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FLAVONOL GLYCOSIDES IN TWO *DIOSPYROS* PLANTS AND THEIR RADICAL SCAVENGING ACTIVITY

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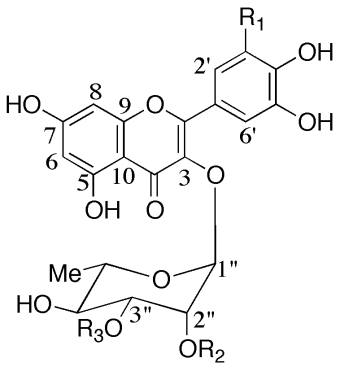
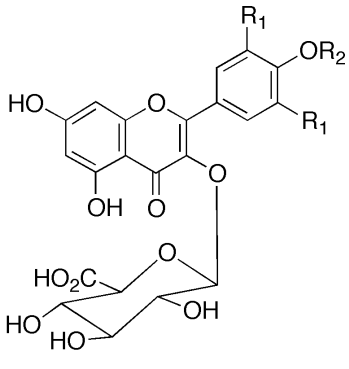
Abstract- Methanol extracts of leaves of *Diospyros glaucifolia* and *D. kaki* were showed the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Chromatographical separation and purification of methanol extracts resulted in isolation of three new flavonol glycosides [2"-(*E*)-*p*-coumaroylmyricitrin (**3**), 3"-(*E*)-*p*-coumaroylmyricitrin (**4**) and mearsetin 3-*O*-β-glucronopyranoside (**11**)] along with known phenolic compounds. These structures were determined by means of spectroscopic analysis. The scavenging activities of DPPH radical of the isolated compounds were examined.



2: R_1= OH, R_2= R_3= H
3: R_1= OH, R_2= *E*-*p*-coumaroyl, R_3= H
4: R_1= OH, R_2= H, R_3= *E*-*p*-coumaroyl
5: R_1= OH, R_2= galloyl, R_3= H
6: R_1= OH, R_2= H, R_3= galloyl
7: R_1= R_2= R_3= H



8: R_1= OH, R_2= H
11: R_1=OH, R_2= Me
12: R_1= R_2= H

Figure 1

The genus *Diospyros* belongs to the family Ebenaceae and comprises about 500 species distributing in the tropical and temperate zone. Wood of some species such as *D. ebonum* is called ebony and used as furniture, building materials, industrial art objects *etc*. Some species have been used as traditional medicines.[1] For instance, leaves of *D. kaki* have been used for remedy of hypertension. Several flavonol glycosides have been characterized as active principle.[2,3] In the previous phytochemical studies of *Diospyros*, triterpenoids and naphthoquinones have been isolated.[4]

Naphthoquinones among them, in particular, show various biological activities such as antitumor,[5-7] antimicrobial[8-10] and ichthyotoxic activity.[11] Although the genus *Diospyros* is interesting in the source of bioactive compounds, there have been a few studies of the constituents in leaves. *D. glaucifolia* distributes mainly in Sichuan Province of China and the leaves have traditionally been used as same as *D. kaki*.[12] In the present paper we describe the isolation and structural determination of flavonol glycosides including three new compounds in leaves of *D glaucifolia* and *D. kaki*. The scavenging activities of DPPH radical of the isolated compounds were also investigated.

The dried leaves of *D. glaucifolia* and *D. kaki* were extracted as described in EXPERIMENTAL. The respective extracts were separated by ODS and silica gel column chromatography, followed by Sephadex LH 20 column chromatography and preparative TLC to afford ten compounds (**1**-**10**) from *D. glaucifolia* and six compounds (**11**-**16**) from *D. kaki*.

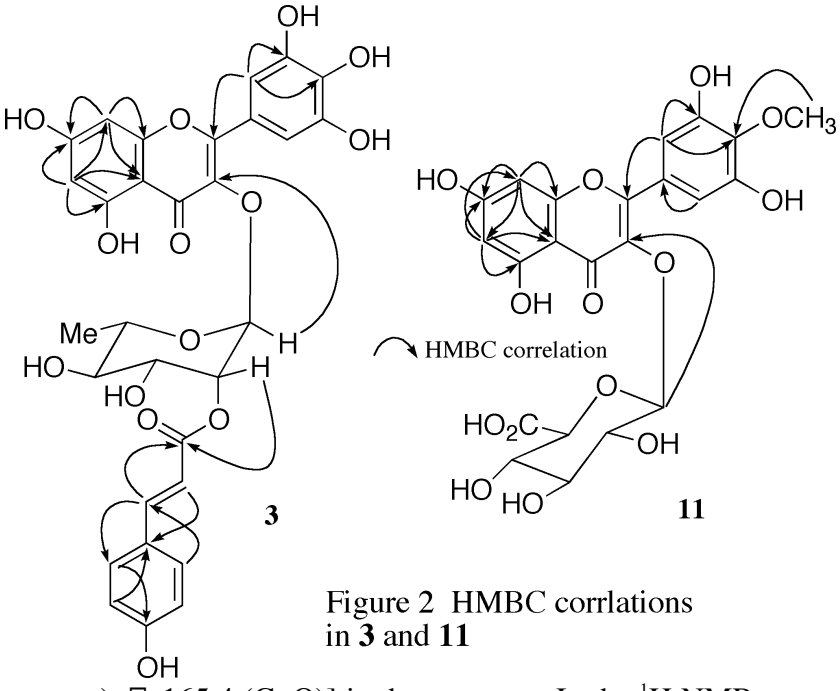


Figure 2 HMBC corrlations in **3** and **11**

Compound (**3**), a pale yellow amorphous powder, showed the $[M-H]^-$ at *m/z* 609, indicating the molecular formula $C_{30}H_{26}O_{14}$. The ^1H and ^{13}C NMR spectral data suggested that **3** was also a derivative of myricitrin (**2**). The spectral data indicated the presence of an (*E*)-*p*-coumaroyl moiety [δ_H 6.89, 7.56 (2H, each d, *J*= 8.5 Hz), 6.38, 7.64 (1H, each d, *J*= 15.8 Hz, *trans* olefinic protons); δ_C 165.4 (C=O)] in the structure. In the ^1H NMR spectrum, the rhamnosyl prontons (H-1", H-2" and H-3") were shifted to lower field by 0.44, 1.27 and 0.48 ppm, respectively by comparison with **2**. To the contrary, C-1" and C-3" of the rhamonsyl moiety were shifted to upper field and C-2" to lower field in the ^{13}C NMR spectrum. These shifts observed suggested that the *p*-coumaroyl group was connected at C-2-OH on the rhamnose in **2**. The connection was finally confirmed by the analysis of HMBC, that is, a significant correlation was observed between H-2" and C-9"' in the HMBC spectrum (Figure 2). Therefore, the structure of **3** was characterized as 2"-(*E*)-*p*-coumaroylmyricitrin.

Compound (**4**), a pale yellow amorphous powder, gave the same molecular formula as **3**. The ^1H and ^{13}C NMR spectral data were closely similar to those of **3** except for the rhamnosyl part. In the ^1H NMR spctrum, the rhamnosyl prontons (H-2", H-3" and H-4") were shifted to lower field by 0.26, 1.43 and 0.23 ppm by comparison with **2**. C-2" and C-4" of the rhamonsyl moiety were shifted to upper field and C-3"

to lower field, respectively in the ^{13}C NMR spectrum. Then, the structure of **4** was determined to 3"-(*E*)-*p*-coumaroylmyricitrin.

Compounds (**5**) and (**6**) were identified to myricitrin 2"-, and 3"-gallate, respectively by comparison with authentic spectral data. Compound (**5**) (desmanthin-1) has been isolated from several plants as a growth inhibitor of tobacco bud worm,[13] a tyrosinase inhibitor[14] and a xanthin oxidase inhibitor.[15] Compound (**6**) has also been isolated from *Myrica esculenta* along with desmanthin-1.[16] In our best knowledge, this is a first instance of isolation of **5** and **6** from the genus *Dyospyros* plants.

Compounds (**1-2**) and (**7**-**10**) were determined to be myricetin (**1**), myricetin 3-*O*-α-rhamnopyranoside (myrcitirin, **2**), 3-*O*-β-rhamnopyranoside (**7**), myricetin 3-*O*-β-glucuronide (**8**), scopoletin (**9**) and gallic acid (**10**), respectively by comparison with authentic spectral data. Myricetin 3-*O*-β-glucronide has been isolated as an anti-inflammatory constituent of *Epilobium angustifolium*.[17]

Compound (**11**), obtained from polar part as a pale yellow amorphous powder showed the $[M-H]^-$ *m/z* 507 in the negative ion FAB MS, indicating the molecular formula $C_{22}H_{20}O_{14}$. The ^1H and ^{13}C NMR spectral data showed the presence of a glucuronopyranose moiety in **11**. The ^1H and ^{13}C NMR spectral data were closely similar to **8** except for the presence methoxyl group (δ_H 3.87; δ_C 60.7). Based on the symmetrical B ring protons [δ 7.33 (2H, s, H-2' and H-6')] and the chemical shift of the methoxyl carbon signal (δ_C 60.7),[18] the B ring was 3',5'-dihydroxy-4'-methoxyl substitution. A cross peak was observed between the methoxyl proton and C-4' in the HMBC spectrum (Figure 2). Thus the aglycone of **11** was confirmed to be myricetin-4'-methyl ether (mearsetin). Another cross peak between H-1" (anomeric proton) and C-3 was observed in the HMBC spectrum. Therefore, the structure of **11** was characterized as mearsetin 3-*O*-β-glucuronopyranoside.

Table 1. Scavenging activity of DPPH

compounds	Sc_{50} (μM)
1	1.6
2	2.8
3	11.2
4	10.5
5	1.9
7	2.7
8	1.9
10	1.0
11	*
12	*
13	7.1
14	2.2
15	*
16	2.1
α-tocopherol	5.2

*>50 μM

Compound (**12**) was determined to kaempferol 3-*O*-glucuronide[19] by means of spectral analysis. Compounds (**13**-**16**), which have already isolated from *D. kaki,* were identified as kaempferol, quercetin, astragalin (kaempferol 3-*O*-β-glucopyranoside) and isoquercitrin (quercetin 3-*O*-β-glucopyranoside), respectively. Although flavonol glucuronides such as **8, 11** and **12** were isolated from various plants, it is first isolation from the genus *Diospyos* plants.

The scavenging activities (Sc_{50}) of MeOH extract of *D. glaucifolia* and *D. kaki* were 9.1 μg/mL and 27.5 μg/mL, respectively**.** The DPPH scavenging activities of the isolated compounds except for **6** and **9** are listed in Table 1. The decrease in the activity caused by 3-*O*-glycosylation (**12** and **15**) and 4'-*O*-methylation (**11**) in a flavonol skeleton. The differences of activity of two methanol

extracts may be caused by the contents of flavonoids.

EXPERIMENTAL

General Method

^{1}H and ^{13}C NMR spectra were measured on a Lambda 300 (^{1}H at 300 and ^{13}C at 75 MHz, respectively) spectrometer. Chemical shifts were given in □ values (ppm) relative to trimethylsilane (TMS) as an internal reference. Negative FABMS and EIMS were recorded on a JMSDA spectrometer equipped with a JMA 3500 data analysis system (JEOL). UV spectra were recorded on a UV-2200 spectrophotometer (Shimadzu). Silica gel 60 (70-230 mesh, Merck), Chromatorex ODS DM102T (Fuji Silysia), ODS Sep-PakC18 (Waters) and Sephadex LH 20 (Amersham Biosciences) were used for column chromatography. Kiesel-gel 60 F_{254} (0.25 and 0.5 mm) (Merck) was used for analytical and preparative TLC, respectively. Absorption in the experiment of DPPH radical scavenging activity was measured at 520 nm on a Labsystene Multiskan MS (Thermo Bio Analysis Japan Co.). DPPH was used a TOKYO KASEI's product.

Plant Material. Leaves of *Diospyros glaucifolia* cultivated in the botanical garden of Setsunan University were collected at Aug. 2002, and the voucher specimen was deposited in the University, Hirakata, Japan. Leaves of *D. kaki* were collected in Gifu, Japan at Aug. 2002. The voucher specimen was deposited in the Institute of Health and Environmental Sciences, Kakamigahara, Japan.

Extraction and isolation of flavonoids from *D. glaucifolia.* The dried leaves (600 g) of *D. glaucifolia* were extracted with chloroform (5 L x 1 week x 2 times), followed by MeOH (5 L x 1 week x 3 times) at rt. The methanol extract (65 g) was subjected on ODS column eluting with methanol-water mixture to give fractions containing flavonoids. The 30% methanol fraction was concentrated *in vacuo* and solved in MeOH. Compound (**2**) was obtained as a pale yellow powder from the MeOH solution. The resulting filtrate was further chromatographed on Sephadex LH 20 eluting with acetone to divide nine fractions by monitoring on TLC. The fractions 1 and 2 were combined and the mixture was further purified by preparative TLC to afford **1** (11 mg) and **9** (15 mg), respectively. Fractions 3-6 were re-chromatographed over ODS using 35 % methanol as solvent, followed by preparative TLC [EtOAc-$CHCl_3$-MeOH-water (15 : 12 : 5 :1)] to afford **2** (430 mg), **3** (8 mg), **4** (7 mg), **5** (10 mg), **6** (3 mg), **7** (24 mg), **10** (150 mg), respectively as pure form. The fraction 9 which contained a most polar flavonoid was purified with Sephadex LH 20 column chromatography (MeOH) to afford **8** (15 mg).

Extraction and isolation of flavonoids from *D. kaki.* The dried leaves of *D. kaki* (2 kg) were extracted with MeOH (6 L x 1 week x 3) at rt. The MeOH extract (350 g) was subjected to column chromatography on silica gel eluting with $CHCl_3$-MeOH mixture and finally with MeOH. The $CHCl_3$-MeOH (10 : 1) and (5 : 1) fractions were further purified with silica gel, Sephadex LH 20 column chromatography, and

preparative TLC to afford **13** (5 mg), **14** (8 mg), **15** (30 mg) and **16** (30 mg), respectively. The MeOH fraction (80 g) was chromatagraphed over ODS eluting with MeOH-water mixture. The 10 % MeOH elute was further fractionated by Sephadex LH 20 column (50 % acetone) to divide 20 sub-fractions. Sub-fractions 6 and 7, which contained polar flavonoids, were combined and partitioned between EtOAc and water. The water layer was subjected on Sep Pack (10 g) eluting with MeOH-water mixture. Finally, flavonoids fraction was purified by Sephadex LH 20 (30 % MeOH), followed by preparative TLC ($CHCl_3$-MeOH-water= 15 : 10 : 1) to afford **11** (3 mg) and **12** (5 mg), respectively.

Myricitrin (2). A pale yellow amorphous powder. Negative ion FABMS $[M-H]^-$ *m/z* 463; EIMS *m/z* (rel. int): 318 (100), 302 (35), 286 (10), 153 (40); ^{1}H NMR (300 MHz, acetone-d_6): δ 0.96 (3H, d, *J*= 6.3 Hz, H-6"), 3.36 (1H, t, *J*= 9.9 Hz, H-4"), 3.54 (1H, dq, *J*= 9.9, 5.7 Hz, H-5"), 3.80 (1H, dd, *J*= 9.9, 3.6 Hz, H-3"), 4.23 (1H, dd, *J*= 3.6, 1.5 Hz, H-2"), 5.30 (1H, d, *J*= 1.5 Hz, H-1"), 6.19 (1H, d, *J*= 1.8 Hz, H-6), 6.36 (1H, d, *J*= 1.8 Hz, H-8), 6.95 (2H, s, H-2', 6'); 12.53 (1H, s, C_5-OH); ^{13}C NMR (75 MHz, acetone-d_6): δ 158.2 (C-2), 135.7 (C-3), 179.2 (C-4), 163.0 (C-5), 99.3 (C-6), 164.8 (C-7), 94.3 (C-8), 157.7 (C-9), 105.6 (C-10), 121.7 (C-1'), 109.1 (C-2', 6'), 146.2 (C-3', 5'), 136.8 (C-4').

2"-(*E*)-*p*-Coumaroylmyricitrin (3). A pale yellow amorphous powder. Negative ion FABMS $[M-H]^-$ *m/z* 609; UV (nm, MeOH): 265, 300sh, 315; ^{1}H NMR (300 MHz, acetone-d_6): δ 1.10 (3H, d, *J*= 5.2 Hz, Me, H-6"), 3.42-3.60 (2H, m, H-4", 5"), 4.28 (1H, br d, *J*= 9.5 Hz, H-3"), 5.50 (1H, br s, H-2"), 5.74 (1H, br s, H-1"), 6.25 (1H, d, *J*=1.8 Hz, H-6), 6.38 (1H, d, *J*= 15.8 Hz, H-8"'), 6.48 (1H, d, *J*= 1.8 Hz, H-8), 6.89 (2H, d, *J*= 8.5 Hz, H-3'", 5'"), 7.14 (2H, s, H-2',6'), 7.56 (2H, d, *J*= 8.5 Hz, H-2"', 6"'), 7.64 (1H, d, *J*= 15.8 Hz, H-7'"), 12.56 (1H, s, C_5-OH); ^{13}C NMR (75 MHz, acetone-d_6): δ 157. 0 (C-2), 133.6 (C-3), 177.7 (C-4), 161.8 (C-5), 98.3 (C-6), 163.7 (C-7), 93.1 (C-8), 156.5 (C-9), 104.3 (C-10), 120.1 (C-1'), 107.9 (C-2', 6'), 144.6 (C-3', 5'), 136.0 (C-4'), 98.4 (C-1"), 72.0 (C-2"), 69.3 (C-3"), 71.2 (C-4"), 70.2 (C-5"), 16.5 (C-6"), 125.6 (C-1"'), 129.6 (C-2"', 6"'), 115.3 (C-3'", 5'"), 159.2 (C-4"'), 145.2 (C-7"'), 114.0 (C-8"'), 165.4 (C-9"').

3"-(*E*)-*p*-Coumaroylmyricitrin (4). A pale yellow amorphous powder. Negative ion FABMS $[M-H]^-$ *m/z* 609; UV (nm, MeOH): 263, 301sh, 316: ^{1}H NMR (300 MHz, acetone-d_6): δ 0.95 (3H, d, *J*= 5.5 Hz, Me, H-6"), 3.59 (1H, t, *J*= 9.9 Hz, H-4"), 3.62 (1H, m, H-5"), 4.49 (1H, br s, H-2"), 5.23 (1H, br d, *J*= 9.9 Hz, H-3"), 5.44 (1H, br s, H-1"), 6.21 (1H, d, *J*= 1.8 Hz, H-6), 6.38 (1H, d, *J*= 15.9 Hz, H-8"'), 6.49 (1H, d, *J*= 1.8 Hz, H-8), 6.86 (2H, d, *J*= 8.5 Hz, H-3"', 5"'), 7.64 (1H, d, J= 15.9 Hz, H-7"'), 7.14 (2H, s, H-2', 6'), 7.52 (2H, d, *J*= 8.5 Hz, H-2"', 6"'), 12.66 (1H, s, C_5-OH); ^{13}C NMR (75 MHz, acetone-d_6): δ 157.8 (C-2), 134.4 (C-3), 177.8 (C-4), 161.8 (C-5), 98.1 (C-6), 163.7 (C-7), 93.1 (C-8), 156.6 (C-9), 104.2 (C-10), 120.2 (C-1'), 108.1 (C-2',6'), 144.4 (C-3',5'), 101.6 (C-1"), 68.2 (C-2"), 74.1 (C-3"), 68.9 (C-4"), 70.5 (C-5"), 16.5 (C-6"), 125.6 (C-1"'), 129.6 (C-2"',6"'), 115.4 (C-3"',5"'), 159.2 (C-4"'), 145.1 (C-7"'), 114.4 (C-8"'), 166.5 (C-9'").

2"-Galloylmyricitrin (5). A pale yellow amorphous powder. Negative ion FABMS $[M-H]^-$ *m/z* 615; UV (nm, MeOH): 270, 302sh, 349; ^{1}H NMR (300 MHz, acetone-d_6): δ 1.03 (3H, d, *J*= 6.2 Hz, Me, H-6"), 3.50 (1H, t, *J*= 9.9 Hz, H-4"), 3.51 (1H, m, H-5"), 4.08 (1H, dd, *J*= 3.6, 9.9 Hz, H-3"), 5.65 (1H, dd, *J*= 1.5, 3.6 Hz, H-2"), 5.74 (1H, d, *J*= 1.5 Hz, H-1"), 6.26 (1H, d, *J*= 1.8 Hz, H-6), 6.38 (1H, d, *J*= 1.8 Hz, H-8), 7.13 (2H, s, H-2"', 6"'), 7.15 (2H, s, H-2', 6'), 12.53 (1H, s, C_5-OH); ^{13}C NMR (75 MHz, acetone-d_6) δ: 158.3 (C-2), 134.9 (C-3), 178.9 (C-4), 162.5 (C-5), 99.4 (C-6), 164.8 (C-7), 94.4 (C-8), 157.8 (C-9), 105.5 (C-10), 121.5 (C-1'), 110.0 (C-2', 6'), 146.4 (C-3", 5"), 137.1 (C-4"), 99.5 (C-1"), 72.7 (C-2"), 70.4 (C-3"), 73.5 (C-4"), 71.5 (C-5"), 17.1 (C-6"), 121.5 (C-1'"), 109.2 (C-2'", 6'"), 145.9 (C-3"', 5'"), 138.4 (C-4'"), 165.9 (C-7"', C=O).

3"-Galloylmyricitrin (6). A pale yellow amorphous powder. Negative ion FABMS $[M-H]^-$ *m/z* 615; UV (nm, MeOH): 269, 301sh, 349; ^{1}H NMR (300 MHz, acetone-d_6): δ1.02 (3δ, d, *J*= 6.2 Hz, Me, H-6"), 3.50 (1H, m, H-5"), 3.75 (1H, t, *J*= 9.9 Hz, H-4"), 4.51 (1H, dd, *J*= 1.5, 3.6 Hz, H-2"), 5.30 (1H, dd, J= 3.6, 9.9 Hz, H-3"), 5.51 (1H, d, *J*= 1.5 Hz, H-1"), 6.26 (1H, d, *J*= 1.8 Hz, H-6), 6.48 (1H, d, *J*= 1.8 Hz, H-8), 7.15 (2H, s, H-2"', 6"'), 7.19 (2H, s, H-2', 6'), 12.52(1H, s, C_5-OH).

Mearsetin 3-*O*-β-glucuronide (11). A pale yellow amorphous powder. Negative ion FABMS: $[M-H]^-$ *m/z* 507; UV (nm, MeOH): 264, 346. ^{1}H NMR (300 MHz, methanol-d_4): δ 3.87 (3H, s, OMe), 5.39 (1H, d, *J*= 7.3 Hz, H-1"), 6.17 (1H, d, *J*= 2.0 Hz, H-6), 6.33 (1H, d, *J*= 2.0 Hz, H-8), 7.33 (2H, s, H-2', 6'); ^{13}C NMR (75 MHz, methanol-d_4) δ: 157.8 (C-2), 136.3 (C-3), 179.2 (C-4), 162.9 (C-5), 100.7 (C-6), 167.0 (C-7), 95.2 (C-8), 158.4 (C-9), 105.2 (C-10), 126.6 (C-1'), 110.2 (C-2', 6'), 151.4 (C-3', 5'), 139.2 (C-4'), 104.3 (C-1"), 75.6 (C-2"), 78.2 (C-3"), 73.4 (C-4"), 77.6 (C-5"), 176.4 (C-6"), 60.7 (OMe).

Scavenging activity of DPPH. The DPPH scavenging activity was examined by the method described in the literatures.[20,21]

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