

Communication

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Communication

Pyrogallol Plays a Central Role in Anti-Allergic and Anti-Inflammatory Activities of Rosebud Extracts of Newly Crossbred Roses

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Abstract

Since chemical anti-allergic compounds have adverse effects, many investigators pay attention on relatively-safe natural products. In order to fulfill such a requirement, we investigated anti-allergic, anti-inflammatory, and antioxidative activities of the extracts of newly-crossbred rosebuds *in vitro* and *in vivo*. Twenty-four candidate rosebuds were extracted with 80% ethanol, and analyzed for polyphenols, flavonoids, tannins, proanthocyanidins, and pyrogallol (1,2,3-benzenetriol). The extracts' *in vitro* antioxidative, anti-allergic, and anti-inflammatory activities were analyzed through 1,1-diphenyl-2-picrylhydrazyl radical-scavenging capacity as well as inhibitory effects on the β -hexosaminidase release from Compound 48/80-stimulated RBL-2H3 mast cells and nitric oxide production from lipopolysaccharide-activated RAW 264.7 macrophages, respectively. The *in vivo* activity was assessed via protection against lethality and itching (scratching) symptoms in mice challenged with Compound 48/80. Among candidates, Lover Shy, Pretty Velvet, Ice Wing, Red Perfume, Onnuri, Jaemina Red, and Hanggina were found to possess high concentrations of antioxidant components and antioxidative activity. By comparison, Pretty Velvet, Red Perfume, Jaemina Red, Hanggina, Onnuri, and Ice Wing were highly effective in anti-allergic and anti-inflammatory activities *in vitro*, in parallel with their concentrations of pyrogallol. Their anti-allergic effects were confirmed in mice: The 6 extracts protected against Compound 48/80-induced mortality and scratching behaviors in a dose-dependent manner. The allergen-induced increases in serum IgE and histamine as well as inflammatory cytokines, tumor-necrosis factor- α and interleukin-1 β , were remarkably attenuated following treatment with the rosebud extracts. These findings demonstrate that the extracts and active ingredients from cross-bred rosebuds exert anti-allergic and anti-inflammatory activities through their high levels of antioxidants and pyrogallol, and that could be promising candidates to overcome allergic responses such as atopic dermatitis.

Keywords: rosebud extract; antioxidant; pyrogallol (1,2,3-benzenetriol); antioxidative activity; anti-allergic activity; anti-inflammatory activity

1. Introduction

As an excessive immune function of the body, hypersensitivity reactions are classified into four types [1]. Among them, type 1 hypersensitivity reactions occur when the immunoglobulin E (IgE), which has been produced excessively through repeated exposure to allergens, binds to mast cells,

and cause systemic anaphylactic shock or local itching and inflammation as granules containing histamines and chemotactic factors are released [2]. Representative type 1 hypersensitivity reactions include atopic dermatitis and respiratory asthma, which are mediated by Th2 cytokines. Atopic dermatitis is a chronic relapsing inflammatory skin disease, showing the highest prevalence in childhood. The number of atopic dermatitis patients in Korea was over 1 million in 2022 (Korea Health Insurance Review and Assessment Service) and has been increasing sharply every year. And the worldwide incidence of atopic dermatitis has increased by 2-3 times over the last 30 years [3].

The cause of atopic dermatitis is not clear, but genetic and environmental factors are involved. For example, atopic dermatitis is often caused by hypersensitivity reactions to dust, mites, bacteria, and fungi in the house, pollens, environmental chemicals, and chemicals added to food [4]. According to a review [5], allergic inflammations occur when external triggers, called allergens, penetrate the impaired skin barriers and reach subcutaneous mast cells. Many inflammatory mediators, including histamine and chemotactic factors released from mast cells, cause itching symptoms. Itching symptoms lead to scratching behaviors and tissue damage, exacerbating skin inflammation so that an 'itching-scratching' vicious cycle is formed. In addition, diverse factors aggravate atopic dermatitis: dry skin, ambient temperature and humidity, strenuous exercise and sweat, strong skin scrubbing, skin irritation by wool and fibers, food, drugs, house dust, mites, animal hair, irritant chemicals, infections, and mental stress. In addition to the above external factors, internal factors such as immune dysfunction or hypersensitivity to injured tissues can also cause atopic dermatitis [4].

In clinical practice, antihistamines and steroids are commonly prescribed as therapeutic agents. Although antihistamines suppress itching symptoms caused by urticarial [6], they play only a limited role in the treatment of atopic dermatitis. Since long-term use of antihistamines can cause adverse effects, interest in natural products with high therapeutic effects and low side effects is increasing. The search for new functional substances from natural products has been accelerating after the enactment of the Korea Food and Drug Administration's notification on safety and efficacy evaluation standards and approval procedures for functional foods and functional cosmetics [7].

Roses with various colors and shapes are a dicotyledonous plant belonging to the genus *Rosa* in the Rosaceae family. Rose flowers have been widely used for ornamental purposes and rose oil has been widely used as a raw material for perfumes and basic cosmetics. By comparison with scent and oils, the nutritional and pharmacological activities of roses were not studied enough. Roses were suggested as a therapeutic agent, because they contain various phytochemicals including antioxidants [8]. In fact, it was found that rose petals contain vitamins, malic acid, cratgolic acid, flavonoids, and lactones, so have a high antioxidative potential [9]. In addition, anthocyanins possessing anti-aging, cardioprotective, anti-cancer, and anti-diabetes activities were rich in rose extracts [10–12]. As additional advantages, roses are caffeine-free [13], and exhibit various antibacterial activities [14].

Recently, we reported strong antioxidative and anti-inflammatory effects of rosebud extracts from new 24 rose cultivars [15]. In addition, we demonstrated that they exerted neuroprotective, anti-microbial, skin-whitening, wrinkle-improving, and anti-allergic properties [16,17]. Notably, it was found out that pyrogallol is a major antioxidant displaying neuroprotection and anti-allergic effects, in which it suppressed interleukin-9 (IL-9) gene expression and calcineurin/nuclear factor of activated T-cells (NFAT) signaling [18–21]. In this context, we extended analyses of antioxidants and pyrogallol in 24 rosebud extracts, and assessed their anti-allergic and anti-inflammatory activities *in vitro* and *in vivo*, demonstrating protective potentials against systemic and local skin anaphylactic responses.

2. Materials and Methods

2.1. Preparation of Rosebud Extracts

Dried rosebuds from 24 new cultivars (*Rosa hybrida*) crossbred in Gumi Smart Agriculture Research Institute (Gumi, Republic of Korea) in 2021 were used: i.e., (1) Lover Shy, (2) Lovely Scarlet,

(3) Loving Heart, (4) Red Perfume, (5) Luminus, (6) Mirinae Gold, (7) Betty, (8) Bichina, (9) Aileen, (10) Onnuri, (11) Yunina, (12) Jaemina Red, (13) Jinseonmi, (14) Chilbaegri, (15) Tamina, (16) Tamnari, (17) Pretty Velvet, (18) Peach Grace, (19) Pink Love, (20) Pink Perfume, (21) Hanaro, (22) Hanaram, (23) Hanggina, and (24) Ice Wing (Figure 1).

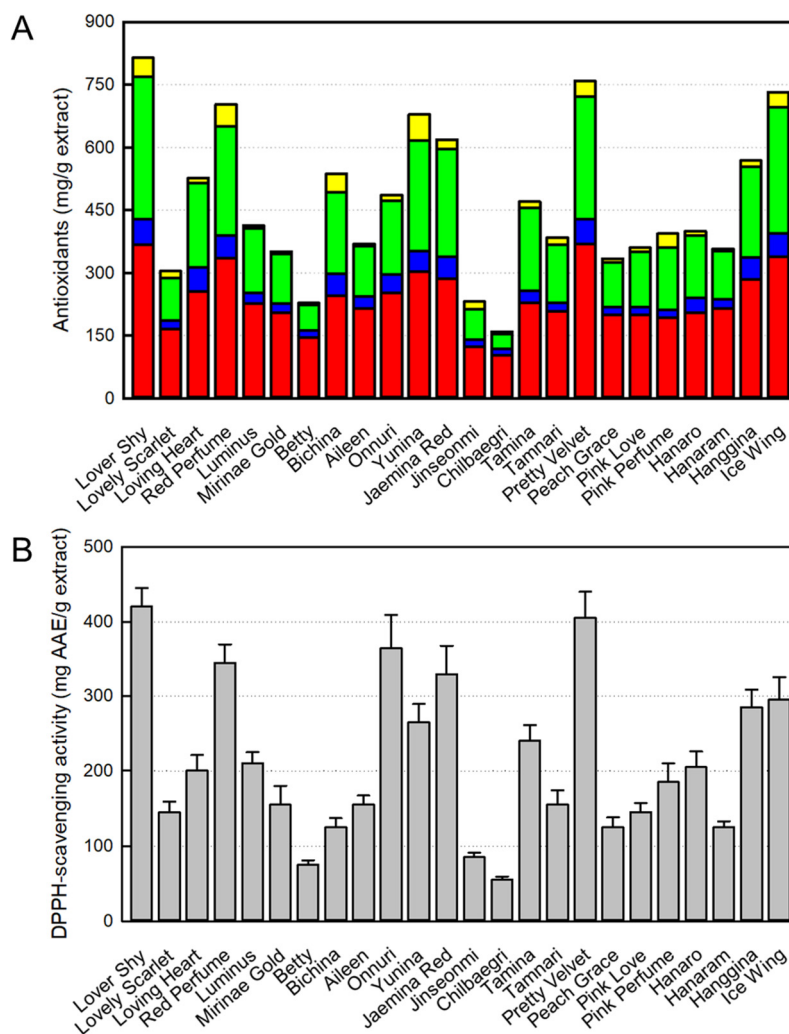


Figure 1. Antioxidant contents (A) and antioxidative activity (B) of 24 rosebud extracts (n = 3). Red: polyphenols, blue: flavonoids, green: tannins, yellow: protoanthocyanidins.

Based on our previous studies, the rosebuds were extracted with 80% ethanol to achieve high antioxidant contents [15,22]. The dried rosebuds were pulverized in a rotor mill (Laval Lab Inc., Laval, QC, Canada), and immersed in 80% ethanol in an ultrasonic water bath, in which the extraction solvent/solid ratio was set to 49 : 1 (980 mL 80% ethanol/20 g dried rosebuds). The bath was heated at 60-70°C for 2 hours, and then ultrasonic-extracted for 1 hour. After extraction, the mixture was cooled at room temperature, filtered, and then concentrated to 60 brix under a reduced pressure using a vacuum evaporator (Rotary Vacuum Evaporator N-N series; Eyela, Tokyo, Japan). The yields were found to be 20-28%. The samples were stored at 4°C until use in a week.

2.2. Analysis of Antioxidative Components

2.2.1. Analysis of Total Polyphenols

The total polyphenol content of the rosebud extracts was measured in triplicates according to the method of Dewanto et al. [15,23]. That is, the total polyphenol content was measured according

to the principle that Folin-Ciocalteu's phenol reagent is reduced by polyphenolic compounds in the extracts to develop a molybdenum color. The concentration of the samples was adjusted to 1 mg/mL, 2 mL of 2% Na₂CO₃ was added for reaction for 3 min, 100 µL of 50% Folin-Ciocalteu's phenol reagent was added, the mixture was left alone for 30 min, and the absorbance of the reaction solution was measured at 750 nm. A standard calibration curve was prepared using gallic acid (Sigma-Aldrich, St. Louis, USA) diluted to 10, 20, 30, 40 and 50 times as standard materials, and the polyphenol content was expressed as mg of gallic acid in 1 g of the sample.

2.2.2. Analysis of Flavonoids

The total flavonoid content of the rosebud extracts was analyzed in triplicates using the method of Zhishen et al. [24] with some modification. The concentration of the samples was adjusted to 1 mg/mL, 1 mL of distilled water and 75 µL of 5% sodium nitrite were added to 250 µL of the sample solution, 150 µL of 10% aluminum chloride hydrate was added 5 min later, 500 µL of 1 M NaOH was added 6 min later, the mixture was left alone for 11 min, and the absorbance was measured at 510 nm. A standard calibration curve was prepared with (+)-catechin hydrate (Sigma-Aldrich), and the flavonoid content was expressed as mg of catechin in 1 g of the sample.

2.2.3. Analysis of Tannins

The total tannin content of the rosebud extracts was measured in triplicates according to the method of Duval and Shetty [25]. The concentration of the sample was adjusted to 1 mg/mL, 1 mL of 95% ethanol and 1 mL of distilled water were added to 1 mL of the sample solution, the mixture was shaken well, 1 mL of 5% Na₂CO₃ solution and 0.5 mL of 1 N Folin-Ciocalteu's reagent (Sigma-Aldrich) were added to the mixture, the color was developed at room temperature for 60 min, and the absorbance was measured at 725 nm. A standard calibration curve was prepared with tannic acid (Sigma-Aldrich), and the tannin content was expressed as mg of tannic acid in 1 g of the sample.

2.2.4. Analysis of Proanthocyanidins

The total proanthocyanidin content of the rosebud extracts was measured in triplicates using the vanillin-sulfuric acid method modified according to the method of Takahama et al. [26]. The concentration of the sample was adjusted to 1 mg/mL, 0.5 mL of 1.2% vanillin solution and 0.5 mL of 20% sulfuric acid were added to 0.2 mL of the sample solution, the mixture was left alone for 20 min, and the absorbance was measured at 500 nm using an ELISA reader (UV-1650PC; Shimadzu, Kyoto, Japan). A standard calibration curve was prepared with (+)-catechin (Sigma-Aldrich), and the proanthocyanidin content was expressed as mg of catechin in 1 g of the sample.

2.2.5. Analysis of Pyrogallol (1,2,3-Benzenetriol)

The pyrogallol content of the rosebud extracts was analyzed in triplicates by HPLC (ACME 9000 system; Younglin, Anyang, Korea) [18]. Separation was performed on a YMC-Triart C18 column (4.6 × 250 mm; YMC, Kyoto, Japan). The mobile phase consisted of acetonitrile containing 0.1% acetic acid (A) and water (B), delivered under gradient elution conditions. The flow rate was set at 1.0 mL/min, and a 20 µL aliquot of each sample was injected. Detection was carried out using a UV detector at a wavelength of 280 nm. Pyrogallol (extra-pure grade) was obtained from Daejung Chemicals & Metals (Siheung, Korea). Quantification was performed by comparison with a calibration curve constructed using a pyrogallol standard.

2.3. Analysis of Antioxidative, Anti-Allergic, and Anti-Inflammatory Activities

2.3.1. Measurement of DPPH-Scavenging Activity

Antioxidative efficacy of the rosebud extracts was measured in triplicates as described previously [27], based on the scavenging potential of 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-

Aldrich). That is, 0.2 mL rose extract or distilled water (blank) was added into 0.8 mL DPPH solution (0.2 mM) to adjust to 0.1 mg/mL. After 30-min incubation, absorbance was measured at 520 nm. DPPH radical-scavenging activity, i.e., electron-donating ability, was expressed as mg AAE in 1 g extract with the difference in absorbance according to the addition and non-addition (distilled water) of the test substance.

2.3.2. Measurement of Mast Cell Degranulation

The cell line RBL-2H3 (KCLB No. 22256, Lot No. 43077) was from the Korean Collection for Type Cultures of the Korea Research Institute of Bioscience and Biotechnology (Jeongeup, Korea). The cells were cultivated in a 5% CO₂ incubator (37°C), using a minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic (Gibco, Grand Island, USA).

Mast cell degranulation was measured in triplicates with the release of β -hexosaminidase, which is a granule marker [16,28]. The cells were separated from the culture flask by treatment with an enzyme (trypsin-EDTA), and the cell suspension was centrifuged at 500 g for 5 min. Cell suspension was prepared at 2×10^6 cells/mL, and aliquoted (100 μ L) into a 96-well cell culture plate, and stabilized for at least 3 hours.

After discarding culture medium, the wells were treated with the new culture medium containing anti-dinitrophenyl (DNP)-IgE (10 ng/mL), and cultivated overnight. The cells were treated with rosebud extracts (final concentrations of 10, 32 or 100 μ g/mL), and incubated for 30 min. Thereafter, the cells were stimulated with DNP-human serum albumin (HAS; 10 μ g/mL) to induce mast cell degranulation. After 1-hour incubation, 60 μ L of the supernatant was taken, mixed with 15 μ L of substrate buffer, and made to react at 37°C for 2 hours. The stop solution (150 μ L) was added, and the absorbance was measured at 405 nm.

2.3.3. Measurement of Nitric Oxide (NO) Production

The inflammatory response of macrophages was measured in triplicates with NO production according to cell activation [15]. The cell line RAW 264.7 (ATCC TIB-71, KCLB No. 40071) used in the test was bought from the Korean Collection for Type Cultures of the Korea Research Institute of Bioscience and Biotechnology (Jeongeup, Korea). The cells were cultivated overnight in a 96-well plate at a concentration of 1×10^7 cells/mL. In order to activate cells, the cells were treated with lipopolysaccharide (LPS; 1 μ g/mL). Rosebud extracts were added at concentrations of 10, 32 or 100 μ g/mL, and incubated for 24 hours. An equal volume of Griess reagent (Promega, Madison, USA) was mixed, and the absorbance was measured at 540 nm 10 min later for NO measurement. A standard calibration curve was prepared with sodium nitrite, and the NO concentration in the culture medium was calculated.

2.4. Analysis of Correlation Between Ingredients and Activities

2.4.1. Correlation Between Antioxidant Content and Antioxidative Activity

The correlations between antioxidant (polyphenol, flavonoid, tannin or protoanthocyanidin) contents and antioxidative activities were analyzed. Linear regression coefficients were calculated for the estimation of relationship. Linear correlation was calculated with a SigmaPlot program.

2.4.2. Correlation Between Pyrogallol Content and Anti-Allergic Activity

The correlation between pyrogallol content and anti-allergic activity was analyzed. Linear regression coefficient was calculated described above.

2.4.3. Correlation Between Pyrogallol Content and Anti-Inflammatory Activity

The correlation between pyrogallol content and anti-inflammatory activity was analyzed. Linear regression coefficient was calculated described above.

2.5. Measurement of Mouse Allergic Reaction

2.5.1. Animals

Male ICR mice (6 weeks old, 25-28 g) for the induction of systemic and local hypersensitivity reactions were procured from Daehan Biolink (Eumseong, Korea) and housed in polysulfone cages (3 animals/cage). Only healthy animals were used after 1-week acclimation to the laboratory environment. The environment of the animal laboratory was adjusted to a temperature of $23 \pm 2^\circ\text{C}$, relative humidity of $55 \pm 10\%$, ventilation frequency of 12 times/hour, lighting cycle of 12 hours (07:00~19:00), and illumination of 150~300 lux. They were fed standard rodent chow and purified water *ad libitum*. The animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of Chungbuk National University, and according to the Standard Operation Procedures (SOP) of the same institution.

2.5.2. Measurement of Systemic Allergic Reaction (Death from Shock)

The anti-allergic activity of rosebud extracts on systemic anaphylactic shock was assessed in mice ($n=6/\text{group}$ under random grouping) challenged Compound-48/80, a mast cell degranulator. Rose extracts were diluted in saline and intraperitoneally administered to ICR mice at doses of 10, 30 or 100 mg/kg. Thirty min later, a lethal dose (8 mg/kg) of Compound 48/80 was intraperitoneally injected to induce anaphylactic reaction, and thereafter the mortality rate of the mice was recorded for 60 min [16,29].

2.5.3. Measurement of Local Allergic Reaction (Skin Itching)

In order to investigate the anti-allergic activity of rosebud extracts on atopic dermatitis, the extracts were diluted in purified water, orally administered to ICR mice ($n=6/\text{group}$ under random grouping) at doses of 10, 30 or 100 mg/kg. Thirty min later, Compound 48/80 (50 $\mu\text{g}/\text{site}$, 50 μL at 1 mg/mL) was subcutaneously injected between the two shoulder blades [16,29,30]. Thereafter, the number of scratching behaviors was recorded to 60 min.

2.5.4. Measurement of Blood IgE and Histamine

After deep anesthesia with diethyl ether, blood was collected from the abdominal vena cava, into a Vacutainer (BD 367986; BD, Seoul, Korea), and centrifuged at 3,000 rpm for 15 min to obtain serum. Serum IgE and histamine were determined via ELISA according to the manufacturer's instructions. Briefly, serum was loaded into ELISA wells together with antibodies specific for IgE (K3231081; Komabiotech, Seoul, Korea) or histamine (ab213975; Abcam, Cambridge, UK), and incubated for 0.5 - 1 hour at room temperature. After washing, the secondary antibody was treated and incubated for 30 min. Following treatment with a substrate for color development, absorbance was measured at 450 nm [31].

2.5.5. Measurement of Blood Cytokines

As inflammatory cytokines, the levels of serum TNF- α and IL-6 were measured using mouse Quantikine immunoassay kits (R&D Systems, Minneapolis, MN, USA) according to the ELISA procedures provided by the manufacturer (Molecular Devices) [31,32].

2.6. Statistical Analysis

Data are presented as mean \pm standard deviation. Statistical analysis was performed with SPSS version 26.0 program (SPSS Inc., Chicago, IL, USA). Differences among groups were analyzed with one-way ANOVA, followed by Tukey's HSD at a level of $P < 0.05$. Correlations between ingredients and their activities were analyzed with SigmaPlot Version 12.5 (Grafiti, Palo Alto, CA, USA).

3. Results

3.1. Antioxidant Contents in Rosebud Extracts and Their Antioxidative Activities

We analyzed extensive antioxidants including polyphenols, flavonoids, tannins, and proanthocyanidins from 24 rose cultivars. Among 4 ingredients, polyphenols were very rich in Pretty Velvet (368.5 ± 1.5 mg/g), Lover Shy (367.5 ± 5.6 mg/g), Ice Wing (338.4 ± 2.2 mg/g), Red Perfume (335.2 ± 2.1 mg/g), Yunina (302.4 ± 4.7 mg/g), and Jaemina Red (286.5 ± 2.1 mg/g) (Figure 1A; Supplementary Table S1). Flavonoids were lower than polyphenols, but Lover Shy (61.3 ± 2.0 mg/g), Pretty Velvet (60.0 ± 5.1), Loving Heart (57.0 ± 3.3 mg/g), Ice Wing (55.3 ± 1.4 mg/g), Red Perfume (54.0 ± 0.8 mg/g), Bichina (52.3 ± 0.3 mg/g), and Haggina (52.0 ± 1.3 mg/g) were found to contain relatively-high concentrations. Tannins were high in Lover Shy (339.6 ± 1.3 mg/g), Ice Wing (302.3 ± 3.8 mg/g), Pretty Velvet (293.6 ± 4.2 mg/g), Yunina (263.7 ± 0.2 mg/g), Red Perfume (261.3 ± 1.3 mg/g), and Jaemina Red (258.5 ± 1.4 mg/g). Protoanthocyanidins were relatively low among other components, but Yunina (62.1 ± 0.8 mg/g), Red Perfume (52.0 ± 2.5 mg/g), Lover Shy (45.6 ± 3.5 mg/g), Bichina (43.8 ± 1.7 mg/g), Ice Wing (36.7 ± 1.4 mg/g), and Pretty Velvet (36.5 ± 2.1 mg/g) had high protoanthocyanidin concentrations.

There were big differences in total antioxidant contents ranging from 814.0 mg/g (Lover Shy) to 159.9 mg/g (Chilbaegri). Among 24 rosebud extracts, Lover Shy (814.0 mg/g), Pretty Velvet (758.6 mg/g), Ice Wing (723.1 mg/g), Red Perfume (698.2 mg/g), Onnuri (678.5 mg/g), Jaemina Red (618.4 mg/g), and Haggina (568.3 mg/g) were found to contain high concentrations of the antioxidants.

Notably, the overall antioxidative activities of 24 rosebud extracts showed a similar trend of their antioxidative contents (Figure 1B). The correlation between antioxidants and their antioxidative activities was described below (Figure 6).

3.2. Identification and Content of Pyrogallol in Rosebud Extracts

Next, we analyzed pyrogallol, as an anti-allergic ingredient, in 24 rosebud extracts. As a result of HPLC analysis. Pyrogallol standard was detected at a retention time of 8.5450 min, and pyrogallol was detected at the same time in a rosebud extract (Figure 2).

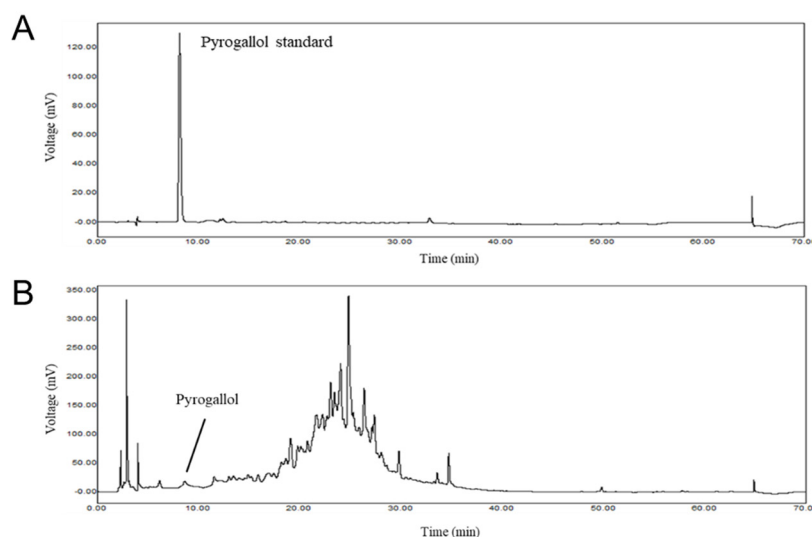
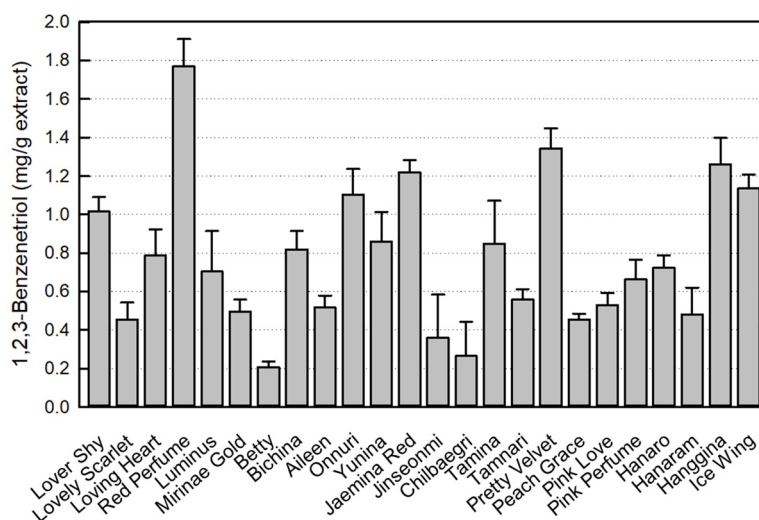


Figure 2. HPLC chromatogram of pyrogallol standard (A) and a rosebud extract (B).

There was a difference in pyrogallol concentration ranging from 1.77 ± 0.14 mg/g (Red Perfume) to 0.21 ± 0.03 mg/g (Betty) (Figure 3). Among 24 rose cultivars, the concentration of pyrogallol was highest in Red Perfume (1.77 ± 0.14 mg/g), Pretty Velvet (1.34 ± 0.10 mg/g), Hanggina (1.26 ± 0.14 mg/g), Jaemina Red (1.22 ± 0.06 mg/g), Ice Wing (1.14 ± 0.07 mg/g), Onnuri (1.10 ± 0.13 mg/g), and Lover Shy (1.02 ± 0.07 mg/g). Notably, the order of pyrogallol concentration was similar with the antioxidant content, although Lover Shy showed relatively-low pyrogallol level compared with highest antioxidants.

**Figure 3.** Pyrogallol (1,2,3-benzenetriol) content in 24 rosebud extracts (n = 3).

3.3. Inhibition of Mast Cell Degranulation

In order to assess allergic reaction, we analyzed β -hexosaminidase, instead of histamine, released from mast cells sensitized with IgE, since both of them are secreted by allergic reactions such as asthma, rhinitis, and atopic dermatitis [16]. β -Hexosaminidase release from RBL-2H3 cells markedly increased following sensitization with anti-DNP IgE (Figure 4A). However, such an IgE-mediated increase in β -hexosaminidase release was attenuated by rosebud extracts (100 μ g/mL), in which Red Perfume, Onnuri, Jaemina Red, Pretty Velvet, Hanggina, and Ice Wing were the most effective, reducing to a normal level. Notably, the anti-allergic activity was in parallel with the concentration of pyrogallol in the rosebud extracts (Figure 3). Further, we assessed the concentration-effectiveness at 10, 30, and 100 μ g/mL. As results, the selected 6 extracts, Red Perfume, Onnuri, Jaemina Red, Pretty Velvet, Hanggina, and Ice Wing, displayed concentration-dependent anti-allergic activities (Figure 4B).

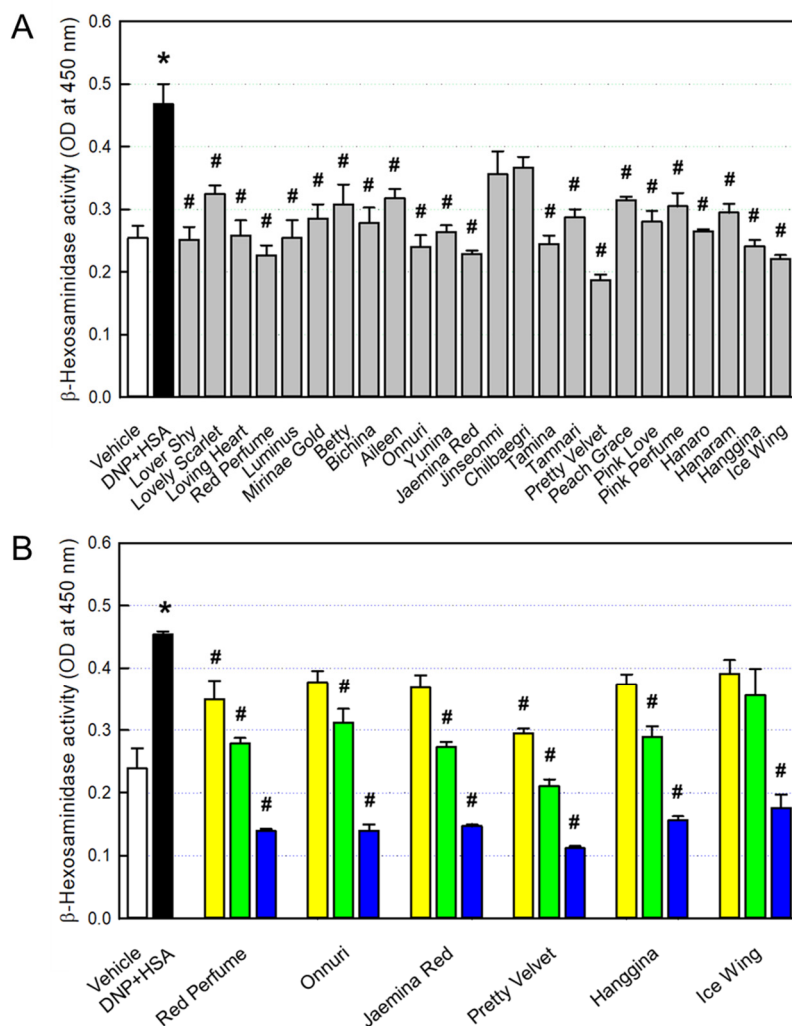


Figure 4. Inhibition by rosebud extracts of β -hexosaminidase release from RBL-2H3 mast cells (n = 3). (A) Anti-allergic activities of 24 rosebud extracts (100 μ g/mL). (B) Concentration-dependent activities of selected rosebud extracts at concentrations of 10 (yellow), 32 (green) or 100 (blue) μ g/mL. *Significantly different from Vehicle control ($P < 0.05$). #Significantly different from DNP-HSA alone ($P < 0.05$).

3.4. Inhibition of Macrophage NO Production

As an inflammatory parameter, we analyzed NO production from macrophages activated with LPS. Treatment of RAW 264.7 cells with LPS greatly increased NO release (Figure 5A). However, all 24 rosebud extracts inhibited the NO production, wherein Red Perfume, Onnuri, Jaemina Red, Pretty Velvet, Hanggina, and Ice Wing were highly effective. Interestingly, the anti-inflammatory activity was in parallel with the concentrations of antioxidants and pyrogallol in the rosebud extracts (Figures 1 and 3). Furthermore, the selected 6 extracts, Red Perfume, Onnuri, Jaemina Red, Pretty Velvet, Hanggina, and Ice Wing, exhibited concentration-dependent anti-inflammatory activities at 10, 30, and 100 μ g/mL (Figure 5B).

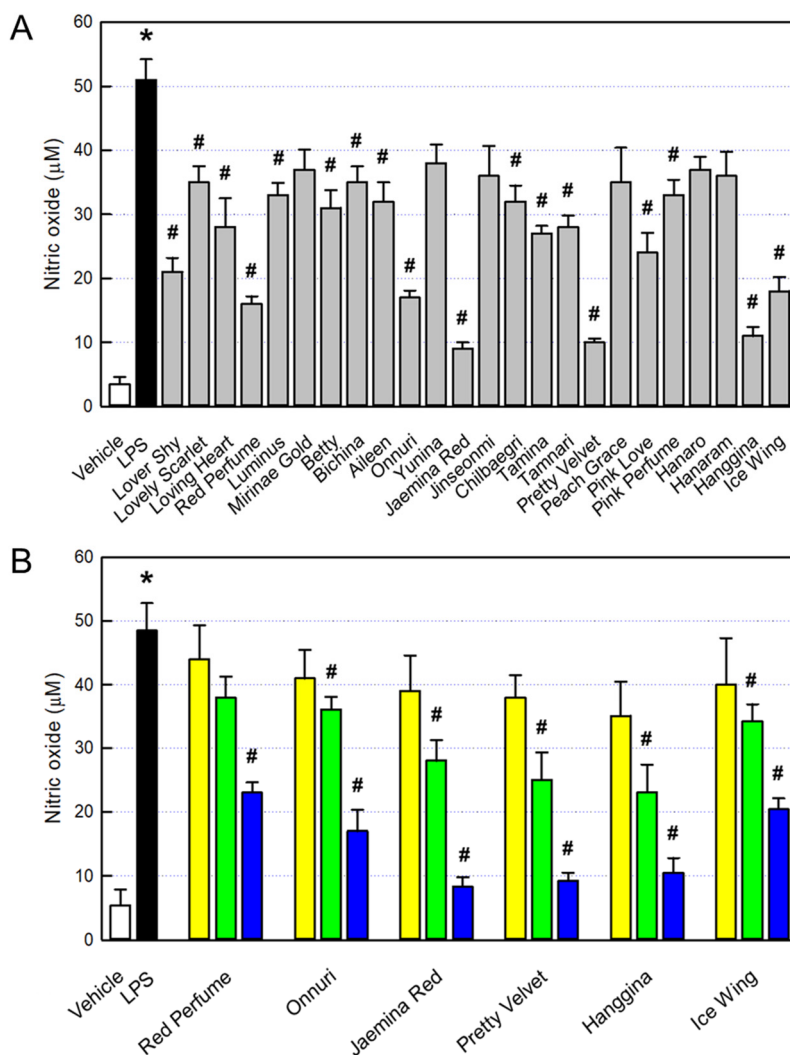


Figure 5. Inhibition by rosebud extracts of nitric oxide production from RAW 264.7 macrophages (n = 3). (A) Anti-inflammatory activities of 24 rosebud extracts (100 µg/mL). (B) Concentration-dependent activities of selected rosebud extracts at concentrations of 10 (yellow), 32 (green) or 100 (blue) µg/mL. *Significantly different from Vehicle control (P < 0.05). #Significantly different from LPS alone (P < 0.05).

3.5. Analysis of Correlation Between Ingredients and Activities

We analyzed correlations between antioxidant contents and DPPH radical-scavenging activities to understand which ingredients are closely related to the antioxidative effect (Figure 6). As a result of linear regression analysis, polyphenol content displayed a good correlation ($r^2 = 0.8069$). In addition, flavonoids ($r^2 = 0.6500$) and tannins ($r^2 = 0.7976$) exhibited high relationship, but proanthocyanidins showed a relatively-low correlation ($r^2 = 0.3048$).

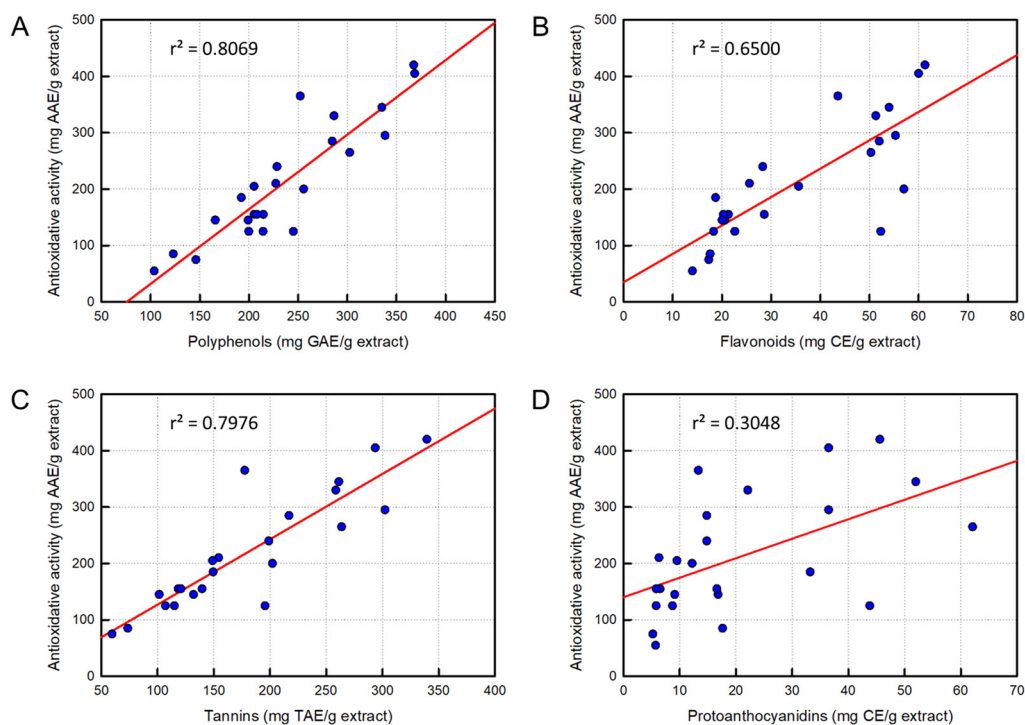


Figure 6. Correlations between 4 antioxidants and antioxidative activity. (A) Linear regression of correlation between polyphenol content and DPPD-scavenging activity at 100 $\mu\text{g/mL}$. (B) Linear regression of correlation between flavonoid content and DPPD-scavenging activity. (C) Linear regression of correlation between tannin content and DPPD-scavenging activity. (D) Linear regression of correlation between protoanthocyanidin content and DPPD-scavenging activity.

In the linear regression analysis between pyrogallol content and anti-allergic activity, a good correlation ($r^2 = 0.7331$) was attained (Figure 7A). NO production-inhibitory activity was also closely related to the pyrogallol content ($r^2 = 0.6097$) (Figure 7B).

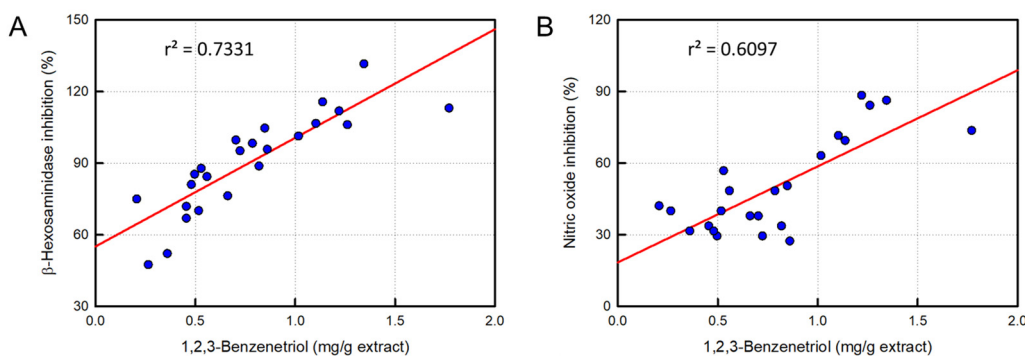


Figure 7. Correlations between pyrogallol (1,2,3-benzenetriol) and anti-allergic or anti-inflammatory activity. (A) Linear regression of correlation between pyrogallol content and β -hexosaminidase release-inhibiting activity at 100 $\mu\text{g/mL}$. (B) Linear regression of correlation between pyrogallol content and nitric oxide production-inhibiting activity.

3.6. Inhibition of Systemic Allergic Reaction

All the mice ($n = 6/\text{group}$) intraperitoneally challenged with a lethal dose (8 mg/kg) of Compound-48/80 died within 60 min due to a systemic shock reaction (Figure 8). Notably, 30-min intraperitoneal pretreatment with 6 rosebud extracts (10, 30 or 100 mg/kg) protected against the Compound-48/80-induced mortality in a dose-dependent manner. In a relative potency, Pretty

Velvet, Onnuri, and Ice Wing were somewhat more effective than Jaemina Red, Hanggina, and Red Perfume, although at the high dose (100 mg/kg) of each rosebud extract all the mice survived.

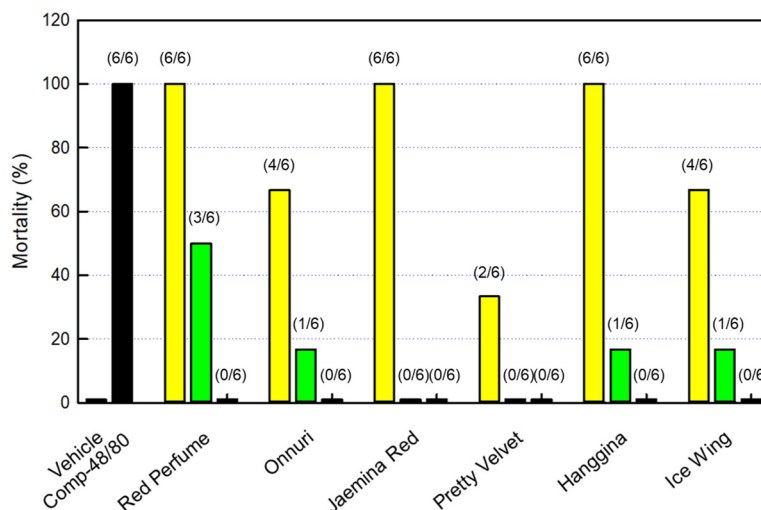


Figure 8. Protection by rosebud extracts against systemic anaphylactic shock (mortality) of Compound-48/80-challenged mice (died/challenged) (n = 6). Yellow: 10 mg/kg, green: 30 mg/kg, blue: 100 mg/kg.

3.7. Inhibition of Skin Allergic Reaction

Based on the systemic anti-allergic activity of rosebud extracts, we tried to confirm their effects on skin allergic reaction. The mice subcutaneously injected with Compound-48/80 (50 μ g/site) showed severe itching symptom, scratching 68.0 ± 5.5 times for 60 min (Figure 9). However, 30-min oral pretreatment with the 6 rosebud extracts, Red Perfume, Onnuri, Jaemina Red, Pretty Velvet, Hanggina, and Ice Wing, significantly attenuated the scratching behavior in a dose-dependent manner.

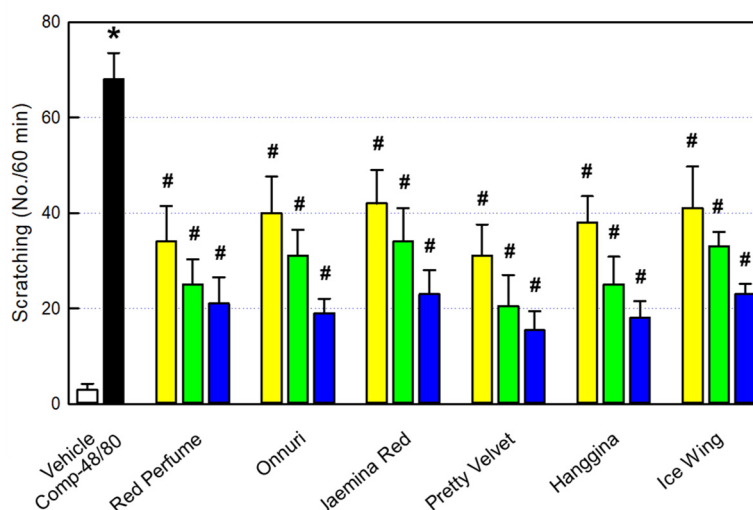


Figure 9. Inhibition by rosebud extracts of skin allergic reaction (scratching behaviors) in Compound-48/80-challenged mice (n = 6). Yellow: 10 mg/kg, green: 30 mg/kg, blue: 100 mg/kg. *Significantly different from Vehicle control ($P < 0.05$). #Significantly different from Compound-48/80 alone ($P < 0.05$).

3.8. Inhibition of Blood IgE and Histamine

According to the anti-allergic activity of the rosebud extracts, we analyzed blood IgE and histamine to clarify underlying mechanisms. The serum IgE level of the mice markedly increased after challenge with Compound-48/80 (Figure 10A). As a related mediator, serum histamine also significantly increased as an allergic response (Figure 10B). It is interest to note that the 6 rosebud extracts inhibited both the IgE and histamine levels, especially to lower blood concentrations than normal levels at high doses.

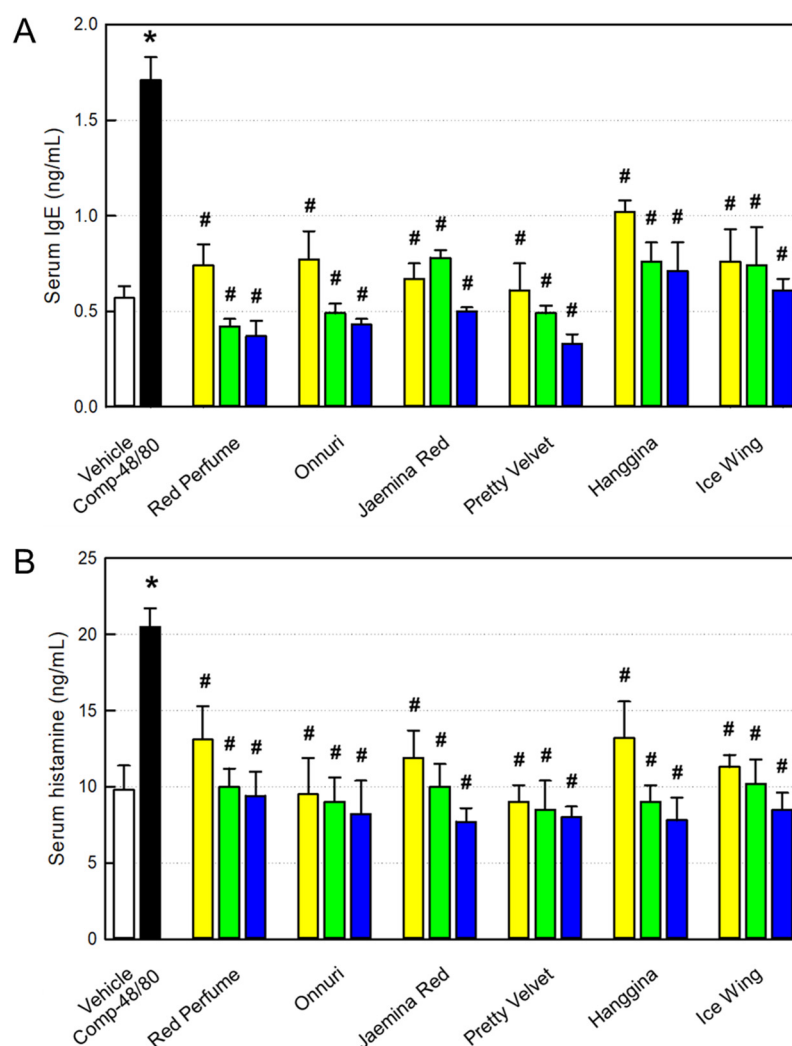


Figure 10. Inhibition by rosebud extracts of blood IgE (A) and histamine (B) in Compound-48/80-challenged mice (n = 6). Yellow: 10 mg/kg, green: 30 mg/kg, blue: 100 mg/kg. *Significantly different from Vehicle control (P < 0.05). #Significantly different from Compound-48/80 alone (P < 0.05).

3.9. Inhibition of Blood Inflammatory Cytokines

As markers of inflammation, we analyzed blood TNF- α and IL-1 β , the major inflammatory cytokines. As expected, both the cytokines were greatly enhanced by challenge with Compound-48/80 (Figure 11). However, the 6 rosebud extracts were found to lower both the blood TNF- α and IL-1 β levels, to normal levels at high doses, as inferred from the inhibitory effects on the NO production from RAW 264.7 macrophages (Figure 5).

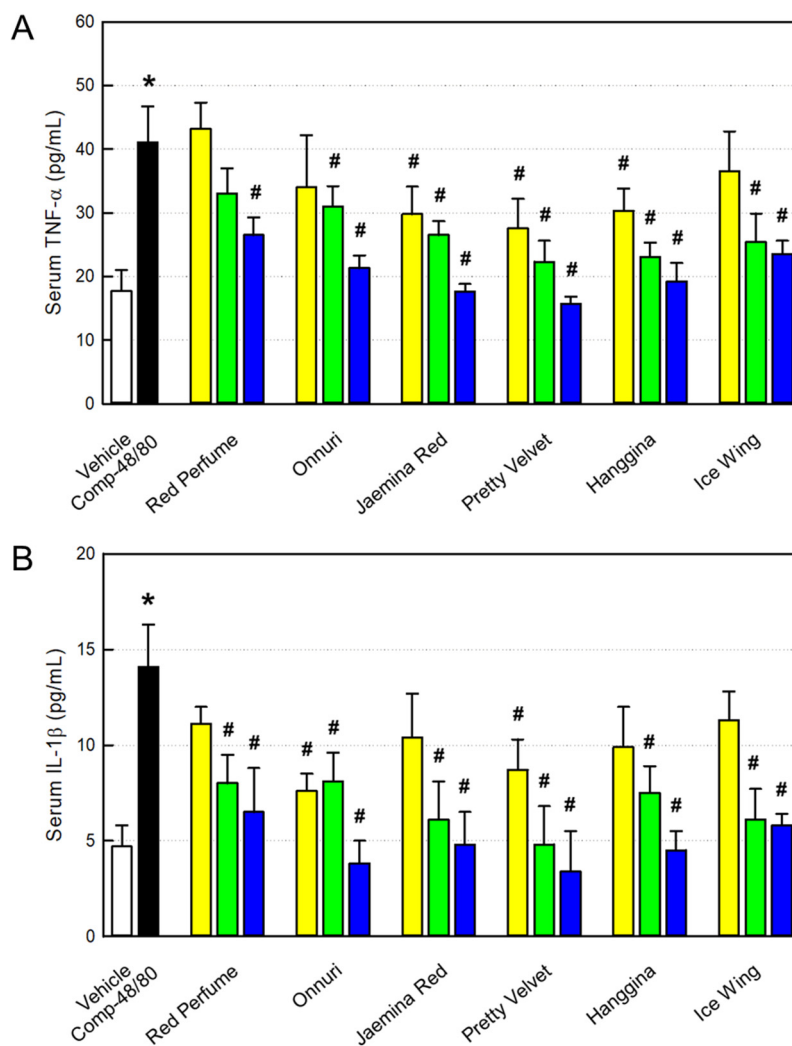


Figure 11. Inhibition by rosebud extracts of blood tumor-necrosis factor- α (TNF- α ; A) and interleukin-1 β (IL-1 β ; B) in Compoud-48/80-challenged mice (n = 6). Yellow: 10 mg/kg, green: 30 mg/kg, blue: 100 mg/kg. *Significantly different from Vehicle control ($P < 0.05$). #Significantly different from Compound-48/80 alone ($P < 0.05$).

4. Discussion

Most animals consuming oxygen in metabolism inevitably suffer from tissue injury and aging due to the generation of reactive oxygen species (ROS), called oxidative stress [33]. In order to counteract ROS toxicity, living organisms synthesize or ingest antioxidant molecules. When oxidative stress exceeds the antioxidant defense system, theoretically, the oxidative damage can be attenuated by ingesting antioxidants.

Antioxidative compounds present in plants, that is, phytochemicals, are largely divided into phenolics, carotenoids, alkaloids, and organosulfur- or nitrogen-containing compounds. Among them, polyphenols, that is, phenolics account for the largest part. In particular, among phenolics, flavonoids that have strong antioxidant activity are well known to prevent and delay aging by playing a role in preventing oxidative stress through the inhibition of lipid peroxidation [3]. Flavonoids are mainly composed of anthocyanidins, flavonols, flavones, catechins, and flavanones, and it has been demonstrated that most of the flavonoids have strong antioxidative and anti-bacterial activities depending on their structures [34]. Tannins are a sort of polyphenols that have an astringent taste. Originally, tannin was a word referring to substances used as preservatives when skins of animals are made into leather, but it is now a general term for large polyphenolic compounds that have sufficient hydroxyl groups and bind strongly to proteins or other macromolecules [35].

Proanthocyanidins and anthocyanins, well known as red pigments in plants, reduce oxidative stress, aging, and heart dysfunction with their free radical-scavenging abilities [36].

In our previous study, Colorado rose petal extract was found to contain high concentrations of polyphenols and flavonoids, so exhibited potential antioxidative and anti-inflammatory activities [18,22]. As expected, the extract protected against epileptic brain damage of mice challenged with excitotoxic kainic acid. Thereafter, we prepared rosebud extracts from 24 newly-crossbred roses, and showed good correlations between polyphenol content and antioxidative potency as well as between polyphenol concentration and anti-inflammatory activity *in vitro* [15]. Especially, an extract from Pretty Velvet rose exhibited strong *in vivo* anti-inflammatory effects in a subcutaneous air-pouch inflammation model through steroid- and NSAID-like activities.

In the present study, we extended analyses to polyphenols, flavonoids, tannins, and proanthocyanidins, and demonstrated their anti-inflammatory effects, showing good relationship with the antioxidant contents. Indeed, there were good correlations between antioxidant contents and their antioxidative activities in the order of polyphenols ($r^2 = 0.8069$) > tannins ($r^2 = 0.7976$) > flavonoids ($r^2 = 0.6500$) >> proanthocyanidins ($r^2 = 0.3048$). Notably, the content of polyphenols was the highest, followed by tannins, flavonoids, and protoanthocyanidins, although there were some differences in rose cultivars (Supplementary Table S1). Therefore, polyphenols and tannins are believed to mainly contribute to the antioxidative activity.

Tissue damage caused by ROS is involved in most inflammatory reactions, and also promotes immune hypersensitivity reactions, that is, systemic and local allergic reactions. Nowadays, opportunities to be exposed to various allergens are rapidly increasing, for example, fine dust from reckless development and combustion of petrochemicals, house dust mites due to carpet culture, animal hair due to the increase in companion animal population, spring pollen, and new foreign fruits and foods. [1,37–40]. Among them, asthma and atopic dermatitis, which are type 1 hypersensitivity reactions, are emerging as social problems. Mast cells are distributed in most organs and tissues, and are important triggering cells for immune reactions such as allergy and anaphylaxis. In particular, the anaphylaxis reaction is induced by histamine and chemotactic factors released from mast cells as well as various inflammatory cytokines from other inflammatory cells [39,41,42].

Steroids are widely prescribed to treat atopic dermatitis, which is emerging as a major disease of modern people. But, they should be used in an appropriate dose and in good balance because of their wide adverse effects such as osteoporosis, osteonecrosis, myopathy, peptic ulcer, arteriosclerosis, dyslipidemia, acne, and skin atrophy [43]. In addition, the US Food and Drug Administration (FDA) required carcinogenicity warnings to be attached to Elidel® and Protopic®, which are non-steroidal atopy treatments, and the Ministry of Food and Drug Safety (MFDS) of Korea restricts the use of them in young patients. Therefore, social demand for the development of safe therapeutic agents is rapidly increasing, and natural products may fulfill this requirement.

Earlier, we demonstrated that a rose petal extract has a strong anti-allergic property, inhibiting β -hexosaminidase release from RBL-2H3 cells as well as systemic and cutaneous anaphylactic reactions in animals [16]. Notably, we found out that pyrogallol is a major antioxidant displaying neuroprotection in an animal model of ischemic stroke [18,19]. Actually, pyrogallol was found to have anti-allergic activity, alleviating nasal symptoms including sneezing in toluene-2,4-diisocyanate (TDI)-sensitized rats by suppressing Th2 cytokine expression calcineurin/nuclear factor of activated T-cells (NFAT) signaling [20,21].

In the present study, therefore, we analyzed pyrogallol contents in 24 rosebud extracts, and assessed their β -hexosaminidase- and NO-blocking activities. Indeed, there were good correlations between pyrogallol content and their anti-allergic ($r^2 = 0.7331$) and anti-inflammatory ($r^2 = 0.6097$) potentials. Therefore, it was confirmed that pyrogallol play major roles in anti-allergic and anti-inflammatory activities.

Next, we selected 6 extracts (Red Perfume, Onnuri, Jaemina Red, Pretty Velvet, Hanggina, and Icw Wing) showing strong anti-allergic and anti-inflammatory activities *in vitro*, and investigated their effects on systemic and local (skin) anaphylactic reactions *in vivo*. As inferred from the blocking

activity on β -hexosaminidase release, the 6 rosebud extracts protected against Compound-48/80 lethality in a dose-dependent manner. Furthermore, the extracts markedly attenuated skin scratching behaviors in mice challenged with Compound-48/80, as shown in a previous study [16]. Such anti-allergic effects of rosebud extracts were supported by blood IgE, histamine and inflammatory cytokines. Indeed, serum IgE, histamine, TNF- α , and IL-1 β were reduced following the rosebud extracts in parallel with the dose-dependent patterns in itching symptoms.

Roses have been widely used for decoration or as a component of perfumes because of their beautiful appearance and strong scent. In particular, Turkey, Bulgaria, Morocco, and China produce rose oil in large quantities and supply it as a raw material for cosmetics all over the world, and more recently, petals are used as jam or dessert [44]. However, the pharmacological activities of rose oil are not well demonstrated. Rather, we have reported various effects such as antioxidation, anti-allergy, skin whitening, wrinkle improvement, anti-bacterial, anti-inflammatory, and neuroprotection of non-oil solvent extracts [15–18,22,45,46]. In the present study, we extended insights into the major component (pyrogallol) as an anti-allergic molecule of 24 new cultivars manufactured in Gumi Smart Agriculture Research Institute, Korea. Based on the research results, in spite of research limitations on clear underlying mechanisms, it is expected that we can develop more effective medicinal plants possessing higher pyrogallol content for the management of allergic diseases such as atopic dermatitis.

5. Conclusions

We demonstrated that 24 rosebud extracts displayed anti-allergic, anti-inflammatory and antioxidative effects in parallel with their ingredients including pyrogallol and/or antioxidant phytochemicals. Especially, pyrogallol was found to play a central role for anti-anaphylactic activity. Therefore, it is proposed that the extracts and active ingredients from cross-bred Korean rosebuds exert beneficial roles through their high levels of pyrogallol and antioxidants, and that could be promising candidates to overcome allergic responses such as atopic dermatitis.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Antioxidants in rosebud extracts; Table S1.

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Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries should be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AAE	ascorbic acid equivalent
DNP	anti-dinitrophenyl
DPPH	1,1-diphenyl-2-picrylhydrazyl

ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FDA	Food and Drug Administration
HSA	human serum albumin
IACUC	Institutional Animal Care and Use Committee
IgE	immunoglobulin E
IL-1 β	interleukin-1 β
IL-9	interleukin-9
LPS	lipopolysaccharide
MEM	minimal essential medium
MFDS	Ministry of Food and Drug Safety
NFAT	nuclear factor of activated T-cells
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drugs
ROS	reactive oxygen species
SOP	Standard Operation Procedures
TDI	toluene-2,4-diisocyanate
TNF- α	tumor-necrosis factor- α

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