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Molluscicidal saponins from *Phytolacca icosandra*

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Abstract

Five monodesmosidic serjanic acid saponins and a monodesmosidic spergulagenic acid saponin were isolated from an aqueous extract of the berries of *Phytolacca icosandra*. A methanol extract of the berries furnished three bidesmosidic serjanic acid glycosides. Their structures were established by spectroscopic (ES–MS, ^{1}H NMR, COSY, HSQC, HMBC, ^{13}C NMR) and chemical methods. The molluscicidal, spermicidal and haemolytic properties of the saponins were investigated. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The discovery of the potent molluscicidal properties of the berries of *Phytolacca dodecandra* L'Hérit (Phytolaccaceae) from Ethiopia (Lemma, 1965) has prompted a large amount of work on plant-derived compounds which show toxicity to schistosomiasis-transmitting snails (Marston and Hostettmann, 1985). Known as "endod" or soapberry, *P. dodecandra* contains up to 25% (w/w) saponins in the dried berries. Both molluscicidal monodesmosidic oleanolic acid saponins (Parkhurst et al., 1974) and inactive bidesmosidic glycosides of oleanolic acid, hederagenin and bayogenin (Domon and Hostettmann, 1984; Dorsaz and Hostettmann, 1986) have been identified in the berries. Several field trials with aqueous extracts of the berries of *P. dodecandra* have been performed in Ethiopia in order to control *Schistosoma mansoni* by killing the intermediate snail host of this parasite (see, for example, Goll et al., 1983).

In an attempt to evaluate the molluscicidal potential of other species of the genus *Phytolacca*, the berries from *P. icosandra* L., found in Indonesia, were investigated for their saponin content.

P. icosandra is a shrub native to tropical America (from Mexico to Peru and found also on the Caribbean islands) but which is now naturalized in the hilly and mountainous regions of central and east Java. It is a noxious weed in Australia, New Zealand and South Africa.

In the present paper, we report the isolation of eight glycosides of serjanic acid (**1–5**, **7–9**) from the fruits of *P. icosandra*, together with a glycoside of spergulagenic acid (**6**).

2. Results and discussion

The berries of *P. icosandra* were extracted sequentially with dichloromethane and methanol. A second batch was extracted with water alone, since it is known in the case of *P. dodecandra* that aqueous extraction converts bidesmosidic saponins into monodesmosidic saponins, with a consequent increase in molluscicidal activity (Domon and Hostettmann, 1984).

The methanol and water extracts showed molluscicidal activity at 200 μg/ml and 25 μg/ml, respectively, after 24 h, against the snail *Biomphalaria glabrata*.

The aqueous extract was partitioned between water and *n*-butanol. The molluscicidal *n*-butanol fraction was separated on a silica gel column using a chloroform-methanol-water gradient, to give 14 fractions (I–XIV). Further chromatography of these fractions on silica gel and Sephadex LH-20, together with low-pressure LC (LPLC) on Lobar RP-18 columns, gave saponins **1–6**.

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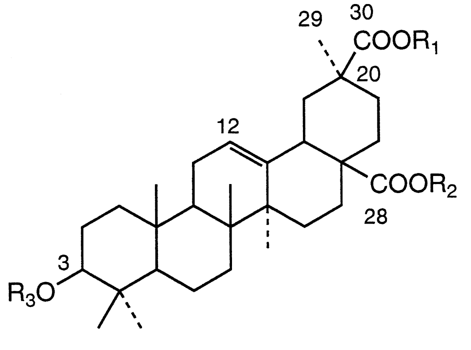
After partition of the methanol extract of the berries between *n*-butanol and water, the *n*-butanol fraction was separated by centrifugal partition chromatography (CPC) into eight fractions (I–VIII). Gel filtration on Sephadex LH-20 and silica gel column chromatography yielded saponins **7**–**9**.

The aglycone part of compounds **1**–**5** and **7**–**9** gave signals in the ^{13}C NMR spectrum which corresponded to serjanic acid (methyl spergulagenate) (Kohda et al., 1992) (Table 1). The signal for C-30 in all cases was at δ 176.9–177.1, confirming the presence of a methyl ester at this position. In **1**–**5**, C-28 appeared at δ 179.7–180.0, suggesting a free acid function, while C-28 appeared at δ 176.0–176.1 in **7**–**9**, implying esterification at this position.

For saponin **6**, the signals for the aglycone in the ^{13}C NMR spectrum corresponded to spergulagenic acid (Haraguchi et al., 1988) (Table 1).

Saponin **1** gave an ion at *m*/*z* 661 $[M-H]^-$ in the electrospray (ES) mass spectrum. MS^2 fragmentation of this ion gave the deprotonated aglycone at *m*/*z* 499 $[A-H]^-$, corresponding to the loss of a hexosyl unit. Signals of the monosaccharide moiety in the ^{13}C NMR spectrum were attributed to glucose. The anomeric signal at δ 106.9 showed the glucose to be attached at C-3 and not at C-28. Thus the structure of **1** was established as 3-*O*-β-D-glucopyranosylserjanic acid. Saponin **1** has previously been obtained as a partial hydrolysis product of a saponin from *P. thyrsiflora* (Haraguchi et al., 1988).



1 R_1 = -CH$_3$, R_2 = -H, R_3 = -Glc
2 R_1 = -CH$_3$, R_2 = -H, R_3 = -Glc^3-Gal
3 R_1 = -CH$_3$, R_2 = -H, R_3 = -Glc^2-Glc
4 R_1 = -CH$_3$, R_2 = -H, R_3 = -Glc^3-Glc^3-Glc
5 R_1 = -CH$_3$, R_2 = -H, R_3 = -Glc^2-Glc^2-Rha
6 R_1 = -H, R_2 = -H, R_3 = -Glc^2-Glc^2-Rha
7 R_1 = -CH$_3$, R_2 = -Glc, R_3 = -Glc^3-Gal
8 R_1 = -CH$_3$, R_2 = -Glc, R_3 = -Glc^2-Glc
9 R_1 = -CH$_3$, R_2 = -Glc, R_3 = -Glc^2-Glc^2-Rha

Signals at δ 106.2 and 106.4 for the anomeric carbons of the sugar moieties in the ^{13}C NMR spectrum of **2** suggested the presence of a disaccharide moiety attached at C-3. Comparison of spectral data with literature values (Haraguchi et al., 1988) proved the saponin to be 3-*O*-(β-D-galactopyranosyl-(1→3)-β-D-glucopyranosyl)serjanic acid.

The ES mass spectrum of **3** gave a pseudomolecular ion at *m*/*z* 823 $[M-H]^-$, indicating the presence of two hexosyl units attached to the aglycone (serjanic acid). An MS^2 experiment on this ion gave two fragments at *m*/*z* 661 and 499, corresponding to the sequential loss of the two sugars. Acid hydrolysis of the glycoside gave glucose as the only sugar. Comparison of the ^{13}C NMR spectrum of **3** with that of **1** showed a downfield shift of C-2 of the inner hexosyl moiety from δ 75.8 to 83.4. The part of the ^{13}C NMR spectrum corresponding to the sugar moiety was virtually identical to that of other diglucosides substituted at position C-2, as found for example in *Astragalus sieberi* (Verotta et al., 1998). Consequently, the structure of **3** is 3-*O*-(β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl)serjanic acid.

Table 1
^{13}C NMR spectral data for the aglycone parts of saponin **3** (serjanic acid) and saponin **6** (spergulagenic acid) (125 MHz in pyridine-d_5)

Carbon	**3**	**6**
1	38.6	38.6
2	26.6	26.4
3	89.1	89.6
4	39.4	39.7
5	56.0	55.9
6	18.5	18.5
7	33.2	33.2
8	39.7	39.6
9	47.9	47.9
10	36.9	36.9
11	23.7	23.7
12	123.1	123.3
13	144.2	144.3
14	42.0	42.0
15	28.2	28.4
16	23.9	23.9
17	46.2	46.3
18	42.6	43.0
19	43.3	43.3
20	44.2	44.0
21	30.7	31.1
22	34.5	34.7
23	28.4	28.4
24	17.5	17.3
25	15.4	15.4
26	16.9	16.8
27	26.0	26.1
28	179.7	180.0
29	28.5	29.0
30	177.1	179.1
–OCH$_3$	51.7	–

A pseudomolecular ion at *m*/*z* 985 $[M-H]^-$ was observed in the ES mass spectrum of **4**. Fragments at *m*/*z* 823, 661 and 499 in an MS^2 experiment corresponded to the sequential loss of three hexosyl moieties from the molecular ion. These were identified as glucose after acid hydrolysis. The anomeric proton resonances of **4** were at *δ* 4.88 (*d*, *J* = 7.8 Hz), 5.27 (*d*, *J* = 7.3 Hz) and 5.39 (*d*, *J* = 7.8 Hz). Proof of attachment of the inner glucose residue to C-3 of the aglycone was shown by the correlation of the anomeric proton at *δ* 4.88 with the signal at *δ* 89.1 in the HMBC spectrum. The 1→3 interglycosidic linkage with the second glucose moiety was deduced from the correlation in the HMBC experiment of the H-1″ signal (*δ* 5.27) with the C-3′ at *δ* 88.9. The terminal glucose was attached to the C-3 position of the middle sugar, as shown by the HMBC correlation between H-1‴ (*δ* 5.39) and C-3″ (*δ* 84.4). Accordingly, **4** is 3-*O*-(β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl)serjanic acid.

The ES mass spectrum of **5** showed a pseudomolecular ion at *m*/*z* 969 $[M-H]^-$, while MS^2 generated fragments at *m*/*z* 823, 661 and 499 which were attributed to the sequential loss of a deoxy sugar, followed by two hexosyl units. Acid hydrolysis of **5** gave glucose and rhamnose. Consequently a linear trisaccharide with a rhamnosyl terminal moiety is attached to the serjanic acid aglycone. The interglycosidic linkages and the linkage position of the sugar chain to the aglycone were solved by concerted use of 2D NMR spectroscopic techniques. The ^{1}H–^{1}H COSY experiment allowed sequential assignment of the proton resonances, while the HMQC spectrum led to unambiguous assignment of the carbon shifts (Table 2). In the HMBC spectrum, the anomeric proton signals at *δ* 6.42 (s, terminal rhamnose), 5.85 (*d*, *J* = 7.3 Hz, glucose) and 4.96 (*d*, *J* = 7.3 Hz, inner glucose) exhibited correlations with the carbon signals at *δ* 78.5 (C-2 of second glucose), 79.0 (C-2 of inner glucose) and 89.6 (C-3 of aglycone), respectively. Saponin **5** is thus 3-*O*-(α-L-rhamnopyranosyl-(→)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl) serjanic acid.

In the case of **6**, the ES mass spectrum gave a pseudomolecular ion at *m*/*z* 955 $[M-H]^-$, MS^2 fragments of this ion at *m*/*z* 809, 647 and MS^3 at *m*/*z* 647, 485, corresponding to the cleavage of a deoxy sugar, followed by two hexosyl moieties. The signal at *m*/*z* 485 was due to the deprotonated $[A-H]^-$ ion of spergulagenic acid. Acid hydrolysis revealed the presence of glucose and rhamnose in the sugar portion, as in **5**. In fact the ^{1}H and ^{13}C NMR spectra (Tables 2 and 3) of the sugar moieties of **5** and **6** were virtually identical. The interglycosidic linkages of **6** were confirmed to be the same as those found in **5** by an HMBC experiment, in which the anomeric proton signals at *δ* 6.41 (*s*, terminal rhamnose) and 5.84 (*d*, *J* = 7.8 Hz, middle glucose) exhibited correlations with the carbon signals at *δ* 78.5 (C-2 of middle glucose) and 79.0 (C-2 of inner glucose), respectively. Thus, saponin **6** is 3-*O*-(α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl) spergulagenic acid.

Saponins **7–9** gave pseudomolecular ions at *m*/*z* 985, 1131 and 985, respectively, in their ES mass spectra. All three were bidesmosidic saponins since basic hydrolysis with 0.1 M KOH gave the corresponding monodesmosidic saponins of spergulagenic acid (the methyl ester at C-30 was also hydrolysed) and glucose. Their structures were confirmed by analysis of the ^{1}H, ^{13}C and 2D NMR spectra. Saponin **7** was identified as 3-*O*-(β-D-galactopyranosyl-(1→3)-β-D-glucopyranosyl)serjanic acid 28-*O*-β-D-glucopyranoside and **9** as 3-*O*-(α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl) serjanic acid 28-*O*-β-D-glucopyranoside, both first isolated from *Phytolacca bogotensis* (Nielsen et al., 1995). Saponin **8** was identified as 3-*O*-(β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl) serjanic acid 28-*O*-β-D-glucopyranoside, found for the first time in the fruits of *P. thyrsiflora* (Haraguchi et al., 1988).

Saponins **7**, **8** and **9** are the corresponding bidesmosidic glycosides of **2**, **3** and **5**, respectively. Since all the major bidesmosides present in the methanol extract were isolated, the corresponding bidesmosides of **1**, **4** and **6** were either absent from this extract or only present in trace amounts. At the same time, saponins **1–6** were only found in the aqueous extract and not in the

Table 2
^{13}C NMR spectral data for the sugar moieties of saponins **3**–**6** (125 MHz in pyridine-d_5)

Carbon	3	4	5	6
1′ (Glc)	104.9	105.9	105.0	105.0
2′	83.4	74.6	78.9	79.0
3′	78.4	88.9	79.4	79.5
4′	71.7	69.7	72.8	72.8
5′	78.0	77.9	77.9	77.9
6′	62.7	62.6	62.6	63.4
1″ (Glc)	106.0	106.3	102.0	102.0
2″	77.2	72.0	78.5	78.5
3″	78.1	84.4	79.4	79.4
4″	71.6	69.5	72.6	72.6
5″	78.3	77.0	77.5	77.5
6″	62.8	62.0	62.7	62.8
1‴ (Glc)		106.2		
2‴		75.8		
3‴		78.3		
4‴		71.5		
5‴		78.6		
6‴		62.5		
1‴ (Rha)			101.9	101.9
2‴			72.4	72.4
3‴			71.9	71.9
4‴			74.3	74.4
5‴			69.5	69.5
6‴			19.0	19.0

methanol extract (TLC and HPLC analysis). The glycosides **3**–**5** have not previously been reported in the literature.

The isolated saponins were tested for their molluscicidal, spermicidal and haemolytic activities (Table 4). The three bidesmosidic saponins (**7**–**9**) from the methanol extract were all inactive against *Biomphalaria glabrata* snails. Saponins with molluscicidal activity were only isolated from the aqueous extract. All the glycosides from this extract (**1**–**6**) were monodesmosidic saponins. Of these, **1** and **2** were the most active, with minimum concentrations of 3.1 μg/ml required to kill the snails. Saponins **3** and **4** were also toxic to snails at the same concentration as that shown by the reference compound, 3-*O*-(β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl)bayogenin, one of the most active triterpene glycosides isolated from *P. dodecandra* (Domon and Hostettmann, 1984; Dorsaz and Hostettmann, 1986). Glycoside **5**, a trisaccharide with rhamnose as the terminal sugar, gave borderline toxicity to the snails. Saponin **6**, differing only from **5** in having a free C-30 carboxyl group, was inactive in the bioassay. The haemolytic activities of the saponins parallelled very closely the molluscicidal activities (Table 4). Again glycosides **1** and **2** showed the highest activities. The pattern was not as clear for the spermicidal activities (Table 4). The disaccharide **3** was more active than the monosaccharide derivative **1**. However, saponins **1**–**4** still remained the only compounds which gave positive results in this test, as in the case of haemolytic activity. It is interesting to note that the spergulagenic acid glycoside **6** was inactive in all three biological tests.

The high molluscicidal activity of the aqueous extract (25 μg/ml) can thus be attributed to the presence of monodesmosidic saponins of serjanic and spergulagenic acids. No monodesmosidic glycosides were isolated from the methanol extract and the corresponding weak molluscicidal activity (200 μg/ml) reflects the very low concentration of monodesmosidic saponins in this extract. At least in certain cases (**2**, **3**, **5**), the formation of monodesmosidic glycosides presumably occurs during extraction of the berries by water as a result of activation of enzymes present and cleavage of the glycoside chains at C-28 (**7**–**9**) (Domon and Hostettmann, 1984).

In view of the potent molluscicidal activity of the aqueous extract of the berries of *P. icosandra*, this could be a promising candidate plant for the local control of schistosomiasis in south-east Asia.

3. Experimental

3.1. General procedures

Optical rotations were measured with a Perkin-Elmer 241 MC polarimeter. ESI–MS were conducted using a Finnigan-MAT LCQ ion trap mass spectrometer in the negative ion mode. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were run on a Varian Inova Unity 500 spectrometer, in C_5D_5N. 2D experiments ^{1}H–^{1}H DQF-COSY (double quantum filtered direct chemical shift correlation spectroscopy), inverse detected ^{1}H–^{13}C HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multiple bond connectivity) were obtained using Varian VNMR software. LPLC was performed on Lobar RP-18 size B columns (Merck) and semi-prep. HPLC on a Waters PREPLC assembly with 2 Nova-Pak columns (PrepHR C18, 6 μm, 100×40 mm). For gel filtration, Sephadex LH-20 (Pharmacia) was used, with MeOH as eluent.

3.2. Plant material

Berries of *P. icosandra* were collected near Purwodadi in Java, Indonesia in October 1992. Voucher specimens have been deposited at the Herbarium Bogoriensis, Bogor, Indonesia and at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland.

3.3. Extraction and isolation

Dried berries (50 g) of *P. icosandra* were ground and extracted with H_2O (3×500 ml). After partition of this extract (15.7 g) between *n*-BuOH and H_2O, a part (4.4 g) of the *n*-BuOH fraction (4.5 g) was chromatographed on silica gel, with a $CHCl_3$–MeOH–H_2O (40:10:1→13:7:1) gradient, to give 14 fractions (I–XIV). LPLC of fr. II (MeOH–H_2O 70:30→75:25) gave saponin **1** (55 mg), while LPLC of fr. IX under the same conditions gave **5** (102 mg). Gel filtration of fr. IV yielded **2** (164 mg). Saponins **3** (4 mg, from fr. V) and **4** (32 mg, from fr. VII) were purified by a combination of silica gel chromatography ($CHCl_3$–MeOH–H_2O) and semi-prep HPLC (MeOH–H_2O 70:30). Fr. XIV contained **6** (20 mg), which was further purified by gel filtration (MeOH) and LPLC with MeOH–H_2O 60:40.

A second batch of berries (400 g) was extracted first with CH_2Cl_2 (3×2.5 l) and then with MeOH (3×2.5 l). The MeOH extract (33 g) was partitioned between *n*-BuOH and H_2O, and a portion (3 g) of the *n*-BuOH fraction (19 g) was chromatographed by CPC on a Pharma-Tech CCC-1000 instrument (capacity 650 ml). Elution with the solvent $CHCl_3$–MeOH–*i*-PrOH–H_2O 5:6:1:4 (lower phase as mobile phase) at a flow-rate of 3 ml/min gave a total of seven fractions (I–VII). Saponin **7** (236 mg) was obtained from fr. II after gel filtration, saponin **8** (23 mg) from fr. III after silica gel chromatography ($CHCl_3$–MeOH–H_2O 65:35:5) and saponin **9** (100 mg) from fr. VI after silica gel chromatography with the same solvent.

3.4. Saponin **1**

White amorphous powder. $[\alpha]_{\mathrm{D}}^{23}+62°\mathrm{C}$ (MeOH, *c* 0.1). Negative ion mode ES–MS *m/z*: 661 $[\mathrm{M-H}]^-$; MS^2 of *m/z* 661: *m/z* 499 $[\mathrm{A-H}]^-$.

3.5. Saponin **2**

White amorphous powder. $[\alpha]_{\mathrm{D}}^{23}+60°\mathrm{C}$ (MeOH, *c* 0.1). Negative ion mode ES–MS *m/z*: 823 $[\mathrm{M-H}]^-$; MS^2 of *m/z* 823: *m/z* 661, 499 $[\mathrm{A-H}]^-$.

3.6. Saponin **3**

White amorphous powder. $[\alpha]_{\mathrm{D}}^{23}+49°\mathrm{C}$ (MeOH, *c* 0.1). ^{13}C NMR: Tables 1 and 2. ^{1}H NMR: Table 3. Negative ion mode ES–MS *m/z*: 823 $[\mathrm{M-H}]^-$; MS^2 of *m/z* 823: *m/z* 661, 499 $[\mathrm{A-H}]^-$.

3.7. Saponin **4**

White amorphous powder. $[\alpha]_{\mathrm{D}}^{23}+47°\mathrm{C}$ (MeOH, *c* 0.1). ^{13}C NMR: Table 2. ^{1}H NMR: Table 3. Negative ion mode ES–MS *m/z*: 985 $[\mathrm{M-H}]^-$; MS^2 of *m/z* 985: *m/z* 823, 661, 499 $[\mathrm{A-H}]^-$.

3.8. Saponin **5**

White amorphous powder. $[\alpha]_{\mathrm{D}}^{23}+21°\mathrm{C}$ (MeOH, *c* 0.1). ^{13}C NMR: Table 2. ^{1}H NMR: Table 3. Negative ion mode ES–MS *m/z*: 969 $[\mathrm{M-H}]^-$; MS^2 of *m/z* 969: *m/z* 823, 661, 499 $[\mathrm{A-H}]^-$.

3.9. Saponin **6**

White amorphous powder. $[\alpha]_{\mathrm{D}}^{23}+22°\mathrm{C}$ (MeOH, *c* 0.1). ^{13}C NMR: Tables 1 and 2. ^{1}H NMR: Table 3. Negative ion mode ES–MS *m/z*: 955 $[\mathrm{M-H}]^-$; MS^2 of *m/z* 955: *m/z* 809, 647; MS^3 *m/z* 647, 485 $[\mathrm{A-H}]^-$.

3.10. Acid hydrolysis of saponins

Saponin (1 mg) was heated at reflux with 5 ml of 2 N HCl–MeOH (1:1) for 2 h. The MeOH was evaporated and the aqueous solution was extracted with EtOAc. The aglycone in the EtOAc layer was analysed by TLC [(i-Pr)$_2$O–acetone 75:30]. The aqueous layer was evaporated to dryness and monosaccharides were identified by comparison with authentic samples on TLC (i-PrOH–H$_2$O 85:15), after detection with *p*-anisidine phthalate.

Table 3
^{1}H NMR spectral data for the sugar moieties of saponins **3**–**6** (500 MHz in pyridine-d_5)[a]

Proton	**3**	**4**	**5**	**6**
1′ (Glc)	4.94 *d* (7.8)	4.88 *d* (7.8)	4.96 *d* (7.3)	4.95 *d* (7.3)
2′	4.37 *dd* (8.8, 7.8)	4.03 *dd* (8.9, 7.8)	4.43 *dd* (8.9, 7.3)	4.42 *dd* (8.8, 7.3)
3′	4.29 *m*	4.32 *dd* (8.9, 8.9)	4.56 *dd* (8.9, 8.9)	4.55 *dd* (8.8, 8.8)
4′	4.17 *dd* (8.8, 8.8)	4.04 *dd* (8.9, 8.9)	4.10 *dd* (8.9, 8.9)	4.09 *dd* (8.8, 8.8)
5′	3.94 *m*	4.01 *m*	3.94 *m*	3.92 *m*
6′a	4.57 *dd* (12.0, 2.5)	4.52 *dd* (11.5, 2.0)	4.51 *dd* (12.0, 2.5)	4.51 *dd* (12.0, 2.5)
6′b	4.39 *dd* (12.0, 5.5)	4.31 *m*	4.35 *dd* (12.0, 6.0)	4.34 *dd* (12.0, 6.0)
1″ (Glc)	5.39 *d* (7.8)	5.27 *d* (7.3)	5.85 *d* (7.3)	5.84 *d* (7.8)
2″	4.14 *dd* (8.8, 7.8)	4.70 *dd* (9.0, 7.3)	4.30 *dd* (8.9, 7.3)	4.28 *dd* (8.9, 7.8)
3″	4.20 *m*	4.31 *m*	4.25 *dd* (8.9, 8.9)	4.24 *dd* (8.9, 8.9)
4″	4.33 *dd* (8.8, 8.8)	4.40 *dd* (9.0, 9.0)	4.08 *dd* (8.9, 8.9)	4.07 *dd* (8.9, 8.9)
5″	3.96 *m*	3.93 *m*	3.87 *m*	3.83 *m*
6″a	4.50 *dd* (11.5, 2.4)	4.52 *dd* (12.0, 2.2)	4.48 *dd* (12.0, 2.2)	4.46 *dd* (12.0, 2.5)
6″b	4.49 *dd* (11.5, 5.6)	4.26 *dd* (12.0, 6.0)	4.32 *dd* (12.0, 6.0)	4.31 *dd* (12.0, 6.0)
1‴ (Glc)		5.39 *d* (7.8)		
2‴		4.09 *m*		
3‴		4.26 *dd* (8.9, 8.9)		
4‴		4.22 *dd* (8.9, 8.9)		
5‴		4.11 *m*		
6‴a		4.70 *dd* (12.0, 2.5)		
6‴b		4.39 *dd* (12.0, 6.0)		
1‴ (Rha)			6.42 *s*	6.41 *s*
2‴			4.76 *br s*	4.75 *br d* (3.4)
3‴			4.71 *dd* (9.2, 3.1)	4.69 *dd* (9.3, 3.4)
4‴			4.36 *dd* (9.2, 9.2)	4.36 *dd* (9.3, 9.3)
5‴			5.06 *dq* (9.2, 6.3)	5.04 *dq* (9.3, 6.3)
6‴			1.82 *d* (6.3)	1.81 *d* (6.3)

[a] Assignments based on COSY, HMQC and HMBC spectra.

Table 4
Molluscicidal, spermicidal and haemolytic activities of saponins isolated from *Phytolacca icosandra*

Saponin	1	2	3	4	5	6	7	8	9	Control[a]
Molluscicidal activity[b]	3.1	3.1	10.0	12.5	50.0	> 50	> 50	> 50	> 50	12.5
Spermicidal activity[c]	700	500	250	1333	> 2000	> 2000	> 2000	> 2000	> 2000	500
Haemolytic activity[d]	1.9	3.8	7.5	7.5	> 60	> 60	> 60	> 60	> 60	7.5

[a] 3-*O*-(β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl)bayogenin.

[b] Minimum concentration required to kill 100% of snails (*Biomphalaria glabrata*) after 24 h (μg/ml).

[c] Minimum concentration required to cause 100% inhibition of the motility of human spermatozoids after 3 min (μg/ml).

[d] Minimum concentration for total haemolysis of bovine erythrocytes (μg/ml).

3.11. Alkaline hydrolysis of bidesmosidic saponins

Saponin (1 mg) was heated at reflux with 10 ml of 0.1 N KOH for 1 hour. The cooled reaction mixture was then neutralised with 1 N HCl and then extracted with *n*-BuOH. The aqueous layer was analysed for sugars (as above) and the *n*-BuOH layer was evaporated to dryness and checked by TLC with the solvent CHCl$_3$–MeOH-H$_2$O 65:35:5.

3.12. Molluscicidal activity

Molluscicidal activity was determined by the method of Hostettmann et al. (1982). Extracts were initially tested at 400 μg/ml and pure compounds at 50 μg/ml in water. Toxicity to *Biomphalaria glabrata* snails was established by observing heart beat under a microscope after 24 h. Successive dilutions of the test solutions were made in order to find the minimum concentration required to kill snails.

3.13. Spermicidal activity

Pure saponin (1.2 mg) was dissolved in 0.9% NaCl (600 μl) to give a concentration of 2000 μg/ml. To 100 μl of this solution was added 20 μl of fresh human sperm. One drop of the resulting mixture was placed in a Neubauer-Improved cell (Assistent A.G., Altnau, Switzerland) and motility of the sperm was observed under a microscope (40×magnification) after 3 min. In the event of a positive result, geometric dilutions were tested to establish the minimum concentration of saponin sufficient for total inactivation of sperm motility. Tests were repeated three times for each concentration, with sperm from three different donors (Waller et al., 1980).

3.14. Haemolytic activity

The method of Romussi et al. (1980) was used to determine the lowest concentration of saponin required to produce total haemolysis. Pure saponin (0.6 mg) was dissolved in 0.9% NaCl (10 ml) to give an initial concentration of 60 μg/ml and then geometric dilutions were tested with bovine erythrocytes until haemolysis was no longer observed. The suspension of erythrocytes was prepared by washing bovine blood (5 ml) with 0.9% isotonic solution (5 ml) and centrifuging. The supernatant was removed and the washing procedure repeated until the supernatant remained colourless. These erythrocytes were mixed with an equal volume of 0.9% NaCl and 50 μl of this suspension was added to the solutions under test. After 1 h, the mixture was centrifuged and the transmittance of the supernatant was measured by a spectrophotometer at 540 nm, using the supernatant resulting after the washing procedure as blank.

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