



## Effect of atmospheric pressure dielectric barrier discharge plasma on the biological activity of naringin



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### ABSTRACT

The biological activity of naringin treated with atmospheric pressure plasma was evaluated to investigate whether exposure to plasma can be used as a method to improve the biological activity of natural materials. Naringin was dissolved in methanol (at 500 ppm) and transferred to a container. A dielectric barrier discharge (DBD) (250 W, 15 kHz, ambient air) was then generated. Treatment with the plasma for 20 min increased the radical-scavenging activity, FRAP value, and the total phenolic compound content of naringin from 1.45% to 38.20%, from 27.78 to 207.78  $\mu\text{M/g}$ , and from 172.50 to 225.83 ppm, respectively. Moreover, the tyrosinase-inhibition effect of naringin increased from 6.12% to 83.30% upon plasma treatment. Naringin treated with plasma exhibited antimicrobial activity against foodborne pathogens, especially *Salmonella* Typhimurium; an activity that was absent before plasma treatment. Structural modifications induced in the naringin molecule by plasma might be responsible for improving the biological activity of naringin.

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### 1. Introduction

In recent years, interest in functional foods has grown because of the physiological benefits that these foods provide. In addition to nutrition and energy: functional foods exhibit, for example, antioxidant, anticancer and antihypertensive effects (Goldberg, 1996). Functional materials in this food category can be defined as materials that produce a beneficial effect in one or more physiological functions, increase wellbeing, and/or reduce the risk of suffering from specific diseases (Herrero, Cifuentes, & Ibañez, 2006).

Among the functional materials available, consumers strongly prefer materials derived from natural products over synthetic chemicals, because natural materials are effective against diverse types of diseases and injuries, and also produce fewer side effects due to little internal toxicity (Jung, 2004). Despite these beneficial effects, natural materials have been consumed mostly by drinking them with boiling water, even though several have been tested in trials for use in food or cosmetics. This is mainly because the colour and flavour of natural materials might make it challenging to use

suitable amounts of these materials in food or cosmetics (Jo, Son, Lee, & Byun, 2003). Thus, to promote the functional use of natural materials, applications based on non-thermal technology including high hydrostatic pressure and irradiation are being studied (Roldán-Marín, 2009).

Plasma treatment has been investigated as a non-thermal technology in food processing. Plasma, which is an ionised quasi-neutral gas (also known as the fourth state of matter) generated by electrical discharge (Bogaerts, Neyts, Gijbels, & van der Mullen, 2002), consists of electrons, ions, free radicals, atomic species, UV photons, and others (Gaunt, Beggs, & Georghiou, 2006). Because it contains an abundance of reactive species, plasma has demonstrated bactericidal, fungicidal and virucidal effects (Kim, Yong, Park, Choe, & Jo, 2013; Lee et al., 2012; Moreau, Orange, & Feuilletoy, 2008).

Dielectric barrier discharge (DBD) plasma is generated by using a single, or two, different electrodes covered by dielectric layers, which prevent a sudden large increase of discharge current and streamer formation (Moreau et al., 2008). The advantages of DBD are its simplicity, and the availability of efficient and affordable power supplies. Fridman et al. (2007) reported that the negative effects of DBD on living targets are minimised when the plasma is used under appropriate conditions.

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Numerous studies have reported the use of plasma to increase food safety. However, no study has yet attempted to use plasma to enhance the biological activity of a natural material for inclusion in food and cosmetic compositions. Therefore, in this study, the changes in the biological activities of naringin (a major flavonoid in citrus fruits) induced by DBD were evaluated, to examine whether plasma technology can be used to improve the functional effects of natural materials.

## 2. Materials and methods

### 2.1. Sample and plasma treatment

Naringin was purchased from Sigma Co. (St. Louis, MO, USA). Naringin solutions (500 ppm, dissolved in methanol) were prepared for each experiment and diluted before use.

An encapsulated DBD plasma source was fabricated using a rectangular, parallelepiped plastic container (137 × 104 × 53 mm) (Fig. 1). The actuator was made of copper electrodes and a polytetrafluoroethylene sheet and attached to the inner walls of the container. A bipolar square-waveform voltage at 15 kHz was applied to one electrode while the other electrode was grounded. The plasma was generated inside the container with an input power of 250 W. The naringin samples were placed in a glass dish at the bottom of the container and treated with plasma for 0, 5, 10, and 20 min.

### 2.2. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

Free radical scavenging activity was estimated using the method of Blois (1958) with slight modifications. Samples (1 ml) were added to test tubes containing 0.2 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Sigma), with methanol serving as a blank, and the mixtures were shaken and left to stand for 30 min at room temperature, after which absorbance was measured at 517 nm using a spectrophotometer (DU 530; Beckman Instruments Inc., Fullerton, CA, USA). The DPPH radical-scavenging activity was calculated (as percentages) using the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = [1 - (A_1/A_0)] \times 100$$

where  $A_0$  is the absorbance of the blank and  $A_1$  is the absorbance of the sample.

### 2.3. Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed as previously described (Benzie & Strain, 1996) using a spectrophotometer (Beckman Instruments Inc.). In the FRAP assay, reductants in the sample reduce the Fe(III)/tripyrilidyltriazine ( $\text{Fe}^{3+}$ -TPTZ) complex, (present in stoichiometric excess), to the blue ferrous form, which increases the absorbance at 593 nm. Absorbance was measured after 0.5 s and

once every 30 s thereafter during the monitoring period of 5 min; the readings at 4 min were used as the FRAP value ( $\mu\text{M/g}$ ) (Benzie & Strain, 1996).

### 2.4. Tyrosinase-inhibition effect

The tyrosinase-inhibition effect was measured spectrophotometrically by monitoring the increase in the absorbance caused by the production of dopachrome from L-3,4-dihydroxyphenyl-alanine (L-DOPA) (Jimenez, Chazarra, Escribano, Cabanes, & Garcia-Carmona, 2001). Samples (0.2 ml) were added to 0.4 ml of the reaction mixture containing 10 mM L-DOPA (Sigma), 0.1 M potassium phosphate buffer (pH 6.8), and mushroom tyrosinase (100 U/ml, Sigma). Methanol was used as the control. The reaction mixture was incubated at 25 °C for 15 min. The amount of dopachrome produced in the reaction mixture was measured at 475 nm using a spectrophotometer (Beckman Instruments Inc.). The tyrosinase-inhibition effect (%) was calculated as follows:

$$\text{Tyrosinase-inhibition effect (\%)} = [1 - (A_1/A_0)] \times 100$$

where  $A_0$  is the absorbance of the control solution and  $A_1$  is the absorbance of the sample.

### 2.5. Total phenolic content

The total phenolic content of samples was estimated colorimetrically using the Folin–Ciocalteu method (Subramanian, Padmanaban, & Sarma, 1965). Samples (0.1 ml) were added to the Folin–Ciocalteu reagent (0.2 ml) and then 3 ml of a 5% sodium carbonate solution was added. The reaction samples were mixed thoroughly by vortexing and after incubating the mixtures for 2 h at room temperature, the absorbance at 765 nm was measured using a spectrophotometer (Beckman Instruments Inc.). The results were quantified based on a standard curve generated using gallic acid.

### 2.6. Antimicrobial activity

#### 2.6.1. Test microorganisms

*Escherichia coli* O157:H7 (ATCC 43889), *Listeria monocytogenes* (KCTC 3569), *Salmonella* Typhimurium (KCTC 1925), and *Staphylococcus aureus* (KCTC 1916) were obtained from the Korean Collection for Type Culture (KCTC, Daejeon, Korea). *E. coli* O157:H7 and *S. aureus* were cultivated in tryptic soy broth. *L. monocytogenes* and *S. Typhimurium* were cultivated in tryptic soy broth containing 0.6% yeast extract and nutrient broth, respectively. Sterilized broths were inoculated using agar slant cultures of microorganisms, and after incubating for 24 h at 37 °C, 0.1 ml of culture was transferred to new broth and cultivated for 18 h. The cultures were centrifuged at 3,000 rpm for 15 min at 4 °C in a refrigerated centrifuge (UNION 32R, Hanil Science Industrial, Co., Ltd., Korea). Culture pellets were washed twice with sterile saline solution and resuspended in sterile saline solution at a final cell density of  $10^7$ – $10^8$  CFU/ml.

#### 2.6.2. Paper disc diffusion assay

The antimicrobial activity was determined using the paper disc diffusion assay. Briefly, 0.1 ml aliquots of the test organisms were spread over the surface of agar plates. Sterilized filter-paper discs were saturated with 50  $\mu\text{l}$  of each of the samples (filtered using 0.22- $\mu\text{m}$  syringe filters) and incubated for 48 h, after which the diameters of the microbe-free clear zones around the disc on the culture plates were measured. The control sample was prepared using methanol.

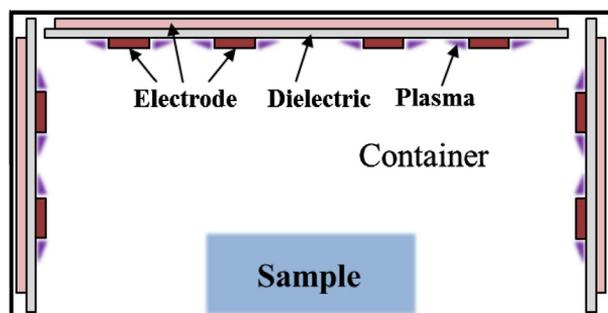


Fig. 1. Schematic diagram for the experimental system for plasma generation.

### 2.7. High performance liquid chromatography (HPLC) analysis

An Agilent HPLC system 1100 equipped with a diode-array detector was used for the chromatographic separation of naringin treated by the plasma. HPLC analysis was performed using a YMC-Pack ODS A-302 column (4.6 mm i.d. × 150 mm; YMC Co., Kyoto, Japan), and the solvent system consisted of a linear gradient that started with 10% (v/v) acetonitrile in 0.1% formic acid/distilled water (detection: 280 nm; flow rate: 1.0 ml/min; temperature: 40 °C), increased to 75% acetonitrile over 25 min, and then increased to 100% acetonitrile over 5 min.

### 2.8. Statistical analyses

Data were analysed using SAS software (Release 8.01, SAS Institute, Inc., Cary, NC, USA). Statistical analysis was performed using one-way Analysis of Variance (ANOVA). When significant differences were detected, the differences among the mean values were determined by using Duncan's multiple-comparison test at a confidence level of  $p < 0.05$ . Mean values and standard errors of the mean are reported.

## 3. Results and discussion

### 3.1. DPPH radical-scavenging activity

The DPPH radical-scavenging activities of naringin treated by the plasma are shown in Table 1. The original DPPH radical-scavenging activity of naringin was 1.45%, and increased to 38.20% after plasma treatment for 10 min.

Naringin is a major flavonoid in oranges, grape fruits, lemons and limes. It exhibits little antioxidant activity (Miyake et al., 2003). Naringin lacks radical-scavenging activity because of the glycosylation at the 7-hydroxyl group in the A-ring (Sadeghipour, Terreux, & Phipps, 2005). Naringin has been reported to be transformed into its aglycone, (naringenin), by enterobacteria or by the enzymes alpha-ramnosidase and beta glucosidase, which possesses antioxidative activity (Sadeghipour et al., 2005).

Energy input has been widely reported to induce structural modifications in natural molecules. Kang, Kim, Yamabe, and Yokozawa (2006) indicated that the double bond at carbon-20(22), or the OH group at carbon-20 that is geometrically close to the OH at carbon-12, increased the OH scavenging activity of ginsenosides upon heat processing. Park et al. (2013) and Kim, Kim, Ito, and Jo (2011) reported that radiolytic transformation of rotenone and curcumin induced by irradiation, generated new degradation products. These new derivatives, including rotenosins A and B or curculactones A and B, inhibited pancreatic lipase activity considerably more strongly than the parent rotenone and curcumin. The major derivatives of naringin that are produced following treatment with plasma are currently being investigated.

**Table 1**  
Antioxidant activity and total phenolic content of naringin treated by plasma.

Treatment time (min)	DPPH radical scavenging activity (%)	FRAP value ( $\mu\text{M/g}$ )	Total phenolic content (ppm)
0	1.45 <sup>d</sup>	27.78 <sup>d</sup>	172.50 <sup>d</sup>
5	22.41 <sup>c</sup>	40.00 <sup>c</sup>	181.17 <sup>c</sup>
10	32.46 <sup>b</sup>	60.56 <sup>b</sup>	211.50 <sup>b</sup>
20	38.20 <sup>a</sup>	207.78 <sup>a</sup>	225.83 <sup>a</sup>
SEM <sup>A</sup>	0.750	4.691	2.472

<sup>a-d</sup> Values with different letters within the same column differ significantly ( $p < 0.05$ ).

<sup>A</sup> Standard error of the mean ( $n = 12$ ).

### 3.2. FRAP value

The FRAP value of naringin increased after plasma treatment in a time-dependent manner (Table 1). A substantial increase in FRAP value was observed after 20-min of treatment, when compared with treatments of shorter times. Antioxidants could have functioned here as reductants. Inactivation of oxidants by reductants can be described as redox reactions in which one reactive species (oxidant) is reduced at the expense of the oxidation of another species, the antioxidant (Lee et al., 2009). The FRAP assay is commonly used to assess antioxidant activity because the assay is highly sensitive, rapid, and inexpensive. At low pH (optimal pH 3.6), the  $\text{Fe}^{3+}$ -TPTZ complex is reduced by antioxidants to its intensely blue-coloured form,  $\text{Fe}^{2+}$ -TPTZ, which has a maximal absorbance at 593 nm (Lee et al., 2009). In this investigation, the FRAP value of naringin increased substantially upon plasma treatment. Ahn, Kim, Jo, Kim, and Byun (2004) reported that the FRAP value of phytic acid showed a dose-response pattern in all samples exposed to gamma irradiation, as exposure removed the phosphorus from the phytic acid structure.

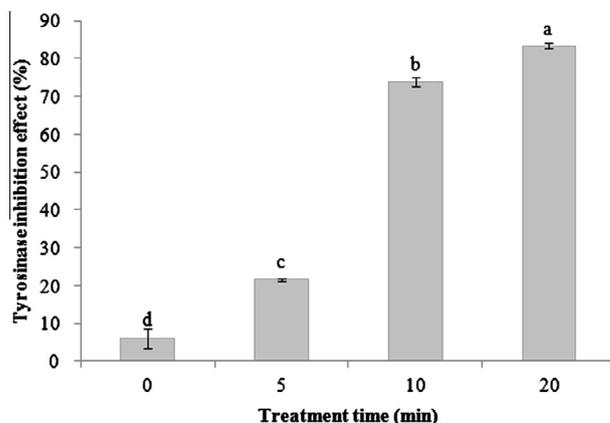
### 3.3. Tyrosinase-inhibition effect

Tyrosinase, which is responsible for skin ageing and damage, stimulates the melanin pigmentation that occurs in skin exposed to UV light (Jung et al., 2012). Tyrosinase is a key enzyme required for eumelanin and pheomelanin synthesis. Eumelanin is the typical biological form of melanin, a brown-black pigment, whereas pheomelanin is a yellow-red pigment. Enzymes such as tyrosinase-related protein 1 and 2 regulate eumelanogenesis (Kobayashi et al., 1995).

The tyrosinase-inhibition effect of naringin was originally low (6.12%); however, after treatment with plasma for 10 min, the activity increased to 83.30% (Fig. 2). The tyrosinase-inhibition effects of green tea byproducts have been widely reported to increase following irradiation (Lee, Jo, Shon, Kim, & Byun, 2006). Based on measuring flavonoid content, Lee et al. (2011) demonstrated that irradiation can increase tyrosinase-inhibition activity of *Schizandra chinensis* extracts by increasing the levels of flavonoids (derived from the breakdown of Cya-3-O-xylrut) that specifically inhibit tyrosinase. However, so far, no study has shown that tyrosinase-inhibition activity is enhanced by plasma treatment.

### 3.4. Total phenolic content

One of the most important components in natural substances is polyphenol. Much of the literature refers to the single group of



**Fig. 2.** Tyrosinase-inhibition effect of naringin treated by plasma. Letters indicate statistically significant differences ( $p < 0.05$ ).

phenolics, the flavonoids, which have applications, such as antioxidants, antimicrobials, anti-ulcer and anti-inflammatory agents (Viswanath, Urooj, & Malleshi, 2009).

The content of phenolic compounds in naringin was 172.50 ppm. After naringin was treated with DBD plasma for 10 min, the concentration of phenolic compounds increased to 225.83 ppm (Table 1). This increase can be attributed to the release of phenolic compounds from the glycosidic components and the degradation of larger phenolic compounds into smaller ones. Harrison and Were (2007) obtained similar results with almond skin extracts after gamma irradiation.

The phenolic content and antioxidant activity of natural substances are correlated. Velioglu, Mazza, Gao, and Oomah (1998) reported a strong correlation between total phenolic content and antioxidant activity in certain fruits, vegetables, and grains. Fan, Toivonen, Rajkowski, and Sokorai (2003) showed that the phenolic content and antioxidant activity in lettuce displayed a highly similar response to irradiation and storage. Jeong et al. (2004) determined that phenolic compounds could be released from citrus peel upon heating. Breitfellner, Solar, and Sontag (2002) reported that radiolysis of the phenolics in aqueous solutions led to their efficient degradation, and to a notable hydroxylation; however, in the complex matrix of the food, no hydroxylation products were formed and only the concentration of 4-hydroxybenzoic acid was affected by irradiation. These results indicated that water or other food components might affect the synthesis or degradation of the phenolics (Ahn et al., 2005).

Naringin and hesperidin, so-called citrus flavonoids, are 2 flavanone glycosides present in citrus fruits (Abeyasinghe et al., 2007). In this study, the phenolic content of naringin increased substantially after treatment with DBD plasma.

### 3.5. Antimicrobial activity

Disc diffusion methods are extensively used to investigate the antibacterial activity of natural substances and plant extracts. These assays are based on the use of discs as reservoirs containing solutions of substances to be examined (Bartner, Pfeiffer, & Bartner, 1994).

In this study, 4 well-known pathogens were used to monitor the possible increase in the antimicrobial activity of naringin after exposure to plasma (Table 2). Naringin did not initially exhibit any antimicrobial activity, however, it increased with an increase in the time of plasma treatment. The diameters of clear zones were 32, 21, 18, and 16 mm against *S. Typhimurium*, *L. monocytogenes*, *S. aureus*, and *E. coli* O157:H7, respectively, after 20-min treatment of naringin with plasma. Polyphenols are generally highly stable but can undergo thiolysis upon reaction with strong acid (McGraw, Steynberg, & Hemingway, 1993). An et al. (2004) reported that the chemical structure of irradiated polyphenols might not change, but the activity of functional groups, such as –OH or –COOH might change, resulting in higher antimicrobial activity.

**Table 2**

Antimicrobial activity of naringin treated by plasma, measured using the paper disc diffusion method.

Pathogens	Diameter (mm)				
	Methanol	0 min	5 min	10 min	20 min
<i>Salmonella Typhimurium</i>	– <sup>a</sup>	–	19 <sup>b</sup>	25	32
<i>Listeria monocytogenes</i>	–	–	9	16	21
<i>Staphylococcus aureus</i>	–	–	–	14	18
<i>Escherichia coli</i> O157:H7	–	–	–	–	16

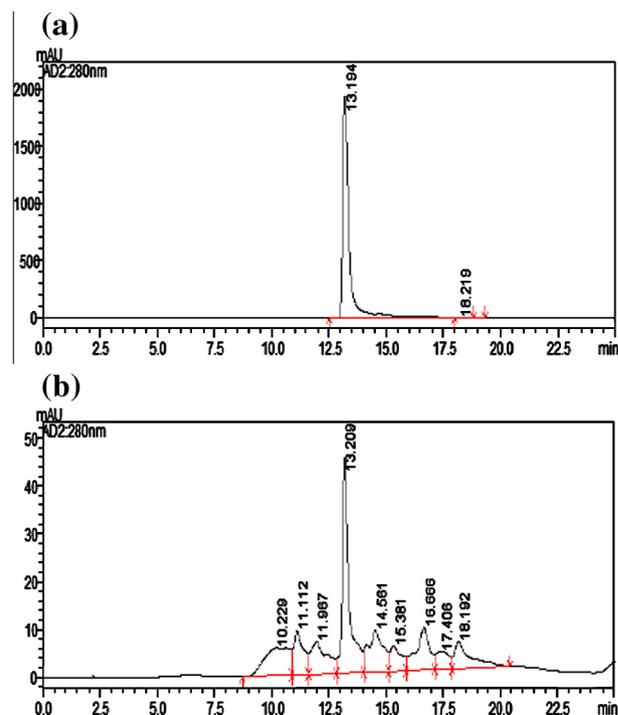
<sup>a</sup> No inhibition.

<sup>b</sup> Clear-zone diameter (disc diameter = 8 mm).

### 3.6. HPLC analysis

The HPLC chromatograms of naringin collected before and after treatment by plasma (Fig. 3) showed that plasma treatment degraded naringin, and that the degradation rate and extent increased when the treatment time was extended to 20 min. Considering the peak area of non-treated naringin to be 100%, only 62% of naringin was present after plasma treatment for 20 min, whereas 6 new peaks were detected, each with <10% peak area (Table 3). The chromatogram indicates that naringin was physically or chemically trapped by other components during plasma treatment. In our laboratory, the plasma-induced compounds are being isolated, their molecular structures are being identified, and their individual biological activities are being investigated.

Bactericidal agents generated in plasma are known to include reactive oxygen species (ROS), UV photons, energetic ions, and electrons. The ROS in plasma generated in ambient air have been reported to be ozone, atomic oxygen, superoxide, peroxide, and hydroxyl radicals (Gaunt et al., 2006). The hydroxyl radical is a powerful oxidising agent, whereas electrons and hydrogen atoms are reducing agents; therefore, all foods containing water are likely



**Fig. 3.** HPLC chromatograms of naringin treated by plasma. (a) Control; (b) sample treated for 20 min.

**Table 3**

Relative peak area percentages of compounds generated from naringin after plasma treatment.

Retention time (min)	Peak area (%)
11.113	5.73
11.967	4.53
13.208 (naringin)	61.82
14.561	6.96
15.343	2.29
16.663	9.49
18.190	9.18
Total	100

to undergo both oxidation and reduction reactions during non-thermal treatments, such as irradiation (Stewart, 2001). These ROS could be mainly responsible for the structural modification of naringin caused by plasma treatment (Knake, Reuter, Niemi, Schulz-von der Gathen, & Winter, 2008).

#### 4. Conclusion

Naringin is a well-known natural compound found in citrus fruits that exhibits limited biological activity. The results presented here indicated that the biological activities of naringin, including antioxidative, tyrosinase-inhibition, and antimicrobial activities, can be improved substantially upon treatment with atmospheric pressure plasma.

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