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Chemical Constituents of *Hedyotis dichotoma* and their Biological Activity

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ABSTRAK

Dua sebatian, isoviteksin dan asid ursolik, telah diasingkan daripada daun dan batang *Hedyotis dichotoma*. Isoviteksin menunjukkan keaktifan terhadap fungus dan kandida. Struktur kedua-dua sebatian tersebut dikenalpasti melalui analisis spektroskopi.

ABSTRACT

Two compounds, isovitexin and ursolic acid, were isolated from the aerial parts of *Hedyotis dichotoma*. Anti-microbial assays indicated that isovitexin was active against fungus and candida. The structures of both compounds were assigned using modern spectroscopic techniques.

Keywords: *Hedyotis dichotoma*, Rubiaceae, isovitexin, ursolic acid, anti-fungal, anti-candida

INTRODUCTION

Hedyotis dichotoma is a small herb of the family Rubiaceae (0.1 – 0.2 m tall) commonly found in open sandy places throughout Malaysia, especially near the sea. Being a soft and easily pulped plant, it is used as a poultice (Burkill 1936). Previous phytochemical studies on this genus include *H. verticillata* (Hamzah *et al.* 1994), *H. chrysotricha* (Fang *et al.* 1992), *H. diffusa* (Wu *et al.* 1991), and *H. lawsoniae* (Nishihama *et al.* 1981; Matsuda *et al.* 1984; Ho *et al.* 1986) 1991. This paper reports the results on the isolation and identification of isovitexin and ursolic acid, and the biological activity studies of isovitexin.

MATERIALS AND METHODS

Plant Material

Hedyotis dichotoma was collected from Gebeng, near Kuantan, Malaysia and a voucher specimen was deposited at the herbarium of Biology Department, Universiti Pertanian Malaysia.

Method

Melting points were determined using Kofler hot stage apparatus and were uncorrected. UV spectra were recorded on Shidmadzu UV-VIS 160, and IR spectra on Perkin Elmer 1600 FTIR spectrometers. Mass spectra were recorded on Finnigan MAT SSQ710 spectrometer with ionization induced by electron impact at 70 eV. ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL GX-500 spectrometer measured at 500 and 125 MHz, respectively. Column chromatography and analytical tlc utilized Merck 7734 and Merck DC-Plastikfollen 60 F_{254}, respectively.

Extraction of Plant Materials

A general extraction method was employed on the plant sample. The fresh leaves and twigs (600 g) were soaked in methanol for 48 h. The methanol extract was filtered and evaporated under reduced pressure. Fresh methanol was then added to the plant and the same extraction procedure was repeated twice. The concentrated extracts were combined and further dried to yield 2.9 g of crude methanol extract. The crude extract was then partitioned successively between water and ethyl acetate, and between water and butanol.

Isolation of Isovitexin (1)

The crude butanol extract (4.8 g) was subjected to column chromatography using ethyl acetate with increasing ratios of methanol. Thirty fractions were collected, of which fractions 8-14 contained the major compound. The yellowish amorphous solid (3.2 g) melts at 212-214° C (isovitexin, lit. m.p. 223-224°C, Buckingham 1994). UV λ_{max} nm (log ε) MeOH: 344.5(1.68), 269.5(1.55), 229.5(1.53), 288.5(1.24), 248 (1.07); IR ν cm^{-1} (KBr disk): 3738, 3398, 1652, 1626, 1611, 1493, 1357, 1180, 835, 782, 618, 569;
^{1}H-NMR δ (500 MHz, CD_3OD): 7.81 (d, 2H, $J_{2',3'} = J_{6',5'} = 8.7$ Hz, H-2′,6′), 6.90 (d, 2H, $J_{3',2'} = J_{5',6'} = 9.1$ Hz, H-3′,5′), 6.59 (s, 1H, H-3), 6.50 (s, 1H, H-8), 4.8 (d, 1H, J = 9.1 Hz, C-1′), 3.40 – 4.00 (m, 6H, sugar protons);
^{13}C-NMR (125.65 MHz, CD_3OD; DEPT-EXPERIMENT): 184.0 (C-4), 166.2 (C-2), 164.8 (C-7), 162.2 (C-4′), 162.0 (C-5), 158.7 (C-9), 129.4 (C-2′, C-6′), 123.1 (C-1′), 117.0 (C-3′, C-5′), 109.1 (C-6), 105.2 (C-10), 103.8 (C-3), 95.2 (C-8), 82.6 (C-5″), 80.1 (C-3″), 75.2 (C-1″), 72.5 (C-2″), 71.7 (C-4″), 62.8 (C-6″);

MS m/z (%): 283 (69), 270 (8), 165(41), 137 (18), 123 (31), 121 (20), 91 (40), 73 (69), 60 (100).

Isolation of Ursolic Acid (II)

The crude chloroform extract (4.5 g) was subjected to column chromatography using chloroform with increasing ratios of methanol as the eluant. Ten fractions (each 50 ml) were collected, of which fractions 2 – 5 showed a similar pattern on analytical tlc. These fractions were combined and then subjected to another column chromatography using $CHCl_3$/EtOAc (9:1) as the solvent system. Ten fractions were collected, of which fractions 2 and 3 consisted of the major product. The two fractions were combined and then decolourized using activated carbon to give a white powder (3.5 g), m.p. 272-275°C (ursolic acid, lit. m.p. 266-267°C, Takagi *et al.* 1979).

UV λ_{max} nm (log ε) MeOH: 470 (0.14), 441 (1.18, 421 (0.17), 462 sh (0.13), 430 sh (0.16);

IR ν cm^{-1} (KBr disk): 3750, 3432, 1692, 1540, 127, 1092, 996;

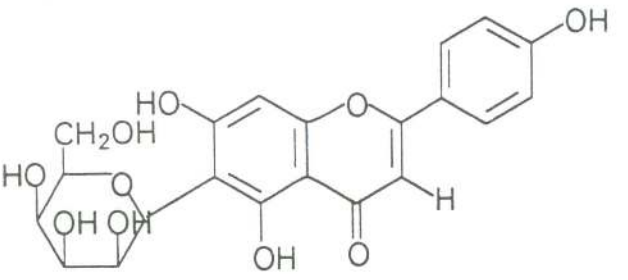
^{1}H-NMR δ (500 MHz, CD_3OD): 5.21 (m, 1H, H-12), 3.14 (m, 1H, H-3), 2.20 (d, 1H, $J_{18,19}$ = 11.3 Hz, H-18), 2.02-1.15 (m, 22H), 1.10 (s, 3H, C-27 Me), 0.96 (s, 3H, C-23 Me), 0.95 (s, 3H, C-25 Me), 0.93 (d, 3H, C-30 Me), 0.87 (d, 3H, C-29 Me), 0.83 (s, 3H, C-24 Me), 0.76 (s, 3H, C-26 Me);

^{13}C-NMR δ (125.5 MHz, CD_3OD, DEPT-experiment): 181.6 (C-28), 139.6 (C-13), 126.8 (C-12), 79.6 (C-3), 56.7 (C-18), 54.3 (C-5), 47.6 (C-17), 47.2 (C-9), 42.8 (C-14), 40.7 (C-8), 40.4 (C-20), 40.4 (C-19), 39.9 (C-4), 39.8 (C-22), 38.1 (C-1), 34.3 (C-7), 31.7 (C-21), 29.2 (C-15), 28.7 (C-23), 27.8 (C-2), 25.3 (C-16), 24.3 (C-11), 24.0 (C-27), 21.5 (C-30), 19.4 (C-6), 17.8 (C-29), 17.6 (C-26), 16.3 (C-25), 16.0 (C-24);

MS m/z (%): 456 (M^+), 248 (100), 219 (7.7), 207 (27), 203 (42.6), 189 (10.1), 133 (32.2), 119 (11.3), 69 (13.3).

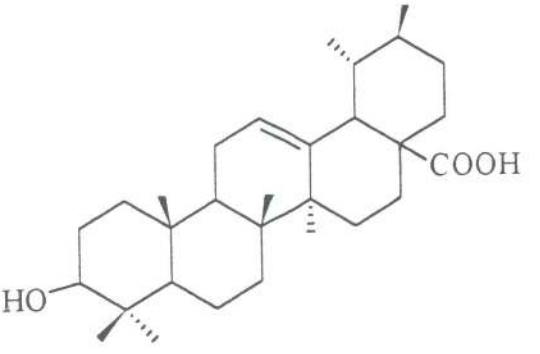
RESULTS AND DISCUSSION

Extraction of the leaves and twigs of *Hedyotis dichotoma* followed by extensive chromatography afforded isovitexin and ursolic acid. Isovitexin, a yellowish powder extracted from butanol fractions is a *C*-glycosyl type flavonoid. This is the first time that the compound has been isolated from this genus. The UV spectrum of the compound displayed strong absorption bands at 344.5 and 269.5 nm, which is typical of a flavone-type skeleton. The former is due to the B ring of the flavone, whereas the latter is due to the A ring. The spectrum showed a bathochromic shift of 68.5 nm upon addition of 2M NaOH, which indicates the presence of a hydroxyl group at C-7 (of ring B) and also a shift of 8 nm (due to ring A), which indicates the the presence of free OH at C-7. The addition of NaOH also showed the formation of a new band at 331 nm which indicates the existence of free 4′-OH in the B ring



(*I*) isovitexin

Compound 1



(*II*) ursolic acid

Compound 2

(Markham 1982; Agrawal 1989). The IR spectrum showed strong absorptions at 1652 cm^{-1} and 1611 cm^{-1} which correspond to the C=O and aromatic stretchings, respectively. A broad band at 3000-3500 cm^{-1} suggested the presence of hydrogen bonded OH group in the compound.

The aromatic region of the ^{1}H-NMR spectrum showed the signals characteristic of an apigenin skeleton. Two doublets at 7.81 and 6.90 ppm, both integrating for two protons, are assigned to protons H-3′ and 5′ and H-2′ and 6′ of the B ring. Signals at 6.59 and 6.50 ppm are due to protons attached at C-6 and C-3 which are part of the A ring in the apigenin skeleton. Signals of the sugar protons integrated for seven protons resonating at 3.41 to 4.87 ppm. The assignments of these protons were accomplished using 2-D (PHSQC) technique. These techniques managed

to identify a cross peak at 75.2 ppm and a proton at 4.8-4.9 ppm, which suggested that the proton attached to the carbon is connected to the C-C bond. To confirm this, the spectrum was measured using the HMG mode, i.e. by removing the solvent peak due to methanol-d_4 whereby the existence of a doublet under this solvent was then observed. This signal is due to the anomeric proton of the sugar which is coupled to the C-2 proton of the sugar moiety. The high J value (9.1 Hz) for this proton suggested that it is in a β-configuration. Further support can be obtained from the ^{13}C-NMR spectrum which gave a signal at 75.2 ppm, indicating that the anomeric carbon is attached to another C-atom and not to the usual oxygen of the agylcone or the sugar unit. The anomeric carbon actually resonates at higher field compared to the normal *C-O*-glycosides where the anomeric carbon resonate at 102.0 ppm. Further assignment of the signals of the flavonoid was accomplished based on the correlation of the ^{13}C-NMR, values which clearly showed the presence of an epigenin skeleton together with the carbons due to the glucose residue. The position of the sugar, whether at C-8 or C-6, was confirmed by comparing the ^{13}C-NMR values with those of vitexin (sugar at C-8) and of isovitexin (sugar at C-6) (Harborne and Mabry 1981; Agrawal 1989).

The second compound, ursolic acid, was isolated from the chloroform extract of the plant. The UV spectrum showed absorptions at 472, 444 and 421 nm and the IR spectrum exhibited absorptions at 3400 cm^{-1} (hydroxyl) and 1692 cm^{-1} (carbonyl). The mass spectrum showed a strong molecular ion peak at m/z 456, which corresponds to molecular formula $C_{30}H_{48}O_3$. A base peak at m/z 248 is typical for an α- or β-amyrin type triterpenes. Comparison of the ^{13}C-NMR signals with the literature values especially those for C-12, C-13, C-18, C-19 and C-20, indicated that the compound was of the α-amyrin type (Doddrell *et al.* 1974). This conclusion was further supported by the ^{1}H-NMR spectrum where a doublet at 2.20 ppm with J = 11.3 Hz indicated that H-18 and H-19 are *trans* to one another. This characteristic can only occur if it is an α-type triterpene and not a β-amyrin type.

Anti-microbial assays, including those for anti-fungal, anti-candidal and anti-bacterial activities, were carried out on isovitexin using the pour plate method. The standards used were streptomycin sulphate for *Pseudomonas aerogenosa* and *Bacillus cereus* and nystatin for microorganisms *Aspergillus ochraceus*, *Aspergillus niger* and *Candida lipolytica*. A standard concentration of 50 µg/disc was used for all samples throughout the study. The results of the bioassay are given in Table 1.

The results showed that isovitexin is active towards fungi and candida but not towards bacteria.

TABLE 1
Anti-microbial activities of isovitexin

Organism	Diameter of inhibition (mm) (50 µg/disc)
Pseudomonas aerogenosa	0
Bacillus cereus	0
Aspergillus ochraceus	17
Aspergillus niger	18
Candida lipolytica	19
Streptomycin sulphate (20 µg/disc)	25
Nystatin (50 µg/disc)	22

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