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Extraction and Structural Characterization of Flavonoids from Twigs of *Sophora japonica*

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Flavonoids represent a large group of polyphenols that have been recognized to exert a wide range of biological effects, such as anticancer, inflammation inhibition, anti-aging, and neuroprotective activities. In the forest industry, tree twigs have been treated as residues; however, tree twigs could be a rich source of high-value added compounds, which have been relatively unexplored. In this study, an investigation into the chemical constituent of extractives from *S. japonica* twigs resulted in the isolation of a new (*Z*)-caffeoyl flavonol glycoside that was elucidated as myricetin 3-*O*-(4′′-(*Z*)-caffeoyl)-*α*-rhamnopyranoside (**IV**). The structure of the new compound was established mostly on the basis of extensive spectroscopic techniques and other physiochemical evidences. Among the three known flavonoids extracted in this work, including isoquercitrin (**I**), isorhamnetin 3′-*O*-*β*-D-glucopyranoside (**II**), and myricitrin (**III**), **II** and **III** have never been previously reported in the *Sophora* genus.

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INTRODUCTION

Flavonoids include a multitude of diverse and widespread tree extractives, occupying a prominent position among the natural polyphenols (Cook and Samman 1996). Currently, over 4,000 different flavonoids have been identified (Shafaghat *et al*. 2014). Interest in flavonoids results particularly from the conspicuous vivid and beautiful colors these pigments impart to various parts of plants, as well as from the importance of these compounds in the tanning of leather, the fermentation of tea, the manufacture of cocoa, and the flavoring and nutritional quality of foodstuffs (Harborne and Williams 2000). In addition, flavonoids exhibit a broad spectrum of biological and pharmaceutical effects, including anti-aging, anti-inflammatory, hepatoprotective, anti-cancer, anti-bacterial, anti-estrogenic, and analgesic activities (Gautam and Jachak 2009).

Tree twigs have long been regarded as a kind of forest residue or waste, which could be a rich source of novel biologically or pharmaceutically active compounds. The investigation of such naturally bioactive compounds isolated from the forest is a permanent attraction and challenge, which still leads to new discoveries (Si *et al*. 2013, 2014).

Sophora japonica (Leguminosae), a deciduous tree with luxuriant branches and twigs, is grown widely in China, Korea, and Japan (Wang *et al*. 2003). Buds and seeds of *S. japonica* have long been used as hemostatic agents in traditional Chinese medicine. Its flowers are used as a folk remedy to prevent against paralysis in patients with high blood pressure. Stem bark of *S. japonica* is also used to treat inflammatory diseases and as an analgesic agent (Wang *et al*. 2003; Zhang *et al*. 2013). The chemical investigations on stem barks, flowers, and seeds of *S. japonica* have shown them to be rich in several classes of compounds, among which flavonoids were the main constituents (Zhang *et al*. 2013). However, chemical extractives of *S. japonica* twigs are far from fully studied. As part of our research on the comprehensive utilization of forest residues, this preliminary study reports the extraction and structure elucidation of a new flavonol glycoside, myricetin 3-*O*-(4''-(Z)-caffeoyl)-α-rhamnopyranoside (**IV**), as well as three known ones, isoquercitrin (**I**), isorhamnetin 3'-*O*-β-D-glucopyranoside (**II**), and myricitrin (**III**), from the twigs of *S. japonica*.

EXPERIMENTAL

Plant Material

Twigs of *S. japonica* were collected during tree pruning at Tianjin University of Science and Technology, China, on June 5, 2014. A voucher specimen (No. 140610) was deposited in the Herbarium of College of Papermaking Science and Technology, Tianjin University of Science and Technology, China.

Instruments

The melting points (mp), uncorrected, were obtained using an Electro Thermal 9100 apparatus (Bibby Scientific Limited, Staffordshire ST15 OSA, UK). The optical rotations were determined using a JASCO DIP-1000 digital polarimeter in MeOH (JASCO, Japan). Infrared spectra were recorded using the KBr disk method on a BX FTIR spectrometer (Perkin-Elmer Inc., Waltham, MA, USA). Ultra-violet (UV) spectra were recorded using a Jenway 6405 spectrophotometer, and positive FAB-MS spectra were done on a Micromass Autospec M363 spectrometer (Micromass, UK). The 1D and 2D nuclear magnetic resonance (NMR) spectra were recorded in CD_3OD with tetramethylsilane (TMS) as an internal standard, using a Bruker Advance DPX 400 spectrometer (Bruker Corp., Germany). Tetramethylsilane experiments were performed on DC-Plastikfolien Cellulose F (Merck and Co., USA) plates and developed with *t*-BuOH-HOAc-H_2O (3:1:1, v/v/v, solvent A) and HOAc-H_2O (3:47, v/v, solvent B). The TLC spots were detected using UV light (365 and 254 nm) and by spraying with a 1.0% ethanolic $FeCl_3$ solution, followed by heating. Sephadex LH-20 (Merck and Co., Germany) and silica gel (Qingdao Haiyang Chemical Co., China) were used for open column chromatography (OCC).

Extraction and Chromatographic Purification

The air-dried and finely smashed twigs of *S. japonica* (3.82 kg) were extracted with 70% acetone (4 × 40 L), each for 96 h at room temperature. This resulted in a yield of 217.26 g of crude extract after evaporation of the solvent under a vacuum. The extract was then suspended in distilled water, and sequentially partitioned with *n*-hexanes,

CHCl$_3$, EtOAc, and *n*-BuOH. A portion of the resultant EtOAc fraction (37.49 g) was loaded over silica gel OCC using a gradient of CHCl$_3$-acetone (29:1→3:1, v/v, 4600 mL) as the solvent system to create seven fractions (F$_1$ to F$_7$), which were grouped, and their compositions were monitored using TLC experiments. F$_3$ (6.05 g) was also subjected to Sephadex LH-20 OCC with MeOH-H$_2$O (2:1, v/v, 1600 mL), which was used as an eluting solvent to further purify the five fractions (F$_{31}$ to F$_{35}$). The second fraction, F$_{32}$, (3.17 g) was rechromatographed through Sephadex LH-20 open columns with MeOH-H$_2$O (1:3, v/v, 900 mL) and EtOH-*n*-hexane (1:1 and 1:4, each 600 mL) to yield two yellow amorphous compounds: **I** (122.4 mg) and **III** (42.7 mg). The F$_{34}$ was successively resubjected to a Sephadex LH-20 open column wash and eluted with MeOH-H$_2$O (1:2, 1:4, and 1:6, each 300 to 600 mL) to yield 52.3 mg of **II** (a brown amorphous compound). The F$_5$ (5.62 g) was reapplied to a Sephadex LH-20 open column using MeOH-H$_2$O (3:1, v/v, 1800 mL) as eluants for further purification to obtain four fractions: F$_{51}$ to F$_{54}$. F$_{53}$ (2.84 g) was further chromatographed on a Sephadex LH-20 open column by eluting with MeOH-H$_2$O three times (1:1, 2:5, and 1:5, v/v, each with 250 to 800 mL), resulting in a yellow amorphous compound (**IV**, 40.3 mg).

Compound **IV**, with the characteristics of yellowish amorphous powder; a melting point of 188 to 190 ℃; $[\alpha]_D^{20}$ -93.4° (MeOH, *c* 0.5); UV λmax (MeOH) nm: 260, 332; IR (KBr) ν_{max} cm^{-1} 3390 (OH), 1650 (conjugated ketone C=O), 1605 (aromatic C=C); R$_f$ 0.37 (solvent A), and 0.48 (solvent B); Positive FAB MS: [M+Na]$^+$ at *m/z* 649 and [M+H]$^+$ at *m/z* 627, molecular weight of 626, and calculated for C$_{30}$H$_{26}$O$_{15}$. The ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data in CD$_3$OD are shown in Table 1.

RESULTS AND DISCUSSION

Identification of Extracted Compounds

Repeated Sephadex LH-20 and silica gel chromatography of the 70% acetone extracts of *S. japonica* twigs led to the purification of four flavonol glycosides, which included two quercetin derivatives (**I** and **II**), and two myricetin derivatives (**III** and **IV**). On the basis of analyzing their spectroscopic data and a detailed comparison with literature values, the three known flavonoids were elucidated as isoquercitrin (**I**), isorhamnetin 3'-*O*-*β*-D-glucopyranoside (**II**), and myricitrin (**III**) (Agrawal 1989; Bousetla *et al*. 2013), as shown in Fig. 1.
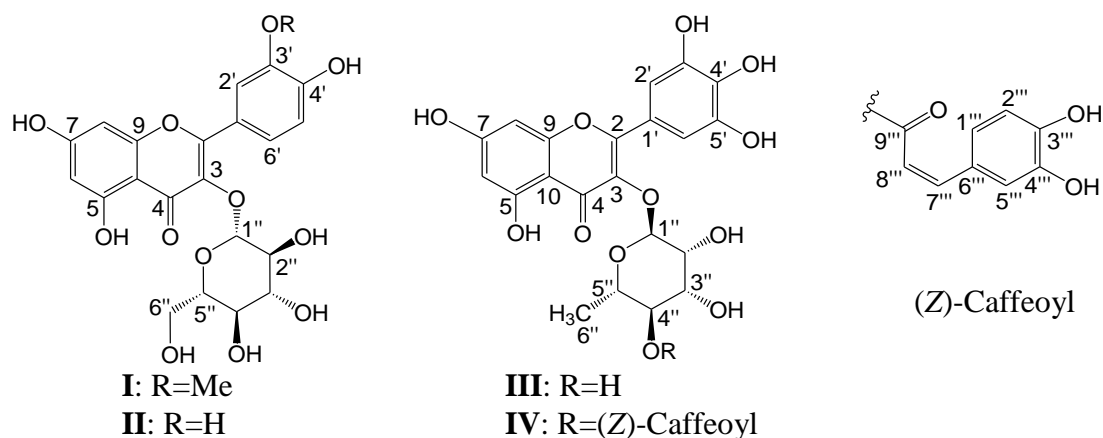
Fig. 1. Structures of the identified and novel flavonoids from twigs of *S. japonica*: Isoquercitrin (**I**), isorhamnetin 3'-*O*-*β*-D-glucopyranoside (**II**), myricitrin (**III**), and myricetin 3-*O*-(4''-(*Z*)-caffeoyl)-*α*-rhamnopyranoside (**IV**)

Previous studies suggest that flavonoids **I** to **III** are high-value-added extractives that exhibit various biological activities and functions. Isoquercitrin (**I**) is widely used in medicines because of its anti-asthmatic (Fernandez *et al*. 2005), anti-oxidant (Li *et al*. 2011), and thiamine-decomposing properties (Nakabayashi 1955). Isorhamnetin 3'-*O*-*β*-D-glucopyranoside (**II**) can be used in diet food for its anti-adipogenic activity, as well as protecting against oxidation-induced cell damage (Kong and Seo 2012). Myricitrin (**III**) is currently used as a flavor modifier in snack foods, dairy products, and beverages, and offers a variety of potential health benefits including, anti-mutagenic, anti-oxidant, anti-inflammatory, and anti-nociceptive effects in experimental models (Hobbs *et al*. 2015).

Compound **I** has previously been reported from *S. japonica* (Tang *et al*. 2013). However, to the best of our knowledge, **II** and **III** have never been extracted in the *Sophora* genus. Compound **IV** is a novel natural compound purified and structurally elucidated in the current work for the first time.

Table 1. NMR Spectra Data for Compound **IV** in CD$_3$OD

Position	δ ^{1}H (m *J* (Hz))	δ ^{13}C	DEPT
Myricetin			
2	–	159.5	C
3	–	136.5	C
4	–	179.6	C
5	–	163.3	C
6	6.17 (1H, *d*, *J* = 1.9 Hz)	99.9	CH
7	–	165.9	C
8	6.37 (1H, *d*, *J* = 1.9 Hz)	94.8	CH
9	–	158.7	C
10	–	105.9	C
1'	–	121.9	C
2'/6'	7.21 (1H, *s*)	110.7	CH
3'/5'	–	146.9	C
4'	–	137.9	C
α-Rhamnosyl			
1''	5.27 (1H, *br s*)	103.7	CH
2''	4.10 (1H, *br s*)	72.4	CH

3″	3.52 (1H, *m*)	71.0	CH
4″	5.02 (1H, *dd*, *J*=9.6 & 9.4 Hz)	75.5	CH
5″	4.32 (1H, *m*)	69.8	CH
6″	1.01 (3H, *d*, *J*=6.2 Hz)	17.8	CH₃
(Z)-Caffeoyl			
1‴	–	127.9	C
2‴	7.35 (1H, *d*, *J*=2.2 Hz)	116.3	CH
3‴	–	148.2	C
4‴	–	145.4	C
5‴	6.68 (1H, *d*, *J*=8.0 Hz)	118.3	CH
6‴	7.06 (1H, *dd*, *J*=2.2 & 8.0 Hz)	125.1	CH
7‴	6.78 (1H, *d*, *J*=12.2 Hz)	145.4	CH
8‴	5.77 (1H, *d*, *J*=12.2 Hz)	115.5	CH
9‴	–	168.2	C

Compound **IV** was extracted as a yellowish amorphous powder, providing an optical rotation of $[\alpha]_D^{20}$ -93.4° (MeOH, *c* 0.5) and a melting point (mp) of 188 to 190 °C. The UV spectrum of **IV** presented absorptions at 260 and 332 nm, and its IR spectrum revealed characteristic absorption bands for the aromatic double bond (1605 cm^{-1}), conjugated carbonyl (1650 cm^{-1}), and hydroxyl groups (3390 cm^{-1}). Its molecular formula was deduced as $C_{30}H_{26}O_{15}$ by examining its positive fast atom bombardment (FAB) MS spectrum. Consequently, the [M+Na]$^+$ and [M+H]$^+$ ions were found at *m/z* 649 and 627, respectively, corresponding to a molecular weight of 626. The presence of phenolic hydroxyl group in compound **IV** was observed in chromogenic experiments of TLC by the grey-green color when spraying 1.0% FeCl₃ (R$_f$ indexes were 0.37 and 0.48 for solvents A and B, respectively) (Imakura *et al*. 1985).

The ^{1}H NMR spectra data of compound **IV** afforded a pair of doublets of *meta* coupling (*J*=1.9 Hz) at *δ* 6.17 and 6.37, assignable to H-6 and H-8, respectively. The singlet at *δ* 7.21 integrated to two protons that were ascribed to a pair of symmetric protons H-2' and a 6' of the B ring. In the ^{13}C NMR spectrum, the flavonol skeleton of compound **1** was confirmed by characteristic signaling at *δ* 159.5 (C-2), 136.5 (C-3), and 179.6 (C-4) (Semmar *et al*. 2002). Therefore, the aglycone was established as myricetin.

In the ^{1}H NMR spectrum of **IV**, the rhamnosyl residue at the *α*-configuration was recognized by an anomeric proton resonating at *δ* 5.27 (1H, H-1″) as a broad singlet, and a secondary methyl group typically irradiating at *δ* 1.01 (3H, *J*=6.2 Hz, H-6″) as a doublet (Si *et al*. 2013). In the ^{1}H-^{1}H correlation spectroscopy spectrum, other proton signals, including 4.10 (1H, *br s*, H-2″), 3.52 (1H, *m*, H-3″), 5.02 (1H, *dd*, *J*=9.6 & 9.4 Hz, H-4″), and 4.32 (1H, *m*, H-5″) were assigned by the inner cross correlations. In the HMBC (^{1}H→^{13}C) spectrum of compound **IV**, long-range correlations were found between sugar anomeric H-1″ (*δ* 5.27) and the C-3 (*δ* 136.5) of myricetin, as shown in Fig. 2. This confirmed the rhamnosyl sugar was linked to the C-3 position of the aglycone.
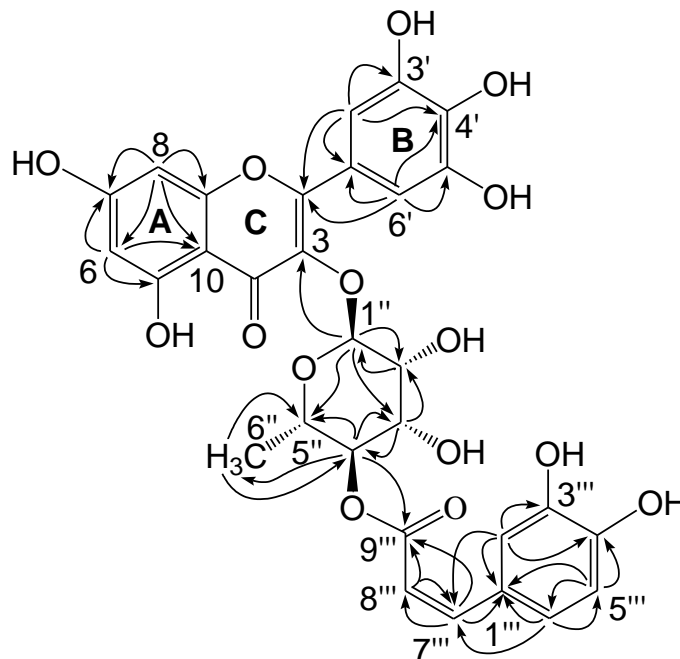
Fig. 2. Heteronuclear multiple-bond correlations (HMBCs) observed in myricetin 3-*O*-(4"-(*Z*)-caffeoyl)-*α*-rhamnopyranoside (**IV**)

The proton signals as doublets at δ 6.78 (H-7''') and 5.77 (H-8'''), with a coupling constant of *J* =12.2 Hz, suggested the presence of (*Z*)-configuration olefinic protons of the AB type on the caffeoyl group (Yahagi *et al*. 2012). A *meta* coupled doublet at δ 7.35 (1H, *d*, *J*=2.2 Hz, H-2'''), double doublets at δ 7.06 (1H, *dd*, *J*=8.0 Hz and *J*=2.2 Hz, H-6'''), and an *ortho* coupled doublet at δ 6.68 (1H, *d*, *J*=8.0 Hz, H-5''') characterized the ABX coupling protons. Furthermore, the HMBC spectrum of **IV** revealed interactions between δ 6.78 (H-7''') and δ 168.2 (C-9'''), δ 6.78 (H-7''') and δ 127.9 (C-1'''), δ 5.77 (H-8''') and δ 168.2 (C-9'''), δ 7.35 (H-2''') and δ 145.4 (C-7'''), and δ 7.06 (H-6'''), and δ 145.4 (C-7''') to confirm the presence of a (*Z*)-caffeoyl residue. The HMBC spectrum provided correlations between δ 5.02 (H-4'') and δ 168.2 (C-9'''), which supported that the (*Z*)-caffeoyl moiety was linked at the C-4'' (δ 75.5) position of the *α*-rhamnosyl residue.

The distortionless enhancement by polarization transfer (DEPT) spectra data of **IV**, summarized in Table 1, was divided into 30 carbon peaks: 14 methine, 1 methyl, and 15 quaternary carbon signals. Thus, compound **IV** was elucidated as myricetine 3-*O*-(4"-(*Z*)-caffeoyl)-*α*-rhamnopyranoside (Fig. 1), which is a novel natural extractive, and has not been previously found from any other plant species.

CONCLUSIONS

1. In this work, extractives from the EtOAc fraction of *S. japonica* twigs were systematically isolated and separated by column chromatography and TLC, which led to the purification of four flavonoids.

2. Based on spectra analysis, physiochemical evidences, and comparison with published data, structures of the extractives were elucidated as isoquercitrin (**I**), isorhamnetin 3'-*O*-*β*-D-glucopyranoside (**II**), myricitrin (**III**), and myricetin 3-*O*-(4"-(*Z*)-caffeoyl)-*α*-

rhamnopyranoside (**IV**) unambiguously.

3. Compound **IV** is a new natural compound and has never been reported previously. While, isorhamnetin 3'-*O-β*-D-glucopyranoside (**II**) and myricitrin (**III**) were isolated in *Sophora* genus for the first time.

4. Investigations on the bioactivities of the new compound **IV** are being carried out, and the current work laid a foundation for further utilization of the forest residues of *S. japonica* twigs.

ACKNOWLEDGMENTS

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