SHORT REPORT

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Wound Healing Effect of Flavonoid Glycosides from *Afgekia mahidolae* B.L.Burtt & Chermsir. Leaves

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Abstract: Five known flavonoid glycosides namely, juglanin (**1**), astragalin (**2**), nicotiflorin (**3**), isoquercetin (**4**) and apigenin-7-*O*-*β*-glucuronide (**5**), and a phenolic glucoside, arbutin (**6**), were isolated for the first time from the leaves of *Afgekia mahidolae* (Fabaceae). Their structures were elucidated using one- and two-dimensional NMR spectroscopic methods and comparison with the literature. The ability of **1**-**4** to promote the migration of CCD-1064sk fibroblasts into a scratch-wound area was evaluated. The results indicated that juglanin (**1**) and nicotiflorin (**3**) significantly increased the migration of these cells and, hence, supporting the wound healing effect of flavonol glycosides.

Keywords:*Afgekia mahidolae*; fibroblasts; flavonoid glycosides; scratch-wound healing.

1. Plant Source

Leaves of *A. mahidolae* B.L.Burtt & Chermsir. were collected from the Phu-kae Botanical Garden, Saraburi, Thailand in July 2012. The plant was identified by one of the authors (R. Suttisri). A voucher specimen (No. 5182) has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. Previous Studies

Afgekia (Fabaceae) is a small Asian genus of perennial climbers which comprises only three species (*A. filipes*, *A. mahidolae* and *A. sericea*); all of them can be found in Thailand. *A. mahidolae* is a

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rare plant distributed in deciduous forests of Laos, Vietnam and southwestern Thailand. No medicinal use of this plant has been recorded. A previous study has demonstrated radical scavenging activity of the methanol extract of its leaves [1], and our preliminary screening using similar extract indicated its promoting activity on the migration of human dermal fibroblasts (CCD-1064sk). Since radical scavenging capacity of plant extracts or compounds has been shown to promote wound healing [2-3], this prompted us to identify the constituents with wound healing potential from the leaves of *A. mahidolae*.

3. Present Study

Dried and powdered leaves of *A. mahidolae* (1.1 kg) were macerated with MeOH (3 × 10 L, 3 days each), then combined and evaporated to obtain the MeOH extract (172 g). The extract was suspended in distilled water (1.5 L) and successively partitioned with EtOAc (3 × 1.5 L) and saturated *n*-BuOH (3 × 1.5 L) to afford EtOAc (7 g), *n*-BuOH (33 g) and aqueous (96 g) extracts. EtOAc and n-BuOH extracts were selected for further fractionation. Aqueous extract was excluded from the study because it had no interesting chemical compositions to explore.

Chromatographic Isolation of Compounds: The EtOAc extract (7 g) was loaded onto a silica gel column (350 g, 4.5 × 40 cm) and eluted with a gradient solvent system of $CHCl_3$/MeOH (9:1 → 0:1). The eluates were examined by TLC and then pooled into eight fractions: A-H. Fraction B (437 mg) was purified on a Sephadex LH-20 column (2.5 × 170 cm) washed down with a gradient solvent system of MeOH/H_2O (2:3, 1.6 L; 3:2, 1.6 L, 4:1, 1.0 L and 1:0, 1.0 L) to afford compound **1** (5.2 mg). Separation of fraction C (477 mg) over a Sephadex LH-20 column (2.5 × 170 cm) by elution with a gradient solvent system of MeOH/H_2O (1:1, 1.4 L; 3:2, 0.8 L; 4:1, 0.8 L and 1:0, 0.8 L), yielded compound **2** (11.4 mg). Fraction F (632 mg) was further separated on a Sephadex LH-20 column (2.5 × 170 cm) by elution with a gradient solvent system of MeOH/H_2O (3:2, 1.0 L; 7:3, 0.8 L; 4:1, 0.8 L; 9:1, 0.8 L and 1:0, 0.8 L), into eighteen subfractions: F1-F18. Subfraction F11 (87.2 mg) was purified on a reversed-phase preparative HPLC column (Atlantis Prep T3 OBD™, 5 μm, 19 × 250 mm) using a gradient solvent system of MeOH/H_2O (3:7 → 7:3) at a flow rate of 10 mL/min for 45 min to afford compound **3** (13.0 mg), whereas subfraction F15 yielded compound **4** (7.4 mg). Fraction H (103 mg) was chromatographed on a Sephadex LH-20 column (2.5 × 170 cm) by elution with a gradient solvent system of MeOH/H_2O (3:2, 1.0 L; 7:3, 0.8 L; 4:1, 0.8 L; 9:1, 0.8 L and 1:0, 0.8 L) to afford compound **5** (2.1 mg). A portion of the *n*-BuOH extract (14.6 g) was separated on two successive silica gel columns (260 g, 4 × 28 cm) by elution with gradient solvent systems of CH_2Cl_2/MeOH (4:1 → 0:1 and 3:1 → 1:4, respectively). Final purification on a Sephadex LH-20 column (2.5 ×100 cm) washed down with CH_2Cl_2/MeOH (1:1) afforded compound **6** (6.1 mg).

Juglanin (*Kaempferol 3-O-α-L-arabinopyranoside*) (***1***): yellow amorphous powder; positive ESI-MS *m/z* 419 $[M+H]^+$; IR (KBr plate, ν) 3440, 1655, 1607, 1571, 1206, 1178 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H (ppm): 12.62 (1H, s, OH-5), 8.08 (2H, d, *J* = 9.2 Hz, H-2′ and H-6′), 6.88 (2H, d, *J* = 9.2 Hz, H-3′ and H-5′), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 5.34 (1H, d, *J* = 4.4 Hz, H-1″), 3.55 (1H, dd, *J* = 11.2, 6.0 Hz, H-5″a), 3.20 (1H, dd, *J* = 11.2, 2.8 Hz, H-5″b); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C (ppm): 177.5 (C-4), 164.6 (C-7), 161.2 (C-5), 160.0 (C-4′), 156.3 (C-2), 156.1 (C-9), 133.5 (C-3), 130.9 (C-2′ and C-6′), 120.6 (C-1′), 115.2 (C-3′ and C-5′), 103.8 (C-10), 101.2 (C-1″), 98.8 (C-6), 93.7 (C-8), 71.5 (C-3″), 70.7 (C-2″), 66.0 (C-4″), 64.2 (C-5″).

Astragalin (*Kaempferol 3-O-β-D-glucopyranoside*) (***2***): yellow amorphous powder; positive ESI-MS *m/z* 449 $[M+H]^+$; IR (KBr plate, ν) 3434, 1660, 1609, 1506, 1205, 1181 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H (ppm): 12.61 (1H, s, OH-5), 8.08 (2H, d, *J* = 9.2 Hz, H-2′ and H-6′), 6.88 (2H, d, *J* = 9.2 Hz, H-3′ and H-5′), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 5.46 (1H, d, *J* = 7.6 Hz, H-1″); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C (ppm): 177.5 (C-4), 164.1 (C-7), 161.2 (C-5), 159.9 (C-4′), 156.4 (C-2), 156.2 (C-9), 133.2 (C-3), 130.9 (C-2′ and C-6′), 120.9 (C-1′), 115.1 (C-3′ and C-5′), 104.0

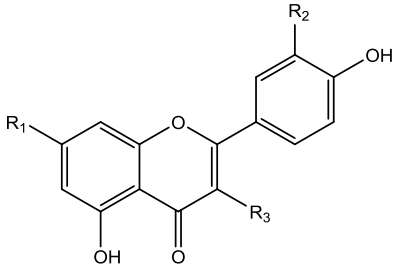
(C-10), 100.8 (C-1″), 98.7 (C-6), 93.6 (C-8), 77.5 (C-5″), 76.4 (C-3″), 74.2 (C-2″), 69.9 (C-4″), 60.8 (C-6″).

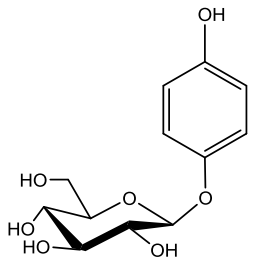
Nicotiflorin (*Kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside*) (***3***): yellow amorphous powder; positive ESI-MS *m/z* 595 $[M+H]^+$; IR (KBr plate, ν) 3428, 1655, 1606, 1507, 1208, 1181 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H (ppm): 12.50 (1H, s, OH-5), 7.98 (2H, d, *J* = 8.8 Hz, H-2′ and H-6′), 6.87 (2H, d, *J* = 8.4 Hz, H-3′ and H-5′), 6.39 (1H, d, *J* = 2.4 Hz, H-8), 6.19 (1H, d, *J* = 2.4 Hz, H-6), 5.30 (1H, d, *J* = 7.6 Hz, H-1″), 4.40 (1H, br s, H-1‴), 0.98 (3H, d, *J* = 6.4 Hz, H-6‴); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C (ppm): 177.3 (C-4), 164.5 (C-7), 161.2 (C-5), 159.9 (C-4′), 156.5 (C-2), 156.7 (C-9), 133.2 (C-3), 130.8 (C-2′ and C-6′), 120.9 (C-1′), 115.1 (C-3′ and C-5′), 104.8 (C-10), 101.4 (C-1″), 100.7 (C-1‴), 98.8 (C-6), 93.8 (C-8), 76.3 (C-3″), 75.7 (C-5″), 74.1 (C-2″), 71.8 (C-4‴), 70.6 (C-3‴), 70.3 (C-2‴), 69.9 (C-4″), 68.2 (C-5‴), 66.8 (C-6″), 17.7 (C-6‴).

Isoquercetin (*Quercetin 3-O-β-D-glucopyranoside*) (***4***): yellow amorphous powder; positive ESI-MS *m/z* 465 $[M+H]^+$; IR (KBr plate, ν) 3269, 1655, 1606, 1496, 1201, 1169 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H (ppm): 7.70 (1H, d, *J* = 2.2 Hz, H-2′), 7.57 (1H, dd, *J* = 7.2, 2.2 Hz, H-6′), 6.84 (1H, d, *J* = 7.2 Hz, H-5′), 6.40 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 5.45 (1H, d, *J* = 7.2 Hz, H-1″); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C (ppm): 177.4 (C-4), 164.1 (C-7), 161.2 (C-9), 156.3 (C-2 and C-5), 148.4 (C-4′), 144.8 (C-3′), 133.4 (C-3), 121.7 (C-1′), 121.2 (C-6′), 116.2 (C-5′), 115.3 (C-2′), 104.0 (C-10), 100.8 (C-1″), 98.6 (C-6), 93.6 (C-8), 77.6 (C-5″), 76.5 (C-3″), 74.1 (C-2″), 69.9 (C-4″), 61.0 (C-6″).

Apigenin-7-O-*β-glucuronide* (***5***): yellow amorphous powder; positive ESI-MS *m/z* 447 $[M+H]^+$; IR (KBr plate, ν) 3419, 1656, 1609, 1497, 1174 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3OD) δ_H (ppm): 7.85 (1H, d, *J* = 8.5 Hz, H-2′ and H-6′), 6.91 (2H, d, *J* = 8.5 Hz, H-3′ and H-5′), 6.81 (1H, d, *J* = 1.8 Hz, H-8), 6.60 (1H, s, H-3), 6.48 (1H, d, *J* = 1.8 Hz, H-6), 5.09 (1H, d, *J* = 6.0 Hz, H-1″); ^{13}C-NMR (125 MHz, CD_3OD) δ_C (ppm): 184.1 (C-4), 172.2 (C-6″), 166.8 (C-2), 164.9 (C-7), 163.0 (C-5), 162.7 (C-4′), 158.9 (C-9), 129.6 (C-2′ and C-6′), 122.9 (C-1′), 117.1 (C-3′ and C-5′), 107.1 (C-10), 104.0 (C-3), 101.5 (C-6 and C-1″), 96.1 (C-8), 77.6 (C-5″), 76.5 (C-3″), 74.5 (C-2″), 73.4 (C-4″).

Arbutin (*Hydroquinone β-D-glucopyranoside*) (***6***): white amorphous powder; positive ESI-MS *m/z* 273 $[M+H]^+$; IR (KBr plate, ν) 3338, 1650, 1603, 1513, 1213 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3OD) δ_H (ppm): 6.96 ppm (2H, br d, *J* = 8.8 Hz, H-2 and H-6), 6.69 (2H, br d, *J* = 8.8 Hz, H-3 and H-5), 4.73 (1H, d, *J* = 7.0 Hz, H-1′), 3.87 (1H, br d, *J* = 11.5 Hz, H-6″b), 3.69 (1H, dd, *J* = 11.5, 5.0 Hz, H-6″a); ^{13}C-NMR (125 MHz, CD_3OD) δ_C (ppm): 153.8 (C-1), 152.4 (C-4), 119.6 (C-2 and C-6), 116.3 (C-3 and C-5), 103.7 (C-1′), 78.0 (C-3′ and C-5′), 75.0 (C-2′), 71.4 (C-4′), 62.6 (C-6′).





6

1: R_1 = -OH, R_2 = -OH, R_3 = -O-α-L-Ara
2: R_1 = -OH, R_2 = -H, R_3 = -O-β-D-Glc
3: R_1 = -OH, R_2 = -H, R_3 = -O-β-D-Glc^6-α-L-Rha
4: R_1 = -OH, R_2 = -OH, R_3 = -O-β-D-Glc
5: R_1 = -O-β-D-GlcA, R_2 = H, R_3 = H

Figure 1. Compounds isolated from *Afgekia mahidolae* leaves

The methanolic extract of *A. mahidolae* leaves was partitioned into EtOAc, *n*-BuOH and aqueous extract. Chromatographic separation of the EtOAc extract yielded five flavonoid glycosides. Based on analyses of their NMR and mass spectral data, these flavonoids were identified as juglanin (**1**) [4], astragalin (**2**) [5], nicotiflorin (**3**) [6], isoquercetin (**4**) [7] and apigenin-7-*O*-*β*-glucuronide (**5**) [7], (Figure 1). In addition, purification of the *n*-BuOH extract led to the isolation of an antioxidant phenolic glucoside, arbutin (**6**) [8-9], as its major component. This is the first report of these phenolic compounds as constituents of *A. mahidolae*. The presence of flavonoids in the leaves and their abundance in the extracts is not surprising considering that antioxidant flavonoids are associated with chloroplasts and play an important role as their stabilizers by scavenging singlet oxygen [10]. Some classes of flavonoids are known to modulate wound healing. Anthocyanins have been shown to stimulate the migration of both human dermal fibroblasts and keratinocytes [11-12], whereas different concentrations of catechins can either enhance or decrease the proliferation of fibroblasts [13]. Quercetin, a well-known flavonol, exhibited inhibitory activity on the proliferation of fibroblasts [14]. However, its 3-*O*-methyl ether and two glycosylated and acylated flavonols have been found to be active in a scratch-wound healing assay on HaCaT keratinocytes [15]. In this study, we subjected flavonol glycosides **1**-**4** to a similar assay on CCD-1064sk fibroblasts to evaluate their ability to promote the migration of these human dermal cells into the wound area. The flavone glycoside **5** was not tested because an insufficient quantity was isolated.

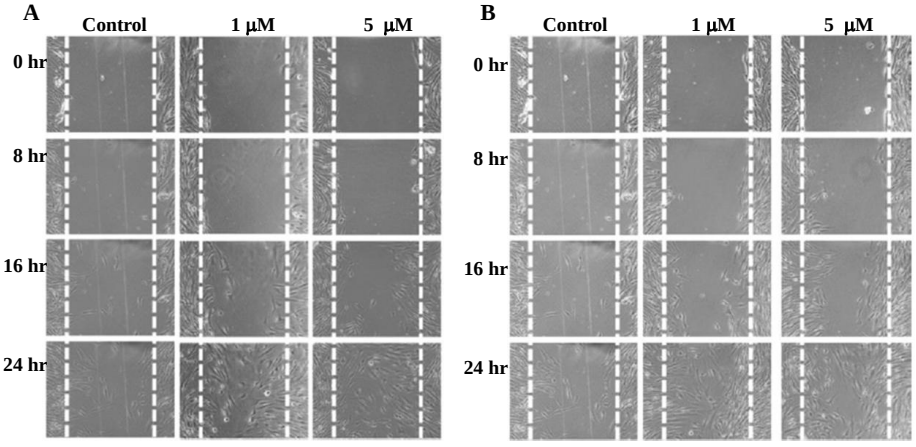


Figure 2. Ability of juglanin (**1**) (A) and nicotiflorin (**3**) (B), at concentrations of 1 and 5 μM, to promote fibroblast migration into the scratch-wound at 0, 8, 16 and 24 hr.

Cell Viability Assay: Human dermal fibroblasts (CCD-1064sk) were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum 1% L-glutamine and 1% penicillin/streptomycin (Gibthai, Bangkok, Thailand) under fully humidified atmosphere containing 5% CO_2 at 37°C. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described with a slight modification [8]. Fibroblasts were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated for 24 h. Individual compounds, at concentrations of 1, 5, 10, 20, 40 and 80 μM, were then added, and the cells were incubated for an additional 24 h before the addition of 0.5 mg/mL MTT solution, which was followed by a 4 h incubation. DMSO was used to dissolve the formazan crystals. Living cells were quantified by measuring the absorbance of the formazan crystals at 570 nm.

Scratch-wound Healing Assay: The ability of the compounds to promote the migration of fibroblasts (CCD-1064sk) into the wound area was evaluated using a scratch-wound assay [16]. Fibroblasts (1×10^5 cells/well) were seeded into 24-well plates and incubated for 24 h. To create a scratch, a confluent monolayer was scraped in a straight line using a 10 μL sterile pipette tip. Debris and suspended cells were

removed by washing with phosphate-buffered saline. All samples were diluted with media, and added into each well. Medium without sample served as a negative control. At 0, 8, 16 and 24 h after the addition of test samples, fibroblasts were photographed using an inverse phase contrast microscope (Olympus IX51, Japan). The percentage of wound closure was analyzed using Image J software. All the statistical analyses were performed using Microsoft Excel and SPSS 17.0. The significance of differences between experimental groups was determined with one way ANOVA followed by Turkey's *post-hoc* test.

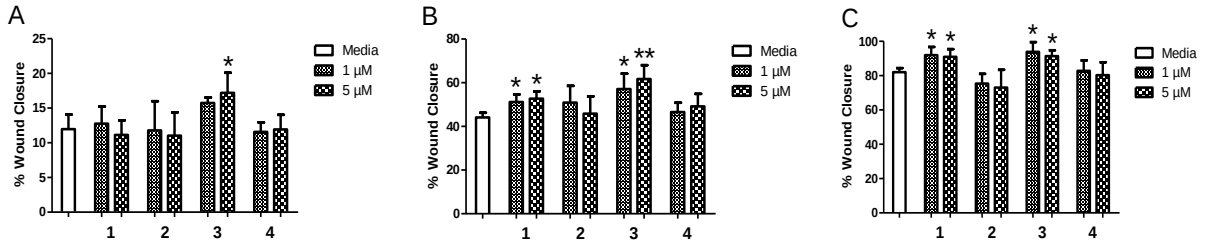


Figure 3. Effect of flavonoid glycosides **1**-**4**, at 1 and 5 µM, on the migration of fibroblasts into the scratch-wound area after (A) 8 h, (B) 16 h and (C) 24 h of incubation, compared to media as the negative control. Percentages of wound closure are expressed as the mean ± SD values from three experiments. *Significant at p <0.05, **Significant at p <0.01.

A cytotoxicity assay using the MTT method was performed first to select suitable concentrations of the test compounds for the scratch-wound healing test. The results showed that all four glycosides were not toxic to the fibroblasts at concentrations of less than 20 µM. Therefore, the wound healing assay was performed using 1 and 5 µM of the test compounds. These flavonol glycosides from *A. mahidolae* leaves are derivatives of either kaempferol (**1**-**3**) or quercetin (**4**). At 5 µM, nicotiflorin (**3**), which is kaempferol conjugated to the disaccharide rutinose, significantly enhanced the migration of fibroblasts into the scratch-wound area as early as 8 h after incubation and thus appeared to be the most effective flavonoid tested (Figure 2). After 16 h, juglanin (**1**), which has the same aglycone as **3** but a different sugar moiety, was also observed to promote the migration of fibroblasts but was slightly less active. The difference in their effects on the fibroblasts was less pronounced after 24 h of incubation (Figure 3). However, the 3-*O*-glucosides of kaempferol and quercetin (**2** and **4**, respectively) or kaempferol itself (data not shown) were not able to stimulate progression of these dermal cells into the wound. Therefore, the type of sugars in these flavonol 3-*O*-glycosides appeared to be important for their ability to promote fibroblast migration during the early proliferative stage of wound repair. Flavonol glycosides have previously been shown to stimulate fibroblast proliferation and collagen synthesis [17-18], enhance the production and accumulation of ECM [2] and promote re-epithelialization by keratinocytes [14]. Thus, our findings on the fibroblast migration promoting activity of the flavonol glycosides obtained from *A. mahidolae* leaves further support the wound healing property of this class of flavonoids.

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