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Increase in nitrite content and functionality of ethanolic extracts of *Perilla frutescens* following treatment with atmospheric pressure plasma

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ABSTRACT

This study investigated the effect of atmospheric pressure plasma (APP) treatment on nitrite content and functionality of plant extracts. Ethanolic extracts of *Perilla frutescens* (EEP) were prepared and treated with APP for 60 min. Nitrite content increased from 0 to 45.8 mg/l in EEP after APP treatment for 60 min. Antimicrobial activity of EEP against *Clostridium perfringens* and *Salmonella* Typhimurium was increased by APP with no influence on antioxidative activity ($p < 0.05$). Lyophilized EEP (LEEP) treated with APP for 60 min contained 3.74 mg/g nitrite. The control (LEEP without APP) contained no nitrite. The minimum inhibitory concentration (MIC) of LEEP for *C. perfringens* was 200 μg/ml. The control did not inhibit *C. perfringens* growth between 25 and 1000 μg/ml. MICs of LEEP and the control against *S.* Typhimurium were 25 and 50 μg/ml, respectively. New nitrite sources with increased antimicrobial activity can be produced from natural plants by APP treatment.

1. Introduction

Nitrite (NO_2^-) has been used as a medicine and preservative in the pharmaceutical and food industries, respectively (Parthasarathy & Bryan, 2012). The biological and functional roles of nitrite are based upon the conversion to nitric oxide (Lundberg, Weitzberg, & Gladwin, 2008; Parthasarathy & Bryan, 2012). The use of nitrite and the subsequent donation of nitric oxide results in vasodilation and wound healing in the human body (Lundberg et al., 2008). Nitrite has multi-functional roles in food, especially in cured meat products. Nitrite can control *Clostridium botulinum* and its spores, as well as spoilage and pathogenic microorganisms. It is also involved in the development of cured colour and flavour, and in the inhibition of lipid oxidation in cured meat products (Parthasarathy & Bryan, 2012).

Both synthetic and natural sources of nitrite have been used in food industries. Synthetic nitrite sources, such as sodium or potassium nitrite, are generally used in industry because of their convenience of use. However, an increase in consumer concern regarding the use of chemically synthetic compounds has resulted in their gradual exclusion from industries (Sebranek & Bacus, 2007). Natural nitrite sources can be derived from natural plants that contain nitrate (NO_3^-). The conversion of nitrate in plant extracts to nitrite by nitrate reductase is a well-known process (Parthasarathy & Bryan, 2012; Sebranek, Jackson-Davis, Myers, & Lavieri, 2012). However, the inherent variability of nitrate levels in plants makes them difficult to control in natural nitrite sources. Previous studies have reported that nitrate levels in plants vary depending on the harvest conditions, including area and season (Amr & Hadidi, 2001; Chang, Yang, & Riskowski, 2013). In addition, candidate plants for use as natural nitrite sources are limited. The antimicrobial and antioxidative properties of additives derived from natural plants are important as natural preservatives when used in foods that are perishable and sensitive to the lipid oxidation (Lee et al., 2015). However, natural plants that do not contain nitrate are not candidate natural nitrite sources, even though they have strong antimicrobial and antioxidative activities.

Plasma is an ionized gas that can be generated by supplying energy to gas. Plasma, especially non-thermal atmospheric pressure plasma (APP), has received considerable interest because of its availability for the non-thermal sterilization of materials and foods, and for medical applications (Attri et al., 2015; Yong et al.,

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2015; Yun et al., 2010). Recently, plasma has been receiving renewed interest as a means of producing functional materials via the interaction of plasma and liquid. Discharges in the gas phase include reactive oxygen and nitrogen species, such as hydroxyl radicals, superoxide, hydrogen peroxide, ozone, and nitric oxides (Ercan, Smith, Ji, Brooks, & Joshi, 2016; Lukes, Dolezalova, Sisrova, & Clupek, 2014). In the gas-liquid environment, discharges in the gas phase can penetrate into liquid and cause various chemical processes. A previous study reported that water treated with plasma showed antimicrobial activity with the generation of various nitrogen compounds including nitrite as well as nitrate and peroxynitrite in water (Ercan et al., 2016; Oehmigen et al., 2010; Traylor et al., 2011). In addition, plasma treatment of naringin dissolved in methanol resulted in increased antioxidative and antimicrobial activity with the structural modification of naringin (Kim et al., 2014).

We hypothesized that novel sources of nitrite derived from a natural plant can be produced. Through such a process, an increase in the nitrite level of plant extracts can be expected following plasma treatment regardless of their inherent nitrate level, which may be accompanied by an increase in the antimicrobial and antioxidative activity of plant extracts. Therefore, the aim of this study was to investigate the nitrite level, antimicrobial activity, and antioxidative activity of plant extracts of *Perilla frutescens* (L.) Britton var. *acuta* Kudo (red perilla) following treatment with atmospheric pressure dielectric barrier discharge (DBD) plasma. Red perilla is a natural edible plant cultivated in Asia. The leaves of red perilla have been traditionally used as medicine for relieving allergies, intestinal disorders, and skin lesions, and as a food ingredient to improve flavour and shelf-life (Jung & Lee, 2000; Kim, Seo, & Bae, 2004; Lee et al., 2015; Makino et al., 2003).

2. Materials and methods

2.1. Reagents

Potassium hexacyanoferrate(II) trihydrate, zinc sulfate heptahydrate, dibasic potassium phosphate (PubChem CID: 24450), sodium nitrite (PubChem CID: 23668193), tetrabytylammonium hydrogen sulfate (PubChem CID: 94433), Folin & Ciocalteu's phenol reagent, gallic acid and 1,1-diphenyl-2-picrylhydrazyl radical were purchased from Sigma-Aldrich (St. Louis, MO, USA). High performance liquid chromatography (HPLC)-grade methanol (PubChem CID: 887) was purchased from J.T. Baker Co. (Center Valley, PA, USA). Sodium hydroxide (food grade) and 70% ethanol (food grade) were purchased from Youngjin Co. (Bucheon, Korea) and Duksan Co. (Yongin, Korea), respectively.

2.2. Preparation of plant extract

Fresh *Perilla frutescens* (L.) Britton var. *acuta* Kudo (red perilla) was purchased from a local farm and used in this study. Leaves (2 kg) of red perilla were mixed with 6 l 70% EtOH and then homogenized with a vacuum blender (Hanssem Co., Seoul, Korea). The homogenate was centrifuged at 6710*g* for 30 min (CR 20B2, Hitachi Koki Co., Ltd. Fukuoka, Japan) and the supernatant was filtered through Whatman No. 4 filter paper (Whatman Inc., Maidstone, England). The pH of the ethanolic extract of red perilla (EEP) was adjusted to 9.0 using 5 N NaOH. EEP (2 litres) and the mixture then subjected to APP treatment.

2.3. APP treatment

APP treatment was carried out using a DBD plasma system (Plasmapp PCS-20N, Plasmapp Co., Daejeon, Korea), composed of a main chamber for mixing plant extracts, a plasma chamber with a cooling system, a plasma power supply and a gas circulating module (Fig. 1). The main chamber stored EEP and provided a sealed space, and plasma was discharged by the power supply in the plasma chamber, which was connected to the main chamber. The discharged gas was supplied into the EEP in the main chamber by the gas circulating module, in which a diaphragm pump of the module received the activated gas from the main chamber and provided it to the plasma chamber. The plasma generator in the plasma chamber included 16 DBD modules and the two plasma electrodes, which had different polarities, were coated with silver (Ag) on the opposite sides of the rectangular alumina (Al_2O_3) plate of each DBD module. The modules were supported by copper (Cu) blocks at both ends of the ceramic plate, and electrodes were connected in parallel to the plasma power supply, which had an input power and frequency of 550 W and 25 kHz, respectively. The power generated a strong electric field near the boundary lines of the electrodes, and the plasma was discharged from the lines on both sides of the module. Ambient gas consisting mainly of nitrogen and oxygen was excited by the discharged plasma to produce reactive nitrogen species, which were supplied into the EEP in the main chamber. APP treatment of EEP was conducted for 60 min and EEP was collected at 10-min intervals. EEP treated with APP for 60 min was lyophilized (Ilshin Co., Seoul, Korea), and stored until analysis in a −70 °C deep freezer. APP treatment of EEP was performed in triplicate.

2.4. pH measurement

The pH of EEP treated with APP was measured using a pH meter (SevenEasy, Mettler-Toledo Inti Inc., Schwerzenbach, Switzerland).

2.5. Nitrite measurement by HPLC

The nitrite content in EEP and the lyophilized EEP (LEEP) was measured using ion-pair chromatography. EEP and LEEP were prepared for HPLC analysis according to the method described by Shah, Petroczi, James, and Naughton (2013). EEP (5 ml) or the solution of LEEP (1 g/100 ml DI water) was mixed with 20 ml deionized water in a test tube, and then placed in a boiling water bath at 80 °C for 20 min. After the test tube was cooled to room temperature, 2 ml of Carrez-I solution [3.6% potassium hexacyanoferrate(II) trihydrate] was added and the samples were mixed. Carrez-II solution (2 ml; 7.2% zinc sulfate heptahydrate) was added followed by mixing. The test tube was centrifuged at 2090*g* for 15 min (Union 32R, Hanil Co., Ltd., Incheon, Korea). The supernatant was collected and made up to 50 ml with deionized water. The sample solution was passed through a 0.2-μm PVDF syringe filter (Whatman), and the filtrate was collected into a vial. The nitrite content was then analyzed by HPLC (ACME 9000, Younglin Instruments Inc., Korea). HPLC was performed using a Eurosil Bioselect 300-5 C18 column (4.0 × 125 mm, KNAUER Co., Berlin, Germany) with a mobile phase consisting of 5 mM tetrabutylammonium hydrogen sulfate (pH was adjusted to 6.5 with 1 M dibasic potassium phosphate). The isocratic flow rate of the mobile phase was 0.7 ml/min, and the injection volume was 10 μl. The column temperature was maintained at 40 °C and the UV/VIS detector was set to 220 nm. The concentration of nitrite in the sample solution was calculated using a standard curve of sodium nitrite.

2.6. Antioxidative activity measurement

2.6.1. Total phenolic content

Total phenolic content was estimated by the Folin-Ciocalteu method (Subramanian, Padmanaban, & Sarma, 1965). EEP (0.1 ml) or LEEP (100 mg/100 ml DI water) was added to the

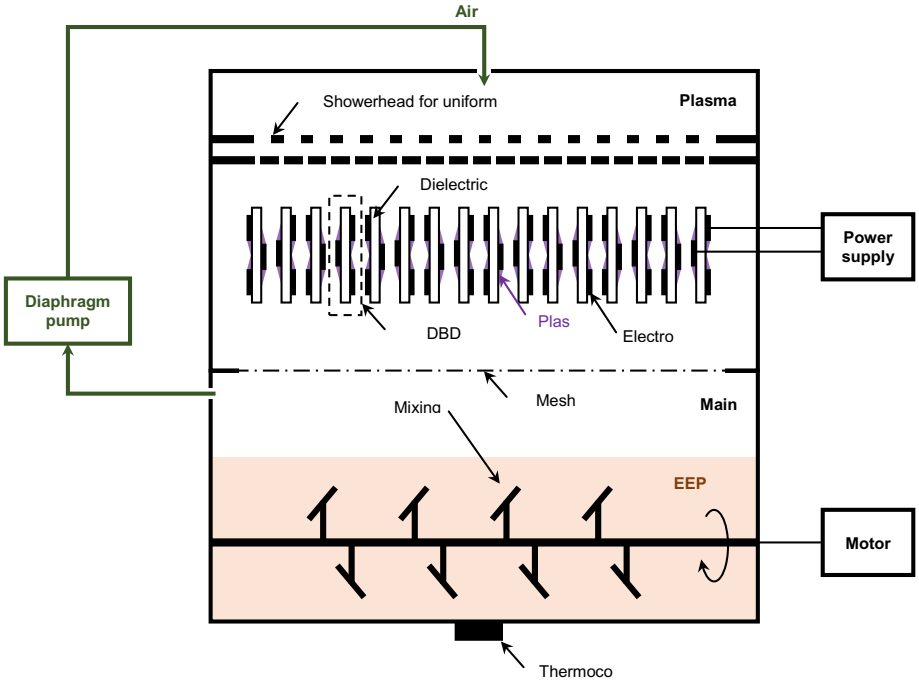

Fig. 1. Atmospheric pressure plasma system used in this study.

Folin-Ciocalteu phenol reagent (0.2 ml), followed by the addition of 3 ml 5% sodium carbonate solution. The reaction mixture was vortexed and the absorbance was measured with a spectrophotometer (DU 530, Beckman Instruments Inc., Fullerton, CA, USA) at 765 nm after incubation for 1 h at 23 °C. Phenolics were quantified based on a standard curve generated with the use of gallic acid, and expressed as gallic acid equivalent.

2.6.2. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of EEP or LEEP was estimated according to the method described by Blois (1958). A 200 μl sample of EEP or LEEP (50, 40, 30, 20 and 10 mg/100 ml DI water) was added to 800 μl deionized water and 1 ml of methanolic DPPH solution (0.2 mM). The mixture was vortexed and left to stand at room temperature (20–22 °C) for 30 min. A tube containing 1 ml of distilled water and 1 ml of methanolic DPPH solution (0.2 mM) served as the control. The absorbance of the solution was measured at 517 nm using a spectrophotometer (Beckman). The percentage of DPPH radical scavenging was obtained from the following equation:

$$\text{Radical scavenging activity} = [1 - (\text{absorbance of sample}/\text{absorbance of control})] \times 100.$$

The half maximal effective concentration (EC_{50}) of LEEP for DPPH radical scavenging was calculated by interpolation from the data.

2.7. Antimicrobial activity measurement

2.7.1. Paper disc diffusion assay

Escherichia coli 0157:H7 (NCTC 12079), *Listeria monocytogenes* (KCTC 13064), *Salmonella* Typhimurium (SL 1344), and *Clostridium perfringens* (NCTC 8239) were stored in a deep freezer in the laboratory at −80 °C. *E. coli* O157:H7 and *S.* Typhimurium were cultivated in Lruia-Bertani (LB) medium containing 0.5% NaCl (Difco, Detroit, MI, USA). *L. monocytogenes* and *C. perfringens* were cultivated in Brain Heart Infusion (BHI) broth and Reinforced Clostridial Medium (RCM) broth (Difco), respectively. Each strain was streaked on the media and the plates were incubated under aerobic or anaerobic condition for 16 h at 37 °C. Seed cultures were prepared by inoculating single colonies in 5 ml broth and were then incubated for 16 h at 37 °C and 200 rpm. The antimicrobial activity of EEP was determined using the paper disc diffusion assay. Aliquots (0.1 ml) of *E. coli* O157:H7, *L. monocytogenes,* and *S.* Typhimurium were spread over the surface of agar plates, and 0.1-ml aliquots of *C. perfringens* were spread over the surface of Mueller-Hinton agar plates. Sterilized filter-paper discs were saturated with 50 μl of the plant extract (filtered using a 0.2-μm syringe filter) and aerobically incubated for 8 h at 37 °C or anaerobically incubated for 10 h at 37 °C (*C. perfringens*). The diameters of microbe-free clear zones around the disc on the culture plates were measured using a Digimatic caliper (model 500-181-20, Mitutoy Co., Kawasaki, Japan). EtOH (70%) and 70 μg/ml sodium nitrite in 70% EtOH were used as control samples.

2.7.2. Minimum inhibitory concentration (MIC) assay

The antimicrobial activity of LEEP against *S.* Typhimurium (SL 1344), and *C. perfringens* (NCTC 8239) was measured by a MIC assay. The strains were inoculated aerobically or anaerobically into 5 ml of medium for 16 h at 37 °C. A 20-μl culture was transferred to 20 ml of new broth and cultivated until the absorbance of the cultures at 600 nm was 0.8. A 150-μl culture sample was dispensed into an individual well of a 96-well microplate with a diluted solution of LEEP. The microplates were aerobically or anaerobically incubated for 12 h at 37 °C, respectively. The absorbance of each well was measured at a wavelength of 600 nm by a microplate reader (Epoch, Bio Tek Instruments, Inc., Winooski, USA). The lowest concentration that significantly inhibited bacterial growth was defined as the MIC.

2.8. Statistical analysis

All experiments in this study were performed in in triplicate. A general linear model was generated using the raw data, and

Tukey's multiple range test was used to compare significant differences between least square mean values ($p < 0.05$). Least square mean values and standard errors of the least square means (SEM) are reported. SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

3. Results and discussion

3.1. pH of EEP after APP treatment

The intrinsic pH of the ethanolic extract from the leaves of red perilla (EEP) was around 6.5. After APP treatment, the pH of EEP gradually decreased until it reached 2.3 (data not shown). The acidification of liquid following APP treatment in an atmospheric air environment is a common phenomenon, and occurs because of the diffusion of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in gas-phase discharges into liquid, resulting in the release of hydrogen ions (Ercan et al., 2016; Kojtari et al., 2013; Oehmigen et al., 2010; Sakiyama, Graves, Chang, Shimizu, & Morfill, 2012). However, nitrite that is unstable under acidic conditions decomposes to form nitrous acid ($pK_a = 2.8 - 3.2$) and subsequently nitrogen oxides via the following reactions (1)–(4) (Rayson, Mackie, Kenndy, & Dlugogorshi, 2012; Thomas & Vanderschuren, 1997).

$$NO_2^- + H^+ HNO_2 \quad (1)$$

$$HNO_2 + H^+ \rightarrow H_2NO_2^+ \rightarrow NO^+ + H_2O \quad (2)$$

$$2HNO_2 \rightarrow NO + NO_2 + H_2O \quad (3)$$

$$3HNO_2H^+ + NO_3^- + 2NO + H_2O \quad (4)$$

The decomposition of nitrite into nitrous acid can be prevented under alkaline conditions by the deprotonation of nitrous acid via reaction (1) (Jung et al., 2015a; Lukes et al., 2014). Therefore, in the present study, alkalization of EEP was required before APP treatment for the utilization of EEP as a nitrite source. Plant extracts contain various phenolic compounds, which act as antioxidant and antimicrobial agents. Phenolic compounds are sensitive to high pH and are adversely affected by pH values above 9 (Friedman & Jurgens, 2000). Therefore, in the present study, the pH of EEP before APP treatment was adjusted to 9 to ensure maximum alkalization of EEP and prevent damage to the phenolic compounds in EEP.

The pH of EEP gradually decreased with increasing APP treatment time ($p < 0.05$, Fig. 2). This was followed by the generation of nitrous acid and nitric acid in EEP by APP treatment. The pH values of EEP treated with APP for 50 and 60 min were 6.16 and 5.89, respectively. Braida and Ong (2000) reported that nitrite in solution was decomposed into nitrogen oxides when the pH of the solution was less than 6. Therefore, APP treatment to EEP in the present study was conducted for up to 60 min.

3.2. Nitrite content of EEP with APP treatment

Nitrite was not detected in EEP without APP treatment (Fig. 3). Following APP treatment, the nitrite content of EEP was significantly increased, up to 43.9 mg/l, with increasing treatment time up to 50 min ($p < 0.05$). Various studies have reported an increase in the nitrite content in liquid following APP treatment under atmospheric air conditions (Ercan et al., 2016; Jung et al., 2015a, 2015b; Kojtari et al., 2013; Oehmigen et al., 2010). Nitrite can be formed in liquid by post-discharge reactions following APP treatment. APP treatment in atmospheric air produces ROS and RNS in gas-phase discharges. Of the ROS and RNS generated, O_3, H_2,

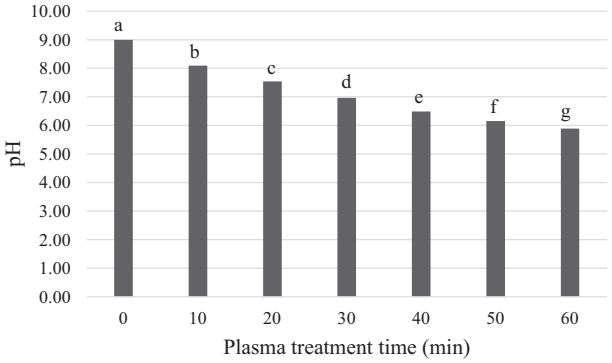

Fig. 2. The pH of ethanolic extracts of red perilla leaf following treatment with atmospheric pressure plasma. Standard error of the mean = 0.052 (n = 21). [a–g] Different letters represent significant differences ($p < 0.05$).

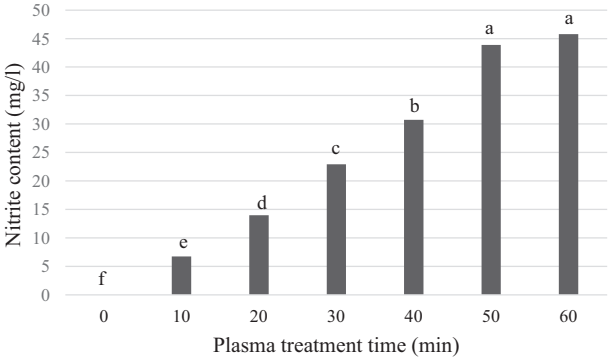

Fig. 3. Nitrite content in ethanolic extracts of red perilla leaf following treatment with atmospheric pressure plasma. Standard error of the mean = 0.464 (n = 21). [a–f] Different letters represent significant differences ($p < 0.05$).

H_2O_2, NO_2, NO_3, N_2O, N_2O_3 and N_2O_5 are relatively stable and dominant species (Sakiyama et al., 2012). Nitrogen oxides from gas-phase discharges diffuse into liquid and react with water molecules, producing nitrites with the release of hydrogen ions via reactions (5)–(8) (Lukes et al., 2014; Rayson et al., 2012; Thomas & Vanderschuren, 1997). In addition, oxygen dissolved in liquid can participate in the production of nitrite via reaction (9) (Lukes et al., 2014; Oehmigen et al., 2011)

$$NO + NO_2 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (5)$$

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (6)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (7)$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (8)$$

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (9)$$

The nitrite content of EEP with APP treatment for 60 min was 45.8 mg/l, which was not significantly different from that of EEP treated with APP for 50 min ($p > 0.05$). The decomposition of nitrite into nitrous acid or nitrogen oxides in solution occurred below pH 6 (Braida & Ong, 2000). The pH of EEP treated with APP for 60 min was 5.89. Therefore, it seemed that the decomposition of nitrite into nitrous acid was initiated following APP treatment for 50 min. In addition, many researchers have found that the hydrogen peroxide content was increased in liquid after APP treatment (Ercan et al., 2016; Lukes et al., 2014; Oehmigen et al., 2011). Therefore, it is also possible that the nitrite generated in EEP was

decreased following reaction with hydrogen peroxide (10). Furthermore, generated peroxynitrous acid ($pK_a = 6.8$) was decomposed into hydroxyl radical and nitrogen dioxide via reaction (11).

$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \quad (10)$$

$$ONOOH \rightarrow OH + NO_2 \quad (11)$$

3.3. Antioxidative and antimicrobial activity of EEP with APP treatment

Red perilla, which was used in this study, is a natural edible plant cultivated in Asia. The leaves of red perilla have been traditionally used as medicine for relieving allergies, intestinal disorders, and skin lesions, and as a food ingredient to improve flavour and shelf-life (Jung & Lee, 2000; Kim et al., 2004; Lee et al., 2015; Makino et al., 2003). The water and ethanol extract from leaves of red perilla contain various phenolic compounds and have shown antioxidative and antimicrobial activity in *in vitro* assays and foods (Kim, Kang, Lee, Kwoen, & Choi, 2007; Meng, Lozano, Bombarda, Gaydou, & Li, 2009; Lee et al., 2015).

Recent studies have reported that the antioxidative activity of the phenolic compound naringin solubilized in methanol was increased by APP treatment (Kim et al., 2014, 2015). These authors also found that APP treatment of methanol containing naringin increased the total phenolic content with the generation of new naringin derivatives such as narinplasmins A and B. However, in the present study, the antioxidative activity of EEP was not enhanced by APP treatment (Fig. 4). In addition, there were no changes in the total phenolic content and DPPH radical scavenging activity of EEP with increasing APP treatment time ($p > 0.05$). Kim et al. (2014) reported that degradation of naringin into smaller derivatives in methanol following APP treatment might be attributed to energy input, especially the absorption of ROS from gas-phase discharges into methanol. It is well known that ROS and RNS generated in gas-phase discharges are key factors for sterilizing the surface of target materials (Attri et al., 2015; Yong et al., 2015; Yun et al., 2010). However, the life times of most ROS generated in the gas phase after APP treatment were less than 2.7 μs (Attri et al., 2015). Previous studies have reported an increase in the polyphenol content in liquid in response to plasma treatment when the distance between the discharge layer and the liquid surface is less than 1.5 cm (Garofulic et al., 2015; Kim et al., 2014). Conversely, in the plasma system used in the present study, the distance between the discharge layer in the plasma chamber and the surface of EEP was over 25 cm. Therefore, it is thought that the ROS in gas-phase discharges were rarely absorbed in the EEP and did not lead to the structural modification of polyphenols in EEP.

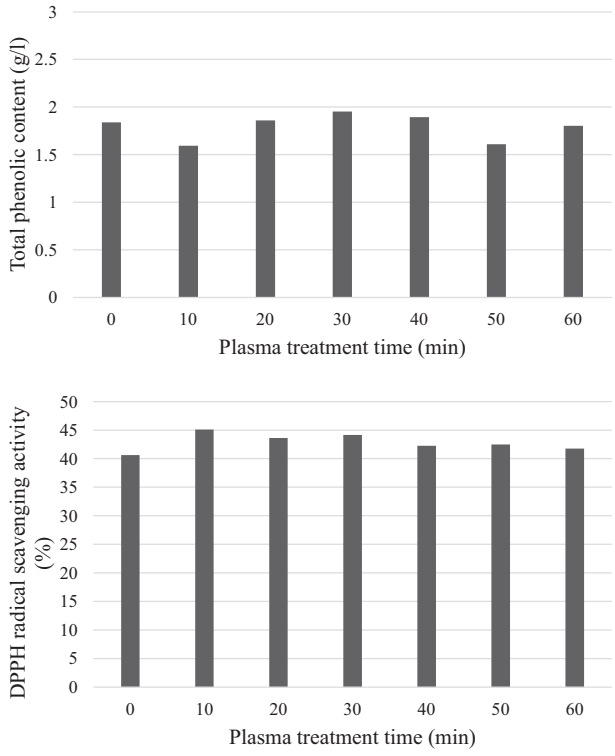

Fig. 4. The antioxidative activity of ethanolic extracts of red perilla leaf following treatment with atmospheric pressure plasma. Standard error of the mean for total phenolic content = 0.412 (n = 21). Standard error of the mean for DPPH radical scavenging activity = 3.677 (n = 21).

The antimicrobial activity of EEP was influenced by APP treatment (Table 1). The clear zones of *C. perfringens* in response to 70% EtOH and 70 μg/ml sodium nitrite in 70% EtOH were 9.7 and 10.7 mm, respectively, with no significant difference between the two ($p > 0.05$). The antimicrobial activity of EEP against *C. perfringens* following APP treatment for 10 min was not significantly different from that with 70% EtOH, 70 μg/ml sodium nitrite, and EEP without APP treatment ($p > 0.05$). However, the *C. perfringens* clear zone in response to EEP after APP treatment for 20 min was significantly larger than that of the other treatments ($p < 0.05$). The largest clear zone of *C. perfringens* was found when EEP was treated with APP for 60 min. Cammack et al. (1999) reported that the growth of *C. perfringens* could be controlled by nitrite. In the present study, sodium nitrite at a concentration of 70 μg/ml (42 μg/ml as nitrite) did not inhibit the growth *C. perfringens* when compared to 70% EtOH. However, EEP with over 20 min APP treatment exhibited increased inhibition of *C. perfringens* growth, although EEPs contained a nitrite level lower than or similar to that of 70 μg/ml sodium nitrite. *L. monocytogenes* growth can also be controlled by nitrite (Cammack et al., 1999). The inhibitory effect of nitrite on *L. monocytogenes* growth was also observed following treatment with 70 μg/ml sodium nitrite, while no such effect was observed with 70% EtOH. EEPs showed a similar inhibitory effect against *L. monocytogenes* when the nitrite level reached that of 70 μg/ml sodium nitrite, which can be achieved by APP treatment for 50 and 60 min. The inhibitory effect of nitrite was reported to be inadequate for gram-negative pathogens (Sebranek, 2009). The clear zone of *S.* Typhimurium with 70 μg/ml sodium nitrite was 10.3 mm, which was not significantly different from that with 70% EtOH. Clear zones of *S.* Typhimurium were not found with EEP without APP treatment or with APP treatment for up to 20 min. However, APP treatment for longer than 40 min significantly increased the inhibitory effect of EEP on the growth of *S.* Typhimurium ($p < 0.05$). EEP given APP treatment for 60 min inhibited *E. coli* O157:H7 growth, while no clear zones of *E. coli* O157:H7 were found when treated with other EEPs, as well as 70% EtOH and 70 μg/ml sodium nitrite. These results indicate that there was a marked increase in the antimicrobial activity of EEP following APP treatment on *C. perfringens* and *S.* Typhimurium, although EEP given APP treatment for 60 min had improved antimicrobial activity against all pathogens tested in this study. Previous studies have shown increased antimicrobial activity of liquid after APP treatment. Kim et al. (2014) reported that APP treatment of naringin solubilized in methanol increased antimicrobial activity by increasing phenolic compounds via the degradation of naringin into smaller molecules. However, the total phenolic content of EEP was not changed following APP treatment in the present study. Nitrite, hydrogen peroxide, and peroxynitrite have been reported as key elements involved in the antimicrobial activity of plasma-treated liquids (Ercan et al., 2016; Oehmigen et al., 2011).

Table 1
Antimicrobial activity of ethanolic extracts from red perilla leaf following treatment with atmospheric pressure plasma as measured using the paper disc diffusion assay.

	Pathogens			
	Clostridium perfringens	*Listeria monocytogenes*	*Salmonella* Typhimurium	*Escherichia coli* O157:H7
70% EtOH	9.7[e][2]	–[c][3]	10.3[c]	–[b]
Sodium nitrite	10.7[e]	9.7[b]	10.3[c]	–[b]
0 min	10.8[e]	–[c]	–[d]	–[b]
10 min	10.5[e]	–[c]	–[d]	–[b]
20 min	17.3[d]	–[c]	–[d]	–[b]
30 min	20.7[c]	–[c]	10.0[c]	–[b]
40 min	20.8[c]	–[c]	12.7[b]	–[b]
50 min	27.3[b]	9.3[b]	15.7[a]	–[b]
60 min	30.8[a]	11.0[a]	15.7[a]	11.3[a]
SEM[1]	0.64	0.16	0.25	0.11

[1] Standard error of the mean (n = 27).
[2] Clear-zone diameter (disc diameter = 8 mm).
[3] No inhibition.
[a–e] Different letters within the same column represent significant differences ($p < 0.05$).

Cytotoxic molecules, such as nitrogen monoxide and nitrogen dioxide, are produced from nitrite via multiple reactions (1, 3, 10, and 11). However, for this reaction, acidic conditions are required. Therefore, in the present study, the compound responsible for increasing the antimicrobial activity of EEP could differ depending on the pH of EEP. Peroxynitrite is a highly cytotoxic molecule that easily diffuses through the cell membrane (Ercan et al., 2016). The generation of peroxynitrite in EEP by a post-discharge reaction was not possible when the pH of EEP was alkaline; however, the generation of peroxynitrite in the gas-discharge phase is possible via reactions 12 and 13, and consequently diffused into EEP (Lukes et al., 2014). In addition, peroxynitrite has a life time of days under alkaline conditions (Beckman & Koppenol, 1996).

$$O_2^- + NO \rightarrow ONOO^- \quad (12)$$

$$OH + NO_2 \rightarrow ONOO^- + H^+ \quad (13)$$

Conversely, the highest antimicrobial activity of EEP (pH 5.89) observed following APP treatment for 60 min was thought to be related to the generated nitrogen monoxide, nitrogen dioxide and peroxynitrous acid. Of the predicted cytotoxic molecules in EEP, nitrogen dioxide might be important because of its long life time of several days, while those of nitrogen monoxide and peroxynitrous acid are a few seconds (Lukes et al., 2014; Oehmigen et al., 2011). However, the elucidation of increased antimicrobial activity of plant extracts following APP treatment is more complex than that of plasma-treated pure water or simple buffer investigated in previous studies. Plant extracts contain various compounds, including phenolic acid and flavonoids. Therefore, the synergistic effect of different compounds in plant extracts with substances generated by APP treatment could also be responsible for the increased antimicrobial activity. In addition, the substances generated by APP treatment in plant extracts could lead to complex chemical processes involving compounds inherently present in plant extracts. Therefore, further study is required for a clear understanding of the effect of APP treatment on the antimicrobial activity of plant extracts.

3.4. Properties of LEEP after APP treatment

The nitrite content, antioxidative activity and antimicrobial activity of LEEP given APP treatment for 60 min (LEEP) were compared with that of LEEP with no APP treatment (control). LEEP contained as much as 3.74 mg/g nitrite, while the control contained no nitrite (Table 2). There was no significant difference in the total phenolic content and EC_{50} value of DPPH radical scavenging between the control and LEEP. *C. perfringens* growth was not significantly inhibited following control treatment from 25 to 1000 μg/ml. However, the MIC of LEEP for *C. perfringens* was 200 μg/ml. In addition, the MIC of LEEP for *S.* Typhimurium was 25 μg/ml, while that of the control was 50 μg/ml.

Table 2
Properties of lyophilized ethanolic extracts from red perilla leaf (LEEP) following treatment with atmospheric pressure plasma.

	Control[1]	LEEP[2]	SEM[3]
Nitrite content (mg/g)	–[b]	3.74[a]	0.019
Antioxidative activity			
Total phenolic content (mg/g)	160.6	157.3	4.86
EC50 value of DPPH radical scavenging (μg/ml)	275.3	278.3	4.20
Antimicrobial activity			
MIC[4] for *C. perfringens*	–[5]	200	
MIC for *S.* Typhimurium	50	25	

[1] Lyophilized ethanolic extract from red perilla leaf without treatment with atmospheric pressure plasma.
[2] Lyophilized ethanolic extract from red perilla leaf following treatment with atmospheric pressure plasma for 60 min.
[3] Standard error of the mean (n = 6).
[4] Minimum inhibitory concentration that significantly inhibited bacterial growth.
[5] No inhibition was found at concentrations from 25 to 1000 μg/ml.
[a,b] Different letters within the same column represent significant differences ($p < 0.05$).

Although there was no effect of APP treatment on antioxidative activity, the APP treatment of plant extract (70% ethanolic extract of *Perilla frutescens* (L.) Britton var. *acuta* Kudo leaves [EEP]) resulted in the generation of nitrite and an increase in the antimicrobial activity of plant extracts. Consequently, powdered plant extracts that contain nitrite and exhibit improved antimicrobial activity can be obtained by APP treatment. Recently, APP has received considerable interest and has been accepted as an eco-friendly technology. In addition, Kim et al. (2016) reported no toxicity of plasma-treated water. Therefore, we conclude that the production of a new nitrite source from natural plants, regardless of their inherent nitrate level, is possible with APP treatment. In addition, natural plants have strong antioxidative and antimicrobial activity and may be good candidates as new nitrite sources following an increase in antimicrobial activity by APP treatment. However, the effect of APP treatment can be affected by the plasma system in terms of discharge gases, the distance between the plasma chamber and the target surface, and input power.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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