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Cytotoxic Investigation of *Cynara sibthorpiana*

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Bioassay guided fractionation of the different extracts of *Cynara sibthorpiana* against L1210 mouse leukemia cell line, led to the isolation of twelve phenolic compounds identified as caffeic acid **1**, esculetin **2**, apigenin **3**, luteolin **4**, quercetin **5**, apigenin 7-O-glucoside **7**, apigenin 7-O-rutinoside (isorhoifolin) **8**, luteolin 7-O-glucoside (cynaroside) **10**, luteolin 7-O-rutinoside (scolymoside) **11**, apigenin 7-O-rhamnoside **6**, scutellarein 7-O-rhamnoside **9**, and quercetin 7-O-glucoside **12**. The last three flavonoid glycosides are reported in this species for the first time. Caffeic acid, quercetin and quercetin 7-O-glucoside showed significant activity compared to the other isolated compounds showing the importance of the free 3-OH group for the cytotoxic activity.

Key Words: ***Cynara sibthorpiana*, L1210 Cell line, Leukemia, Quercetin, Caffeic acid.**

INTRODUCTION

Cynara is a genus of seven annual species belonging to the family Asteraceae, found mainly in the Mediterranean region[1, 2]. The genus has been used in therapy for its strong choleretic activity causing substantial increase in the amount of bile extract[3], as well as for its diuretic, hypochloestrolemic and antilipidemic properties[4–10].

Cynara sibthorpiana Boiss ed Helder is a wild plant endogenous to Egypt[11]. It is known for its medicinal value as antihepatotoxic and in the treatment of renal diseases[12]. Previous studies on the leaves of *C. sibthorpiana* reported the isolation of a terpenoid alcohol vomifoliol (blumenol A) and three cytotoxic guaianolide sesquiterpene lactones: zaluzanin-C, solstitalin and sibthorphine[13]. Four flavonoid glycosides: apigenin 7-O-glucoside and rutinoside, as well as luteolin 7-O-glucoside and ruthinoside were also reported[14], in addition to the isolation of other constituents: taraxasterol acetate, pseudotaraxasterol, lupeol acetate, taraxasterol, stigmasterol, β-sitosterol, palmitic, stearic, linoleic and arachidic acids[15].

In the current study, bioassay guided fractionation against the L1210 mouse leukemia cell line led to the isolation and identification of twelve phenolic compounds. All the isolated compounds were tested for their cytotoxicity, among which caffeic acid, quercetin and quercetin 7-O-glucoside were found to be the most active compounds as anti-leukemic agents.

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EXPERIMENTAL

Caffeic acid, esculetin, apigenin, luteolin and quercetin were obtained from Sigma (St. Louise, MO, USA). ^1H and ^{13}C NMR spectra were recorded on a Varian 500 spectrometer at 500 and 100 MHz, respectively, in acetone-d_6. ESI (both +ve and –ve modes) were recorded on a Micromass ZAB-SE spectrometer (Waters Corporation, Beverly, MA, USA) in the Mass Spectrometry Laboratory of the School of Chemical Sciences, University of Illionis, Urbana-Champaign. Vacuum liquid chromatography was done using silica gel for TLC (Merck-Darmstadt, Germany). Column chromatography was performed either on silica gel for column 70–230 mesh size (Merck-Darmstadt, Germany) or on Lipophilic Sephadex LH-20 (Fluka, Switzerland) with various column dimensions, solvent systems and flow rates. Thin layer chromatography was carried out on pre-coated plates, silica gel 60 with F_{254} fluorescent indicator (Sigma-Aldrich Corporation, Germany) using benzene/ EtOAc 8 : 2 and $CHCl_3$/MeOH 9 : 1 as solvent systems and the plates were visualized under UV and sprayed with 5% $FeCl_3$.

Cynara sibthorpiana Boiss ed Helder aerial parts were collected from the north coast of Egypt in spring 2002. It was air dried and finely ground. A voucher specimen was kindly identified by Dr. Sherif El-Khanagry, Lecturer of Plant Flora Taxonomy Department, Egyptian Museum of Agriculture and kept in the herbarium of the National Research Center, Cairo, Egypt.

Extraction and isolation

Cynara sibthorpiana Boiss ed Helder aerial parts (700 g) were extracted with 70% MeOH by percolation. The aqueous layer after evaporation of methanol under vacuum was extracted successively with petroleum ether (yield 7.3 g), CH_2Cl_2 (yield 5.0 g) and EtOAc (7.4 g). A portion (4g) of the CH_2Cl_2 concentrate was applied to a silica gel column (120 g, ϕ 2.0 cm × 75 cm), eluting with hexane and increasing proportions of CH_2Cl_2 and MeOH (1–5%), collecting 20 fractions 250 mL each. Similar fractions were pooled together according to TLC profiles to give four main fractions. Fraction A (0.15 g) was fatty matter, fraction B (0.75 g) was a mixture of sterols. Fraction C (0.12 g) was further fractionated on preparative TLC to give compounds **1** (20 mg) and **2** (15 mg). Fraction D (0.18 g) was further separated on Sephadex column (15.0 g, ϕ 1.5 cm × 60 cm) using EtOH to give compounds **3** (20 mg), **4** (28 mg) and **5** (35 mg). An outline of the separation procedure is shown in Fig. 1. EtOAc concentrate (4 g) was chromatographed on Whatman paper No. 3 using butanol : acetic acid : water (BAW) (4 : 1 : 5) and 15% acetic acid[16] as eluting systems. The separated bands were extracted with methanol and purified on Sephadex columns using EtOH as eluting solvent. Paper chromatograms were visualized under UV before and after exposure to ammonia vapour and sprayed with $FeCl_3$ and Naturstoff reagent[17]. Seven flavonoid glycosides could be isolated: **6** (35 mg), **7** (40 mg), **8** (33 mg), **9** (45 mg), **10** (30 mg), **11** (25 mg) and **12** (40 mg). All the isolated compounds were tested for their cytotoxic activity against L1210 mouse leukemia cell line.

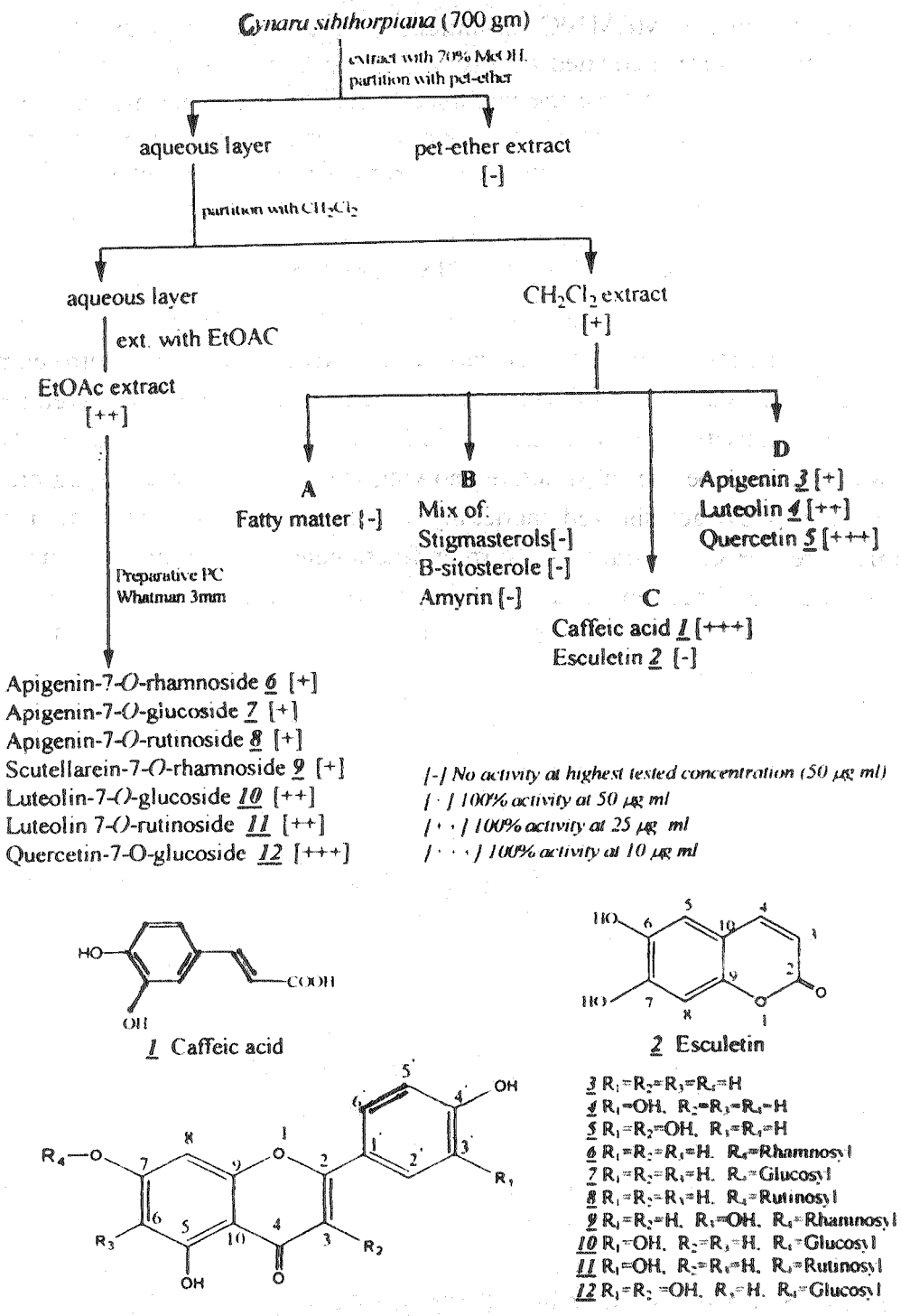

Fig. 1. Flow scheme of the bioassayed fractionation of *Cynara sibthorpiana* extracts and the chemical structures of the isolated phenolic compounds

L1210 Bioassay

A modified method[18–24] was used, where experimental samples were made up in MeOH (lab grade) solution and the measured amounts were dried in 18 wells of 24-well plate, the other six wells being reserved as negative controls. Sufficient amounts to make final concentrations of 50, 25, 10, 5, 2.5 and 1.0 μg/mL of each sample were added to the experimental wells. After the solvent had evaporated, one thousand L1210 cells, taken from a flask in exponential growth, were added

to each well in 1 mL of MEM10C (minimum essential medium with 10% calf serum). The plates were incubated for about 72 h until there were 8000 cells in the control wells, at which time the numbers of cells in the experimental wells was estimated by visual observation under an inverted optics phase-contrast microscope and inhibition, the difference between 8000 and the numbers present in the experimental wells, was expressed as a per cent of 8000.

RESULTS AND DISCUSSION

The aerial parts of *Cynara* were extracted with 70% MeOH and the concentrated aqueous alcoholic extract was partitioned successively with petroleum ether, CH_2Cl_2 and finally with EtOAc. The obtained extracts were bioassayed for their cytotoxic activity against L1210 mouse leukemia cell line. The EtOAc extract was found to be the most active showing 100% inhibition at 25 µg/mL. Also the CH_2Cl_2 extract showed moderate activity giving 100% inhibition at 50 µg/mL. The CH_2Cl_2 extract was further fractionated by column chromatography on silica gel. Fraction A (fatty matter) showed no cytotoxic activity at the highest tested concentration (50 µg/mL), thus it was neglected. Fraction B was identified as a mixture of stigmasterol, β-sitosterol and amyrin (compared with authentic samples), also showed no cytotoxic activity at the highest tested concentration (50 µg/mL). Fractions C and D showed cytotoxic activity (100% inhibition at 50 µg/mL), thus they were purified by preparative TLC to give compounds **1–5**. The five compounds were identified by their UV spectral data, MS data and also by comparison with authentic samples as: the phenolic caffeic acid **1** (R_f $S_1 = 0.79$, $S_2 = 0.26$, UV and UV/NH_3 blue, λ_{max} 243, 326 nm, $M^{+1} = 181$ and $M^{-1} = 179$); a coumarin aglycone esculetin **2** (R_f $S_1 = 0.78$, $S_2 = 0.28$, UV blue, λ_{max} 230, 260, 303, 351 nm, $M^{+1} = 179$ and $M^{-1} = 177$); and three flavonoid aglycones: apigenin **3** (R_f $S_1 = 0.87$, $S_2 = 0.11$, UV deep purple and UV/NH_3 yellowish green, λ_{max} 267, 296 sh, 336 nm, $M^{+1} = 271$ and $M^{-1} = 269$); luteolin **4** (R_f $S_1 = 0.77$, $S_2 = 0.08$, UV deep purple and UV/NH_3 yellow, λ_{max} 242 sh, 253, 267, 291 sh, 349 nm, $M^{+1} = 287$ and $M^{-1} = 285$); and quercetin **5** (R_f $S_1 = 0.57$, $S_2 = 0.03$, UV and UV/NH_3 yellow, λ_{max} 255, 269 sh, 301 sh, 370 nm, $M^{+1} = 303$ and $M^{-1} = 301$).

When the isolated five compounds were tested against L1210, caffeic acid **1** and quercetin **5** showed significant activity which gave 100% inhibition at 10 µg/mL, luteolin **4** and apigenin **3** showed lower activity (100% inhibition at 25 µg/mL and 50 µg/mL, respectively), while esculetin **2** showed no activity.

The EtOAc extract showing cytotoxic activity (100% inhibition at 25 µg/mL), was separated by preparative paper chromatography to give seven flavonoid glycosides **6–12** which were further purified on sephadex columns. The purified flavonoid glycosides were identified as apigenin-7-O-rhamnoside **6**, apigenin-7-O-glucoside **7**, apigenin-7-O-rutenoside **8**, scutellarin-7-O-rhamnoside **9**, luteolin 7-O-glucoside **10**, luteolin 7-O-rutinoside **11** and quercetin 7-O-glucoside **12**. The purified flavonoid glycosides (**6–12**) were identified by their spectral data; UV

TABLE-1
CHROMATOGRAPHIC PROPERTIES AND UV SPECTRAL DATA OF ISOLATED FLAVONOID GLYCOSIDES 6–12 FROM *CYNARA SIBTHORPIANA* BOISS ED HELDER

Compound	R_f values		Fluorescence		Spray reagents		UV data λ_{max} (MeOH), nm					
	S_1	S_2	UV	UV/NH_3	$FeCl_3$	NA/PE	MeOH	+NaOAc	+H_3BO_3	+$AlCl_3$	+HCl	+NaOMe
6	0.55	0.14	Purple	Yellowish green	Green	Grayish yellow	268 333	256 sh, 267 355, 387	267 340	276, 300 348, 386	277, 299 341, 382	245 sh, 269 301 sh, 386
7	0.61	0.23	Purple	Yellowish green	Green	Grayish yellow	268 333	256 sh, 267 355, 387	267 340	276, 300 348, 386	277, 299 341, 382	245 sh, 269 301 sh, 386
8	0.52	0.49	Purple	Yellowish green	Green	Grayish yellow	268 333	257 sh, 267 354, 387	267 341	275, 300 348, 382	276, 299 341, 380	245 sh, 267 300 sh, 386
9	0.43	0.16	Purple	Yellowish green	Green	Grayish yellow	232sh, 286 334	286 383	287, 301 sh 370, 428 sh	240 sh, 290 sh, 303 366	240 sh, 288 sh, 303 361	274 378
10	0.26	0.30	Deep purple	Yellow	Green	Orange	255, 267sh 348	259, 266 sh 365, 405	259 372	274, 298 sh 329, 432	273, 294 sh 358, 387	263, 300 sh 394
11	0.77	0.08	Deep purple	Yellow	Green	Orange	255, 265sh 349	259, 266 sh 365, 403	258 370	272, 296 sh 331, 432	272, 295 359, 389	263, 299 sh 394
12	0.61	0.23	Yellow	Fluorescent yellow	Green	Orange	256, 269sh 372	286 378, 269 sh (decompe)	261, 289 sh 386	259 sh, 273 339, 458	268, 303 sh 365, 426	241 sh, 291 367, 457 (decomp)

S_1 = butanol/acetic acid/water (BAW 4 : 1 : 5), S_2 = 15% acetic acid in water, Na/PE = Naturstoff reagent, NaOAc = sodium acetate

TABLE-2

^{1}H NMR SPECTRAL DATA OF ISOLATED FLAVONOID GLYCOSIDES **6–12** (δ, DMSO-d_6) FROM *CYNARA SIBTHORPIANA* BOISS ED HELDER

Compound **6**	Compound **7**	Compound **8**	Compound **9**	Compound **10**	Compound **11**	Compound **12**
7.90, *d*, J = 8.8 Hz (2H-2′ & 6′)	7.90, *d*, J = 8.8 Hz (2H-2′ & 6′)	7.90, *d*, J = 8.8 Hz (2H-2′ & 6′)	7.97, *d*, J = 8.8 Hz (2H-2′ & 6′)	7.45, *d*, J = 2.1 Hz (1H-2′)	7.38, *d*, J = 2.1 Hz (1H-2′)	7.56, *d*, J = 2.1 Hz (1H-2′)
6.90, *d*, J = 8.8 Hz (2H-3′ & 5′)	6.90, *d*, J = 8.8 Hz (2H-3′ & 5′)	6.90, *d*, J = 8.8 Hz (2H-3′ & 5′)	7.00, *s*, (1H-8)	7.42, *dd*, J = 8.8 & 2.1 Hz (1H-6′)	7.35, *dd*, J = 8.8 & 2.1 Hz (1H-6′)	7.54, *dd*, J = 8.8 & 1.9 Hz (1H-6′)
6.83, *d*, J = 1.9 Hz (1H-8)	6.83, *d*, J = 1.9 Hz (1H-8)	6.83, *d*, J = 1.9 Hz (1H-8)	6.94, *d*, J = 8.8 Hz (2H-3′ & 5′)	6.91, *d*, J = 8.8 Hz (1H-5′)	6.90, *d*, J = 8.8 Hz (1H-5′)	6.85, *d*, J = 8.8 Hz (1H-5′)
6.80, *s*, (1H-3)	6.80, *s*, (1H-3)	6.80, *s*, (1H-3)	6.82, *s*, (1H-3)	6.71, *d*, J = 1.9 Hz (1H-8)	6.70, *d*, J = 1.9 Hz (1H-8)	6.83, *d*, J = 1.9 Hz (1H-8)
6.46, *d*, J = 1.9 Hz (1H-6)	6.46, *d*, J = 1.9 Hz (1H-6)	6.46, *d*, J = 1.9 Hz (1H-6)	5.53, *d*, J = 1.9 Hz (1H-1‴)	6.63, *s*, (1H-3)	6.64, *s*, (1H-3)	6.19, *d*, J = 1.9 Hz (1H-6)
5.25, *d*, J = 1.9 Hz (1H-1″)	5.00, *d*, J = 7.4 Hz (1H-1″)	5.09, *d*, J = 7.4 Hz (1H-1″)	3.17–3.94 *m*, (–2″, 3″, 4″, 5″)	6.40, *d*, J = 1.9 Hz (1H-6)	6.38, *d*, J = 1.9 Hz (1H-6)	5.04, *d*, J = 7.4 Hz (1H-1″)
3.12–3.99 *m*, (–2″, 3″, 4″, 5″)	3.71–3.16 *m*, (–2″, 3″, 4″, 5″)	4.40, *d*, J = 1.9 Hz (1H-1‴)	1.14, *d*, J = 6.1 Hz (3H-6″)	5.07, *d*, J = 7.4 Hz (1H-1″)	5.09, *d*, J = 7.4 Hz (1H-1″)	3.71–3.16 *m*; (–2″, 3″, 4″, 5″)
0.89, *d*, J = 6.2 Hz (3H-6″)		3.18–2.75 *m*, (rutinosyl protons)		3.71–3.16, *m*, (–2″, 3″, 4″, 5″)	4.40, *d*, J = 1.9 Hz (1H-1‴)	
		1.08, *d*, J = 6.1 Hz (3H-6‴)			3.18–3.75 *m*, (rutinosyl protons)	
					1.08, *d*, J = 6.1 Hz (3H-6″)	

(using different shift reagents[17], Table-1), ^{1}H NMR (Table-2), ^{13}C NMR (Table-3) and MS where +ve and –ve ESI-MS data showed that the M of **6** = 416, **7** and **9** = 432, **10** = 448, **12** = 464, **8** = 578 and **11** = 594, confirmed by the appearance of corresponding free aglycone peaks when the compounds lose rhamnose units (M-146), glucose units (M-162) or both (M-308). Also flavonoid glycosides were identified chemically by acid hydrolysis[16], where the aglycones detected in the organic fractions were apigenin in compounds **6, 7** and **8**, luteolin in **10** and **11**, scutellarin in **9** and quercetin in **12**. While rhamnose sugar was detected in the aqueous fractions of compounds **6** and **9**, glucose was detected in compounds **7, 10** and **12** and both were detected in compounds **8** and **11**. Also the obtained data was compared with those reported[17, 25–28].

TABLE-3

^{13}C NMR SPECTRAL DATA OF ISOLATED FLAVONOID GLYCOSIDES 6–12 (δ, DMSO-d_6) ISOLATED FROM *CYNARA SIBTHORPIANA* BOISS ED HELDER

Assignments	Chemical shift δ (ppm)						
	6	**7**	**8**	**9**	**10**	**11**	**12**
C-2	164.0	164.0	164.4	164.1	164.5	163.0	147.9
C-3	102.8	102.8	103.3	102.4	103.2	103.5	135.9
C-4	181.0	181.6	181.8	182.2	181.6	182.0	175.9
C-5	161.0	161.0	161.2	147.0	161.1	161.0	160.3
C-6	99.7	99.7	99.6	131.0	99.7	99.8	98.9
C-7	162.7	162.7	162.6	150.7	162.9	164.7	162.7
C-8	94.6	94.6	94.8	94.5	94.9	95.2	94.5
C-9	156.6	156.6	157.0	149.0	156.9	157.1	155.7
C-10	105.1	105.1	105.6	105.7	105.5	105.6	104.7
C-1′	120.7	120.7	121.2	121.3	121.6	121.5	121.9
C-2′	128.3	128.3	128.4	128.4	113.7	113.6	115.5
C-3′	115.7	115.7	116.1	116.0	145.7	145.7	145.0
C-4′	160.8	160.8	161.2	161.1	149.7	150.0	147.8
C-5′	115.7	115.7	116.1	116.0	116.6	116.2	115.4
C-6′	128.3	128.3	128.4	128.4	119.0	119.5	120.1
C-1″	99.3	99.3	101.1	99.3	100.0	101.1	100.3
C-2″	70.1	73.0	73.2	70.1	73.2	73.2	73.2
C-3″	69.9	77.0	76.5	69.9	76.5	76.5	75.6
C-4″	71.8	69.4	69.7	71.8	69.7	69.7	69.9
C-5″	69.9	76.3	75.7	69.9	77.2	75.7	77.2
C-6″	17.8	60.5	66.2	17.8	60.8	66.2	60.9
C-1‴			100.6			100.6	
C-2‴			70.5			70.5	
C-3‴			70.9			70.9	
C-4‴			72.2			72.2	
C-5‴			68.5			68.5	
C-6‴			18.0			18.0	

When the isolated seven flavonoid glycosides (**6–12**) were tested for their cytotoxic activity against L1210 cell line, the flavonol quercetin-7-O-glucoside (**12**), which is isolated for the first time from this species, showed significant activity giving 100% activity at 10 μg/mL. Luteolin and its 7-O-glucoside and rutinoside showed lower activity (100% inhibition at 25 μg/mL), while the other flavone compounds were less active showing 100% activity at 50 μg/mL. It can be said that in this case the free 3-OH group is essential for the cytotoxic activity of flavonoids, which accords with reported data of structural activity relationship of flavonoids[29]. Also the presence of the 3′-OH may enhance the cytotoxic activity when comparing luteolin to apigenin glycosides, though they both show similar proliferative and cytotoxic effect on leukemic cell line (K562, DG75, BB58, B95) and blasts of patients with actute lymphoblastic leukemia[30].

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