Flavonoids from *Andrographis viscosula*

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Phytochemical investigation of the whole plant of *Andrographis viscosula* has led to the isolation of three new 2′-oxygenated flavonoids, (2*R*)-5-hydroxy-7,2′,3′-trimethoxyflavanone (1), 7,2′,5′-trimethoxyflavone (2), 5,7,2′,3′-tetramethoxyflavone (3), and eight known flavones, 5,7,2′-trimethoxyflavone (4), 5,7,2′,4′,5′-pentamethoxyflavone (5), echioidinin (6), 5,2′,6′-trihydroxy-7-methoxyflavone (7), 5-hydroxy-7,2′-dimethoxyflavone (8), echioidin (9), echioidinin 5-*O*-glucoside (10), and 5,2′,6′-trihydroxy-7-methoxyflavone 2′-*O*-glucoside (11). The structures of 1—11 were elucidated by physical and spectral methods, including extensive 2D NMR studies. The presence of 2′-oxygenated flavonoids is apparently restricted to *Andrographis* species in Acanthaceae. Therefore, 2′-oxygenated flavonoids are regarded as chemotaxonomic markers of *Andrographis* genus in the Acanthaceae family.

Key words *Andrographis viscosula*; Acanthaceae; 2′-oxygenated flavonoid; 5-deoxyflavone; traditional Indian medicine

Andrographis (Acanthaceae) is a genus of about 40 species several members of which enjoy a reputation in indigenous medicine. In the traditional Indian medicine some of the *Andrographis* species were used in the treatment of dyspepsia, influenza, malaria and respiratory infections, and as astringent and antidote for poisonous stings of some insects.[1,2] About 21 species of *Andrographis* have been reported to occur in India. *A. viscosula* Nees is an erect herb found widely in the Hills of Tinnevelly, south India.[3] The phytochemistry of this genus has been investigated quite well in view of its importance in Indian traditional medicine and reported to contain several flavonoids[4,5] and labdane diterpenoids.[6—11] As part of our program to study the flavonoid constituents of *Andrographis* species,[12—18] we have investigated the whole plant of *A. viscosula*. In previous paper, we have reported the isolation and structural elucidation of two new and three known flavones from this plant.[18] Further investigation of extracts of the whole plant has led to the isolation of a new flavanone, (2*R*)-5-hydroxy-7,2′,3′-trimethoxyflavanone (**1**), a new 5-deoxyflavone, 7,2′,5′-trimethoxyflavone (**2**), and a new flavone, 5,7,2′,3′-tetramethoxyflavone (**3**) together with eight known flavonoids (**4**—**11**). This paper deals with the isolation and structural elucidation of **1**—**3**.

Results and Discussion

Compound **1** was isolated as colorless solid and gave a positive ferric chloride test. The high resolution electron impact mass spectrum (HR-EI-MS) of **1** exhibited the molecular ion at *m/z* 330.1105 consistent with the molecular formula $C_{18}H_{18}O_6$. This was further corroborated by the 18 carbon signals in ^{13}C-NMR spectrum, which include a conjugated carbonyl, seven nonprotonated, six methine, one methylene, and three methoxyl carbons. The UV spectrum of **1** in MeOH showed absorption maxima at 286 and 329 (sh) nm, typical of a flavanone derivative.[19] A bathochromic shift of 40 nm in its UV absorption maxima on addition of $AlCl_3$ and $AlCl_3$/HCl suggested the presence of a chelated hydroxyl group at C-5 position. The IR absorption bands at 3425 and 1647 cm^{-1} indicated the presence of hydroxyl and carbonyl functions, respectively. Accordingly, the ^{1}H-NMR spectrum of **1** revealed a D_2O exchangeable downfield signal at δ 12.08 assignable to a chelated hydroxyl group at C-5. An AMX system with resonances at δ 5.76 (1H, dd, J=13.2, 2.7 Hz), 3.04 (1H, dd, J=17.2, 13.2 Hz), and 2.80 (1H, dd, J=17.2, 2.7 Hz) were characteristic of H-2, H-3$_{ax}$, and H-3$_{eq}$, respectively, of a flavanone moiety.[20] Two *meta* coupled doublets (J=2.1 Hz) each integrating for one proton at δ 6.08 and 6.05 were assigned to H-6 and H-8, respectively. A typical ABC system at δ 7.13 (2H, d, J=8.1 Hz, H-4′, -6′) and 6.94 (1H, t, J=8.1 Hz, H-5′) for three consecutive protons, H-4′, H-5′, and H-6′ established the 2′,3′-dioxygenated B-ring in the molecule.[21] The ^{1}H-NMR spectrum also showed signals due to three methoxyl groups (each 3H, s) at δ 3.89, 3.87, and 3.80. The EI-MS fragmentation of the molecular ion at *m/z* 330 [M]$^+$ of **1** in its *retro* Diels–Alder fragmentation at ring C yielded diagnostic peaks at *m/z* 166 and 164 indicating the presence of a hydroxyl and a methoxyl group in ring A, and two methoxyl groups in ring B, respectively. Of the three methoxyl groups in **1**, the one at δ 3.80 was placed at C-7 on the basis of its ^3J correlation with C-7 at δ 167.8 in the heteronuclear multiple bond connectivity (HMBC) spectrum and two strong NOEs with H-6 and H-8 in the nuclear Overhauser enhancement spectroscopy (NOESY). The chemical shift of C-2 in 2′-unsubstituted flavanones usually appears at δ 79.0. However, in compound **1**, the C-2 signal appeared at an upfield position (δ 74.5), indicating the presence of oxygenation at C-2′.[22] Thus the methoxyl group at δ 3.87 was attached to C-2′, as it displayed ^3J HMBC correlation with C-2′ at δ 146.1. The remaining methoxyl group at δ 3.89 was assigned to C-3′, as evidenced by the HMBC connectivity with C-3′ at δ 152.5, and two strong NOEs with H-4′ and OCH_3-2′. The absolute configuration at C-2 was determined to be *R* by the negative Cotton effect at 330 nm and a positive Cotton effect at 289 nm in its circular dichroism (CD) spectrum.[23] From these findings, the structure of **1** was elucidated as (2*R*)-5-hydroxy-7,2′,3′-trimethoxyflavanone.

Compound **2**, obtained as yellow amorphous powder, showed [M]$^+$ peak at *m/z* 312.1000 in its HR-EI-MS corresponding to the molecular formula $C_{18}H_{16}O_5$, which was further supported by the appearance of 18 carbon signals in the

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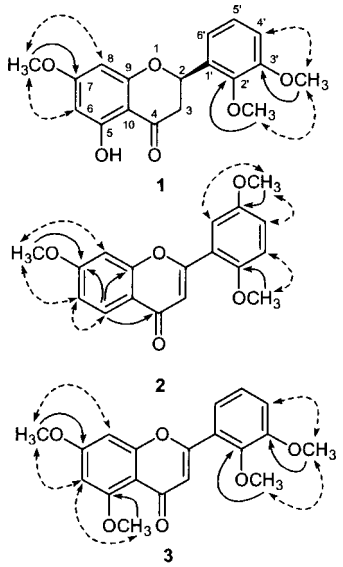

Fig. 1. Key HMBC (Dark Arrow) and NOESY (Dotted Double-Headed Arrow) Correlations of **1**—**3**

^{13}C-NMR spectrum. The UV absorption maxima of **2** in MeOH at 260 and 303 nm were characteristic of a 5-deoxyflavone skeleton.[24] Addition of $AlCl_3$ and NaOAc caused no shift in the UV absorption maxima, also indicating the absence of free hydroxyls at the 5- and 7-positions, respectively. The IR absorption bands at 2956, 1657, 1614 and 1504 cm^{-1}, and negative ferric chloride test inferred that compound **2** could be a nonphenolic flavone. The ^{1}H-NMR spectrum exhibited a set of deshielded ABX protons at δ 8.13 (1H, d, J=8.8 Hz), 6.96 (1H, dd, J=8.8, 2.4 Hz), and 6.93 (1H, d, J=2.4 Hz) typical for H-5, H-6, and H-8, respectively of a 5-deoxyflavone.[25] In the hetero nuclear single quantum coherence (HSQC) spectrum, a sharp one proton singlet at δ 7.23 correlating with C-3 (δ 112.1) was ascribed to H-3 of a 2′-oxygenated flavone.[26] The ^{1}H-NMR spectrum also showed a typical ABX system for three aromatic protons at δ 7.43 (1H, d, J=2.8 Hz, H-6′), 7.04 (1H, dd, J=6.8, 2.8 Hz, H-4′), and 7.01 (1H, d, J=6.8 Hz, H-3′) characteristic of a 2′,5′-dioxygenated flavone.[27] This was further supported by the chemical shift of ring-B carbons, which were closely resemble to the literature values of 2′,5′-dioxygenated flavones.[28] The ^{1}H-NMR spectrum of **2** revealed the signals for three aromatic methoxyl groups at δ 3.92, 3.89, and 3.85. The EI-MS of **2** showed two *retro* Diels–Alder fragments at *m*/*z* 150 and 162 consistent with the presence of one methoxyl group in ring-A and two methoxyl groups in B-ring, respectively. The methoxyl group at δ 3.92 was attached to C-7 based on an HMBC correlation of these protons with C-7 at δ 164.3 and two NOE crosspeaks with H-6 and H-8 in its NOESY spectrum. Therefore, the other two methoxyl groups at δ 3.89 and 3.85 should be present at the 2′ and 5′ positions. This was confirmed by the HMBC connectivities of these protons with C-2′ (δ 153.4) and C-5′ (δ 152.4), and NOESY correlations with H-3′, and H-4′ and H-6′, respectively. Thus, the structure of **2** was established as 7,2′,5′-trimethoxyflavone.

Compound **3** was obtained as yellowish solid. Its HR-EI-MS displayed molecular ion peak at *m*/*z* 342.1106, which was in accord with the molecular formula $C_{19}H_{18}O_6$ and was also corroborated by the 19 carbon signals in ^{13}C-NMR spectrum. The UV spectrum of **3** in MeOH at 261 and 304 nm suggested a flavone structure.[29] Its UV spectrum was unaffected by the shift reagents NaOAc and $AlCl_3$/HCl indicating the absence of free hydroxyls at the C-7 and C-5 positions. The IR absorptions at 2928, 1661, 1614 and 1471 cm^{-1}, and negative ferric chloride test also indicated that compound **3** had no free hydroxyl groups. The ^{1}H-NMR spectrum of **3** showed the presence of four methoxyl groups at δ 3.93, 3.89, 3.88, and 3.87. A set of *meta* coupled doublets (J=2.0 Hz) at δ 6.35 and 6.50 were assigned to H-6 and H-8, respectively. A sharp singlet integrating for one proton at δ 6.89 correlated with C-3 in its HSQC spectrum was characteristic of C-3 proton of a 2′-oxygenated flavone.[26] Two of the four methoxyl groups at δ 3.93 and 3.88 were placed at C-5 and C-7, respectively as indicated by ^3J HMBC correlations with carbons at δ 160.8 (C-5) and 164.1 (C-7). These assignments were further substantiated by NOE crosspeaks of C-5 methoxyl protons with H-6, and C-7 methoxyl protons with H-6 and H-8 in NOESY studies. The EI-MS fragments at *m*/*z* 180 and 162 formed after *retro* Diels–Alder cleavage, suggested that **3** has two methoxyl groups in ring-A and other two methoxyl groups in ring-B. This fixes the placement of the remaining two methoxyl groups at C-2′ (δ 3.87) and C-3′ (δ 3.89), further supported by HMBC connectivities of these methoxyl protons with C-2′ (δ 147.9) and C-3′ (δ 153.3), respectively. This was also inferred by the NOEs, OCH_3-2′/OCH_3-3′, OCH_3-3′/H-4′, and H-5′/H-4′, H-6′ in NOESY experiment. Moreover, a typical ABC pattern signals appeared at δ 7.30 (1H, d, J=8.0 Hz, H-6′), 7.14 (1H, t, J=8.0 Hz, H-5′), and 7.03 (1H, d, J=8.0 Hz, H-4′) established the presence of three adjacent aromatic protons in ring-B.[21] From the foregoing spectral analyses, the structure of compound **3** was characterized as 5,7,2′,3′-tetramethoxyflavone.

The known isolates from the whole plant of *A. viscosula* were identified as 5,7,2′-trimethoxyflavone (**4**),[18] 5,7,2′,4′,6′-pentamethoxyflavone (**5**),[18] echioidinin (**6**),[13] 5,2′,6′-trihydroxy-7-methoxyflavone (**7**),[12] 5-hydroxy-7,2′-dimethoxyflavone (**8**),[30] echioidin (**9**),[30] echioidinin 5-*O*-glucoside (**10**),[13] and 5,2′,6′-trihydroxy-7-methoxyflavone 2′-*O*-glucoside (**11**)[12] by comparison of their physical and spectral data with literature values.

Incidentally, the occurrence of **2**, constitutes the first report of a 2′-oxygenated flavonoid without 5-oxygenation in the genus, *Andrographis*. Isolation of three new and eight known 2′-oxygenated flavonoids, which occur rarely in nature, from *A. viscosula* provided strong evidence for the statement, "*Andrographis* species are noted for profuse production of 2′-oxygenated flavonoids." Accordingly, 2′-oxygenated flavonoids met with in Acanthaceae so far were confined to *Andrographis* species only. This shows promise of being a useful chemotaxonomic marker of *Andrographis* in Acanthaceae.

The isolates **1**—**4**, **8**, and **9** were assayed for the α,α-diphenyl-β-picrylhydrazyl free radical (DPPH) scavenging and antityrosinase activities using the reported procedures.[31] None of these compounds showed significant antioxidant and antityrosinase properties. It is conceivable to be the cause of these that these compounds cannot possess in their structures an *o*-dihydroxy group which is the putative radical target site.

Experimental

General Experimental Methods Melting points were measured on Yanaco MP-S3 micro-melting point apparatus and uncorrected. ^{1}H- and ^{13}C-NMR spectra were obtained on the Bruker Avance-300 NMR spectrometer, with tetramethylsilane (TMS) as internal standard. IR spectra were determined as KBr discs on a Perkin-Elmer FT-IR Paragon-500 spectrophotometer, and UV spectra were recorded in MeOH on a Hitachi UV-3210 spectrophotometer. EI- and HR-EI-MS were measured with a 70 eV direct inlet system on a VG70-250S spectrometer. Optical rotations were recorded on a Jasco DIP-370 digital polarimeter. CD spectra were recorded with a Jasco J-720 spectropolarimeter.

Plant Material The whole plant of *A. viscosula* Nees, used in this study was collected in December, 1998 from the Hills of Tinnevelly, South India and authenticated by Dr. Madhava Chetty. A voucher specimen (CVR-989) has been deposited in the Herbarium of the Department of Botany, Sri Venkateswara University, Tirupati, India.

Extraction and Separation The whole plant of *A. viscosula* (2.5 kg) was shade dried, powdered, and extracted with *n*-hexane (51×3), Me_2CO (51×3), and MeOH (51×3), successively. The *n*-hexane extract (30 g) was column chromatographed over silica gel using *n*-hexane and EtOAc step gradient as eluents. The *n*-hexane and EtOAc, 8 : 2, 7 : 3, and 1 : 1 eluates were purified individually by repeated columns over silica gel followed by prep. TLC (developed with benzene/EtOAc, 9 : 1) to yield **1** (12 mg), **2** (10 mg), **3** (9 mg), **4** (23 mg), and **5** (26 mg). The Me_2CO extract (90 g) was defatted with *n*-hexane and the residue obtained was separated by using conventional isolation techniques (*n*-hexane–ethyl acetate mixtures) to obtain **6** (30 mg), **7** (24 mg), and **8** (11 mg). The MeOH extract (30 g) on purification over silica gel columns repeatedly, employing EtOAc/MeOH step gradient eluents afforded **9** (15 mg), **10** (5 mg), and **11** (8 mg).

(2*R*)-5-Hydroxy-7,2′,3′-trimethoxyflavanone (**1**): Colorless solid (MeOH); mp 148—150 °C. $[\alpha]_D^{25}$: −16.6° (c=0.1, MeOH). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 2.80 (1H, dd, J=17.2, 2.7 Hz, H-3β), 3.04 (1H, dd, J=17.2, 13.2 Hz, H-3α), 3.80 (3H, s, OCH_3-7), 3.87 (3H, s, OCH_3-2′), 3.89 (3H, s, OCH_3-3′), 5.76 (1H, dd, J=13.2, 2.7 Hz, H-2), 6.05 (1H, d, J=2.1 Hz, H-8), 6.08 (1H, d, J=2.1 Hz, H-6), 6.94 (1H, t, J=8.1 Hz, H-5′), 7.13 (2H, d, J=8.1 Hz, H-4′, 6′), 12.08 (1H, s, D_2O exchangeable, OH-5). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 42.6 (C-3), 55.6 (OCH_3-7), 55.7 (OCH_3-3′), 61.0 (OCH_3-2′), 74.5 (C-2), 94.1 (C-8), 94.9 (C-6), 103.1 (C-10), 112.6 (C-5′), 118.2 (C-6′), 124.4 (C-4′), 132.2 (C-1′), 146.1 (C-2′), 152.5 (C-3′), 163.0 (C-9), 164.1 (C-5), 167.8 (C-7), 196.2 (C-4). IR (KBr) cm^{-1}: 3425 (OH), 2918 (OCH_3), 1647 (C=O), 1633, 1576, 1500, 1480, 1447, 1357, 1280, 1159. UV λ_{max} (MeOH) nm (log ε): 329 (sh) (1.93), 286 (2.73); (+$AlCl_3$) 369, 311; (+$AlCl_3$+HCl) 369, 309. CD (c=0.00049, MeOH): $[\theta]_{330}$ −162, $[\theta]_{312}$ 0, $[\theta]_{289}$ +225, $[\theta]_{270}$ 0, $[\theta]_{264}$ −571. HR-EI-MS m/z: 330.1105 (Calcd for $C_{18}H_{18}O_6$: 330.1103). EI-MS m/z: 330 (M^+), 315, 299, 268, 193, 167, 166, 164, 149, 138, 121, 95.

7,2′,5′-Trimethoxyflavone (**2**): Yellow amorphous solid (MeOH); mp 136—138 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 3.85 (3H, s, OCH_3-5′), 3.89 (3H, s, OCH_3-2′), 3.92 (3H, s, OCH_3-7), 6.93 (1H, d, J=2.4 Hz, H-8), 6.96 (1H, dd, J=8.8, 2.4 Hz, H-6), 7.01 (1H, d, J=6.8 Hz, H-3′), 7.04 (1H, dd, J=6.8, 2.8 Hz, H-4′), 7.23 (1H, s, H-3), 7.43 (1H, d, J=2.8 Hz, H-6′), 8.13 (1H, d, J=8.8 Hz, H-5). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 55.8 (OCH_3-7), 55.9 (OCH_3-2′), 56.1 (OCH_3-5′), 100.2 (C-8), 112.1 (C-3), 113.0 (C-6), 114.6 (C-6′, C-3′), 117.1 (C-10), 117.4 (C-4′), 121.1 (C-1′), 126.9 (C-5), 152.4 (C-5′), 153.4 (C-2′), 158.2 (C-9), 160.6 (C-2), 164.3 (C-7), 178.1 (C-4). IR (KBr) cm^{-1}: 2956 (OCH_3), 1657 (C=O), 1615, 1585, 1504, 1434, 1347. UV λ_{max} (MeOH) nm (log ε): 303 (2.77), 260 (2.95). HR-EI-MS m/z: 312.1000 (Calcd for $C_{18}H_{16}O_5$: 312.0997). EI-MS m/z: 312 (M^+), 297, 281, 269, 162, 151, 150, 122, 119, 107.

5,7,2′,3′-Tetramethoxyflavone (**3**): Yellowish solid (MeOH); mp 152—154 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 3.87 (3H, s, OCH_3-2′), 3.88 (3H, s, OCH_3-7), 3.89 (3H, s, OCH_3-3′), 3.93 (3H, s, OCH_3-5), 6.35 (1H, d, J=2.0 Hz, H-6), 6.50 (1H, d, J=2.0 Hz, H-8), 6.89 (1H, s, H-3), 7.03 (1H, d, J=8.0 Hz, H-4′), 7.14 (1H, t, J=8.0 Hz, H-5′), 7.30 (1H, d, J=8.0 Hz, H-6′). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 55.7 (OCH_3-2′), 56.0 (OCH_3-3′), 56.3 (OCH_3-5), 60.8 (OCH_3-7), 92.7 (C-6), 96.0 (C-8), 108.9 (C-10), 113.3 (C-3), 114.8 (C-4′), 120.5 (C-6′), 124.1 (C-5′), 126.1 (C-1′), 147.9 (C-2′), 153.3 (C-3′), 159.2 (C-2), 160.1 (C-9), 160.8 (C-5), 164.1 (C-7), 177.7 (C-4). IR (KBr) cm^{-1}: 2928 (OCH_3), 1661 (C=O), 1614, 1571, 1471, 1419, 1338, 1261, 1219, 1116. UV λ_{max} (MeOH) nm (log ε): 304 (2.55), 261 (2.89). HR-EI-MS m/z: 342.1106 (Calcd for $C_{19}H_{18}O_6$: 342.1103). EI-MS m/z: 342 (M^+), 314, 311, 296, 181, 180, 166, 162, 151, 137, 119.

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