องค์ประกอบทางเคมีและความเป็นพิษต่อเซลล์ของหญ้าลิ้นงู

CHEMICAL CONSTITUENTS AND CYTOTOXIC ACTIVITY OF *HEDYOTIS CORYMBOSA* LAMK

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บทคัดย่อ

การศึกษาองค์ประกอบทางเคมีของหญ้าลิ้นงู (*Hedyotis corymbosa* Lamk.) โดยนำมาแยกด้วยวิธีโครมาโทกราฟี พบสารประกอบ 7 ชนิด คือ aurantiamide acetate **(1)**, 3β-acetylaleuritolic acid **(2)**, salicylic acid **(3)**,β-sitosteryl-3-*O*-β-*D*-glucopyranoside **(6)**,1,5-anhydroglucitol **(7)** และสารผสมของ 22,23-dihydrochondrillasterol **(4)** และ chondrillasterol **(5)** สารประกอบ **(1)** เป็นสารไดเปปไทด์ที่แยกได้ครั้งแรกจากพืชสกุลนี้ การพิสูจน์โครงสร้างของสารใช้เทคนิคทางสเปกโทรสโกปี นำสารสกัดและสารบริสุทธิ์ที่แยกได้มาทดสอบความเป็นพิษต่อเซลล์มะเร็งเม็ดเลือดขาว P388

คำสำคัญ : หญ้าลิ้นงู, รูบิเอซิอี, ความเป็นพิษต่อเซลล์, ออเรนไทเอไมด์แอซิเตต

Abstract

Chemical investigation of *Hedyotis corymbosa* Lamk. was carried out using chromatographic methods. This led to the isolation of seven known compounds, namely aurantiamide acetate (1), 3β-acetylaleuritolic acid (2), salicylic acid (3), β-sitosteryl-3-*O*-β-*D*-glucopyranoside(6), 1,5-anhydroglucitol (7) and a mixture of 22,23-dihydrochondrillasterol (4) and chondrillasterol (5). Among the compounds isolated, dipeptide (aurantiamide acetate 1) was isolated for the first time from this genus. Structures of these compounds were identified on the basis of spectroscopic techniques. The crude extracts and isolated compounds were evaluated for cytotoxic activity against leukemia (P388) cell lines.

Keywords: Hedyotis corymbosa, Rubiaceae, cytotoxic activity, aurantiamide acetate

Introduction

Several species of *Hedyotis* (family Rubiaceae) have been used in traditional medicine in a number of Asian countries including Thailand, and have been used for the treatment of cancer, dysentery, diarrhea, wounds and snake-bite. Phytochemical studies on some *H*edyotis species have shown to be chemically diverses, yielding among other, β--carloline alkaloids [1,2], flavonol glycosides [3,4] and anthraquinones [5].

Hedyotis corymbosa Lamk. is a weedy herb with small white flowers in axilliary cluster [6], widely distributed throughout Thailand. It is known in Thai as Ya-Lin-Ngu. *H. corymbosa* has been used as traditional medicine for anticancer, antiinfection, antimalaria and anti-inflammatory [7]. Hepatoprotective effect of the methanolic extract of *H. corymbosa* on paracetamol overdose-induced liver damage in Wistar rats had been reported [8].

Previous phytochemical investigations of *H. corymbosa* showed the presence of iridoid glucosides [9,10]. We investigated the chemical constituents and cytotoxic activity of this plant, resulted in a diverse group of compounds, including one dipeptide **(1),** four triterpenoids **(2,4-6)**, one phenolic **(3)** and one sugar **(7)**. Compound **1** was identified for the first time from the genus *Hedyotis*. In addition, the cytotoxic activity of the crude extracts and isolated compounds **1-7** were also tested.

Material and method

General experimental procedures

All commercial grade solvents were distilled prior to use. Melting points were determined on a Griffin melting point apparatus. IR spectra were reported on a Perkin-Elmer FT-IR spectrum BX spectrophotometer. NMR spectra were recorded on Bruker Avance 300 FT-NMR spectrometer. EIMS spectra were obtained on Finigan MAT 90 instrument. Column chromatography was carried out using Merck silica gel 60 (particle size 70-230 and 230-400 mesh). For TLC analysis, precoated sililca gel plates (Merck Kieselgel 60 GF_{254}) were used. Spots on TLC were visualized under UV light at 254 and 365 nm, and by spraying with anisaldehyde-H_2SO_4 solution followed by heating.

Plant material

The whole plant of *H. corymbosa* (4 kg) was collected from Singburi Province, Thailand, in March 2003. It has been identified by comparison with voucher specimen (BKF 128757) and was deposited at the Forest Herbarium, National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment, Thailand.

Extraction and Isolation

Dried whole plant of *H. corymbosa* (4 kg) was ground and extracted with hexane (12L× 7days×2). The extracts were filtered and evaporated to dryness under reduced pressure to obtain hexane extract (129 g). Similary, the extraction was carried out using ethyl acetate and methanol to give the corresponding ethyl acetate

extract (95 g) and methanol extract (104 g), respectively.

The hexane extract (47 g) was subjected to silica gel column chromatography eluted with hexane-ethyl acetate mixtures of increasing polarity as eluent to give 9 fractions (H1-H9). Fraction H8 (15.2 g) was loaded on silica gel column chromatography eluted with chloroform-ethanol (9:1) to give 5 fractions (H8.1-H8.5). Fraction H8.2 was further chromatographed on silica gel, eluting with chloroform to give aurantiamide acetate (**1**,15.1 mg).

The ethyl acetate extract (68 g) was chromatographed on silica gel column and eluted with gradients of chloroform-ethanol to give 7 fractions (E1-E7). Fraction E4 was rechromatographed on silica gel column, using gradients of chloroform-ethyl acetate and further recrystallization of subfractions [E4.2-E4.4] afforded 3β-acetylaleuritolic acid (**2**,21 mg), salicylic acid (**3**, 21 mg) and a mixture of 22,23-dihydrochondrillasterol **(4)** and chondrillasterol (**5**) (12 mg).

The methanol extract (75 g) was directly chromatographed on silica gel column and eluted with gradients of ethyl acetate-ethanol to give 4 fractions (M1-M4). Fraction M1 was further purified by elution through silica gel column using chloroform-ethanol (9:1), yielded β-sitosteryl-3-*O*-β-*D*-glucopyranoside (**6**, 320 mg). Fraction M2 was recrystallized from ethanol to yield 1,5-anhydroglucitol (**7**, 2.85 g).

Identification of compounds

Aurantiamide acetate (1): white amorphous powder; m.p. 183-184°C (lit.185-186°C [11]); FTIR n_{max}(KBr): 3313, 1726, 1662, 1632, 1261, 746, 698 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.72 (2H, m, H-2‴ and H-6‴), 7.53(1H, m, H-4‴), 7.44 (2H, m, H-3‴ and H-5‴), 7.25 (6H, m, H-3′, H-5′, H-2″, H-3″, H-5″, and H-6″),7.15 (2H, m, H-4′, and H-4″), 7.07 (2H, m, H-2′ and H-6′), 6.76 (1H, d, *J* = 7.6 Hz, H-9), 5.98 (1H, d, *J* = 8.5 Hz, H-6), 4.77 (1H, m, H-8), 4.35 (1H, m, H-5) , 3.93 (1H, dd, *J* = 11.4, 4.9 Hz, H-4a), 3.82 (1H, dd, *J* = 11.4, 4.9 Hz, H-4b), 3.22 (1H,dd, *J* = 13.7, 8.5 Hz, H-7″a), 3.07 (1H, dd, *J* = 13.7, 8.5 Hz, H-7″b), 2.75 (2H, m, H-7′), 2.03 (3H, s, H-1); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 170.2 (C-2,7), 167.1 (C-10), 137.0 (C-1′), 136.6 (C-1″), 133.6 (C-1‴), 131.9 (C-4‴), 129.2 (C-3″, 5″), 129.1 (C-3′, 5′), 128.8(C-2″,6″), 128.6 (C-2′, 6′), 128.6 (C-3‴, 5‴), 127.1 (C-4′), 127.0 (C-2‴, 6‴), 126.7 (C-4″), 64.6 (C-4), 55.0 (C-8), 49.4 (C-5), 38.4 (C-7″), 37.4 (C-7′), 20.8 (C-1); EIMS m/z (% relative intensity): 444 (M^+, 3), 323 (1), 252 (61), 224 (28), 105 (100), 91 (9), 77 (38), 51 (10).

3β-Acetylaleuritolic acid (2): white amorphous powder; m.p. 299-300°C (lit. 298-300°C [12]); FTIR n_{max}(KBr): 3300, 1734, 1687, 1242 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$):

δ 5.50 (1H, dd, *J* = 3.0, 7.6 Hz, H-15), 4.45 (1H, t, *J* = 7.8 Hz, H-3), 2.02 (3H, s, $COOCH_3$), 1.23 (3H, s, H-26), 0.93 (3H, s, H-27), 0.90 (3H, s, H-30), 0.86 (3H, s, H-24), 0.83 (3H, s, H-23); ^{13}C-NMR (75 MHz, $CDCl_3$); δ 184.1 (C-28, COOH), 171.0 ($COOCH_3$), 160.5 (C-14), 116.8 (C-15), 80.9 (C-3), 55.6 (C-5), 51.5 (C-17), 49.1 (C-9), 41.4 (C-18), 40.8 (C-7), 39.0 (C-8), 37.9 (C-10), 37.7 (C-4), 37.4 (C-13), 37.3 (C-1), 35.3 (C-19), 33.7 (C-12), 31.9 (C-29), 33.3 (C-21), 31.3 (C-16), 30.7 (C-22), 29.3 (C-20), 28.6 (C-30), 27.9 (C-23), 26.2 (C-26), 23.5 (C-2), 22.5 (C-27), 22.0($COOCH_3$), 18.7 (C-6), 17.3 (C-11), 16.6 (C-24), 15.6 (C-25); EIMS m/z (% relative intensity): 498 (M^+,7), 329 (10), 269 (15), 234 (55), 189 (100).

Salicylic acid (3): white needles; m.p. 154-156°C (lit. 157-159°C [13]); FTIR n_{max} (KBr): 3238-2596, 1654, 1295 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 10.40 (1H, s, COOH), 7.92 (1H, d, *J* = 7.7 Hz, H-6), 7.5 (1H, t, *J* = 8.2, 7.4 Hz, H-4), 7.0 (1H, d, *J* = 8.2 Hz, H-3), 6.93 (1H, t, *J* = 7.8,7.4, H-5); ^{13}C-NMR (75 MHz, $CDCl_3$): d174.3 (CO), 162.2 (C-2), 137.0 (C-4), 130.9 (C-6), 119.6 (C-5), 117.8 (C-3), 111.2 (C-1); EIMS m/z (% relative): 138 (M^+,32), 120 (100), 92 (94), 63 (41).

A mixture of 22,23-dihydrochondrillasterol(4) and chondrillasterol (5): white amorphous powder; m.p. 156-158 °C [14]; FT IR V_{max} (KBr): 3422, 2959, 1458, 1383 cm^{-1}; ^{1}H-NMR (300 Hz, $CDCl_3$): d 5.25 (1H, br., H-7), 5.15 (1H,dd, *J* = 12.3, 8.3 Hz, H-23), 5.01 (1H, dd, *J* = 12.3, 8.3 Hz, H-22), 3.55 (1H. M, H-3), 1.03 (3H, d, *J* = 6.5 Hz, H-21), 0.82 (6H, d, *J* = 6.0 Hz, H-26, H-27), 0.80 (3H, d, *J* = 6.5 Hz, H-29), 0.78 (3H, s, H-19), 0.53 (3H, s, H-18); ^{13}C-NMR (75 MHz, $CDCl_3$): d 139.6 (C-8), 138.2* (C-22), 129.4*(C-23), 117.5 (C-7), 71.1 (C-3), 56.1 (C-17), 55.0 (C-14), 51.2 (C-24), 49.5 (C-9), 43.4 (C-13), 40.3 (C-5), 39.6 (C-12), 37.1 (C-1), 36.6 (C-20), 34.2 (C-10), 33.9 (C-22), 33.9 (C-4), 31.7 (C-25), 29.6 (C-6), 28.5 (C-16), 28.0 (C-2), 26.2 (C-23), 23.1 (C-28), 23.0 (C-15), 21.5 (C-11), 21.4 (C-27), 21.1 (C-21), 19.0 (C-26), 13.0 (C-19), 12.2 (C-29), 12.0 (C-18) [* $\Delta^{22,23}$ of **5**]; EIMS m/z (% relative intensity) : 414 (M^+, 72), 412 (M^+, 17), 399 (33), 397 (10), 273 (43), 271 (100), 255 (52), 231 (41).

***β*-Sitosteryl-3-*o*-*β*-D-glucopyranoside (6)**: white amorphous powder; m.p.278-280 °C (lit.290°C [15]); FTIR V_{max} (KBr): 3385, 2938, 1458, 1368, 1630 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 5.05(1H, d, *J* = 4.51 Hz, H-6), 3.55 (1H, m, H-3), 2.90-4.90 (7H, m, sugar moiety H), 0.85 (3H, s, H-19), 0.78 (3H, d, *J* = 6.5 Hz, H-21), 0.70 (3H, t, *J* = 7.1 Hz, H-29), 0.65 (3H, s, H-27), 0.42 (3H, s, H-18); ^{13}C-NMR (75 MHz, DMSO-d_6): d 140.8 (C-5), 121.8 (C-6), 102.4 (C-1′), 78.5 (C-3′), 78.4 (C-5′), 78.0 (C-3), 75.2

(C-2′), 71.6 (C-4′), 62.7 (C-6′), 56.7 (C-14), 56.1 (C-17), 50.2 (C-9), 45.9 (C-24), 42.4 (C-13), 39.8 (C-16), 39.2 (C-4), 37.4 (C-1), 36.8 (C-10), 36.3 (C-20), 34.1 (C-23), 32.0 (C-7), 31.9 (C-8), 30.1 (C-2), 29.3 (C-25), 28.4 (C-12), 26.2 (C-28), 24.4 (C-15), 23.3 (C-22), 21.2 (C-11), 19.9 (C-21), 19.3 (C-27), 19.1 (C-26), 18.9 (C-19), 12.4 (C-18, C-29); EIMS m/z (% relative intensity) : 576 (M^+,5), 414 (40), 399 (15), 397 (100), 396 (60), 273 (15), 271 (30), 255 (55), 231 (15), 229 (30), 213 (10).

1,5-Anhydroglucitol (7): yellow crystal; m.p. 139-140°C (lit.142-143°C [16]); FTIR n_{max} (KBr): 3329, 2936, 1105, 1077 cm^{-1}; ^1H -NMR (300 MHz, DMSO-d_6): δ 4.90 (1H, d, *J* = 5.7 Hz, OH-2), 4.88 (1H, d, *J* = 4.9 Hz, OH-3), 4.85 (1H, d, *J* = 4.7 Hz, OH-4), 4.47 (1H, t, *J* = 5.9 Hz, OH-6), 3.68 (1H, dd, *J* = 10.9, 5.30 Hz, H-1b), 3.62 (1H, dd, *J* = 11.5, 5.9 Hz, H-6a), 3.35 (1H, dd, *J* = 11.5, 5.9 Hz, H-6b), 3.05 (1H, m, H-3), 3.20 (1H, m, H-2), 2.95 (3H, m, H-1a, H-4, H-5); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 82.7 (C-5), 79.5 (C-3), 71.4 (C-4), 70.9 (C-2), 70.6 (C-1), 62.5 (C-6); EIMS m/z (% relative intensity) : 164 (M^+,72), 147 (100), 133 (20).

Cytotoxic assay

Cytotoxic activity against the P388 (leukemia cells) cell lines was assayed by the cell count method. The P388 cells were grown in RPMI-1640 supplemented with 10% newborn calf serum under a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 4 days. The cultured cells were treated with various concentrations of the test compounds dissolved in DMSO and incubated for 4 days in the above conditions. The cell concentration was determined by counting the P388 cells in a hematocytometer, using 5-fluorouracil as a positive control. The activity was expressed as ED_{50} (the effective dose that inhibits 50% of cell growth).

Results and Discussion

The hexane, ethyl acetate and methanol extracts of whole plant of *H. corymbosa* were subjected to silica gel column chromatography using mixtures of hexane-ethyl acetate, chloroform-ethanol and ethyl acetate -ethanol, respectively. Further purification by repeated column chromatography and recrystallization gave seven known compounds, aurantiamide acetate (**1**), 3β-acetylaleuritolic acid (**2**), salicylic acid (**3**), a mixture of 22,23-dihydrochondrillasterol (**4**) and chondrillasterol (**5**), β-sitosteryl-3-*O*-β-*D*-glucopyranoside (**6**) and 1,5-anhydroglucitol (**7**). The structures of compounds were determined by comparison of their spectroscopic data with those reported in the literatures as shown in Figure 1.

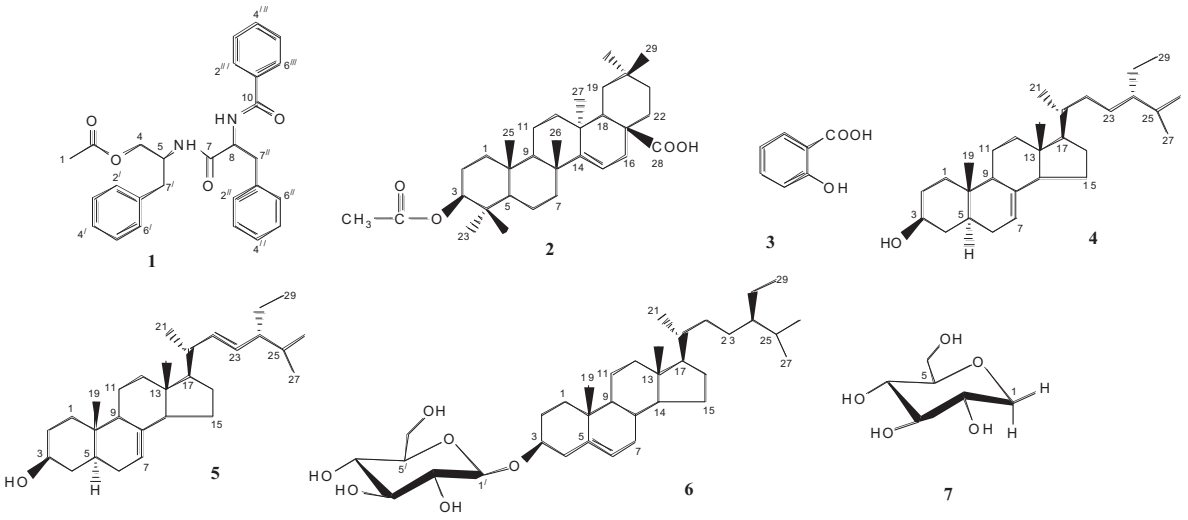

Figure 1. Structures of the compounds **1-7**.

The isolated compounds had been reported to possess diverse bioactivities as follows; compound **1** is a dipeptide that has been reported previously from red alga [17], *Moringa oleifera* [18] and other sources [11,19-20]. Although the compound **1** was previously isolated from other plants, it has been first found in genus *Hedyotis* by our group. It had been reported to display significant inhibitory activity on the release of β-glucuronidase and to a lesser effect on histamine [17]. In addition, it showed inhibition effect on tumor necrosis factor-α (TNF-α) and interleukin (IL)-2 [18]. Compound **2** had been found earlier in several plants, such as *Neoboutonia glabrescens* [21], *Cleidion spiciflorum* [22], *Phytolacca esculenta* [23] and *Mallotus* species [24]. It was reported to show strong activity against *Staphytococcus aureus* and *Salmonella typhimurium* [25] and inhibition on vitality of adult male worms of *Onchocerca gutturosa* [26]. Compound **3** was previously obtained from plants (white willow back and wintergreen leaves) and was also synthesized. It possesses bacteriostatic, fungicidal, and keratolytic actions [27]. Compounds **4** and **5** had been isolated from *Cucurbita maxima* [14], *Cucurbitaceae* [28] and *Gambeya boukokoensis* [29]. Compound **6** had been isolated from several plants, and recently from *Ficus chlamydocarpa* and *Ficus cordatd* [30]. Compound **7** was first discovered in the plant *Polygala senega* and has been used clinically in Japan for over a decade to monitor short-term glycemic control [31].

The cytotoxic activity of the crude extracts and isolated compounds **1-7** were tested *in vitro* against P388 cell lines. The ethyl acetate extract showed cytotoxic activity with ED_{50} value of 20 μg/mL. Other crude extracts and pure compounds **1-7** were inactive. It is interesting to observe that the ethyl acetate extract showed cytotoxic activity whereas the pure compounds from this fraction were inactive. This may be due to

synergistic effect of some or all the compounds present in the extract. According to National Cancer Institute guidelines, the extracts and pure compounds with ED_{50} values $\leq$ 20 μg/mL and $\leq$ 4 μg/mL, respectively, were considered cytotoxic [32,33].

Acknowledgements

The authors wish to thank the Faculty of Science, Srinakharinwirot University and National Cancer Institute of Thailand for facilities and supports.

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