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Research Article

Flavonoids from *Leucanthemopsis trifurcatum* Leaves and their Cytotoxic Activity

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ABSTRACT
A radical scavenging guided phytochemical study on the leaf of *Leucanthemopsis trifurcatum* afforded twelve flavonoids (1-12). The structures of the isolated compounds were elucidated on the basis of spectroscopic evidences and correlated with known compounds. The cytotoxic activity of the isolated compounds was evaluated against human Hela and MCF-7 cell lines. Compound 1 displays the strongest cytotoxic activity with IC_{50} values of 10.3 (Hela) and 12.2 μM (MCF-7).

Keywords: *Leucanthemopsis trifurcatum*; *Chrysanthemum macrocephalum*; Flavonoids; cytotoxicity.

INTRODUCTION

The genus *Leucanthemopsis* belongs to the family Asteraceae, with only 6 species widespread mainly in Southwestern Europe, and North Africa[1,2]. *Leucanthemopsis trifurcatum* (Desf.) Alavi. is a sub-shrub distributed mainly in Tunisia, Morocco, Algeria and Libya and is known by the following synonyms: *Chrysanthemum trifurcatum* Desf.; *Chrysanthemum macrocephalum* Viv.; and *Pyrethrum trifurcatum* (Desf.) Willd[3]. Previous phytochemical reports of the genus *Leucanthemopsis* revealed the isolation and identification of phloroglucinol derivatives and sesquiterpenoid lactones[1,2,4,5]. To the best of our knowledge, no hits were reported about the chemical constituents of *L. trifurcatum* which encourage us to investigate its active constituents and potential biological activities. We report here the isolation and identification of 12 flavonoids[1-12], which were isolated for the first time from this plant. The structures of these compounds were deduced by comparison of their spectral data with those reported in the literature. In our continuous interest to search for drug leads from natural sources, we had the opportunity to work on the leaves of *L. trifurcatum* to investigate its chemical constituents and their potential biological activities.

MATERIALS AND METHODS

Apparatus and Chemicals

UV spectra were obtained on a Cary 50 spectrophotometer, Varian, Inc. NMR spectra were recorded at 23 °C with a Varian Inova 600 MHz NMR spectrometer. Column chromatography (CC) was performed using a silica gel (Kieselgel 60 Å, 40-63 µM mesh size, Fluorochem, UK). RP-HPLC were carried out using Phenomenex Luna C18 (2) (250 × 4.6 mm) (5 μm) on Shimadzu HPLC-LC-20 AD series binary gradient pump with Shimadzu SPD-M20A detector (Tokyo, Japan). All flash chromatography was performed on Sepacore Flash Chromatography System, Buchi Labortechnik AG, Netherlands. TLC was done using pre-coated silica-gel 60 F254 (0.25 mm, ALUGRAM® SIL G/UV254, Macherey-Nagel, Germany) and RP-18 F254 plates (0.25 mm, Merck, Germany).

Plant material

The plant was collected at the end of April 2010 from the northeastern region of Libya. The plant material was identified by the members of Plant Taxonomy Department, College of Science, Assiut University.

Extraction and isolation

400 g of the air-dried powdered leaves were extracted by maceration (72 h x 3) with 70% EtOH till complete exhaustion (2L x 3). The alcoholic extract was concentrated and the solvent free residue (29 g, 7.3%) was mixed with 500 mL of distilled H_2O, and subjected to successive solvent fractionation with *n*-hexane and chloroform till complete exhaustion in each case to give *n*-hexane fraction (7.3 g), chloroform fraction (5.5 g), and aqueous fraction (13.8 g). The chloroform fraction was subjected to flash chromatography on silica gel column using $CHCl_3$–MeOH mixtures in a manner of increasing polarities. Thirty-two fractions (20 mL each) were collected and monitored on TLC (silica gel) using $CHCl_3$–

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Table 1: ^{1}H NMR spectroscopic data of compounds 1-12 in DMSO-d_6 (600 MHz).

S. No.	4 δ^{1}H/ppm, mult. (*J*)	5 δ^{13}C/pp, mult. (*J*)	6 δ^{13}C/ppm, mult. (*J*)	7 δ^{13}C/pp m, mult. (*J*)	8 δ^{1}H/ppm , mult. (*J*)	9 δ^{13}C/pp m, mult. (*J*)	10 δ^{13}C/ppm, mult. (*J*)	11 δ^{13}C/ppm, mult. (*J*)	12 δ^{13}C/ppm , mult. (*J*)
3	6.87, s	6.86, s	6.79, s	-	-	-	-	6.74, s	6.74, s
6	6.46, brs	6.46, brs	6.46, brs	6.18, brs	6.19, brs	6.20, brs	6.20, brs	6.46, brs	6.47, brs
8	6.84, brs	6.90, brs	6.92, brs	6.38, brs	6.36, brs	6.40, brs	6.42, brs	6.74, brs	6.80, brs
2′	7.97, d (8.7)	7.44, brs	7.44, brs	7.76, d (8.4)	7.77, d (8.6)	7.75, d (8.4)	8.05, d (8.0)	7.44, brs	7.46, brs
3′	6.95, d (8.7)	-	-	6.93, d (8.4)	6.92, d (8.6)	6.91, d (8.4)	6.89, d (8.0)	-	-
5′	6.95, d (8.7)	6.93, d (8.5)	6.92, d (8.7)	6.93, d (8.4)	6.92, d (8.6)	6.91, d (8.4)	6.89, d (8.0)	6.92, d (8.7)	6.93, d (8.5)
6′	7.97, d (8.7)	7.58, d (8.5)	7.50, d (8.7)	7.76, d (8.4)	7.77, d (8.6)	7.75, d (8.4)	8.05, d (8.0)	7.46, d (8.7)	7.48, d (8.5)
3′-OCH$_3$	-	3.85, s	-	-	-	-	-	-	-
Sugar I	*Glucose*	*Glucose*	*Glucose*	*Rhamnose*	*Glucose*	*Rhamnose*	*Glucose*	*Glucose*	*Glucose*
1″	5.06, d (7.4)	5.03, d (7.4)	5.01, d (7.4)	5.30, brs	5.02, d (7.4)	5.30, brs	5.45, d (7.4)	5.10, d (7.4)	5.10, d (7.3)
2″	3.27, m	3.25, m	3.25, m	4.01, m	3.26, m	3.99, m	3.25, m	3.60, m	3.65, m
3″	3.30, m	3.31, m	3.31, m	3.15, m	3.31, m	3.14, m	3.23, m	3.47, m	3.44, m
4″	3.19, m	3.20, m	3.20, m	3.12, m	3.21, m	3.11, m	3.11, m	3.38, m	3.35, m
5″	3.44, m	3.42, m	3.42, m	3.48, m	3.400, m	3.49, m	3.12, m	3.41, m	3.40, m
6″	a. 3.73, d (11.5) b. 3.48, m	a. 3.72, d (11.5) b. 3.50, m	a. 3.73, d (11.5) b. 3.51, m	0.81, d (6.1)	a. 3.70, d (11.5) b. 3.51, m	0.81, d (6.0)	a. 3.57, d (11.6) b. 3.35, m	a. 3.84, d (11.6) b. 3.49, m	a. 4.20, d (11.2) b. 3.68, m
Sugar II								*Rhamnose*	*Glucose*
1‴	-	-	-	-	-	-	-	4.56, brs	3.96, d (9.0)
2‴	-	-	-	-	-	-	-	3.65, m	3.67, m
3‴	-	-	-	-	-	-	-	3.44, m	3.45, m
4‴	-	-	-	-	-	-	-	3.39, m	3.33, m
5‴	-	-	-	-	-	-	-	3.49, m	3.37, m
6‴	-	-	-	-	-	-	-	1.08, d (6.1)	a. 3.69, m b. 3.31, m
5-OH	12.96, brs	12.96, brs	12.98, brs	12.41, brs	12.54, brs	-	-	13.00, brs	13.00, brs

MeOH (90:10), $CHCl_3$–MeOH–H_2O (80:20:2) and (70:30:3) as solvent systems and 10% v/v H_2SO_4 in EtOH and/or 0.2% DPPH in MeOH as spraying agents. Similar fractions on TLC were combined to yield 8 groups. The selected groups were subjected to HPLC Phenomenex Luna C18 (2) (250 × 4.6 mm) (5 μm) using a gradient of 10–100% CH_3CN–H_2O over 30 min to give 3 compounds. Identified compounds are (1) (12.7 mg) from group 3; (2) (16.1 mg) from group 4; and (3) (9.5 mg) from group 6. The aqueous fraction (13.8 g) was subjected to Diaion-HP_{20} CC using H_2O and MeOH (4 L each). The methanolic elute was concentrated under reduced pressure to yield a fraction (8.6 g). The methanolic fraction was subjected to flash chromatography on silica gel column using $CHCl_3$–MeOH mixtures in a manner of increasing polarities. Fifty-eight fractions (20 mL each) were collected and monitored on TLC (silica gel) using $CHCl_3$–MeOH–H_2O (80:20:2), (70:30:3) and (55:40:5), as well as on RP-C18 using H_2O–MeOH (70:30), (50:50) and (30:70) as solvent systems and 20% v/v H_2SO_4 in EtOH and/or 0.2% DPPH in MeOH as spraying reagents. Similar fractions on TLC were combined to yield eleven groups. Groups (3, 6, 7 and 9) were subjected to HPLC (Phenomenex Luna C18 (2), 5 μm, 4.6 x 250 mm) using a gradient of 10–70% CH_3CN–H_2O over 35 min to give nine compounds. Identified compounds are (4) (7.2 mg) from group 3; (5) (4.3 mg) and

Table 2: ^{13}C NMR spectroscopic data of compounds 4-7 and 9-12 in DMSO-d_6 (150 MHz).

S. No.	4 $\delta^{13}C$/ppm, mult.	5 $\delta^{13}C$/ppm, mult.	6 $\delta^{13}C$/ppm, mult.	7 $\delta^{13}C$/ppm, mult.	9 $\delta^{13}C$/ppm, mult.	10 $\delta^{13}C$/ppm, mult.	11 $\delta^{13}C$/ppm, mult.	12 $\delta^{13}C$/ppm, mult.
2	164.3, s	164.3, s	164.3, s	161.7, s	161.7, s	161.1, s	164.1, s	165.0, s
3	103.1, d	103.2, d	103.1, d	134.5, s	134.6, s	133.2, s	103.7, d	103.5, d
4	182.1, s	182.5, s	182.3, s	178.0, s	178.1, s	177.3, s	182.3, s	182.3, s
5	161.1, s	161.3, s	161.3, s	157.4, s	157.6, s	156.4, s	161.7, s	161.6, s
6	99.9, d	100.1, d	100.0, d	99.4, d	99.3, d	98.8, d	100.2, d	100.1, d
7	163.0, s	163.1, s	163.1, s	160.4, s	160.4, s	164.8, s	163.3, s	163.4, s
8	94.9, d	95.1, d	94.8, d	94.9, d	94.2, d	93.7, d	95.2, d	95.2, d
9	157.0, s	157.1, s	157.0, s	157.0, s	157.0, s	156.1, s	157.4, s	157.5, s
10	105.4, s	105.5, s	105.2, s	105.6, s	104.4, s	103.7, s	105.9, s	105.9, s
1′	121.0, s	121.3, s	122.0, s	121.0, s	121.0, s	120.9, s	121.9, s	121.9, s
2′	128.7, d	111.2, d	113.5, d	131.0, d	131.0, d	130.7, d	114.1, d	114.1, d
3′	116.0, d	152.2, s	145.9, s	115.8, d	115.9, d	115.0, d	146.2, s	146.2, s
4′	161.5, s	148.5, s	146.5, s	161.4, s	160.4, s	159.9, s	150.4, s	150.4, s
5′	116.0, d	116.0, d	116.0, d	115.8, d	115.9, d	115.0, d	116.5, d	116.5, d
6′	128.7, d	121.8, d	121.8, d	131.0, d	131.0, d	130.7, d	119.7, d	119.7, d
3′-OCH_3	-	56.2, q	-	-	-	-	-	-
Sugar I	*Glucose*	*Glucose*	*Glucose*	*Rhamnose*	*Rhamnose*	*Glucose*	*Glucose*	*Glucose*
1″	100.0, d	100.0, d	100.0, d	102.2, d	102.2, d	101.0, d	100.1, d	101.0, d
2″	73.1, d	72.9, d	73.0, d	71.0, d	71.0, d	73.3, d	73.6, d	72.9, d
3″	76.5, d	76.7, d	76.3, d	70.8, d	70.8, d	76.4, d	76.8, d	76.6, d
4″	69.5, d	70.0, d	69.6, d	71.5, d	71.6, d	69.9, d	70.7, d	71.0, d
5″	77.2, d	77.5, d	77.2, d	70.5, d	70.5, d	77.3, d	77.6, d	76.6, d
6″	60.6, t	60.5, t	60.6, t	17.9, q	17.9, q	60.9, t	68.8, t	68.1, t
Sugar II							*Rhamnose*	*Glucose*
1‴	-	-	-	-	-	-	100.4, d	103.5, d
2‴	-	-	-	-	-	-	71.4, d	73.5, d
3‴	-	-	-	-	-	-	71.2, d	76.1, d
4‴	-	-	-	-	-	-	72.5, d	67.6, d
5‴	-	-	-	-	-	-	70.8, d	76.1, d
6‴	-	-	-	-	-	-	18.3, q	65.0, t

Table 3: Cytotoxic activity of isolated compounds (1-12) against different cell lines.

Compound/extracts	IC_{50} (μM)	
	Hela	MCF-7
1	10.3	12.2
2	15.7	20.0
3	23.1	29.4
4	39.2	45.1
5	53.3	66.5
6	46.6	58.2
7	61.1	88.0
8	56.4	73.7
9	74.9	>100
10	69.2	>100
11	>100	>100
12	>100	>100
Doxorubicin	1.1	1.65

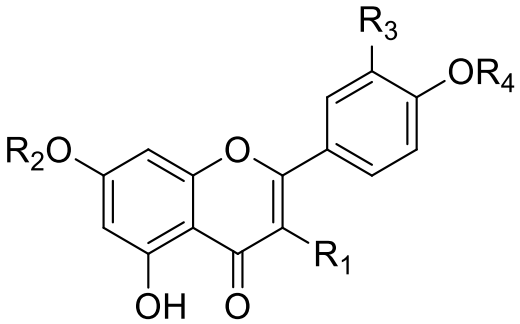
(6) (18.8 mg) from group 6; (7) (5.5 mg) and (8) (2.1 mg) from group 7; (9) (7.5 mg) and (10) (14.7 mg) from group 8; and finally (11) (8.3 mg) and (12) (21.2 mg) from group 10.

DPPH Radical Scavenging Assay

Radical scavenging activity of the isolated compounds against DPPH* was performed with a rapid TLC screening method using 0.2% DPPH in MeOH. 30 min after spraying, the active compounds appear as yellow spots against purple background[6].

Cytotoxicity assays

The *in vitro* cancer growth inhibitory activity of the isolated compounds was determined using MTT colorimetric assay against HeLa (human cervix carcinoma) and MCF-7 (human breast adenocarcinoma) cell lines. MCF-7 and Hela Cells was obtained from ATCC (American Type Culture Collection) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose (Sigma–Aldrich), supplemented with 5% fetal bovine serum (Gibco) and 1% of a penicillin–streptomycin mixture (10.000 UI/ml and 10 mg/ml, respectively)[16]. Cells were maintained at 37 ºC in 5% CO_2 and 98% humidity. The cytotoxicity was determined based on the cell viability and measured by the colorimetric MTT [3-



1 R_1=H, R_2=H, R_3=H, R_4=H
2 R_1=H, R_2=H, R_3=OH, R_4=H
3 R_1=OH, R_2=H, R_3=OH, R_4=H
4 R_1=H, R_2=Glc, R_3=H, R_4=H
5 R_1=H, R_2=Glc, R_3=OCH_3, R_4=H
6 R_1=H, R_2=Glc, R_3=OH, R_4=H
7 R_1=OH, R_2=Rha, R_3=H, R_4=H
8 R_1=OH, R_2=Glc, R_3=H, R_4=H
9 R_1=ORha, R_2=H, R_3=H, R_4=H
10 R_1=OGlc, R_2=H, R_3=H, R_4=H
11 R_1=H, R_2=Glc (6-1) Rha, R_3=OH, R_4=H
12 R_1=H, R_2=Glc (6-1) Glc, R_3=OH, R_4=H

Figure 1: Chemical Structure of the isolated compounds (1-12).

(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. The next day, drugs pre-dissolved in DMSO at different concentration were added to the culture. Untreated and blank groups were set as controls. After 48-hour exposure, the MTT solution (5 mg/ml in phosphate buffered saline, PBS) was added to the wells and the plates were incubated for an additional 4 h in a CO_2 incubator at 37 ºC, then the supernatant was discarded, each well was added 150 µL of DMSO. The absorbance was measured at 570 nm in a plate reader (Bio-Tek, USA). The% cell inhibition was determined using the following formula[7-9].

Percentage cell inhibition = 100− Abs (Sample) / Abs (Control) x 100

RESULTS AND DISCUSSION

The phytochemical investigations of the leaf extract lead to isolation and identification of 12 known flavonoids (1-12) (Fig. 1). The flavonoids were identified as apigenin (1)[10], luteolin (2)[11], quercetin (3)[12], apigenin-7-*O*-β-D-glucopyranoside (4)[13], chrysoeriol-7-*O*-β-D-glucoside (5)[14-16], luteolin-7-*O*-β-D-glucoside (6)[17], Kaempferol-7-*O*-α-L-rhamnopyranoside (7)[18], Kaempferol-7-*O*-β-D-glucoside (8)[19], Kaempferol-3-*O*-α-L-rhamnopyranoside (9)[20], Kaempferol-3-*O*-β-D-glucoside (10)[21], luteolin-7-*O*-rutinoside (11)[22,23], and luteolin-7-*O*-[β-D-Glucopyranosyl-(1→6)-β-D-glucopyranoside] (luteolin-7-*O*-gentiobioside) (12)[24]. All physical and spectral data (Table 2) of these compounds were in agreement with the respective published data. The antiproliferative effects for the isolated compounds were evaluated using MTT colorimetric assay[9,25,26] on human Hela and MCF-7 (breast) cell lines using doxorubicin as a positive control. As shown in Table 3, the isolated non-glycosidic flavonoids exhibited more cytotoxicity than the glycosidic forms. The highest inhibitory activity was observed for compound 1 (apigenin) against Hela (10.3 μM) and MCF-7 (12.2 μM) cell lines. Thus, Luteolin can be considered as promising candidate agent for treatment of cancer. Generally, flavonoids have reported growth inhibitory effects on different kinds of cancer cells. Flavonoids can penetrate *in vitro* cultured cells, and modulate the cellular metabolic activities in addition to reduction of oxidative damage, induction of cell cycle arrest and apoptosis, inhibition of proliferation, promotion of differentiation, inactivation of carcinogens, suppression of metastasis and impairment of tumour angiogenesis[9,27-29].

CONCLUSIONS

In this paper, twelve flavonoids were isolated from the leaf extracts of *Leucanthemopsis trifurcatum* and recorded for the first time. The structural determination was accomplished using extensive NMR as well as HRESIMS analysis. The isolated compounds were evaluated for them *in vitro* growth inhibitory activity against different cell lines. Flavonoids free aglycones (1-3) showed promising cytotoxic activity and the highest activity was observed with compound 1 on Hela and MCF-7 cell lines.

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