

# Physiological Functions of 70% Ethanol Extracts of 6 Edible Flowers *in Vitro*: A Comparative Study

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## Abstract

The physiological functions of 70% ethanol extracts of 6 edible flowers of different colors—*Chrysanthemum morifolium* (Chry., yellow), *Rosa miniature* (Rosa, red), *Dendrobium phalaenopsis* (Dend., purple), *Viola x wittrockiana* cv. fluna (Fluna, white), *Viola x wittrockiana* cv. pansy (Pansy, orange), and *Primula x juliana* (Prim., blue)—were evaluated *in vitro*. Anti-oxidant activity was evaluated based on the DPPH radical scavenging activity. Anti-diabetic activity, anti-hypertensive activity, and anti-adiposity activity were evaluated based on  $\alpha$ -amylase/ $\alpha$ -glucosidase, ACE, and lipase inhibitory activity, respectively. The extracts showed 2 - 3-fold higher anti-oxidative activity than  $\alpha$ -tocopherol, which was not in accordance with their polyphenol content and colors. The anti-diabetic activity of the extracts showed a unique pattern: the extracts from Rosa and Dend. showed only  $\alpha$ -amylase inhibitory activity, where as those of Chry. and Prim. selectively showed  $\alpha$ -glucosidase activity. The extracts from Fluna and Pansy showed both  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activity. Except for the extract from Dend., the ACE inhibitory activity of the extracts was relatively high, and that of Chry. showed the highest inhibition with an  $IC_{50}$  value of 69.1 ng/mL. None of the extracts showed inhibitory activity of lipase. These results suggested that daily consumption of the edible flowers used in this study would help prevent diabetes and hypertensive diseases.

## Keywords

Edible Flower, Anti-Oxidant,  $\alpha$ -Amylase,  $\alpha$ -Glucosidase, ACE, Lipase

## 1. Introduction

Edible flowers are used to add special colors and flavors to dishes. The color of

edible flowers is an important factor that influences consumer purchasing [1]. Many countries have dietary cultures that utilize edible flowers, and we can see them in side dishes, cakes, drinks, and so on. The use of edible flowers is very popular in European countries, such as England, France, Spain, Italy, etc. In Japan, the yellow-colored *Chrysanthemum morifolium* is a very popular edible flower that is used as an ornament or is sometimes cooked by boiling and seasoned with soy source.

Studies on edible flowers have mainly analyzed the chemical compositions [2] [3], identified the chemical structures that result in their distinct colors [4] [5], and evaluated their physiological functions [6] [7] [8] [9]. For example, Rop *et al.* researched the chemical compositions of 12 edible flowers and found that *Chrysanthemum*, *Dianthus*, and *Viola* have the highest mineral content, of such minerals as potassium or sodium [2]. Pires *et al.* prepared hydromethanolic extracts from four flowers of different colors: *Dahlia mignon*, *Rosa damascene*, *Calendula officinalis* L., and *Centaurea cyanus* L., and identified the main compounds as naringenin-3-*O*-glucoside, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, and apigenin-glucuronide, respectively [4]. In the same reports, they also found that their bioactive potentials, referring to the anti-oxidant, anti-proliferative, and anti-bacterial capacities, were different based on the polyphenolic compositions.

Concerning the physiological functions of edible flowers, anti-oxidative activity has been well documented, while other activities have been less reported [10] [11] [12]. In the few reports regarding—physiological functions other than the anti-oxidative activity—anti-bacterial [4] [6], anti-adipogenic [7], anti-allergic [8], and anti-tumor activities [9] of edible flowers have been reported. Especially, as the interest in human health increases every year, it will be important to research the effects of edible flowers on lifestyle-related diseases, such as diabetes, hypertension, and adiposity.

As an abrupt increase in the blood glucose level is one of the risks of becoming diabetic, inhibition of  $\alpha$ -amylase or  $\alpha$ -glucosidase activities, which produce glucose from polysaccharides in the small intestine, is one of the effective strategies to protect against this disease. Hypertension is partially induced by the renin-angiotensin-aldosterone system. Angiotensin I converting enzyme (ACE) is a rate-controlling enzyme. It hydrolyzes angiotensin I to produce angiotensin II, which promotes the secretion of aldosterone in the adrenal gland. Aldosterone increases sodium reabsorption in the kidney, which results in hypertension. Lipase hydrolyzes adipose (triacylglycerol) to release monoacylglycerol and free fatty acids. As the absorption of adipose starts with the hydrolysis of triacylglycerol in the small intestine, the inhibition of lipase activity leads to suppression of the absorption of adipose.

In this study, 70% ethanol extracts were prepared from 6 edible flowers of different color: *Chrysanthemum morifolium* (Chry. yellow), *Rosa miniature* (Rosa, red), *Dendrobium phalaenopsis* (Dend., purple), *Viola x wittrockiana* cv. fluna

(Fluna, white), *Viola x wittrockiana* cv. pansy (Pansy, orange), and *Primula x juliana* (Prim., blue). These flowers are available in commercial. In addition, the physiological functions of the extracts for their anti-diabetics, anti-hypertensive, and anti-adiposity, as well as the anti-oxidative activities were evaluated and compared *in vitro*.

## 2. Experimental Procedures

### 2.1. Materials

#### 2.1.1. Edible Flowers

Chry. was purchased from a local supermarket in Shobara, Hiroshima, Japan. Rosa and Dend. were obtained by mail-order from Marche Co., Ltd. (Shizuoka, Japan). Fluna, Pansy, and Prim. were obtained by mail-order from Maro Co., Ltd (Osaka, Japan).

#### 2.1.2. Enzymes

$\alpha$ -Amylase from *Bacillus subtilis* (200 U/mg) and  $\alpha$ -glucosidase from yeast (75 U/mg) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ACE from rabbit lung (2.0 U/mg) and lipase from porcine pancreas (100 - 500 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

#### 2.1.3. Chemicals

Phenol reagent (Folin-Ciocalteu reagent) was purchased from the Kishida Chemical Co., Ltd. (Osaka, Japan). 1,1-diphenyl-2-picrylhydrazyl (DPPH),  $\alpha$ -tocopherol, starch, hippuryl-histidyl-leucine, *p*-nitrophenyl laurate, chlorogenic acid, l-carnosine and captopril were purchased from Wako Pure Chemical Industries, Ltd. Ethyl gallate, 3,5-dinitrosalicylic acid, 4-nitrophenyl  $\alpha$ -D-glucopyranoside, and *o*-phthalaldehyde were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Triton®X100 was purchased from nacalai tesque (Kyoto, Japan). Tannic acid was purchased from Sigma Chemical Co. Other reagents and solvents used in this study were of analysis grade.

## 2.2. Methods

### 2.2.1. Preparation of 70% Ethanol Extracts

Each flower petal (5 g) was cut smaller than 1 cm<sup>2</sup> in area, and 100 mL of 70% ethanol was added. The mixture was stirred with a magnetic stirrer for 3 h in the dark. The solution was filtered with filter paper (Advantec® No.1), and the filtrate was evaporated to obtain the extract. The extract was dissolved in 70% ethanol and adjusted to an adequate concentration.

### 2.2.2. Polyphenol Content

The polyphenol content of the extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent, using a modified method of Folin and Ciocalteu [13].

About 0.5 mL of each extract was mixed with 0.2 mL of the Folin-Ciocalteu reagent (1:1 v/v in distilled water) and 0.5 mL of 20% of sodium carbonate. Next,

4.3 mL of distilled water was added to each sample. After 30 min, the absorbance was measured at 765 nm with a spectrophotometer (U-2001, Hitachi High-Technologies Co., Tokyo, Japan). Ethyl gallate was used as a standard for the calibration curve ( $y = 0.073x + 0.0475$ ,  $R^2 = 0.9967$ ), showing the relation of polyphenol content of sample solution and the absorbance of sample solution at 765 nm.

### 2.2.3. Polyphenol Composition

The polyphenol composition of the extracts was analyzed by high performance-liquid chromatography (HPLC) [14]. The HPLC system consisted of a Waters 2695 Separations module and a Waters 2487 Dual  $\lambda$  Absorbance Detector. An Inert Sustain C18 (4.6  $\times$  250 mm, 5  $\mu$ m, GL Science) column was used. The extracts were eluted with a gradient elution of mobile phase A (5% acetonitrile in 0.035% trifluoroacetic acid) and B (80% acetonitrile in 0.025% trifluoroacetic acid), where B increased from 10% in 10 min to 50% by 30 min, maintained at 50% for 5 min, returned to the initial condition (10%) in 5 min, and remained there for 5 min before injection. The flow rate was 0.7 mL/min, and the column temperature was 30°C. Elutes were detected at 280 nm.

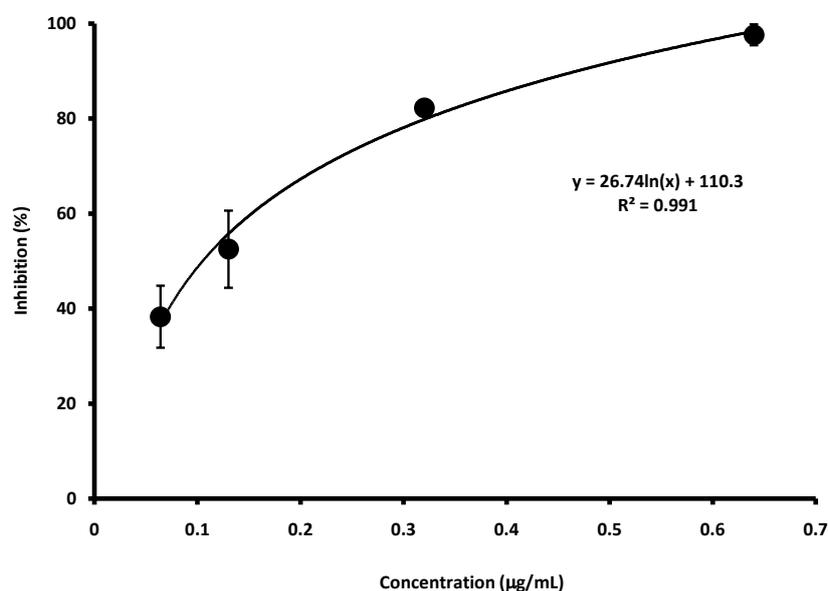
### 2.2.4. Anti-Oxidative Activity

The anti-oxidative activity was evaluated as the ability to trap a DPPH radical. In this assay [15], 2 mL of extract was mixed with 2 mL of 0.1 M acetate buffer (pH 5.5) and 1 mL of 0.5 mM DPPH ethanol solution. Next, the solutions were allowed to stand in a dark place at room temperature for 30 min. Absorbance was measured at 517 nm with a spectrophotometer (U-2001). The relative DPPH radical scavenging activity of the extract was evaluated and converted into the  $\alpha$ -tocopherol equivalent ( $\alpha$ -tocopherol = 1) with a calibration curve ( $y = -0.0067x + 0.8109$ ,  $R^2 = 0.9969$ ).

### 2.2.5. Anti-Diabetic Activity

The anti-diabetic activity was evaluated as the ability to inhibit  $\alpha$ -amylase [16] and  $\alpha$ -glucosidase activities [17]. For the  $\alpha$ -amylase inhibition system, 50  $\mu$ L of extract was mixed with 50  $\mu$ L of  $\alpha$ -amylase solution (6 U/mL). After 10 min of incubation at 30°C, 800  $\mu$ L of 0.5% starch/1.0% NaCl in 0.1 M phosphate buffer (pH 6.8) was added. After 20 min of incubation at 30°C, 125  $\mu$ L of 2 M NaOH and 125  $\mu$ L of 3,5-dinitrosalicylic acid was added. After 10 min of incubation at 99°C, the absorbance of the solution at 540 nm was measured using a micro-plate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA). The inhibition was calculated from Equation (1), where S, B, Cs, and Cb means the absorbance of the sample solution, the solution without enzyme, the solution without sample, and the solution without enzyme and sample, respectively. The inhibition activity was expressed as the IC<sub>50</sub> from the inhibition curve (Figure 1).

$$\text{Inhibition (\%)} = \left(1 - \frac{S - B}{C_s - C_b}\right) \times 100 \quad (1)$$



**Figure 1.**  $\alpha$ -Glucosidase-inhibition curve obtained from the extract from Chry.  $IC_{50}$  calculated from the equation inserted in this figure was 0.41 ( $\mu\text{g/mL}$ ).

For the  $\alpha$ -glucosidase inhibition system, 10  $\mu\text{L}$  of extract was mixed with 40  $\mu\text{L}$  of  $\alpha$ -glucosidase solution (32 mU). After 5 min incubation at 37°C, 950  $\mu\text{L}$  of 0.7 mM 4-nitrophenyl  $\alpha$ -D-glucopyranoside in 0.1 M phosphate buffer (pH 6.8) containing 100 mM NaCl was added. After 15 min of incubation at 37°C, 500  $\mu\text{L}$  of 1 M Tris was added, and the absorbance of the solution at 400 nm was measured using a micro-plate reader (Varioskan Flash). Calculation of the  $IC_{50}$  was the same as the  $\alpha$ -amylase inhibition system.

### 2.2.6. Anti-Hypertensive Activity

The anti-hypertensive activity was evaluated as ability to inhibit ACE [18]. Fifty microliters of the extract was mixed with 100  $\mu\text{L}$  of ACE solution (10 mU/mL). After 10 min of incubation at 37°C, 25  $\mu\text{L}$  of 25 mM hippuryl-histidyl-leucine was added. After 40 min of incubation at 37°C, 50  $\mu\text{L}$  of 1 M NaOH was added, followed by 10  $\mu\text{L}$  of 0.2% *o*-phthalaldehyde. After 15 min of incubation at room temperature, 15  $\mu\text{L}$  of 3.6 M phosphate was added, and the fluorescence intensity (Ex: 360 nm, Em: 460 nm) was measured using a micro-plate reader (Varioskan Flash). Calculation of the  $IC_{50}$  was the same as the  $\alpha$ -amylase inhibition system.

### 2.2.7. Anti-Adiposity Activity

The anti-adiposity activity was evaluated as the ability to inhibit lipase [19]. Fifty microliters of the extract was mixed with 50  $\mu\text{L}$  of lipase solution (2 mg/mL) in 50 mL Tris-HCl buffer (pH 8.5). After 5 min of incubation at room temperature, 50  $\mu\text{L}$  of 5 mM *p*-nitrophenyl laurate in dimethyl sulfoxide was added. After 5 min of incubation at 37°C, 50  $\mu\text{L}$  of 10% Triton®X100 was added, and the absorbance of the solution at 410 nm was measured using a micro-plate reader (Varioskan Flash). Calculation of the  $IC_{50}$  was the same as the  $\alpha$ -amylase inhibition system.

### 2.2.8. Statistical Analysis

All experiments were conducted in triplicate. Data are shown as the means  $\pm$  standard deviation. A significant difference was determined by Scheffe's test ( $P < 0.01$ ).

## 3. Results and Discussion

### 3.1. Yield, Colors, and Polyphenol Content of the Extracts

The yield, colors, and polyphenol content of the extracts are summarized in **Table 1**. The yields varied from 3.0% - 4.8%, based on their origin. As this study used fresh flowers, the yields were much lower those that have used dried flowers. For example, an approximate ~17% yield was obtained from a water or ethanol extraction of the dried daylily flower [10]. In addition, a 22% yield was obtained by methanol extraction of dried *Rosa damascena* [6]. However, Loizzo *et al.* reported 4.4% - 10.5% yield that was obtained by the ethanol extraction of several fresh edible flowers [20]. These values were slightly higher than those in this study.

The colors of the extracts were in accordance with their petal color before extraction, except for that of Fluna. As Fluna is primarily white color, but has a yellow color in the center of the petal, the yellow appeared in the extracts.

The polyphenol content of the extracts was highest for Fluna (18.6%) and lowest for Rosa (10.8%). Rocio *et al.* [3] reported that a polyphenol content of 15.2% was obtained for the *Viola x wittrockiana* cv. pansy, which is the same as the results of this study (14.4%). On the other hand, Özkan *et al.* reported a polyphenol content of 27.6% for *Rosa damascena* extracts [6]. It has been suggested that the polyphenol content differs even for the same genus.

### 3.2. Anti-Oxidative Activity of the Extracts

All of the extracts exerted more than 2-fold higher  $\alpha$ -anti-oxidative activity, compared to  $\alpha$ -tocopherol (**Figure 2**). Especially, the extracts from Fluna, which had highest polyphenol content (**Table 1**), exerted the highest activity. However, among the other extracts, there was no significant anti-oxidative activity. As the results showed that the polyphenol content of these extracts were varied in significance (**Table 1**), it was suggested that the composition of these polyphenols was varied. For example, Rosa, which had lowest polyphenol content, had the kind of polyphenols that have higher radical scavenging activity than the others.

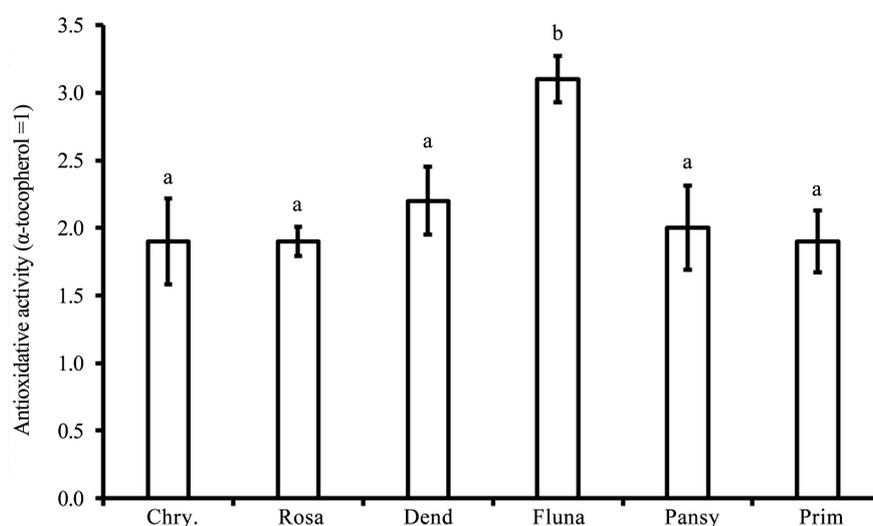
### 3.3. Anti-Diabetics, Anti-Hypertensive, and Anti-Adiposity Activities of the Extracts

The anti-diabetic, anti-hypertensive, and anti-adiposity activities of the extracts were evaluated as the activity of inhibition of the corresponding enzyme. For anti-diabetes, the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase was evaluated. For anti-hypertension and anti-adiposity, the inhibition of ACE and lipase was evaluated, respectively. **Table 2** summarizes corresponding enzyme-inhibitory activities of the extracts as  $IC_{50}$  values.

**Table 1.** Yields, colors, and polyphenol content of the extracts from 6 edible flowers.

Samples (color)	Yield (%)	Color	Polyphenol (%)
Chry. (yellow)	4.6	yellow	13.4 ± 0.7 <sup>ad</sup>
Rosa (red)	4.6	red	10.8 ± 0.6 <sup>bf</sup>
Dend. (purple)	3.2	purple	15.5 ± 0.5 <sup>af</sup>
Fluna (white)	3.0	slightly yellow	18.6 ± 0.5 <sup>c</sup>
Pansy (orange)	4.8	yellow	14.4 ± 0.4 <sup>ad</sup>
Prim. (blue)	3.8	blue	12.7 ± 0.2 <sup>bd</sup>

<sup>ad</sup>Different letters are significantly different in each other (Scheffe's test,  $p < 0.01$ ).

**Figure 2.** Anti-oxidative activity of the extracts from 6 edible flowers. a, b: different letters are significantly different in each other (Scheffe's test,  $p < 0.01$ ).**Table 2.** Enzyme-inhibitory activities ( $IC_{50}$ ) of the extracts from 6 edible flowers.

Samples	Enzymes to inhibit			
	$\alpha$ -Amylase	$\alpha$ -Glucosidase	ACE	Lipase
Chry.	N.D.	0.41 $\mu$ g/mL	69.1 ng/mL	N.D.
Rosa	231.9 $\mu$ g/mL	N.D.	60.9 $\mu$ g/mL	N.D.
Dend.	161.9 $\mu$ g/mL	N.D.	N.D.	N.D.
Fluna	267.5 $\mu$ g/mL	80.3 $\mu$ g/mL	4.6 $\mu$ g/mL	N.D.
Pansy	183.2 $\mu$ g/mL	133.9 $\mu$ g/mL	6.2 $\mu$ g/mL	N.D.
Prim.	N.D.	79.4 $\mu$ g/mL	97.2 $\mu$ g/mL	N.D.
Positive control	20.9 $\mu$ g/mL (tannic acid)	4.4 $\mu$ g/mL (chlorogenic acid)	76.2 $\mu$ g/mL (carnosine)	3.3 $\mu$ g/mL (chlorogenic acid)

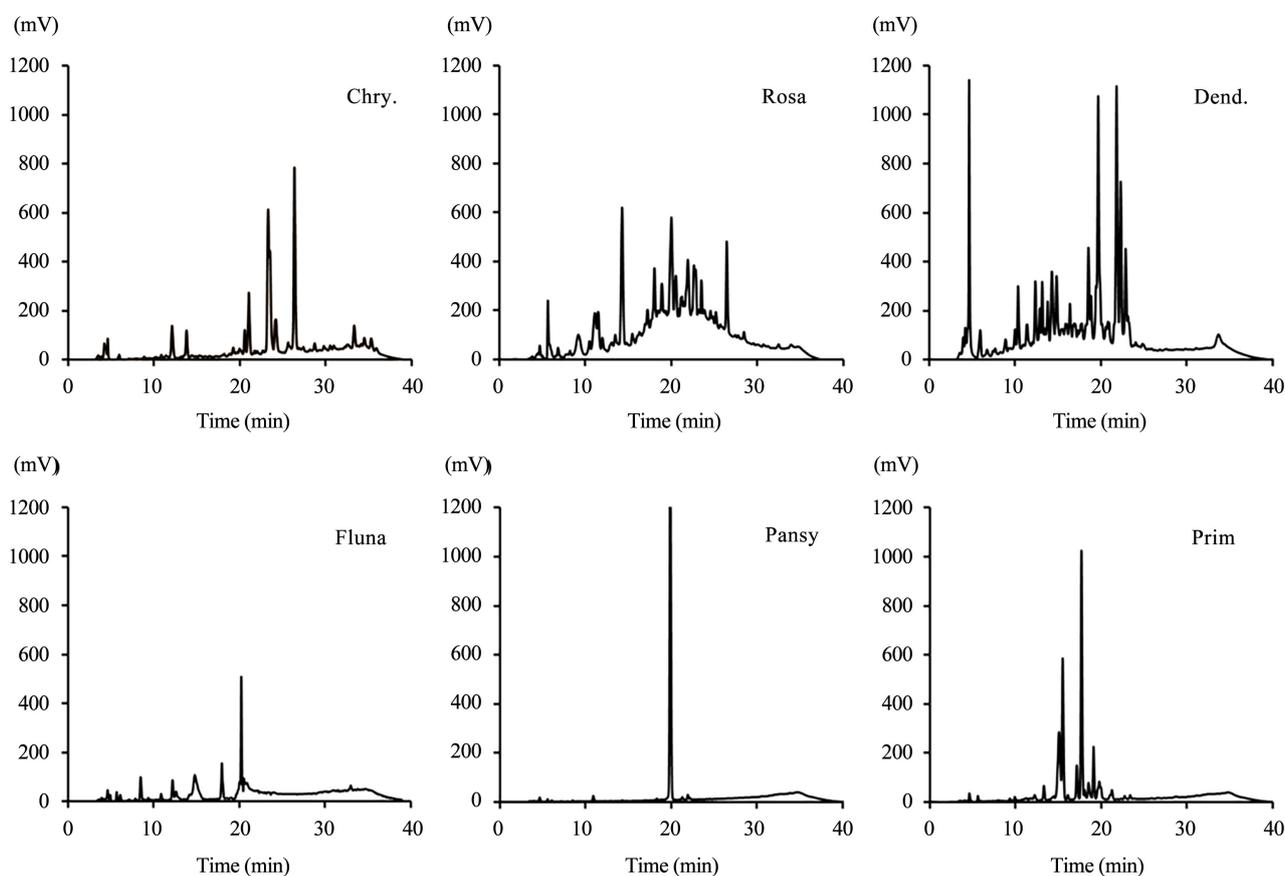
For anti-diabetes, the extracts from Rosa, Dend., Fluna, and Pansy showed dose-dependent inhibition  $\alpha$ -amylase in which, Dend. showed the highest inhibition with an  $IC_{50}$  value of 161.9  $\mu\text{g/mL}$ , although it was only 1/8 of the activity of tannic acid, a positive control. On the other hand, Chry., Fluna, Pansy, and Prim. showed a dose-dependent inhibition of  $\alpha$ -glucosidase, in which Chry. showed the highest inhibition with an  $IC_{50}$  value of 0.41  $\mu\text{g/mL}$  that was 10-fold higher than chlorogenic acid, a positive control. Loizzo *et al.* evaluated the  $\alpha$ -amylase/ $\alpha$ -glucosidase inhibitory activities of 8 edible flower extracts and reported that 7 of them excluding *Sambucus nigra*, showed inhibition of both of the two enzymes [20]. In this study, it was unique that Rosa and Dend. showed selective  $\alpha$ -amylase inhibitory activity, and Chry. and Prim. showed selective  $\alpha$ -glucosidase inhibitory activity. The inhibition mode of methanol/ethanol extracts of *Rosa damascena* Mill. and banana flower on  $\alpha$ -glucosidase were non-competitive mode [21] [22]. Further studies are required to determine the mechanism of inhibition of the extracts used in this study.

For anti-hypertension, the extracts, except for the extract from Dend., showed dose-dependent inhibition of ACE, in which Chry. showed the highest inhibition with an  $IC_{50}$  value of 69.1  $\text{ng/mL}$  that was 1000-fold higher than carnosine, a positive control. As carnosine is a food derivative peptide, we evaluated the activity of captopril, an ACE-inhibitory drug and revealed that its  $IC_{50}$  was 1.9  $\text{ng/mL}$ . The inhibitory activity of peptides for ACE is well known, and a lot of corresponding peptide sequences have also been identified [23]. However, the inhibition level of these peptides is in the order of  $\mu\text{g/mL}$ . To our knowledge, few papers describing the inhibitory activity of *Chrysanthemum* toward ACE have been published. Kim *et al.* [24] reported the ACE inhibitory activity of a water extract from *Chrysanthemum boreale* Makino with an  $IC_{50}$  value of 440  $\text{ng/mL}$ . They identified its structure as guanosine. Further studies are required to identify the chemical structures and inhibition mechanism of the compounds evaluated in this study.

For anti-adiposity, none of the extracts showed inhibitory activity for lipase in this assay.

### 3.4. Polyphenol Compositions of the Extracts

The HPLC chromatograms obtained from detection at 280 nm, showed the polyphenol composition of the extracts were different for each original flower (Figure 3). However, Fluna and Pansy, which are of the same genus, were alike in the chromatogram pattern. As these two flowers have different colors, but showed a similar pattern of physiological function (Table 2), the peak at a retention time of 20 min may be a compound that exerts the physiological function. Of course, it is difficult to identify the compounds present in each chromatogram by only this HPLC analysis. It is necessary to identify the corresponding compound in a future study.



**Figure 3.** HPLC chromatograms obtained from each sample. Concentration of the samples applied into HPLC were 4.6 mg/mL (Chry.), 0.46 mg/mL (Rosa), 3.2 mg/mL (Dend.), 0.6 mg/mL (Fluna), 0.24 mg/mL (Pansy), and 0.38 mg/mL (Prim.), respectively.

#### 4. Conclusion

As the color of edible flowers is an important factor that influences consumer purchasing, this study selected 6 edible flowers of different colors (yellow, red, orange, white, purple, and blue) and compared the physiological functions of their 70% ethanol extracts *in vitro*. Their anti-oxidative activities were 2 - 3-fold higher than  $\alpha$ -tocopherol, which was not related to their color. All of the extracts from the edible flowers used in this study showed  $\alpha$ -amylase/ $\alpha$ -glucosidase inhibitory activity. Except for the extract from Dend., the extracts showed ACE inhibitory activity. Especially, Chry. showed strong inhibitory activity of ACE. Finally, none of them showed lipase inhibitory activity. These results suggested that daily consumption of the edible flowers used in this study will help prevent from diabetes and hypertensive diseases.

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