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Abstract

The first reported study of the isolation and identification of compounds from the bark and fruit of *Rothmannia wittii* yielded two new iridoids, 6 β -hydroxy-10-O-acetylgenipin (1) and 10-O-acetylmacrophyllide (2) together with six known iridoids; 6 β -hydroxygenipin (3), genipin (4), garjamine (5), cerbinal (6), and mixture of β -gardiol (7) and α -gardiol (8); benzoic acid (9); vanillic acid (10); and stigmasterol (11). Their structures were elucidated by spectroscopic methods. Iridoid 1 showed antimycobacterial activity against *Mycobacterium tuberculosis* with a MIC value of 12.50 μ g/mL. Iridoid 2 showed cytotoxicity against the NCI-H187 cancer cell line with an IC₅₀ value of 6.82 μ g/mL. In addition, 2 and 5 exhibited weak cytotoxic activity against KB and MCF-7 cell lines, while 4 was active against the NCI-H187 cancer cell line.

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The first reported study of the isolation and identification of compounds from the bark and fruit of *Rothmannia wittii* yielded two new iridoids, 6 β -hydroxy-10-*O*-acetylgenipin (**1**) and 10-*O*-acetylmacrophyllide (**2**) together with six known iridoids; 6 β -hydroxygenipin (**3**), genipin (**4**), garjasmine (**5**), cerbinal (**6**), and mixture of β -gardiol (**7**) and α -gardiol (**8**); benzoic acid (**9**); vanillic acid (**10**); and stigmasterol (**11**). Their structures were elucidated by spectroscopic methods. Iridoid **1** showed antimycobacterial activity against *Mycobacterium tuberculosis* with a MIC value of 12.50 μ g/mL. Iridoid **2** showed cytotoxicity against the NCI-H187 cancer cell line with an IC₅₀ value of 6.82 μ g/mL. In addition, **2** and **5** exhibited weak cytotoxic activity against KB and MCF-7 cell lines, while **4** was active against the NCI-H187 cancer cell line.

Keywords: *Rothmannia wittii*; iridoid; macrophyllide; genipin; garjasmine; cerbinal

1. Introduction

Rothmannia wittii (Craib) Bremek. (RUBIACEAE) is a tree of 4-10 m in height and found in deciduous dipterocarp and mixed deciduous forest of Thailand. It is a Thai traditional medicinal plant and called “Muk Mo” in Thai. It has been used for venereal disease treatment and as an anti-fever agent [1]. However, the phytochemical study of the bark and fruit of this plant has never been reported. We report herein the first isolation and identification of the chemical constituents from the bark and fruit of *R. wittii*.

2. Results and discussion

The n-hexane and EtOAc extracts obtained from the bark and the MeOH extract from the fresh fruit of *R. wittii* were separated on silica gel column chromatography to yield two new iridoids, 6 β -hydroxy-10-*O*-acetylgenipin (**1**) and 10-*O*-acetylmacrophyllide (**2**), and nine known compounds. Their structures were identified using physical and spectroscopic data (¹H NMR, ¹³C NMR and 2D NMR) as well as comparing the data with those reported for the known compounds in the literature to be 6 β -hydroxygenipin (**3**) [2], genipin (**4**) [3,4], garjasmine (**5**) [5], cerbinal (**6**) [6], β -gardiol (**7**), α -gardiol (**8**) [5], benzoic acid (**9**), vanillic acid (**10**) [7] and stigmasterol (**11**) [8] as shown in Figure 1.

Figure 1. Structures of isolated compounds **1-11**

Compound **1** was obtained as a colourless viscous liquid. The molecular formula ($C_{13}H_{16}O_7$) was deduced from the HR-ESI-TOF-MS, m/z 307.0798 $[M + Na]^+$ (calcd for $C_{13}H_{16}O_7 + Na$, 307.0794), indicating 6 degrees of unsaturation. The UV spectrum showed absorption maximum at 239 nm which correspond to those of iridoids, genipin [9] and 10-acetylgenipin skeletons [10]. The IR spectrum showed the presence of hydroxyl (3379 cm^{-1}), ester (1730 cm^{-1}), α,β -unsaturated ester (1676 cm^{-1}), and alkene (1629 cm^{-1}) functionality. The ^1H NMR spectrum of **1** showed resonances for two olefinic protons at δ 7.56 (1H, s, H-3) and 5.83 (1H, brs, H-7), oxygenated methylene protons at δ 4.75 (2H, m, H-10), two oxygenated methine protons at δ 4.72 (1H, d, $J = 8.4\text{ Hz}$, H-1) and 4.53 (1H, m, H-6), two methine protons at δ 2.95 (1H, m, H-5) and 2.78 (1H, m, H-9), methoxy protons at δ 3.75 (3H, s, OMe-11) and acetyl protons at δ 2.09 (3H, s, MeCOO-10). The ^{13}C NMR and HMQC spectra showed resonances for alkene carbons at δ 155.0 (C-3), 142.4 (C-8), 132.4 (C-7) and 109.9 (C-4), oxygenated carbons at δ 98.3 (C-1) and 82.8 (C-6), methine carbons at δ 48.9 (C-9) and 46.8 (C-5), an oxygenated methylene carbon at δ 63.3 (C-10), carbonyl carbon at δ 170.8 (C-11) and a methoxy group at δ 52.1 which agreed well with those of the related iridoid, 6 β -hydroxygenipin [2]. The HMBC spectrum of **1** displayed correlations of H-1 with C-5 and C-8; H-3 with C-1, C-4, C-5 and carbonyl (C-11); H-5 with C-1, C-3, C-4, C-6, C-9 and carbonyl (C-11); H-6 with C-8; H-7 with C-5, C-6, C-8, C-9, and C-10; H-9 with C-1, C-

5, C-6, C-7 and C-8; H-10 with C-7 C-8, C-9 and the carbonyl of the acetate group (COO-10); OMe with C-11; and methyl protons of the acetate group (MeCOO-10) with the carbonyl carbon (COO-10) confirming the structure of **1** (Figure 2).

Figure 2. Selected HMBC correlations of **1**.

The relative configuration of **1** was assigned from the NOESY and coupling constant of these protons. The NOESY spectrum showed correlations of H-1 with H-9 and 10; H-5 with H-9; and H-7 with H-6 and 10. The NOESY correlation between H-5 and H-9 revealed their *syn* relationship. Further, the absence of a NOESY correlation between H-5 and H-6 indicated the opposite (*anti*) orientation of these protons. The large coupling constant of H-1 with H-9 (8.4 Hz) indicating a *trans* disposition between these protons [2]. Moreover, the ^{13}C NMR chemical shift of the resonance associated with C-6 (δ_{C} 82.8) was comparable to an analogue, scandoside (δ_{C} 82.4) with a 6β -hydroxy orientation [11]. Hence, by analogy the configurations at C-1 and C-6 were tentatively assigned as $1R$ and $6R$, respectively. On the basis of the above data, **1** was deduced as a new iridoid, 6β -hydroxy-10-*O*-acetylgenipin.

Compound **2** was obtained as a white amorphous solid. The molecular formula $\text{C}_{12}\text{H}_{12}\text{O}_6$ was deduced from the molecular ion peak at m/z 275.0535 $[\text{M} + \text{Na}]^+$ by the HR-ESI-TOF-MS (calcd for $\text{C}_{12}\text{H}_{12}\text{O}_6 + \text{Na}$, 275.0532), indicating 7 degrees of unsaturation. The IR spectrum showed the presence of lactone (1768 cm^{-1}), ester (1737 cm^{-1}) and alkene (1645 cm^{-1}) functionality. The ^1H NMR spectrum of **2** showed resonances for an olefinic proton at δ 6.16 (1H, brs, H-7), three oxygenated methine protons at δ 5.83 (1H, s, H-1), 5.50 (1H, d, $J = 3.6$ Hz, H-3) and 4.97 (1H, dd, $J = 2.2, 4.4$ Hz, H-6), three methine protons at δ 3.37 (1H, m, H-5), 3.24 (1H, d, $J = 8.0$ Hz, H-9) and 3.21 (1H, dd, $J = 3.6, 5.2$ Hz, H-4), two methylene protons at δ 4.79 and 4.67 (2H, d, $J = 14.1$ Hz, H-10a and H-10b), and acetyl protons at δ 2.08 (3H, s, CH_3). ^{13}C NMR and HMQC spectra showed resonances for alkene carbons at δ 144.2 (C-8) and 133.9 (C-7); two ketal carbons at δ 102.0 (C-3) and 98.3 (C-1); an oxygenated methine carbon at δ 86.4 (C-6); three methine carbons at δ 53.4 (C-9), 48.3 (C-4)

and 43.2 (C-5); an oxygenated methylene carbon at δ 62.2 (C-10); lactone carbonyl at δ 171.4 (C-11). These resonances agreed well with those of the related case-like iridoid, macrophyllide [12] except for an additional acetate group at C-10. The COSY experiments showed correlations of H-1 with H-9; H-4 with H-3 and H-5; H-5 with H-4, H-6 and H-9; H-6 with H-7; H-7 with H-10 (allylic coupling) (Figure 3).

Figure 3. COSY (arrow line) and NOESY (arrow dashed line) correlations of **2**.

The absence of coupling between H-1 and H-9 in the ^1H NMR spectrum suggested that the dihedral angle of these two protons is nearly 90° . The HMBC spectrum of **2** showed correlations of H-1 with C-3, C-5, C-9 and C-11; H-3 with C-1, C-5 and C-6; H-4 with C-5, C-9, and C-11; H-5 with C-1, C-3, C-4, C-6 and C-9; H-6 with C-3, C-8 and C-9; H-7 with C-5, C-6, C-8, C-9 and C-10; H-9 with C-1, C-5, C-6, C-7 and C-8; H-10 with C-7, C-8, C-9 and COO-10; and methyl protons with COO-10 confirming the structure of **2** (Figure 4).

Figure 4. selected HMBC correlations of **2**.

The HMBC showed cross-peaks between H-3 to C-6 and H-6 to C-3. This evidence confirmed that this compound contained an ether bridge between these carbons. Another one bridge linkage was also observed based on the HMBC correlation between H-1 and C-11. Hence, **2** contained two bridge linkages as describe above. The NOESY spectrum showed correlations of H-1 with H-9 and H-10, H-3 with H-4, H-6 with H-4 and H-7, and H-5 with H-9 confirmed the structure of **2** (Figure 4). Based on the above evidence, the relative configurations of C-1 C-3, C-4 and C-6 were assigned. This assignment is reasonable for the stability of such a rigid molecule. Thus **2** was deduced as a new iridoid acetate, 10-*O*-acetylmacrophyllide.

Compound **2** may arise via an intramolecular oxo-Michael addition reaction starting from either the unknown C-6 epimer of **1** or the known iridiod 6 α -hydroxygenipin (the C-6 epimer of **3**), [2] followed by lactone formation by cyclization of the C-1 hydroxyl group onto the C-11 carbonyl. If 6 α -hydroxygenipin was the biosynthetic precursor to **2** then acetylation of the primary C-10 hydroxyl would also be required.

It has been known that iridoids exhibit a wide range of bioactivities for example 6 β -hydroxygenipin (**3**) has inhibitory effects on nitric oxide production; genipin (**4**) has inhibitory effects on nitric oxide production [13], effect to liver diseases [14] and has antitumor activity [15]; garjasmine (**5**) has antimicrobial and antiplasmodial activities [16]; and cerbinal (**6**) has potent antifungal activities to some plant fungi [6]. Therefore, the isolated iridoids were evaluated for others biological activities. It was found that iridoid **1** had antimycobacterial activity against *M. tuberculosis* with a MIC value of 12.50 $\mu\text{g/mL}$. Iridoid **2** showed cytotoxicity against NCI-H187, KB, and MCF-7 cell lines with IC₅₀ values of 6.82, 30.32 and 23.38 $\mu\text{g/mL}$, respectively. Iridoid **4** exhibited cytotoxicity against the NCI-H187 cancer cell with an IC₅₀ value of 18.28 $\mu\text{g/mL}$. In addition, iridoid **5** showed cytotoxicity against KB and MCF-7 cancer cell lines with IC₅₀ values of 30.34 and 14.02 $\mu\text{g/mL}$, respectively (Table 1). These results indicated the biological activity potential of iridoids from this plant.

Table 1. Biological activity of isolated compounds **1–6**.

compound	antiTB ^a (MIC, µg/mL)	Cytotoxicity IC ₅₀ (µg/mL)		
		KB ^b	MCF-7 ^c	NCI-H187 ^d
1	12.50	>50	>50	>50
2	>50	30.32	23.38	6.82
3	>50	>50	>50	>50
4	>50	>50	>50	18.28
5	>50	30.34	14.02	>50
6	>50	>50	>50	>50
isoniazid ^e	0.05			
kanamycin sulfate ^e	2.5			
doxorubicin ^e		0.60	9.48	0.092

^a*Mycobacterium tuberculosis*.

^bhuman epidermoid carcinoma in the mouth.

^cHuman breast adenocarcinoma.

^dHuman small cell lung cancer.

^ePositive control substances.

The isolation of iridoids from *R. wittii* show natural characteristics of the Rubiaceae plant [17]. There is no iridoid containing acetate group at C-10 that has been found in other species of *Rothmannia* [17]. Therefore, this unique 10-*O*-acetate connecting group could be used as a marker for *R. wittii*.

3. Experimental

3.1. General experimental procedures

Melting points were determined using Electrothermal IA9200 digital melting point apparatus (Bibby Scientific Limited, Staffordshire, UK). Optical rotations were measured on a JASCO DIP-1000 digital polarimeter (JASCO Inc., USA) and UV spectra were recorded using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). IR spectra were obtained using a Bruker Tenser 27 spectrophotometer (Bruker, Germany). NMR spectra were recorded on a Varian Mercury Plus 400 spectrometer using (Varian Inc., USA) CDCl₃ and CD₃OD as solvents. The internal standards were referenced from the residue of those solvents. The HR-ESI-TOF-MS were recorded on a Bruker micrOTOF mass

spectrometer (Brucker, Germany). Column chromatography was carried out on MERCK silica gel 60 (230–400 mesh) (Merck, Darmstadt, Germany). Thin-layer chromatography was carried out with pre-coated MERCK silica gel 60 PF254 (Merck, Darmstadt, Germany); the spots were visualised under UV light (254 and 365 nm) and further by spraying with anisaldehyde and then heating until charred.

3.2. Plant material

The bark and fruit of *R. wittii* were collected from Ubolratana district, Khon Kaen province, Thailand, in January 2014 and was identified by Prof. Pranom Chantaranothai, Department of Biology, Khon Kaen University, Thailand, where a voucher specimen (voucher number S. Kanokmedhakul- 20) has been deposited.

3.3. Extraction and isolation

Air-dried bark of *R. wittii* (7.64 kg) was ground and extracted successively with *n*-hexane (3 X 19.7 L) and EtOAc (3 X 19.7 L) at room temperature. Removal of solvents from each extract under reduced pressure gave crude *n*-hexane (34.5 g, 0.45%) and EtOAc (52.1 g, 0.68%) extracts, respectively.

The *n*-hexane extract (34.5 g) was separated on a silica gel flash column chromatography (FCC), gradient eluting with *n*-hexane-EtOAc 100/0 to 0/100 and EtOAc-MeOH 100/0 to 0/100 by increasing polarity to give four fractions, BH₁-BH₄. Fraction BH₃ was then subjected to silica gel FCC eluting with *n*-hexane-EtOAc 100/0 to 0/100 and EtOAc-MeOH 100/0 to 0/100 to provide three subfractions, BH_{3,1}-BH_{3,3}. Fraction BH_{3,3} was further purified by using silica gel FCC eluting with EtOAc-*n*-hexane (30:70) to give stigmasterol (**11**) as colourless needles (220.1 mg, 0.64%).

The EtOAc extract (52.1 g) was purified by silica gel FCC, eluted with a gradient system of *n*-hexane-EtOAc 100/0 to 0/100 and EtOAc-MeOH 100/0 to 0/100 to give ten fractions, BE₁-BE₁₀. Fraction BE₂ was further separated using silica FCC by using EtOAc:CH₂Cl₂ (10:90) as a solvent system to give cerbinal (**6**) as orange-yellow needles (12.0 mg, 0.02%) and benzoic acid (**9**) as a colourless needles (11.1 mg, 0.02%). Fraction BE₄ was subjected to silica gel FCC using a gradient system of *n*-hexane-acetone 100/0 to 0/100 and acetone-MeOH 100/0 to 0/100 by increasing of polarity to give three subfractions, BE_{4,1}-BE_{4,3}. Subfraction BE_{4,3} was then purified using FCC, a solvent system of EtOAc:CH₂Cl₂ (15:85) as an eluent, to provide garjamine (**5**) as a white solid (23.6 mg, 0.04%). Fraction BE₅ was subjected to silica gel FCC, eluted with acetone:CH₂Cl₂ (30:70), to

afford vanillic acid (**10**) as pale yellow powder (17.4 mg, 0.03%). Fraction BE₆ was purified using silica gel FCC, eluted with a gradient system of *n*-hexane-EtOAc 100/0 to 0/100 and EtOAc-MeOH 100/0 to 0/100 to give two subfractions, BE_{6,1} and BE_{6,2}. Subfraction BE_{6,1} was then purified by using silicagel FCC, eluted with EtOAc:*n*-hexane (30:70) to give 10-*O*-acetylmacrophyllide (**2**) as a white amorphous solid (7.7 mg, 0.01%). Fraction BE₈ was further subjected to silica gel FCC, eluted with a gradient system of *n*-hexane-EtOAc 100/0 to 0/100 and EtOAc-MeOH 100/0 to 0/100 to give two subfractions, BE_{8,1} and BE_{8,2}. Subfraction BE_{8,1} was then purified by using silica gel FCC with EtOAc:*n*-hexane (40:60) was used as an eluent to afford genipin (**4**) as a colourless oil (7.3 mg, 0.01%). The subfraction BE_{8,2} was separated using silica gel FCC, eluting with EtOAc:*n*-hexane (40:60), and then subjected to preparative thin layer chromatography (PLC), eluting with MeOH:CH₂Cl₂ (3:97), to give 6 β -hydroxy-10-*O*-acetylgenipin (**1**) as a viscous liquid (11.4 mg, 0.02%). Fraction BE₁₀ was purified using silica gel FCC, eluting with acetone:*n*-hexane (50:50) to provide the mixture of β -gardiol (**7**) and α -gardiol (**8**) as an amorphous solid in the ratio of 1:1 (155.9 mg, 0.29%).

Fresh fruit of *R. wittii* (12.0 kg) was ground and extracted with MeOH (3 X 13.5L) at room temperature. Removal of solvents from the extract under reduced pressure gave crude MeOH (641.6 g, 5.35%).

The crude MeOH extract (50.0 g) was separated on a silica gel FCC, eluted with a gradient system of *n*-hexane-EtOAc 100/0 to 0/100 and EtOAc-MeOH 100/0 to 0/100 to give six fractions, FM₁-FM₆. Fraction FM₂ was purified using silica gel FCC, eluted with a gradient system of CH₂Cl₂-MeOH 100/0 to 0/100 to give an amorphous solid mixture of β -gardiol (**7**) and α -gardiol (**8**) in the ratio of 2:1 (733.3 mg, 1.46%). Fraction FM₄ was then purified using silica gel FCC, eluted with a gradient system of CH₂Cl₂-MeOH 100/0 to 0/100 to give three subfractions, FM_{4,1} and FM_{4,3}. Subfraction FM_{4,2} was separated using silica gel FCC, eluting with MeOH:CH₂Cl₂:*n*-hexane (6:54:40), to provide 6 β -hydroxygenipin (**3**) as a viscous liquid (26.2 mg, 0.05%).

6 β -Hydroxy-10-*O*-acetylgenipin (**1**): colourless viscous liquid; $[\alpha]_D^{25} + 126$ (*c* 0.1, MeOH); *R*_f: 0.62 (acetone-CH₂Cl₂, 1:1); UV λ_{max} (log ϵ): 239 (3.97); IR (neat) ν_{max} (cm⁻¹): 3379, 2924, 2860, 1730, 1676, 1629, 1441, 1377, 1297, 1234, 1150, 1111, 1034, 937, 877, 800, 765, 691; HR-ESI-TOF-MS: *m/z* 307.0798 [M + Na]⁺ (calcd for C₁₃H₁₆O₇ + Na, 307.0794); ¹H NMR (400 MHz, CD₃OD): δ 7.56 (1H, s, H-3), 5.83 (1H, brs, H-7), 4.75 (2H, m, H-10), 4.72 (1H, d, *J* = 8.4 Hz, H-1), 4.53 (1H, m, H-6), 3.75 (3H, s, OMe-11), 2.95 (1H, m, H-5), 2.78 (1H, m, H-9), 2.09 (3H, s, MeCOO-10); ¹³C NMR (100 MHz, CD₃OD): δ

172.4 (MeCOO-10), 170.8 (C-11), 155.0 (C-3), 142.4 (C-8), 132.4 (C-7), 109.9 (C-4), 98.3 (C-1), 82.8 (C-6), 63.3 (C-10), 52.1 (OMe-11), 48.9 (C-9), 46.8 (C-5), 20.7 (MeCOO-10).

10-*O*-Acetylmacrophyllide (**2**): white amorphous solid; m.p. = 102-104 °C; $[\alpha]_D^{25} + 0.83$ (*c* 1.0, MeOH); *R*_f: 0.63 (EtOAc-n-hexane, 1:1.5); IR (neat) $\nu_{\max}$ (cm⁻¹): 2943, 2917, 2849, 1768, 1737, 1682, 1645, 1596, 1511, 1463, 1431, 1365, 1328, 1210, 1174, 1142, 1096, 1032, 940, 917, 896, 877, 805, 727; HR-ESI-TOF-MS: *m/z* 275.0535 [M + Na]⁺ (calcd for C₁₂H₁₂O₆ + Na, 275.0532); ¹H NMR (400 MHz, CD₃OD): δ 6.16 (1H, brs, H-7), 5.83 (1H, s, H-1), 5.50 (1H, d, *J* = 3.6 Hz, H-3), 4.97 (1H, dd, *J* = 2.0, 4.4 Hz, H-6), 4.79 and 4.67 (2H, d, *J* = 14 Hz, H-10), 3.37 (1H, m, H-5), 3.24 (1H, d, *J* = 8.0 Hz, H-9), 3.21 (1H, dd, *J* = 3.6, 5.2 Hz, H-4), 2.08 (3H, s, Me); ¹³C NMR (100 MHz, CD₃OD): δ 172.2 (acetyl C=O), 171.4 (C-11), 144.2 (C-8), 133.9 (C-7), 102.0 (C-3), 98.3 (C-1), 86.4 (C-6), 62.2 (C-10), 53.4 (C-9), 48.3 (C-4), 43.2 (C-5), 20.6 (Me)

3.4. Biological activity procedures

3.4.1. Antimycobacterial assay.

The antimycobacterial activity was assessed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) described by Collins and Franzblau [18]. Standard drugs, isoniazid and kanamycin sulfate, were used as the reference compounds.

3.4.2. Cytotoxicity assays

Cytotoxicity assays against human epidermoid carcinoma (KB), human breast adenocarcinoma (MCF7) and human small cell lung cancer (NCI-H187) were performed employing the Resazurin microplate assay described by O'Brien et al. [19]. The reference substance was doxorubicin.

4. Conclusions

A phytochemical investigation of the Thai herbal medicinal plant *R. wittii* resulted in the isolation of eight iridoids, two of them were new compounds, 6 β -hydroxy-10-*O*-acetylgenipin (**1**) and 10-*O*-acetylmacrophyllide (**2**). Stigmasterol was isolated as the major constituent 0.64%, while the mixture of β -gardiol (**7**) and α -gardiol (**8**) (155.9 mg, 0.29%) in the ratio of 1:1 are the major component of iridoid isolates. Moreover, this iridoid mixture (733.3 mg, 1.46%) in the ratio of 2:1 was also obtained as the major component of the fruit extract. Iridoids have been reported to possess a wide range of biological activities. Our findings also support the bioactive potential of several isolated iridoids such as anti-TB and

cytotoxic activity against cancer cells. Therefore, the *R. wittii* is a valuable source of iridoid compounds.

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