The Contents of Polyphenols in *Perilla frutescens* (L.) Britton var. *frutescens* (Egoma) Leaves are Determined by Vegetative Stage, Spatial Leaf Position, and Timing of Harvesting during the Day

Yuba Raj Gaihre¹*, Keisuke Tsuge², Hiroshi Hamajima¹, Yasuo Nagata¹,³, and Teruyoshi Yanagita¹,⁴

¹ Saga Foods & Cosmetics Laboratory, Division of Research and Development Promotion, Saga Regional Industry Support Center, 114 Yaemizo, Nabeshima-machi, Saga 849-0932, JAPAN
² Industrial Technology Center of Saga, 114 Yaemizo, Nabeshima-machi, Saga 849-0932, JAPAN
³ Center for Industry, University and Government Cooperation, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, JAPAN
⁴ Department of Applied Biochemistry and Food Science, Saga University, 1-Honjo, Saga 840-8505, JAPAN

Abstract: The leaf of *Perilla frutescens* (L.) Britton var. *frutescens* (egoma) is a rich source of polyphenolic compounds, including rosmarinic acid. However, there is still a lack of detailed information concerning the content of phenolic compounds in these leaves. Since some flavonoids were found as a conjugated form, leaves were used untreated or hydrolyzed using β-glucuronidase for analysis. Enzymatic hydrolysis method successfully identified some polyphenols, which have not been reported before. Scutellarin, a flavone glucuronide with a molecular mass similar to that of luteolin 7-O-glucuronide, was present in egoma leaves. Scutellarin was the second most abundant polyphenolic compound, after rosmarinic acid. Egoma leaves at the top of the plant contained a higher amount of rosmarinic acid and scutellarin compared to that in the leaves below. The difference in plant growth stage also influenced the rosmarinic acid and scutellarin contents, while the time of harvesting during the day did rosmarinic acid contents only. This is the first time that scutellarin, a traditional Chinese medicine, widely used for the treatment of cerebrovascular disease, was quantitatively determined in egoma leaves. The present study may help adding value to egoma leaves, developing dietary supplements, functional foods, and cosmetics.

Key words: perilla, egoma, β-glucuronidase, rosmarinic acid, scutellarin

1 Introduction

*Perilla frutescens* (L.) Britton is an annual plant belonging to the mint family Lamiaceae and native to regions of the Himalayas up to eastern Asia. It is cultivated throughout China, Japan, South Korea, Vietnam, India, and Nepal¹,². *Perilla frutescens* (L.) Britton is classified into two varietal groups, var. *crispa* Deane for vegetable crops (Shiso in Japanese), and var. *frutescens* for oilseed crops (egoma in Japanese and Silam in Nepal)³. Leaves of egoma are used as fresh vegetable or processed for pickles, whereas red and green leaves of shiso are more often used in China and Japan for its medicinal properties, food flavoring, and fish garnish⁴. Egoma contains α-linolenic acid-rich oil, which is used as anti-inflammatory, but has many other medicinal applications. Its global demand as a dietary supplement has been increasing recently⁵.

Leaves as well as seeds of the mint family Lamiaceae are rich in rosmarinic acid and have been drawing wide attention as functional food. Polyphenols are present in various molecular structures and could provide diverse biomedical benefits. The main phenolic compounds reported in leaves in the mint family Lamiaceae are rosmarinic acid (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2 enoyl]oxypropanoic acid), glucoside and glucuronides of luteolin (2-(3,4-dihydroxyphenyl)-, apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one) and scutellarin (5,6,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one)⁴,⁶,⁷.

*Correspondence to: Yuba Raj Gaihre, Saga Foods & Cosmetics Laboratory, Division of Research and Development Promotion, Saga Regional Industry Support Center, 114 Yaemizo, Nabeshima-machi, Saga 849-0932, JAPAN*

E-mail: yubarajgaihre@gmail.com  ORCID ID: Yuba Raj Gaihre (https://orcid.org/0000-0003-2743-0791), Teruyoshi Yanagita (https://orcid.org/0000-0001-8187-2924), Yasuo Nagata (https://orcid.org/0000-0001-8995-1109)

Accepted February 8, 2021 (received for review October 17, 2020)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

http://www.jstage.jst.go.jp/browse/jos/  http://mc.manuscriptcentral.com/jjocs
However, there is still a lack of detailed quantitative information of other phenolic compounds except rosmarinic acid in the mint family Lamiaceae leaves, which are presumably influenced by the timing of harvesting during the day and plant growth.

Rosmarinic acid is a secondary metabolite in egoma plants, which has antioxidant, antiinflammatory, immune system enhancing, antiviral, antibacterial, hepatoprotective, antimicrobial, antiinociceptive, and antiinflammatory properties. Scutellarin (25S,3S,4S,5R,6S)-6-[5,6-dihydroxy-2-(4-hydroxyphenyl)-4-oxochromen-7-yl]oxy-3,4,5-trihydroxyoxan-2-carboxylic acid) has a broad range of cardiovascular benefits. Despite a limited number of reports on scutellarin content in the mint family Lamiaceae, no other quantitative result has been reported so far. We assume that other flavonoids are present, with a molecular weight similar to luteolin 7-O-glucuronide. Thus, β-glucuronidase could be used to hydrolyze conjugated form of polyphenols for analysis.

The aim of this study was to determine the most abundant phenolic compounds other than rosmarinic acid in egoma leaves, using enzymatic hydrolysis as a pretreatment step. Applying HPLC, the phenolic content was quantified at different plant growth stages, at specific harvesting times of the day, and using leaves of different spatial positions.

2 Materials and Methods

2.1 Materials

Leaves of egoma were obtained from Ecobito Farm & Company (Kanzaki, Japan). Rosmarinic acid and bovine-liver-derived β-glucuronidase were purchased from Sigma-Aldrich GK. (Tokyo, Japan). Scutellarin and scutellarein were obtained from Toronto Research Chemicals (Toronto, Canada) and Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), respectively.

2.2 Preparation of egoma leaf powder

Egoma leaves were rinsed in water, and subsequently blanched, simmering in boiling water (ratio of 1:5) for 10 s. The blanched leaves were drained of water and cooled to room temperature. All the blanched samples were vacuum-dried (EYELA vacuum oven, VOS-451SD, Tokyo Rikakikai Co., Ltd). The dried leaves were crushed and ground to make a leaf powder using a CMT Vibrating Sample Mill T1-100 (CMT Co., Ltd., Fukushima, Japan).

2.3 Egoma leaf extracts for quantification of phenolic compounds

An aliquot of egoma leaf powder (0.1 g) was extracted twice with 10 mL of solvent (acetone/water/acet acid (60/39.8/0.2 v/v), heated to 80°C for 1 h using a heat block (Dry Thermo unit DTU-2CN, Taitec, Saitama, Japan), and then centrifuged with a Kubota 5100 centrifuge (Kubota Corporation, Tokyo, Japan) at 1600 × g for 15 min. The combined extract was transferred to a measuring flask and filled up to 20 mL. The leaf extracts were filtered through a 0.45 μm filter for HPLC analysis.

2.4 Egoma leaf extracts for hydrolysis

An aliquot of leaf powder (0.1 g) was extracted twice with 10 mL of 80% ethanol, shaken at room temperature for 1 h using a tube mixer (CM-1000 Cute Mixer, EYELA, Tokyo, Japan), and then centrifuged with a Kubota 5100 centrifuge (Kubota Corporation, Tokyo, Japan) at 1600 × g for 15 min. The combined extract was poured into a measuring flask and filled up to 20 mL. The extract was then transferred to a tube and dried by centrifugal evaporation (MiVac Quatro Concentrator, Genevac Ltd., Ipswich, Suffolk, UK). The dried perilla extract was finally dissolved in 80% ethanol (50 mg/mL).

2.5 Hydrolysis of egoma leaf extracts

Fifty μL (50 mg/mL) of leaf extract and 50 μL of 100 U/mL of β-glucuronidase were mixed with 400 μL of an acetate buffer of pH 5.0 in a 2 mL screw-capped glass vial. After tightening the caps firmly, the vials were incubated at 37°C for 24 h. Then, the hydrolysis process was terminated, heating the vials up to 100°C for 5 min. The hydrolyzed samples were precipitated by adding 500 μL of 100% acetonitrile and then filtered through a 0.45 μm filter for HPLC analysis.

2.6 Determination of phenolic compounds

HPLC analysis was carried out with a Dionex™ UltiMate™ 3000 HPLC system (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA). Prior to the HPLC analysis, the egoma extracts were filtered through a 0.45 μm filter. Then, 3 μL of hydrolyzed and unhydrolyzed perilla leaf extracts were injected onto an analytical Unison UK-C18 (Intakt Crop 20 × 250 mm) column at 40°C. The mobile phases were composed of 0.1% (v/v) of formic acid in water (eluent A) and 0.1% (v/v) of formic acid in acetonitrile (eluent B). The gradient program was as follows: 10% B (0 min), 50% B (20 min), 100% B (21 to 27 min), and 10% B (28 min to 40 min). The total run-time was 40 min. Absorbance at 330 nm was measured to detect phenolic compounds. A standard with known retention time was used to calculate the phenolic content by comparing the peak area with that of the standard. The concentration of the standards ranged from 6.25 to 100 μg/mL.

2.7 Statistics

All values were expressed as means ± SE. Statistical analysis was performed by one-way ANOVA with Tukey-Kramer’s post hoc test, and a p-value < 0.05 was consid-
3 Results and Discussion

Leaves of egoma were collected at different spatial positions and growth stages of the plant. The growth cycle of perilla is defined as the period from leaf formation to leaf senescence. Classified growth stages were: early vegetative, vegetative, before flowering, during flowering, seed maturation time, and senescence. Phenolic compounds, extracted from egoma leaves with or without enzymatic hydrolysis, were analyzed with HPLC. Their chromatograms are shown in Fig. 1. Six peaks were detected before hydrolysis. One major peak was rosmarinic acid. We first attempted to identify unknown phenolic compounds using available standards, comparing retention times, UV and LC-MS spectra of the leaf extracts. Four peaks in the unhydrolyzed samples disappeared and three new peaks of aglycon (apigenin, luteolin, and scutellarein) developed after hydrolysis. This suggests that polyphenolic compounds in egoma leaves consist mainly glucuronides of apigenin, luteolin, and scutellarein. Scutellarein was noticeable as the most abundant aglycon obtained after hydrolyzation.

Scutellarin is a flavone glucuronide predominantly detected in *Erigeron breviscapus* (vant.) Hand. Mazz., utilized in pharmacological applications such as antioxidants, anti-inflammatory, or antitumor agents\(^1\), \(^2\). Scutellarin has also been used for clinical treatments of cerebrovascular and cardiovascular diseases, such as strokes, and exerts protective effects on brain ischemia or ischemia/reperfusion\(^3\), \(^4\). This suggests that egoma leaves extracts could also be used as supplements to prevent cardiovascular diseases.

Quantitative analysis of rosmarinic acid and scutellarin in leaves of different plant growth stages was performed with HPLC, as presented in Fig. 2. The highest rosmarinic acid content (81.9 ± 2.4 mg/g dry weight (dw)) was determined at the time of flowering, while a significantly higher scutellarin content was analyzed at the early vegetative stage. We observed further that the total polyphenol content was highest during flowering time (data not shown), when polyphenols act as antioxidants to protect plants from insects and oxidative stress\(^5\). It is further suggested that polyphenol synthesis is induced in leaves during the reproductive stage.

Rosmarinic acid and scutellarin remained in senescent leaves at the time of harvesting the seeds. The amounts detected were 53.3 ± 3.3 mg/g dw and 14.2 ± 0.45 mg/g dw,
respectively, indicating that senescent egoma leaves could also be an alternative source of health-promoting supplements.

Specific spatial leaf positions seem to play a vital role in accumulating polyphenolic compounds during the vegetative stage. As shown in Fig. 3, the levels of rosmarinic acid in the first pair of leaves at the top of the plant (66.7 ± 1.04 mg/g dw) were higher than those of the third and fourth pairs of leaves. The first pair of leaves had higher scutellarin levels (17.7 ± 0.24 mg/g dw) than those of the second, third, and fourth pairs. Thus, regular harvesting of the upper fully-grown leaves during the vegetative stage resulted in egoma leaves rich in rosmarinic acid and scutellarin.

To determine the best harvesting time of the day, the first pair of leaves was picked four times daily, continuously for three days. The effect of harvesting time on accumulated phenolics is presented in Fig. 4. The rosmarinic acid levels were higher in the samples collected at 06:00 am and 10:00 am compared to those collected at 02:00 pm and 06:00 pm. In contrast, no harvesting time effect was found for scutellarin. The contents of rosmarinic acid and scutellarin in the mornings were 51.4 ± 3.79 mg/g dw and 16.9 ± 0.58 mg/g dw, respectively, and decreased to 42.1 ± 3.97 mg/g dw and 13.0 ± 1.43 mg/g dw, respectively, in the afternoon. This indicates that perilla leaves should be picked in the morning to obtain higher amounts of phenolic compounds.

4 Conclusion

Scutellarin is the second most abundant polyphenolic compound in egoma leaves. Glucuronides of apigenin and luteolin were also present. Higher amounts of rosmarinic acid and scutellarin, both of which are known to have physiological properties such as anti-inflammatory action, were detected in the upper leaves during the vegetative stage, with a higher scutellarin content observed at the
early vegetable stage. The content of rosmarinic acid was highest at the time of flowering. Scutellarin and rosmarinic acid extracts may find potential value adding applications as dietary supplements, functional foods, or as cosmetic ingredient.

Acknowledgements
We would like to thank the Ecobito Farm & Company (Kanzaki, Japan) for providing the leaf of *Perilla frutescens* (L.) Britton var. *frutescens* (egoma).

Conflict of Interest
The authors declare no competing financial interests.

References
1) Hu, Y.; Sun, L.W.; Neo, M.C.; Zhang, Y.X.; Wen, C.X.; Xie, X.L.; Liu, Y.J. Primary identifications and palynological observations of *Perilla* L. in China. *J. Syst. Evol.* 48, 133-145 (2010).
2) Zhou, X.J.; Yan, L.L.; Yin, P.P.; Shi, L.L.; Zhang, J.H.; Liu, Y.J.; Ma, C. Structural characterization and antioxidant activity evaluation of phenolic compounds from cold-pressed *Perilla frutescens* var. arguta seed flour. *Food Chem.* 164, 150-157 (2014).
3) Nitta, M.; Ohnishi, O. Genetic relationships among two perilla crops, shiso and egoma, and the weedy type revealed by RAPD markers. *Genes Genet. Syst.* 74, 43-48 (1999).
4) Meng, L.; Lozano, Y.F.; Gaydou, E.M.; Li, B. Antioxidant activities of polyphenols extracted from Perilla. *Molecules* 14, 133-140 (2009).
5) Lu, N.; Bernardo, E.L.; Tippayadarapanich, C.; Takagaki, M.; Kagawa, N.; Yamori, W. Growth and accumulation of secondary metabolites in perilla as affected by photosynthetic photon flux density and electrical conductivity of the nutrient solution. *Front. Plant Sci.* 8, 708 (2017).
6) Asif, M. Phytochemical study of polyphenols in *Perilla frutescens* as an antioxidant. *Avicenna J. Phytomed.* 2, 169-178 (2012).
7) Lee, Y.H.; Kim, B.; Kim, S.; Kim, M.S.; Kim, H.; Hwang, S.R.; Kim, K.; Lee, J.H. Characterization of metabolite profiles from the leaves of green perilla (*Perilla frutescens*) by ultra-high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry and screening for their antioxidant properties. *J. Food Drug Anal.* 25, 776-788 (2016).
8) Mikami-Konishide, I.; Murakami, S.; Nakanishi, K.; Takahashi, Y.; Yamaguchi, M.; Shiyoa, T.; Watanabe, J.; Hino, A. Antioxidant capacity and polyphenol content of extracts from crops cultivated in Japan, and the effect of cultivation environment. *Food Sci. Technol. Res.* 19, 69-79 (2013).
9) Uritu, C.M.; Mihai, C.T.; Stanciu, G.D. Medicinal plants of the family Lamiaceae in pain therapy: A review. *Pain Res. Manag.* 2018, 7801543 (2018).
10) Hase, T.; Shirshido, S.; Yamamoto, S.; Yamashita, R.; Nukima, H.; Taira, S.; Toyoda, T.; Abe, K.; Hamaguchi, T.; Ono, K.; Noguchi-Shinohara, M.; Yamada, M.; Kobayashi, S. Rosmarinic acid suppresses Alzheimer’s disease development by reducing amyloid β aggregation by increasing monoamine secretion. *Sci. Rep.* 9, 8711 (2019).
11) Wang, L.; Ma, Q. Clinical benefits and pharmacology of Scutellarin: A comprehensive review. *Pharmacol. Ther.* 190, 105-127 (2018).
12) Zhu, J.; Chen, L.; Qi, Y.; Feng, J.; Zhu, L.; Bai, Y.; Wu, H. Protective effects of *Erigeron brevisscapus* Hand.-Mazz. (EBHM) extract in retinal neurodegeneration models. *Mol. Vis.* 24, 315-325 (2018).
13) Wang, W.; Ma, X.; Han, J.; Zhou, M.; Ren, H.; Pan, Q.; Zheng, C.; Zheng, Q. Neuroprotective effect of scutellarin on ischemic cerebral injury by down regulating the expression of angiotensin-converting enzyme and AT1 receptor. *PLoS ONE* 11, e0146197 (2016).
14) Gao, J.; Chen, G.; He, H.; Liu, C.; Xiong, X.; Li, J.; Wang, J. Therapeutic effects of breviscapine in cardiovascular diseases: A review. *Front. Pharmacol.* 8, 289 (2017).
15) Suwa, R.; Tajima, H.; Gima, S.; Uehara, N.; Watanabe, K.; Yabuta, S.; Toninag, J.; Kawamitsu, Y. Polyphenol production in *Peucedanum japonicum* Thunb. varies with soil type and growth stage. *Hort. J.* 87, 382-388 (2018).