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Isolation, Identification and Cytotoxic Activity of Triterpenes and Flavonoids from Green Walnut (*Juglans regia* L.) Pericarps

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Abstract: Three α-amyrine type triterpenes (**1-3**) in addition to oleanolic acid (**4**) and six known flavonoids, namely apigenin (**5**) and its 7-O-β-D-glucuronide (**9**), 5,6,4′-trihydroxy-7,3′-dimethoxy-flavone (**6**), cirsilineol (**7**), sudachitin (**8**) and cilicione-b (**10**) were isolated from the dichloromethane extract of the pericarps of *Juglans regia*. The structures of the compounds were established on the basis of spectroscopic analyses (UV, IR, HRESIMS and 1D & 2D NMR). Compounds **2**, **3** and **6-10** were isolated from *J. regia* for the first time. To the best of our knowledge, compound **3** is a new natural product. All isolated compounds, as well as the dichloromethane extract were evaluated for their cytotoxic activity against the human cancer cell lines, MCF-7, HCT-116, HeLa, K562, Raji and THP-1. Compounds (**1**) and (**5**) showed the most potent anticancer activity against the cancer cells assessed. Interestingly, the concentration of compound (**5**) that was cytotoxic to cancer cells did not induce human peripheral blood mononuclear cell apoptosis. These results suggest that the structure of compound (**5**) may eventually serve as a lead for developing novel anticancer drugs with limited side effects against normal cells.

Keywords: *Juglans regia*; pericarps; pentacyclic triterpenes; flavonoids; cytotoxic activity.

1. Introduction

Walnut (*Juglans regia* L. Juglandaceae) has been used in human nutrition since ancient times providing macronutrients and micronutrients, as well as many bioactive constituents [1]. Different parts of several *Juglans* species have been used in folk medicine for the treatment of cancer, rheumatic pains and eczema [2].

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Particularly, green pericarps of *J. regia* have been widely used as a traditional herbal medicine in China, Korea and Japan to treat cancer and dermatosis [3]. A plethora of compounds belonging to different phytochemical groups has been reported including mainly tetralones, diarylheptanoids, terpenoids and phenolic compounds [4-8]. Of these tetralones and diarylheptanoids could partially contribute to the cytotoxic and anticancer properties of the green walnut husks [5, 9, 10] and are found in several *Juglans* species [10-14]. Concerning the flavonoid content of *Juglans* species, quercetin and myricetin derivatives have been mainly identified [8, 15]. Moreover, green pericarps are characterized by the presence of triterpenes [5]. Oleane, ursane and lupane triterpenes have been also isolated from *J. sinensis* and were reported to exert anti-proliferative effects [16].

Although previous investigations have been focused on the napthoquinone and diarylheptanoid content of the green pericarps, the present study revealed the presence of pentacyclic triterpenes and flavonoids in significant amounts, some of which showed significant cytotoxic activity. The crude dichloromethane extract as well as the isolated secondary metabolites have been screened for their cytotoxic/cytostatic effects against human cancer cell lines including breast, colon, cervix and leukemic. The two most prominent were also tested for apoptosis induction against normal cells.

2. Materials and Methods

2.1. Plant Material

Fresh green pericarps of *Juglans regia* L. were collected (May, 2008) in Chalkida (Greece).

2.2. Extraction and Isolation

The fresh plant material (2.9 kg) was extracted at room temperature with cyclohexane, dichloromethane, methanol, successively. The obtained extracts were concentrated in vacuo. Part of the dichloromethane extract (23.8 g) was prefractionated by CC over Sephadex LH-20 (30 x 6 cm; MeOH) yielded seven fractions (A-G) of 350 mL each one. Further CC over Sephadex LH-20 (30 x 4 cm; MeOH) of fraction C (12.71 g) afforded 51 fractions (50 mL each one) combined in 9 groups (C_A-C_I). Subfraction C_E (5.15 g) was subjected to CC over Sephadex LH-20 (30 x 4 cm) and afforded 53 fractions (50 mL each one) combined in 7 groups (C_{EA}-C_{EG}). CC over silica gel (20.0 x 2.5 cm) of subfraction C_{EC} (1.02 g) using CH_2Cl_2–EtOAc-MeOH mixtures of increasing polarity as eluents yielded compounds **3** (8.6 mg; eluted with CH_2Cl_2–EtOAc-MeOH 95:5:0) and **4** (14.4 mg). Subfraction C_{EE} (159.2 mg) was purified by RP-HPLC using as mobile phase ACN-2%-CH_3COOH 20:80 and afforded pure compound **10** (0.2 mg; Rt = 24.3min). Subfraction C_F (802.2 mg) subjected to CC over Sephadex LH-20 (25.0 x 3.0 cm) with elution with MeOH afforded 77 fractions (10 mL each one) combined to 17 groups (C_{FA}-C_{FR}). Subfractions C_{FI} (33.2 mg) was further purified by RP-HPLC using as mobile phase MeOH-5%-CH_3COOH 65:35, and afforded the pure compounds **7** (8.2 mg; Rt = 29.1 min), and **8** (16.7 mg; Rt = 17.7 min). Subfraction C_{FJ} (52.4 mg) subjected to RP-HPLC using as mobile phase MeOH-5%-CH_3COOH 55:45, and afforded compound **6** (2.2 mg; Rt = 35.0 min). Subfraction C_{FN} (28.7 mg) subjected to RP-HPLC using as mobile phase MeOH-5% -CH_3COOH 55:45 and afforded compound **5** (17.5 mg; Rt = 94.2 min). Fraction E (4.22 g) was applied to Sephadex LH-20 (33 x 6 cm; MeOH) afforded 38 fractions (25 mL each one) combined to 5 groups (E_A-E_E). Subfraction Ec (271.7 mg) was applied to CC over silica gel (10.5 x 2.5 cm) with mixtures of cyclohexane-EtOAc-MeOH and yielded compound **1** (11.7 mg; eluted with cyclohexane-EtOAc-MeOH 70:30:0) and **2** (0.9 mg; cyclohexane-EtOAc-MeOH 70:30:0). Subfraction E_E (560 mg) was subjected to CC over Sephadex LH-20 (36.0 x 4.0 cm; MeOH) and afforded 42 fractions (10 mL each one; E_{EA}-E_{EE}). Subfraction E_{EB} (230.0 mg) was further purified by RP-HPLC using as mobile phase ACN-2%-CH_3COOH 25:75 and afforded pure compound **9** (7.9 mg; Rt = 20.4 min).

2.3. Cytotoxic effects on cancer cell lines

A panel of six cancer cell lines was used: MCF-7 (breast), HeLa (cervix), HCT-116 (colon), K562 (erythroleucemic), Raji (Burkitt's lymphoma) and THP-1 (monocytic leukemia). The cell lines were maintained in RPMI-1640 (Lonza Ltd, Switzerland), supplemented with 10% heat inactivated foetal calf serum (FBS, Lonza), 10 mM Hepes (Lonza), 50 mM mercaptoethanol (Sigma-Aldrich Chemical Co., St Louis, MO, USA), 103 U/mL penicillin (Lonza), 1.0 mg/mL streptomycin (Lonza) and 5 mg/mL gentamycin (Lonza) (thereafter referred to as complete medium) at 37 °C in a humidified 5% CO_2 incubator.

Compounds were prepared as a stock solution of 10.0 mg/mL in DMSO and prior use, were diluted in RPMI-1640. Cytotoxicity was evaluated by the MTT reduction assay [17] that allows estimating the number of metabolically active cells present in culture and determining the effect of treatment with an exogenously added agent on the viability of the cell population. Adenocarcinoma cells were plated in 96-well plates ($5x10^3$ cells/well) and incubated at 5% CO_2, 95% air at 37°C for 24 h, in order to adhere. Leukemic cells that grow in suspension were plated at $5x10^3$ cells/well prior the assay. All cells were incubated with the compounds for 72 h. After addition of the MTT reagent (1 mg/mL in PBS; 100 μL/well), the plates were further incubated for 4 h at 37°C in a 5% CO_2 incubator. The formazan crystals formed were dissolved by adding 0.1 N HCl% in 2-propanol (100 μL/well) and absorption was measured by an enzyme-linked immunosorbent assay (ELISA) reader at 545 nm. All cultures were set in triplicate, whereas cells incubated in complete medium or in medium containing the equivalent amount of DMSO, as well as cells incubated in the presence of doxorubicin (Sigma-Aldrich) were used as negative and positive controls, respectively. The IC_{50} was calculated according to the formula: $100(A_0 - A)/A_0 = 50$, where A and A_0 are optical densities of wells exposed to the compounds and control wells, respectively.

The compounds were tested at a concentration range of 200.0 to 12.5 μg/mL and the extract at 500.0 to 7.8 μg/mL. All experiments were performed at least in triplicate.

2.4. Flow cytometry analysis

PBMCs were isolated from normal donors' peripheral blood as previously described [18] seeded into 24-well plates ($3x10^5$/mL; 2 mL/well) and incubated with the mean IC_{50} values of compounds **1** (20 μg/mL) and **5** (10 μg/mL) for 24 h. Cells were collected, centrifuged in cold PBS (1500 rpm; 5 min) and stained with the Annexin V-FITC Apoptosis Detection Kit (BioLegend, Fell, Germany), according to the manufacturers' instructions. In brief, PBMCs were re-suspended in binding buffer, annexin V-FITC (5 μL) and PI (5 μL) were added, mixed and incubated with the cells for 15 min in the dark at room temperature. The volume was adjusted to 500 μL with binding buffer and the cell suspension was analyzed in a FACSCanto II (BD Biosciences, San Diego, CA) using FACSDiva software (BD Biosciences).

3. Results and Discussion

We report, herein, the isolation and structure elucidation of one novel pentacyclic triterpene (**3**), along with ursolic acid (**1**), 21α-hydroxy-ursolic acid (**2**) and oleanolic acid (**4**), as well as of six known flavonoids, namely apigenin (**5**) [19], 5,6,4′-trihydroxy-7,3′-dimethoxy-flavone (**6**) [20-21], cirsilineol (**7**) [19], sudachitin (**8**) [22], apigenin 7-O-*β*-D-glucuronide (**9**) [19] and cilicione-b (**10**) [23] from *J. regia* pericarps.

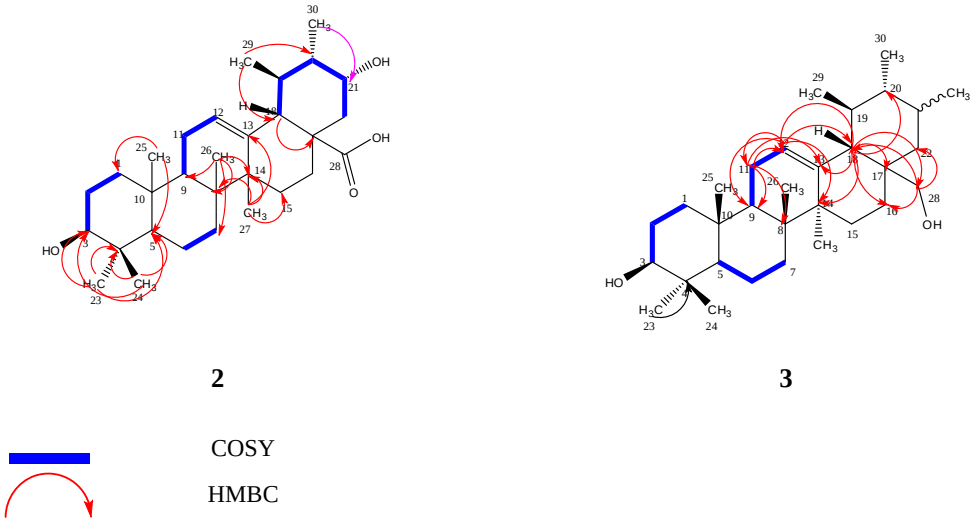

Figure 1. ^{1}H-^{1}H COSY correlations and the selected HMBC correlations of compounds **2**, **3**.

3.1. Structure elucidation

Compound **2** was obtained as whitish amorphous powder. $[a]_D^{20}$ -25.00 (*c*, 0.002, CH_2Cl_2); IR (CaF_2) ν_{max} 3600-3300, 2940, 1695 cm^{-1}; ESIMS *m/z* 471.3470 compatible with the molecular formula $C_{30}H_{48}O_4$ $[M-H]^+$ [calcd $[M]^+$ 472.3554]. The 1D and 2D-NMR data (Table 1) indicated that it belongs to the group of Δ^{12} pentacyclic triterpenes with an ursane skeleton [24-25]. The central skeleton of **2** was deduced by comparing the chemical shifts and the coupling constants between vicinal protons with those reported for ursolic acid (**1**). C-17 bears a carboxyl moiety as **1** since its chemical shift at δ_C 47.5 is in accordance with those reported in literature [25]. The main difference was an additional carbon shift (δ_C 71.5, C-21) assigned to a hydroxyl substituted carbon, which showed a HSQC correlation to a broad quartet (J = 3.4 Hz) in the ^{1}H NMR spectrum (Table 1, δ_H 3.85, H-21). The HMBC cross peak of CH_3-30 at δ_H 1.00 (3H, *d*, J = 6.7 Hz) with C-21 confirmed the allocation of this additional hydroxyl group. Furthermore, the placement of the hydroxyl group at C-21 is in accordance with the literature [26] and the observed downfield-shifted carbons C-20, C-21, C-22 (comparing to **1**) (Table 1). Due to its HRESIMS spectrum, which showed an increase by 16 mass units to *m/z* 471.3470 $[M - H]^-$ referring to a molecular formula of $C_{30}H_{48}O_4$ (calcd. for 472.3554), compound **2** might differ from **1** only in an additional hydroxyl group. Although NOESY spectrum was poor in signals, the pattern and the coupling constants of H-3 and H-21 (*dd*, J= 11.0, 4.7 Hz; *brq*, J= 3.4 Hz, respectively), as well as the chemical shifts of their carbons (δ_C 78.7 for C-3; δ_C 71.5 for C-21) evidenced the presence of 3β-OH and 21α-OH [27-28]. According to Mahato and Kundu [24] carbons bearing an β-OH are deshielded *vs* carbons possessing an α-OH. Thus, compound **2** was identified due to its spectroscopic data as 3β, 21α-dihydroxy-urs-12-en-28-oic acid. It was previously isolated from *Amaracus dictamnus* [29], where its semi-synthetic derivative, the 21α-hydroxy-methyl ursolate, was described. Compared to these literature data [29], we should note that the chemical shift of C-30 is at δ_C 23.7 (HSQC & HMBC spectra) instead of the reported δ_C 17.2. In addition, compound **2** was isolated from *Meconopsis punicea* Maxim [30], where in the ^{1}H NMR spectrum, only the methyl groups are described. Moreover, we should note that C-13 resonates at δ_C 137.2, a shift encountered in all ursane derivatives instead of the reported δ_C 144.3 [30]. Moreover, based on 2D experiment (HSQC & HMBC spectra), we should note that the chemical shifts of C-18 up to C-21 and of C-29 are significantly different, compared to the published data [30]. Therefore, we report here the physical and spectroscopic data (Table 1) of compound **2**.

Compound **3** was obtained as amorphous powder; $[a]_D^{20}$ +48.8 (*c*, 0.01, CH_2Cl_2). IR (CaF_2) ν_{max} 3600-3300, 2935, 1.700 cm^{-1}. Its ESIMS showed a pseudo-molecular peak of $[M + H]^+$ *m/z* 457.3676 compatible with the molecular formula $C_{31}H_{52}O_2$ [calcd 456.3970 $[M]^+$]. The ^{13}C-NMR (Table 1), DEPT/^{13}C-NMR spectra and HSQC experiments revealed 31 carbons including eight tertiary methyl groups, nine methylenes (eight aliphatic and one exocyclic), eight methines (seven aliphatic and one olefinic) and six quaternary carbons (five aliphatic and one olefinic). A thorough study of the ^{1}H-NMR spectrum (Table 1) showed one upfield chemical shift at δ_H 0.71 (*dd*, *J* = 11.8, 1.6, H-5) and eight methyl signals, five of them singlets at δ_H 0.97, 0.76, 0.91, 0.96, 1.08, one doublet at δ_H 0.92 (*J* = 6.8 Hz) and one doublet at δ_H 0.78 (*J* = 5.8 Hz). The signals at δ_H 5.12 (*t*, *J* = 3.5 Hz, H-12), 3.20 (*m*, H-3) together with the HSQC data (C-12 at δ_C 124.6, C-3 at δ_C 79.0) and the cross peaks in the HMBC spectrum between H-12 with the carbons δ_C 23.4 (C-11), δ_C 41.9 (C-14), δ_C 47.6 (C-9) and δ_C 54.0 (C-18) strongly suggested a structure of a Δ^{12} pentacyclic triterpene with an ursane skeleton [24-25]. The up field resonance of H-18 at δ_H 1.36 *vs* δ_H 2.16 in **1** gives evidence of a different substitution pattern of the ring E. Two doublets at δ_H 3.50 and 3.19 with large coupling constant (*J* = 11.0 Hz), which showed a HSQC correlation at δ_C 69.9, were assigned to a pair of geminal protons attached to an oxygenated carbon (CH_2-28). Key HMBC cross peaks of H-18 (δ_H 1.36) with C-28, as well as with the carbons at δ_C 138.3 (C-13), 124.6 (C-12), 41.9 (C-14), 39.4 (C-20), 23.4 (CH_2-16) and 37.7 (C-17), in addition to the cross-peaks of the CH_2-28 with C-18 (δ_C 54.0) gave evidence that the hydroxyl-methylene group should be placed at C-17 in the junction of the rings D and E. Moreover, the resonance of C-17 at δ_C 37.7 *vs* δ_C 47.5 (**1**) confirmed this assignment. The COSY correlation of H-18 with a doublet at δ_H 0.78 (*J* = 5.8 Hz) in combination to the long ^{1}H-^{13}C correlations (HMBC) of this doublet with the carbons at δ_C 54.0 (C-18), δ_C 39.4 (C-20) and δ_C 38.1 (C-19) enabled us to assign CH_3-29. The doublet at δ_H 0.92 integrating for 6 protons showed HSQC correlations at δ_C 17.8 and 21.3. The latter signal was assigned to CH_3-30 [25]. Moreover, in the HMBC spectrum, the doublet at δ_H 0.92 and CH_2-28 were correlated with C-22 at δ_C 35.2 (J^3); thus, the cross peak of the doublet should be attributed to another methyl group, as CH_3-30 is definitely excluded. Consequently, these data suggest that this second methyl group (δ_C 17.8) should be located at position C-21. The coupling patterns and the magnitude of the coupling constants of H-5 and H-6 (*dd*, *J* = 11.8, 1.6 Hz) were in full agreement with the α stereochemistry for H-5 and a *trans*-disposition of H-5/H-6 [31]. Moreover, NOESY correlations between H-3 and H-5 confirmed the α stereochemistry of H-5. Hence, compound **3** was identified as 28-hydroxymethylene-21-methyl-urs-12-ene. To the best of our knowledge, compound **3** is a new natural product.

The known pentacyclic triterpenes **1** and **4**, as well as the flavonoids **5**-**10** were identified by comparing their spectroscopic data with previously published. The ^{1}H and ^{13}C NMR data of **1** and **4** are reported, herein (see Supplementary Data; Tables 3 and 4), based on 2D NMR spectra for comparison with the other two isolated triterpenes. Compounds **2**, **3** and **6**-**10** were isolated from *J. regia* for the first time.

3.2. Antiproliferative activity

Using the MTT dye reduction assay, the dichloromethane extract and the aforementioned purified secondary metabolites (compounds **1**-**10**) were screened for their cytotoxicity against six human cancer cell lines, three adenocarcinomas of the breast (MCF-7), colon (HCT-116) and cervix (HeLa), and three leukemic, i.e., K562, Raji and THP-1.The 50% inhibitory concentrations (IC_{50}) of the samples on each cell line are presented in Table 2 (see Supporting Information for the structures of compounds **1**-**10**). Doxorubicin was used as a standard cytotoxic agent and showed IC_{50} values $\leq$ 0.2 μg/mL in all cell lines tested. As noticed, differential patterns of inhibition were observed, with some compounds exhibiting remarkable to strong activity against tumor cell lines, whereas others showed moderate or no cytotoxicity.

Table 1. ^{1}H NMR and ^{13}C NMR data of compounds **2** and **3** (at 400 MHz in $CDCl_3$, δ in ppm, *J* in Hz).

	2		**3**	
Position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	1.65 *m*	38.1	1.37 *m*	38.8
2	1.59-1.52 *m*	26.9	1.59-1.52 *m*	27.2
3	3.18 *dd* (11.0, 4.7)	78.7	3.20 *m*	79.0
4		38.5		39.1
5	0.70 *dd* (11.8, 1.2)	55.2	0.71 *dd* (11.8, 1.6)	55.1
6	1.30 *m*	18.6	1.43 *m*	18.3
	1.40 *m*		1.46 *m*	
7		33.1	1.51 *m*	32.9
8		39.1		40.6
9	1.47 *m*	47.2	1.54-1.49	47.6
10		37.8		38.0
11	1.89 *dd* (8.5, 3.2)	23.2	1.89 *dd* (9.0, 3.6)	23.4
12	5.25 *t* (3.8)	126.1	5.12 *t* (3.5)	124.6
13		137.2		138.3
14		42.2	*	41.9
15	*	28.3	1.19 *m*	26.1
16	*		*	23.4
17		47.5		37.7
18	2.22 br*d* (11.3)	52.6	1.36 *m*	54.0
19	1.83 *m*	32.0	*	38.1
20	1.97	42.2	0.99	39.4
21	3.85 br*q* (3.4)	71.5	*	*
22	1.77 *dd* (14.1, 3.5)	42.6	*	35.2
23	0.95 *s*	27.8	0.97 *s*	28.1
24	0.75 *s*	15.6	0.76 *s*	15.7
25	0.90 *s*	15.5	0.91 *s*	15.6
26	0.77 *s*	16.1	0.96 *s*	17.8
27	1.07 *s*	22.7	1.08 *s*	23.5
28		*	3.50 *d* (11.0)	69.9
			3.19 *d* (11.0)	
29	0.83 *d* (6.7)	16.5	0.78 *d* (5.8)	16.8
30	1.00 *d* (6.7)	23.7	0.92 *d* (6.8)	21.3
31			0.92 *d* (6.8)	17.8

* overlapping signals

More specifically, the dichloromethane extract exhibited cytotoxic activity with an IC_{50} range of 26.0 ± 2.83 to 101.0 ± 1.41 μg/mL (Table 2). Based upon these data, we further proceeded to the screening of the isolated natural products against the same human cancer cell lines. The IC_{50} values calculated for each cell line are presented in Table 2. Among the purified compounds **1-10** the most prominent was **5** which demonstrated a considerable cytotoxic activity against Raji, MCF-7, HCT-116 and HeLa cell lines, with IC_{50} values at 5.0 ± 0.00 μg/mL, 5.5 ± 0.71, 12.5 ± 3.54 μg/mL and 15.5 ± 4.95 μg/mL respectively. A striking decrease in the IC_{50} values between **5** and its glucuronide **9** was

noticed; the latter showing 6-35 fold higher IC_{50} values than **5**. Comparison of the structures and cytotoxicity results suggests a possible structure activity relationship. The differential cytotoxicity can be attributed to the attachment of a glucuronyl moiety at OH-7, which is in accordance with previously reported data [32]. Compounds **7** and **8** lacking an *ortho*-OH substitution in the flavonoid skeleton proved to be more effective in killing K562 cells (IC_{50} 18.5 ± 0.71 and 26.5 ± 4.95 μg/mL, respectively) compared to **6** and **10** (IC_{50} ≥100 μg/mL for both compounds). Against the other cell lines tested, compounds **7** and **8** exhibited moderate cytotoxicity, whereas the anticancer activity of **6** and **10** was marginal.

Table 2. *In vitro* cytotoxicity of the dichloromethane extract and isolated compounds from *J. regia* pericarps on human cancer cell lines.

	IC_{50} ± SD (in μg/mL)[a]					
Compounds	**MCF-7**	**HCT-116**	**HeLa**	**K-562**	**Raji**	**THP-1**
D*	92.3 ± 6.66	96.0 ± 5.65	101.0 ± 1.41	29.5 ± 6.36	26.0 ± 2.83	98.5 ± 14.84
1	19.0 ± 3.61	18.0 ± 2.83	21.0 ± 1.41	10.5 ± 2.12	6.5 ± 0.71	19.5 ± 3.54
2	84.3 ± 6.02	112.5 ± 3.54	120.0 ± 2.83	164.0 ± 1.41	39.0 ± 1.41	>200
3	30.0 ± 0.01	32.5 ± 2.12	45.0 ± 4.24	23.5 ± 2.12	28.0 ± 2.83	46.5 ± 2.12
4	47.5 ± 4.90	74.5 ± 7.78	106.5 ± 2.12	62.0 ± 9.80	29.0 ± 5.66	75.0 ± 7.07
5	5.5 ± 0.71	12.5 ± 3.54	15.5 ± 4.95	30.0 ± 11.31	5.0 ± 0.00	>200
6	82.5 ±10.60	79.5 ± 6.36	133.5 ± 12.02	101.0 ± 0.01	130.0 ± 0.01	>200
7	37.5 ± 3.54	40.0 ± 0.01	51.5 ± 4.95	18.5 ± 0.71	34.5 ± 0.71	33.5 ± 9.19
8	42.5 ± 3.54	45.5 ± 2.12	47.5 ± 6.36	26.5 ± 4.95	43.0 ± 7.07	>200
9	127.5 ± 10.10	139.0 ± 1.41	137.0 ± 7.07	165.0 ± 14.14	178.5 ± 0.71	>200
10	131.0 ± 1.41	137.5 ± 10.61	138.5 ± 4.95	>200	145.0 ± 2.43	>200

* D: dichloromethane extract

[a] IC_{50} values were determined after 72 h of exposure to each compound and represent means ± standard deviation (SD) of three independent experiments performed; Doxorubicine was used as positive control and showed $IC_{50} \leq 0.20$ μg/mL for all cell lines assayed.

Among the triterpenes, **1** induced significant loss of cell viability in the Raji cell line with an IC_{50} value of 6.5 ± 0.7 μg/mL, while **2** also bearing a carboxyl group at C-17 as **1**, showed moderate cytotoxicity with IC_{50} value at 39.0 ± 1.41 μg/mL against the same cell line. The importance of compound **1** and its derivatives as cytotoxic and potent anticancer agents has been previously reviewed, since they were shown to suppress cancer cells' growth and differentiation [33]. Moreover, according to Shao et al. [34] hydrogen donor groups at positions 3 or/and 17 are essential for cytotoxic activity, nonetheless **2** despite the presence of 3-OH, 21-OH and 17-COOH exhibited moderate cytotoxic activity (Table 2). It is remarkable that **3** revealed moderate, but almost consistent cytotoxicity against all cancer cell lines tested. **3** is a new natural product and its structure resembles to uvaol [35], which also presents weak cytotoxic activity [36]. Finally, compound **4** exhibited moderate cytotoxicity against the cell lines tested, but was significantly cytotoxic against Raji cells (IC_{50} 29.0±5.66 μg/mL).

3.3. Comparative cytotoxic effect on normal cells

Based on the cytotoxicity results, we further tested whether the two compounds with the higher anticancer activity, compounds **1** and **5**, were toxic against normal cells, i.e., peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals. PBMCs were incubated for 24 h with the IC_{50} of **1** (20 μg/mL) and **5** (10 μg/mL), stained with annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI) and analyzed by flow cytometry. As shown in Fig. 2, marginal percentages of early apoptotic (annexin V+PI-), late apoptotic (annexin V+PI+) or necrotic (annexin V-PI+) cells were detected in PBMCs incubated in culture medium (control). Upon incubation with doxorubicin (40 μg/mL), the majority of PBMCs were driven to necrosis (73.7%). Accordingly, incubation with the IC_{50} of compound **1** resulted in significant PBMC necrosis (70.4%), indicating that **1** is cytotoxic against both cancer and normal cells. On the contrary, PBMC incubation with the IC_{50} of compound **5**, shown to be cytotoxic to cancer cells, induced early and late apoptosis and necrosis in significantly lower number of cells (4.3, 2.8 and 23%, respectively). The observed selective cytotoxicity against cancer cell lines has been previously proved by Das et al., whereas apigenin (**5**) showed little or no cytotoxicity against normal peripheral blood mononuclear cells (PBMC) [37].

The results suggest that the plant possesses cytotoxic activity due not only to its naphthoquinone content, but also attributed to others constituents, such as pentacyclic triterpenes and flavonoids.

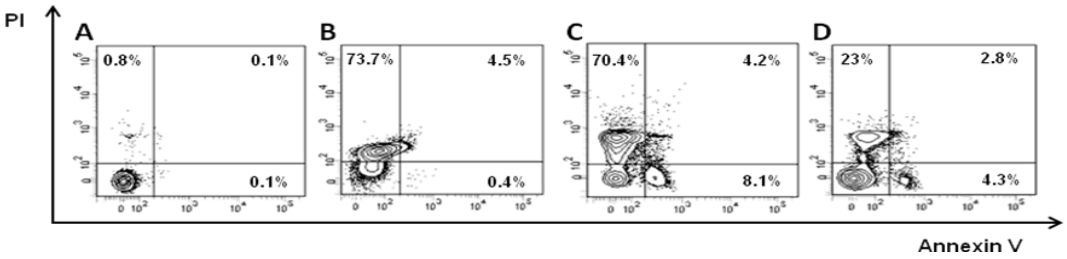

Figure 2. Normal donor PBMCs were incubated in complete medium (A), in the presence of doxorubicin (40 μg/mL; B), with the mean IC_{50} of compounds **1** (20 μg/mL; C) and **5** (10 μg/mL; D) and further stained with annexin V-FITC and PI. Numbers in quadrants indicate percentages of positive cells for each marker. Shown data are from one representative donor of 3 tested with similar results.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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