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Variation of the Contents of Triterpenoids and Tannins Depending on Growth and Infection in the Leaves of *Rubus crataegifolius* and *Rubus parvifolius*

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Abstract – Several 19α-hydroxyursane-type triterpenoids and hydrolysable tannins have beneficial effects on human health. *Rubus crataegifolius* (Rosaceae) has the cleft simple leaf whereas *R. parvifolius* has pinnate compound leaves. This research was aimed to find the variation in the contents of the triterpenoids and tannins between the infected versus uninfected leaves of *R. coreanus* and *R. parvifolius* and between young versus mature leaves. Triterpenoids and tannins were quantitatively analyzed by HPLC. Six triterpenoids including tormentic acid, euscaphic acid, 23-hydroxytormentic acid, coreanoside F_1, kaji-ichigoside F_1 and niga-ichigoside F_1 were used for standard compounds. Gallotannins and ellagitannins were quantitatively evaluated using the indicatives of methyl gallate and ellagic acid. The infected leaves of *R. crataegifolius* contained higher levels of triterpenoids and tannin than the uninfected leaves; however, lower quantity of total tannin was observed in the mature leaves than in the young leaves. Although the pinnate compound leaves of *R. parvifolius* exhibited similar tendency of those compositional variation with *R. crataegifolius* each other, its contents of triterpenoids do not considerably vary. Variation of the contents of triterpenoids and tannins were particularly distinct in *R. crataegifolius* by growth and infection.

Keywords – *Rubus crataegifolius*, *Rubus parvifolius*, Rosaceae, Triterpenoid, Tannin, HPLC

Introduction

The edible fruits of *Rubus crataegifolius* and *Rubus parvifolius* (both Compositae family) have been used as functional foods or medicinal herbs for treatment of diarrhea, diabetes mellitus and sexual disinclination.[1] *Rubus coreanus* was previously reported on anti-nociceptive and anti-inflammatory effects[2] and anti-rheumatic and gastro-protective effects.[3] The bioactive compounds responsible for those effects are niga-ichigoside F_1 and its aglycone 23-hydroxytormentic acid. Those compounds isolated from *R. crataegifolius* and *R. parvifolius* also exert the same functional activity.

Although the two plants are used as resources of red raspberry, the morphology is quite different each other. *R. crataegifolius* which is an upright perennial herb has the cleft simple leaf. By contrast, *R. parvifolius* which is a creeping perennial plant has the pinnate compound leaf with each three leaflets. Higher plants accumulate a large number of different primary metabolites such as amino acids, fats and carbohydrates as well as secondary metabolites such as phenolics, terpenoids and alkaloids.[4] The plants respond to a variety of stresses by producing many secondary metabolic compounds that can exert defensive actions against predation, competition and diseases.[5] For example, plants that are under attack often produce and accumulate secondary metabolites known as phytoalexins, which act as toxins to the attacking organisms.[5]

The niga-ichigoside F_1 may be synthesized from the precursor tormentic acid via 23-hydroxytormentic acid in the leaves of *Rubus* species. These triterpenoids have the 3β-OH group, whereas euscaphic acid and kaji-ichigoside F_1 have 3α-OH group, as shown in Fig. 1. The biosynthesis and accumulation of the triterpenoids with 3α- or 3β-OHs may increase under severe stresses. As hydrolyzable tannins generally include gallotannin and ellagitannin, methyl gallate and ellagic acid can be used for quantitative indices of total tannin.[6] Tannins, polyphenolic compounds, are mainly located in the vacuoles or surface wax of the plants. These sites are where tannins do not interfere with plant metabolism, and it is only after cell breakdown and death that the tannins are active in metabolic effects during plant-pathogen interaction.[5] In

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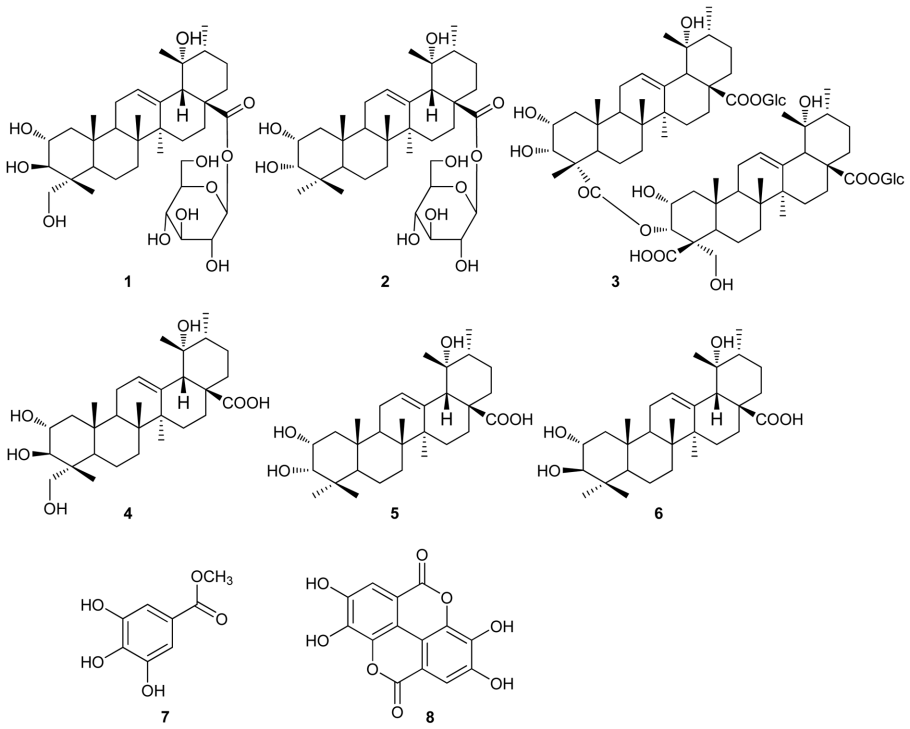

Fig. 1. Structure of triterpenoids, ellagic acid, and methyl gallate for HPLC analysis.
Niga-ichigoside F_1 (**1**), kaji-ichigoside F_1 (**2**), coreanoside F_1 (**3**), 23-hydroxytormentic acid (**4**), euscaphic acid (**5**), tormentic acid (**6**), methyl gallate (**7**), and ellagic acid (**8**).

addition, they are an important concentration determinant in growing plant parts such as the secondary vascular tissue and the layer between the cortex and epidermis.[7]

Leaf spot caused by the fungus *Mycosphaerella fragariae* is one of the most common and widespread diseases of *Rubus* species in Korea and strawberry in America. Since many major cultivars are not completely resistant to leaf spots, it is the most agriculturally important disease. The fungus *Colletotrichum acutatum*, a causal agent of a notorious fruit rot known as anthracnose, also attacks the leaves and petioles of *Rubus* plants.[8,9] In this study, we compared the quantitative changes of secondary metabolites in the infected versus the uninfected leaves and young versus mature leaves.

Experimental

Instruments and Reagents – HPLC was performed using Varian Prostar 210 solvent delivery module, Prostar 325 UV-Vis detector, and 20 μL sample loop. Separation was achieved on Shiseido Capcell Pak C18 column (5 μL, 250 mm × 4.6 mm I.D.). All the solvents used for analysis were HPLC grade. The six triterpenoids used for the present analysis are the compounds that have been isolated from *R. coreanus* (Choi et al., 2003; Nam et al., 2006). The structures are shown in Fig. 1.

Plant material – The *Rubus crataegifolius* (natchem# 46) and *Rubus parvifolius* (natchem# 48) were collected over June - July on a mountain near Sangji University in Wonju city, Gangwon-do, Korea. The voucher specimens (natchem #46 and 48) were identified by Prof. Sang-Cheol Lim (Dept. of Horticulture and Landscape Artchtecture, Sangji University, Korea). The collected leaves were divided into four classes as follows; young leaves (less than 4.0 × 3.3 cm), mature leaves (less than 11.1 × 9.7 cm), uninfected- and infected leaves of the mature ones. Here, infected- and uninfected leaves are designated as the mature leaves with and without leaf spots, respectively. The mature leaves highly damaged by the leaf spot were chosen for the infected leaves; but, clean leaves without leaf spots were chosen for the uninfected leaves. They were dried and pulverized for HPLC analysis.

Extraction – One gram of the pulverized plant material was sonicated in methanol (MeOH) (40 mL) at 40 ºC, filtered, concentrated to dryness in a rotatory centrifuge evaporator, and further dried by a freeze-dryer. The concentrates were weighed and diluted for HPLC analysis.

HPLC conditions triterpenoid quantification – The samples and standard compounds were dissolved in 80% aqueous MeOH and the mixture was filtered through a

0.50 μm syringe for injection. The UV detector was fixed at 206 nm. The mobile phase was a mixed solvent of 1.25% phosphoric acid (solvent A) - MeOH (solvent B) in water (solvent A : solvent B = 30 : 70). The HPLC was run for 30 min at a flow rate of 0.70 mL/min. Linear calibration curves were generated at 100, 250, 500 and 1000 μg/mL (R^2 = 0.999). Each calibration curve equation and retention time (t_R) are as follows: niga-ichigoside F_1 (**1**, $y = 162.1x - 6469$, t_R 4.6 min), kaji-ichigoside F_1 (**2**, $y = 108.2x + 1784$, t_R 6.3 min), coreanoside F_1 (**3**, $y = 116.8x - 6144$, t_R 7.1 min), 23-hydroxytormentic acid (**4**, $y = 356.9x - 24495$, t_R 9.2 min), euscaphic acid (**5**, $y = 110.4x - 1290$, t_R 21.4 min) tormentic acid (**6**, $y = 89.85x - 1439$, t_R 22.7 min), where x is concentration (μg/mL) and y is peak area. The equations were determined from the peak areas measured at 100, 200, 500, and 1,000 ml of each compounds.

HPLC analysis for total tannin – Total tannin was quantitated using the indicators, ellagic and gallic acid following the treatment with anhydrous methanolic HCl as previously described.[6] Using this method, HPLC was performed for analysis of total tannin. Twenty mg of ground samples was extracted in a screw-cap tube (10 mL) containing 5 mL anhydrous methanolic HCl. The tubes were placed in Muti-Position (Lab. Tech) containing reflux condenser at 100 °C for 60 min. The mixtures were cooled to room temperature and filtered (0.50 μm). The filtrates were evaporated to dryness and the residues were dissolved again in 5 mL of 80% aqueous methanol. The filtrate was injected into the same HPLC column used for triterpenoid analysis. Ellagic acid and methyl gallate levels were detected at the wavelength of 252 nm and 280 nm, respectively.

The mobile phase was methanol (solvent A) and 0.2% aqueous trifluoroacetic acid (solvent B). The gradient was: 0 min, 0% A : 100% B; 0 - 40 min, 100% A : 0% B; 40 - 41 min, 0% A : 100% B; 41 - 50 min, 0% A : 100% B. Run time was 40 min and the flow rate was 0.75 mL min^{-1}. The two standard compounds dissolved in 80% aqueous methanol were used to plot the calibration curve (R^2 = 0.999). Each calibration curve equation and retention time (t_R) are as follows: methyl gallate (**7**, $y = 408.3x + 512.6$, t_R 18.5 min), ellagic acid (**8**, $y = 3238x + 4347$, t_R 27.0 min).

Results and Discussion

This research was aimed to find the difference of the contents of triterpenoids and tannins between the leaves of *R. crataegifolius* with the cleft simple leaf and *R. parvifolius* with the pinnate compound leaf. In addition, Rubus plants are known to be infected by two types of fungi, *M. fragariae* and *C. acutatum*; leaf spot infected by the fungus *M. fragariae* is one of the most common in Korea. Therefore, the leaves with the leaf spot due to the tissue injury were also subjected to HPLC analysis.

The types of triterpenoids and hydrolysable tannins that were examined are shown in Fig. 1, and all these compounds were clearly resolved and identified on HPLC chromatograms (Fig. 2 and 3). The resulting quantitative data are shown in Table **1** and **2**. We would target the size of the collected leaves according to the degrees of infection and maturity as judged by the sizes of the collected leaves: the uninfected and infected leaves; mature (less than 7.9 × 8.0 cm) and young (less than 5.4 × 5.0 cm) leaves.

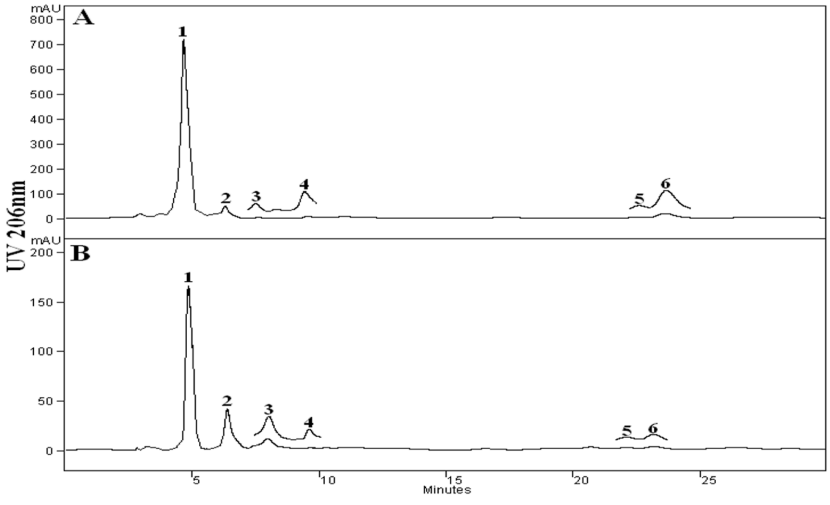

Fig. 2. HPLC chromatogram of triterpenoids in mature leaves of *R. crataegifolius* (A) and *R. parvifolius* (B).

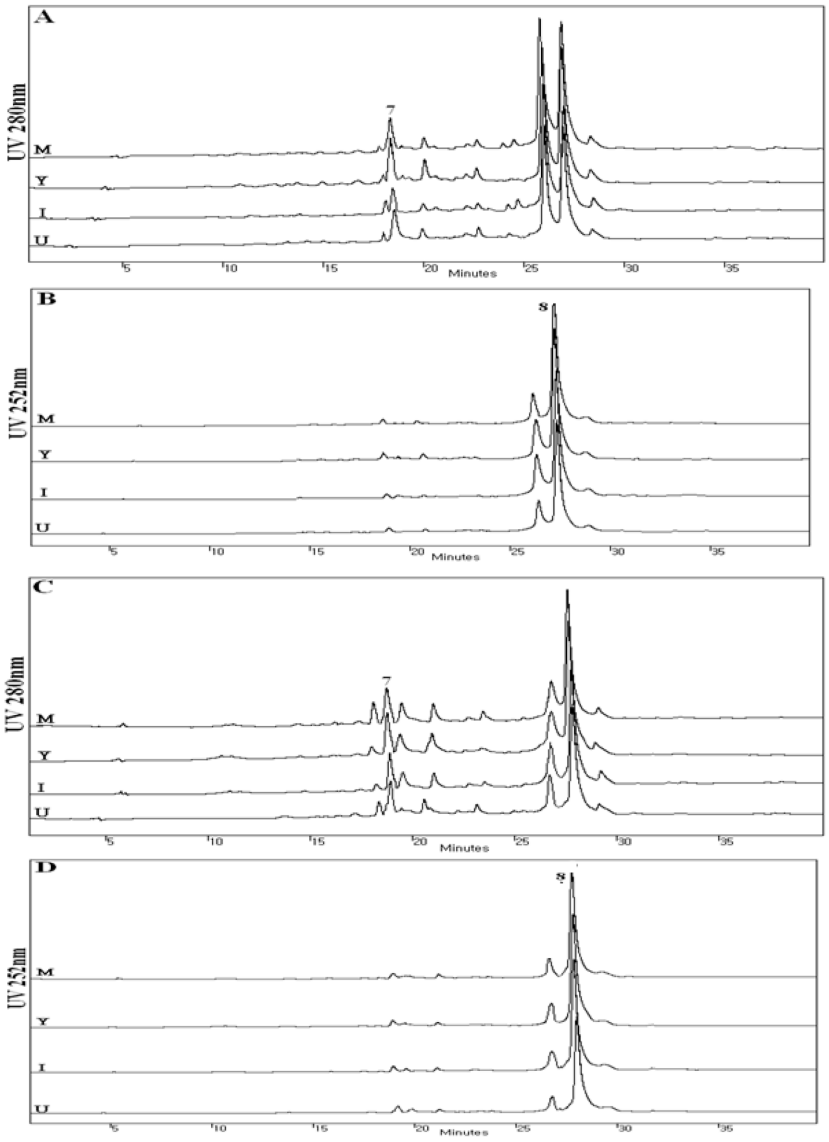

Fig. 3. HPLC chromatogram of methyl gallate (A, C) and caffeic acid methyl ester and ellagic acid (B, D) in *R. crataegifolius* (A, B) and *R. parvifolius* (C, D) leaves.

It is notable that niga-ichigoside F_1 content was significantly higher than any other triterpenoid contents and that *R. crataegifolius* accumulated much higher levels of total triterpenoid than *R. parvifolius* (Table 1). Infection resulted in about 2-fold increase in glycoside in the leaf of *R. crataegifolius*; the relative percentages of niga-ichigoside F_1 in uninfected leaves (23.17 mg/g niga-ichigoside F_1 of 47.5 mg/g total triterpenoids) versus infected ones (40.22 mg/g niga-ichigoside F_1 of 55.8 mg/g total triterpenoids) were 48.8 : 72.0 (Table 1 and Fig. 4). *R. crataegifolius* also showed a significant difference in niga-ichigoside F_1 quantity between the mature and young leaves, but *R. parvifolius* did not (Table 1). In *R. crataegifolius*, infection of the leaf was accompanied by the simultaneous increase in niga-ichigoside F_1 and decrease in tormentic acid, a precursor of niga-ichigoside F_1, On the other hand, maturation of the leaf led to the increased accumulation of both niga-ichigoside F_1 and tormentic acid. These results suggest that the accumulated tormentic acid is rapidly consumed to synthesize more niga-ichigoside F_1 during the infection, whereas the pool of tormentic acid is steadily increased during developmental maturation. In the leaves of *R. parvifolius*, the contents of triterpenoid glycoside were affected not by growth but by infection.

Gallotannins and ellagitannins have been isolated from *Rubus* species.[10] As gallic acid or hexahydroxydiphenic acid are generally esterified to monosaccharides to form tannins, the tannins of this type can produce methyl gallate

Table 1. Triterpenoid quantity (mg/g) in the leaves of *R. crataegifolius* and *R. parvifolius*

Variety	Group	$NigaF_1$	$KajiF_1$	$CorF_1$	Sum	23-HTA	EA	TA	Sum
		Glycoside				Genin			
R. crataegifolius	Mature	50.01 ± 0.62[a,b]	0.53 ± 0.07	2.37 ± 0.27	52.90 ± 0.85	3.06 ± 0.29	1.03 ± 0.12	4.64 ± 0.39	8.73 ± 0.53
	Young	37.43 ± 0.78	7.13 ± 0.26	2.79 ± 0.16	47.35 ± 0.77	3.03 ± 0.19	0.83 ± 0.12	1.25 ± 0.46	5.11 ± 0.76
	Infected	40.22 ± 0.90	5.38 ± 0.37	4.32 ± 0.15	49.91 ± 0.91	3.51 ± 0.51	nd[c]	2.37 ± 0.06	5.88 ± 0.57
	Uninfected	23.17 ± 0.09	0.01 ± 0.01	2.77 ± 0.46	25.95 ± 0.46	3.07 ± 0.05	3.06 ± 0.30	15.38 ± 0.26	21.51 ± 0.36
R. parvifolius	Mature	10.22 ± 0.53	2.76 ± 0.27	4.16 ± 0.30	17.14 ± 1.06	2.86 ± 0.21	0.93 ± 0.07	1.47 ± 0.28	5.26 ± 0.42
	Young	9.08 ± 0.49	tr[d]	3.46 ± 0.39	12.54 ± 0.72	2.84 ± 0.25	1.88 ± 0.45	3.53 ± 0.44	8.25 ± 0.99
	Infected	8.75 ± 0.58	tr	3.20 ± 0.10	11.95 ± 0.52	2.81 ± 0.15	1.57 ± 0.20	4.33 ± 0.23	8.70 ± 0.13
	Uninfected	7.71 ± 0.33	nd	3.96 ± 0.27	11.67 ± 0.58	nd	2.04 ± 0.13	3.04 ± 0.58	5.08 ± 0.62

Abbreviation : $NigaF_1$, Niga-ichigoside F_1; $KajiF_1$, Kaji-ichigoside F_1; $CorF_1$, Coreanoside F_1; 23-HTA, 23-hydroxytormentic acid; EA, Euscaphic acid; TA, Tormentic acid.
[a]Values represent mean ± S.D. based on three experiments. [b] Reported in mg/g. [c] not detected.
[d]trace (peak have not integral value at 206 nm).

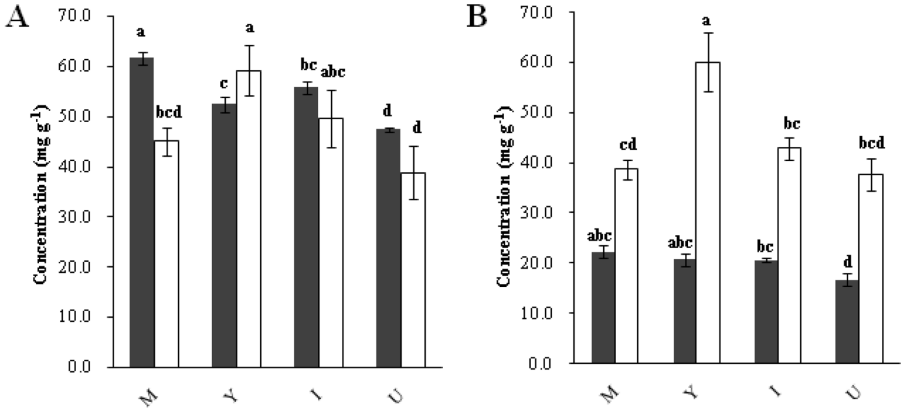

Fig. 4. Comparison of total content of triterpenoids and tannins in the *R. parvifolius* (**A**) and *R. crataegifolius* (**B**).
■, total triterpenoid; □, total tannin; abbreviation M (mature leaves), Y (young leaves), I (infected leaves), U (uninfected leaves).
The value of concentration is expressed mean ± S.D. for three experiments. Concentrations with the same letter on the bar are not significantly different each other ($p < 0.05$) by Duncan's multiple range test among black bars or among the white bars

or ellagic acid on methanolysis. Thus, total tannin can be quantified using the markers of ellagic acid and methyl gallate capable of being produced by methanolysis.[6] As shown in Table 2, no considerable changes in ellagitannin concentrations were observed between each of the groups. On the other hand, marked differences in gallotannin concentrations were found except for the infected and uninfected groups of *R. parvifolius*. Unlike the mature leaves, the infected leaves contained higher level of methyl gallate than the uninfected leaves in *R. crataegiolius*, suggesting that the infection also leads to an increased accumulation of tannin. However, the increase of tannin content followed by tissue infection was very weak in the leaves of *R. parvifolius*.

The two plants have quite different morphology: *R. craetaegifolius* is an upright plant with the cleft simple leaves and *R. Parvifolius* is a creeping plant with pinnate compound leaves. Variations of the contents were more significant in *R. crataegifolius* than in *R. parvifolius*. The

Table 2. Total tannin quantity (mg/g) in the leaves of *R. crataegifolius* and *R. parvifolius*

Variety	Group	Methyl gallate	Ellagic acid
R. crataegifolius	Mature	8.35 ± 0.03[a,b]	32.19 ± 0.49
	Young	12.83 ± 0.77	37.21 ± 1.50
	Infected	12.87 ± 0.34	30.12 ± 1.64
	Uninfected	6.42 ± 0.31	26.41 ± 1.16
R. parvifolius	Mature	9.02 ± 0.30	26.43 ± 1.07
	Young	13.72 ± 0.69	39.72 ± 1.78
	Infected	7.78 ± 0.06	30.49 ± 0.55
	Uninfected	7.53 ± 0.22	26.37 ± 0.30

[a]Values represent mean ± S.D. based on three experiments.
[b]Reported in mg/g

different morphology may affect the tendency of responses to the pathogen. Thus, taken all the results together, the developmental stage may influence the levels of gallotanninin with a similar accumulation tendency in the two *Rubus* species. Ellagic and gallic acid play an important role in human nutrition, along with antioxidant,[11] anti-inflammatory,[12] anticancer and anti-atherosclerotic[13] effects. Recently, the simultaneous quantification of gallic and ellagic acid also has been used for routine quality evaluation of herbal raw materials[14] with respect to free radical scavenging, lipid peroxidation inhibition, and bacteriocidal activities.[15]

In conclusion, the cleft simple leaves of *R. crataegifolius* displayed an accumulation tendency of triterpenoid and tannin depending on developmental stage or infection. Although the pinnate compound leaves of *R. parvifolius* exhibited similar tendency of those compositional variation with *R. crataegifolius*, the contents of triterpenoids do not considerably vary.

Acknowledgments

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