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## Quercetin 3–glucoside and Quercetin 3–rhamnoside Found in the Reddish–purple Blotch Areas of *Rhododendron simsii* Planch. Flowers

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The flowers of *Rhododendron simsii* have reddish–purple blotches in the upper inside of the red petals. HPLC analysis indicated that two major flavonols present in the blotch areas besides anthocyanins. Quercetin 3–glucoside and quercetin 3–rhamnoside were identified by co–chromatographic TLC, HPLC and LC–MS analyses.

**Key words:** Flavonols, quercetin 3–glucoside, quercetin 3–rhamnoside, *Rhododendron simsii*

### INTRODUCTION

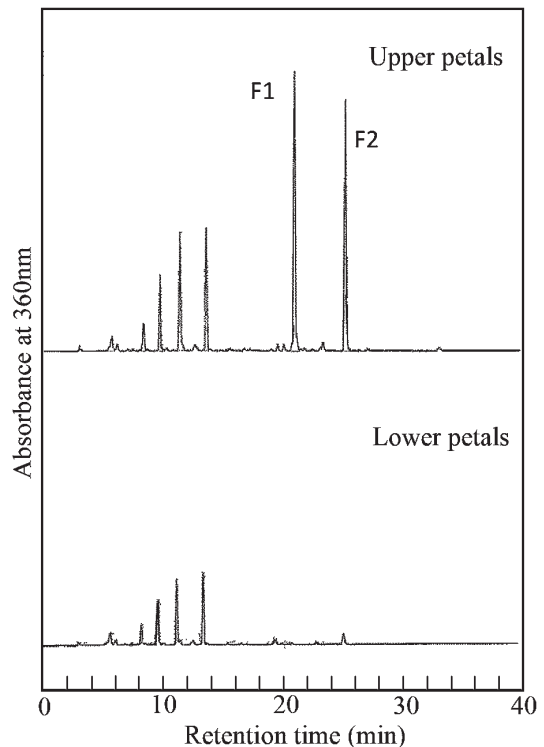
In Vietnam, some accessions of wild *Rhododendron simsii* are found in northern and central mountainous area, where this species grow near riverside or streamside at 800–1,400 m altitude (Ho, 1991). All of these accessions show only red colored funnel shaped flowers with reddish–purple blotches in upper inside of the petals (Fig. 1). In our continued work on pigmentation of *R. simsii* flowers, co–pigmentation of anthocyanin–flavonol, and the effect on flavonol for coloration of reddish–purple blotches of *R. simsii* flowers was revealed

(Huyen *et al.*, 2016). In this report, two major flavonols were detected in the upper petals of *R. simsii* flowers (Fig. 2). However, the flavonols isolated from reddish–purple blotches of *R. simsii* flowers have not yet been identified.

Flavonols of some wild evergreen azalea species were effectively analyzed by High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) (Harborne, 1959; Miyajima *et al.*, 1997, 2001; Sakata *et al.*, 1991). In this study, chromatographic analyses, and the flavonol properties in the flowers of several red flowered evergreen azalea species were discussed.



**Fig. 1.** *Rhododendron simsii* flower having red corolla with reddish–purple blotches on the upper inside of the petals. Bar indicates 1 cm.



**Fig. 2.** HPLC profiles of flavonols extracted from the upper petals of *R. simsii*.

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## MATERIALS AND METHODS

**Plant materials**

Fresh flowers of wild *R. simsii* distributed in Than Uyen district, Lai Chau province, Vietnam were collected at anthesis. Flowers were separated into upper and lower petals, and each part of petals was boiled at 100°C for 5 seconds, and immediately cooled in water. Then, the samples were dried in a forced convection oven through overnight at 50°C. The dried samples were stored in a desiccator at 4°C until pigment analysis.

**Isolation and purification of major flavonols**

Dried petals (ca. 0.7 g) of *R. simsii* were soaked overnight with 100% MeOH. After filtration, preparative HPLC was performed on an LC-6AD system (Shimadzu, Kyoto, Japan), using a Cosmosil 5C<sub>18</sub> AR column (20  $\phi$   $\times$  250 mm; Nakalai Tesque, Kyoto, Japan) at 40°C with a flow rate of 9 mL·min<sup>-1</sup>, and monitoring at 360 nm for isolation of major flavonol peaks. A linear gradient elution was applied for 40 min from 50 to 85% solvent B (10% formic acid, 40% MeCN in H<sub>2</sub>O) in solvent A (10% formic acid in H<sub>2</sub>O). Major peaks were collected using a fraction collector.

**Co-chromatography by TLC analysis**

After isolating from preparative HPLC, each purified flavonol fraction was dried by evaporator and diluted by drops of MeOH. Each pigment was spotted on 10 cm  $\times$  20 cm cellulose-coated glass plates (Merck, Darmstadt, Germany). In addition, four authentic standard quercetin glycosides: quercetin 3-glucoside (Qu 3Gl, isoquercetin), quercetin 3-galactoside (Qu 3Ga, hyperoside), quercetin 3-rutinoside (Qu 3Rt, rutin) and quercetin 3-rhamnoside (Qu 3Rh, quercitrin) were also spotted in order to compare with two major flavonols. Thin layer chromatography analyses were performed by three mobile phases according to the method of Harborne (1959): BAW (1-BuOH/AcOH/H<sub>2</sub>O, 4:1:2, v/v/v), 100% H<sub>2</sub>O and 15% AcOH. Then all spots were observed under UV light (365 nm) and *R<sub>f</sub>* values were also recorded.

**Co-chromatography by HPLC analysis**

In order to compare the retention times of two major flavonols and four authentic standard quercetin glycosides (same as mentioned above), HPLC analyses

were conducted in different solvent systems: (solvent system I) A (1.5% H<sub>3</sub>PO<sub>4</sub>) and B (1.5% H<sub>3</sub>PO<sub>4</sub>; 20% AcOH; 25% CH<sub>3</sub>CN), B conc.: 20%–85% at 40 min; (solvent system II) A (4% H<sub>3</sub>PO<sub>4</sub>) and B (CH<sub>3</sub>CN), B conc.: 15%–30% in 30 min; (solvent system III) A (0.1 M AcOH) and B (CH<sub>3</sub>CN) with constant flow. The wavelength of the analyses was 360 nm. Other HPLC conditions (pump, column, column oven temperature, and flow rate) were same as described by Huyen *et al.* (2016).

**LC-MS analysis**

Two purified major flavonols were analyzed using LC-MS by TSK gel ODS-80Ts QA 2.0  $\phi$   $\times$  150 mm column. A constant flow was applied for 30 min at 20% solvent B (MeCN) in solvent A (0.1 M AcOH) with a flow rate of 0.2 mL·min<sup>-1</sup> and 40°C of column temperature.

**Flavonol constitution of the flowers in several red flowered evergreen azalea species**

Approximately 50 mg of dried upper and lower petals from *R. simsii* (Iriomotejima Island, Japan), *R. indicum* (Yakushima Island, Japan), *R. oldhamii* (Alishan, Taiwan, ROC) and *R. scabrum* (Okinawa Island, Japan) were soaked overnight with 50% HOAc-H<sub>2</sub>O, respectively. After filtration, analytical HPLC was conducted in the same condition as described by Huyen *et al.* (2016).

## RESULTS AND DISCUSSION

By using preparative HPLC, two major flavonols were isolated and purified. The result of co-chromatography by TLC showed that the *R<sub>f</sub>* value of flavonol 1 (F1) was almost same as Qu 3Gl or Qu 3Ga in three different solvent systems and could not be distinguished. Flavonol 2 (F2) seemed to be quercetin 3-rhamnoside (Table 1). However, HPLC analysis results indicated the differences in retention times between two standard flavonols Qu 3Gl and Qu 3Ga, and the retention time of F1 was close to that of Qu 3Gl. Thus, F1 and F2 were almost coincided with quercetin 3-glucoside and quercetin 3-rhamnoside, respectively (Table 2). This result was supported by the molecular weight analysis. Table 3 showed that F1 [465.09 g/mol (M + H<sup>+</sup>)] and F2 [449.10 g/mol (M + H<sup>+</sup>)] had concurred molecular weight to that of standard Qu 3Gl and Qu 3Rh, respectively. The chemical struc-

**Table 1.** TLC analysis of F1, F2 and four authentic standard quercetin glycosides in different solvent systems

Flavonol glycosides	BAW <sup>z</sup>	H <sub>2</sub> O	15% AcOH
Flavonol 1	69	06	27
Flavonol 2	84	12	40
Quercetin 3-glucoside (isoquercetin)	68	07	29
Quercetin 3-galactoside (hyperoside)	64	09	31
Quercetin 3-rutinoside (rutin)	61	35	53
Quercetin 3-rhamnoside (quercitrin)	84	13	40

<sup>z</sup> 1-butanol: AcOH: DW (4: 1: 2; v/v/v)

**Table 2.** Retention times of F1, F2 and four authentic standard quercetin glycosides in different solvent system of HPLC analysis

Flavonol glycosides	Solvent system <sup>z</sup>		
	I	II	III
Flavonol 1	23.2	13.5	7.2
Flavonol 2	27.8	18.0	11.1
Quercetin 3-glucoside (isoquercetin)	23.9	13.9	7.7
Quercetin 3-galactoside (hyperoside)	21.7	15.1	7.4
Quercetin 3-rutinoside (rutin)	23.3	12.6	6.4
Quercetin 3-rhamnoside (quercitrin)	27.7	17.8	11.4

<sup>z</sup> I: solvent A (1.5% H<sub>3</sub>PO<sub>4</sub>) and solvent B (1.5% H<sub>3</sub>PO<sub>4</sub>; 20% AcOH; 25% CH<sub>3</sub>CN); B conc.: 20%–85% at 40 min.

II: solvent A (4% H<sub>3</sub>PO<sub>4</sub>) and solvent B (acetonitrile); B conc.: 15%–30% at 30 min.

III: solvent A (0.1 M AcOH) and solvent B (acetonitrile); B conc.: 20% (constant flow).

**Table 3.** Molecular weight of flavonol glycosides of F1 and F2

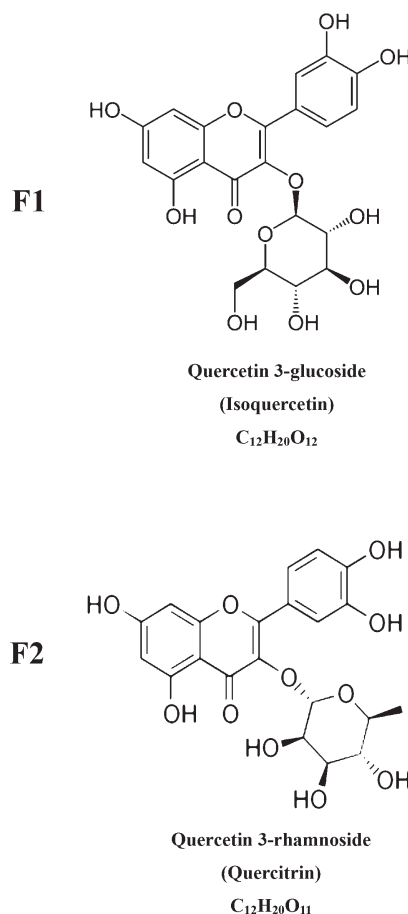
Flavonol glycosides	Molecular weight (g/mol)
Flavonol 1 (M + H <sup>+</sup> )	465.09
Flavonol 2 (M + H <sup>+</sup> )	449.10
Quercetin 3-glucoside (isoquercetin)	464.37
Quercetin 3-rutinoside (rutin)	610.52
Quercetin 3-rhamnoside (quercitrin)	448.38

ture of F1 and F2 was described in Fig. 3.

In *Rhododendron* species, four quercetin glycosides were identified as quercetin 3-arabinoside, 3-rhamnoside, 3-galactoside and the 3-rhamnoside of quercetin 5-methyl ether (Harborne, 1962). De Loose (1969) reported that quercetin glycosides (3-rhamnoside or 3-galactoside) seem to be popular in the flower of Red Wing Azalea (*R. simsii* hybrids). However, this is the first report about the presence of quercetin 3-glucoside and 3-rhamnoside in flowers of wild *R. simsii* distributed in Vietnam.

The flavonol composition of several wild red flowered species was also surveyed by HPLC analysis. The results showed that upper petals of these species also contained two major peaks and coincided with two major flavonols (Qu 3Gl and Qu 3Rh), which were identified in the blotch areas of *R. simsii* flowers from Vietnam in this study (Fig. 4).

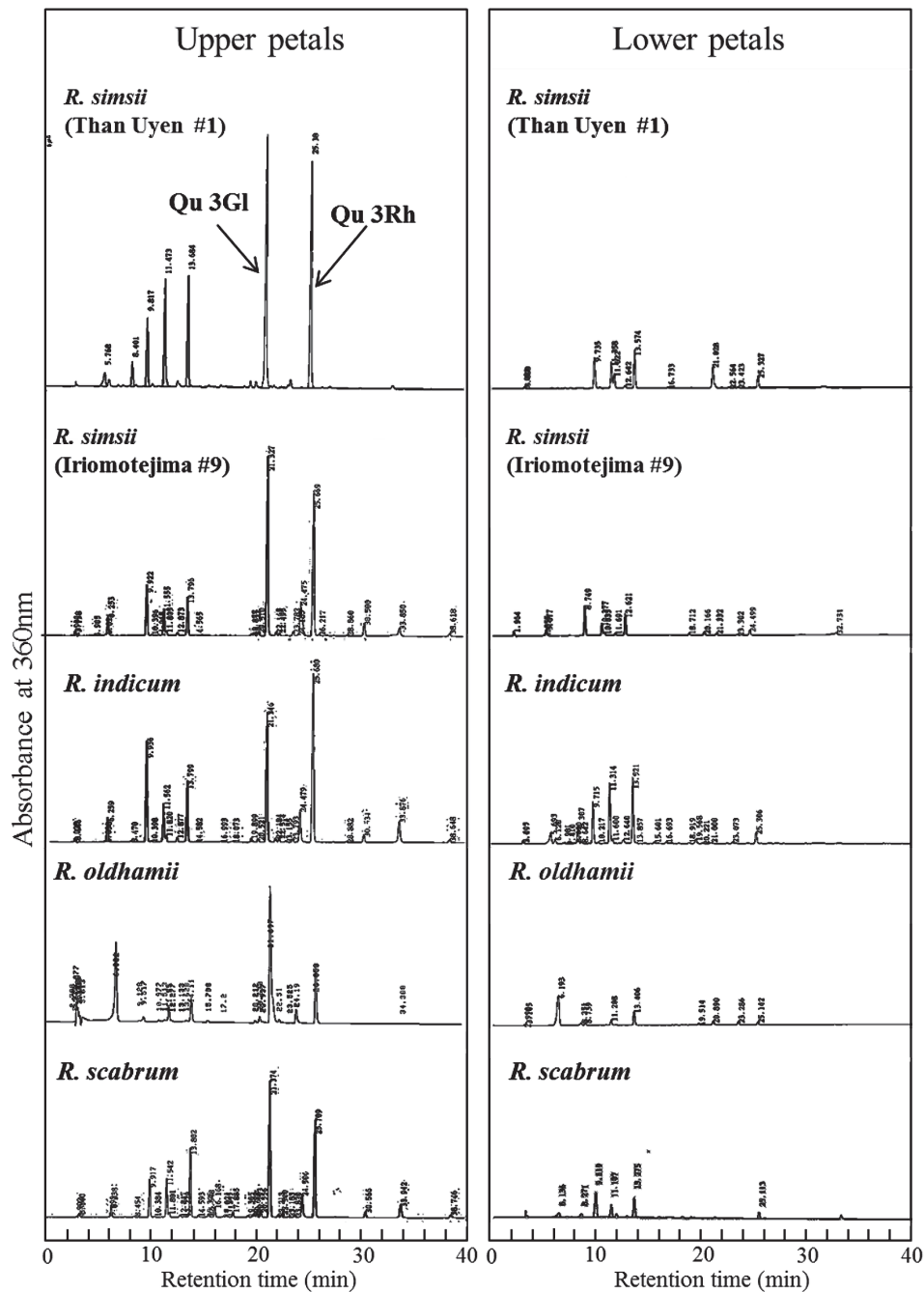
From the results, quercetin 3-glucoside (isoquercetin) and quercetin 3-rhamnoside (quercitrin) present as the two flavonols in the reddish-purple blotch areas of flowers of *R. simsii* and several red flowered species. These two flavonols play an important role on co-pigmentation phenomenon. Reddish-purple color of blotch areas is due to quercetin 3-glucoside and quercetin 3-rhamnoside co-pigmented with cyanidin 3-galactoside and cyanidin 3-arabinoside. This detailed understanding about pigmentation of flowers will contribute necessary knowledge for flower color breeding in the further study using Vietnamese wild *R. simsii* as the important genetic resources.



**Fig. 3.** Chemical structures of flavonol 1 and 2 found in the upper petals of *R. simsii* flowers.

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**Fig. 4.** HPLC profiles of extracts from upper and lower petals of *R. simsii* and several red flowered evergreen azalea species. Qu 3Gl: quercetin 3-glucoside; Qu 3Rh: quercetin 3-rhamnoside.

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