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Haemolytic activity, cytotoxicity and membrane cell permeabilization of semi-synthetic and natural lupane- and oleanane-type saponins

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ABSTRACT

The haemolysis of red blood cells inducing toxicity in most animals including humans is a major drawback for the clinical development of saponins as antitumour agents. In this study, the haemolytic and cytotoxic activities as well as the membrane cell permeabilization property of a library of 31 semi-synthetic and natural lupane- and oleanane-type saponins were evaluated and the structure–activity relationships were established. It was shown that lupane-type saponins do not exhibit any haemolytic activity and membrane cell permeabilization property at the maximum concentration tested (100 μM) independently of the nature of the sugar moieties. While oleanane-type saponins such as β-hederin (**25**) and hederacolchiside A_1 (**27**) cause the death of cancer cell lines by permeabilizing the cellular membranes, lupane-type saponins seem to proceed via another mechanism, which could be related to the induction of apoptosis. Altogether, the results indicate that the cytotoxic lupane-type glycosides **10** and **22** bearing an α-L-rhamnopyranose moiety at the C-3 position represent promising antitumour agents for further studies on tumour-bearing mice since they are devoid of toxicity associated with the haemolysis of red blood cells.

1. Introduction

Saponins are natural compounds found in higher plants and exhibit a wide spectrum of biological activities.[1] Despite a marked interest of the scientific community during the past decade towards the isolation,[2] total synthesis[3] and pharmacological properties[1,4] of these ubiquitous glycosides, the haemolytic activity of saponins inducing toxicity in most animals is a major drawback for their clinical development. Although the exact mechanism of the rupture of erythrocyte membranes (haemolysis) by saponins is not yet clearly understood, it was found to be correlated with their amphiphilic properties. It has been hypothesized that saponins interact with lipid membranes of cells[5] and form insoluble complexes with cholesterol leading to formation of pores,[6] permeabilization of cells[7] and subsequent loss of haemoglobin in the extracellular medium. On the other hand, Winter[8] proposed a different mechanism in which the interaction of saponins with the water channel aquaporin results in an increase of the water transport inside the cells inducing the haemolysis of erythrocytes.

Since some saponins are not haemolytic, structure–activity relationship (SAR) studies were recently undertaken in order to find active compounds such as anticancer agents[9,10] or vaccine adjuvants[11–13] devoid of toxicity towards erythrocytes. It was shown that there is no evident correlation between the haemolysis of red blood cells and cytotoxicity of saponins,[10] and both the nature of the aglycone and the osidic part are important for these biological activities.[9] Voutquenne and co-workers[14] reported that, among 59 tested triterpenoid glycosides, bidesmosidic saponins bearing sugar moieties at both C-3 and C-28 positions were generally less haemolytic than C-3 monodesmosides toward erythrocytes. In another study, it was also pointed out that the presence of non-natural linkages (1,2-*cis*-glycosides) have detrimental effects for both the haemolytic and cytotoxic activities of saponins.[9] However, since the isolation and total synthesis of saponins are usually quite difficult to achieve, the SAR studies are often limited by the possibility of obtaining a broad variety of pure saponins in a convenient quantity for biological assays.

In the past few years, our laboratory has been interested in the synthesis of cytotoxic lupane-type saponins having pentacyclic triterpenoids betulinic acid (**1**) or betulin (**2**) as aglycones. To this date, more than 50 natural and non-natural saponins have been chemically synthesized such as monodesmosides[15–18] and bidesmosides[19] containing various sugar moieties. Our SAR studies have shown that highly potent[19] and/or selective[15] anticancer agents are obtained for lupane-type saponins bearing an α-L-rhamnopyranose moiety at the C-3 position. Thus, we thought that it would be of interest to investigate the haemolytic activity of these lupane-type

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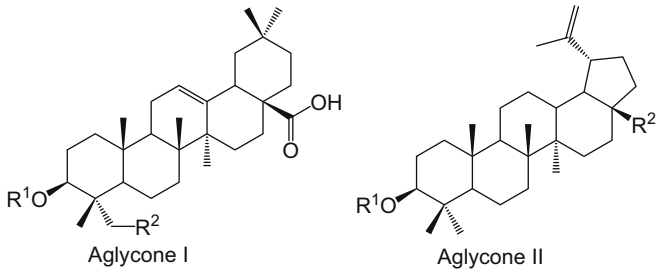
Table 1
Chemical structures of triterpenes **1**–**4** and saponins **5**–**22**



Compound	Aglycone	R^1	R^2	Reference
1[a] (Betulinic acid)	II	H	COOH	–
2[a] (Betulin)	II	H	CH_2OH	15
3[a] (Hederagenin)	I	H	OH	20
4[a] (Oleanolic acid)	I	H	H	—
5	II	α-L-Ara*f*	COOH	17
6	II	α-L-Ara*f*	CH_2OH	17
7[a]	I	β-D-Glc*p*	OH	23
8	II	β-D-Glc*p*	COOH	15
9	II	β-D-Glc*p*	CH_2OH	15
10[a]	II	α-L-Rha*p*	COOH	15
11	II	α-D-Ara*p*	COOH	15
12	II	β-D-Gal*p*	COOH	16
13	II	α-D-Man*p*	COOH	16
14	II	α-D-Man*p*	CH_2OH	16
15	II	β-D-Xyl*p*	COOH	16
16	II	H	COO-β-D-Glc*p*	19
17	II	β-D-Glc*p*	COO-β-D-Glc*p*	19
18	II	β-D-Glc*p*	CH_2O-β-D-Glc*p*	19
19	II	α-L-Rha*p*	COO-β-D-Glc*p*	19
20	II	α-L-Rha*p*	CH_2O-β-D-Glc*p*	19
21	II	α-L-Rha*p*	COO-α-L-Rha*p*	19
22	II	α-L-Rha*p*	CH_2O-α-L-Rha*p*	19

[a] Natural compounds.

saponins in relation with their cytotoxicity profile in order to choose 'lead' compounds devoided of undesirable toxicity for further in vivo studies on tumour-bearing mice. To our knowledge, this is the first study on the haemolytic activity and SAR of saponins having betulinic acid (**1**) or betulin (**2**) as aglycones. As shown in Tables 1 and 2, natural oleanane-type saponins such as hederagenin 3β-*O*-β-D-glucopyranoside (**7**), δ-hederin (**23**), α-hederin (**24**), β-hederin (**25**), hederacolchiside A (**26**) and hederacolchiside A_1 (**27**) isolated from species of the *Hedera* genus[20–23] were also investigated and their activities were compared to lupane-type saponins (**5**, **6**, **8**–**22** and **28**–**35**) and triterpenoid aglycones (**1**–**4**). α-Hederin (**24**) and hederacolchiside A_1 (**27**) were already shown to possess strong haemolytic[9] and anticancer activities[24–30] both in vitro and in vivo which are related to their capacity to interact with biological membranes.[31,32] In this study, we report the in vitro haemolytic and cytotoxic activities as well as the membrane cell permeabilization property of a library of 31 semi-synthetic and natural saponins of the lupane and oleanane family and discuss their SAR.

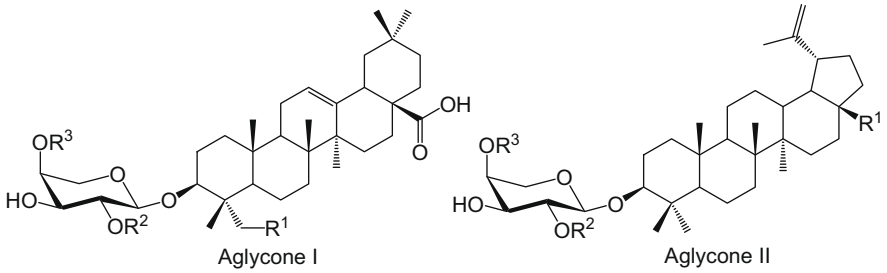
2. Results

2.1. Haemolytic activity

Haemolytic activity of triterpenoids (**1**–**4**) and saponins (**5**–**35**) was evaluated on sheep red blood cells (erythrocytes) as described by Chwalek et al.[9] with slight modifications. The values are expressed as the concentrations inducing 50% of erythrocytes haemolysis (HD_{50}). Saponin mixture (Sigma-Aldrich®) from quillaja bark (20–35% sapogenin) was used as a positive control. Significant differences in values ± standard deviation were calculated in comparison to treated erythrocytes in phosphate buffer saline (PBS).

The results, presented in Figure 1, clearly indicate that tested lupane-type saponins were not haemolytic compared to oleanane-type saponins with HD_{50} values mostly over 100 μM similarly to the negative control. While betulinic acid (**1**), betulin (**2**) and betulin glycosides were completely inactive, only three saponins with betulinic acid (**1**) as aglycone showed slight haemolytic activity significantly different from those of the control and oleanane

Table 2
Chemical structures of saponins **23**–**35** containing α-L-arabinopyranose



Compound	Aglycone	R^1	R^2	R^3	Reference
23[a] (δ-Hederin)	I	OH	H	H	23
24[a] (α-Hederin)	I	OH	α-L-Rha*p*	H	23
25[a] (β-Hederin)	I	H	α-L-Rha*p*	H	20
26[a] (Hederacolchiside A)	I	OH	α-L-Rha*p*	β-D-Glc*p*	21
27[a] (Hederacolchiside A_1)	I	H	α-L-Rha*p*	β-D-Glc*p*	22
28	II	COOH	H	H	17
29	II	CH_2OH	H	H	17
30	II	COOH	α-L-Rha*p*	H	17
31	II	CH_2OH	α-L-Rha*p*	H	17
32[a]	II	COOH	α-L-Rha*p*	β-D-Glc*p*	17
33[a]	II	COO-β-D-Glc*p*	H	H	19
34	II	CH_2O-β-D-Glc*p*	H	H	19
35[a]	II	COO-β-D-Glc*p*	α-L-Rha*p*	H	17

[a] Natural compounds.

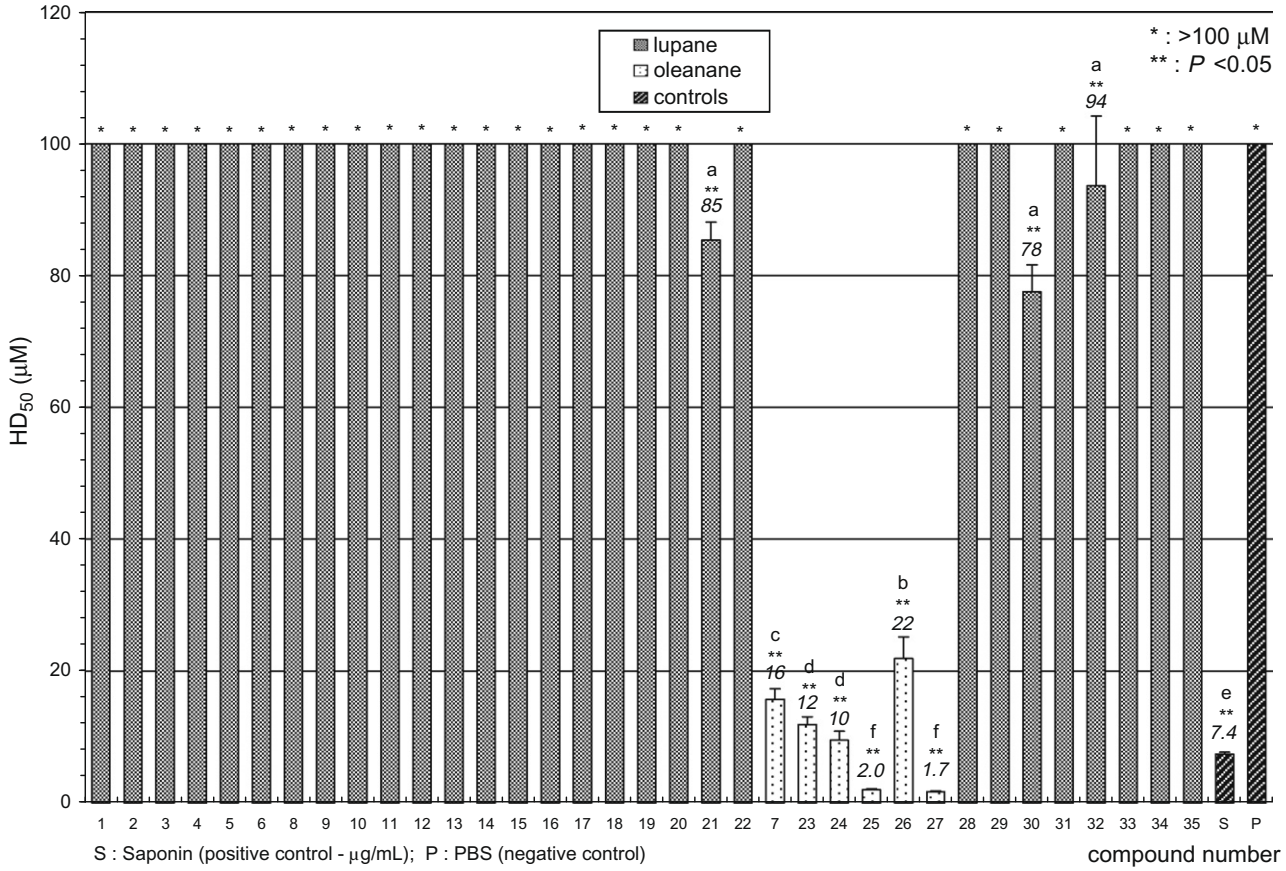


Figure 1. Haemolytic activity of triterpenes **1**–**4** and saponins **5**–**35**. Data represent mean values ± standard deviation of at least two independent experiments made in triplicate. HD_{50} inferior to 100 μM were compared using Kruskal–Wallis One-Way ANOVA on Ranks followed by Student–Newman–Keuls multiple comparisons. Significant difference ($P < 0.05$) are represented by different letters over the columns.

glycosides ($P < 0.05$), that is, saponin **21** bearing two α-L-rhamnopyranose moieties at both C-3 and C-28 (HD_{50} = 85 ± 3 μM), saponin **30** bearing an α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose moiety at C-3 (HD_{50} = 78 ± 4 μM), and saponin **32** bearing an α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranose moiety at C-3 (HD_{50} = 94 ± 11 μM). In contrast, all natural oleanane-type saponins induced strong haemolysis of red blood cells with HD_{50} values ranging from 1.7 to 22 μM. The saponins β-hederin (**25**) and hederacolchiside A_1 (**27**) with oleanolic acid (**4**) as aglycone were the most haemolytic of all tested saponins (HD_{50} = 2.0 ± 0.1 and 1.7 ± 0.1 μM, respectively) and were more active than the positive control. δ-Hederin (**23**) and α-hederin (**24**) showing significantly ($P < 0.05$) similar haemolytic activity (HD_{50} = 12 ± 1 and 10 ± 1 μM, respectively) were approximately five to sixfold less active than saponins **25** and **27**. The activity of hederagenin saponin (**7**) bearing a β-D-glucopyranose moiety at C-3 was significantly higher than saponins **23** and **24** with an HD_{50} value of 16 ± 2 μM. The least potent oleanane-type saponin to induce the haemolysis of erythrocytes was hederacolchiside A (**26**) (HD_{50} = 22 ± 3 μM). The related triterpenoids hederagenin (**3**) and oleanolic acid (**4**) did not exhibit haemolytic activity at the maximum concentration tested (HD_{50} > 100 μM).

2.2. Cytotoxic activity

The in vitro antiproliferative activity of compounds **1**–**35** against lung carcinoma (A549), colorectal adenocarcinoma (DLD-1) and normal skin fibroblasts (WS1) human cell lines was assessed using the resazurin reduction test.[33] Betulinic acid (**1**) was used as a positive control in this assay.[34] The cytotoxicity results presented in Table 3 are expressed as the concentration inhibiting 50% of the cell growth (IC_{50}). They are subdivided into five groups: triterpenoids (**1**–**4**; group I), lupane monodesmosides (**5**, **6**, **8**–**16**; group II), lupane bidesmosides (**17**–**22**; group III), oleanane saponins (**7**, **23**–**27**; group IV), and lupane saponins containing α-L-arabinopyranose (**28**–**35**; group V).

In the first group, betulin (**2**) was the most cytotoxic triterpenoid (IC_{50} = 3.6–6.6 μM) followed by betulinic acid (**1**) (IC_{50} = 10.3–15.0 μM). In contrast, hederagenin (**3**) and oleanolic acid (**4**) were only moderately active against A549 cancer cell lines (IC_{50} = 39 ± 6 and 27 ± 3 μM, respectively) while they did not exhibit any cytotoxicity against DLD-1. As previously reported,[15,16] betulinic acid 3β-*O*-α-L-rhamnopyranoside (**10**, IC_{50} = 2.6–3.9 μM) was the most active monodesmosidic lupane-type saponin of group II against A549 and DLD-1 cancer cell lines followed by betulin 3β-*O*-α-D-mannopyranoside (**14**, IC_{50} = 7.3–10.1 μM), and betulinic acid saponin **11** (IC_{50} = 10–17 μM) having a non-natural α-D-arabinopyranoside moiety at C-3. The saponins bearing glucopyranose moieties at either C-3 or C-28 (**8**, **9** and **16**) or a galactopyranose moiety at C-3 (**12**) were not able to exhibit cytotoxicity at the maximum concentration tested (IC_{50} > 100 μM). Moreover, α-L-arabinofuranosides **5** and **6** were also completely inactive against cancer (IC_{50} > 100 μM). Of all tested compounds in this study, betulin bis-3,28-α-L-rhamnopyranoside (**22**)[19] was the most potent to inhibit the growth of human cell lines with IC_{50} ranging from 1.3 to 1.9 μM. This saponin (**22**) was significantly more cytotoxic than its parent triterpenoid betulin (**2**). Bidesmoside **21** bearing two rhamnose moieties was also strongly active (IC_{50} 4.9–7.3 μM) with a

Table 3
Results of the cytotoxicity (resazurin reduction test) and membrane cell permeabilization (calcein–AM assay) of compounds **1**–**35**

Group[a]	Compound	IC_{50} (μmol L^{-1})[b]					
		Cytotoxicity			Membrane cell permeabilization		
		A549	DLD-1	WS1	A549	DLD-1	WS1
I	**1**	*10.3 ± 0.4*	*15.0 ± 0.3*	*12 ± 1*	>200	>200	>200
	2	*3.8 ± 0.1*	*6.6 ± 0.3*	*3.6 ± 0.1*	>200	>200	>200
	3	39 ± 6	>100	77 ± 3	>200	>200	>200
	4	27 ± 3	>100	>100	>200	>200	>200
II	**5**	>100	>100	>100	>200	>200	>200
	6	>100	>100	>100	>200	>200	>200
	8	>100	32 ± 9	>100	>200	>200	>200
	9	*>100*	*>100*	*>100*	>200	>200	>200
	10	*2.6 ± 0.6*	*3.9 ± 0.4*	*31 ± 3*	>200	>200	>200
	11	*10 ± 2*	*17 ± 3*	*47 ± 5*	>200	>200	>200
	12	*>100*	*>100*	*>100*	>200	>200	>200
	13	*34 ± 4*	*15 ± 1*	*13 ± 3*	>200	>200	>200
	14	*7.3 ± 0.4*	*10.1 ± 0.5*	*5.1 ± 0.6*	>200	>200	>200
	15	*15 ± 2*	*18 ± 2*	*20 ± 1*	>200	>200	>200
	16	*>100*	*>100*	>100	>200	>200	>200
III	**17**	*>100*	*>100*	35 ± 3	>200	>200	>200
	18	*>100*	*27 ± 2*	20 ± 2	>200	>200	>200
	19	*23 ± 1*	*11.0 ± 0.5*	9 ± 1	>200	>200	>200
	20	*16.8 ± 0.9*	*10.6 ± 0.9*	5.3 ± 0.4	>200	>200	>200
	21	*7.2 ± 0.5*	*7.3 ± 0.3*	4.9 ± 0.7	>200	>200	>200
	22	*1.9 ± 0.1*	*1.9 ± 0.1*	1.3 ± 0.1	>200	>200	>200
IV	**7**	59 ± 12	59 ± 8	32 ± 4	65 ± 8	89 ± 16	102 ± 17
	23	58 ± 2	57 ± 15	30 ± 2	40 ± 8	54 ± 10	51 ± 12
	24	33 ± 4	60 ± 17	30 ± 6	28 ± 6	38 ± 11	24 ± 5
	25	15 ± 2	30 ± 6	14 ± 2	3 ± 1	4 ± 1	2.8 ± 0.9
	26	59 ± 16	62 ± 17	58 ± 7	110 ± 12	159 ± 4	86 ± 28
	27	15 ± 4	16 ± 7	5.7 ± 0.7	4 ± 1	4 ± 1	3 ± 1
V	**28**	12 ± 3	11 ± 2	19 ± 1	>200	>200	>200
	29	>100	>100	>100	>200	>200	>200
	30	>100	>100	>100	>200	>200	>200
	31	>100	>100	>100	>200	>200	>200
	32	>50	>50	>50	>200	>200	>200
	33	*76 ± 4*	*60 ± 5*	50 ± 7	>200	>200	>200
	34	*>100*	*19 ± 2*	4.5 ± 0.3	>200	>200	>200
	35	>50	>50	>50	>200	>200	>200

Values in italics were previously reported by our laboratory (see references in Tables 1 and 2).
[a] (I) Triterpenoids, (II) lupane monodesmosides, (III) lupane bidesmosides, (IV) oleanane saponins, (V) lupane saponins containing α-L-arabinopyranose.
[b] Data represent mean values ± standard deviation for at least two independent experiments made in triplicate.

cytotoxicity profile slightly superior than betulinic acid (**1**). In addition, bidesmosidic saponins **21** and **22** were significantly more active against cancer cell lines than corresponding rhamnose-containing saponins **19** and **20** bearing a glucose moiety at the C-28 position. In group IV consisting of oleanane-type saponins, the order of anticancer activity was quite similar to the haemolytic activity. Indeed, there is a significant correlation between both biological activities ($P < 0.05$, Pearson product moment correlation). However, oleanane saponins (**7** and **23**–**27**) were not as cytotoxic as the positive control betulinic acid (**1**) against A549 and DLD-1. The strongest cytotoxicities on human cell lines were exerted by β-hederin (**25**) and hederacolchiside A_1 (**27**) (IC_{50} = 14–30 and 5.7–16 μM, respectively). Other saponins (**7**, **23**, **24** and **26**) with hederagenin (**3**) as aglycone were only moderately active against cancer with IC_{50} ranging from 30 to 62 μM. In the last group, lupane-type saponins (**30**, **31**, **32** and **35**) containing an α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose moiety did not exhibit any cytotoxicity at the maximum concentration tested. The most active saponin of group V was betulinic acid 3β-*O*-α-L-arabinopyranoside (**28**), which exerted a cytotoxicity profile similar to the parent triterpenoid **1** against human cell lines (IC_{50} = 11–19 μM) while the betulin analog **29** was inactive (IC_{50} > 100 μM). The addition of a glucose moiety at the C-28 position of **28** to give bidesmoside **33** had a detrimental effect on the anticancer activity (IC_{50} = 50–76 μM). In contrast, the cytotoxicity against DLD-1 and WS1 was increased when a glucose moiety was added at the C-28 position of **29** to give bidesmoside **34** (IC_{50} = 19 ± 2 and 4.5 ± 0.3 μM, respectively). It is important to note that only monodesmosidic saponins **10**, **11** and **28** were significantly more active against cancer cell lines than against normal skin fibroblasts (WS1).

2.3. Permeabilization of cell membrane

In order to evaluate the propensity of saponins to permeabilize the cellular membrane, we performed the calcein–AM assay as described by Debiton and co-workers.[31] The intracellular calcein–AM is converted by esterase into a green-fluorescent calcein and retained within intact cells. After treatment with drugs, the fluorescence is proportional to the number of cells (A549, DLD-1 and WS1) with undamaged membranes. The results presented in Table 3 are expressed as the drug concentration that decreased calcein fluorescence by 50% (IC_{50}).

As for the haemolytic assay, triterpenoids (**1**–**4**) and lupane-type saponins (**5**, **6**, **8**–**22** and **28**–**35**) did not permeabilize the membrane of A549, DLD-1 and WS1 human cell lines at the maximum concentration tested (IC_{50} > 200 μM). On the other hand, regarding the oleanane-type saponins, β-hederin (**25**) and hederacolchiside A_1 (**27**) were the most active as they strongly permeabilized the membrane of all human cell lines with IC_{50} ranging from

2.8 to 4 μM in function of the type of cells. This membrane activity was significantly higher ($P < 0.05$) than other saponins in this group having hederagenin (**3**) as aglycone such as hederagenin 3β-*O*-β-D-glucopyranoside (**7**, IC_{50} = 65–102 μM), δ-hederin (**23**, IC_{50} = 40–54 μM), α-hederin (**24**, IC_{50} = 24–38 μM) and hederacolchiside A (**26**, IC_{50} = 86–159 μM). Moreover, there is a significant correlation ($P < 0.05$) between the haemolytic activity and the membrane permeabilization potential of oleanane saponins (**7** and **23**–**27**).

3. Discussion

Saponins are well known to exhibit cytotoxic and haemolytic activities,[1] which are strongly correlated with the nature of both the aglycone and sugar side chains.[11,35] However, it was reported that these two properties are not linked since they can proceed by different mechanisms.[9,10] Recent studies demonstrated that several naturally occurring antitumour saponins cause the death of cancer cells by induction of apoptosis via the mitochondrial pathway.[36–38] On the other hand, the haemolysis of erythrocytes was shown to be due to the interaction of saponins with the membrane cholesterol of red blood cells.[39] However, Hu and co-workers reported that the liposomal membrane disrupting activity of oleanolic acid saponins glycosylated at both C-3 and C-28 positions (bidesmosides) can occur without the presence of cholesterol.[40]

The haemolytic activity of saponins is a serious drawback to the further pharmaceutical development of this type of natural product although some saponins show only weak[41] or no haemolytic effect at all.[42] In this SAR study, our results showed that, contrary to the majority of saponins including those of the oleanane family, lupane-type saponins do not exhibit any haemolytic activity at the maximum concentration tested (HD_{50} > 100 μM) independently of the nature of the sugar moieties. In fact, there were no significant differences between the haemolytic activities of lupane-type monodesmosides (**5**, **6** and **8**–**16**), bidesmosides (**17**–**22**) and those containing an α-L-arabinopyranose moiety (**28**–**35**). Similar results were reported by Yoshizumi et al.[43] for lupane-type saponins with a modified ring-A isolated from the leaves of *Acanthopanax sessiliflorus*, which did not lead to any haemolysis. Betulinic acid saponins **21**, **30** and **32** are the sole exceptions since they exerted a weak activity against red blood cells (HD_{50} = 78–94 μM). With regard to saponins **30** and **32**, the slight haemolytic activity could be correlated with the presence of an α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose moiety at the C-3 position of the triterpenoid skeleton. Indeed, this unique disaccharide chain is also present in oleanane-type saponins such as α-hederin (**24**), which have been shown to induce strong haemolysis of sheep erythrocytes.[9] Yamashita and co-workers[44] reported that betulin (**2**) and betulinic acid (**1**) triterpenoids do not exhibit any haemolytic effect even at a concentration of 500 μM as corroborated by our results (HD_{50} > 100 μM). These findings confirm the non-toxicity activity of lupane-type triterpenoids[45,46] and are of great interest for their clinical utilisation as anticancer[34] or chemopreventive[47] agents. Although they are virtually not haemolytic, betulin (**2**) and betulinic acid (**1**) have been reported to induce alterations of erythrocyte membrane shape towards echinocytes,[48] which seems to be related with their capacity to inhibit the growth of *Plasmodium falciparum*.[49] The in vitro antiplasmodial activity was shown to be due to the incorporation of triterpenoids **1** and **2** into the lipid bilayer of erythrocytes.[48]

In this study, the natural oleanane-type saponins (**7** and **23**–**27**) were shown to exhibit strong haemolytic activity (IC_{50} = 1.7–22 μM) compared to lupane-type saponins and natural aglycones **3** and **4** (IC_{50} > 100 μM). On the whole, it appears to be a direct correlation ($P < 0.05$) between the capacity of oleanane saponins to cause the lysis of red blood cells and their membrane cell permeabilization property. Moreover, the order of cytotoxicity seems to be linked with both the membrane alteration and haemolytic activities of oleanane saponins ($P < 0.05$). It is noteworthy that β-hederin (**25**) and hederacolchiside A_1 (**27**) with oleanolic acid (**4**) as aglycone were significantly more haemolytic and cytotoxic than α-hederin (**24**) and hederacolchiside A (**26**) bearing, respectively, the same sugar moieties. These results are in good agreement with those of Barthomeuf et al.[27] who reported that oleanolic acid saponins exhibit higher cytotoxicity than hederagenin saponins. Furthermore, it was pointed out that the structural requirements of potent antitumour oleanane-type saponins including α-hederin (**24**), hederacolchiside A (**26**) and A_1 (**27**) isolated from the roots of *Pulsatilla chinensis*[50] and *P. koreana*[51] are the presence of an α-L-Rha*p*-(1→2)-α-L-Ara*p* moiety at the C-3 position and a free carboxylic acid at the C-28 position.[28] Recently, hederacolchiside A_1 (**27**) was reported to induce membrane injury resulting in the formation of pores and permeabilization of the membrane of human melanoma (MEL-5) cells, which could be related to its anticancer activity.[31,32] Moreover, another study highlighted the apoptosis inducing activity of saponin **27**.[30] These results suggest that the anticancer activity of hederacolchiside A_1 (**27**) and its analogs could be due to the complementary action of both the alteration of cellular membranes and apoptosis induction. Nevertheless, as revealed by the results of the haemolytic assay, it is obvious that the high toxicity towards erythrocytes of these oleanane-type saponins (**7** and **23**–**27**) could hamper their utilisation as pharmaceutical agents.

While lupane-type saponins tested in this study were almost non-haemolytic, their cytotoxic activity were in some cases superior to oleanane-type saponins and greatly varied according to the nature of the sugar moieties linked to C-3 and C-28.[15,16,18,19] Indeed, concerning monodesmosidic saponins (group II), the presence of α-L-Ara*f*, β-D-Glc*p* or β-D-Gal*p* moieties at the C-3 position of the lupane skeleton seems to have a deleterious effect on the anticancer activity. In contrast, saponins bearing α-D-Ara*p*, α-D-Man*p* or β-D-Xyl*p* at the C-3 position exhibited a cytotoxicity profile similar or slightly inferior to the parent triterpenoids **1** and **2**. The addition of an α-L-Rha*p* at C-3 of betulinic acid (**1**) gave the most active monodesmosidic saponin (**10**) against human cancer cell lines (IC_{50} = 2.6–3.9 μM). With regard to lupane-type saponins of group V, it is worth noting that saponins (**30**–**32** and **35**) bearing an α-L-Rha*p*-(1→2)-α-L-Ara*p* moiety at the C-3 position were not able to inhibit the growth of cancer cell lines at the maximum concentration tested. Thus, our results suggest that this unique sugar moiety, which has been previously identified as a basic sequence for the antitumour activity of oleanolic acid and hederagenin monodesmosides[24,26–28] such as saponins **24**–**27**, does not improve the in vitro cytototoxicy of lupane-type saponins. Notably, betulinic acid saponin **28** bearing an α-L-Ara*p* without the presence of an α-L-Rha*p* linked at C-2' exhibited a cytotoxicity profile slightly superior to **1** against colorectal adenocarcinoma cell lines. As shown for monodesmosides **8** and **9**, the presence of a β-D-Glc*p* moiety at the C-3 position of the lupane skeleton has a detrimental effect on the cytotoxicity of bidesmosidic saponins **17** and **18**. These results may be explained by the high polarity of the glucose moiety, which could prevent the diffusion of the saponins into the cellular cytoplasm. In contrast, bidesmosidic saponins (**19**–**22**) containing α-L-Rha*p* moieties strongly inhibit the growth of cancer cell lines, the most active compound being betulin bis-3,28-α-L-rhamnopyranoside (**22**) (IC_{50} = 1.3–1.9 μM). It was postulated that the high anticancer activity of rhamnose-containing saponins may be attributable to their interaction with lectins, which could recognize rhamnose and facilitate the diffusion of the drug into the cytoplasm.[19,52]

In this SAR study, as for the haemolytic assay, all tested lupane-type saponins were unable to permeabilize the membrane of both human cancer (A549, DLD-1) and healthy (WS1) cell lines as determined by the calcein–AM assay (IC_{50} > 200 μM). Therefore, we can hypothesize that the strong cytotoxicity of lupane-type saponins such as glycosides **10** and **22** may be explained by another mechanism, which should not involve cellular membrane injuries. The parent triterpenoid betulinic acid (**1**) has been shown to induce apoptosis of cancer cells[45] independently of the p53 gene status through the perturbation of mitochondrial membrane potential and production of reactive oxygen species.[53] These processes trigger the release of mitochondrial apoptogenic factors, activation of caspases and subsequent DNA fragmentation.[54] Thus, in a study in progress in our laboratory, we investigated the mechanism of action of some lupane-type saponins. The preliminary results showed that, similarly to betulinic acid (**1**), saponins **10** and **22** could provoke the death of A549 and DLD-1 cancer cell lines through the induction of apoptosis as revealed by both the cleavage of apoptotic factors such as PARP and caspase 3 and the characteristic morphological changes in the cells (data not shown).

In conclusion, the haemolytic and cytotoxic activities as well as the membrane cell permeabilization property of semi-synthetic and natural lupane- and oleanane-type saponins have been investigated and structure–activity relationships have been established. While oleanane-type saponins such as β-hederin (**25**) and hederacolchiside A_1 (**27**) cause the death of cancer cell lines by permeabilizing the cellular membranes, lupane-type saponins seem to proceed according to another mechanism, which could be related to an apoptosis induction. The overall results suggest that lupane-type saponins such as the highly potent glycosides **10** and **22** represent promising anticancer agents for further studies on tumour-bearing mice since they are devoid of toxicity associated with the haemolysis of red blood cells in comparison with oleanane-type saponins. Work is currently in progress in our laboratory in order to elucidate the exact mechanism of the anticancer action of lupane-type saponins and results will be reported in due course.

4. Experimental

4.1. Chemicals

DMSO grade Biotech and saponin from quillaja bark (20–35% sapogenin content), the positive control used for the haemolysis assay, were purchased from Sigma-Aldrich® Canada. Betulinic acid (**1**) was purchased from Indofine Chemical Company Inc. and stored at 4 °C.

4.2. Triterpenes and saponins

Betulin (**2**) was extracted from the outer bark of *Betula papyrifera* Marsh. and purified by silica gel flash chromatography.[15] Lupane-type saponins (**5**, **6**, **8**–**22** and **28**–**35**) were synthesized according to procedures previously reported by our laboratory.[15–17,19] Oleanolic acid (**4**) was kindly provided by Laboratoire de Pharmacognosie-Homéopathie de l'Université de la Méditerranée de Marseille (France). Hederagenin 3β-*O*-β-D-glucopyranoside (**7**), δ-hederin (**23**) and α-hederin (**24**) were isolated from the berries of *Hedera colchica* and purified as described previously.[23] Hederagenin (**3**) and β-hederin (**25**) were obtained by acid and alkaline hydrolysis from hederasaponins C and B, respectively, isolated from the leaves of *Hedera helix*.[20] Hederacolchiside A (**26**) and hederacolchiside A_1 (**27**) were isolated from the leaves of *H. colchica* and purified according to literature.[21,22] The chemical structures (Tables 1 and 2) of all triterpenes and saponins used in this study were confirmed by spectral data (MS, ^1H and ^{13}C NMR).

4.3. Haemolytic assay

Sheep erythrocytes (Laboratoire Quelab, Montréal, QC, Canada) were first centrifuged at 3000 rpm for 5 min and then suspended in PBS to obtain a 1% erythrocytes solution. Samples were dissolved in a mixture of 5:1 DMSO/PBS to obtain a concentration of 500 μg/mL. Concentration gradient were prepared within a range of 500 to 3.9 μg/mL. A 160-μL volume of 1% erythrocyte solution was added to 96-well microplates (Costar, Corning Inc.) and a volume of 40 μL of sample or control solution was added to obtain a final erythrocyte concentration of 0.8% and sample concentration gradient ranging from 100 to 0.78 μg/mL. Microplates were incubated at 37 °C on an orbital shaker for 60 min. Microplates were then centrifuged at 3000 rpm for 5 min, after what the supernatant was transferred to empty microplates. Absorbance of the supernatant was measured at 540 nm with a Varioskan plate reader (Thermo, Waltham, MA, USA). Each experiment was carried out twice in triplicate. HD_{50} was calculated by comparison with the 100% haemolysis caused by the highest saponin (Sigma–Aldrich® Canada) concentration and were expressed as mean ± standard deviation.

4.4. Cells culture

Human lung carcinoma (A549), human colorectal adenocarcinoma (DLD-1) and human normal skin fibroblasts (WS1) cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured in minimum essential medium containing Earle's salts and L-glutamine (Mediatech Cellgro, VA), to which were added 10% foetal bovine serum (Hyclone), vitamins (1×), penicillin (100 IU/mL) and streptomycin (100 μg/mL), essential amino acids (1×), and sodium pyruvate (1×) (Mediatech Cellgro, VA). Cells were kept at 37 °C in a humidified environment containing 5% CO_2.

4.5. Cytotoxicity assay

Exponentially growing cells were plated in 96-well microplates (Costar, Corning Inc.) at a density of 5×10^3 cells per well in 100 μL of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of each compound in biotech DMSO (Sigma–Aldrich) were then added (100 μL per well) and the cells were incubated for 48 h. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cytotoxicity was assessed using resazurin[33] on an automated 96-well Fluoroskan Ascent F1™ plate reader (Labsystems) using excitation and emission wavelengths of 530 and 590 nm, respectively. Fluorescence was proportional to the cellular metabolic activity in each well. Survival percentage was defined as the fluorescence in experimental wells compared to that in control wells after subtraction of blank values. Each experiment was carried out thrice in triplicate. IC_{50} results were expressed as means ± standard deviation.

4.6. Calcein–AM assay

Exponentially growing A549, DLD-1 and WS1 cells were plated in 96-well microplates (Costar, Corning Inc.) at a density of 10^4 cells per well in 100 μL of DMEM and were allowed to adhere for 24 h before treatment. Microplates were then washed with PBS and 16 μM calcein–AM was added in 100 μL of culture medium. Microplates were incubated 60 min at 37 °C and 5% CO_2 for the calcein to be incorporated into cells. Unabsorbed calcein was then removed and microplates were washed with PBS. Growing concentrations of samples or controls were added in 100 μL of DMEM without PBS. Microplates were incubated 30 min after what wells were washed again with PBS. Fluorescence was read with a Fluoroskan Ascent F1™ plate reader (Labsystems) using excitation

and emission wavelengths of 485 and 530 nm, respectively. Fluorescence was proportional to the cell membrane integrity. Each experiment was carried out twice in triplicate. IC_{50} results were expressed as means ± standard deviation.

4.7. Statistical analysis

Calcein-AM assay (IC_{50}), haemolytic test (HD_{50}) and cytotoxic assay (IC_{50}) were determined by polynomial regression of dose–response curve after blank subtraction. Significant differences between samples were determined by Kruskal–Wallis One-Way Analysis of Variance on Ranks followed by post hoc multiple comparisons with Student–Newman–Keuls method. Probabilities (P) inferior to 0.05 were considered significant. Correlation coefficients were determined by Pearson Product Moment Correlation.

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