Evaluation of Cytotoxic Compounds from *Calligonum comosum* L. Growing in Egypt

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Calligonum comosum (Polygonaceae), an Egyptian desert plant, was extracted and fractionated using petroleum ether, methylene chloride, and ethyl acetate. The total methanolic extract and other fractions were tested for their anticancer activity using Ehrlich ascites, brine shrimp and antioxidant assays. Ethyl acetate fraction proved to be the most active in all assays. Eight compounds were isolated, purified, and identified from this fraction as (+)-catechin (**1**), dehydrodicatechin A (**2**), kaempferol-3-*O*-rhamnopyranoside (**3**), quercitrin (quercetin-3-*O*-rhamnopyranoside) (**4**), β-sitosterol-3-*O*-glucoside (**5**), isoquercitrin (quercetin-3-*O*-glucopyranoside) (**6**), kaempferol-3-*O*-glucuronide (**7**), and mequilianin (quercetin-3-*O*-glucuronide) (**8**). All isolated compounds were tested for their cytotoxicity and antioxidant activity. Compound **2** showed the best cytotoxic and antioxidant activity.

Key words: Calligonum, Anticancer, Ehrlich Ascites, Dehydrodicatechin

Introduction

Plants have been the basis of sophisticated medical systems for thousands of years and they have an essential role in healthcare. The World Health Organization has estimated that 80% of the earth's inhabitants rely on traditional medicines for primary healthcare, and the plant products are highly important in the remaining 20% of the population. Natural products have made an enormous impact on the discovery of anticancer compounds. In fact, possibly 60% of all cancer drugs that are used clinically are either natural products or owe their origin to a natural source (Heinrich *et al*., 2004). *Calligonum comosum* (Polygonaceae) is a small leafless shrub, which has reputation in folklore medicine as a stimulant and astringent, under the local names "ghardaq", "rusah" or "arta" (Muschler, 1912). It was reported that *Calligonum comosum* possesses anti-ulcer and anti-inflammatory activity (Liu *et al.*, 2001). Besides, anthraquinones of *Calligonum* showed high antimicrobial potential (Zaki *et al.*, 1984). This study intended to identify and evaluate the anticancer compounds of *Calligonum comosum* using bioguided-fractionation assays.

Results and Discussion

Petroleum ether, methylene chloride, ethyl acetate, and total methanolic extracts were subjected to a battery of biological assays included brine shrimp and antioxidant assays. Ethyl acetate fraction showed the highest activity in comparison to the other tested fractions. Subsequently, ethyl acetate fraction was further purified and fractionated on a silica gel column to afford eight compounds (Fig. 1): (+)-catechin (**1**), dehydrodicatechin A (**2**), kaempferol-3-*O*-rhamnopyranoside (**3**), quercitrin (quercetin-3-*O*-rhamnopyranoside) (**4**), β-sitosterol-3-*O*-glucoside (**5**), isoquercitrin (quercetin-3-*O*-glucopyranoside) (**6**), kaempferol-3-*O*-glucuronide (**7**), and mequilianin (quercetin-3-*O*-glucuronide) (**8**).

The reliable criteria for judging the value of any anticancer drug in the Ehrlich ascites assay are decreasing the number of viable cells of the ascetic fluid as well as decreasing of WBCs from blood. The results of the present study showed an antitumour effect of *Calligonum comosum* extract (50 mg/kg) against EAC in Swiss albino mice. The total WBC count of the treated mice was found to decrease (nearly normal) with an increase in the Hb content and RBC count (Table I). In addition, ALT value was improved and the results were compared with fluorauracil (5-FU, 20 mg/kg).

From Table II it is clear that ethyl acetate fraction exhibited greater cytotoxic activity in the brine shrimp assay due to a low LC_{50} value than the methanol and total methanolic extract.

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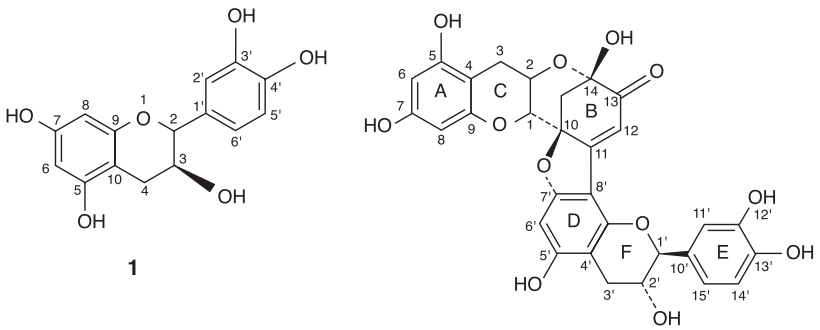

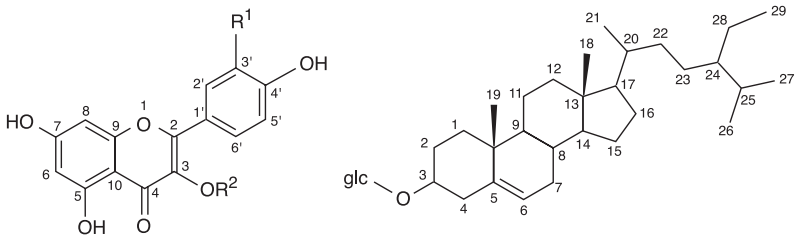

	R^1	R^2
3	H	rhamnose
4	OH	rhamnose
6	OH	glucose
7	H	glucuronic acid
8	OH	glucuronic acid

Fig. 1. Compounds isolated from the biologically active fraction (ethyl acetate) of *Calligonum comosum* L. herb.

Table I. Effect of total methanolic extract of *Calligonum comosum* and 5-fluorouracil on viable cells count and hematological and biochemical parameters.

Parameter	Normal	Control (Ehrlich only)	Total (50 mg/kg)	5-Fluorouracil (20 mg/kg)
Viable cells [count/100 μl]	–	192.8×10^6	99.2×10^6	83.6×10^6
Hb [g%][a]	13.4	5.7	11.1	15.6
RBCs [10^6 mm^{-3}][b]	5.1	3.2	6.0	5.9
Total WBCs [10^3 mm^{-3}][c]	4.4	47.4	9.5	14.7
ALT $\times$ 0.1 [IU/l][d]	3.7	14.4	6.7	5.2

[a] Hemoglobin. [b] Red blood corpuscles. [c] White blood corpuscles. [d] Alanine aminotransferase.

Both petroleum ether and methylene chloride fractions showed weak cytotoxic activities. In the erythrocyte hemolysis assay, both total extract and other fractions showed nearly equal results of complete hemolysis of blood erythrocytes with distilled water (absorbance about 0.8 nm). Ethyl acetate fraction exhibited the best activity.

Table II. Results of brine shrimp lethality assay of total methanolic extract and different fractions.

Fraction	LC_{50} [mg/ml]
Total methanolic extract	56.1
Petroleum ether	174.8
Methylene chloride	147.5
Ethyl acetate	41.8
Methanol	53.2

Table III. Results of brine shrimp lethality assay of isolated compounds.

Compound	LC_{50} [mg/ml]
1	61.6
2	35.1
3	80.1
4	145.3
5	261.0
6	105.5
7	106.6
8	103.8

Table IV. Results of ABTS antioxidant activity screening assay.

Compound	Average absorbance[a]	Average inhibition (%)
Control	0.7	0.0
Ascorbic acid (2%)	0.14	80.0
Total methanolic extract	0.21	70.0
Ethyl acetate fraction	0.16	77.14
1	0.2	71.43
2	0.30	57.14
3	0.49	30.0
4	0.22	68.57
5	0.69	1.43
6	0.19	72.86
7	0.50	28.57
8	0.40	42.86

[a] Absorbance of test solution at λ_{max} = 734 nm.

From Table III it is clear that compound **2** had the highest cytotoxic activity of the isolated compounds to brine shrimp nauplii. Both compounds **1** and **3** possessed remarkable activities. Compounds **4**, **6**, **7** and **8** exhibited moderate activitiy. Compound **5** retained the lowest activity among all tested compounds.

From Table IV it was found that the ABTS antioxidant activity of the ethyl acetate fraction was greater than that of individual isolated compounds at the same concentration, suggesting a synergism between the different compounds. Compounds **1**, **2**, **4** and **6** showed remarkable activities. Compound **5** showed no activity (1.4%) compared to ascorbic acid (80% inhibition). Total methanolic extract showed significant reduction of viable tumour cells.

The full ^{13}C NMR data for all compounds are presented in Table V.

Compound **1** was isolated as a yellowish brown powder (130 mg), m.p. 150 °C, soluble in methanol, sparingly soluble in chloroform and insoluble in petroleum ether. Its UV spectrum in methanol indicated that it has a dihydroflavonol nucleus (Markham, 1982); ^1H NMR , ^{13}C NMR, EI-mass spectra confirmed the identity of compound **1** as (+)-catechin.

Compound **2** was isolated as yellow needles (76 mg), m.p. 300 °C, soluble in methanol, sparingly soluble in chloroform and insoluble in petroleum ether.

Table V. ^{13}C NMR data (500 MHz) for compounds **1**, **3**, **4**, **6**, **7**, **8**.

C #	**1** (DMSO)	**3** (CD_3OD)	**4** (CD_3OD)	**6** (CD_3OD)	**7** (DMSO)	**8** (CD_3OD)
2	81.5	157.9	157.1	157.6	157.4	157.8
3	66.7	134.8	134.8	134.3	134.3	134.4
4	28.3	178.2	178.2	178.0	177.7	178.1
5	156.6	161.8	161.7	161.7	161.0	161.6
6	95.72	98.5	98.5	102.9	99.8	98.6
7	156.8	164.6	164.5	165.4	165.5	164.7
8	94.2	93.4	93.4	93.5	94.5	93.4
9	155.8	157.1	157.9	157.2	156.8	157.1
10	99.6	104.5	104.5	104.2	103.9	104.3
1′	131.1	121.2	121.6	121.7	120.4	131.1
2′	114.9	130.5	115.0	114.7	131.5	114.8
3′	145.3	115.1	145.0	144.6	115.9	144.5
4′	145.3	160.2	148.4	148.6	160.1	148.5
5′	115.6	115.1	115.6	116.2	115.9	116.7
6′	119.0	130.5	121.6	121.9	131.5	121.4
1″		102.1	102.1	103.1	102.8	102.9
2″		70.7	70.70	74.4	72.0	72.0
3″		70.7	70.75	76.8	73.6	74.2
4″		71.8	71.9	69.9	76.3	76.4
5″		70.5	70.5	77.0	76.7	76.7
6″		16.3	16.3	61.2	173.8	174.9

The spectroscopic data were in full agreement with those reported for dehydrodicatechin A (Shimomura *et al*., 1989). It is reported here for the first time from Polygonaceae .

Compound **3** was isolated as yellow needles (45 mg), soluble in methanol, sparingly soluble in chloroform and insoluble in petroleum ether. The spectroscopic data showed that compound **3** is a flavonol glycoside with free 5-, 7-, 4′-hydroxy groups (Mabry *et al*., 1970; Agrawal, 1989). Compond **3** was found to be kaempferol-3-*O*-α-L-rhamnopyranoside. It is reported here for the fist time from *Calligonum.*

Compound **4** was isolated as yellow needles (80 mg), soluble in methanol, sparingly soluble in chloroform and insoluble in petroleum ether. The data indicated that compound **4** has a 3-OH substituted flavonol nucleous (Markham, 1982). The spectral data of compound **4** confirmed its identity as quercitrin (quercetin-3-*O*-α-rhamnopyranoside). It is reported here for the fist time from *Calligonum.*

Compound **5** was isolated as a white amorphous powder (120 mg), m.p. 294–296 °C. It is odorless. It is sparingly soluble in diethyl ether, chloroform, ethyl acetate and methanol. Compond **5** was identified as β-sitosterol-3-*O*-glucoside.

Compound **6** was isolated as yellow crystals (20 mg), m.p. 220–222 °C, soluble in methanol, slightly soluble in ethyl acetate and insoluble in chloroform, diethyl ether and benzene. Compound **6** was identified as isoquercitrin (quercetin-3-*O*-glucopyranoside) which has been previously isolated from the herb of *Calligonum comosum* L. (El-Sayyad and Wagner, 1978).

Compound **7** was isolated as a yellow amorphous powder (23 mg), soluble in methanol, sparingly soluble in ethyl acetate insoluble in chloroform and petroleum ether. EI-mass spectra showed $[M]^+$ at *m/z* 462, suggesting the molecular formula $C_{21}H_{18}O_{12}$, and the main fragment at 286, suggesting the aglycone $C_{15}H_{10}O_6$ The data suggested that compound **7** is kaempferol-3-*O*-glucuronide which has been previously isolated from the herb of *Calligonum comosum* L. (El-Sayyad and Wagner, 1978).

Compound **8** was isolated as a yellow amorphous powder (25 mg), soluble in methanol, sparingly soluble in ethyl acetate insoluble in chloroform and petroleum ether. The combined data confirmed the identity of compound **8** as mequilianin (quercetin-3-*O*-glucuronide) which is reported here for the first time from the genus *Calligonum*, but it was reported from other plants of the Polygonaceae family such as *Polygonum viviparum* and *Eriogonum nudum* (Wagner *et al.,* 1970).

Materials and Methods

The brine shrimp assay was performed according to Solis *et al.* (1993) and the ABTS assay according to Lissi *et al*. (1999).

Ehrlich ascites (Fujita *et al*., 1988).

Animals were divided into groups (7 each) as follows: (1) tumour-bearing mice, (2) tumour-bearing mice treated for the first 9 d, and (3) control mice (normal).

Ehrlich ascites carcinoma cells, 2×10^6 cells/mouse, were inoculated intraperitoneally (i. p.) into experimental animals. From 24 h after inoculation, each sample at a given amount, as a solution in saline, was injected into mice i. p. once a day for 9 consecutive days. The control group was treated with the same volume of 0.9% sodium chloride solution. The mice were observed for 30 d. The activity was assessed using peritoneal cell count and hematological studies.

Plant material

The plant (*Calligonum comosum* L.) was collected by Dr. Mohamed R. Akl from deserts near Rosetta (Rashid), Egypt, on May 3, 2006 in the afternoon (during flowering stage). The authenticity of the collected plant was confirmed by Prof. Dr. Ibrahim Mashaly, Faculty of Science, Mansoura University, Egypt. The freshly collected plant material was air-dried in shade at room temperature. The aerial parts (stem, flowers, and leaves) of this plant were used for investigations.

Extraction

The aerial parts of *Calligonum comosum* (powdered, 7 kg) were extracted by maceration in a glass jar with cold distilled methanol (7×10 l). The combined methanolic extract was concentrated to a syrupy consistency under reduced pressure and then allowed to dry in a desiccator over anhydrous $CaCl_2$ to a constant weight (1.3 kg). The dried alcoholic extract was dissolved in a small amount of methanol, then diluted with the same volume of distilled water in a separating funnel. Using the liquid-liquid partitioning method, it was extracted

successively till exhaustion with petroleum ether, methylene chloride and ethyl acetate. The extracts, in each case, were evaporated to dryness under reduced pressure and kept for further investigation. The obtained fractions were weighed to obtain the petroleum ether fraction (250 g), methylene chloride fraction (50 g) and ethyl acetate fraction (70 g) and finally the methanol fraction (remaining aqueous mother liquor) (930 g).

Isolation and identification

Only the ethyl acetate fraction was investigated as explained before. The ethyl acetate extract (70 g) was applied onto the top of a glass column (60 × 5 cm i. d.) previously packed with silica gel (350 g; 70–230 mesh; Machery-Nagel) in ethyl acetate. The extract was gradiently eluted with ethyl acetate containing an increasing proportion of methanol. The effluent was collected in 200 ml fractions. Each fraction was concentrated to a small volume and monitored by TLC. Fractions 1–18 (F_A) revealed the presence of four spots (compounds **1**, **2**, **3**, **4**). Fractions 19–32 (F_B) revealed the presence of two spots (compounds **5**, **6**). Fractions 33–50 (F_C) revealed the presence of two spots but were not investigated due to poor yield. Fractions 51–90 (F_D) revealed the presence of two spots (compounds **7**, **8**).

The full detailed methods for purification, isolation, and identification will be provided upon request.

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