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Triterpene glycosides of *Lupinus angustifolius*

Girma M. Woldemichael[a,b], Michael Wink[a,*]

[a]*Institut für Pharmazeutische Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany*
[b]*Department of Pharmacognosy, School of Pharmacy, Addis Ababa University, P.O.Box 1176, Addis Ababa, Ethiopia*

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Abstract

Investigation of whole seeds of *Lupinus angustifolius* L. (Leguminosae) yielded the two triterpenoid saponins with branched monosaccharide chain 3β,21β,22β,24-tetrahydroxyolean-12-en-3-*O*-α-L-rhamnopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside (**3**) and 3β,21β,22β,24-tetrahydroxyolean-12-en-3-*O*-α-L-rhamnopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl-21-*O*-α-L-rhamnopyranoside (**4**) along with the known compounds soyasaponin I (**1**) and 3β,21β,22β,24-tetrahydroxyolean-12-en-3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl-21-*O*-α-L-rhamnopyranoside (**2**). The structures of the compounds were elucidated using hydrolysis, FAB–MS and extensive NMR experiments. Compounds **2–4** showed moderate antifungal activity against *Candida albicans* with MIC values of 25, 25 and 30 μg/ml, respectively. Only soyasaponin I was found weakly hemolytic (HC_{50} > 500 μg/ml).

Keywords: *Lupinus angustifolius*; Leguminosae; Triterpenoid saponin; Antifungal activity; Soyasapogenol glycosides; *Candida albicans*

1. Introduction

As part of an investigation into plants traditionally used in the preparation of food for people with diabetes, the genus *Lupinus* was selected for study. This choice was based on reports that the seed flour of its various species is incorporated in marmalades as part of the diabetic diet in several countries (Kusmenoglu et al., 1998; Villarroel et al., 1996; Mohamed et al., 1993). Investigation of any possible antidiabetic effect of lupins in general has until now mainly focused on the quinolizidine alkaloids and a series of alkaloids have been reported as characteristic constituents and are generally thought to be responsible for the hypoglycemic activity of the seed powder. Some reports show that lupin alkaloids such as lupanine, sparteine, multiflorine and *N*-methylcytisine in fact do possess hypoglycemic activity (Kubo et al., 2000; Mohamed et al., 1993). In addition to up to 2% alkaloids, however, many lupin seeds (including those of *Lupinus angustifolius*) are also known to contain up to 5% saponins (Wink, 1993, 1998). Pereire et al. (2001) reported that aqueous extracts of debittered lupin seeds (with very low alkaloid levels) appear to potentiate glucose-induced insulin release. Thus, the antidiabetic activity could be due to lupin saponins. Attempts at identification of the saponins present in the seeds of *L. angustifolius* based on their chromatographic properties have so far indicated that soyasaponins I and VI were present (Ruiz et al., 1995a,b), but there remained a few triterpene glycoside constituents that awaited further investigation. This work was undertaken to identify saponins present in *L. angustifolius*. Investigation of antidiabetic activity of the isolated lupin saponins is currently underway.

2. Results and discussion

Methanolic extract of the seed powder of *L. angustifolius* gave saponins **1**, **2**, **3** and **4**. Saponins **1** and **2** were identified as soyasaponin I and 3β,21β,22β,24-tetrahydroxyolean-12-en 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-21-*O*-α-L-rhamnopyranoside based on their FAB–MS and NMR spectra. These two compounds are already known to be present in *Glycine max* (Hosny and Rosazza, 1999) and *Medicago sativa* (Bialy et al., 1999), respectively.

* Corresponding author. Tel.: +49-6221-544880/81; fax: +49-6221-544884.
E-mail address: wink@uni-hd.de (M. Wink).

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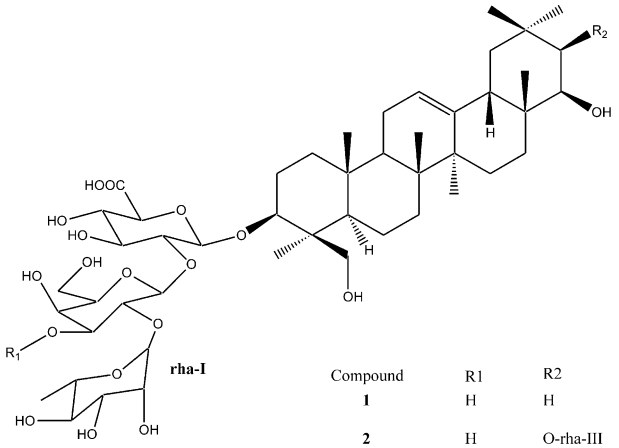

Compound	R1	R2
1	H	H
2	H	O-rha-III
3	rha-II	OH
4	rha-II	O-rha-III

Compound **3** gave a positive Liebermann–Burchard test. As in **2**, $C_{54}H_{88}O_{23}$ was determined as its molecular formula based on negative and positive ion HR–FABMS, which gave $[M-H]^-$ at *m/z* 1103.5560 and $[M+Na]^+$ and $[M+2Na-H]^+$ at *m/z* 1127.5704 and 1149.5493, respectively. The aglycon moiety region in the ^1H and ^{13}C NMR spectra also showed great similarity to that in **2**. Signals for seven tertiary methyl groups (δ 0.65, 0.91, 1.01, 1.28, 1.29, 1.36 and 1.47), one trisubstituted olefin at δ 5.26, (*t*-like, H-12), an oxymethine proton at δ 3.37 (*dd*, $J=11.8$, 3.7 Hz, H-3), two additional oxymethine protons which with selective TOCSY excitation showed coupling only with each other at δ 3.62 (*d*, $J=3.1$, H-22) and 4.07 (*d*, $J=3.1$, H-21), and two geminal oxymethylene protons at δ 3.35 (*d*, $J=11.5$, H-24a) and 4.32 (*d*, $J=11.5$, H-24b), could be discerned in the ^1H spectrum. Chemical shifts for two olefinic carbons at δ 122.5 (C-12) and 144.3 (C-13), characteristic of a Δ^{12}-β-amyrin skeleton, were also observed in the ^{13}C spectrum (Connolly and Hill, 1991). The foregoing data thus enabled the identification of the aglycon of **3** as 3β,21β,22β,24-tetrahydroxyolean-12-en or soyapogenol A, a compound already isolated from *Pueraria radix* (Arao et al., 1997).

On acid hydrolysis, **3** afforded D-glucuronic acid, D-galactose and L-rhamnose, which were identified by co-TLC and co-injection as their trimethylsilyl derivatives on GC–MS with authentic samples. However, the

Table 1
^{13}C chemical shift assignments and corresponding ^1H NMR correlation data of the aglycon region of compounds **3** and **4** in pyridine-d_5[a]

Position[b]	**3**		**4**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1 (CH_2)	38.5	0.84 *m*, 1.32 *m*	38.5	0.81 *m*, 1.33 *m*
2 (CH_2)	26.5	1.89 overlapped, 2.32 *m*	26.4	1.93 *m*, 2.19 *m*
3 (CH)	91.5	3.37 *dd* (11.8, 3.7)	91.1	3.38 *dd* (11.9, 3.7)
4 (C)	43.6		43.8	
5 (CH)	56.0	0.88 *t* (12.8)	55.9	0.87 *t* (13.0)
6 (CH_2)	18.4	1.25 overlapped, 1.57 *m*	18.4	1.25 overlapped, 1.54 *m*
7 (CH_2)	32.8	1.25 overlapped, 1.49 *m*	32.8	1.26 overlapped, 1.48 *m*
8 (C)	40.1		40.1	
9 (CH)	47.6	1.62 *d* (6.0)	47.7	1.60 *d* (6.1)
10 (C)	36.6		36.6	
11 (CH_2)	24.0	1.81 *d* (6.1)	24.0	1.80 *dd* (6.1, 0.7)
12 (CH)	122.5	5.26 *t*-like	122.5	5.26 *t* (0.7)
13 (C)	144.3		144.3	
14 (C)	42.0		42.0	
15 (CH_2)	26.5	0.93 *m*, 1.86 overlapped	26.4	0.99 overlapped, 1.83 *m*
16 (CH_2)	27.4	1.10 *m*, 1.95 *m*	27.4	1.09 *m*, 1.95 *m*
17 (C)	39.3		39.3	
18 (CH)	43.4	2.37 *dd* (13.1, 2.0)	44.0	2.53 *dd* (13.5, 1.9)
19 (CH_2)	47.0	1.44 *t* (13.1), 1.52 *dd* (13.1, 2.0)	47.4	1.26 *dd* (13.5, 1.9), 2.01 *t* (13.5)
20 (C)	36.3		36.8	
21 (CH)	74.6	4.07 *d* (3.1)	84.2	3.82 *d* (2.8)
22 (CH)	79.3	3.62 *d* (3.1)	78.8	3.91 *d* (2.8)
23 (CH_3)	22.7	1.47 *s*	22.9	1.44 *s*
24 (CH_2)	63.3	3.35 *d* (11.5), 4.32 *d* (11.5)	63.5	3.26 *d* (11.5), 4.26 *d* (11.5)
25 (CH_3)	15.5	0.65 *s*	15.7	0.67 *s*
26 (CH_3)	16.7	0.91 *s*	16.7	0.92 *s*
27 (CH_3)	26.6	1.29 *s*	26.8	1.29 *s*
28 (CH_3)	22.0	1.28 *s*	22.0	1.27 *s*
29 (CH_3)	31.0	1.01 *s*	31.2	1.01 *s*
30 (CH_3)	21.7	1.36 *s*	21.8	1.36 *s*

[a] Assignments based on ^1H, ^{13}C, DEPT, HSQC, HMBC and selective 1D-TOCSY experiments.
[b] Multiplicities as determined by DEPT.

^{1}H NMR spectrum showed four anomeric protons at δ 5.02, 5.86, 6.26 and 6.38. The corresponding anomeric carbons were located at δ 105.5, 102.1, 102.7 and 102.3 using their HMQC correlations. This suggested, as in **2**, the presence of two L-rhamnose residues. Confirmation was obtained by the appearance of a signal for a methyl doublet at δ 1.80 (6H, *d*, $J = 6.8$ Hz). The site of attachment and the sequence in the monosaccharide chain was determined through the analysis of the HMBC spectrum of **3** and comparison with that from **2**. These showed great similarity in that the presence of the same rha-(1→2)-gal-(1→2)-glcA-(1→3) could also be discerned in **3** at C-3. Long range correlations between H-3 and glcA C-1, between gal C-1 and glcA H-2, and between rha-I C-1 and gal H-2 were observed in the HMBC spectrum. However, additional glycosylation at the gal C-3 position was established by the appearance of HMBC correlation between rha-II C-1 and gal H-3. NOESY correlations of anomeric protons of each monosaccharide were also in agreement with these HMBC observations. Spin systems of individual monosaccharide units, as shown in Table 2, were identified using selective irradiation of easily identifiable anomeric proton signals of each monosaccharide along with irradiation of other non-overlapping proton signals of individual monosaccharides in a series of selective 1D-TOCSY and HSQC-TOCSY experiments. These also enabled complete assignment of ^{1}H and ^{13}C chemical shifts for all monosaccharide units including identification of the multiplicity pattern of individual protons. Anomeric configurations for the monosaccharide units in **3** were first inferred from the chemical shift values of the anomeric carbons and protons. These suggested that both the glucuronic acid unit and the galactose unit had a β-configuration (Miyao et al., 1996). This was also supported by the observed coupling constants ($J = 7.2$ Hz, glcA; $J = 7.6$ Hz, gal) of the anomeric protons of both monosaccharides. The C-5 signals of the two rhamnosyl units at δ 69.3 indicated their α-configuration (Seo et al., 1978). Hence, the foregoing enabled the characterization of **3** as 3β,21β,22β,24-tetrahydroxyolean-12 - en - 3 - *O* - α - L - rhamnopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside.

Table 2
^{13}C chemical shift assignments and corresponding ^{1}H NMR correlation data of the monosaccharide region of compounds **2** and **3** in pyridine-d_5[a]

Position	**3**		**4**	
	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]
GlcA				
1	105.5	5.02 *d* (7.2)	105.6	4.99 *d* (7.2)
2	77.8	4.64 *dd* (8.6, 7.2)	77.8	4.65 *dd* (8.2, 7.2)
3	78.5	4.38 *t* (8.6)	78.8	4.40 *t* (8.2)
4	73.9	4.51 *dd* (8.6, 8.0)	73.9	4.47 *dd* (9.0, 8.2)
5	76.7	4.60 *d* (8.0)	76.7	4.59 *d* (9.0)
6	172.8		172.8	
Gal				
1	102.1	5.86 *d* (7.6)	101.9	5.77 *d* (7.6)
2	78.0	4.50 *dd* (9.0, 7.6)	78.0	4.54 *dd* (9.0, 7.6)
3	79.3	4.26 *dd* (9.0, 1.6)	79.3	4.24 *dd* (9.0, 1.4)
4	78.0	3.62 *dd* (3.4, 1.6)	78.0	3.60 *dd* (3.1, 1.4)
5	76.7	4.08 *td* (6.2, 3.4)	76.8	4.09 *td* (6.1, 3.1)
6	61.5	4.30 *dd* (11, 6.2), 4.43 *dd* (11, 6.2)	61.7	4.34 *dd* (12, 6.1), 4.42 *dd* (12, 6.1)
Rha-I				
1	102.7	6.26 *br s*	102.7	6.26 *br s*
2	72.3	4.70 *d* (3.1)	72.5	4.70 *d* (3.2)
3	72.7	4.81 *dd* (9.5, 3.1)	72.6	4.78 *dd* (9.6, 3.2)
4	74.3	4.32 *dd* (9.5, 9.5)	74.5	4.34 *dd* (9.7, 9.6)
5	69.3	5.00 *dq* (9.5, 6.8)	69.6	5.01 *dq* (9.7, 7.0)
6	19.2	1.80 *d* (6.8)	19.2	1.79 *d* (7.0)
Rha-II				
1	102.3	6.38	102.3	6.38 *br s*
2	72.4	4.69 *d* (3.1)	72.5	4.68 *d* (3.2)
3	72.8	4.79 *dd* (9.5, 3.1)	72.6	4.76 *dd* (9.6, 3.2)
4	74.3	4.32 *dd* (9.5, 9.5)	74.0	4.35 *dd* (9.7, 9.6)
5	69.3	5.01 *dq* (9.5, 6.8)	69.6	5.02 *dq* (9.7, 6.9)
6	19.2	1.80 *d* (6.8)	19.2	1.79 *d* (6.9)
Rha-III				
1			104.7	5.50 *br s*
2			72.6	4.69 *d* (3.1)
3			72.9	4.61 *dd* (9.3, 3.1)
4			74.6	4.32 *dd* (9.4, 9.3)
5			69.6	4.52 *dq* (9.4, 6.0)
6			18.7	1.65 *d* (6.0)

[a] Assignments based on ^{1}H, ^{13}C, DEPT, selective 1D-TOCSY and HSQC-TOCSY experiments.
[b] Multiplicities as determined by selective 1D-TOCSY.

The negative and positive ion HR–FABMS of **4** established its molecular formula as $C_{60}H_{98}O_{27}$ through quasimolecular ions observed at m/z 1249.6151 $[M-H]^-$ and at m/z 1273. 6242 $[M+Na]^+$ and 1295.6067 $[M+2Na-H]^+$, respectively. This, together with the ^{1}H and ^{13}C NMR spectra and comparison of the same with those of **3** suggested the presence of a third deoxyhexose residue in addition to the triterpene aglycon and the tetrasaccharide residue found in **3**. The aglycon moiety region in both the ^{1}H and ^{13}C spectra of **4** was identical to that of **2** and differed from **3** in that a marked downfield shift was observed for C-21. This implied glycosylation at this position. Acid hydrolysis of the intact saponin gave soyasapogenol A as the aglycon and glucuronic acid, galactose and rhamnose as the monosaccharide residues comprising **4**. However, also observed in both the ^{1}H and ^{13}C spectra were five anomeric proton and carbon signals at δ 4.99, 5.50, 5.77, 6.26 and 6.38 in the ^{1}H and at δ 101.9, 102.3, 102.7, 104.7 and 105.6 in the ^{13}C spectrum, respectively. As in **3**, HMBC correlations between glcA C-1 and H-3, between gal C-1 and glcA H-2, between rha-I C-1 and gal H-2, and between rha-II C-1 and gal H-3 were also observed. On the other hand, as in **2**, long-range

correlation between rha-III C-1 and H-21 was also seen in the HMBC spectrum. Here too, NOESY correlations of glcA H-1 with H-3, gal H-1 with glcA H-2, rha-I H-1 with gal H-2, rha-II H-1 with gal H-3, and rha-III H-1 with H-21 were in agreement with the HMBC findings. These assignments led to the determination of the structure of **4** as 3β,21β,22β,24-tetrahydroxyolean-12-en-3-*O*-α-L-rhamnopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl-21-*O*-α-L-rhamnopyranoside.

3. Bioactivity evaluation

The ability of **1**, **2**, **3** and **4** to hemolyse sheep erythrocytes and their activity against *Candida albicans* were evaluated in vitro as described in Experimental. The hemolysis test showed that while **1** was weakly hemolytic (HC_{50} > 500 μg/ml), **2**, **3** and **4** did not exhibit any hemolytic effect. However, in antifungal tests against *C. albicans* **2**, **3** and **4** showed moderate antifungal activity with MICs of 25, 25 and 30 μg/ml, respectively, while **1** was found inactive at the concentrations tested. The positive control used, amphotericin B, was found to inhibit growth of *C. albicans* at 1 μg/ml.

4. Experimental

4.1. General

CC: silica gel 60 (Macherey-Nagel) 0.063–0.2 mm and Sephadex LH-20 (Pharmacia Biotech). TLC: silica gel 60 F254 (Merck). Optical rotation: Jasco P1020 digital polarimeter. IR: Perkin–Elmer FTIR 1600 (film). GLC-MS: Carlo Erba HRGC 4160 with OV-1 fused capillary column (0.32 mm i.d., 0.25 μm × 30 m) and Finnigan MAT 4500 mass spectrometer (at 45 eV). HPLC: Knauer model 64 system with Lichrospher 100, RP-18 (5 or 10 μm), either 250 × 4 mm or 250 × 8 mm i.d. using CH_3CN – 0.1% trifluoroacetic acid in H_2O linear gradient (80 to 50% in 40 min), detection at 200 nm. HR–FABMS: Jeol JMS 700 spectrometer with either PEG 100 or NBA as the matrix. ^1H, ^{13}C, DEPT-135, DEPT-90, DQF-COSY, NOESY (mixing time 300 and 700 ms), 1D-TOCSY (for monosaccharide residues, mixing time 60.9, 71.0 and 81.2 ms), HSQC-TOCSY (mixing time 70 ms), HMQC and HMBC NMR spectra: Brüker DRX 500 or DRX 600 spectrometer in pyridine-d_5 and TMS internal standard.

4.2. Plant material

The seeds of *L. angustifolius*, available commercially, were purchased in Germany in February 1996. Voucher specimen has been kept at the Institute of Pharmaceutical Biology, University of Heidelberg.

4.3. Extraction and isolation

The powdered seeds of *L. angustifolius* (1 kg) were extracted successively with petroleum ether (40–60 °C) and 70% EtOH at room temperature with constant stirring. The EtOH extract was then concentrated to a brown viscous mass under reduced pressure at 37 °C. This was suspended in H_2O and partitioned with water-saturated *n*-BuOH. Solvent was removed from the *n*-BuOH fraction and the residue dissolved in a small amount of MeOH, applied onto a Sephadex column and eluted with MeOH. Fractions 7–10 were combined and chromatographed on silica gel (EtOAc–EtOH–H_2O, 7:2:2, upper) to give **1** (30 mg) and a mixture of **2**, **3** and **4**. The mixture was then separated using HPLC to yield **2** (14 mg), **3** (2 mg) and **4** (2 mg).

4.4. Acid hydrolysis of *1–3*

An amount of the saponin (1–5 mg) and 1 ml of 1N HCl (dioxane–H_2O, 1:1) were placed in an ampoule. The ampoule was sealed and placed in an oven at 90 °C for 2 h. The ampoule was cooled, its seal broken and its contents dried under a stream of nitrogen. Contents of the ampoule were then suspended in H_2O (3 ml), the aqueous hydrolysate neutralized with Ag_2CO_3 and then washed with EtOAc (3 × 3 ml). Removal of the solvent after collection of the EtOAc fraction yielded the aglycon while centrifugation followed by removal of H_2O from the aqueous portion gave monosaccharides. Sugars were analyzed by co-chromatography with a standard on TLC [$CHCl_3$–MeOH–H_2O (15:6:2 lower layer)–HOAc (9:1)] and also after silylation [*N*-methyl-*N*-(trimethylsilyl)-2,2,2-trifluoroacetamide, Sigma] using GC–MS.

4.5. Compound *3*

A white amorphous solid, $[\alpha]_D^{25}$ −2.9° (MeOH: *c* 0.83). HR–FABMS $[M-H]^-$ at *m/z* 1103.5560 (calc. 1103.5613), $[M+Na]^+$ at *m/z* 1127.5704 (calc. 1127.5589), and $[M+2Na-H]^+$ at 1149.5493 (calc. 1149.5409). IR ν_{max} (film) cm^{-1}: 3358 (OH), 2958 (CH), 1649 (COOH). ^{13}C NMR: see Tables 1 and 2. ^1H NMR NMR: see Tables 1 and 2.

4.6. Compound *4*

A white amorphous solid, $[\alpha]_D^{25}$ −8.4° (MeOH: *c* 0.83). HR–FABMS $[M-H]^-$ at *m/z* 1249.6151 (calc. 1249.6189), $[M+Na]^+$ at *m/z* 1273.6242 (calc.1273.6165), and $[M+2Na-H]^+$ at 1295.6067 (calc. 1295.5985). IR ν_{max} (film) cm^{-1}: 3385 (OH), 2959 (CH), 1692 (COOH).

^{13}C NMR: see Tables 1 and 2. ^{1}H NMR: see Tables 1 and 2.

4.7. Activity against C. albicans

Compounds **1**, **2**, **3** and **4** were tested for antifungal activity according to a modified NCCLS micro dilution assay procedure. All tests were carried out using sterile 96-well plates and Saburaud dextrose broth-SDB (Sigma). The log phase inoculum was obtained by incubating *C. albicans* (ATCC 10231) for 48 h at 30 °C on Saburaud dextrose agar plates. The compounds were dissolved in either H_2O or MeOH–H_2O combination. Solutions of each test compound were further diluted with SDB yielding their final concentration. Each saponin was evaluated in triplicate in a dose–response format where the final concentrations were 1, 25, 50, 100 and 500 μg/ml and where the final fungal concentration was 2×10^3 cfu. To each well containing double the final saponin dilution, an equal volume of the inoculum suspension containing double the final fungal concentration was added. The minimum inhibitory concentration (MIC), the lowest concentration of the test compound resulting in the complete inhibition of growth, was determined visually. Amphotericin B (Sigma) at 1 μg/ml final concentration was used as a positive control.

4.8. Hemolysis assay

One milliliter of 1% sheep erythrocyte (Oxoid) suspension in phosphate buffer saline (PBS, pH 7.4) was incubated with 100 μl of saponin dilutions (1, 0.25, 0.05, 0.01, 0.005 and 0.001 mg/ml) in PBS at 37 °C for 30 min. This mixture was centrifuged (2500 *g*, 30 min) and the hemoglobin content in the supernatant was measured at 490 nm using a multiwell spectrophotometer (Virion autoReader A) by transferring 200 μl to 96-well microtiter plates. Parallel measurements of saponin dilutions without erythrocytes, erythrocytes without saponin dilutions and buffer without both erythrocytes and saponin dilutions were made. The results were then compared to a positive control sample containing erythrocytes in distilled water. The 50% hemolysing concentrations (HC_{50}) of each saponin were then inferred from the hemoglobin absorbance vs saponin concentration curve.

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