

# Inhibitory Effects on Oral Microbial Activity and Production of Lipopolysaccharides-Induced Pro-Inflammatory Mediators in Raw264.7 Macrophages of Ethanol Extract of *Perilla frutescens* (L.) Britton

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**Background:** The leaves of *Perilla frutescens*, commonly called perilla and used for food in Korea, contain components with a variety of biological effects and potential therapeutic applications. The purpose of this study was to identify the components of 70% ethanol extracted *Perilla frutescens* (EEPF) and determine its inhibitory effects on oral microbial activity and production of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in lipopolysaccharides (LPS)-stimulated Raw264.7 macrophages, consequently, to confirm the possibility of using EEPF as a functional component for improving the oral environment and preventing inflammation.

**Methods:** One kg of *P. frutescens* leaves was extracted with 70% ethanol and dried at -70°C. EEPF was analyzed using high-performance liquid chromatography analysis, and antimicrobial activity against oral microorganisms was revealed using the disk diffusion test. Cell viability was elucidated using a methylthiazolyldiphenyl-tetrazolium bromide assay, and the effect of EEPF on LPS-induced morphological variation was confirmed through microscopic observation. The effect of EEPF on LPS-induced production of pro-inflammatory mediators, NO and PGE<sub>2</sub> was confirmed by the NO assay and PGE<sub>2</sub> enzyme-linked immunosorbent assay.

**Results:** The main component of EEPF was rosmarinic acid, and EEPF showed weak anti-bacterial and anti-fungal effects against microorganisms living in the oral cavity. EEPF did not show toxicity to Raw264.7 macrophages and had inhibitory effects on the morphological variations and production of pro-inflammatory mediators, NO and PGE<sub>2</sub> in LPS-stimulated Raw264.7 macrophages.

**Conclusion:** EEPF can be used as a functional material for improving the oral environment through the control of oral microorganisms and for modulating inflammation by inhibiting the production of inflammatory mediators.

**Key Words:** Oral microbial activity, *Perilla frutescens*, Pro-Inflammatory Mediator, Raw264.7 cells, Rosmarinic acid

## Introduction

The leaves of *Perilla frutescens*, commonly called perilla or Korean perilla, are plants used for food in Asia, especially in Korea, and belong to the mint family Lamiaceae<sup>1</sup>. *P. frutescens* contains antioxidant, anti-tumor and anti-allergic components, and is used for treatment in

traditional medicine<sup>2-5</sup>. The important components that can be extracted from this plant are rosmarinic acid (RA), apigenin, and luteolin<sup>6</sup>. One of the chemical components of perilla leaves, RA ( $\alpha$ -o-caffeoyl-3,4-dihydroxyphenyl-lactic acid), has various effects (anti-oxidative, anti-inflammatory, anti-mutagen, anti-bacterial and anti-viral), and has the potential for use as a therapeutic drug for disease<sup>7,8</sup>.

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Many microorganisms inhabit the oral cavity, but among these, the disease-causing ones are limited by a few strains<sup>9,10</sup>. Caries and periodontal diseases, are caused by infection of pathogenic bacteria isolated from dental plaques<sup>11</sup>. *Streptococcus mutans* plays an important role in the formation of dental plaques and is known to cause tooth caries<sup>12,13</sup>. *Aggregatibacter actinomycetemcomitans* is isolated from the dental plaque of healthy individuals and those with periodontal disease. It is one of the few strains causing aggressive periodontal disease characterized by the rapid destruction of periodontal tissue<sup>14,15</sup>. *Escherichia coli* accounts for 15% of the oral flora and is known to be associated with dry mouth. *Candida albicans* is a representative opportunistic fungus in the oral cavity<sup>13</sup>. The control of these oral microorganisms is important to prevent infectious diseases, such as tooth decay and periodontal disease. Some broad-spectrum antibiotics, such as penicillin, ampicillin, clindamycin, chlorhexidine, and metronidazole, are effective in controlling dental caries and periodontal disease, but have various adverse effects, such as discoloration of teeth, increased calculus, diarrhea, and oral flora imbalance<sup>11</sup>. Therefore, it is important to consider safe and useful chemicals from plant extracts with low adverse effects for the control of infection-causing microorganisms and infectious diseases<sup>11</sup>. Despite various therapeutic properties and interest in perilla cultivated in large quantities in the Korean peninsula, studies on the chemical components of the ethanol extracted *P. frutescens* (EPPF) extract and their anti-microbial activity against oral microorganisms causing oral disease and pro-inflammatory mediators are rare.

The purpose of this study was to identify the useful components of EPPF and to determine their inhibitory effects on oral microbial activity and production of nitric oxide (NO) and prostaglandin E2 (PGE<sub>2</sub>) in lipopolysaccharides (LPS)-stimulated Raw264.7 macrophages, consequently, to confirm the possibility of using EPPF as a functional

substance for improving the oral environment and preventing inflammation.

## Materials and Methods

### 1. Preparation of EPPF leaves

Fresh *P. frutescens* leaves were purchased from the herb markets in Busan, Korea, and one kg was used for ethanol extraction. For ethanol extraction, the perilla leaves were immersed in 1 L of 70% (v/v) ethanol, and ultrasonication was performed thrice in 30 minutes at room temperature. The extracted solvent was filtered with whatman No. 2 filter paper, concentrated at 60°C with a rotary evaporator (Eyela A-1000, Eyela, Tokyo, Japan), and then freeze-dried at -70°C. The powder from the extract was preserved at -20°C until use, and was reconstituted to an appropriate concentration using dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA).

### 2. High-performance liquid chromatography analysis

The powder from the extract was transferred to a 50-ml flask, adjusted to a volume of 70% methanol, and filtered through a syringe filter (0.2 µm; Altech, Beerfield, IL, USA). The filtrate (10 µl) was injected into the high-performance liquid chromatography (HPLC) for quantitative analysis of RA. Ultraviolet detection of RA was made at 330 nm wavelength of the HPLC (Table 1). Elution was performed at a 1.0 ml/min flow rate at 30°C. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was acetonitrile. A ratio of 88% mobile phase A and 12% B was applied for the first 10 minutes. After 50 minutes, a ratio of 60% A and 40% B was used for 5 minutes. Finally, 88% A and 12% B was used after 5 minutes for 15 minutes.

**Table 1.** Results of High-Performance Liquid Chromatography (HPLC) Analysis for 70% Ethanol Extracted *Perilla frutescens* (EPPF)

No.	Compound	UV (nm)	Concentration (mg/L)	Regression equation (y=ax+b)	Correlation coefficient (r <sup>2</sup> )
1				Y=152057x-164534	0.999306
2	EPPF	330	25, 50, 100	Y=157516x-365061	0.999762
3				Y=151398x-171408	0.999895

### 3. Oral microorganisms

*S. mutans* (KCCM 40105), *A. actinomycetemcomitans* (KCTC 2581), *E. coli* (KCTC 1039), and *C. albicans* (KCCM 11282) were purchased from the Korea Microbiological Conservation Center (KCCM) and the gene bank (KCTC) for experiments. *S. mutans* was cultured in Brain Heart Infusion (MB cell Ltd., Seoul, Korea) agar and broth, *A. actinomycetemcomitans* in MRS (MB cell Ltd.) agar and broth, *E. coli* in Luria Bertani (MB cell Ltd.) agar and broth, and *C. albicans* in Potato Dextrose (MB cell Ltd.) agar and broth.

### 4. Disk diffusion test

According to the standardized method<sup>16)</sup>, colonies from each strain were cultured for 24 hours, diluted to  $5 \times 10^6$  CFU/ml with saline solution, and then 100  $\mu$ l spread on an agar plate. After absorption of each concentration of EEPF on sterilized paper discs ( $\phi$ 6 mm; Advantec Toyo Kaisha Ltd., Tokyo, Japan), these discs were placed on a microorganism coated agar plate. After incubation for 24 hours, the diameter of the clear zone formed around the discs was measured. Ampicillin (10 IU; Oxoid Ltd., Hampshire, United Kingdom) and penicillin G (10 mcg, Oxoid Ltd.) antibiotic discs were used as controls.

### 5. Cell culture and EEPF treatment

The RAW264.7 macrophages (KCLB, Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Seoul, Korea) containing 10%

fetal bovine serum (Gibco-BRL) and 1% antibiotic-antimycotic solution (Gibco-BRL) in a 5% CO<sub>2</sub> incubator at 37°C. The prepared cells were treated with LPS (100 ng/ml) or EEPF (50, 100, 200, 300, 400  $\mu$ g) and were incubated.

### 6. Methylthiazolyldiphenyl-tetrazolium assay

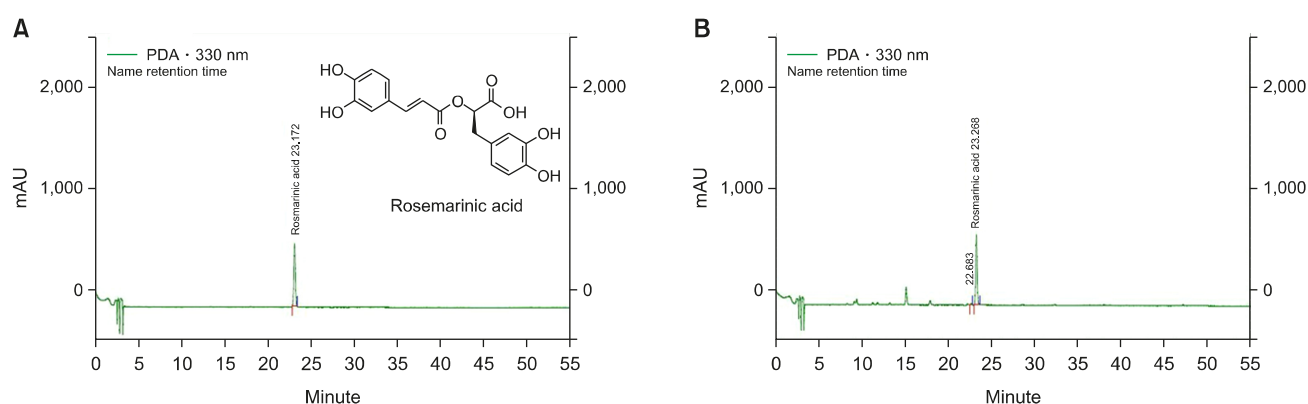
Cell viability as an EEPF effect was elucidated using the methylthiazolyldiphenyl-tetrazolium (MTT) assay. LPS and EEPF treated Raw264.7 macrophages were added to 0.5 mg/ml of MTT (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and were wrapped in foil to block light and then reacted in an incubator for 2 hours. The cells were treated with DMSO and 200  $\mu$ l of the solution was used for measurement at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

### 7. Microscopic observation

Prepared cells were fixed with 2.5% glutaraldehyde (MERCK, Frankfurter, Germany) in phosphate-buffered saline (PBS, pH 7.4), and washed thrice with PBS, and then examined using an inverted microscope (Olympus, Japan) to explore the morphological variation of the cells.

### 8. NO assay and PGE<sub>2</sub> ELISA assay

NO was measured at an absorbance of 540 nm with an ELISA reader after treatment according to the manufacturer's method using a NO assay kit (R&D



**Fig. 1.** High performance liquid chromatography (HPLC) profile of ethanol extracts of *Perilla frutescens* (EEP) Leaves. (A) The chromatogram of standard of rosmarinic acid (RA). (B) Chromatograms from EEPF. The quantitative data chromatogram from HPLC analysis of EEPF showed the highest peak in RA.

Systems, Minneapolis, MN, USA). PGE<sub>2</sub> was measured at an absorbance of 490 nm after treatment according to the manufacturer’s method using a PGE<sub>2</sub> ELISA kit (R&D Systems).

### 9. Statistical analysis

All data from triplicate experiments are expressed as mean±standard deviation and were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Statistically significant differences (p<0.05) were analyzed using the Student’s t-test.

## Results

### 1. Contents of bioactive materials of EEPF

One kg of Perilla leaves was extracted with 70% ethanol and the crude extract was used for HPLC analysis. The HPLC chromatogram showed the highest peak at 23.270

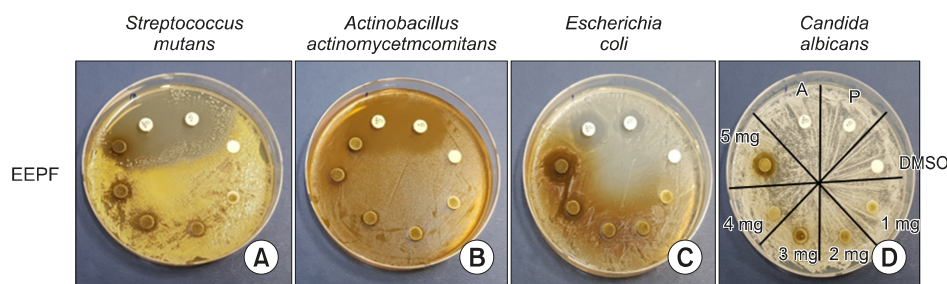
minutes retention time, which was RA (Fig. 1). The peak area of RA was based on the HPLC analysis shown in Table 1, and the RA extracted from EEPF was 49.967±0.153 g/kg (Table 2).

### 2. Anti-microbial activity of EEPF against oral microorganisms

In order to confirm the antimicrobial activity of EEPF against *S. mutans*, *A. actinomycetemcomitans*, *E. coli*, and *C. albicans*, microorganisms living in the oral cavity, a disk diffusion test was performed (Fig. 2, Table 3). EEPF (4 and 5 mg) had weak anti-bacterial activity against *S. mutans*, *A. actinomycetemcomitans*, and *E. coli*. In addition, 5 mg EEPF showed a weak antifungal effect against *C. albicans*. From the above results, EEPF has weak anti-bacterial and anti-fungal effects against microorganisms living in the oral cavity.

**Table 2.** Results from High-Performance Liquid Chromatography (HPLC) Analysis of 70% Ethanol Extract of *Perilla frutescens* Leaves for Rosmarinic Acid

Compound	Trial	Sample weight (g)	Retention time (min)	Area	Sample Con (g/kg)	Mean±standard deviation (g/kg)
Rosmarinic acid	1	0.1021	23.138	7617032	50.1	49.967
	2	0.1000	23.250	7535870	49.8	±0.153
	3	0.1040	23.268	7709009	50.0	



**Fig. 2.** Inhibitory effects on microbial activity of ethanol extracts of *Perilla frutescens* (EEPf) against oral bacteria and fungi. A: ampicillin, P: penicillin G, DMSO: dimethyl sulfoxide.

**Table 3.** Anti-Microbial Activity of 70% Ethanol Extract of *Perilla frutescens* (EEPf) Leaves against Oral Microorganisms

Microorganism	EEPf (mg)						Ampicillin (10 IU)	Penicillin G (10 mcg)
	0	1	2	3	4	5		
<i>Streptococcus mutans</i>	-	-	+	+	+	+	+++	++
<i>Aggregatibacter actinomycetemcomitans</i>	-	-	-	-	+	+	+++	++
<i>Escherichia coli</i>	-	-	-	-	+	+	++	+
<i>Candida albicans</i>	-	-	-	-	-	+	-	-

-: resistant (<5 mm), +: susceptible (5~14 mm), ++: more susceptible (15~24 mm), +++: most susceptible (>25 mm).

### 3. Effects of EEPF on the cell viability and morphology of LPS-stimulated Raw264.7 cells

In order to confirm the change in cell viability due to EEPF, the MTT assay was conducted on cells treated with LPS and EEPF (Fig. 3A). Raw264.7 macrophages were treated with LPS (100 ng/ml) for 1 hour and then treated with EEPF according to the concentration. Cell viability of the EEPF-treated groups was similar to that of the control, and that of the 300  $\mu\text{g}$ -treated group was slightly increased, confirming that EEPF had no cytotoxic effects. Based on this result, the highest concentration of EEPF for the experiment was 300  $\mu\text{g}$ . The variations in cell morphology of Raw264.7 cells treated with LPS and each concentration of EEPF were confirmed using a microscope (Fig. 3B). In the LPS-treated group, Raw264.7 cells had increased sharp protrusions, but the formation of sharp protrusions gradually decreased as the concentration of EEPF increased. From the above results, EEPF was not

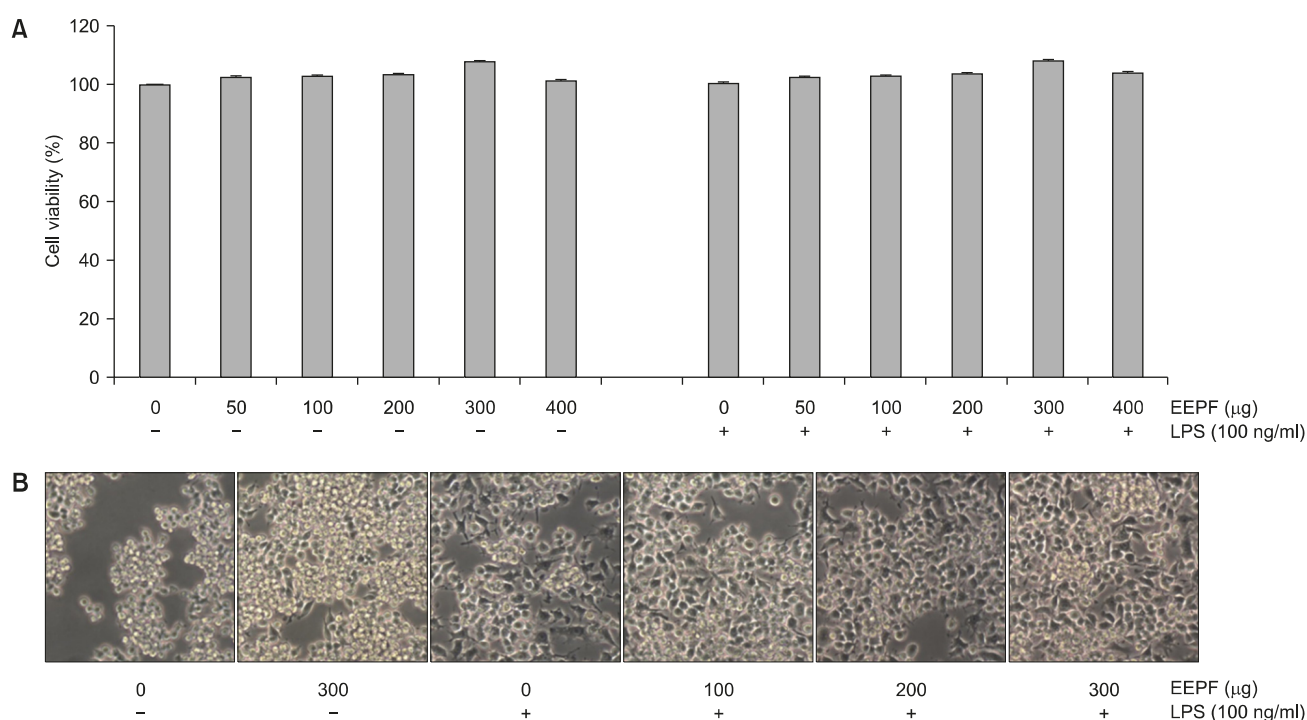
toxic to Raw264.7 macrophages and alleviated the morphological variations of LPS-stimulated cells.

### 4. Effects of EEPF on NO and PGE<sub>2</sub> production in LPS-stimulated Raw264.7 cells

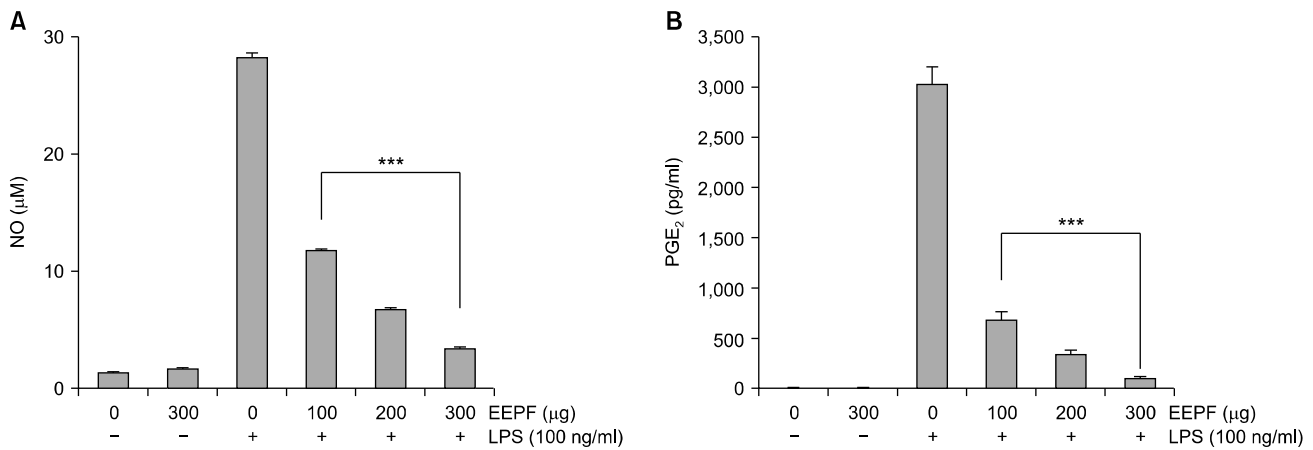
Changes in the production of NO and PGE<sub>2</sub> were confirmed in Raw264.7 macrophages following treatment with LPS and EEPF (Fig. 4). The production of NO and PGE<sub>2</sub> was significantly increased in the LPS-treated group, but significantly decreased as the concentration of EEPF increased in the group treated with LPS and EEPF. Therefore, EEPF inhibited the LPS-induced production of NO and PGE<sub>2</sub>, inflammatory mediators in RAW264.7 cells.

## Discussion

Dental caries and periodontal diseases, which are important diseases of the oral cavity, are caused by



**Fig. 3.** Effects of EEPF on cell viability and morphological variation of RAW264.7 macrophages. Raw264.7 macrophages were treated with LPS (100 ng/ml) before 1 hour and then treated with EEPF according to the concentration. MTT assay was performed to evaluate cell viability (A) and observed using a microscope to evaluate morphological variation (B). Cell viability of the EEPF-treated groups was similar to that of the control and those of 300  $\mu\text{g}$ -treated group was slightly increased, confirming that EEPF had not cytotoxic effects. The LPS-stimulated Raw264.7 cells had increased sharp protrusions, but as the treatment concentration of EEPF increased, the formation of sharp protrusions gradually decreased. EEPF: ethanol extracts of *Perilla frutescens*, LPS: lipopolysaccharides, MTT: methylthiazolodiphenyl-tetrazolium.



**Fig. 4.** Inhibition effects of EEPF on production of LPS-induced pro-inflammatory mediators production in Raw264.7 macrophages. (A) NO, (B) PGE<sub>2</sub>. The production of NO and PGE<sub>2</sub> was significantly increased in the LPS-treated group compared to the control, but the production of NO and PGE<sub>2</sub> in the group treated with LPS and EEPF significantly decreased as the concentration of EEPF increased, compared to the group treated with only LPS (\*\*p < 0.01, \*\*\*p < 0.001). EEPF: ethanol extracts of *Perilla frutescens*, PGE<sub>2</sub>: prostaglandin E<sub>2</sub>, NO: nitric oxide, LPS: lipopolysaccharides.

infection of pathogenic bacteria that are isolated from dental plaque<sup>11</sup>. *S. mutans* plays an important role in the formation of dental plaque which is the basis for colonization of pathogenic bacteria that can cause oral disease and induce dental caries<sup>12,13</sup>. Periodontal tissues, including the cementum, periodontal ligaments, and alveolar bone surrounding the tooth root, are plagued by bacterial infection as acute and chronic periodontal diseases<sup>17</sup>. If periodontal disease is left untreated, excessive inflammatory reactions and alveolar bone resorption progress, leading to tooth loss<sup>17</sup>. Periodontal disease is initiated by the overgrowth of special gram-negative anaerobic bacteria such as *A. actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, and the number increases significantly during the inflammatory conditions of periodontal tissues<sup>15</sup>. *A. actinomycetemcomitans*, isolated from the dental plaque of healthy individuals and those with periodontitis, is one of the representative bacteria causing aggressive periodontal disease characterized by rapid destruction of periodontal tissue<sup>12,14,15</sup>. *E. coli*, although transiently present, accounts for 15% of the oral flora, and the frequency increases with age and is known to be related to the induction of dry mouth<sup>13</sup>. *C. albicans* is a representative opportunistic fungus that causes candidiasis in people with weak immunity<sup>13</sup>. The control of these microorganisms in the oral cavity is important for the

treatment of infectious diseases such as tooth caries and periodontal disease. EEPF, which contains RA as a major component, weakly inhibited the growth of oral pathogenic bacteria *S. mutans*, *A. actinomycetemcomitans*, *E. coli*, and *C. albicans* belonging to the fungus, and showed anti-bacterial and anti-fungal activities (Fig. 2, Table 3). Although there is paucity of data on the effect of perilla on oral microorganisms, RA has weak antimicrobial activity against oral streptococci and relatively strong antimicrobial activity against *P. gingivalis*<sup>9</sup> and has no antimicrobial activity against fungi (data not shown). The above results showed that another component of EEPF can control oral fungi. Therefore, it is necessary to analyze other components of the EEPF in future studies.

LPS from gram-negative oral bacteria is an important component of the outer membrane of bacteria<sup>17</sup>, and induces the up-regulation and secretion of pro-inflammatory enzymes, such as nitric oxide synthase (NOS) and cyclooxygenase (COX) from immune cells (monocytes, macrophages and neutrophils)<sup>17,18</sup>. iNOS and COX-2, as inflammatory inducers, induce the secretion of large amounts of inflammatory mediators, such as NO and PGE<sub>2</sub>, and inflammatory cytokines such as tumor necrosis factor alpha and interleukin 1 beta, respectively<sup>18,19</sup>. They play an important role in the destruction of periodontal tissue during the progression of periodontal disease and induce activation of immune and inflammatory responses<sup>15,17,20</sup>.

Plant-derived phytochemicals capable of modulating inflammatory mediators are used for the relief and treatment of acute and chronic inflammatory diseases<sup>21,22</sup>. From our results, the main component of EEPF was RA (Fig. 1, Table 2) and EEPF reduced the morphological variations and the production of NO and PGE<sub>2</sub>, inflammatory mediators induced by LPS, in Raw264.7 macrophages (Fig. 4).

Therefore, EEPF component RA was confirmed to be an important element in the HPLC analysis that had anti-bacterial and anti-fungal activity against microorganisms living in the oral cavity. In addition, EEPF has an inhibitory effect on the morphological variation and the production of NO and PGE<sub>2</sub> in LPS-stimulated Raw264.7 macrophages. These results indicate that EEPF can be used as a functional substance for improving the oral environment and preventing inflammation.

## Notes

### Conflict of interest

No potential conflict of interest relevant to this article was reported.

### Ethical approval

This article is not necessary for IRB screening.

### Author contributions

Conceptualization: Moon-Jin Jeong, Soon-Jeong Jeong, Data acquisition: Do-Seon Lim, Myoung-Hwa Lee, Kyungwon Heo, Han-Hong Kim. Formal analysis: Do-Seon Lim, Myoung-Hwa Lee, Kyungwon Heo, Han-Hong Kim. Funding: Moon-Jin Jeong. Supervision: Soon-Jeong Jeong. Writing-original draft: Soon-Jeong Jeong. Writing-review & editing: Moon-Jin Jeong, Soon-Jeong Jeong.

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