



# High antiallergic activity of 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone and 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone from eau de cologne mint (*Mentha × piperita citrata*)

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## ABSTRACT

The following compounds with higher antiallergic activities were isolated from eau de cologne mint leaves: 5,6,4'-trihydroxy-7,8-dimethoxyflavone (**6**), 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**7**), 5,6-dihydroxy-7,3',4'-trimethoxyflavone (**8**), 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone (**9**), and 5,6-dihydroxy-7,8,4'-trimethoxyflavone (**10**).

The IC<sub>50</sub> values of compounds **6–10** against RBL-2H3 cells were 6.7, 2.4, 5.6, 3.0, and 6.1 μM. Compounds **7** and **9** (IC<sub>50</sub> 2.4 μM and 3.0 μM) had the highest antiallergic activities among the flavonoids previously reported. The amounts of **7**, **9**, and **10** isolated were fairly high, at 177.7 mg/kg, 278.0 mg/kg, and 179.7 mg/kg in the mint, respectively. LD<sub>50</sub> value (index of toxicity) and LD<sub>50</sub>/IC<sub>50</sub> ratio of **7** and **9** indicate that the safety is greater than that of luteolin, a typical antiallergic substance. The extract containing powerful antiallergic flavones, **6–10** with higher hydrophobicity could be selectively separated from the extract containing luteolin and other flavonoid glycosides by partition with dichloromethane and water. Therefore, compounds **7** and **9** in mint, and the dichloromethane extract would be the most potent and preventive resources against type I allergy.

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## 1. Introduction

Nowadays, allergies are becoming a worldwide health problem, because allergic rhinitis currently affects up to 40% of the world population [1]. Type I allergy, common as food allergy and pollen allergy, can be explained by the progress of

the following mechanisms [2]. After primary exposure to allergens, IgE antibodies produced in plasma cells bond to FcεR1 (fragment, crystallizable epsilon region R1) of mast cells or basophil cells (sensitization). Under the cross-link with the allergen and IgE, chemical mediators such as histamine and β-hexosaminidase are released from mast cells or basophil cells (degranulation). Finally, allergic symptoms appear. RBL-2H3 rat basophilic leukemia cells have been widely used in antiallergy research [3,4] as a model of IgE-mediated degranulation of mast cells, because degranulation of RBL-2H3 cells is linked to the release of β-hexosaminidase and histamine.

Food ingredients such as phenylpropanoids [5], rosmarinic acid [6], caffeoylquinic acids [7], curcuminoids [8], and flavonoids [9] are reported to prevent type 1 allergic reactions. Thus, the potential of finding antiallergic compound-containing plants is still high, as well as for preventing and protecting

**Abbreviations:** RBL-2H3, rat basophilic leukemia cell; UV, ultraviolet; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy; DEPT, distortionless enhancement by polarization transfer; COSY, correlated spectroscopy; HETCOR, hetero-nuclear correlation; HMBC, hetero-nuclear multiple-bond connectivity; MS, mass spectrometry; WST, water soluble tetrazolium salts.

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against food and pollen allergies. Recently, *O*-methylated catechin in green tea and oleuropein aglycone in olive fruit were found to be potent chemicals for preventing allergies [10,11]. The most important phytochemicals for antiallergic activity were reported to be luteolin [12,13], and apigenin [14] in the flavonoid family. The flavonoid aglycone is especially well-known as a powerful antiallergic substance [15]. Some flavonoids with high activity would be leading compounds for the design of new antiallergic drugs. In future, the bioavailability of such flavonoids should be considered when we design the leading compounds.

Mint has a wide range of uses such as in cooking as a spicy herb, aromatherapy as a fragrance, in the manufacture of candy, toothpaste and gum [16], and in Chinese medicine. Mint leaves have some biological activities such as antioxidant [17,18], anti-inflammatory [19], and antibacterial activities [20]. However, there are few reports about the antiallergic substances of the mint family even though we can understand the amount of flavonoids in mint family [21].

In this study, we report two highly active substances in eau de cologne mint leaves for allergy suppression to be 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone and 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone.

## 2. Materials and methods

### 2.1. Mint leaves

Eau de cologne mint (10 seedlings) was purchased from Saijo Seed Co. Ltd. (Kagawa, Japan) in March 2012 and planted at University Farm of Kagawa University. The leaves (50 g) harvested in August 2012 were frozen at  $-28^{\circ}\text{C}$  and then used as necessary in this research.

### 2.2. Chemicals and reagents

Dulbecco's modified Eagle's medium (D-MEM), PBS (–), dimethylsulfoxide (DMSO) and TritonX-100 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Equitech-Bio (Kerrville, TX, USA). Antibiotic–antimycotic 100 $\times$  was obtained from Invitrogen (Grand Island, NY, USA). Mouse monoclonal anti-dinitrophenyl antibody, albumin dinitrophenyl (HSA), Tyrode's salt solution, and *p*-nitrophenyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (*p*-NAG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Luteolin 7-*O*-glucoside, hesperidin, apigenin 7-*O*-glucoside, luteolin and rosmarinic acid were purchased from EXTRASYNTHÈSE (Genay Cedex, France) and Sigma-Aldrich (St. Louis, MO, USA), and used without further purification.

### 2.3. Preparation of the media and samples

DMEM including 10% FBS and 1% antibiotic–antimycotic 100 $\times$  was used as a base medium. Modified Tyrode (MT) buffer was prepared by dissolving HEPES (4.76 g/L) and BSA (1 g/L) in Tyrode's salt solution. Anti-DNP IgE solution (anti-body) was prepared by diluting mouse monoclonal anti-dinitrophenyl antibody with the base medium (50 ng/mL). HSA solution (antigen) was prepared by dissolving HSA in MT buffer

(2.5  $\mu\text{g/mL}$ ). The samples tested were dissolved in DMSO, and the solutions were added to MT buffer (final DMSO concentration was adjusted at 0.1%).

### 2.4. Extracts from eau de cologne mint leaves

Eau de cologne mint leaves (50 g) were cut using scissors. The cut leaves were soaked in 100% ethanol (100 mL) four times. The solvent (400 mL) was removed under vacuum. Finally, 1.58 g of ethanol extract was obtained from the ethanol solution.

### 2.5. Fractionation of mint compounds by liquid–liquid partition

The ethanol extract (1.58 g) was fractionated using four different combinations of liquid–liquid partitions with acetonitrile–hexane and dichloromethane–water mixtures as illustrated in Fig. 1. Thus, ethanol extract (1.5 g) was firstly partitioned with a bilayer solution of an acetonitrile–hexane mixture. Acetonitrile was removed to get the active extract (1.2 g). The active extract (1.2 g) was partitioned again with a bilayer solution of a water (40 mL)–dichloromethane (40 mL) mixture. By triplicate extractions with 40 mL dichloromethane, the combined solution (120 mL) was afforded to evaporation to give the extract (146.4 mg) as described in Fig. 1. Identification of known chemicals was done by comparing the retention times and the UV spectra obtained from a photodiode array detector with the five authentic chemicals as shown in Fig. S1.

### 2.6. Isolation of polymethoxyflavones from dichloromethane extract

Dichloromethane extract (130 mg), showing higher antiallergic activity was selected as the most important fraction for further experiments. Major components in this fraction were clarified by the HPLC analysis to be five polymethoxyflavones such as 5,6,4'-trihydroxy-7,8-dimethoxyflavone (**6**), 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**7**), 5,6-dihydroxy-7,3',4'-trimethoxyflavone (**8**), 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone (**9**) and 5,6-dihydroxy-7,8,4'-trimethoxyflavone (**10**). Thus, five compounds were carefully separated and purified by using an HPLC 5  $\mu$ -ODS column with 60% methanol aqueous solution at a flow rate of 1 mL/min and at a UV wavelength of 334 nm. Finally, 2.2 mg of pure 5,6,4'-trihydroxy-7,8-dimethoxyflavone (**6**) with 95.6% purity, 3.1 mg of pure 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**7**) with 95.8% purity, 2.3 mg of pure 5,6-dihydroxy-7,3',4'-trimethoxyflavone (**8**) with 91.9% purity, 8.1 mg of pure 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone (**9**) with 97.8% purity, and 4.7 mg of pure 5,6-dihydroxy-7,8,4'-trimethoxyflavone (**10**) with 91.8% purity were isolated after drying under vacuum as shown in Fig. 2.

**5,6,4'-Trihydroxy-7,8-dimethoxyflavone (6).** Color reaction by  $\text{FeCl}_2$  showed green [22,23]. Other data were shown as follows: UV  $\lambda_{\text{max}}$ , 295 nm and 335 nm in  $\text{CH}_3\text{CN}$ , 295 nm and 335 nm in  $\text{CH}_3\text{OH}$ , 315 nm and 371 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3$ , 311 nm and 359 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3 + 0.1\% \text{HCl}$ ; ESI-Q-TOF-MS, positive mode,  $m/z$  331.0774 ( $[\text{M} + \text{H}]^+$ ,  $\text{C}_{17}\text{H}_{15}\text{O}_7$ , Cal: 331.0818).

**5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone (7).** Color reaction by  $\text{FeCl}_2$  showed green [22,23]. Other data were shown

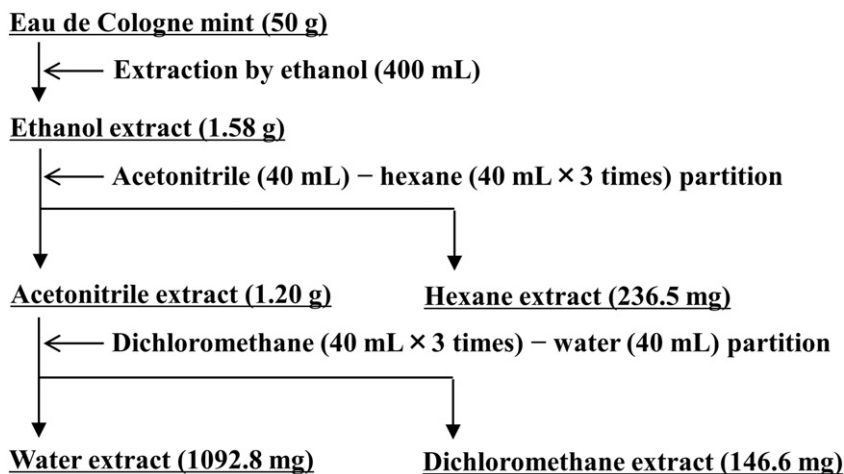


Fig. 1. Partitionary separation of ethanol extracts of eau de cologne mint leaf.

as follows: UV  $\lambda_{\text{max}}$ , 287 nm and 347 nm in  $\text{CH}_3\text{CN}$ , 291 nm and 343 nm in  $\text{CH}_3\text{OH}$ , 307 nm and 383 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3$ , 303 nm and 371 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3 + \text{HCl}$ ; ESI-Q-TOF-MS, positive mode,  $m/z$  361.0891 ( $[\text{M} + \text{H}]^+$ ,  $\text{C}_{18}\text{H}_{17}\text{O}_8$ , Cal: 361.0923).

**5,6-Dihydroxy-7,3',4'-trimethoxyflavone (8).** Color reaction by  $\text{FeCl}_2$  showed green [22,23]. Other data were shown as follows: UV  $\lambda_{\text{max}}$ , 283 nm and 339 nm in  $\text{CH}_3\text{CN}$ , 283 nm and 339 nm in  $\text{CH}_3\text{OH}$ , 295 nm and 375 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3$ , 295 nm and 363 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3 + \text{HCl}$ ; ESI-Q-TOF-MS, positive mode,  $m/z$  345.0949 ( $[\text{M} + \text{H}]^+$ ,  $\text{C}_{18}\text{H}_{17}\text{O}_7$ , Cal: 345.0974).

**5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone (9).** Color reaction by  $\text{FeCl}_2$  showed green [22,23]. Other data were shown as

follows: UV  $\lambda_{\text{max}}$ , 291 nm and 343 nm in  $\text{CH}_3\text{CN}$ , 291 nm and 339 nm in  $\text{CH}_3\text{OH}$ , 303 nm and 375 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3$ , 303 nm and 367 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3 + \text{HCl}$ ; ESI-Q-TOF-MS, negative mode,  $m/z$  373.0893 ( $[\text{M} - \text{H}]^-$ ,  $\text{C}_{19}\text{H}_{17}\text{O}_8$ , Cal: 373.0923).

**5,6-Dihydroxy-7,8,4'-trimethoxyflavone (10).** Color reaction by  $\text{FeCl}_2$  showed green [22,23]. Other data were shown as follows: UV  $\lambda_{\text{max}}$ , 295 nm and 331 nm in  $\text{CH}_3\text{CN}$ , 295 nm and 331 nm in  $\text{CH}_3\text{OH}$ , 315 nm and 367 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3$ , 311 nm and 355 nm in  $\text{CH}_3\text{OH} + \text{AlCl}_3 + \text{HCl}$ ; ESI-Q-TOF-MS, negative mode,  $m/z$  343.0786 ( $[\text{M} - \text{H}]^-$ ,  $\text{C}_{18}\text{H}_{15}\text{O}_7$ , Cal: 343.0818).

UV spectra, NMR data, and chemical structures of polymethoxyflavones are shown in Table 1, Figs. 3, S2, and S3.

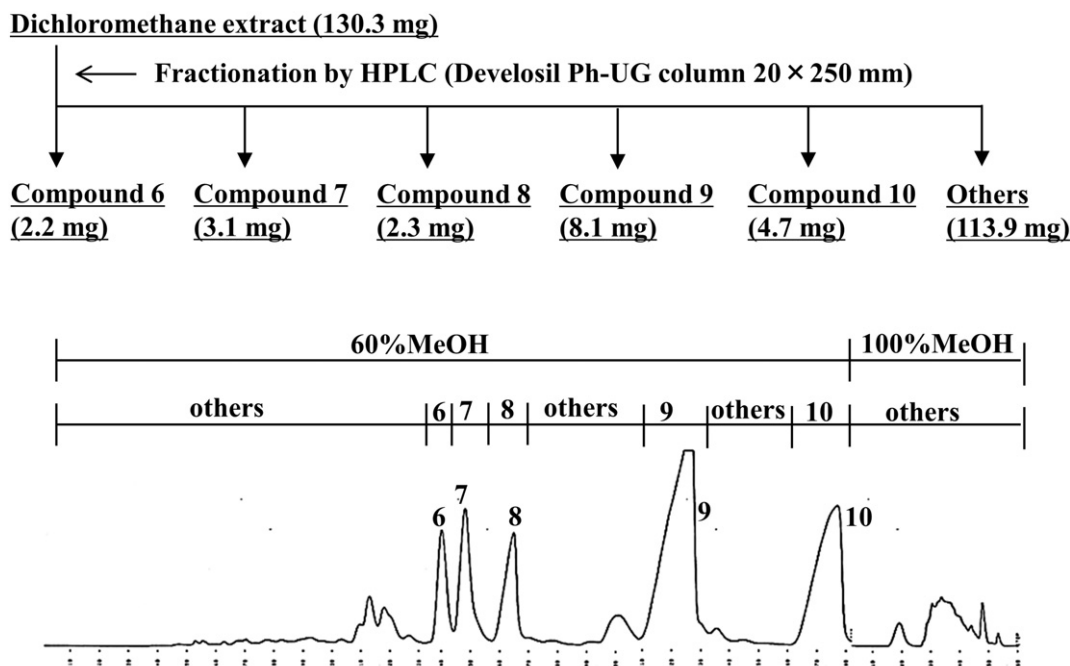


Fig. 2. HPLC isolation of compounds 6, 7, 8, 9, and 10 from dichloromethane extract.

**Table 1**  
Chemical shifts ( $\delta$ ) of polymethoxyflavones in eau de cologne mint leaves.

| Atom                | Compound 6                  |                          | Compound 7                  |                          | Compound 8                  |                          | Compound 9                  |                          | Compound 10                 |                          |
|---------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|
|                     | $\delta^{13}\text{C}$ [ppm] | $\delta^1\text{H}$ [ppm] | $\delta^{13}\text{C}$ [ppm] | $\delta^1\text{H}$ [ppm] | $\delta^{13}\text{C}$ [ppm] | $\delta^1\text{H}$ [ppm] | $\delta^{13}\text{C}$ [ppm] | $\delta^1\text{H}$ [ppm] | $\delta^{13}\text{C}$ [ppm] | $\delta^1\text{H}$ [ppm] |
| 2                   | 164.66                      |                          | 164.46                      |                          | 164.04                      |                          | 164.07                      |                          | 164.24                      |                          |
| 3                   | 102.92                      | 6.71 (s)                 | 103.22                      | 6.80 (s)                 | 103.89                      | 6.82 (s)                 | 103.76                      | 6.84 (s)                 | 103.52                      | 6.77 (s)                 |
| 4                   | 183.08                      |                          | 183.10                      |                          | 182.74                      |                          | 183.12                      |                          | 183.11                      |                          |
| 5                   | 143.53                      |                          | 143.52                      |                          | 146.58                      |                          | 146.58                      |                          | 143.53                      |                          |
| 6                   | 134.63                      |                          | 134.61                      |                          | 130.48                      |                          | 130.64                      |                          | 134.69                      |                          |
| 7                   | 148.50                      |                          | 148.50                      |                          | 154.98                      |                          | 148.55                      |                          | 148.58                      |                          |
| 8                   | 133.46                      |                          | 133.40                      |                          | 91.79                       | 6.88 (s)                 | 133.39                      |                          | 133.48                      |                          |
| 9                   | 142.40                      |                          | 142.41                      |                          | 150.30                      |                          | 142.43                      |                          | 142.44                      |                          |
| 10                  | 106.56                      |                          | 106.57                      |                          | 105.61                      |                          | 106.61                      |                          | 106.61                      |                          |
| 1'                  | 121.89                      |                          | 122.23                      |                          | 123.59                      |                          | 123.51                      |                          | 123.44                      |                          |
| 2'                  | 128.93                      | 7.86 (d, $J$ = 8.94 Hz)  | 110.46                      | 7.48 (s)                 | 109.94                      | 7.50 (s)                 | 109.69                      | 7.47 (s)                 | 128.76                      | 7.95 (d, $J$ = 6.84 Hz)  |
| 3'                  | 116.71                      | 6.92 (d, $J$ = 8.94 Hz)  | 148.61                      |                          | 149.57                      |                          | 149.55                      |                          | 115.31                      | 7.09 (d, $J$ = 6.84 Hz)  |
| 4'                  | 161.75                      |                          | 151.33                      |                          | 152.68                      |                          | 152.79                      |                          | 163.01                      |                          |
| 5'                  | 116.71                      | 6.92 (d, $J$ = 8.94 Hz)  | 116.53                      | 6.92 (d, $J$ = 8.22 Hz)  | 112.37                      | 7.07 (d, $J$ = 8.22 Hz)  | 112.46                      | 7.09 (d, $J$ = 8.28 Hz)  | 115.31                      | 7.09 (d, $J$ = 6.84 Hz)  |
| 6'                  | 128.93                      | 7.86 (d, $J$ = 8.94 Hz)  | 120.90                      | 7.51 (d, $J$ = 8.22 Hz)  | 120.67                      | 7.68 (d, $J$ = 8.28 Hz)  | 120.56                      | 7.60 (d, $J$ = 8.28 Hz)  | 128.76                      | 7.95 (d, $J$ = 6.84 Hz)  |
| 7-OCH <sub>3</sub>  | 61.52                       | 3.90 (s)                 | 61.53                       | 3.91 (s)                 | 56.87                       | 3.87 (s)                 | 61.52                       | 3.91 (s)                 | 61.52                       | 3.91 (s)                 |
| 8-OCH <sub>3</sub>  | 62.42                       | 3.86 (s)                 | 62.37                       | 3.88 (s)                 |                             |                          | 62.34                       | 3.87 (s)                 | 62.41                       | 3.86 (s)                 |
| 3'-OCH <sub>3</sub> |                             |                          | 56.36                       | 3.83 (s)                 | 56.45                       | 3.83 (s)                 | 56.29                       | 3.81 (s)                 |                             |                          |
| 4'-OCH <sub>3</sub> |                             |                          |                             |                          | 56.29                       | 3.80 (s)                 | 56.24                       | 3.80 (s)                 | 56.09                       | 3.80 (s)                 |

## 2.7. Analytical HPLC

HPLC analysis was performed under the following conditions in accordance with Areias et al. [21]. The HPLC system comprised a 250 × 4.6 mm COSMOSIL 5C<sub>18</sub>-ARII column, coupled to a JASCO MD 2010 Plus photodiode array detector and double JASCO PU-980 pumps. Chromatograms were obtained from UV absorbance at 290 nm. The column temperature was set at 40 °C. The flow rate was 1 mL/min; the mobile phase used was a mixture of 10% acetonitrile and 1% phosphoric acid in water (A), and a mixture of 90% acetonitrile and 1% phosphoric acid in water (B). The total analytical time was 40 min and the gradient was done as follows: 100% A to 0% A for 35 min and 0% A was kept for 5 min. For preparative HPLC, the HPLC system consisted of a 20 × 250 mm DEVELOSIL Ph-UG column, coupled to a JASCO 875-UV detector (334 nm) with a JASCO PU-980 pump at 1 mL/min. For the preparative HPLC, 60% methanol and 100% methanol were used as a mobile phase. Luteolin 7-O-glucoside (**1**), hesperidin (**2**), apigenin 7-O-glucoside (**3**), rosmarinic acid (**4**), and luteolin (**5**) were dissolved in dimethylsulfoxide and then diluted to prepare a standard solution (0.067 µg/mL to 0.5 µg/mL) to make the calibration curve of each chemical.

## 2.8. Color reactions of polymethoxyflavones by aluminum chloride and ferric chloride

Color reactions were observed under the following conditions in accordance with previous methods [22,23]. Compounds **6–10** were dissolved in three solutions (methanol, methanol containing 0.5% aluminum chloride, and methanol containing 0.5% aluminum chloride and 0.1% hydrochloric acid) at a concentration of 30 µM. UV spectra of each solution were measured using the JASCO MD 2010 Plus photodiode array detector. Ferric chloride ethanol solution (1%) was sprayed at each spot of polymethoxyflavone on the Advantec Toyo chromatography paper grade no. 50, and each spot color change was observed.

## 2.9. $\beta$ -Hexosaminidase release assay of RBL-2H3 cells

The amount of  $\beta$ -hexosaminidase released by RBL-2H3 rat basophilic leukemia cells was determined using a previous method with some modifications [3,7]. RBL-2H3 cells were obtained from RIKEN BioResource Center Cell Bank (Ibaraki, Japan). The RBL-2H3 cells in the base medium (D-MEM containing 10% FBS and 1% antibiotic–antimycotic 100×) were dispensed into a 24-well plate (2.5 × 10<sup>5</sup> cells/well) and were incubated overnight at 37 °C under a humidified 5% CO<sub>2</sub> atmosphere. After washing with 1 mL PBS, the cells were incubated with 500 µL antibody solution (mouse monoclonal anti-dinitrophenyl antibody, 50 ng/mL dissolved in base medium) for 2 h sensitization. A test sample solution of 490 µL was added to the well after washing twice 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 was used as control instead of the sample. The test sample was dissolved in DMSO and diluted with MT buffer (the final concentration of DMSO was set at 0.1%). After 10 min incubation, albumin dinitrophenyl (10 µL, final concentration = 50 ng/mL) was added to each well and the cells were incubated for 30 min to evoke allergic reactions (degranulation). The reaction was stopped by cooling

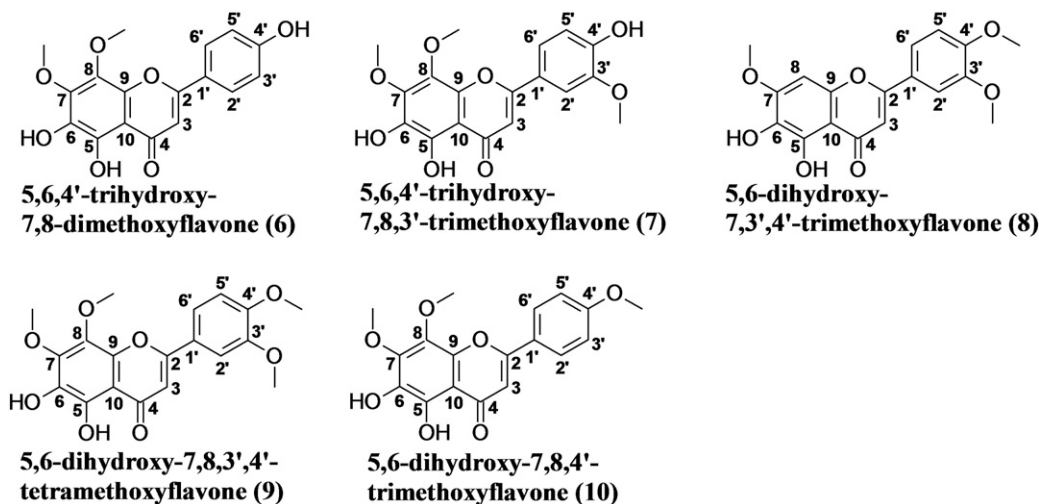


Fig. 3. Chemical structure of compounds **6**, **7**, **8**, **9**, and **10**.

in an ice bath for 10 min. The supernatant (50  $\mu$ L) was transferred to a 96-well plate and incubated with 100  $\mu$ L substrate (3.3 mM *p*-nitrophenyl-2-acetamide-2-deoxy- $\beta$ -D-glucopyranoside) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 25 min. The reaction was terminated by adding 100  $\mu$ L of stop solution (2 M glycine buffer, pH 10.0). The absorbance (OD) was measured at 405 nm using a microplate reader (Thermo Scientific Multiskan FC, Yokohama, Japan). The gain in OD reflects  $\beta$ -hexosaminidase release. The calculation was performed using Eq. (1) and (2) shown below. To obtain a valid value, the factors that are not typically induced by samples need to be excluded. In “blank”, neither antibody solution nor sample was added to the cells to confirm spontaneous  $\beta$ -hexosaminidase release from the cells. In “control”, MT buffer instead of samples was added to the cells to confirm  $\beta$ -hexosaminidase release from the cells in conditions without a sample. In “total”, the cells were lysed in 0.1% Triton X-100 in MT buffer to confirm the total amount of  $\beta$ -hexosaminidase contained in the cells. In “sample”, both antibody solution and samples were added to the cells to confirm  $\beta$ -hexosaminidase release from the cells in these conditions.

$$\text{ratio of } \beta\text{-hexosaminidase release (\%)} = \frac{(\text{OD}_{\text{control or sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{total}} - \text{OD}_{\text{blank}})} \times 100. \quad (1)$$

The ratio of  $\beta$ -hexosaminidase release (%) of “control” against “total” should be greater than 25% if the cells are healthy.

$$\beta\text{-hexosaminidase release (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100. \quad (2)$$

Finally,  $\beta$ -hexosaminidase release (%) was calculated as the ratio of  $\beta$ -hexosaminidase release of “sample” against that of “control” described above (2).

#### 2.10. $\beta$ -Hexosaminidase inhibitory activity assay

$\beta$ -Hexosaminidase inhibitory activity was measured following previous methods with some modifications [5,8,9]. RBL-2H3 cells were grown in base medium. The cells were lysed in 0.1% Triton X-100 ( $5.0 \times 10^5$  cells/mL). The cell lysate (25  $\mu$ L) was transferred to a 96-well plate. The tested solutions were diluted in different concentrations with MT buffer and then added to the sample well (25  $\mu$ L); MT buffer was added to the control well (25  $\mu$ L). Following that, 100  $\mu$ L of 0.1 M citrate buffer (pH 4.5) containing 3.3 mM *p*-NAG (substrate) was added to each well and incubated for 25 min at 37 °C. The reaction was stopped by adding 100  $\mu$ L of 2 M glycine buffer (the stop buffer solution) at pH 10.0 to each well. The absorbance (OD) of each reaction was measured at 405 nm using a microplate reader (Thermo Scientific Multiskan FC, Yokohama, Japan). The inhibitory activity of  $\beta$ -hexosaminidase was calculated using the following equation.

$$\beta\text{-hexosaminidase inhibitory (\%)} = \left(1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100. \quad (3)$$

#### 2.11. Cell viability assay (CCK-8 assay)

Cell viability against pure chemical was measured following previous methods with some modifications [24,25]. The cell viability was examined using cell counting kit-8 (CCK-8) from Dojindo. WST-8 substrate is reduced by dehydrogenases in the living cells to afford an orange product (formazan). RBL-2H3 cells ( $2.5 \times 10^4$  cells/well) were exposed to solutions containing compounds **5**, **6**, **7**, **8**, **9**, and **10** at different concentrations (5–20  $\mu$ M) in 96-well plates for 48 h. DMSO served as the solvent control. After the tested compounds were exposed, 10  $\mu$ L of CCK-8 solution was added to each well, and the 96-well plate was continuously incubated at 37 °C for 4 h, then the OD values for each well were read at wavelength 450 nm using a microplate reader (Thermo Scientific Multiskan

FC, Yokohama, Japan). Cell viability was expressed as the percent viability of treated cells compared with the untreated control.

## 2.12. NMR analysis

$^1\text{H}$ ,  $^{13}\text{C}$ , DEPT-45, DEPT-90, DEPT-135, COSY, HETCOR, and HMBC NMR spectra were recorded with a 600 MHz JNM-ECA spectrometer, dissolving samples in deuterated DMSO with tetramethylsilane (TMS, Isotec; Taiyo Nippon Sanso Corp. Ltd., Tokyo, Japan) as an internal standard. Temperature was set at 40 °C.

## 2.13. MS analysis

The mass spectra of the polymethoxyflavones were measured using liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry (LC/ESI-Q-TOF-MS) analysis using an Acquity UPLC/Xevo QTOF MS system (Waters, USA). Chromatographic separations were conducted using an Acquity UPLC BEH  $\text{C}_{18}$  (1.7  $\mu\text{m}$ , 2.1  $\times$  50 mm) column (Waters, USA).

## 2.14. Statistical analysis

Statistical analysis was carried out using EZR (version 1.23) with the Tukey test (one-way ANOVA). The values represent means  $\pm$  standard deviation (SD) in triplicate at least. Significant difference was set at  $p < 0.05$ .

# 3. Results and discussion

## 3.1. Isolation and identification of antiallergic active compounds

The ethanol extract from eau de cologne mint leaves inhibited  $\beta$ -hexosaminidase release (Fig. 4). The ethanol extract (1.57 g) isolated was further separated into three extracts using a stepwise liquid–liquid partition with different solvents (Fig. 1), yielding 236.5 mg hexane extract from the hexane–acetonitrile partition, followed by 146.6 mg dichloromethane extract from the dichloromethane–water partition of

the acetonitrile concentrates. Finally, 1092.8 mg water extract was obtained after evaporation of the residual water layer. The antiallergic activities of the three extracts shown above were tested at a concentration of 40  $\mu\text{g}/\text{mL}$ . It was found that the dichloromethane extract exhibited extremely low  $\beta$ -hexosaminidase release ( $3.1 \pm 0.5\%$ ), followed by the ethanol extract ( $39.2 \pm 0.6\%$ ), with significant differences ( $p < 0.05$ ), while hexane extract exhibited  $\beta$ -hexosaminidase release over 80% (Fig. 4). The residues of water layer (data not shown) and the hexane extracts did not show any antiallergic activity. This result means that the partition procedure mentioned above showed good separation efficiency in activity.

Moreover, the HPLC chromatogram (Fig. 5) of the dichloromethane extract contained marked peaks corresponding to compounds **6**, **7**, **8**, **9**, and **10**. The chemical structures of the antiallergic active compounds in this extract were confirmed by UV spectra (Fig. S3), MS spectra, and NMR analyses (Table 1 and Fig. S2).

## 3.2. Antiallergic activities of compounds in *Mentha $\times$ piperita citrata*

A comparison of  $\text{IC}_{50}$  values of the antiallergic activity of five chemicals (compounds **1–5**) purchased and five compounds (**6–10**) isolated from eau de cologne mint with some extracts from eau de cologne mint is shown in Table 2. The activity of the ethanol extract of eau de cologne mint was fairly high ( $\text{IC}_{50}$ :  $32.4 \pm 0.2 \mu\text{g}/\text{mL}$ ). Hesperidin (**2**) did not have antiallergic activity at a concentration of 800  $\mu\text{g}/\text{mL}$ . Luteolin 7-*O*-glucoside (**1**), apigenin 7-*O*-glucoside (**3**), and rosmarinic acid (**4**) had moderate antiallergic activities, with  $\text{IC}_{50} > 180 \mu\text{g}/\text{mL}$ .

On the other hand, dichloromethane extract ( $\text{IC}_{50} = 7.5 \pm 0.3 \mu\text{g}/\text{mL}$ ) containing 5,6,4'-trihydroxy-7,8-dimethoxyflavone (**6**), 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**7**), 5,6-dihydroxy-7,3',4'-trimethoxyflavone (**8**), 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone (**9**), and 5,6-dihydroxy-7,8,4'-trimethoxyflavone (**10**) showed the highest antiallergic activity (0.8 to 2.2  $\mu\text{g}/\text{mL}$  in  $\text{IC}_{50}$ ) without enzyme inhibition. This extract should contribute to the high activity of the entire plant extract. The highest activities were observed in compounds **7** and **9**. Compounds **6–10** were abundant in the dichloromethane

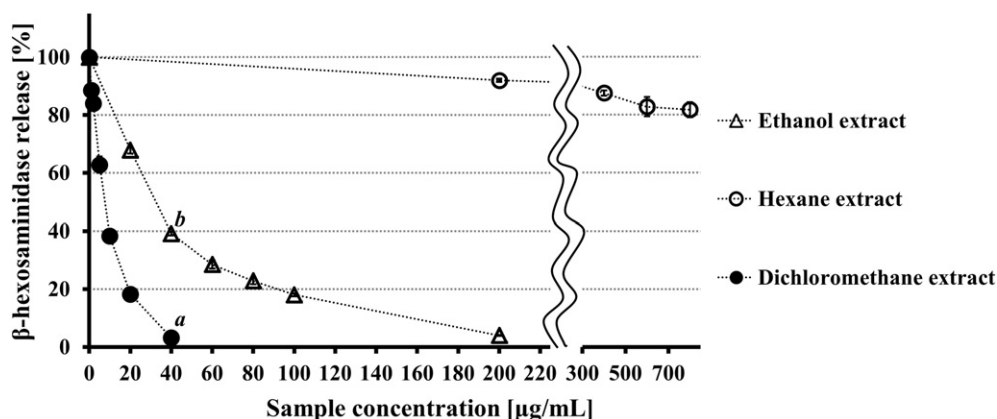


Fig. 4. Antiallergic activities of each extract from liquid–liquid partitions. Each value represents mean  $\pm$  SD ( $n = 4$ ). Means with different letters are significantly different ( $p < 0.05$ ).

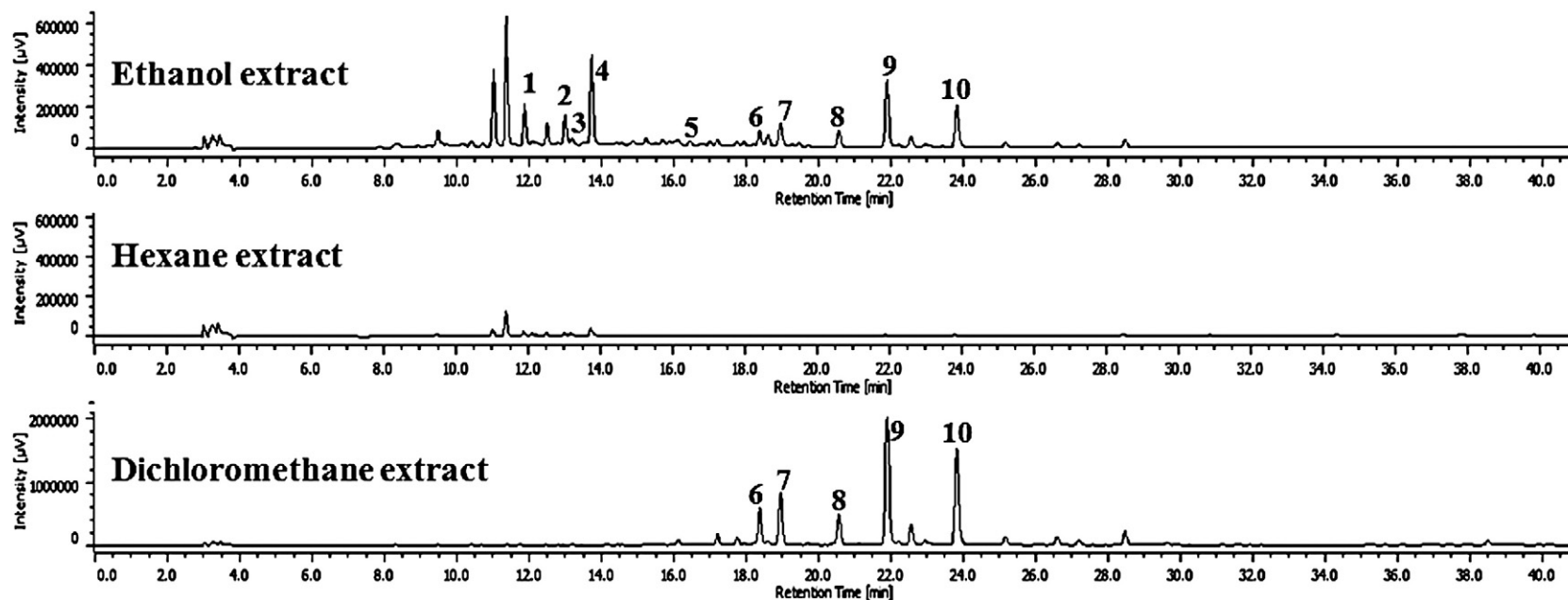


Fig. 5. HPLC chromatograms of extracts of eau de cologne mint leaves ( $\lambda = 290$  nm). Compounds isolated by HPLC analyses were identified as luteolin 7-O-glucoside (1), hesperidin (2), apigenin 7-O-glucoside (3), rosmarinic acid (4) and luteolin (5) by comparing their retention times and UV spectra of each compound with those of authentic compounds. Compounds 6–10 mainly found in ethanol extract and dichloromethane extract were identified using two-dimensional NMR techniques, high-resolution MS, and color reactions.

**Table 2**

Antiallergic activities of extract and substances found in eau de cologne mint leaves.

| Sample                                                   | IC <sub>50</sub> [μg/mL] | IC <sub>50</sub> [μM]      | Enzyme inhibition % <sup>a</sup> |
|----------------------------------------------------------|--------------------------|----------------------------|----------------------------------|
| Ethanol extract                                          | 32.4 ± 0.2               | –                          | –                                |
| Hexane extract                                           | No activity              | No activity                | –                                |
| Dichloromethane extract                                  | 7.5 ± 0.3                | –                          | –                                |
| Luteolin 7-O-glucoside ( <b>1</b> )                      | 186.9 ± 7.4              | 416.9 ± 16.5 <sup>f</sup>  | 22.0 ± 2.5                       |
| Hesperidin ( <b>2</b> )                                  | No activity              | No activity                | –                                |
| Apigenin 7-O-glucoside ( <b>3</b> )                      | 544.4 ± 3.2              | 1259.1 ± 7.4 <sup>g</sup>  | 85.7 ± 5.7                       |
| Rosmarinic acid ( <b>4</b> )                             | 555.2 ± 30.5             | 1540.9 ± 84.7 <sup>h</sup> | 4.1 ± 3.6                        |
| Luteolin ( <b>5</b> )                                    | 0.7 ± 0.1                | 2.6 ± 0.1 <sup>ab</sup>    | –3.2 ± 7.9                       |
| 5,6,4'-Trihydroxy-7,8-dimethoxyflavone ( <b>6</b> )      | 2.2 ± 0.1                | 6.7 ± 0.2 <sup>e</sup>     | 0.6 ± 11.9                       |
| 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone ( <b>7</b> )  | 0.8 ± 0.1                | 2.4 ± 0.1 <sup>a</sup>     | 2.4 ± 7.2                        |
| 5,6-Dihydroxy-7,3',4'-trimethoxyflavone ( <b>8</b> )     | 1.9 ± 0.1                | 5.6 ± 0.1 <sup>c</sup>     | 0.1 ± 3.7                        |
| 5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone ( <b>9</b> ) | 1.1 ± 0.1                | 3.0 ± 0.2 <sup>b</sup>     | –6.5 ± 11.2                      |
| 5,6-Dihydroxy-7,8,4'-trimethoxyflavone ( <b>10</b> )     | 2.1 ± 0.1                | 6.1 ± 0.1 <sup>d</sup>     | –9.9 ± 1.7                       |
| others in dichloromethane extract                        | 42.0 ± 0.9               | –                          | –                                |

<sup>a</sup> Values indicate enzyme inhibition (%) against β-hexosaminidase. Sample concentrations: compound **1**, 800 μM; compounds **3** and **4**, 2000 μM; compounds **5–10**, 20 μM. Each value represents mean ± SD (n = 4). Means with different letters are significantly different (p < 0.05).

extract (19.5%) compared with the ethanol extract (2.8%) and the leaves (0.1%) as shown in Table 3 and Fig. 5.

### 3.3. Structure–activity relationship of various kinds of flavonoids, and flavonoids found in *Mentha × piperita citrata*

Table 4 shows the significance of functional groups of various flavonoids for their antiallergic activities. The correlation coefficient values between the activity and total number of OCH<sub>3</sub> and OH groups, and between the activity and the number of OH groups were sufficiently high (0.62\* and 0.61\*, respectively, n = 20, p\* < 0.01). Therefore, the greater the number of hydroxyl groups and/or the total number of OCH<sub>3</sub> and OH groups, the higher the activity of flavonoids. Actually, flavonoids that have high activity (IC<sub>50</sub> < 3.0 μM) bear more than two hydroxyl groups or more than four of the total number of OCH<sub>3</sub> and OH groups. For example, IC<sub>50</sub> values of typical flavonoids with higher activity become lower in the following order: flavanone (44 μM) < 7-hydroxyflavone (33 μM) < 5,7-dihydroxyflavone (20 μM) < 5,6,7-trihydroxyflavone (16 μM). Matsuda et al. [9] reported the following structural requirements of flavonoids for higher activity: (1) the 2–3 double bonds of flavones and flavonols are essential for the activity; (2) the 3- or 7-glycoside moiety reduced the activity; (3) as the hydroxyl groups at the 3', 4', 5-, 6-, and 7-positions increased in number, the inhibitory activities become

stronger; (4) the flavonols with a pyrogallol-type moiety (the 3',4',5'-trihydroxyl groups) at the B ring exhibited less activity than those with a phenol-type moiety (the 4'-hydroxyl group) or catechol-type moiety (the 3',4'-dihydroxyl groups) at the B ring; (5) the activities of flavones were stronger than those of flavonols; and (6) methylation of flavonols at the 3-position reduced the activity. However, (7) several flavones and flavonols with the 4'- and/or 7-methoxyl groups did not follow rules (3), (4), and (5). There was no activity information on poly-substituted flavones before this report. Therefore, we should reconsider the multiple effects of some functional groups. Among compounds **6–10**, trihydroxyflavones with one methoxy substituent and dihydroxyflavones with trimethoxy substituents were certainly essential for the high activities, and the position of the hydroxyl and methoxy groups on the flavone molecule also affected the activities as seen from the evidence shown in Table 4.

### 3.4. Comprehensive antiallergic activities and toxicities of six flavones found in *Mentha × piperita citrata*

Cell cytotoxicity (lethal concentration 5% is expressed as LD<sub>5</sub> and it is defined as the limitation of a non-toxic concentration for RBL-2H3 cells) of antiallergic flavones against RBL-2H3, and the amounts in eau de cologne mint leaves are shown in Table 3. The IC<sub>50</sub> values of compounds **7** and **9** were at

**Table 3**Cell cytotoxicity and comprehensive antiallergic activities of compounds **6–10** in eau de cologne mint leaves.

| Sample                                                   | Cell cytotoxicity                 | Amount <sup>b</sup>                  |                              |                           | Total activity <sup>c</sup> |
|----------------------------------------------------------|-----------------------------------|--------------------------------------|------------------------------|---------------------------|-----------------------------|
|                                                          | LD <sub>5</sub> [μM] <sup>a</sup> | mg/100 mg of dichloromethane extract | mg/100 mg of ethanol extract | mg/kg of leaves           |                             |
| 5,6,4'-Trihydroxy-7,8-dimethoxyflavone ( <b>6</b> )      | >20                               | 2.270.07                             | 0.34 ± 0.03                  | 108.9 ± 7.1 <sup>c</sup>  | 49,058                      |
| 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone ( <b>7</b> )  | >20                               | 3.40 ± 0.14                          | 0.56 ± 0.04                  | 177.7 ± 9.7 <sup>b</sup>  | 204,333                     |
| 5,6-Dihydroxy-7,3',4'-trimethoxyflavone ( <b>8</b> )     | >20                               | 2.24 ± 0.09                          | 0.44 ± 0.01                  | 141.1 ± 2.6 <sup>c</sup>  | 73,536                      |
| 5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone ( <b>9</b> ) | >20                               | 6.33 ± 0.25                          | 0.88 ± 0.10                  | 278.0 ± 24.4 <sup>a</sup> | 246,070                     |
| 5,6-Dihydroxy-7,8,4'-trimethoxyflavone ( <b>10</b> )     | >20                               | 4.63 ± 0.11                          | 0.56 ± 0.07                  | 179.7 ± 16.2 <sup>b</sup> | 85,170                      |
| Luteolin ( <b>5</b> )                                    | 11.5 ± 0.8                        | –                                    | –                            | 56.1 ± 0.3 <sup>d</sup>   | 74,920                      |

Each value represents mean ± SD (n = 4). Means with different letters are significantly different (p < 0.05).

<sup>a</sup> Lethal concentration 5% (LD<sub>5</sub>) values is the dose required to kill 5% of RBL-2H3 cells and was calculated from dose–response curves after RBL-2H3 cells were exposed with tested samples for 48 h.

<sup>b</sup> Amount was calculated from the chromatograms of the dichloromethane extract and ethanol extract.

<sup>c</sup> Total activity was estimated using the following formula: total activity = amount [mg/kg of leaves] / IC<sub>50</sub> [mg/mL].

**Table 4**

Tendency of flavonoids with different antiallergic activities.

| Chemical                                        | Activity                    |                      | Functional groups |    |                  |                  |                  |                  | Number of functional groups |                  |              |              |          |
|-------------------------------------------------|-----------------------------|----------------------|-------------------|----|------------------|------------------|------------------|------------------|-----------------------------|------------------|--------------|--------------|----------|
|                                                 | IC <sub>50</sub> [ $\mu$ M] | 100/IC <sub>50</sub> | 5                 | 6  | 7                | 8                | 3'               | 4'               | OH                          | OCH <sub>3</sub> | OR in A-ring | OR in B-ring | Total OR |
| 5,7,3',4'-Tetramethoxyflavone                   | 68                          | 1.5                  | OCH <sub>3</sub>  | H  | OCH <sub>3</sub> | H                | OCH <sub>3</sub> | OCH <sub>3</sub> | 0                           | 4                | 2            | 2            | 4        |
| 5-Hydroxy-7-methoxyflavone                      | 49                          | 2.0                  | OH                | H  | OCH <sub>3</sub> | H                | H                | H                | 1                           | 1                | 2            | 0            | 2        |
| Flavanone                                       | 44                          | 2.3                  | H                 | H  | H                | H                | H                | H                | 0                           | 0                | 0            | 0            | 0        |
| 7-Hydroxyflavone                                | 33                          | 3.0                  | H                 | H  | OH               | H                | H                | H                | 1                           | 0                | 1            | 0            | 1        |
| 3',4'-Dihydroxyflavone                          | 30                          | 3.3                  | H                 | H  | H                | H                | OH               | OH               | 2                           | 0                | 0            | 2            | 2        |
| 5,7-Dihydroxy-8-methoxyflavone                  | 29                          | 3.4                  | OH                | H  | OH               | OCH <sub>3</sub> | H                | H                | 2                           | 1                | 3            | 0            | 3        |
| 5,3'-Dihydroxy-7,4'-dimethoxyflavone            | 28                          | 3.6                  | OH                | H  | OCH <sub>3</sub> | H                | OH               | OCH <sub>3</sub> | 2                           | 2                | 2            | 2            | 4        |
| 7,4'-Dihydroxyflavone                           | 22                          | 4.5                  | H                 | H  | OH               | H                | H                | OH               | 2                           | 0                | 1            | 1            | 2        |
| 5,7-Dihydroxyflavone                            | 20                          | 5.0                  | OH                | H  | OH               | H                | H                | H                | 2                           | 0                | 2            | 0            | 2        |
| 5-Hydroxy-7,3',4'-trimethoxyflavone             | 19                          | 5.3                  | OH                | H  | OCH <sub>3</sub> | H                | OCH <sub>3</sub> | OCH <sub>3</sub> | 1                           | 3                | 2            | 2            | 4        |
| 5,6,7-Trihydroxyflavone                         | 16                          | 6.3                  | OH                | OH | OH               | H                | H                | H                | 3                           | 0                | 3            | 0            | 3        |
| 7,3',4'-Trihydroxyflavone                       | 13                          | 7.7                  | H                 | H  | OH               | H                | OH               | OH               | 3                           | 0                | 1            | 2            | 3        |
| 5,6,4'-Trihydroxy-7,8-dimethoxyflavone (6)      | 6.7                         | 14.9                 | OH                | OH | OCH <sub>3</sub> | OCH <sub>3</sub> | H                | OH               | 3                           | 2                | 4            | 1            | 5        |
| 5,7,4'-Trihydroxyflavone                        | 6.1                         | 16.4                 | OH                | H  | OH               | H                | H                | OH               | 3                           | 0                | 2            | 1            | 3        |
| 5,6-Dihydroxy-7,8,4'-trimethoxyflavone (10)     | 6.1                         | 16.4                 | OH                | OH | OCH <sub>3</sub> | OCH <sub>3</sub> | H                | OCH <sub>3</sub> | 2                           | 3                | 4            | 1            | 5        |
| 5,6-Dihydroxy-7,3',4'-trimethoxyflavone (8)     | 5.6                         | 17.9                 | OH                | OH | OCH <sub>3</sub> | H                | OCH <sub>3</sub> | OCH <sub>3</sub> | 2                           | 3                | 3            | 2            | 5        |
| 5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone (9) | 3.0                         | 33.3                 | OH                | OH | OCH <sub>3</sub> | OCH <sub>3</sub> | OCH <sub>3</sub> | OCH <sub>3</sub> | 2                           | 4                | 4            | 2            | 6        |
| 5,7,3',4'-Tetrahydroxyflavone (5)               | 2.6                         | 38.5                 | OH                | H  | OH               | H                | OH               | OH               | 4                           | 0                | 2            | 2            | 4        |
| 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone (7)  | 2.4                         | 41.7                 | OH                | OH | OCH <sub>3</sub> | OCH <sub>3</sub> | OCH <sub>3</sub> | OH               | 3                           | 3                | 4            | 2            | 6        |
| 5,7,3'-Trihydroxy-4'-methoxyflavone             | 2.1                         | 47.6                 | OH                | H  | OH               | H                | OH               | OCH <sub>3</sub> | 3                           | 1                | 2            | 2            | 4        |
| Correlation coefficient value                   | –                           | 1.00                 | –                 | –  | –                | –                | –                | –                | 0.61**                      | 0.24             | 0.45*        | 0.49*        | 0.62**   |

IC<sub>50</sub> of chemicals other than compounds **5–10** are reference data [9]. OR: OH group and OCH<sub>3</sub> group.

Pearson's correlation coefficient between antiallergic activities and the number of functional groups.

\* Significantly different at  $p < 0.05$ .\*\* Significantly different at  $p < 0.01$ .

least 8.3 to 6.7 times lower than LD<sub>5</sub>. On the other hand, the IC<sub>50</sub> of luteolin was 4.4 times lower than LD<sub>5</sub>. The poly-substituted flavones (**6–10**) do not affect cell viability (LD<sub>5</sub>) at 20  $\mu$ M, but luteolin (**5**) suppressed cell viability to 95% at  $11.5 \pm 0.8 \mu$ M. Luteolin should be toxic for RBL-2H3 cells rather than the poly-substituted flavones. On the other hand, the amount of compounds **6** to **10** in the dichloromethane extract (2.2 – 6.3 mg/100 mg of the dichloromethane extract) is higher than that in the ethanol extract (0.3–0.8 mg/100 mg of the ethanol extract). Thus, isolating the powerful antiallergic poly-substituted flavones can be easily attained by dichloromethane extraction. The amount of each antiallergic poly-substituted flavone (**6–10**) in the leaves was in the range 108.9–278.0 mg/kg dry leaves (Table 3). The abundance in the leaves of the five flavones was also higher than luteolin (**5**,  $56.1 \pm 0.3$  mg/kg dry leaves). Finally, total activity was defined and expressed as the following unit:

Total activity unit

= (the amount of a flavonoid in leaves)/(IC<sub>50</sub> of a flavonoid).

Consequently, the total activity of 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone (**9**) was the highest (unit = 246,070) in units, followed by 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**7**, unit = 204,333) in the eau de cologne mint leaves. The total activities of both poly-substituted flavones were three times higher than luteolin (**5**, unit = 74,920), which is the most popular natural antiallergic substance.

Therefore, it is concluded that 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone (**9**) and 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone (**7**) are the most effective and useful main antiallergic substances in eau de cologne mint leaves.

So far it has been said that *Mentha piperita* L. has high antiallergic activity [26], but it has been reported that some simple flavonoids as active substances such as ericitrin, narirutin, hesperidin, luteolin 7-O-rutinoside, diosmin and rosmarinic acid were candidates for the activity. However, it was found that these simple flavonoids have comparatively weak activity as shown in Table 2. Some patents on peppermint extracts support the antiallergic activity [27,28]. Furthermore, flavonoids isolated from eau de cologne mint can be found in other mints [21,29,30]. However, the patents and papers did not report the active substances in those mints.

Moreover, as the powerful antiallergic substances, **6–10** with higher hydrophobicity are selectively found in dichloromethane extract, the separation of these substances from the mint family such as *Mentha aquatic*, *Mentha citrata*, and other mints might be easily attained by partition with dichloromethane and water of those plant extracts in future. Tomas-Barberan and Wollenweber (1990) reported that *Acinos*, *Calamintha*, *Mentha*, *Micromeria*, *Origanum*, *Satureja*, and *Thymus* in the Labiatae family contained 5,6-dihydroxy-7,8-dimethoxyflavones with some substituted B-rings as characteristic secondary metabolites [31]. *Salvia*, *Rosmarinas*, and *Ocimum* in other Labiatae plants contained 5,7-dihydroxy-6-methoxyflavones with some substituted B-rings as different type of flavonoids. Chemotaxonomic differentiation of flavonoids is observed in the Labiatae

family even though the authors did not show any evidence of antiallergic activity of those flavonoids. Moreover, nobiletin is a typical polymethoxyflavanone in rutaceous plants. IC<sub>50</sub> of nobiletin was nearly equal to 500  $\mu$ M [32]. Therefore, the antiallergic activity of this compound is fairly weak. So, the mint families might be good targets for antiallergic resources.

### Conflict of interest

The authors declare that they have no conflicts of interest with this study.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.fitote.2015.02.003>.

### References

- Izquierdo-Domínguez A, Valero AL, Mullol J. Comparative analysis of allergic rhinitis in children and adults. *Curr Allergy Asthma Rep* 2013;13:142–51.
- Kumar S, Verma AK, Das M, Dwivedi PD. Molecular mechanism of IgE mediated food allergy. *Int Immunopharmacol* 2012;13:432–9.
- Sugiura Y, Torii T, Matsuda K, Yamada Y. Anti-allergic effects of extracts from commercial products of cooked burdock. *Food Sci Technol Res* 2009;15:423–6.
- Chen BH, Wu PY, Chen KM, Fu TF, Wang HM, Chen CY. Antiallergic potential on RBL-2H3 cells of some phenolic constituents of *Zingiber officinale* (Ginger). *J Nat Prod* 2009;72:950–3.
- Matsuda H, Morikawa T, Managi H, Yoshikawa M. Antiallergic principles from *Alpinia galanga*: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF- $\alpha$  and IL-4 in RBL-2H3 cells. *Bioorg Med Chem Lett* 2003;13:3197–202.
- Zhu F, Asada T, Sato A, Koi Y, Nishiwaki H, Tamura H. Rosmarinic acid extract for antioxidant, antiallergic, and  $\alpha$ -glucosidase inhibitory activities, isolated by supramolecular technique and solvent extraction from *Perilla* leaves. *J Agric Food Chem* 2014;62:885–92.
- Shimoda H, Tanaka J, Yamada E, Morikawa T, Kasajima N, Yoshikawa M. Anti type I allergic property of Japanese butterbur extract and its mast cell degranulation inhibitory ingredients. *J Agric Food Chem* 2006;54:2915–20.
- Matsuda H, Tewtrakul S, Morikawa T, Nakamura A, Yoshikawa M. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF- $\alpha$  and IL-4 in RBL-2H3 cells. *Bioorg Med Chem* 2004;12:5891–8.
- Matsuda H, Morikawa T, Ueda K, Managi H, Yoshikawa M. Structural requirements of flavonoids for inhibition of antigen-induced degranulation, TNF- $\alpha$  and IL-4 production from RBL-2H3 cells. *Bioorg Med Chem* 2002;10:3123–8.
- Maeda-Yamamoto M, Ema K, Shibuichi I. In vitro and in vivo anti-allergic effects of 'benifuuki' green tea containing O-methylated catechin and ginger extract enhancement. *Cytotechnology* 2007;55:135–42.
- Sato A, Shinozaki N, Tamura H. Secoiridoid type of antiallergic substances in the pomace, wasting materials in three varieties of Japanese olive (*Olea europaea*). *J Agric Food Chem* 2014;62:7787–95.
- Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clin Exp Allergy* 2000;30:501–8.
- Ueda H, Yamazaki C, Yamazaki M. Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. *Biol Pharm Bull* 2002;25:1197–202.
- Park J, Kim SH, Kim TS. Inhibition of interleukin-4 production in activated T cells via down-regulation of NF-AT DNA binding activity by apigenin, a flavonoid present in dietary plants. *Immunol Lett* 2006;103:108–14.
- Murata K, Takano S, Matsuda M, Iinuma M, Matsuda H. Anti-degranulating activity in rat basophil leukemia RBL-2H3 cells of flavanone glycosides and their aglycones in citrus fruits. *J Nat Med* 2013;67:643–6.
- Asekun OT, Grierson DS, Afolayan AJ. Effects of drying methods on the quality and quantity of the essential oil of *Mentha longifolia* L. subsp. *Capensis*. *Food Chem* 2007;101:995–8.
- Kanatt SR, Chander R, Sharma A. Antioxidant potential of mint (*Mentha spicata* L.) in radiation-processed lamb meat. *Food Chem* 2007;100:451–8.
- Biswas AK, Chatli MK, Sahoo J. Antioxidant potential of curry (*Murraya koenigii* L.) and mint (*Mentha spicata*) leaf extracts and their effect on colour and oxidative stability of raw ground pork meat during refrigeration storage. *Food Chem* 2012;133:467–72.
- Arumugam P, Gayatri Priya N, Subathra M, Ramesh A. Anti-inflammatory activity of four solvent fractions of ethanol extract of *Mentha spicata* L. investigated on acute and chronic inflammation induced rats. *Environ Toxicol Pharmacol* 2008;26:92–5.
- Tassou C, Koutsoumanis K, Nychas GJE. Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. *Food Res Int* 2000;33:273–80.
- Areias FM, Valentao P, Andrade PB, Ferreres F, Seabra RM. Phenolic fingerprint of peppermint leaves. *Food Chem* 2001;73:307–11.
- Markham KR. Ultraviolet–visible absorption spectroscopy. In: Markham KR, editor. *Techniques of flavonoid identification*. New York: Academic Press Inc. Ltd; 1982. p. 36–51.
- Saito N, Ootani S. The color reaction of plant tissue extract. In: Hayashi K, editor. *Plant pigments — an introduction to research and experiments —*. Tokyo: Yokendo Ltd; 1980. p. 81–5.
- Siriwan D, Naruse T, Tamura H. Effect of epoxides and  $\alpha$ -methylene- $\gamma$ -lactone skeleton of sesquiterpenes from yacon (*Smallanthus sonchifolius*) leaves on caspase-dependent apoptosis and NF- $\kappa$ B inhibition in human cervical cancer cells. *Fitoterapia* 2011;82:1093–101.
- Trisonthi P, Sato A, Nishiwaki H, Tamura H. A new diterpene from *Litsea cubeba* fruits: structure elucidation and capability to induce apoptosis in HeLa cells. *Molecules* 2014;19:6838–50.
- Inoue T, Sugimoto Y, Matsuda H, Kamei C. Antiallergic effect of flavonoid glycosides obtained from *Mentha piperita* L. *Biol Pharm Bull* 2002;25:256–9.
- Setoguchi Y, Nakade Y, inventors; Sekisui Chemical Co. Ltd., assignee. Medical tape containing anti-I type allergic agent. Japan Kokai Tokkyo Koho JP 07 — 033648A. 1995 Feb 3.
- Watanabe K, inventor; Watanabe K, assignee. Supplement drink for pollinosis. Japan Kokai Tokkyo Koho JP 2001 — 348339A. 2001 Dec 18.
- Voirin B, Bayet C, Faure O, Jullien F. Free flavonoid aglycones as markers of parentage in *Mentha aquatic*, *M. citrata*, *M. spicata* and *M. × piperita*. *Phytochemistry* 1999;50:1189–93.
- Zaidi F, Voirin B, Jay M, Viricel MR. Free flavonoid aglycones from leaves of *Mentha pulegium* and *Mentha suaveolens* (Labiatae). *Phytochemistry* 1998;48:991–4.
- Tomas-Barberan FA, Wollenweber E. Flavonoid aglycones from the leaf surfaces of some *Labiatae* species. *Plant Syst Evol* 1990;173:109–18.
- Kobayashi S, Tanabe S. Evaluation of the anti-allergic activity of *Citrus unshiu* using rat basophilic leukemia RBL-2H3 cells as well as basophils of patients with seasonal allergic rhinitis to pollen. *Int J Mol Med* 2006;17:511–5.

# Supplementary data

**High antiallergic activity of 5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone and 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone from Eau de Cologne mint (*Mentha × piperita citrata*)**

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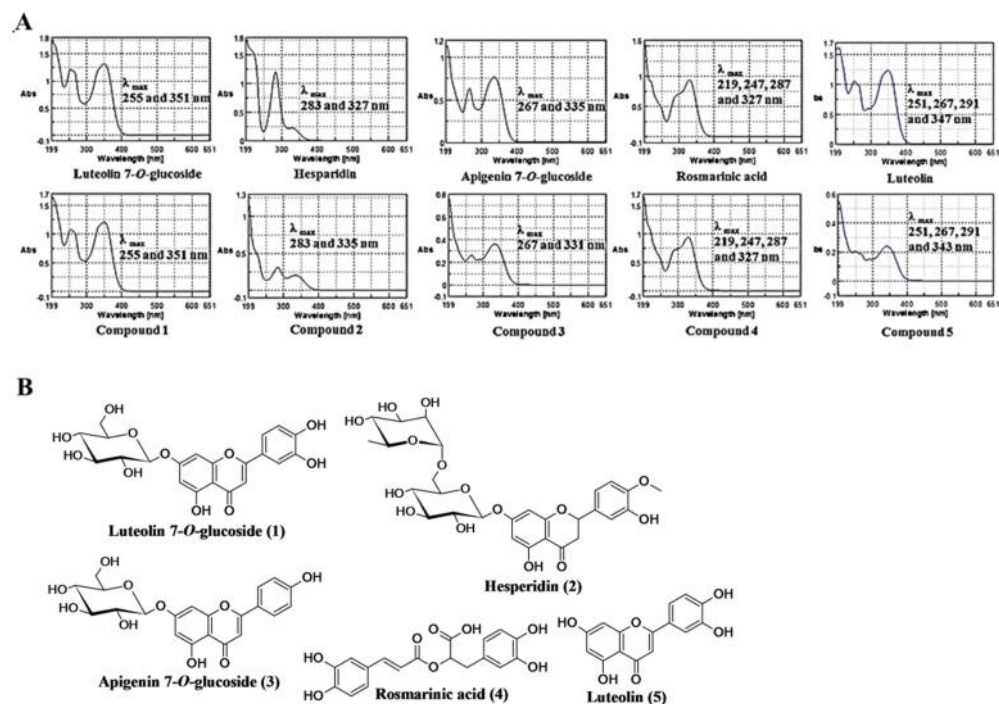


**Fig. S1. (A)** UV absorption spectra and **(B)** chemical structures of the antiallergic substances in Eau de Cologne mint from the PDA detector of HPLC analyses.  $\lambda_{\text{max}}$  of each compound is as follows: luteolin 7-*O*-glucoside, 255 and 351 nm; compound **1**, 255 and 351 nm; hesperidin, 283 and 327 nm; compound **2**, 283 and 335 nm; apigenin 7-*O*-glucoside, 267 and 335 nm; compound **3**, 267 and 331 nm; rosmarinic acid, 219, 247, 287, and 327 nm; compound **4**, 219, 247, 287, and 327 nm; luteolin, 251, 267, 291, and 347 nm; and compound **5**, 251, 267, 291, and 343 nm.

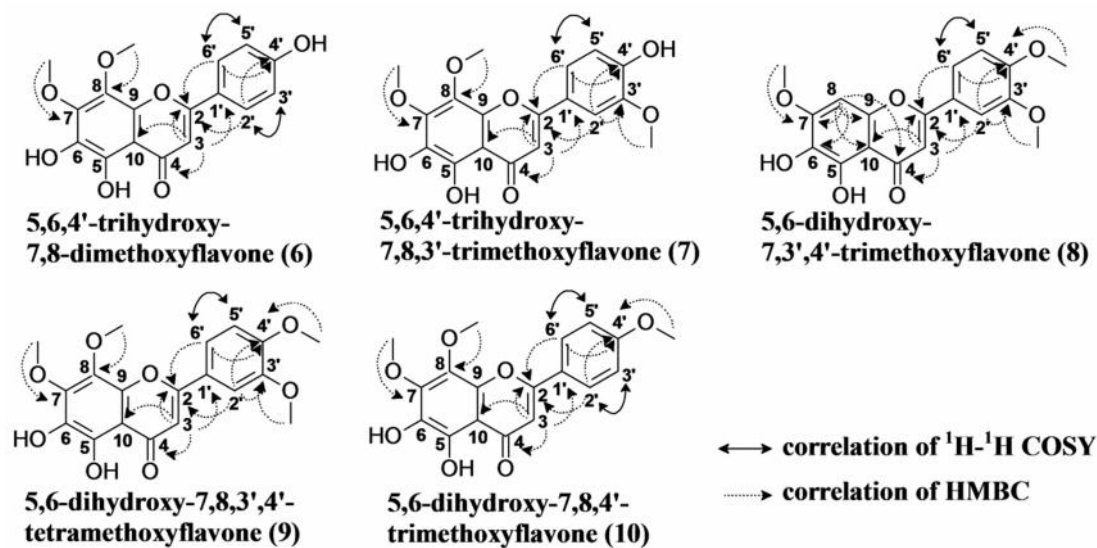
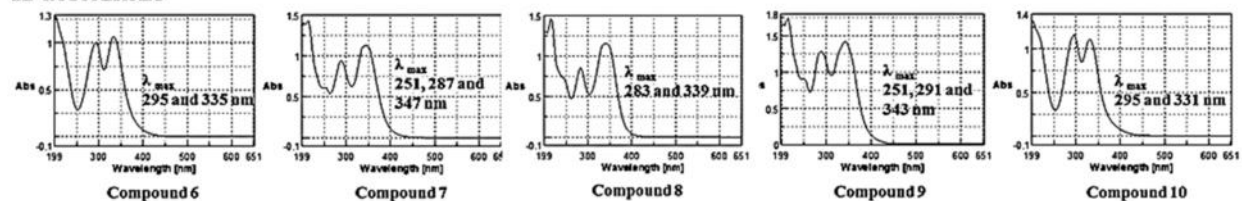
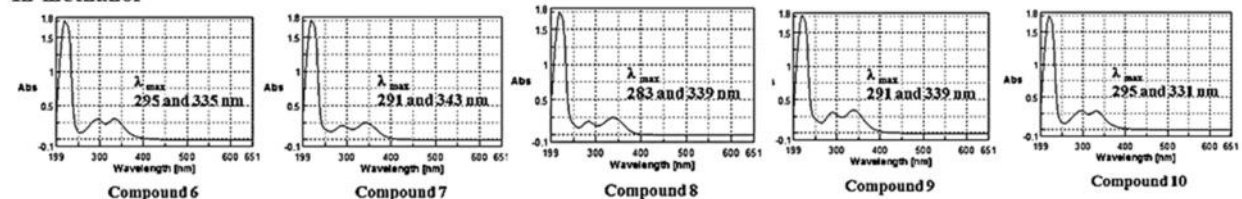


**Fig. S2.** Chemical structure of compounds **6**, **7**, **8**, **9**, and **10** and proton-proton and proton-carbon relationship using two-dimensional NMR analyses.

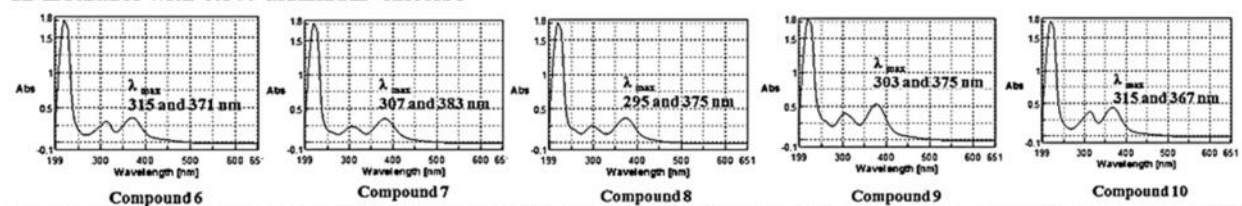
**In acetonitrile**



**In methanol**



**In methanol with 0.5% aluminum chloride**



**In methanol with 0.5% aluminum chloride and 0.1% hydrochloric acid**

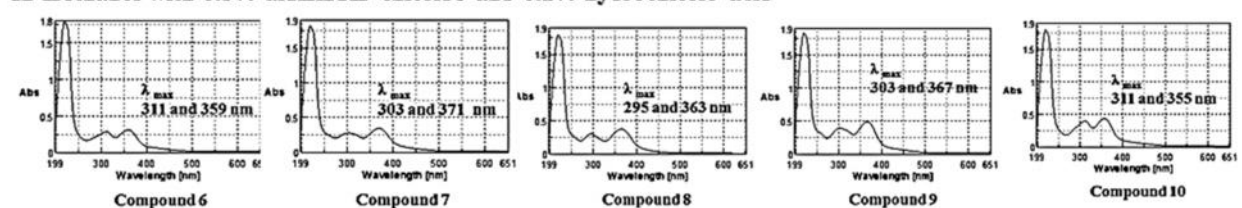


**Fig. S3.** UV absorption spectra of compounds **6**, **7**, **8**, **9**, and **10** under different solvent conditions.