum* contains β-caryophyllene, limonene, sabinene, 3-carene, β-pinene, and α-pinene [43]. Kapoor et al. showed that after extraction of *P. nigrum* with the Clevenger and Soxhlet apparaturs, GC-MS revealed that piperine, β-caryophyllene, limonene, β-pinene, and sabinene were the components present in *P. nigrum* [44]. These studies suggest that various compounds besides piperine may contribute to the antioxidant activity of green pepper. Therefore, other components present in green pepper should be identified and quantified by liquid chromatography-MS analysis in future studies.

Inflammation is the major cause of various metabolic diseases such as diabetes, cardiovascular diseases, and brain disease; therefore, inflamed tissues are the major therapeutic target for functional foods as well as medication [9,45–47]. Because green pepper was found to have higher antioxidant activity than black pepper, we further evaluated the anti-inflammatory efficacy and the mechanism of action of GPEs in this study. GPS and GPI inhibited LPS-induced nitrite production by suppressing iNOS expression in RAW 264.7 cells. Abnormal production of NO and expression of iNOS as a response to LPS are considered as an inflammatory phenomenon in mammals. Thus, it can be assumed that green pepper is a promising anti-inflammatory nutraceutical for the treatment of various inflammatory diseases.

As the central mediator in LPS-induced iNOS expression and subsequent NO production, pepper extracts were evaluated for their effects on the phosphorylation of p65, the catalytic subunit of NF-κB.

Interestingly, p65 phosphorylation was not affected by GPS and GPI. Since MAPKs are also a major factor affecting the NF-κB signaling pathways and iNOS expression, we evaluated the effects of GPS and GPI on LPS-induced phosphorylation of MAPKs. GPS and GPI suppressed the LPS-induced phosphorylation of JNK1/2 and p38, while the GPEs only slightly suppressed the phosphorylation of ERK1/2 in RAW 264.7 cells. In fact, NF-κB plays a critical role in LPS-mediated iNOS expression and NO production. In a previous study, we found that seaweed extracts affect the phosphorylation of MAPKs, but not NF-κB signaling pathways by using SR2001, a specific AP-1 inhibitor [48]. We demonstrated that only AP-1 can regulate LPS-induced iNOS expression and NO production in RAW 264.7 cells. Therefore, we further evaluated the effect of GPS and GPI on LPS-induced phosphorylation of c-Jun, a down-stream signaling molecule of JNK, and c-Jun translocation from the cytosol to the nucleus. Western blot assay and immunofluorescence analysis results showed that GPI suppressed the phosphorylation of c-Jun and c-Jun translocation (Figure 4). These results indicate that the inhibitory effect of GPS and GPI on LPS-induced iNOS expression and NO production results from the inhibition of phosphorylation of MAPKs and c-Jun and translocation of c-Jun from the cytosol to the nucleus.

5. Conclusions

The findings of our study showed that GPI and GPS have a greater antioxidant capacity and higher chlorophyll a and b, flavonoid, and phenolic contents than BPI and BPS. Moreover, we investigated the mechanism of action and the anti-inflammatory efficacy of GPI and GPS and we found that green pepper inhibited NO-related inflammation by suppressing LPS-induced phosphorylation of MAPKs and c-Jun phosphorylation and translocation from the cytosol to the nucleus. Further, piperine in pepper has been reported to enhance drug bioavailability because it inhibits glucuronidation by UDP-glucuronosyltransferase. As a nonpolar molecule, piperine may form complexes with polar drugs, thereby facilitating their absorption. Moreover, piperine is absorbed rapidly across the intestinal barrier. However, further studies should be performed on how piperazine inhibits the biological transformation of drugs in the intestine and in the liver in cases wherein the drug is not metabolized effectively. In summary, the findings of our study confirm that green pepper can be considered as a promising antioxidant and anti-inflammatory nutraceutical for the treatment of various inflammatory metabolic diseases.

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