# Antioxidant and anti-inflammatory effects of a mixture of propolis, red bean and tomato extracts

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Received February 26, 2024; Accepted April 3, 2024

DOI: 10.3892/ijfn.2024.35

**Abstract.** The aim of the present study was to investigate the antioxidant and anti-inflammatory effects of a mixture of propolis, red bean and tomato extracts (PRTE). First, the radical scavenging activities of various combinations of PRTE (1:1:1, M1-PRTE; 1.5:1:0.5, M2-PRTE; 1.5:0.5:1, M3-PRTE; and 1.2:0.9:0.9, M4-PRTE) were estimated. M2-PRTE exhibited the optimal 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging efficacy compared to the other ratios. The DPPH and ABTS radical scavenging activities ($IC_{50}$) of M2-PRTE were 192.86±3.34 and 554.28±4.78 $\mu$g/ml, respectively. M2-PRTE also restored the levels of superoxide dismutase, glutathione peroxidase and glutathione which were reduced by fine particulate matter in HaCaT cells. In addition, M2-PRTE suppressed the levels of inflammatory mediators, such as nitric oxide, inducible nitric oxide synthase, cyclo-oxygenase-2 and prostaglandin $E_2$, which were increased by lipopolysaccharide (LPS) stimulation in the RAW264.7 cells, in a dose-dependent manner. In addition, M2-PRTE significantly inhibited the production of pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor-α and IL-6, which were increased by LPS stimulation in RAW264.7 cells. On the whole, the findings of the present study suggest that M2-PRTE is an effective material for alleviating oxidative stress and inflammatory responses caused by environmental pollution, and it has potential for use in health functional foods.

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*Key words:* propolis, red bean, tomato, antioxidant, anti-inflammation

## Introduction

In the modern industrial society, environmental pollutants, including particulate matter (PM) such as fine dust, have a significant impact on public health (1). PM is typically categorized into dust with a diameter of ≤10 $\mu$m and dust with a diameter of ≤2.5 $\mu$m [fine PM ($PM_{2.5}$)]. It is primarily generated from industrial facilities and vehicles, and consists of organic components, such as dioxins and benzene, as well as inorganic components such as nitrates, sulfates and metal compounds (2). PM infiltrates the respiratory and circulatory systems of the human body, leading to various health issues. Particularly, $PM_{2.5}$ is recognized as a key factor causing severe diseases in humans, including respiratory diseases, cardiovascular diseases and cancer (3,4). Health issues related to environmental pollution are exponentially increasing, and pollution has a lethal impact on individuals with respiratory and cardiovascular diseases, including the elderly, resulting in an increase in mortality rates (5). Therefore, there is a marked emphasis on research, not only on the mechanistic aspects of the impact of $PM_{2.5}$ on human health, but also on the development of materials that can effectively control its presence (6).

Oxidative stress in the human body has been reported to play a crucial role in causing genetic mutations in cells and tissues, as well as in exerting lethal effects on cellular organelles, ultimately leading to various human diseases (7). Reactive oxygen species (ROS), a key factor contributing to oxidative stress, are generated not only during physiological conditions such as immune responses, but also due to physical and chemical environmental pollutants. Specifically, $PM_{2.5}$, when inhaled through the respiratory system, has been reported to directly generate large amounts of ROS along the bloodstream, affecting various tissues in the human body and inducing oxidative stress (8,9). Since ROS are considered essential factors in inducing both acute and chronic inflammatory diseases, there is an increasing need to effectively control them. This has led to a concentration of interest not only in the field of biomedicine, but also in the health food sector, prompting numerous researchers to focus on developing natural food materials for effective ROS control (10). Consequently, research in the food industry is actively pursuing the development of natural food materials that can control ROS effectively

with minimal or no side-effects (11,12). However, research on natural food materials specifically aimed at managing and improving oxidative stress and inflammation caused by $PM_{2.5}$ is still insufficiently advanced.

Propolis, a natural resin collected by bees to protect their hives, contains various bioactive components, such as flavonoids, phenolic acids, esters, terpenes, amino acids and vitamins (13-15). These components exhibit potent antioxidant effects by neutralizing ROS and eliminating free radicals. Propolis is well-known for its anti-inflammatory effects, inhibiting the generation of inflammatory mediators and reducing inflammatory responses (16). Red bean (*Vigna angularis*), a 1-year vine plant cultivated in East Asia, has been reported to have anticancer, antioxidant, anti-inflammatory and anti-obesity effects (17). Red beans are rich in polyphenols and flavonoids, which help prevent oxidative damage and contribute to maintaining cellular health (18). Furthermore, polyphenols and flavonoid components derived from red beans have been reported to regulate inflammatory responses and contribute to the prevention and management of chronic inflammatory-related diseases (19). Additionally, tomatoes (*Solanum lycopersicum*) contain antioxidants, such as polyphenols, flavonoids and lycopene. These components protect cells from free radicals, reduce DNA damage and contribute to inhibiting inflammatory responses (20,21). Lycopene, in particular, provides protective effects against various health issues related to oxidative stress (22). Despite the well-known benefits of propolis, red beans and tomatoes, there is a consistent increase in consumer demand due to a growing interest in health. However, despite their efficacy, the utilization of extracts from these three sources is relatively low based on consumer preferences, resulting in a slow growth rate in demand.

Therefore, the aim of the present study was to develop a mixture of propolis, red bean and tomato extracts (PRTE) that could alleviate oxidative stress and inflammatory responses caused by $PM_{2.5}$. The aim was to manufacture PRTE, verify its antioxidant abilities based on the ratios of each extract, and create a novel natural extract. In order to achieve this, optimal ratios were determined, and the antioxidant and anti-inflammatory effects were investigated using keratinocyte cells (HaCaT cells) and macrophages (RAW264.7 cells) following treatment with the developed mixture.

## Materials and methods

*Cells and materials*. HaCaT cells (cat. no. 300493-SF) were acquired from the CLS Cell Lines Service GmbH. RAW264.7 cells (cat. no. TIB-71) were purchased from ATCC. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, RIPA buffer, and trypsin-EDTA were purchased from Thermo Fisher Scientific, Inc. The Quanti-MAX™ WST-8 cell viability assay kit, and TBST buffer was obtained from BIOMAX, Inc. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Griess reagent, lipopolysaccharide (LPS), $PM_{2.5}$ and goat anti-rabbit IgG HRP-conjugated antibody (cat. no. 31458) were purchased from MilliporeSigma. The prostaglandin $E_2$ ($PGE_2$), interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) ELISA kits were obtained from R&D Systems, Inc. The superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione (GSH) assay kits were purchased from Cayman Chemical Company. Inducible nitric oxide synthase (iNOS; sc-7271), cyclooxygenase-2 (COX-2; sc-514489) and β-actin (cat. no. sc-8432) antibodies, along with goat anti-mouse IgG HRP-conjugated antibody (cat. no. sc-2354), were acquired from Santa Cruz Biotechnology, Inc. The Bradford assay reagent and SDS-PAGE sample loading buffer were purchased from Bio-Rad Laboratories, Inc.

*PRTE*. The propolis used in the present study was provided by Unique BioTech Co., Ltd. Red beans and tomatoes were purchased from a local market, and following verification by Professor Hong-Jun Kim at the College of Oriental Medicine, Woosuk University (Wanju-gun, Korea) the samples (voucher specimen; #2023-06-07) were stored at the research laboratory of SIJ at Jeonju University (Jeonju, Korea). Red bean and tomato extracts were prepared by mixing them in a 1:20 ratio with 70% ethanol and subjecting them to vibration extraction at 161 x g for 3 days at 50˚C. The extracts were filtered once through a nylon mesh and twice through a 0.45-$\mu$m filter paper. The filtered extracts were concentrated under a rotary vacuum (A-3S; EYELA) at 50˚C and then freeze-dried to obtain powder samples. The obtained powder samples were stored at -80˚C and used in the following experiments.

*The derivation of the mixture ratio of propolis, red bean and tomato*. PRTE were mixed under four different conditions as follows: Mixture 1 (M1-PRTE) was prepared by combining propolis, red bean and tomato at a ratio of 1:1:1. Mixture 2 (M2-PRTE) was prepared at a ratio of 1.5:1:0.5, mixture 3 (M3-PRTE) at a ratio of 1.5:0.5:1 and mixture 4 (M4-PRTE) at a ratio of 1.2:0.9:0.9. In order to determine the optimal mixture ratio, the radical-scavenging efficacy of each mixture was evaluated, as described below. Based on these results, the optimal mixture was determined.

*DPPH radical scavenging activity*. The DPPH radical scavenging activity experiment was conducted with a slight modification of the method proposed in the study by Blois (23). Each extract and mixture were dissolved in distilled water. Subsequently, 100 $\mu$l of each sample solution and 100 $\mu$l of 0.3 mM DPPH solution were mixed in a 96-well plate and allowed to react at room temperature for 20 min. The absorbance was then measured at 540 nm (Sunrise™, Tecan Group, Ltd.), and the percentage difference in absorbance between the sample solution and the blank solution was calculated.

*ABTS radical scavenging activity*. The ABTS radical scavenging activity was measured according to the method described in the study by Re *et al* (24). A mixture of 7 mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) at a 1:1 ratio was allowed to stand for 24 h at room temperature to generate radicals. The resulting radical solution was diluted with distilled water to achieve an absorbance of 0.70±0.04 at 720 nm. Subsequently, 50 $\mu$l of each extract and mixture were mixed with 950 $\mu$l of the prepared ABTS solution and allowed to react for 30 min at 23˚C. After the reaction, 100 $\mu$l of the mixture were transferred to a 96-well plate, and the

absorbance was measured at 720 nm (Sunrise™, Tecan Group, Ltd.). The percentage difference in absorbance between the sample solution and the blank solution was calculated.

*Cell culture*. The human-derived keratinocyte cell line (HaCaT) was obtained from CLS Cell Lines Service GmbH, and the murine macrophage cell line (RAW264.7) was acquired from ATCC. The cells were cultured in DMEM containing 10% FBS and 1% antibiotics (penicillin and streptomycin) in a humidified atmosphere at 37˚C with 5% $CO_2$.

*Cell viability*. The HaCaT cells were seeded at a concentration of $2x10^5$ cells/ml in a 96-well plate and the cells were cultured at 37˚C and 5% $CO_2$ for 24 h. The cells were then exposed to with various concentrations of $PM_{2.5}$ (0-100 $\mu$g/ml) or M2-PRTE (0-50 $\mu$g/ml). Following 24 h of incubation, the cells were cultured at 37˚C and 5% $CO_2$, WST-8 solution (10 $\mu$l per well) was added, and after 4 h, the absorbance was measured at 450 nm (Sunrise™, Tecan Group, Ltd.) to calculate the cell viability. The RAW264.7 cells were seeded at a final concentration of $2x10^5$ cells/ml in a 96-well plate, then cultured for 24 h at 37˚C with 5% $CO_2$. Subsequently, they were treated with M2-PRTE at concentrations of 25 and 50 $\mu$g/ml, followed by exposed to LPS at a concentration of 1 $\mu$g/ml after 1 h. Following 24 h of incubation, the cells were cultured at 37˚C and 5% $CO_2$, WST-8 solution (10 $\mu$l per well) was added, and after 4 h, the absorbance was measured at 450 nm (Sunrise™, Tecan Group, Ltd.) to calculate cell viability.

*Measurement of SOD and GPx, and determination of the GSH content*. After seeding tbe HaCaT cells in a 60-mm dish at a final concentration of $2x10^5$ cells/ml, the cells were cultured at 37˚C and 5% $CO_2$ for 24 h. Subsequently, the cells were treated with M2-PRTE at concentrations of 25 and 50 $\mu$g/ml. After 1 h, $PM_{2.5}$ was added at a concentration of 100 $\mu$g/ml, and the cells were then cultured at 37˚C and 5% $CO_2$ for an additional 24 h. Subsequently, the cells were washed twice with PBS and protein extraction was performed using RIPA buffer. The extracted proteins were quantified by measuring the absorbance at 595 nm using Bradford protein assay reagent, and the activities of SOD and GPx, as well as the GSH content, were measured according to the manufacturer's instructions.

*Measurement of nitric oxide (NO) production*. After seeding the RAW264.7 cells in a 48-well plate at a final concentration of $2x10^5$ cells/ml, the cells were cultured in an incubator at 37˚C and 5% $CO_2$ for 24 h. Following this, the cells were treated with M2-PRTE at concentrations of 25 and 50 $\mu$g/ml, and 1 h later, LPS was added at a concentration of 1 $\mu$g/ml. After 24 h, a mixture of 100 $\mu$l Griess reagent and 100 $\mu$l cell culture supernatant was prepared in a 96-well plate, and the absorbance was measured at 540 nm using a microplate reader (Tecan Group Ltd.) at room temperature. A standard curve was constructed using sodium nitrate, and the amount of NO production was calculated.

*Western blot analysis*. After seeding the RAW264.7 cells in a 60-mm dish at a final concentration of $2x10^5$ cells/ml, the cells were cultured in an incubator at 37˚C and 5% $CO_2$ for 24 h. Following this, the cells were treated with M2-PRTE at concentrations of 25 and 50 $\mu$g/ml. Subsequently, 1 h later, LPS was added at a concentration of 1 $\mu$g/ml, and the cells were cultured at 37˚C and 5% $CO_2$ for 24 h. Subsequently, the cells were washed twice with PBS and protein extraction was performed using RIPA buffer. The extracted proteins were quantified by measuring the absorbance at 595 nm using Bradford protein assay reagent. The quantified proteins were separated by SDS-PAGE (7.5%) at 100 V for 1 h and transferred onto a PVDF (polyvinylidene difluoride) membrane (Bio-Rad Laboratories, Inc.). The membrane was blocked with 5% skim milk at room temperature for 1 h, followed by three washes with TBST buffer for 10 min each. Primary antibodies for iNOS (1:200), COX-2 (1:100) and β-actin (1:2,000) were then applied, and the membrane was incubated at 4˚C for 24 h. The membrane was then washed three times with TBST for 10 min each. The secondary antibody (mouse IgG HRP; 1:5,000) was applied at room temperature for 2 h, followed by three washes with TBST for 10 min each. Subsequently, images were obtained using a UV imaging system (ALLIANCE LD4; UVITEC). Protein band intensity was analyzed using ImageJ (1.53a) gel analysis software (National Institutes of Health).

*Measurement of TNF-α, IL-1β and IL-6 cytokines, and $PGE_2$ levels*. After seeding the RAW264.7 cells in a 12-well plate at a final concentration of $2x10^5$ cells/ml, the cells were cultured in an incubator at 37˚C and 5% $CO_2$ for 24 h. Following this, the cells were treated with M2-PRTE at concentrations of 25 and 50 $\mu$g/ml. Subsequently, 1 h later, LPS was added at a concentration of 1 $\mu$g/ml. After 24 h, the supernatant was collected, and the levels of TNF-α, IL-1β, IL-6 and $PGE_2$ were measured according to the protocol of the ELISA assay kits provided by the manufacturer.

*High-performance liquid chromatography (HPLC) analysis*. Solvent extracts of propolis, red bean and tomato were filtered using a 0.45-$\mu$m syringe filter and then used for HPLC analysis. HPLC analysis was performed using a Waters e2695 Alliance HPLC System (Waters Corporation) equipped with a binary pump delivery system, degasser (G1379A), autosampler (G1313A) and PDA detector (G1315B) operating at 330 nm. Separation was performed with a gradient elution (0 min-10% B, 13 min-10% B, 20 min-25% B, 24 min-30% B, 28 min-35% B, 32 min-45% B, 35 min-45% B, 40 min-50% B, 43 min-55% B, 47 min-60% B, 50 min-60% B, 55 min-10% B) and flow rate and sample consisting of 0.1% formic acid in acetonitrile and 0.1% acetic acid in distilled $H_2O$ over an Xbridge C18 column (Waters Corporation, 4.6x250 mm, 5 $\mu$m). The injection volume was fixed at 0.5 ml/min and 15 $\mu$l, respectively. The column temperature was 35˚C. Standards were identified based on retention time, and the concentrations of caffeic acid, ferulic acid, chlorogenic acid, caffeic acid phenethyl ester, isoquercetin, rutin and lycopene were calculated by comparing the peak area with that of the standard.

*Statistical analysis*. All experimental values are expressed as the mean ± standard deviation (mean ± SD). Statistical comparisons were performed using IBM SPSS Statistics 22 (IBM Corp.). Comparisons between different experimental groups were conducted using one-way analysis of variance (ANOVA), and post hoc multiple comparisons were carried

Table I. Radical scavenging ability of different ratios of PRTE and each extract.

| Samples | DPPH ($IC_{50}$) | ABTS ($IC_{50}$) |
|---|---|---|
| M1-PRTE | 385.13±7.22[e] | 704.06±5.56[d] |
| M2-PRTE | 192.86±3.34[a] | 554.28±4.78[a] |
| M3-PRTE | 197.54±2.86[a] | 674.75±6.92[c] |
| M4-PRTE | 296.18±4.62[b] | 683.65±5.66[c] |
| Propolis extract | 197.21±4.11[a] | 566.43±9.26[a] |
| Red bean extract | 316.12±3.83[c] | 598.93±8.61[b] |
| Tomato extract | 336.28±11.26[d] | 974.24±8.26[e] |

All extracts were examined in a set of experiments repeated three times. $IC_{50}$ is the concentration of extract ($\mu$g/ml) required to scavenge 50% of DPPH and ABTS radicals. PRTE, propolis, red bean and tomato extracts; M1-PRTE, 1:1:1 ratio; M2-PRTE, 1.5:1:0.5 ratio; M3-PRTE, 1.5:0.5:1 ratio; M4-PRTE, 1.2:0.9:0.9 ratio; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). [a-e]Different lowercase letters indicate statistically significant differences between groups (P<0.05).

out using Tukey's test to identify significant differences among the experimental groups. P-value <0.05 was considered to indicate a statistically significant difference.

## Results and Discussion

*Determination of the ratio of propolis, red bean and tomato mixture, and the measurement of the antioxidant activity.* Prior to assessing the intracellular antioxidant and anti-inflammatory efficacy of PRTE, the PRTE were mixed under four conditions as follows: M1-PRTE was mixed at a 1:1:1 ratio, M2-PRTE at a ratio of 1.5:1:0.5, M3-PRTE at a ratio of 1.5:0.5:1 and M4-PRTE at a ratio of 1.2:0.9:0.9. Free radicals in an unstable state can cause damage to cells within the body, and the antioxidant efficacy of using antioxidant substances can be measured by evaluating radical scavenging ability (25). In the present study, in order to determine the optimal mixture ratio, the DPPH and ABTS radical scavenging abilities of each mixture were evaluated. As presented in Table I, among the four combinations, M2-PRTE at a ratio of 1.5:1:0.5 exhibited the most superior DPPH and ABTS radical scavenging abilities compared to the other ratios. The radical scavenging abilities ($IC_{50}$) of DPPH and ABTS radicals for M2-PRTE were confirmed as 192.86±3.34 $\mu$g/ml and 554.28±4.78 $\mu$g/ml, respectively (Table I). Furthermore, when comparing the radical scavenging abilities of DPPH and ABTS with the individual extracts of propolis, red bean and tomato, M2-PRTE at a ratio of 1.5:1:0.5 exhibited enhanced radical scavenging abilities compared to the individual extracts. Based on these results, M2-PRTE was selected for confirming the antioxidant efficacy in HaCaT keratinocytes.

*Antioxidant effects of M2-PRTE on $PM_{2.5}$-induced oxidative stress.* Before measuring the antioxidant effects, the cytotoxicity of $PM_{2.5}$ and M2-PRTE on the human-derived keratinocyte cell line, HaCaT, was evaluated using the WST-8 assay to assess cell viability. The results revealed no cytotoxicity at all concentrations tested for both $PM_{2.5}$ and M2-PRTE (Fig. 1). Based on these results, subsequent experiments were performed using the HaCaT cells with a non-cytotoxic concentration of $PM_{2.5}$ at 100 $\mu$g/ml and PRTE at concentrations <50 $\mu$g/ml. To investigate the effects of M2-PRTE on the activity of antioxidant enzymes, the HaCaT cells were pre-treated with M2-PRTE (25 and 50 $\mu$g/ml) for 1 h, followed by the induction of oxidative stress with $PM_{2.5}$. Subsequently, the activities of SOD and GPx, as well as the GSH content, were measured. The results revealed that exposure to $PM_{2.5}$ significantly depleted the enzymatic activities of SOD and GPx, and reduced the GSH content compared to the control group (Fig. 2). However, following treatment with M2-PRTE at a concentration of 25 $\mu$g/ml, the GPx activity exhibited no significant change; however, a substantial increase was observed following treatment at a concentration of 50 $\mu$g/ml (Fig. 2A). SOD activity, which decreased in a concentration-dependent manner following exposure to $PM_{2.5}$, was restored and significantly increased at a concentration of 50 $\mu$g/ml M2-PRTE (Fig. 2B). Finally, the GSH content exhibited a modest restorative effect at a concentration of 25 $\mu$g/ml M2-PRTE; notably, at a concentration of 50 $\mu$g/ml M2-PRTE, there was a marked restorative effect in the GSH content (Fig. 2C).

SOD, GPx and GSH are essential antioxidant enzymes that protect cells from oxidative stress and ROS. They significantly contribute to maintaining the health and stability of cells in unique ways (26). SOD, as an endogenous antioxidant enzyme, effectively removes reactive oxygen species such as $O_2$-, thus playing a crucial role in protecting cells from oxidative stress (27). Furthermore, GPx collaborates with GSH to prevent cellular damage by eliminating ROS and organic peroxides (28). GSH, in turn, functions as an antioxidant responding to oxidative stress within cells. It is essential for neutralizing and detoxifying toxic substances, and is known to be involved in the proper response and protection mechanisms of cells (29). Therefore, M2-PRTE appears to be a bioactive material contributing to the restoration of antioxidant enzyme activities, such as SOD and GPx, which are depleted by oxidative stress such as $PM_{2.5}$, as well as the replenishment of the antioxidant substance GSH. Hence, the superior antioxidant effects of M2-PRTE suggest its potential use as a natural antioxidant agent.

*Inhibitory effects of M2-PRTE on NO production.* Prior to confirming whether the superior antioxidant efficacy of M2-PRTE translates to anti-inflammatory effects, the present study first evaluated the NO scavenging ability of PRTE mixtures under four conditions in RAW264.7 cells. This was performed to verify whether the anti-inflammatory efficacy of M2-PRTE aligns with its antioxidant efficacy. The cellular environment has a marked impact on the conditions the cell experiences. In the present study, the RAW 264.7 cells exhibited variable NO production rates that were associated with the number of passages. To ensure accuracy, experiments were repeated with the same number of passages (10-11) to ensure the consistency of NO production levels. The results revealed that the NO scavenging ability followed the order of M2-PRTE > M1-PRTE > M3-PRTE > M4-PRTE, and consistent with the antioxidant efficacy experiments, M2-PRTE exhibited the most

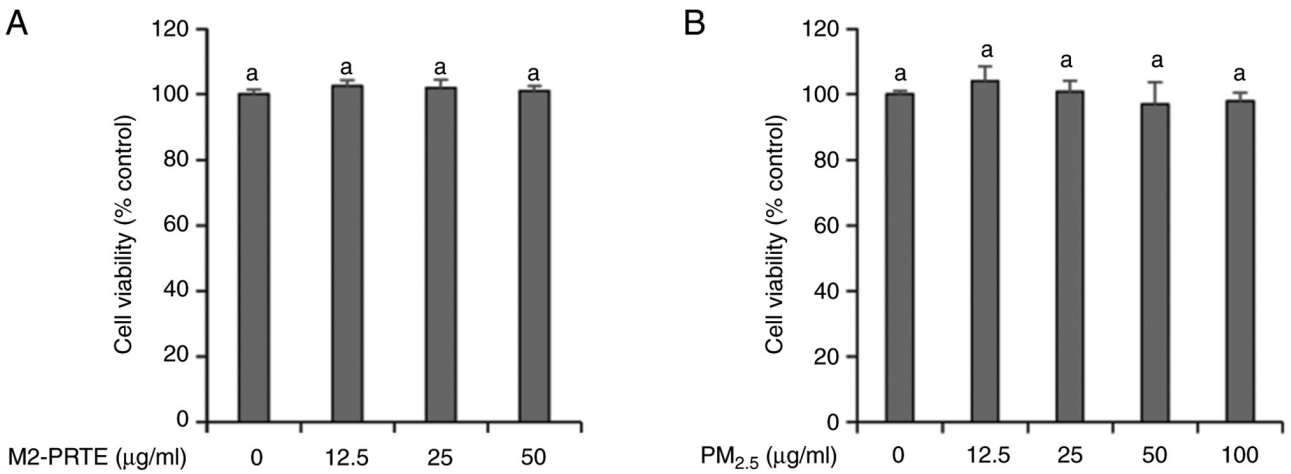


Figure 1. Effect of (A) M2-PRTE and (B) $PM_{2.5}$ on the viability of HaCaT cells. The cells ($2x10^5$ cells/ml) were cultured and treated with various concentrations of $PM_{2.5}$ or M2-PRTE for 24 h, and relative cell viability was assessed using WST-8 assay. The results are presented as the mean ± SD of three different experiments. Bars with the same lowercase letter (a) indicate that there were no statistically significant differences between groups (P>0.05). M2-PRTE, propolis, red bean and tomato extracts at a ratio of 1.5:1:0.5; $PM_{2.5}$, fine particulate matter.

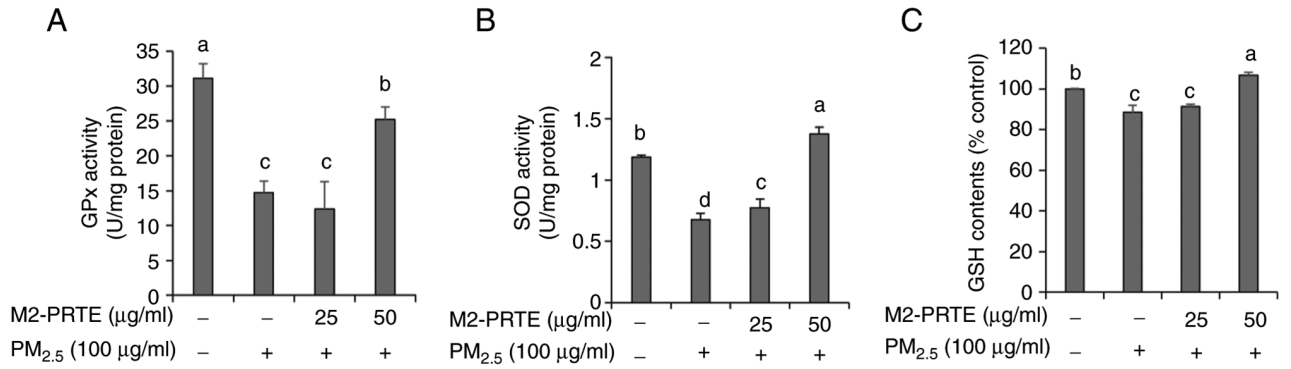


Figure 2. Restorative effects of M2-PRTE on the (A) GPx, (B) SOD and (C) GSH content in $PM_{2.5}$-exposed HaCaT cells. The cells ($2x10^5$ cells/ml) were cultured and pre-treated with 25 or 50 $\mu$g/ml M2-PRTE for 1 h and then exposed to $PM_{2.5}$ (100 $\mu$g/ml) for 24 h. GPx, SOD and GSH were measured in whole cell extracts. The results are presented as the mean ± SD of three different experiments. Bars with different lowercase letters (a-d) indicate statistically significant differences between groups (P<0.05). M2-PRTE, propolis, red bean and tomato extracts at a ratio of 1.5:1:0.5; $PM_{2.5}$, fine particulate matter; GPx, glutathione peroxidase; SOD, superoxide dismutase; GSH, glutathione.

superior performance (Fig. 3A). Additionally, when comparing the NO scavenging ability with the individual extracts of propolis, red bean and tomato, the ratio of M2-PRTE at 1.5:1:0.5 exhibited superior NO scavenging ability compared to the individual extracts (Fig. 3B). Subsequently, to investigate whether the observed NO scavenging ability resulted from toxicity induced by LPS and the extracts, cell toxicity was examined using WST-8. The results revealed no cytotoxicity under all conditions, confirming the absence of toxic effects (Fig. 3C and D). Based on these results, M2-PRTE was selected for further confirmation of its anti-inflammatory efficacy in RAW264.7 mouse macrophages.

The inhibitory effects of M2-PRTE on inflammatory mediators, NO and $PGE_2$, in LPS-stimulated RAW264.7 cells were then investigated. Initially, in the LPS-stimulated RAW264.7 cells, the production of NO and $PGE_2$ significantly increased compared to the untreated control group. However, in the cells pre-treated with M2-PRTE, a concentration-dependent and significant inhibitory effect on both NO and $PGE_2$ production were observed (Fig. 4A and B). In acute inflammation, NO promotes vasodilation and increases blood flow to the inflammatory site, aiding in the defense against invading microorganisms (30). However, the chronic overproduction of NO can lead to tissue damage and inflammation in diseases, such as chronic lung conditions (31). At the same time, the pro-inflammatory mediator, $PGE_2$, is involved in vasodilation, increased vascular permeability and the infiltration of immune cells into the inflammatory site. Environmental pollutants, such as fine dust can induce the excessive production of $PGE_2$, potentially serving as a cause for chronic inflammatory diseases such as chronic bronchitis and atopic dermatitis (32). Therefore, the regulation of the excessive production of NO and $PGE_2$ is considered a crucial therapeutic target in the management of chronic inflammatory conditions. The present study then investigated the mechanisms of action of M2-PRTE in the inhibition of NO and $PGE_2$ production; the effects on the expression of iNOS and COX-2 proteins were examined using western blot analysis. The results revealed an increase in the protein expression of iNOS due to LPS exposure (Fig. 4C). However, following treatment with two

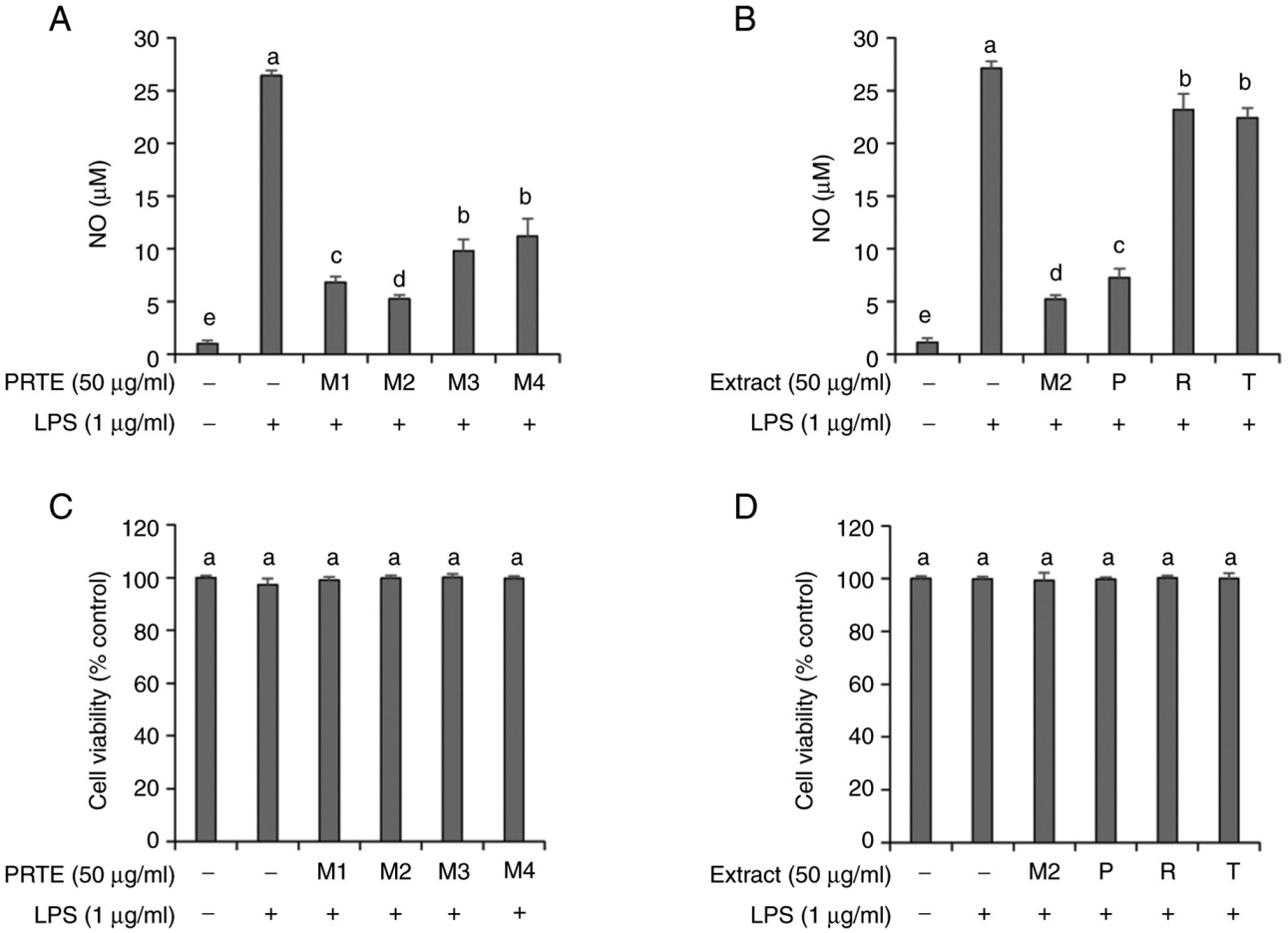


Figure 3. Inhibitory effects of different ratios of PRTE on (A and B) NO levels and (C and D) on the viability of LPS-stimulated RAW264.7 cells. The cells ($2x10^5$ cells/ml) were cultured and pre-treated with 25 or 50 $\mu$g/ml M2-PRTE for 1 h, and then stimulated with LPS (1 $\mu$g/ml) for 24 h. NO levels were examined in the culture supernatants, and relative cell viability was assessed using WST-8 assay. The results are presented as the mean ± SD of three different experiments. Bars with different lowercase letters (a-d) indicate statistically significant differences between groups ($P<0.05$). Bars with the same lowercase letter (a) indicate that there were no statistically significant differences between groups ($P>0.05$). PRTE, propolis, red bean and tomato extracts; M1-PRTE, 1:1:1 ratio; M2-PRTE, 1.5:1:0.5 ratio; M3-PRTE, 1.5:0.5:1 ratio; M4-PRTE, 1.2:0.9:0.9 ratio; NO, nitric oxide; LPS, lipopolysaccharide; P, propolis; R, red bean; T, tomato.

concentrations of M2-PRTE, 25 and 50 $\mu$g/ml, the protein expression of both iNOS and COX-2 significantly decreased (Fig. 4C-E). The activation of iNOS can have negative effects on health by increasing inflammation and oxidative stress. COX-2 plays a crucial role in converting arachidonic acid to $PGE_2$, directly participating in the inflammatory process (33). Therefore, M2-PRTE was found to inhibit the expression of iNOS and COX-2, leading to the suppression of NO and $PGE_2$ production. Consequently, M2-PRTE may be considered as a bioactive food material that can effectively inhibit mediators causing inflammatory diseases in the human body.

*Inhibitory effects of M2-PRTE on IL-1β, TNF-α and IL-6 production*. IL-1β, TNF-α and IL-6 are well-known representative pro-inflammatory cytokines that induce inflammatory responses (34). Therefore, in LPS-stimulated RAW264.7 cells, the present study investigated the inhibitory effects of M2-PRTE on the production of the pro-inflammatory cytokines, IL-1β, TNF-α and IL-6. The results revealed a significant increase in the production of IL-1β, TNF-α and IL-6 in the LPS-exposed RAW264.7 cells; however, pre-treatment with M2-PRTE exerted a concentration-dependent and significant inhibitory effect (Fig. 5). IL-1β promotes immune cell migration to the inflammatory site and triggers important responses, such as fever (35), while TNF-α increases vascular permeability at the inflammatory site, activating the movement of inflammatory cells (36). Furthermore, IL-6 functions as a key factor that activates the immune system when infection or tissue damage occurs (37). Therefore, to improve and treat inflammatory diseases, the effective regulation of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6 is crucial, and there is a need to discover substances that can modulate these inflammatory mediators. From this perspective, M2-PRTE is considered to have the potential to effectively alleviate inflammation by controlling the production of IL-1β, TNF-α and IL-6. The present study demonstrated that the mixture of propolis, tomato and red bean provided prominent antioxidant and anti-inflammatory effects, exerting protective effects against oxidative stress and inflammation induced by $PM_{2.5}$ pollution. With its ability to regulate immune function and alleviate oxidative stress, propolis functions synergistically with tomatoes (38), which are rich in lycopene, a free radical neutralizer, to maintain cellular health (39). The addition of red beans, known for their high antioxidant content, improves

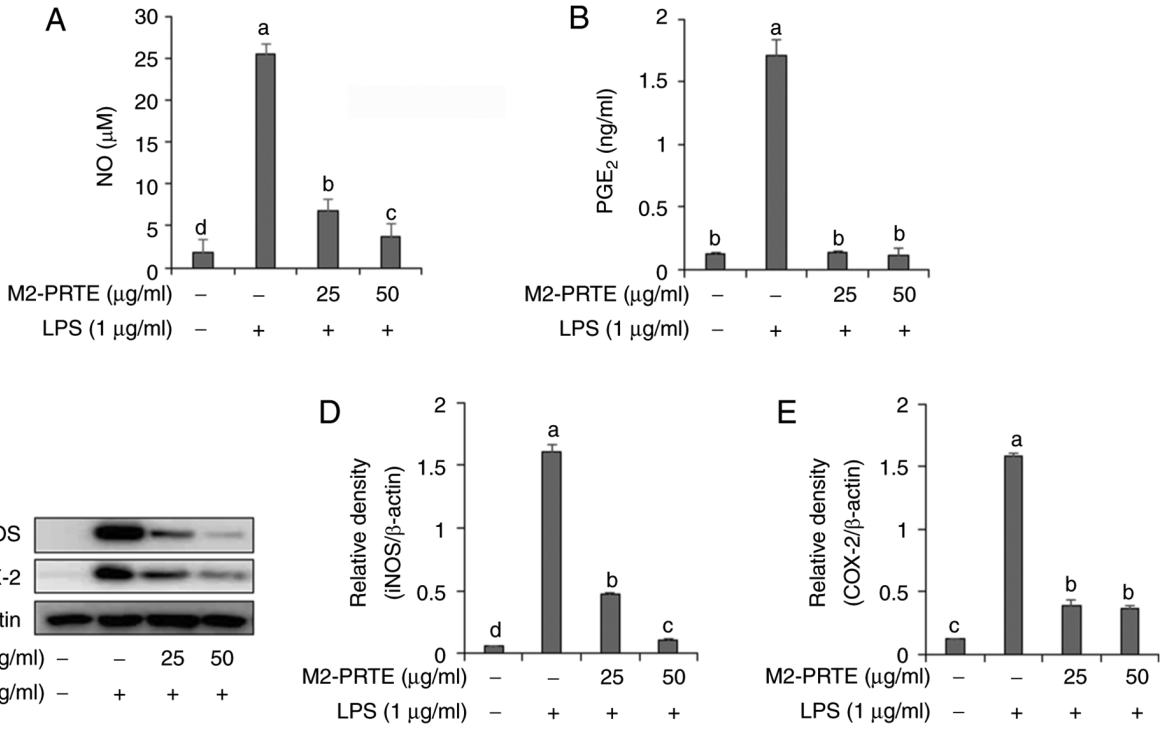


Figure 4. Inhibitory effects of M2-PRTE on (A) NO levels, (B) $PGE_2$ levels, and (C-E) on iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells. The cells ($2x10^5$ cells/ml) were cultured and pre-treated with 25 or 50 $\mu$g/ml M2-PRTE for 1 h, and then stimulated with LPS (1 $\mu$g/ml) for 24 h. The NO and $PGE_2$ levels were examined in the culture supernatants. iNOS and COX-2 expression levels were examined in the total cell extracts, and the relative density of iNOS and COX-2 was then calculated using ImageJ software. The results are presented as the mean ± SD of three different experiments. Bars with different lowercase letters (a-ds) indicate statistically significant differences between groups (P<0.05). M2-PRTE, propolis, red bean and tomato extracts at a ratio of 1.5:1:0.5; LPS, lipopolysaccharide; NO, nitric oxide; $PGE_2$, prostaglandin $E_2$; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

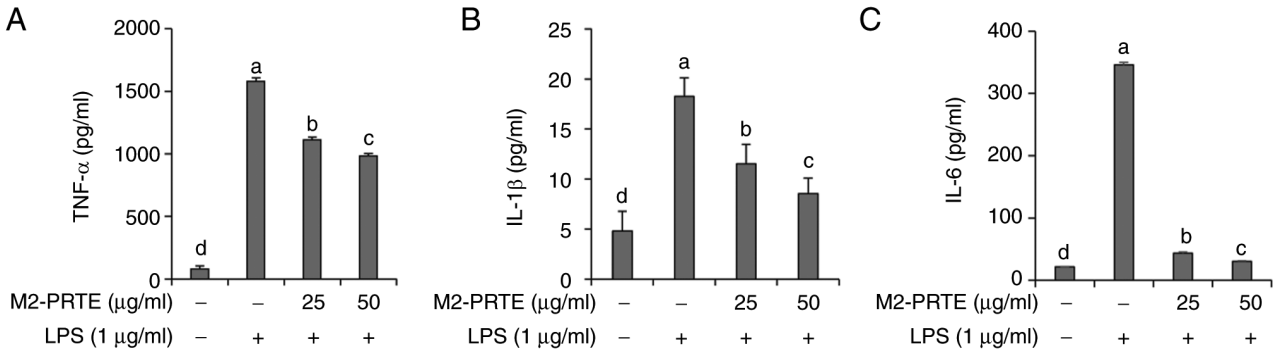


Figure 5. Inhibitory effects of M2-PRTE on (A) TNF-α, (B) IL-1β and (C) IL-6 production in LPS-stimulated RAW264.7 cells. The cells ($2x10^5$ cells/ml) were cultured and pre-treated with 25 or 50 $\mu$g/ml M2-PRTE for 1 h, then stimulated with LPS (1 $\mu$g/ml) for 24 h. TNF-α, IL-1β and IL-6 in the culture supernatants were measured using ELISA kits. The results are presented as the mean ± SD of three different experiments. Bars with different lowercase letters (a-d) indicate statistically significant differences between groups (P<0.05). M2-PRTE, propolis, red bean and tomato extracts at a ratio of 1.5:1:0.5; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor a; IL, interleukin.

the protective efficacy of the mixture against oxidative damage (40). This combination not only highlights the usefulness of natural compounds in reducing health risks associated with $PM_{2.5}$, but also enhances their role in alleviating oxidative stress and inflammation.

*HPLC analysis*. HPLC was conducted to determine the content of chemical compounds contained in the propolis, red bean and tomato extracts. The chemical compounds of the propolis extract were chlorogenic acid (42.68±0.63 $\mu$g/g), caffeic acid (17.81±0.57 $\mu$g/g), ferulic acid (0.28±1.48 $\mu$g/g), and caffeic acid phenethyl ester (18.66±1.24 $\mu$g/g), respectively (Fig. 6A). In the red bean extract, isoquercetin (23.41±0.92 $\mu$g/g) and rutin (1.38±0.58 $\mu$g/g) were detected (Fig. 6B). Moreover, lycopene (10.69±1.17 $\mu$g/g), known as a representative substance in tomato extract, was quantified (Fig. 6C). As aforementioned, as regards the anti-inflammatory and antioxidant effects of PRTE, additional verification for the chemical composition of their extracts was deemed necessary.

In conclusion, the mixture of propolis, red bean, and tomato, known as M2-PRTE, exhibited not only DPPH and ABTS radical scavenging abilities, but also demonstrated

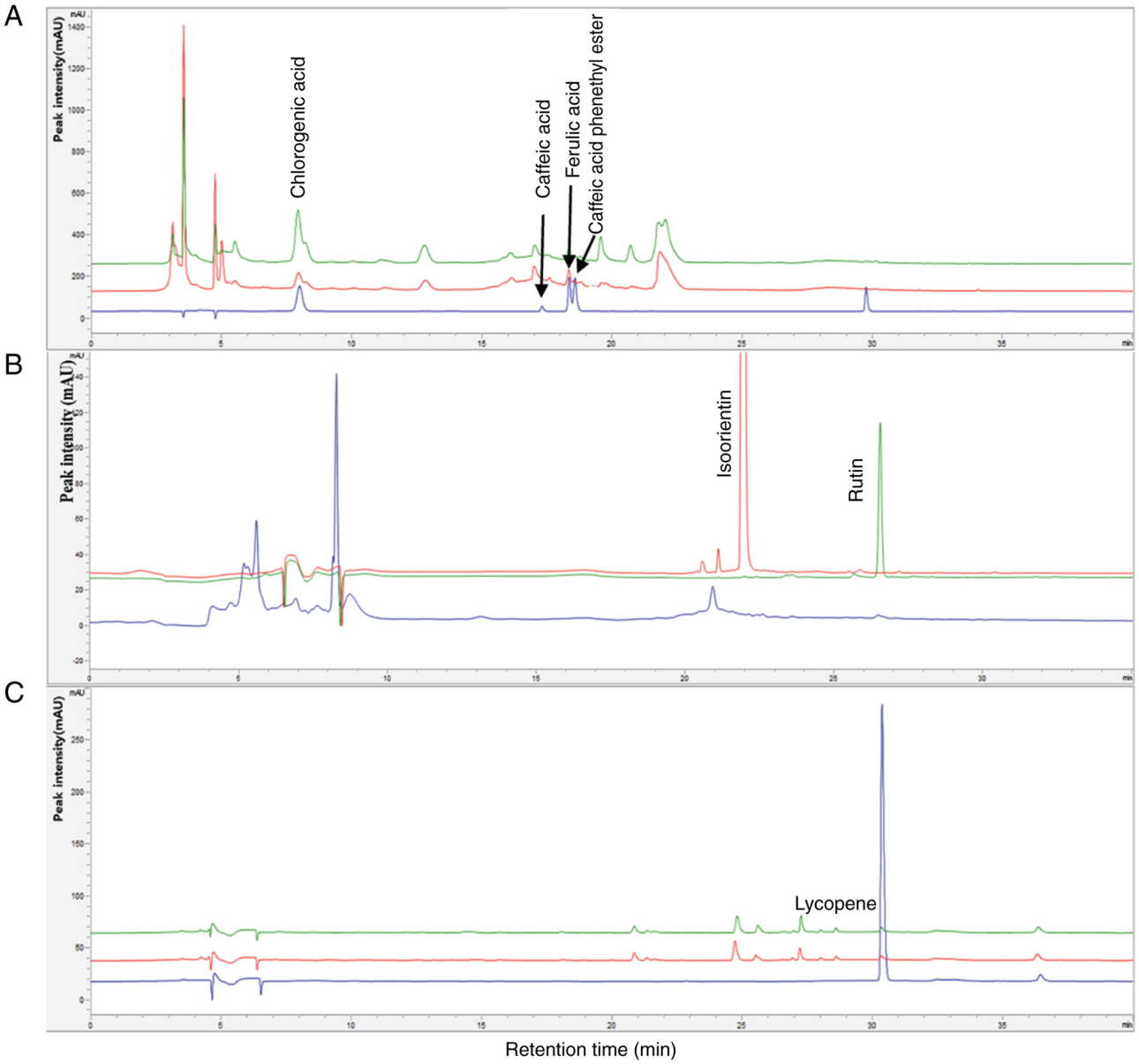


Figure 6. HPLC chromatograms in the propolis, red bean and tomato extracts. The figure presents HPLC chromatograms obtained at 254 nm for propolis extract, red bean extract and tomato extract, each treated with distinct standard compounds. (A) Chromatogram of propolis extract showcasing peaks corresponding to - chlorogenic acid; - caffeic acid; - ferulic acid; -caffeic acid phenethyl ester. (B) Chromatogram of red bean extract displaying peaks for - isoquercetin; - rutin. (C) Chromatogram of tomato extract revealing a single peak for - lycopene. HPLC, high-performance liquid chromatography.

antioxidant activity by inducing the activation of antioxidant enzymes, such as SOD and GPx, as well as by increasing the intracellular GSH levels in HaCaT cells under conditions of oxidative stress induced by $PM_{2.5}$. Additionally, M2-PRTE exerted inhibitory effects on the expression of iNOS and COX-2 molecules in LPS-stimulated RAW264.7 cells, leading to the suppression of NO and $PGE_2$ production. Moreover, M2-PRTE effectively inhibited the production of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6. Therefore, M2-PRTE is anticipated to have high potential as a functional food ingredient for alleviating oxidative stress and inhibiting inflammatory responses. However, further research is required in order to explore the efficacy and molecular mechanisms of M2-PRTE at the physiological level, and additional studies on functional components are warranted for its utilization as a health functional food ingredient.

**Acknowledgements**

Not applicable.

**Funding**

The present study was supported by the Collabo R&D between Industry, Academy, and Research Institute (RS-2023-00224909) funded by the Ministry of SMEs and Startups (MSS, Korea).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

ESK and BOC conceived and designed the experiments. ESK and SYJ participated in the design of the study and in the drafting of the manuscript. ESK and SYJ carried out the experiments. ESK, SYJ, MHJ, MYK, YKH, BOC and SIJ participated in acquisition, analysis and interpretation of the data. SIJ provided resources, reviewed and edited the manuscript, and supervised the study. BOC and SIJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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