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Quantitative Analysis of Fustin and Sulfuretin in the Inner and Outer Heartwoods and Stem Bark of *Rhus verniciflua*

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Abstract – The heartwood of *Rhus verniciflua* Stokes (Anacardiaceae) is widely used for a medicinal plant to treat diabetes mellitus and lingering intoxication in the folkloric society of Korea, while the stem bark has been traditionally used to treat menstrual disorder and helminthiasis. We previously reported that a flavonoid, fustin, isolated from the heartwood of *R. verniciflua* is effective in Alzheimer's disease and rheumatoid arthritis. To explore the possibility to produce more flavonoid-rich fraction from this plant, the MeOH extracts from the plant parts of stem bark, outer heartwood, and inner heartwood were quantitatively analyzed by HPLC. Phenolic or flavonoid compounds (chlorogenic acid, caffeic acid, *p*-coumaric acid, sulfuretin, fustin, fisetin, luteolin and astragalin) were detectable in the HPLC chromatogram. The orange-colored inner heartwood was found to contain the highest levels of fustin (16.96 mg/g) and sulfuretin (2.22 mg/g). Moreover, the inner heartwood accumulated fustin and sulfuretin at least 4-fold higher level as compared to the stem bark and outer heartwood. The levels of total phenolic compounds positively correlated with the extents of antioxidant properties. Therefore, the inner heartwood of *R. verniciflua* could be used to increase fustin concentration of the extract which is capable of treating Alzheimer's disease and rheumatoid arthritis.
Keywords – *Rhus verniciflua* Stokes, Sulfuretin, Fustin, Flavonoid, Phenolic, HPLC

Introduction

Rhus verniciflua is a valuable medicinal tree in Asia that is used to treat gastritis, stomach cancer and atherosclerosis (Kim, 1996). The stem bark of *R. verniciflua* contains a high level of urushiols, which are polymerized to form a lacquer film by a radical-chain reaction (Hirota *et al*., 1998) and are known to have anti-AIDS, strong antioxidant and immune-enhancing activities (Miller *et al*., 1996).

However, urushiols have been concerned in its therapeutic use due to their allergenic activity. Unlike the stem bark extract, the heartwood extract of this plant does not cause the same type of allergenic action, which implies that it does not contain urushiols. The heartwood part has been used for beverages for tonics, cancer prevention and detoxication of smoking or lingering (Choi *et al*., 2003a; Park *et al*., 2004).

In our previous studies, we isolated the flavonoids, sulfuretin, fisetin and fustin from the heartwood of *R. verniciflua* (Park *et al*., 2000) and identified diverse bioactivities of sulfuretin associated with the aging disease (Park *et al*., 2002a; Park *et al*., 2002b). Recently, a variety of bioactivities derived from *R. verniciflua* have been reported. Examples are antioxidant and antitumor properties (Lim *et al*., 2001), the scavenging properties of phenolic compounds such as gallic acid, protocatechuic acid, butein, quercetin, sulfuretin, fustin, and fisetin (Kim, 2003; Lee *et al*., 2002; Son *et al*., 2005). It has been reported that sulfuretin has anti-rheumatoid activity (Choi *et al*., 2003b) and fustin does a potent activity against Alzheimer's disease based on the neuroprotection against 6-hydroxydopamine-induced cell death (Park *et al*., 2007),.

Therefore, it needs to be analyzed to find which part of the *Rhus verniciflua* heartwood is responsible for the high concentration of sulfuretin and fustin. In the present study, we quantified the levels of sulfuretin, fustin, and some other phenolic compounds in the extracts of the stem bark, outer heartwood, and inner heartwood of *R. verniciflua* in which a distinct line of the boundary exists

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between the inner (orange-yellow color) and outer (white) heartwood and between the heartwood and the bark. Here we report that the inner heartwood of *R. verniciflua* contains the highest level of sulfuretin and fustin as well as antioxidant activity.

Experimental

Instruments and Reagents – HPLC was performed on a Varian HPLC system (Walnut Creek, CA, USA) that includes Prostar 210 solvent delivery module, Prostar 325 UV-Vis detector and 20 μL sample loop (Rheodyne, Rohnert Park, CA, USA). Separation was achieved on Shiseido (Chuoku, Tokyo, Japan) Capcell Pak C18 column (5 μm, 250 mm × 4.6 mm I.D.), and injection syringe was purchased from Hamilton (Reno, Nevada, USA). All the solvents used for analysis were the HPLC grade obtained from J.T Baker (Phillipsburg, NJ, USA).

Plant material – *Rhus verniciflua* was collected during September, 2009, in Daehan-ri, Heungeop-myeon, Wonju city, Gangwon-do, Korea, and the wood stem was dissected into the stem bark, the inner heartwood (orange-colored part), and outer heartwood (white-colored part). The samples were dried and pulverized for HPLC analysis.

Standards and calibration curves – Chlorogenic acid, caffeic acid, *p*-coumaric acid, astragalin, luteolin, kaempferol and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fustin, sulfuretin, and fisetin were isolated from *Rhus verniciflua* as previously described (Park *et al.*, 2000). The identity of the compounds was verified by comparison of physicochemical data (mp, $[\alpha]_D$, ^{1}H-NMR and ^{13}C-NMR) with the published data (Park *et al.*, 2000). The purity of those compounds was approximately 98%. All standards were dissolved in 80% aqueous methanol. The standard curves were plotted using 5, 10, 25, and 50 μg/g with high linearities of $R^2 > 0.99$ (Table 1). Their concentrations were calculated by comparing the peak areas of samples with those of the standards. The structures of standard compounds are shown in Fig. 1.

Extraction – Three parts of plant material (1 g) added in the test tube was sonicated in pure MeOH (40 ml) at 40 °C for 2 h, and the extract was filtered and concentrated to dryness on a rotatory evaporator less than 60 °C and finally a freeze dryer at –40 °C. And the concentrated extracts were dissolved in 80% aqueous MeOH and were filtered through a 0.50 μm syringe filter.

MeOH fraction analysis – The plant materials of dried and pulverized 60 g stem bark, 56 g outer heartwood, and 46 g inner heartwood of *R. verniciflua* were extracted two times with MeOH under reflux for 4h. The extracts were filtered and concentrated to dryness on a rotatory evaporator less than 60 °C, to obtain the MeOH extracts; 7 g, 5.53 g, and 2 g, respectively. The 0.1 g of MeOH fraction was dissolved in 2 ml of 80% aqueous MeOH, and the suspension was filtered through a syringe filter (0.50 μm).

HPLC condition for phenolic compounds – The UV-Vis detector was fixed at 280 and 360 nm. The mobile phase was a mixed solvent of 2% acetic acid (solvent A)

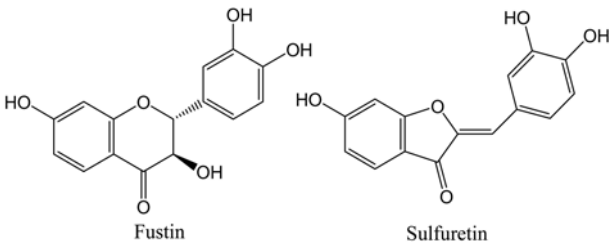

Fig. 1. Structure of fustin and sulfuretin used for quantitative analysis.

Table 1. Calibration curve equation of 9 standards of phenolic compounds

Compound	Retention time	Equation	
Chlorogenic acid	18.7	y = 295.6x – 471.9	R^2 = 0.999
Caffeic acid	20.4	y = 720.6x – 645.7	R^2 = 0.995
Fustin	23.6	y = 274.0x – 57.04	R^2 = 0.999
p-coumaric acid	25.2	y = 1021.x + 103.0	R^2 = 0.999
Astragalin	34.6	y = 437.1x – 1078.	R^2 = 0.997
Sulfuretin	38.1	y = 493.4x – 804.9	R^2 = 0.997
Fisetin	39.1	y = 562.6x – 3807.	R^2 = 0.997
Luteolin	40.4	y = 965.3x – 2188.	R^2 = 0.995
Kaempferol	43.1	y = 917.3x – 7126.	R^2 = 0.998

y (area, μV), x (concentration, μg/mL)

and methanol (solvent B) in water. The gradient system was: 0 min, 95% A: 5% B; 0-10 min, 80% A:20% B ;10-40 min, 40% A:60% B ; 40-50 min, 20% A:80% B, 50-51 min, 95% A:5% B, 51-60 min, 95% A:5% B. Chromatography was performed at the flow rate of 1.00 mL min^{-1} in 60 min.

DPPH radical-scavenging activity – One gram of freeze-dried pulverized sample was mixed with 100 mL of 80% aqueous methanol and was stirred for 24 h at 24 °C. The extract was filtered through Whatman No. 42 paper, evaporated at below 50 °C and freeze-dried at –40 °C. The residues were dissolved in DMSO to make 1% solution (w/v) and filtered through a 0.45 μm syringe filter. The free radical scavenging activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the method of Oh *et al.* (2005). Each 0.25 mL of sample solution was mixed with 2.5 mL of 0.35 mM DPPH in 50% ethanol, and the mixtures were left for 30 min at room temperature in the dark. The DPPH value was measured at 517 nm. DPPH activity was calculated as an inhibition percentage based on the following equation: Free radical scavenging activity (%) = $(1 - Abs_{sample} / Abs_{control}) \times 100$

Results and Discussion

Although a number of natural products have potential biological activities, most of those compounds are often obtained at a low purity or low yield. Therefore, the production of fustin-rich fraction from *R. verniciflua* would be important for practical applications since fustin display a neuroprotection activity against 6-hydroxydopamine-induced cell death (Park *et al.*, 2007). As concentrations of natural products differ depending on the plant part, we dissected *R. verniciflua* wood stem into the three parts and examined the levels of phenolic compounds. Representative HPLC chromatograms monitored at 280 and 360 nm are shown in Fig. 2. Using two different wavelengths for analysis was useful to distinguish each peak of phenolic compounds. All of the nine compounds examined were detectable in the HPLC chromatograms. The quantities of those compounds are listed in Table 2. The concentration of MeOH fraction was represented from mg/g to percentage of each compound. It is notable that fustin and sulfuretin are enriched in the orange inner heartwood at the level of 16.96 mg/g (39.07%) and 2.28 mg/g (5.11%), respectively. In comparison, fustin and sulfuretin accumulated at the level of 1.51 mg/g (1.30%) and 0.51 mg/g (0.44%) in the stem bark, respectively. In the outer heartwood, both of the compounds accumulated below 0.94%.

The inner and outer heartwood contained more chlorogenic acid and *p*-coumaric acid than stem bark. However, the quantities of caffeic acid, astragalin, fisetin, and luteolin were higher in the stem bark than the other two plant parts. As the proportion of fustin in the MeOH fraction of the inner heartwood reaches at the highest rate (39.07%), use of this MeOH extract is preferred for a flavonoid-rich biomaterial that can be used to inhibit 6-hydroxydopamine toxicity in neuron. Moreover, the inner

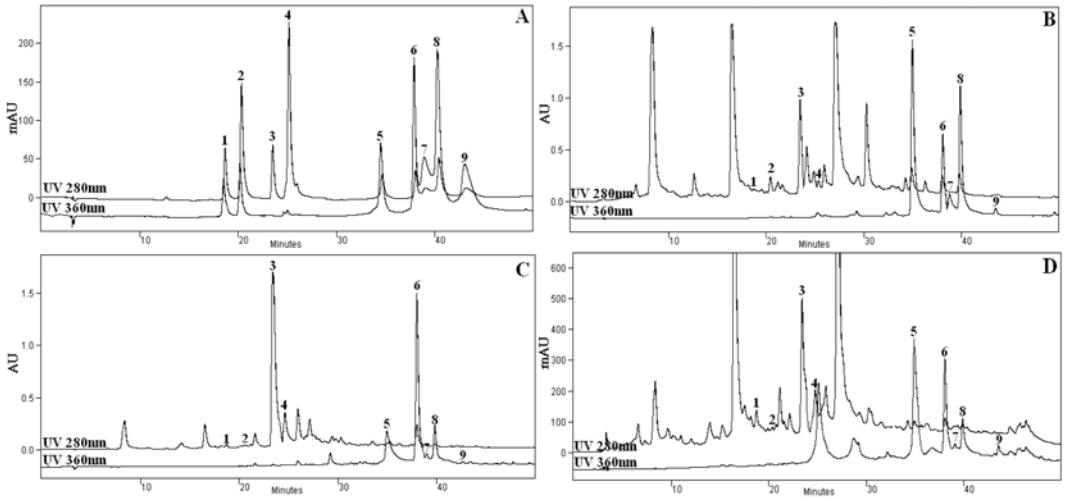

Fig. 2. HPLC chromatograms of a standard mixture (A), stem bark (B), inner heartwood (C), and outer heartwood (D).
Compounds - **1** (chlorogenic acid), **2** (caffeic acid), **3** (fustin), **4** (*p*-coumaric acid), **5** (astragalin), **6** (sulfuretin), **7** (fisetin), **8** (luteolin), **9** (kaempferol)

Table 2. Phenolic compounds contents of the *R. verniciflua* samples

Compounds	Stem bark		Inner heartwood		Outer heartwood	
	mg/g[b]	%[c]	mg/g	%	mg/g	%
Chlorogenic acid	0.19±0.02[a]	0.16±0.01	0.24±0.09	0.55±0.04	0.29±0.01	0.29±0.02
Caffeic acid	0.14±0.01	0.12±0.05	0.09±0.00	0.20±0.01	0.04±0.00	0.04±0.00
Fustin	1.51±0.12	1.30±0.15	16.96±2.48	39.07±3.01	0.94±0.09	0.96±0.10
p-coumaric acid	0.10±0.00	0.08±0.00	0.99±0.11	2.28±0.22	0.13±0.05	0.13±0.07
Astragalin	1.66±0.31	1.42±0.21	1.24±0.08	2.85±0.14	0.32±0.07	0.33±0.16
Sulfuretin	0.51±0.18	0.44±0.10	2.22±0.17	5.11±0.51	0.12±0.04	0.12±0.03
Fisetin	0.23±0.01	0.20±0.07	0.14±0.02	0.32±0.06	0.03±0.01	0.03±0.00
Luteolin	0.57±0.03	0.49±0.05	0.32±0.05	0.73±0.04	0.04±0.01	0.04±0.01
Kaempferol	0.04±0.00	0.03±0.01	0.09±0.01	0.22±0.06	0.02±0.00	0.02±0.00
Sum	4.95	4.24	22.29	51.33	1.93	1.97

Abbreviation: [1]; chlorogenic acid, [2]; caffeic acid, [3]; fustin, [4]; *p*-coumaric acid, [5]; astragalin, [6]; sulfuretin, [7]; fisetin, [8]; luteolin, [9]; kaempferol

[a] Values represent mean ±S.D. based on three experiments. [b] Unit (mg/g) represents mg/g dried plant material.

[c] Unit (%) represents the percentage of each compound in MeOH fraction.

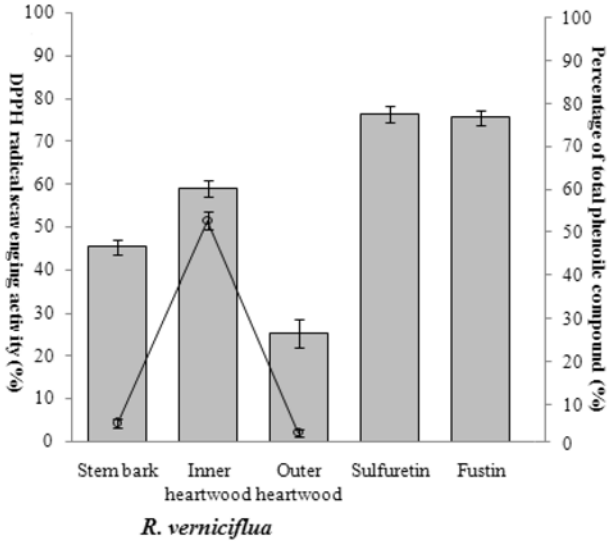

Fig. 3. DPPH free radical scavenging activities and total concentration of phenolic compounds of methanolic *R. verniciflua* fractions.

■ ; DPPH free radical scavenging activities, ○ ; Total concentration of phenolic compounds from MeOH fraction.

heartwood accumulated fustin and sulfuretin flavonoid compounds at least 4-fold higher level as compared to the stem bark and outer heartwood. The dissected heartwood consisted of the orange-colored inner and the white-colored outer part. This color difference might be attributed to the varying levels of total phenolic compounds retained in each part.

Flavonoids are well known to be a pharmacologically active constituent in many medicinal plants. Because the MeOH extract of the inner heartwood contains much higher levels fustin than the other stem parts, the extract prepared from the inner heartwood is very preferred to treat Alzheimer's disease.

To compare the suppression rates of 6-hydroxy-tryptamine toxicity by the extracts of the three different stem parts, DPPH assay was employed in the present experiment instead of that assay, and the results are shown in Fig. 3. The inner heartwood extract exhibited the highest radical scavenging activity of 58.92%, being followed by the stem bark (45.25%) and the outer heartwood was exhibited the lowest activity by (25.31%) which were to some extent correlated with the total phenolic contents. Sufuretin and fustin scavengend DPPH radical by 75.85% and 74.94%, respectively. Therefore, the inner heartwood of *R. verniciflua* could be used to increase fustin concentration of the extract which is capable of treating Alzheimer's disease and rheumatoid arthritis.

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