

Rosmarinic Acid Extract for Antioxidant, Antiallergic, and α -Glucosidase Inhibitory Activities, Isolated by Supramolecular Technique and Solvent Extraction from *Perilla* Leaves

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ABSTRACT: Rosmarinic acid extract with potent biological activities was successfully isolated by supramolecular technique and solvent extraction from *Perilla* leaves. By the supramolecular complex which was formed from flavocommelin and *Perilla* leaf extract as initial materials, the supernatant containing rosmarinic acid was isolated. Rosmarinic acid extract ($62.9 \pm 4.5\%$ purity) was partly purified by partitioning ethyl acetate and water. Rosmarinic acid extract exhibited high total phenolic content of $433.9 \pm 58.6 \mu\text{g}/\text{mg}$ of gallic acid equivalent, effective DPPH radical scavenging activity (SC_{50} of $5.5 \pm 0.2 \mu\text{g}/\text{mL}$), antiallergic activity (IC_{50} of $52.9 \pm 6.7 \mu\text{g}/\text{mL}$), and α -glucosidase inhibitory activity (IC_{50} of $0.23 \pm 0.01 \text{ mg}/\text{mL}$). Rosmarinic acid extract shows high potential for diabetes mellitus and allergy treatments by inhibiting α -glucosidase activity and measuring β -hexosaminidase, related to life-style disease.

KEYWORDS: *Perilla frutescens*, supramolecular formation, rosmarinic acid extract, antioxidant activity, antiallergic activity, α -glucosidase inhibitory activity

INTRODUCTION

Perilla frutescens is usually utilized as a traditional medicine or vegetable in Japan and other countries.¹ *Perilla* leaves with red color are often used as food colorant, due to the presence of major anthocyanins, malonylshisonin (MS) and shisonin (S), and other related anthocyanin compounds. Natural resources of anthocyanins are urgently required for safe colorants and additives in the food industry. Also, it was found that anthocyanins exhibit various biological activities. For example, anthocyanin pigments isolated from Muscat Bailey A grape and *Phaseolus vulgaris* L. inhibit lipid peroxidation² and have an oxygen radical scavenging effect.³ Moreover, phenolic compounds in *Perilla* leaves such as rosmarinic acid (RA), luteolin, apigenin, and α -linolenic acid have antiallergic,^{4,5} antiinflammatory,^{4,5} and antioxidant properties,⁶ have a neuroprotective effect,⁷ act against tumors,⁸ and suppress diabetes mellitus.⁹ RA, a typical phenolic acid in the *Lamiaceae* family, is potentially valuable for improvement of diabetes mellitus and obesity by inhibiting digestive enzymes. Lemon balm extract, containing RA as a major component, inhibited α -amylase¹⁰ and α -glucosidase¹¹ activities while RA inhibited α -glucosidase activity higher than catechol and quercetin.

Supramolecular pigments, found in blue flower petals, such as *Salvia patens*,¹² *Commelina communis*,¹³ and *Salvia uliginosa*,¹⁴ are complexes of anthocyanins, flavonoids, and metals by intermolecular hydrophobic association and metal complexation of their anthocyanins. For example, commelinin, a blue pigment, was isolated from *C. communis* and the structure was determined by X-ray analysis to be self-assembled.¹³ Flavocommelin is recyclable by crystallization after dissociation of anthocyanins and flavocommelin from the complex pigment. Recently, Asada et al.¹⁵ applied aluminum complex formation,

based on supramolecular formation, to selectively purify bilberry anthocyanin 3-glycosides from the crude pigments in order to get biological active anthocyanins.

In this study, supramolecular technology was successfully applied in RA extract separation from *Perilla* leaf extract. RA extract, separated by supramolecular formation and solvent extraction, exhibited greatest activities of DPPH radical scavenging, antiallergy effects, and α -glucosidase inhibition. This is the first report on RA extract, with high potent biological activities, separated from *Perilla* leaves by means of the supramolecular technique and solvent extraction.

MATERIALS AND METHODS

Plant Materials. *Perilla frutescens* (L.) Britt. was planted on the University farm in early May 2012, while the seeds were purchased from Fuji Seed Company (Gifu, Japan). The red leaves (2.4 kg) were harvested in September 2012.

Chemicals. Rosmarinic acid, butylated hydroxytoluene (BHT), *p*-nitrophenyl- α -D-glucopyranoside, α -glucosidase from *Bacillus stearothermophilus*, 2,2-diphenyl-1-picrylhydrazyl (DPPH), albumin dinitrophenyl, and mouse monoclonal anti-dinitrophenyl antibody were supplied by Sigma-Aldrich (St. Louis, MO, USA). Gallic acid was provided by Tokyo Chemical Industry Co. (Tokyo, Japan). Magnesium acetate tetrahydrate, TritonX-100, *p*-nitrophenyl-2-acetamide-2-deoxy- β -D-glucopyranoside, and Dulbecco's modified Eagle medium (D-MEM) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Folin–Ciocalteu phenol reagent was provided by Nacalai Tesque (Kyoto, Japan), and fetal bovine

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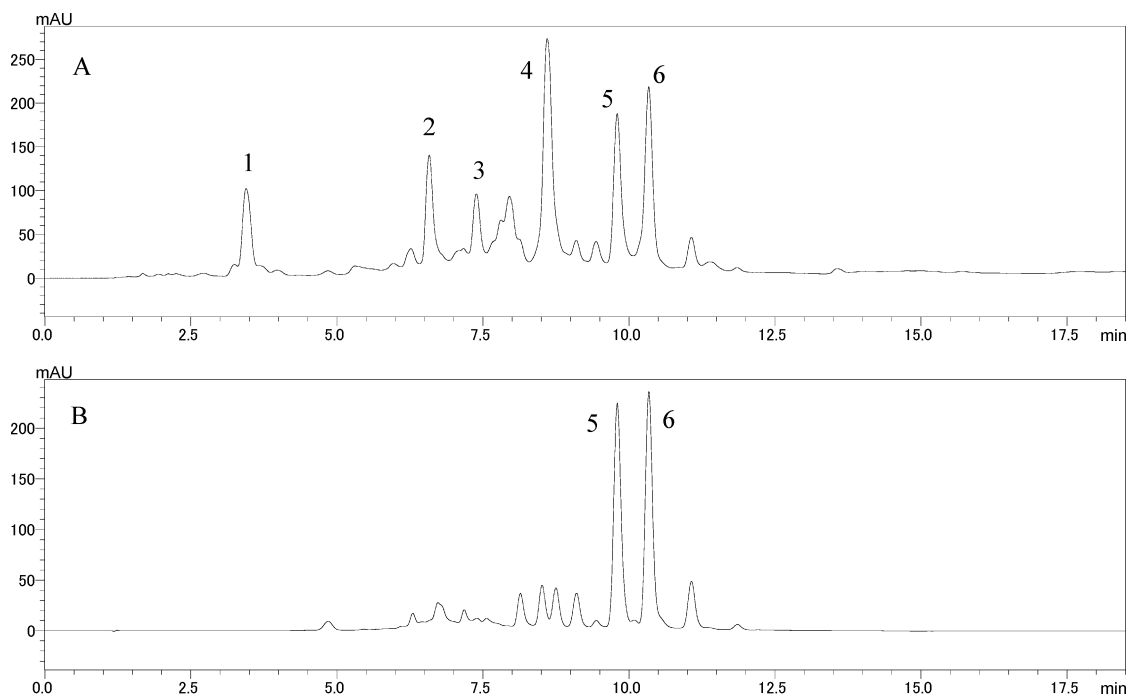


Figure 1. UFLC chromatograms of *Perilla* leaf extract: (A) 280 nm, (B) 520 nm; (1) caffeic acid; (2) luteolin 7-*O*-diglucuronide; (3) apigenin 7-*O*-diglucuronide; (4) rosmarinic acid; (5) shisonin; (6) malonylshisonin.

serum (FBS), antibiotic–antimycotic 100X, bovine serum albumin (BSA) were supplied by Equitech-bio (Kerrville, TX, USA), Invitrogen (Grand Island, NY, USA) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively. All the reagents in the study were of analytical grade.

Ultrafast Liquid Chromatography (UFLC). A Shimadzu UFLC system equipped with two LC-20AD pumps and a SPD-M20A detector was used to analyze test samples. The analysis was performed on a Shim-pack XR ODS column, 2.2 μm (3.0 mm i.d. $\times$ 100.0 mm). The mobile phase was composed of (A) 20% methanolic solution containing 0.5% TFA and (B) 70% methanolic solution containing 0.5% TFA. The injection volume was 1 μL , and the analytical temperature was set at 40 $^{\circ}\text{C}$. The UFLC elution conditions were as follows: 0.0% B (0–0.50 min); 0 $\rightarrow$ 30.0% B (0.50–1.00 min); 30.0 $\rightarrow$ 50.0% B (1.00–6.00 min); 50.0 $\rightarrow$ 70.0% B (6.00–11.50 min); 70.0 $\rightarrow$ 100.0% B (11.50–13.00 min); holding 100% B for further 2.00 min. The flow rate was 0.50 mL/min.

Preparation of *Perilla* Leaf Extract. The red *Perilla* leaves, 2.4 kg, harvested in September 2012, were first smashed by a muller. They were then immersed in 5 L of 10% acetic acid solution and extracted by a modified extractor. After extraction in triplicate with 5 L of 10% acetic acid, the total extraction solution arrived at 15.6 L. Amberlite XAD-7 resin (46.7 cm $\times$ 5.7 cm inner diameter) was filled with the above solution. Three liters of 1% TFA–80% methanol eluent was collected and evaporated to dryness (11.1 g).

The *Perilla* leaf extract obtained above was analyzed by UFLC. As shown in Figure 1A main six peaks were distributed in the UFLC chromatogram at a wavelength of 280 nm. Peaks 5 and 6 were also detected at a wavelength of 520 nm and determined as anthocyanins (S and MS, Figure 1B) (see also Table 1). These six peaks isolated were identified as the following compounds by UV spectra, mass spectra, and previous literature. (1) Caffeic acid: $^{16-18}$ $\lambda_{\text{max}} = 322$ nm, m/z 179 ($[\text{M} - \text{H}]^{-}$). (2) Luteolin 7-*O*-diglucuronide: ^{16} $\lambda_{\text{max}} = 347$ nm, 265 nm, m/z 639 ($[\text{M} + \text{H}]^{+}$). (3) Apigenin 7-*O*-diglucuronide: ^{16} $\lambda_{\text{max}} = 336$ nm, 266 nm, m/z 623 ($[\text{M} + \text{H}]^{+}$). (4) Rosmarinic acid: $^{16-18}$ $\lambda_{\text{max}} = 329$ nm, m/z 359 ($[\text{M} - \text{H}]^{-}$). (5) Shisonin: ^{16} $\lambda_{\text{max}} = 523$ nm, m/z 757 ($[\text{M} + \text{H}]^{+}$). (6) Malonylshisonin: ^{16,19} $\lambda_{\text{max}} = 524$ nm, 278 nm, m/z 843 ($[\text{M} + \text{H}]^{+}$).

Supramolecular Formation and Rosmarinic Acid Extract Isolation. Figure 2 shows the process of supramolecular formation

Table 1. Identification of UFLC Peaks of *Perilla* Leaf Extract Using a PDA Detector of UFLC and Mass Spectra Analysis^a

peak no.	t_{R} (min)	UV λ_{max} (nm)	molecular ion (m/z)	compound identification	refs
1	3.2	322	179 $[\text{M} - \text{H}]^{-}$	caffeic acid	19–21
2	5.5	347, 265	639 $[\text{M} + \text{H}]^{+}$	luteolin 7- <i>O</i> -diglucuronide	19
3	6.8	336, 266	623 $[\text{M} + \text{H}]^{+}$	apigenin 7- <i>O</i> -diglucuronide	19
4	9.2	329	359 $[\text{M} - \text{H}]^{-}$	rosmarinic acid	19–21
5	11.6	523	757 $[\text{M} + \text{H}]^{+}$	shisonin	19
6	12.4	524, 278	843 $[\text{M} + \text{H}]^{+}$	malonylshisonin	19, 22

^aLiquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry (LC/ESI-Q-TOF-MS) analysis was conducted using an Acquity UPLC/Xevo QToF MS system (Waters Corporation).

and RA extraction from *Perilla* leaf. *Perilla* leaf extract (2.0 g), dissolved by 50% aqueous methanol solution (10.0 mL), was neutralized with $\text{NH}_3 \cdot \text{H}_2\text{O}$ (2.0 mL). After the solution was dried by a vacuum, flavocoumarin (Fc; 612.0 mg/12.0 mL) and magnesium acetate aqueous solution (Mg^{2+} ; 714.0 mg/5.0 mL) were added into the above residue. The reaction solution was mixed thoroughly for 20 min. After the solution was evaporated with a rotary evaporator, 8.0 mL of water and 56.0 mL of ethanol were added in order to form a precipitate at -20 $^{\circ}\text{C}$ overnight. The precipitation step was repeated two times. The supernatant and precipitate were separated by centrifugation (3000 rpm $\times$ 10 min) and evaporated to dryness. Both the dried supernatant and precipitate were kept in a desiccator with CaCl_2 for several days, yielding 1.7 and 1.5 g, respectively.

The supernatant described above (50.3 mg) was dissolved in 12.0 mL of 1% TFA aqueous solution, followed by the addition of 4.0 mL of ethyl acetate for extraction. The extraction procedure was repeated three times (12.0 mL of ethyl acetate extract). The ethyl acetate layer and water layer were evaporated to dryness in a vacuum, yielding 10.5 ± 0.5 mg and 44.3 ± 2.9 mg, respectively.

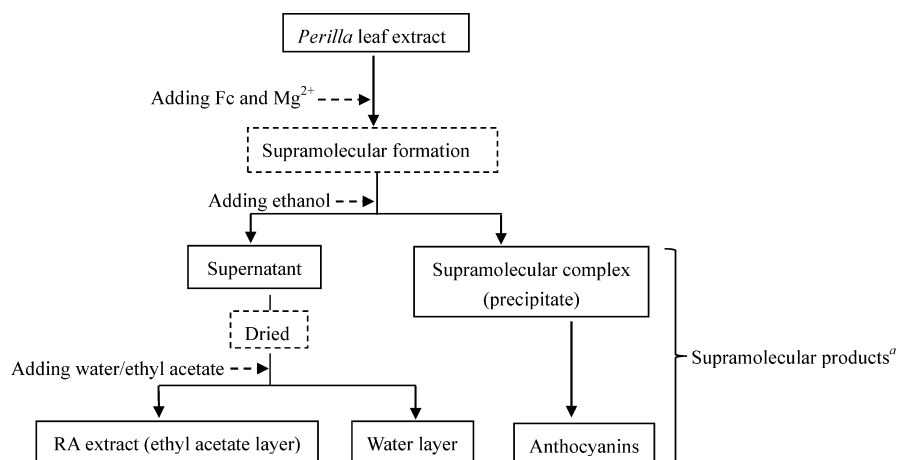


Figure 2. Rosmarinic acid extract and anthocyanins isolated from *Perilla* leaf extract by supramolecular formation. “Supramolecular products are supernatant and supramolecular complex obtained by supramolecular formation, and the related extracts such as RA extract, water layer, and anthocyanins described above.

Total Phenolics (TP). TP was determined by a previous method reported by Cheplick et al.²⁰ Absorbance was monitored at 725 nm. Gallic acid (10, 25, 40, 60, 80 $\mu\text{g/mL}$) was used to establish a standard curve. TP was expressed as gallic acid equivalent (GAE) of a test sample.

DPPH Radical Scavenging Activity (DRSA). DRSA was determined using a previously reported method.¹⁸ A sample solution (0.25 mL) was added into a tube with 0.10 M acetic acid buffer (0.25 mL, pH 5.5). The above solution was mixed with methanol (0.25 mL) and DPPH (0.25 mL, 0.4 mM in methanol). The reaction stood for 30 min in a dark place. DPPH absorbance was measured at 517 nm. Trolox and BHT were used as the standard chemicals. The calculation of DPPH radical scavenging activity (%) was carried out according to eq 1.

$$\text{scavenging activity(\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100 \quad (1)$$

The SC_{50} value which represents the concentration of sample that caused 50% scavenging was determined by GraphPad PRISM 5 from the obtained scavenging activity values.

β -Hexosaminidase Release from the RBL-2H3 Cell Line. β -Hexosaminidase release from the RBL-2H3 cell line (supplied by RIKEN BioResource Center Cell Bank, Ibaraki, Japan) was determined using a previous method with some modifications.²¹ The RBL-2H3 cells in a base medium (D-MEM containing 10% FBS and 1% antibiotic–antimycotic 100X) were dispensed into a 24-well plate (2.5×10^5 cells/well), followed by an incubation at 37 °C in 5% CO_2 atmosphere for one night. The cells were washed by PBS (1 mL) and incubated with 500 μL of antibody solution (mouse monoclonal anti-dinitrophenyl antibody, 50 ng/mL in base medium) for 2 h sensitization. A test sample solution of 490 μL was added to each well after washing two times with 500 μL of modified Tyrode’s buffer (MT buffer, Tyrode’s salt solution containing 1 g/L BSA and 4.76 g/L HEPES). The MT buffer instead of the sample was added into the well as the control. The test samples, dissolved in DMSO, were diluted with MT buffer (final DMSO concentration = 0.1%). After 10 min incubation, albumin dinitrophenyl (10 μL , final concentration = 50 ng/mL) was added to each well. The cells evoked allergic reactions for 30 min (degranulation). The microplate was cooled at 0 °C for 10 min to terminate the reactions. After transference of supernatant (50 μL) into a 96-well plate, 100 μL of *p*-nitrophenyl-2-acetamide-2-deoxy- β -D-glucopyranoside (3.3 mM) in 0.1 M citric acid buffer (pH 4.5) was added into each well with an incubation at 37 °C for 25 min. Finally, 100 μL of glycine buffer (pH 10.0, 2 M) was added and β -hexosaminidase release was quantitatively calculated by the amount of *p*-nitrophenol release from the substrate with a microplate reader at 405 nm. The calculation was carried out using eqs 2 and 3 below. In

“blank”, spontaneous β -hexosaminidase release from cells was checked in the condition without antibody or samples. In “total”, total amount of β -hexosaminidase release from the cells was confirmed by lysing the cells with 0.1% TritonX-100 in MT buffer. In “control”, the amount of β -hexosaminidase released from cells was determined in the condition without a sample. In “sample”, the amount of β -hexosaminidase released from cells was determined in the condition with a sample added.

$$\begin{aligned} \text{ratio of } \beta\text{-hexosaminidase release (\%)} \\ = \frac{(\text{OD}_{\text{control or sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{total}} - \text{OD}_{\text{blank}})} \times 100 \end{aligned} \quad (2)$$

$$\beta\text{-hexosaminidase release (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100 \quad (3)$$

The ratio of β -hexosaminidase release (%) of “control” was between 25% and 38%.

α -Glucosidase Activity. α -Glucosidase activity was assessed according to a previous report.¹¹ A sample solution (50 μL) was mixed with α -glucosidase solution (100 μL , 1 U/mL) in a 96-well plate. After preincubation at 25 °C for 10 min, the reaction was initiated by adding 50 μL of *p*-nitrophenyl- α -D-glucopyranoside (5 mM) in 0.1 M PBS (pH 6.9). The mixed solution was shook at 25 °C for 5 min. At the beginning and the end of the reaction, absorbance at 405 nm was measured by a microplate reader. In control, PBS instead of the sample was added into the well. The α -glucosidase inhibition (%) was calculated by eq 4.

$$\text{inhibition (\%)} = (\Delta A_{\text{control}} - \Delta A_{\text{sample}}) / \Delta A_{\text{control}} \times 100 \quad (4)$$

The sample concentration that causes 50% inhibition (IC_{50} value) was calculated from α -glucosidase inhibition by GraphPad PRISM 5.

Statistical Analysis. Statistical analysis was carried out by GraphPad PRISM 5 with Newman–Keuls test (one-way ANOVA). The value represents mean $\pm$ standard deviation (SD) in triplicate at least. Significant differences of values were statistically $p < 0.05$.

RESULTS AND DISCUSSION

Separation of RA Extract by Supramolecular Formation. There are many resources of RA in nature as biologically active substances. So, isolation techniques have been developed so far. For example, RA was purified up to 38.8%, yielding 43.8% from 1 g of the crude sample by a Sephadex LH-20²² column, after response surface optimized

Table 2. RA Extract and Anthocyanins Separated from *Perilla* Leaf Extract by Supramolecular Formation^a

sample	weight (g)	purity (%)		yield (%)	
		RA	anthocyanins	RA	anthocyanins
<i>Perilla</i> leaf extract	2.0 ^b	21.2	27.5		
supernatant	1.7	16.3	6.5	79.5	18.9
RA extract	10.5 ± 0.5 ^c	62.9 ± 4.5	1.2 ± 0.2	82.9 ± 5.6	3.9 ± 0.3
water layer	44.3 ± 2.9 ^c	1.0 ± 0.1	5.9 ± 0.6	1.6 ± 0.1	79.9 ± 1.8
supramolecular complex	1.5	1.5	18.7	7.4	71.4
anthocyanins	15.6 ± 1.2 ^d	ND ^e	95.5 ± 2.5	ND	83.9 ± 2.3

^aEach value represents mean ± SD in triplicate. ^bWeight of *Perilla* leaf extract for supramolecular formation. ^cAmount of sample (mg) was obtained by water/ethyl acetate extraction from 50.3 mg of supernatant. ^dAmount of anthocyanin (mg) obtained from 94.9 mg of supramolecular complex. ^eNot detected.

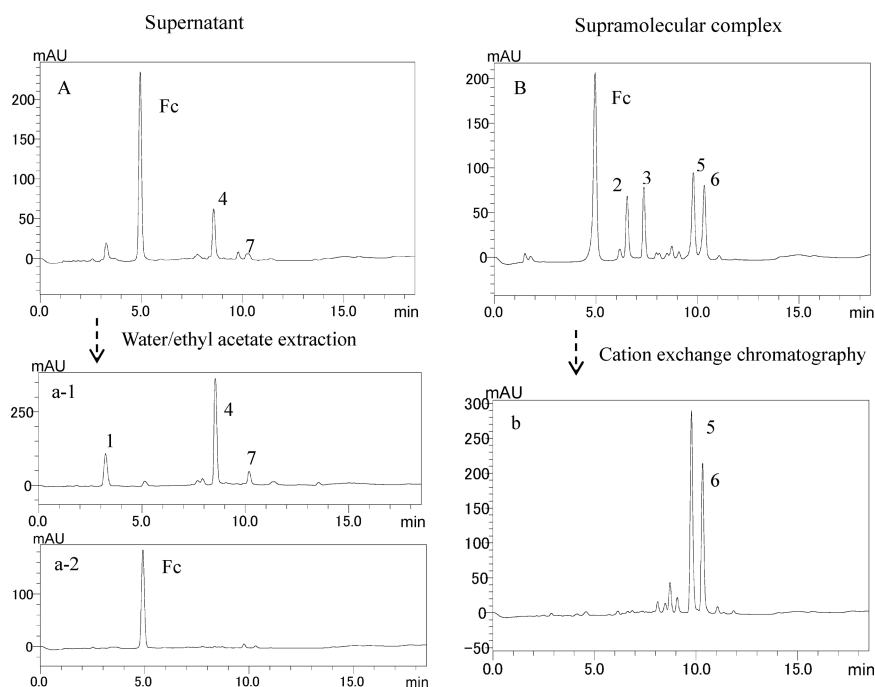


Figure 3. UFLC chromatograms of rosmarinic acid extract from supernatant and anthocyanin from supramolecular complex after supramolecular formation (280 nm): (A) supernatant; (B) supramolecular complex; (a-1) RA extract; (a-2) water layer; (b) anthocyanins. (1) Caffeic acid. (2) Luteolin 7-O-diglucuronide. (3) Apigenin 7-O-diglucuronide. (4) Rosmarinic acid. (5) Shisonin. (6) Malonylshisonin. (7) Rosmarinic acid methyl ester.

extraction from *Melissa officinalis* leaves. Phenolics and RA were purified with macroporous resins (HP-20 and Amberlite XAD-7HP)²³ from *Rabdosia serra* (MAXIM.) HARA leaf, yielding highly concentrated RA (112.5 mg with 62.9% purity for HP-20 and 95.7 mg with 58.8% purity for Amberlite XAD-7HP).

In this experiment, crude extracts (11.1 g) of *Perilla* leaves immersed in 10% acetic acid contained anthocyanins (27.5% purity, MS and S mainly) and RA (21.2% purity) with caffeic acid, luteolin 7-O-diglucuronide, and apigenin 7-O-diglucuronide (Figure 1A). Figure 2 shows the scheme of practical separation of RA extract and *Perilla* anthocyanins by applying the supramolecular formation technique. Through supramolecular formation of anthocyanins in *Perilla* leaf extract (2.0 g) and adding Fc (612.0 mg) and Mg²⁺ (714.0 mg), RA-containing supernatant (1.7 g) was excluded from the supramolecular complex (precipitate, 1.5 g). Thus, purity of RA in the supernatant was 16.3%, including excess amount of Fc in the supernatant. If Fc content is omitted from the calculation of the purity, purity of RA would be 53.4%,

compared with the initial purity of RA (21.2%). RA extract (yield 10.5 ± 0.5 mg, purity 62.9 ± 4.5%) was easily purified and isolated from an ethyl acetate layer through partitioning of water and ethyl acetate from 50.3 mg of supernatant by excluding Fc (Table 2) because RA was able to dissolve in ethyl acetate but Fc could not be dissolved in it at all. With the solubility difference between RA and Fc in ethyl acetate, RA extract, 390.4 mg (purity of 60.9%), could be isolated in ethyl acetate layer with a fairly high purity from 1.5 g of supernatant as a typical example of large scale of purification. Moreover, Fc could be recycled after usage in the experiment.

On the other hand, as supramolecular formation, supramolecular complex (Figure 3B) including anthocyanins (MS and S mainly) and unexpected flavonoids (luteolin 7-O-diglucuronide and apigenin 7-O-diglucuronide) was precipitated by ethanol, which is supported by the report on the alumina complex formation published by Asada et al.¹⁵ In the supramolecular complex, 15.6 mg of anthocyanins (Figure 3b, 95.5 ± 2.5% purity) has been successfully isolated from the supramolecular complex (94.9 mg), after excluding neutral

chemicals such as Fc and flavonoids (Table 2). Thus, by supramolecular technique, both rosmarinic acid and anthocyanins could be easily isolated from *Perilla* leaf extract.

With supramolecular technology, fairly pure rosmarinic acid (60.9%) and highly pure malonylshisonin and shisonin (95.5%), which have various biological activities,^{2–9} as well as RA extract are possible for large scale purifications to supply the extracts and the pure chemicals to the food industry.

Antioxidant Activities of Supramolecular Products.

Polyphenols possess biologically beneficial activities and are frequently utilized as antioxidant,¹⁸ antiallergic,²⁴ and anti-diabetic²⁰ reagents. Phenolics in *Perilla* leaves are well-known compounds, owing to their potent antioxidant activities and other bioactivities.^{4–9} In our study, *Perilla* leaf extract, Fc, and supramolecular products (Figure 2) were tested for antioxidant and other biological activities. Total phenolics (TPs) as an antioxidant activity were 433.9 ± 58.6 mg/g gallic acid equivalent (GAE) for RA extract, 116.9 ± 2.7 mg/g GAE for supernatant, 170.7 ± 8.2 mg/g GAE for supramolecular complex, and 4.1 ± 1.3 mg/g GAE for Fc (Table 3). Fc,

Table 3. Total Phenolics of *Perilla* Leaf Extract, Fc, and Supramolecular Products^a

sample	total phenolics (mg/g GAE) ^b
<i>Perilla</i> leaf extract	215.2 ± 2.9
Fc	4.1 ± 1.3
supernatant	116.9 ± 2.7
RA extract	433.9 ± 58.6
water layer	47.6 ± 1.6
supramolecular complex	170.7 ± 8.2
anthocyanins	189.8 ± 9.8
standard RA	711.8 ± 5.3

^aEach value represents mean $\pm$ SD in triplicate. ^bTotal phenolics expressed as gallic acid equivalent (GAE).

with only one hydroxyl group on the A-ring, hardly contributed to high TP. RA extract exhibited the highest TP, more than two times higher than *Perilla* leaf extract (215.2 ± 2.9 mg/g GAE). It is suggested that TP of RA extract isolated from *Perilla* leaf extract using the supramolecular technique showed the greatest TP. Moreover, the TP of standard RA (711.8 ± 5.3 mg/g GAE) was higher than that of RA extract. The TP of RA extract was calculated to be 61.0% of standard RA. Therefore TP of RA extract was interpreted by the purity of RA ($62.9 \pm 4.5\%$) in

the extract. Compared with the previous report, RA extract and *Perilla* leaf extract have a higher TP than the extracts of oregano, chocolate mint, and other Lamiaceae family herbs.¹¹

DPPH radical scavenging activity (DRSA) is widely applied in assessment of antioxidant activities of plant extracts. A dose-dependent capability of DRSA was observed for *Perilla* leaf extract and supramolecular products (Table 4). RA extract exhibited DRSA at $88.3 \pm 0.7\%$ at a concentration of $10 \mu\text{g/mL}$ while the other supramolecular products exhibited scavenging activity below 50%. RA extract exhibited the highest DRSA with an SC_{50} value of $5.5 \pm 0.2 \mu\text{g/mL}$ (concentration of sample that causes 50% scavenging of free radicals as shown in eq 1), which was more than two times higher DRSA than *Perilla* leaf extract (SC_{50} value, $10.8 \pm 0.1 \mu\text{g/mL}$) and supernatant (SC_{50} value, $21.7 \pm 0.3 \mu\text{g/mL}$). Standard RA (SC_{50} value, $4.6 \pm 0.2 \mu\text{g/mL}$) exhibited higher DRSA than RA extract (SC_{50} value, $5.5 \pm 0.2 \mu\text{g/mL}$), which is consistent with the results for TP. It has been reported that polyphenols are good antioxidants because phenolic hydroxyl groups are able to provide hydrogen to hydroxyl radical, superoxide radical, and other reactive free radicals.²⁵ Comparing DRSA of RA extract with those of the reference chemicals it demonstrated that RA extract exhibited similar DRSA to Trolox and higher than BHT (Table 4).

As many inflammations (induced by TNF- α , IL-6, and other sources) of animal cells including human are triggers of diabetes and cancer,²⁶ anti-inflammatory activities, which can be linked with free radical scavenging activity, of polyphenols including flavonoids and phenylpropanoids have been reported.²⁴ Polyphenolic compounds also provide anticarcinogenic, antimutagenic, and cardioprotective activities, as a consequence of their strong antioxidant properties.²⁷ Polyphenols could be good resources for suppressing allergy²⁴ and treating hyperglycemia associated with type 2 diabetes.²⁰ Because of the observation of high total phenolics, as well as strong DPPH radical elimination ability, among supramolecular products, we expected that effective antiallergic activity would be observed and hyperglycemia could be suppressed by α -glucosidase inhibition.

Antiallergic Activities of Supramolecular Products on the Suppression of β -Hexosaminidase Release from the RBL-2H3 Cell Line. The lower release of β -hexosaminidase, biomarkers of the RBL-2H3 cell line, is necessary to elicit an antiallergic response while the RBL-2H3 cell line is applied as a typical model for the research on mast cell excretion and screening for antiallergic phytochemicals.²⁸ Shimoda et al.²¹

Table 4. DPPH Radical Scavenging Activity of *Perilla* Leaf extract, Fc, and Supramolecular Products^a

sample ($\mu\text{g/mL}$)	scavenging activity (%)					SC_{50}^b ($\mu\text{g/mL}$)
	2.5	5	10	15	20	
<i>Perilla</i> leaf extract	10.7 ± 1.2	23.4 ± 2.0	48.8 ± 0.2 e	71.7 ± 1.6	87.0 ± 0.3	10.8 ± 0.1 c
Fc	NI ^c	NI	NI	NI	NI	>25
supernatant	NI	10.2 ± 0.9	23.5 ± 0.9 f	35.9 ± 0.1	50.5 ± 0.6	21.7 ± 0.3 e
RA extract	21.1 ± 3.9	48.0 ± 2.2	88.3 ± 0.7 b	89.7 ± 0.5	89.8 ± 0.1	5.5 ± 0.2 a
water layer	NI	NI	2.5 ± 0.2 h	6.1 ± 0.5	11.7 ± 2.1	>25
supramolecular complex	7.8 ± 0.6	8.9 ± 0.6	22.7 ± 0.3 f	36.9 ± 0.3	61.1 ± 0.4	17.6 ± 0.2 d
anthocyanins	5.7 ± 0.5	9.0 ± 1.4	20.8 ± 0.5 g	35.8 ± 0.8	45.8 ± 0.8	23.2 ± 0.4 f
standard RA	32.3 ± 0.6	58.3 ± 1.0	90.6 ± 0.7 a	90.9 ± 0.7	92.5 ± 0.5	4.6 ± 0.1 a
Trolox	24.7 ± 0.7	53.4 ± 0.9	85.3 ± 0.7 c	91.6 ± 1.7	94.4 ± 0.3	5.1 ± 0.1 a
BHT	14.0 ± 0.6	41.5 ± 2.9	76.2 ± 0.9 d	84.5 ± 0.6	87.0 ± 0.4	6.6 ± 0.3 b

^aEach value represents mean $\pm$ SD in triplicate. Means with different letters are significantly different ($p < 0.05$). ^b SC_{50} value which represents the concentration of sample that caused 50% scavenging was calculated from scavenging activity values. ^cNo inhibition detected.

reported β -hexosaminidase release suppression by extracts of *Perilla* leaf (*Perilla frutescens*), Japanese butterbur (*Petasites japonicas*, Compositae), and tien-cha (*Rubus suavissimus*). These extracts have a potency for the prevention of pollennosis, seasonal allergic rhinitis, and other allergic reactions.^{29–31} Therefore, *Perilla* leaf extract is becoming more popular in the Japanese market as a folk medicine. However, they did not show the efficient extraction technique of RA extract with great activities.

In this paper, the antiallergic activities of *Perilla* leaf extract, Fc, and supramolecular products, such as supramolecular complex, supernatant, RA extract, anthocyanins, and water layer, were assessed by β -hexosaminidase release suppression of RBL-2H3 cells (Figure 4). The concentrations of 50% β -

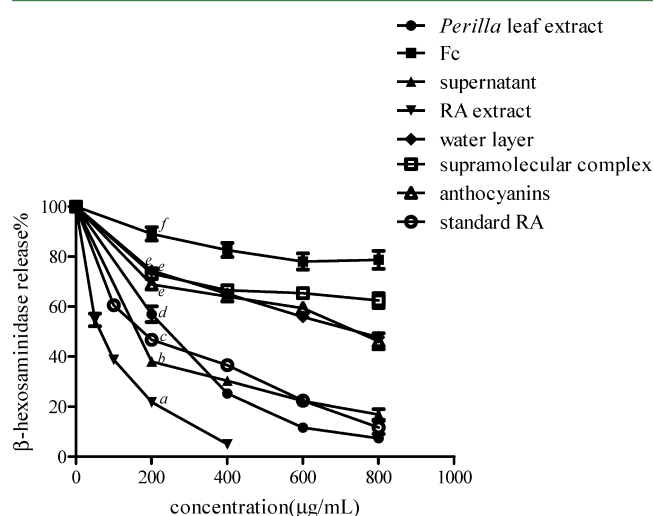


Figure 4. Antiallergic activities of supramolecular products on β -hexosaminidase release suppression from the RBL-2H3 cell line. Each value represents mean $\pm$ SD in quadruplicate.

hexosaminidase release inhibition of standard RA, *Perilla* leaf extract, supernatant, and RA extract were mainly 136.7 ± 12.6 $\mu\text{g/mL}$, 208.4 ± 20.0 $\mu\text{g/mL}$, 81.1 ± 23.3 $\mu\text{g/mL}$, and 52.9 ± 6.7 $\mu\text{g/mL}$ (IC_{50} values), respectively. The suppression of β -hexosaminidase release was dose-dependent. In our study, RA extract suppressed β -hexosaminidase release 3.9 times and 2.6 times more than *Perilla* leaf extract and standard RA, respectively, and exhibited the highest antiallergic activity among the supramolecular products. Also, it is suggested that other compounds as well as RA in RA extract may contribute to antiallergic activity.

RA is reported to possess anti-inflammatory and antiallergic effect and inhibit seasonal allergic rhinoconjunctivitis.^{31,32} RA suppresses inflammatory activity by the decrease of IgE and COX-2 levels.⁵ Therefore, efficient isolation of pure RA and RA extract that has higher antiallergic activity than RA itself is desired in current food and drug markets. Supramolecular formation and solvent partition technique may offer the possibility of the development of new drugs.

In the UFLC chromatogram (Figure 3a-1), caffeic acid ($t_R = 3.2$ min) and rosmarinic acid methyl ester (RA-me, $t_R = 10.2$ min) were found in RA extract as minor components besides RA. The β -hexosaminidase release inhibitory result gave IC_{50} values of caffeic acid and RA-me (synthesized by us) of 132.0 ± 3.6 $\mu\text{g/mL}$ and 9.9 ± 0.8 $\mu\text{g/mL}$, respectively. This result showed that RA-me exhibited higher inhibitory activity than

standard RA. In RA extract the content of RA and RA-me was 62.9% and 6.9% respectively. At IC_{50} of RA extract (52.9 $\mu\text{g/mL}$), it was calculated as 33.3 $\mu\text{g/mL}$ for RA and 3.7 $\mu\text{g/mL}$ for RA-me, respectively, which contributed to 43% β -hexosaminidase release inhibition as an additive effect. Therefore, RA-me, as a component in RA extract, contributed to high β -hexosaminidase release inhibitory activity besides RA. We found the greatest activity of RA-me in RA extract and supernatant as a minor component. So, although the purity of RA (16.3%) in supernatant is a bit lower than that of RA in leaf extract (21.2%), supernatant showed greater values of IC_{50} than that of leaf extract because of not only increasing of Fc in supernatant as the impurity but also increasing of RA-me by the selective isolation with supramolecular technique and solvent partition technique.

Yun et al.²⁴ reported that curcumin, with the highest potential to suppress β -hexosaminidase release among 25 polyphenols, was around 4 times greater than RA. In contrast, RA-me found in RA extract was 13.8 times higher than RA. Therefore, RA-me can suppress β -hexosaminidase release more effectively than curcumin.

In conclusion, RA extract isolated by supramolecular technique showed effective antiallergic activity. We clarified that RA-me, as a minor component in RA extract, contributed to high antiallergic activity with RA as an additive effect.

α -Glucosidase Inhibitory Activities of Supramolecular Products. α -Glucosidase inhibitors, alleviating postprandial hyperglycemia, would have potential to manage diabetes. *Perilla* leaf extract, Fc, and supramolecular products were examined for inhibition effects of α -glucosidase. RA extract inhibited the greatest α -glucosidase activity (IC_{50} of 0.23 ± 0.01 mg/mL) among the supramolecular products (Table 5). Furthermore,

Table 5. α -Glucosidase Inhibitory Activity of *Perilla* Leaf Extract, Fc, and Supramolecular Products

sample	IC_{50}^a (mg/mL)
<i>Perilla</i> leaf extract	0.42 ± 0.01 a
Fc	5.60 ± 0.23 e
supernatant	0.42 ± 0.01 a
RA extract	0.23 ± 0.01 a
water layer	2.74 ± 0.21 d
supramolecular complex	1.54 ± 0.04 b
anthocyanins	1.99 ± 0.01 c
standard RA	0.95 ± 0.01 b

^a IC_{50} values were calculated from dose–response curves of α -glucosidase inhibition. Each value represents mean $\pm$ SD in triplicate. Means with different letters are significantly different ($p < 0.05$).

RA extract inhibited α -glucosidase activity 4.1 times and 1.8 times higher than standard RA (IC_{50} value, 0.95 ± 0.01 mg/mL) and *Perilla* leaf extract (IC_{50} value, 0.42 ± 0.01 mg/mL), respectively, while RA is reported to be a better research target on α -glucosidase inhibitory activity.¹¹ Moreover, RA extract exhibited α -glucosidase inhibitory activity higher than 4,5-dicaffeoylquinic acid (IC_{50} , 0.46 mg/mL), as an effective α -glucosidase inhibitor, isolated from flower buds of *Tussilago farfara* L.³³ and coffee bean.³⁴ Therefore, RA extract has potential to suppress hyperglycemia by inhibiting α -glucosidase activity.

The α -glucosidase inhibitory activity result showed that IC_{50} values of caffeic acid and RA-me were 3.46 ± 0.18 mg/mL and 0.44 ± 0.01 mg/mL, respectively. It showed that RA-me

exhibited greater α -glucosidase inhibitory activity than standard RA. We concluded that RA extract containing RA-me and other minor components as unknown compounds besides RA contributed to high α -glucosidase inhibitory activity, especially unknown compounds might highly contribute to the activity. We found that RA-me and other minor compounds with high α -glucosidase inhibitory activity were contained in RA extract and supernatant. So, although the purity of RA (16.3%) in supernatant was a bit lower than that of RA in leaf extract (21.2%), supernatant showed the same IC_{50} value of α -glucosidase inhibitory activity with that of leaf extract because of not only increasing of Fc in supernatant as the impurity but also increasing of RA-me and other minor compounds by the selective isolation with supramolecular technique and solvent partition technique (Table 5).

Moreover, in the *Perilla* leaf extracted by 10% acetic acid, RA-me was not detected in our experiment though Kang et al.¹⁷ characterized RA-me as one phenolic phytochemical in *Perilla frutescens*. Therefore, we surmise it to be an artificial compound in *Perilla* leaf extract. The generated RA-me and other minor components in RA extract potentially contributed to antiobesity activity by inhibiting α -glucosidase activity. Clarification of the minor components contributing to α -glucosidase inhibitory activity is under investigation.

Through supramolecular formation, RA-containing supernatant was excluded by supramolecular complex and RA extract ($62.9 \pm 4.5\%$ purity) with potent biological activities was obtained. The obtained RA extract had exhibited the three great activities of DPPH radical scavenging, antiallergy effects, and α -glucosidase inhibition. Antiallergic activity and α -glucosidase inhibition of RA extract were greater than those of standard RA. This study shows that RA and other minor components in RA extract contributed both activities of antiallergy effects and α -glucosidase inhibition. We will discuss molecular mechanisms of the activities in detail by linking with the interaction of biologically important chemicals after identification of these active compounds. In conclusion, the RA extract isolated from *Perilla* leaf extract by supramolecular formation and solvent extraction has potential applications in managing diabetes mellitus and other chronic and degenerative diseases. This is the first report on RA extract with potent antioxidant, antiallergic, and α -glucosidase inhibitory activity separated from *Perilla* leaf extract using the supramolecular formation and partition technique.

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ABBREVIATIONS USED

DPPH, 2,2-diphenyl-1-picrylhydrazyl; DRSA, DPPH radical scavenging activity; Fc, flavocommelin; GAE, gallic acid equivalent; MS, malonylshisonin; RA, rosmarinic acid; RA-me, rosmarinic acid methyl ester; S, shisonin; TP, total phenolics; UFLC, ultrafast liquid chromatography

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