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CHEMISTRY AND ANTIPROLIFERATIVE ACTIVITIES OF 3-METHOXYFLAVONES ISOLATED FROM *Varthemia iphionoides*

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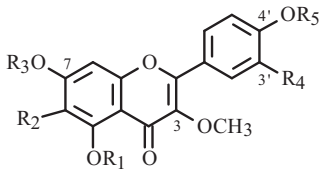
*A new isolated flavone, 4′-hydroxy-3,5,6,7-tetramethoxyflavone (**6**), together with seven known 3-*O*-methylated flavones isolated from the ethanol extract of the aerial parts of* Varthemia iphionoides, *were studied for DPPH free radical-scavenging and cytotoxic activities. Flavones **2**, **3**, **4**, and **8** were the most active DPPH free-radical scavengers with inhibition percentage of 63.5, 42.9, 47.9, and 55.6, respectively, at a concentration of 100 μg/mL. Flavones **2** and **8** were the most active as inhibitor for human leukemia HL-60 cells; the IC_{50} values after 48 h incubation were 20.5 and 23.9 μg/mL, respectively.*

Keywords: *Varthemia iphionoides*, 3-methoxyflavone, antiproliferative activity, human leukemia (HL-60) cells, DPPH.

Varthemia (Compositae) is a large genus with *ca* 90 species [1]. In Jordan, there are three species of *Varthemia* DC. that have been reported and described in the list of Jordanian vascular plants, namely, *V. candicans*, *V. montana,* and *V. iphionoides* [2]. *Varthemia iphionoides* Boiss. & Bl. is the most widely distributed and is commonly used in Jordanian folk medicine for the treatment of diabetes mellitus [3] and gastrointestinal disorders [4]. Phytochemical studies of *V. iphionoides* resulted in the isolation of four flavones: 3,3′-di-*O*-methylquercetin, kumatakenin, xanthomicrol, and jaceidine [5], seven 3-methoxyflavones [6], and eudesmane sesquiterpene [7]. In our previous work, a pronounced cytotoxic effect on human leukemia (HL-60) cells was shown in the hexane, chloroform, and ethanol extracts of the aerial parts of this species with inhibition of 89.0, 68.4, and 62.3%, respectively, at a concentration of 200 μg/mL [8]. In this study, the ethanol extract possessing antiradical and antitumor activities was further fractioned and purified, and the active flavonoids were characterized by different chromatographic techniques.

Isolation and separation of the active components were performed by subjecting the ethanol extract to a variety of open-column chromatography and preparative TLC separation to obtain compounds **1**–**8**.



1 - 8

1: $R_1 = R_2 = R_5 = H$, $R_3 = CH_3$, $R_4 = OCH_3$
2: $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OCH_3$
3: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CH_3$
4: $R_1 = R_2 = R_3 = R_4 = R_5 = H$
5: $R_1 = R_3 = R_5 = CH_3$, $R_2 = OCH_3$, $R_4 = H$
6: $R_1 = R_3 = CH_3$, $R_2 = OCH_3$, $R_4 = R_5 = H$
7: $R_1 = R_4 = R_5 = H$, $R_2 = OCH_3$, $R_3 = CH_3$
8: $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OCH_3$

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TABLE 1. EI-MS Data and DPPH Radical-Scavenging and Antiproliferative Activities of Compounds **1–8**

Compound	EI-MS	DPPH inhibition, %*,**	Antiproliferative activity (IC_{50}, μg/mL)[a]
1	343.9	33.5 ± 2.4	> 150.0 ± 25.0
2	329.9	63.5 ± 2.4	20.5 ± 2.3
3	313.9	42.9 ± 2.7	48.5 ± 3.9
4	299.9	47.9 ± 1.7	39.5 ± 2.5
5	371.9	8.8 ± 2.3	54.0 ± 3.6
6	357.9	16.3 ± 5.7	60.0 ± 2.0
7	343.9	25.1 ± 2.6	118.3 ± 6.5
8	329.9	55.6 ± 2.5	23.9 ± 3.6
BHT	N. d.	81.3 ± 1.7	N. d.

*Results are recorded as means ± SD (n = 3). $P < 0.05$.

**Compounds were tested at a concentration of 100 μg/mL. N. d.: not determined.

Compounds **1–4**, **7**, and **8** were found to be identical with 3,7,3′-tri-*O*-methylquercetin (**1**), 3,3′-di-*O*-methylquercetin (**2**), kumatakenin (3,7-di-*O*-methylkaempferol) (**3**), 3-*O*-methylkaempferol (**4**), penduletin (5,4′-dihydroxy-3,6,7-trimethoxyflavone) (**7**), and 5,7,4′-trihydroxy-3,6-dimethoxyflavone (**8**), respectively, based on a comparison of the physical, NMR (^{1}H, ^{13}C, HMBC, and HMQC), and MS spectral data with those of authentic compounds.

Compound **5** was obtained as colorless needles. Electron impact mass spectrum (EI-MS) showed an $[M]^+$ ion peak at *m/z* 371.9 (Table 1). The ^{1}H NMR spectrum of **5** displayed signals due to an A_2B_2-doublet at δ 7.35 and 8.25 (each 2H), suggesting the presence of a phenol ring in the molecule. In addition, the spectrum displayed an isolated aromatic singlet at δ 7.06 ascribed to a penta-substituted aromatic ring, as well as five methyl protons at δ 3.79, 3.91, 3.97, 4.03, and 4.19 (each 3H) attached to hydroxyl groups, suggesting that the molecule has an *O*-methylated flavonoid structure.

In the ^{13}C NMR spectrum of **5**, there was observed five carbon signals at δ 55.4, 56.4, 59.7, 61.4, and 62.4 arising from five methoxyl carbons, as well as four carbon signals at δ 114.4 (2C), 123.5, 130.3 (2C), and 161.7 arising from the B-ring of the flavone structure. Furthermore, three out of the remaining nine carbon signals at δ 141.0, 153.1, and 173.2 are clearly assigned due to the presence of the C_3-unit of the flavone structure, C-3, C-2, and C-4 (C=O), respectively.

The HMQC and HMBC spectra showed that the flavonol molecule has a trimethoxylated A-ring (δ 97.1, 113.6, 140.6, 152.7, 153.9, 158.1) in which the alternative bond sequence, 5,6,7-trimethoxylated or 5,7,8-trimethoxylated, could not be determined. Comparison with the ^{13}C NMR data of the synthesized methoxyflavones [9] showed that the ^{13}C NMR spectrum of **5** was identical with that of 3,5,6,7,4′-pentamethoxyflavone.

Compound **6** was obtained as amorphous white powder. The ^{1}H NMR spectrum of **6** closely resembled those of **5**; however, the spectrum lacked one signal due to a methoxyl protons when compared with that of **5**. EI-MS supports this lack of a methyl group by showing an $[M]^+$ ion peak at *m/z* 357.9 where 14 mass units were subtracted from that of **5** (Table 1).

In the ^{13}C NMR spectrum of **6**, there were observed downfield shifts of 3′, 5′-C signals at δ 116.5 and of 2′, 6′-C at δ 130.7 in comparison with those of **5**. In contrast, the signals of 1′- and 4′-C at δ 122.1 and 161.3, respectively, were determined to be upfield shifted, suggesting that demethylation at the 4′-hydroxyl position of the B-ring occurred. In the HMBC spectrum of **6**, a long-range correlation was observed between four methyl protons at δ 4.04, 4.18, 3.97, 3.93 and four quaternary carbons at δ 59.6, 62.4, 61.4, 56.4. Thus, the structure of **6** was confirmed to be 4′-hydroxy-3,5,6,7-tetramethoxyflavone. It should be noted that compound **6**, formerly known as the synthesized flavonol, was first isolated from nature.

DPPH Radical-Scavenging and Antiproliferative Activities of the Isolated Compounds. Compounds **1**–**4**, **7**, and **8** were found to exhibit significantly good scavenging activity with inhibition percentage of 33.5, 63.5, 42.9, 47.9, 25.0, and 55.6 at a concentration of 100 μg/mL (Table 1).

The antiproliferative activities (IC_{50}, μg/mL) of the eight isolated 3-methoxyflavones against human leukemia (HL-60) cells are shown in Table 1. Most of the studied flavones inhibited significantly the proliferation of leukemia HL-60 cells in a concentration-dependent manner.

Flavones **2** and **8** showed significant inhibitory effect against human leukemia (HL-60) cells (IC_{50} < 25 μg/mL), whereas flavones **3–6** showed modest inhibitory effect (IC_{50} ≤ 60 μg/mL). Flavones **1** and **7** showed low inhibitory effect (IC_{50} > 100 μg/mL). The pronounced inhibitory effect of *V. iphionoides* extracts against human leukemia (HL-60) cells

in vitro, is possibly due to the synergistic antiproliferative effects of isolated flavones, because plant flavonoids are known to inhibit several biochemical events associated with cellular growth [10].

Although it is not possible to establish a general structure-activity relationship for antiproliferative activity of the isolated compounds, some trends can be observed from these results. All the isolated flavones tested in this study have the C2-C3 double bond with the 4-oxo functionality and 3-methoxyl group in the C ring; in addition, some of them possess polymethoxyl groups on the flavone structure, which are considered an essential functionality for antiproliferative activity [11–13]. The requirements for the A ring may be quite stringent. For example, an increase in the number of hydroxyl groups on the A ring and 7-OH in particular, increases the antiproliferative activity of the isolated flavones (**2**, **4** and **8** > **1**, **3**, **7** and **5**); this finding is consistent with results obtained previously that the IC_{50} of 7-hydroxyflavone against human leukemia HL-60 cells and HeLa cells was less than other hydroxyl group substitutions on the flavone structure [14, 15]. It seems to be that the 5′-OCH_3 substitution in the B ring of flavones possessing the 7-OH is essential for high activity (**2**). The existence of three hydroxyl groups, accompanied by more than one methoxyl group on the flavone structure (**2** and **8**), appeared to have an important role for the activity. For example, compound **4**, which has an almost similar structure as compounds **2** and **8** without the methoxyl group at C-6 or at C-5′, exerted an inhibitory effects almost half of theirs. For greater activity in 3-polymethoxylated flavones, it seems that 5- and 7-methoxyl are advantageous (**5** and **6**). Nevertheless, compound **1** has 5-OH, 4′-OH and 3′-, 3-OCH_3 groups that showed minor inhibitory effect against human leukemia (HL-60) cells, and the unexpected activity value obtained for this compound may be explained by its lower solubility in the test medium.

EXPERIMENTAL

The ^1H, ^{13}C NMR, DEPT, COSY, HMQC, and HMBC were measured at 600 and 150 MHz at 27°C in pyridine-d_5 on a JEOL FX-600 spectrometer using standard pulse sequences and referenced to residual solvent signals. The mass spectra (EI-MS) were taken on a ThermoFinnigan MAT 900 XL instrument (Germany) at 70 eV. Column chromatography (CC) was carried out on silica gel 60 (0.063–0.200 mm, Merck), Sephadex LH-20, and Fuji gel using ODS. Preparative and analytical TLC were carried out on silica gel 60 F_{254} (Merck). All solvents used for isolation and purification were of highest analytical grade, purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Extraction and Isolation. The ground sun-dried aerial parts of *V. iphionoides* (650 g) were extracted successively with hexane, ethyl acetate, and ethanol at 60°C until the refluxed solvent became colorless. The extracts were separately evaporated to dryness at 35°C in a rotary vacuum evaporator (Eyela rotary evaporator NE-series, Tokyo, Japan). The yields were 3.1% (w/w) in the hexane extract, 3.6% in the ethyl acetate extract, and 2.4% in the ethanol extract.

A portion (15 g) of the ethanol extract was dissolved in chloroform and applied to a normal CC packed with 300 g of silica gel using $CHCl_3$. The column was eluted using the following solvent system: $CHCl_3$ and ($CHCl_3$–CH_3OH–H_2O, 9:1:0.1) under gradient conditions. A total of 28 fractions (200 mL each) were collected. $CHCl_3$/($CHCl_3$–CH_3OH–H_2O) (9:1) – fr. 1–5, (8:2) – fr. 6–8, (7:3) – fr. 9–12, (5:5) – fr. 13–16, (3:7) – fr. 17–19, (2:8) – fr. 20–21, (1:9) – fr. 22–24, and (0:10) – fr. 25–28.

Fractions that showed similar components on thin layer chromatography (TLC) were combined. Fractions showing potential antiproliferative activities were further fractionated. The combined frs. 9–12 (2.6 g) were fractionated in column chromatography (CC) using benzene. Two hundred forty-five fractions were collected in 15 mL tubes, and the gradient solvent system used to eluate the column was benzene–acetone. These fractions, based on TLC, were combined as follows: fraction A (fr. 60–65), B (fr. 66–75), C (fr. 76–94), D (fr. 95–121), E (fr. 122–185), and F (fr. 186–245). The combined fraction A was passed through an ODS column and eluted with gradient solvents of water and methanol, started with (CH_3OH–H_2O) (1:1). After subsequent recrystallization from $CHCl_3$–CH_3OH (1:1), it afforded compounds **3** (kumatakenin, 40 mg) [16] and **1** (6 mg) [6, 16].

The combined fraction B (0.9 g) was subjected to open CC packed with 70 g silica gel and eluted with benzene (100%) and benzene–acetone (90:1, 14:1, and 9:2). After further recrystallization from $CHCl_3$–CH_3OH (1:1), it afforded pure compounds **7** (16 mg) [16] and **5** (10 mg) [9, 17].

The combined frs. 13–16 (2.3 g) were fractionated in CC using a gradient solvent system of benzene–acetone. A total of 250 fractions (15 mL each) was collected. These fractions, based on TLC, were combined as follows: fraction A2 (fractions 85–125) after recrystallization from $CHCl_3$–CH_3OH (1:1) afforded pure compound **8** (50 mg) [6, 18], B2 (frs. 126–137) after recrystallization from $CHCl_3$–CH_3OH (1:1) afforded pure compound **2** (21 mg) [6, 16], D2 (frs. 158–169) afforded pure

compound **6** (22 mg) [19] after subsequent recrystallization from $CHCl_3$–CH_3OH (1:1), E2 (fr. 170–207), and F2 (fr. 208–255).

The combined frs. 17–19 (0.8 g) were fractionated in open CC using a gradient solvent system of benzene–acetone. A total of 57 fractions (10 mL each) was collected. Based on TLC, the fractions were combined as follows: fr. A3 (fr. 1–20), B3 (fr. 21–37), and C3 (fr. 38–57). Fraction B3 was subjected to preparative TLC eluting with $CHCl_3$–CH_3OH–H_2O (9:1:0.1), and after subsequent recrystallization from $CHCl_3$–CH_3OH (1:1) afforded pure compound **4** (70 mg) [16].

DPPH Free-Radical-Scavenging Assay. DPPH (1,1-diphenyl-2-picrylhydrazyl) was used to determine the free-radical scavenging activity of the extracts, fractions, and the isolated compounds at a concentration of 100 μg/mL using the Blois method [20]. The absorbance of the extracts, fractions, isolated compounds, standard, and control were measured at 517 nm after 30 min.

The scavenging activity of the extracts was calculated as follows:

$$\% \text{ DPPH radical scavenging} = \frac{\text{C.a. - S.a.}}{\text{C.a.}} \times 100,$$

where C.a. is control absorbance, S.a. is sample absorbance.

Determination of Cytotoxic Activity. Human leukemia (HL-60) cell lines are a valid model and are widely used to determine general antitumor activity [21, 22]. Human leukemia (HL-60) cells were obtained from Cancer Cell Repository (Tohoku University, Japan). The cell lines were grown and maintained in a humidified incubator at 37°C and in 5% CO_2 atmosphere in RPMI 1640 medium containing 100 g/L FBS (fetal bovine serum) and 10 g/L PSG (penicillin–streptomycin–glutamine).

The MTT colorimetric assay Hou method [23], which is based on the reduction of MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase to a purple formazan product, was used to assess the antiproliferative action of *V. iphionoides* extracts and isolated flavonoids in human leukemia HL-60 cells. The cells were suspended at a density of 2×10^4 cells/mL in RPMI 1640 medium containing 100 g/L FBS and 10 g/L PSG, then 100 μL of each was plated into each well of 96-well microtiter plates and incubated for 48 h. Different *V. iphionoides* extracts and isolated compounds were added to the wells to final concentrations of 25, 50, 100, and 200 μg/mL and the whole was incubated at 37°C, 5% CO_2 for 48 h. Two controls were used: one control contained the medium and cells with sterilized water, and the other contained the extracts and medium without cells to check the effect of each extract color. 10 μL (0.5 mg/mL) of MTT solution was then added to each well and incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 100 μL of 0.04 N HCl-isopropanol solution to each well, followed by mixing with a micropipette and measuring the absorbance at 595 nm using a microplate reader (Bio-Rad, Model 550, USA).

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