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Flavonoids from *Thyrsanthera suborbicularis* and Their NO Inhibitory Activity

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Abstract – Further phytochemical investigation on the whole plant of *Thyrsanthera suborbicularis,* collected in Cambodia, led to kaempferol (**1**), vitexin (**2**), apigenin-7-*O*-neohesperidoside (**3**), chrysoeriol-7-*O*-β-D-glucopyranoside (**4**), isorhamnetin 3-*O*-rutinoside (**5**), kaempferol-3-*O*-[α-L-rhamnopyranosyl-(13)-α-L-rhamnopyranosyl-(16)-β-D-galactopyranoside (**6**), kaempferol-3-*O*-α-L-rhamnopyranosyl(12)-*O*-[α-L-rhamnopyranosyl (16)]-β-D-glucopyranoside (**7**), kaempferol-3-*O*-[6"-*O*-(*E*)-*p*-coumaroyl]-β-D-glucopyranoside (**8**), kaempferol-3-*O*-[6"-*O*-(*E*)-*p*-coumaroyl]-β-D-galactopyranoside (**9**), and amentoflavone (**10**). All the structures were confirmed by the interpretation of NMR (1D and 2D) and MS data, and comparison with the published values. Of the isolated compounds **1** - **10**, compounds **8** and **10** displayed the inhibitory activity against NO production in LPS-induced Raw 264.7 cells with IC_{50} values, 3.56 and 15.73 μM, respectively.
Keywords – *Thyrsanthera suborbicularis,* Flavonoids, Nitric oxide inhibition

Introduction

Thyrsanthera suborbicularis Pierre ex Gagnep. (Euphorbiaceae) is the sole member of the genus *Thyrsanthera*, found in Cambodia, Vietnam and Thailand (Pauline, 2000). Traditionally, the twigs with leaves of this plant have been used for mothers after birth and the roots for the treatment of malaria (Pauline, 2000).

Lipopolysaccharide (LPS) is a macromolecular component of Gram-negative bacteria that stimulates a cellular inflammatory response and the release of inflammatory mediator such as nitric oxide (NO) (Palmer et al., 1998). Nitric oxide (NO) is a physiological mediator that mediates a variety of biological functions (Vallance and Leiper, 2002). Previous study on this plant reported the presence of diterpenoid, sesquiterpenoids, and triterpenoids as well as the inhibitory activity of diterpenoid on lipopolysaccharide-induced nitric oxide production (Piseth *et al*., 2011). As part of our ongoing search for phytochemical investigation on this plant, further investigation on the EtOAc and *n*-BuOH-soluble fractions of *T. suborbicularis* was conducted and led to the isolation and identification of ten known flavonoids from this plant for the first time. All the isolates were assessed for their NO production inhibitory activity.

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Experimental

General experimental procedures – Optical rotation were measured with a Jasco P-1020 polarimeter (Jasco corporation, Japan), UV using UV-VIS spectrophotometer 2400 (Shimadzu Co. Ltd., Japan), and FT-IR spectra using a Jasco FT/IR-4200 (Jasco corporation, Japan). NMR spectra were recorded on a Varian UNITY 300 and 400 (Varian, Inc., Palo Alto, CA) FT-NMR spectrometer with the tetramethylsilane as an internal standard. HRESIMS was performed with on a Waters Q-Tof Premier spectrometer (Micromass UK Ltd., Manchester, UK). Sephadex LH-20 (25 - 100 μm, Sigma-Aldrich, Steinheim, Germany), silica gel (230 - 400 mesh, SiliCycle Inc., Quebec, Canada), RP-C18 (Cosmosil $40C_{18}$-PREP, Kyoto, Japan) were used for column chromatography. TLC was performed on precoated Kiesel-gel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany) and Kiesel-gel 60 RP-$18F_{254s}$ (0.25 mm, Merck, Steinheim, Germany).

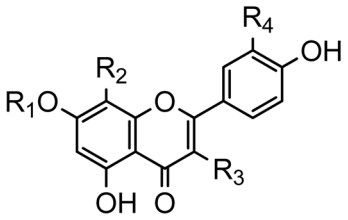
Plant materials – Whole plants of *T. suborbicularis* were collected in Kandal province, Cambodia in February 2007. These plant samples were identified by Prof. Yok Lin, Department of Biology, Faculty of Science, and Royal University of Phnom Penh, Cambodia. A voucher specimen (KRIBB 023087) has been deposited at herbarium of Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea.

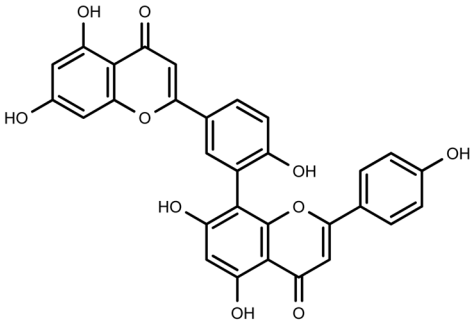
Extraction and isolation – Air-dried whole plants of *T. suborbicularis* (6.0 kg) were extracted with MeOH (3 × 20 L) at room temperature three times to obtain 366.0 g of solid extract. The MeOH extract was suspended in H_2O and then partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, successively, to give 70.0 g of *n*-hexane-soluble extract, 23.4 g of $CHCl_3$-soluble extract, 8.7 g of EtOAc-soluble extract, and 35.0 g of *n*-BuOH-soluble extract, respectively. The EtOAc-soluble extract was subjected to a medium pressure liquid chromatography (MPLC) column of RP C-18 (7.0 × 100 cm, 40C_{18}-PREP), eluted with MeOH-H_2O [2:3 (2 l), 1 : 1 (2 l), 3 : 2 (2 l), 3 : 1 (2 l), 100% MeOH (2 l), v/v] to yield eleven fractions (TS22-1–TS22-11). Fraction TS22-5 (520 mg) was subjected to a silica gel column chromatography (10 × 100 cm, 230 - 400 mesh) with a stepwise gradient mixture of $CHCl_3$-MeOH [5 : 1 (1 l), 4 : 1 (2 l), 3 : 1 (1 l), 1 : 1 (1 l), v/v)] to obtain four fractions (TS22-5-A–TS22-5-E). Fraction TS22-5-B (252 mg) was further fractionated using a Sephadex LH-20 column chromatography eluted with 70% MeOH in H_2O to give five fractions (TS22-5-B-1–TS22-5-B-5). TS22-5-B-3 (27 mg) was purified by RP-HPLC [Cosmosil C_{18}-MS-II 20 × 250 mm column and Varian 150 × 21.2 mm column, MeOH-H_2O (3 : 2), 10 mL/min] to afford compounds **3** (3 mg, t_R = 23.3 min) and **5** (16 mg, t_R = 26.6 min). TS22-5-B-4 (20 mg) was purified by RP-HPLC [Varian 150 × 21.2 mm column, MeOH-H_2O (3:2), 10 mL/min] to afford compound **4** (8 mg, t_R = 29.2 min). Fraction TS22-7 (730 mg) was subjected to a Sephadex LH-20 column chromatography eluted with 70% MeOH in H_2O to give compound **1** (4 mg) and fraction TS22-7-B. TS22-7-B (150 mg) was purified by RP-HPLC [Cosmosil C_{18}-MS-II 20 × 250 mm column and Varian 150 ¥ 21.2 mm column, MeCN-H_2O (1 : 3.3), 10 mL/min] to afford compounds **8** (47 mg, t_R = 48.2 min) and **9** (17 mg, t_R = 45.2 min). Fraction TS22-10 (300 mg) was subjected to a silica gel column chromatography (10 × 100 cm, 230 - 400 mesh) and eluted with $CHCl_3$-MeOH [10 : 1 (2 l), v/v] to give compound **10** (180 mg). The *n*-BuOH-soluble extract (10 g) was subjected to a MPLC column of RP C-18 (7.0 × 100 cm, 40C_{18}-PREP), eluted with MeOH-H_2O (2:3) to yield ten fractions (TS31-1–TS31-10). Fraction TS31-4 (1 g) was subjected to a Sephadex LH-20 column chromatography eluted with 70% MeOH in H_2O to give compound **2** (49 mg). Fraction TS31-5 (1.0 g) was subjected to a Sephadex LH-20 column chromatography eluted with 50% MeOH in H_2O to give seven fractions (TS31-5-A–TS31-5-G). Fraction TS31-5-F (300 mg) was purified by RP-HPLC [Varian 150 × 21.2 mm column, MeOH-H_2O (1 : 1), 10 mL/min] to afford compounds **6** (14 mg, t_R = 19.6 min) and **7** (27 mg, t_R = 25.5 min).

Kaempferol (1) – Yellow powder**,** UV (MeOH) λ_{max} nm (log ε): 247 (4.55), 339 (4.51), HRESIMS (positive mode) at *m/z* 287.0545 $[M + H]^+$ (calcd for $C_{15}H_{11}O_6$, 287.0556), ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 8.04 (2H, d, *J* = 8.8 Hz, H-2',6'), 6.92 (2H, d, *J* = 8.8 Hz, H-3',5'), 6.43 (1H, d, *J* = 1.5 Hz, H-8), 6.18 (1H, d, *J* = 1.5 Hz, H-6), ^{13}C-NMR (75 Hz, DMSO-d_6) δ: 175.9 (C-4), 163.9 (C-7), 160.7 (C-5), 159.2 (C-4'), 156.1 (C-9), 146.8 (C-2), 135.7 (C-3), 129.5 (C-2',6'), 121.7 (C-1'), 115.4 (C-3',5'), 103.1 (C-10), 98.2 (C-6), 93.5 (C-8).

Vitexin (2) – Yellow powder, $[\alpha]_D$ -8.5 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 270 (4.26), 332 (4.29), HRESIMS (positive mode) at *m/z* 433.1121 $[M + H]^+$ (calcd for $C_{21}H_{21}O_{10}$, 433.1135), ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 8.01 (2H, d, *J* = 8.7 Hz, H-2',6'), 6.98 (2H, d, *J* = 8.7 Hz, H-3',5'), 6.77 (1H, s, H-3), 6.25 (1H, s, H-6), 4.68 (1H, d, *J* = 9.6 Hz, H-1"), 3.96 (1H, m, H-2"), 3.75 (1H, m, H-6"a), 3.51 (1H, m, H-6"b), 3.32-3.25 (m, H-3", 4", 5"). ^{13}C-NMR (75 Hz, DMSO-d_6) δ: 182.01 (C-4), 163.8 (C-2), 162.5 (C-7), 161.1 (C-4'), 160.4 (C-5), 156.0 (C-9), 128.9 (C-2',6'), 121.6 (C-1'), 115.8 (C-3',5'), 104.6 (C-8), 103.9 (C-10), 102.4 (C-3), 98.2 (C-6), 81.8 (C-5"), 78.6 (C-3"), 73.4 (C-1"), 70.8 (C-2"), 70.5 (C-4"), 61.2 (C-6").

Apigenin-7-*O*-neohesperidoside;Rhoifolin (3) – Yellow-brown amorphous powder, $[\alpha]_D$ −33.5 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 268 (4.20), 330 (4.26), HRESIMS (positive mode) at *m/z* 579.1681 $[M + H]^+$ (calcd for $C_{27}H_{31}O_{14}$, 579.1714), ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 7.94 (2H, d, *J* = 8.7 Hz, H-2',6'), 6.94 (2H, d, *J* = 8.7 Hz, H-3',5'), 6.87 (1H, s, H-3), 6.78 (1H, d, *J* = 2.2 Hz, H-8), 6.36 (1H, d, *J* = 2.2, Hz, H-6), 5.22 (1H, d, *J* = 7.5 Hz, H-1"), 5.12 (1H, brs, H-1'"), 3.11-3.77 (m , sugar protons), ^{13}C-NMR (75 Hz, DMSO-d_6) d:181.9 (C-4), 164.2 (C-2), 162.5 (C-7), 161.4 (C-4'), 161.1 (C-5), 156.9 (C-9), 128.5 (C-2',6'), 120.9 (C-1'), 115.9 (C-3',5'), 105.4 (C-10), 103.4 (C-3), 100.4 (C-1'"), 99.3 (C-6), 97.8 (C-1"), 94.0 (C-8), 77.4 (C-5"), 77.2 (C-2"), 77.1 (C-3"), 72.4 (C-4'"), 70.5 (C-4", 3'"), 70.2 (C-2'"), 68.3 (C-5'"), 61.5 (C-6"), 18.1 (C-6'").





1 R_1, R_2, R_3, R_4 = H
2 R_1, R_3, R_4 = H, R_2 = glucopyranoside
3 R_2, R_3, R_4 = H, R_1 = neohesperidoside
4 R_2, R_3, = H, R_4 = OCH_3, R_1 = glucopyranoside
5 R_1, R_2 = H, R_4 = OCH_3, R_3 = rutinoside
6 R_2, R_3, R_4 = H, R_3 = *O*-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside
7 R_1, R_2, R_4 = H,, R_3 = *O*-α-L-rhamnopyranosyl(1→2)-*O*-[α–L-rhamnopyranosyl(1→6)]-β-D-glucopyranoside
8 R_1, R_2, R_4 = H, R_3 = *O*-[6''-*O*-*(E)*-ρ-coumaroyl]-β-D-glucopyranoside
9 R_1, R_2, R_4 = H, R_3 = *O*-[6''-*O*-*(E)*-ρ-coumaroyl]-β-D-galactopyranoside

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Fig. 1. Structures **1** - **10** from T. suborbicularis.
(**1**; Kampferol, **2**; Vitexin, **3**; Apigenin-7-*O*-neohesperidoside, **4**; Chrysoeriol-7-*O*-β-D-glucopyranoside, **5**; Isorhamnetin 3-*O*-rutinoside, **6**; Kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside], **7**; Kaempferol-3-*O*-α-L-rhamnopyranosyl(1 → 2)-*O*-[α-L-rhamnopyranosyl(1 → 6)]-β-D-glucopyranoside, **8**; Kaempferol-3-*O*-[6''-*O*-(E)-*p*-coumaroyl]-β-D-glucopyranoside, **9**; Kaempferol-3-*O*-[6''-*O*-(E)-*p*-coumaroyl]-β-D-galactopyranoside , **10**; Amentoflavone).

Chrysoeriol-7-*O*-β-D-glucopyranoside (4) – Yellow powder, $[\alpha]_D$ −1.8 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 266 (3.88), 364 (3.86), HRESIMS (positive mode) at *m/z* 463.1219 $[M+H]^+$ (calcd for $C_{22}H_{23}O_{11}$, 463.1240); ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 7.61 (1H, d, *J* = 2.1 Hz, H-2'), 7.59 (1H, dd, *J* = 8.0, 2.1 Hz, H-6'), 6.97 (1H, s, H-3), 6.94 (1H, d, *J* = 8.0 Hz, H-5'), 6.86 (1H, d, *J* = 2.2 Hz, H-8), 6.44 (1H, d, *J* = 2.2 Hz, H-6), 5.05 (1H, d, *J* = 7.2 Hz, H-1''), 3.89 (s, OCH_3), 3.16-3.47 (m, H-2'', 3'', 4'', 5'', 6''), ^{13}C-NMR (75 Hz, DMSO-d_6) d:182.0 (C-4), 164.2 (C-2), 162.9 (C-7), 161.1 (C-5), 156.9 (C-9), 151.3 (C-3'), 148.1 (C-4'), 121.0 (C-1'), 120.5 (C-6'), 115.8 (C-5'), 110.3 (C-2'), 105.3 (C-10), 103.3 (C-3), 100.0 (C-1''), 99.5 (C-6), 95.0 (C-8), 77.2 (C-3''), 76.4 (C-5''), 73.1 (C-2''), 69.6 (C-4''), 60.6 (C-6''), 55.9 (OMe, C-3').

Isorhamnetin 3-*O*-rutinoside (5) – Yellow amorphous powder, $[\alpha]_D$ −9.7 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 253 (4.95), 349 (4.88), HRESIMS (positive mode) at *m/z* 625.1780 $[M+H]^+$ (calcd for $C_{28}H_{33}O_{16}$, 625.1769), ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 7.84 (1H, d, *J* = 2.2 Hz, H-2'), 7.51 (1H, dd, *J* = 2.2, 8.1 Hz, H-6'), 6.90 (1H, d, *J* = 8.1 Hz, H-5'), 6.42 (1H, d, *J* = 2.1 Hz, H-8), 6.20 (1H, d, *J* = 2.1 Hz, H-6), 5.43 (1H, d, *J* = 7.2 Hz, H-1''), 4.40 (2H, brs, H-1'''), 3.83 (3H, s, 3'-OCH_3), 0.97 (3H, d, *J* = 6.0 Hz, H-6'''). ^{13}C-NMR (75 Hz, DMSO-d_6) δ: 177.3 (C-4), 164.1 (C-7), 161.2 (C-5), 156.4 (C-2, 9), 149.4 (C-3'), 146.9 (C-4'), 133.0 (C-3), 122.3 (C-6'), 121.0 (C-1'), 115.2 (C-5'), 113.3 (C-2'), 103.9 (C-10), 101.2 (C-1''), 100.9 (C-1'''), 98.7 (C-6), 93.7 (C-8), 76.4 (C-3''), 75.9 (C-5''), 74.3 (C-2''), 71.8 (C-4'''), 70.6 (C-3'''), 70.3 (C-2'''), 70.1 (C-4''), 68.3 (C-5'''), 66.8 (C-6''), 55.6 (3'-OCH_3), 17.6 (C-6''').

Kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside] (6) – Yellow powder, $[\alpha]_D$ −35.4 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 266 (3.63), 350 (3.57), HRESIMS (negative mode) at *m/z* 739.2087 $[M-H]^-$ (calcd for $C_{33}H_{39}O_{19}$, 739.2086), ^{1}H-NMR (400 Hz, DMSO-d_6) δ: 8.04 (2H, d, *J* = 8.8 Hz, H-2',6'), 6.86 (2H, d, *J* = 8.8 Hz, H-3',5'), 6.41 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 5.30 (1H, d, *J* = 8.0 Hz, H-1''), 4.72 (1H, brs, H-1''''), 4.40 (1H, brs, H-1'''), 3.11-3.70 (m, sugar protons), 1.06 (3H, d, *J* = 6.0 Hz, H-6'''), 0.96 (3H, d, *J* = 6.0 Hz, H-6''''), ^{13}C-NMR (100 Hz, DMSO-d_6) δ: 177.4 (C-4), 164.2 (C-7), 161.2 (C-5), 160.0 (C-4'), 156.7

(C-9), 156.5 (C-2), 133.4 (C-3), 130.9 (C-2',6'), 120.8 (C-1'), 115.07 (C-3',5'), 103.9 (C-10), 102.4 (C-1''''), 102.1 (C-1'''), 100.1 (C-1'''), 98.7 (C-6), 93.7 (C-8), 78.1 (C-3'''), 73.3 (C-5''), 72.9 (C-3'') 72.1 (C-4''''), 71.1 (C-3''''), 70.9 (C-4'''), 70.5 (C-2''''), 70.4 (C-2''), 70.0 (C-2'''), 68.6 (C-5'''), 68.2 (C-5''''), 68.0 (C-4''), 65.4 (C-6''), 17.9 (C-6'''), 17.6 (C-6'''').

Kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl(1 → 6)]-β-D-glucopyranoside (7) – Yellow powder, $[\alpha]_D$ −40.5 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 266 (4.96), 349 (4.88), HRESIMS (negative mode) at *m/z* 739.2082 $[M - H]^+$ (calcd for $C_{33}H_{39}O_{19}$, 739.2086), ^{1}H-NMR (400 Hz, DMSO-d_6) δ: 7.97 (2H, d, *J* = 8.8 Hz, H-2',6'), 6.88 (2H, d, *J* = 8.8 Hz, H-3',5'), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 5.27 (1H, d, *J* = 8.0 Hz, H-1''), 4.71 (1H, s, H-1''''), 4.36 (1H, s, H-1'''), 3.00 - 3.75 (m, sugar protons), 0.98 (3H, d, *J* = 6.0 Hz, H - 6''''), 0.93 (3H, d, *J* = 6.0 Hz, H-6'''), ^{13}C-NMR (100 Hz, DMSO-d_6) δ: 177.4 (C-4), 164.1 (C-7), 161.1 (C-5), 159.8 (C-4'), 156.9 (C-9), 156.4 (C-2), 133.3 (C-3), 130.8 (C-2',6'), 120.9 (C-1'), 115.1 (C-3',5'), 103.9 (C-10), 102.3 (C-1''''), 101.5 (C-1''), 100.9 (C-1'''), 98.7 (C-6), 93.7 (C-8), 78.0 (C-2''), 76.2 (C-3''), 75.6 (C-5''), 72.1 (C-4''''), 72.0 (C-4'''), 70.8 (C-3''''), 70.5 (C-3'''), 70.4 (C-2''''), 70.1 (C-2'''), 69.9 (C-4''), 68.4 (C-6''), 68.1 (C-5''''), 68.1 (C-5'''), 17.6 (C-6'''), 17.5 (C-6'''').

Kaempferol-3-*O*-[6''-*O*-(*E*)-*p*-coumaroyl]-β-D-glucopyranoside; Tiliroside (8) – Yellow-brown powder, $[\alpha]_D$ −61.6 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 267 (4.36), 315 (4.48), HRESIMS (positive mode) at *m/z* 595.1437 $[M + H]^+$ (calcd for $C_{30}H_{27}O_{13}$, 595.1452) ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 8.00 (2H, d, *J* = 8.7 Hz, H-2',6'), 7.39 (2H, d, *J* = 8.7 Hz, H-2''',6'''), 7.37 (1H, d, *J* = 15.9 Hz, H-7'''), 6.87 (2H, d, *J* = 8.7 Hz, H-3',5'), 6.79 (2H, d, *J* = 8.1 Hz, H-3''',5'''), 6.37 (1H, brs, H-8), 6.14 (1H, brs, H-6), 6.12 (1H, d, *J* = 15.9 Hz, H-8'''), 5.44 (1H, d, *J* = 7.2 Hz, H-1''), 4.28 (1H, d, *J* = 11.4 Hz, H-6''a), 4.03 (1H, dd, *J* = 6.6, 11.4 Hz, H-6''b), 3.21-3.42 (m, sugar protons), ^{13}C-NMR (75 Hz, DMSO-d_6) δ: 177.3 (C-4), 166.1 (C-9'''), 164.6 (C-7), 161.1 (C-5), 159.9 (C-4'''), 159.8 (C-4'), 156.3 (C-2,9), 144.6 (C-7'''), 133.0 (C-3), 130.7 (C-2''',6'''), 130.1 (C-2',6'), 124.9 (C-1'''), 120.7 (C-1'), 115.7 (C-3''',5'''), 115.1 (C-3',5'), 113.6 (C-8'''), 103.7 (C-10), 101.0 (C-1''), 98.9 (C-6), 93.6 (C-8), 76.2 (C-3''), 74.2 (C-5''), 74.1 (C-2''), 69.9 (C-4''), 62.9 (C-6'').

Kaempferol-3-*O*-[6''-*O*-(*E*)-*p*-coumaroyl]-β-D-galactopyranoside (9) – Yellow-brown powder, $[\alpha]_D$ −32.4 (*c* 0.1, MeOH), UV (MeOH) λ_{max} nm (log ε): 267 (4.00), 315 (4.36), HRESIMS (positive mode) at *m/z* 595.1420 $[M + H]^+$ (calcd for $C_{30}H_{27}O_{13}$, 595.1452), ^{1}H-NMR (300 Hz, DMSO-d_6) δ: 8.04 (2H, d, *J* = 8.8 Hz, H-2', 6'), 7.34 (2H, d, *J* = 8.0 Hz, H-2''',6'''), 7.32 (1H, d, *J* = 15.8 Hz, H-7'''), 6.85 (2H, d, *J* = 8.8 Hz, H-3',5'), 6.77 (2H, d, *J* = 8.0 Hz, H-3''',5'''), 6.40 (1H, brs, H-8), 6.14 (1H, d, *J* = 15.8 Hz, H-8'''), 6.08 (1H, brs, H-6), 5.40 (1H, d, *J* = 8.1 Hz, H-1''), 4.09 (1H, d, *J* = 6.0 Hz, H-6''a), 3.70 (1H, dd, *J* = 11.6, 6.0 Hz, H-6''b), 3.10-3.72 (m, sugar protons), ^{13}C-NMR (75 Hz, DMSO-d_6) δ: 177.5 (C-4), 166.1 (C-9'''), 164.1 (C-7), 161.1 (C-5), 159.9 (C-4'''), 159.7 (C-4'), 156.3 (C-2,9), 144.6 (C-7'''), 133.2 (C-3), 130.9 (C-2''',6'''), 130.1 (C-2',6'), 124.9 (C-1'''), 120.8 (C-1'), 115.7 (C-3''',5'''), 115.1 (C-3',5'), 113.6 (C-8'''), 103.8(C-10), 101.6 (C-1''), 98.8 (C-6), 93.6 (C-8), 72.9 (C-5''), 72.8 (C-3''), 70.9 (C-2''), 68.2 (C-4''), 63.2 (C-6'').

Amentoflavone (10) – Yellow powder, UV (MeOH) λ_{max} nm (log ε): 337 (4.47), 269 (4.49), HRESIMS (positive mode) at *m/z* 539.3596 $[M + H]^+$ (calcd for $C_{30}H_{19}O_{10}$, 539.0978) ^{1}H-NMR (300, Hz, DMSO-d_6) δ: 8.01(1H, d, *J* = 2.1 Hz, H-2'), 8.00 (1H, dd, *J* = 8.7, 2.1 Hz, H-6'), 7.56 (2H, d, *J* = 8.7 Hz, H-2''',6'''), 7.16 (1H, d, *J* = 8.7, H-5'), 6.83 (1H, s, H-3), 6.79 (1H, s, H-3''), 6.71 (2H, d, *J* = 8.7 Hz, H-3''',5'''), 6.46 (1H, d, *J* = 1.5 Hz, H-8), 6.40 (1H, s, H-6''), 6.18 (1H, d, *J* = 2.1 Hz, H-6), ^{13}C-NMR (75 Hz, DMSO-d_6) δ: 182. 1 (C-4''), 181.7 (C-4), 164.1 (C-2''), 163.8 (C-2), 163.7 (C-7), 161.8 (C-7''), 161.4 (C-5), 161.1(C-4''') 160.5 (C-5''), 159.5 (C-4'), 157.3 (C-9), 154.5 (C-9''), 131.4 (C-6'), 128.2 (C-2''' and 6'''), 127.8 (C-2'), 121.4 (C-3'), 120.9 (C-1'''), 119.9 (C-1'), 116.1 (C-5'), 115.7 (C-3''' and 5'''), 103.9 (C-8''), 103.7 (C-10), 103.6 (C-10''), 102.9 (C-3), 102.6 (C-3''), 98.8 (C-6''), 98.6 (C-6), 94.0 (C-8).

Measurement of nitric oxide (NO) production – NO production was assayed by measuring nitrite in supernatants of cultured RAW 264.7 cells (Chae *et al*., 2009). Cells (1×10^6/mL) were seeded in 96 well culture plates. After pre-incubation of RAW 264.7 cells for 18 hours, cells were pretreated with compounds **1 - 10**, MeOH extract (2.5, 5, 10 and 20 μg/mL) for 30 min and then stimulated LPS (1 μg/mL) for 24 hours. The supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 5 min. The concentration of nitrite was measured at 570 nm.

Results and Discussion

Column chromatographic separation on the EtOAc-soluble extract of the *T. suborbicularis* yielded seven flavonoids **1**, **3 - 5**, and **8 - 10**. From *n*-BuOH-soluble extract, three flavonoids **2**, **6**, and **7** were obtained. From

^{1}H-NMR of **1**, two *ortho*-coupled signals at δ_H 8.04 and 6.92, and two meta-coupled signals at δ_H 6.43 and 6.18 implied the presence of kaempferol skeleton. Further spectroscopic data including ^{13}C-NMR, HRESIMS, and UV data confirmed compound **1** as kaempferol (Kim *et al*., 2002). When compared with compound **1**, compound **2** has additional signals belonging to *C*-glycoside at δ_H 4.68 (1H, d, J = 9.6 Hz, H-1"), 3.96 (1H, m, H-2"), 3.75 (1H, m, H-6"a), 3.51 (1H, m, H-6"b), and 3.32-3.25 (m, H-3", 4", 5"). Hence, this structure was assumed to be vitexin, which was consitent with the published values (Burns *et al*., 2007). The ^{1}H and ^{13}C NMR data of **3** were similar to those of kaempferol except for the presence of two sugars moities [neohesperidoside moity] (rhamnose : δ_C 100.4 (C-1'"), 70.2 (C-2'"), 70.5 (C-3'"), 72.4 (C-4'"), 68.3 (C-5'"), 18.1 (C-6'") and glucose: δ_C 97.8 (C-1"), 77.2 (C-2"), 77.1 (C-3"), 70.5 (C-4"), 61.5 (C-6"). The HMBC correllations between 5.22 (1H, d, J = 7.5 Hz, H-1") to 162.5 (C-7) enabled to locate the neohesperidoside at C-7 via an ether linkage. Hence, compound was identified as rhoifolin (Djoukeng et al., 2008). It was inferred that compound **4** had an isorhamnetin skeleton based on the observed signals at δ_H 7.60 (1H, s, H-6'), 7.57 (1H, s, H-2'), 6.93 (1H, d, J = 9.0 Hz, H-5'), and 3.89 (OCH_3), corresponding to an 1,3,4-trisubsttituted B-ring with a methoxy group at C-3'. The present sugar in **4** was identified as a glucose by comparision with the literature and was placed at C-7 by the observation of HMBC correlation between δ_H 5.05 (1H, d, J = 7.2 Hz, H-1") and δ_C 162.9 (C-7). Thus, the structure of **4** was identified as chrysoeriol-7-*O*-β-D-glucopyranoside (Dinda *et al*., 2006). The ^{1}H and ^{13}C NMR data of compound **5** had closely resemblance to those of compound **4** except that there was a rhamnopyranoside affixed to glucopyranoside. Therefore, the structure of **5** was turned out to be isorhamnetin 3-*O*-rutinoside (Fukunaga *et al*., 1988). Also, the kaempferol triglycosides (compounds **6** and **7**) isolated from this plant were identified as kaempferol-3-*O*-[α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyrano-side] (**6**) and kaempferol-3-*O*-α-L-rhamnopyranosyl(1 → 2)-*O*-[α-L-rhamnopyranosyl (1 → 6)]-β-glucopyranoside (**7**), by the interpretation of 1D (^{1}H, ^{13}C) and 2D (COSY, HMQC, HMBC) NMR data and the comparison with the published values (Lakenbrink *et al*., 2000; Yoshitama *et al*., 1997).

The ^{1}H and ^{13}C NMR data of compound **8** displayed the signals accounting for a kaempferol skeleton, a coumaroyl group, and a glucopyranoside. All linkage between these groups were unambiguously completed by aid of HMBC correlations, leading to kaempferol-3-*O*-[6"-*O*-(*E*)-*p*-coumaroyl]-β-D-glucopyranoside as the structure of compound **8**. The ^{1}H and ^{13}C NMR data of compound **9** were superimposable to those of compound **8**. Only difference was observed at sugar region. In compound **9**, a galactopyranoside was present instead of a glucopyranoside in **8**. Thus, compound **9** was assigned as kaempferol-3-*O*-[6"-*O*-(*E*)-*p*-coumaroyl]-β-D-galactopyranoside (Zhang *et al.*, 2007). The ^{1}H-NMR spectral data of compound **10** showed the presence of six OH groups and twelve aromatic protons, indicating a biflavone. When compared with the published values, this compound was identified as amentoflavone (Dora et al., 1991).

All the compounds **1** - **10** were tested in LPS-induced NO production assay using Raw 264.7 cell. Tiliroside (**8**) and amentoflavone (**10**) were found to inhibit NO production (IC_{50} 3.56 and 15.73 μM, respectively) in this assay system while other compounds deemed inactive (IC_{50} > 20 μg/mL). Previously, tiliroside (**8**) was investigated for inhibitory activity of NO production using the primary cells obtained from mouse peritoneal macrophages and reported to possess the inhibitory activity against NO production (Rao *et al*., 2005). In the current investigation, tiliroside demonstrated to inhibit NO production in LPS-induced Raw 264.7 cells, consistent with the preceding study. Amentoflavone has been reported to inhibit NO production via suppression of NO synthase and nucler factor κB in LPS-induced Raw 264.7 cells. The present study demonstrated the same inhibitory activity as the former study (Woo *et al*., 2005).

In conclusion, the present study disclosed ten flavonoids as the chemical constituents of *T. suborbicularis* for the first time and of the isolates, two flavonoids, tiliroside and amentoflavone, were found to exert the inhibitory activity against NO production in LPS-induced Raw 264.7 cells.

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