

Soyasaponins and Related Glycosides of *Desmodium canadense* and *Desmodium illinoense*

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Abstract: Seed extracts of *Desmodium canadense* (L.) DC., a native Canadian legume, were examined by HPLC and electrospray ionization mass spectrometry (LC/MS) for the presence of triterpenoid saponins of the oleanene type. An aqueous methanol extract, fractionated by use of a Diaion™ HP-20 macroporous resin, was found to possess soyasaponin III (soyasaponin Bb') and the 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one conjugate of soyasaponin III (DDMP S-III) as major saponin components. Minor components were identified by LC/MS as C-24 aldehyde (sandosapogenol) derivatives, S-III al and DDMP S-III al. Soyasaponin I (soyasaponin Bb), soyasaponin VI (soyasaponin βg) and dehydrosoyasaponin I (soyasaponin Be) were also found as minor components. These components were also detected in extracts obtained by Soxhlet extraction with water followed by partitioning into *n*-butanol. Extracts from foliage of greenhouse and field origins were essentially free of these saponins. Soyasaponin VI was the main seed saponin of *Desmodium illinoense* A. Gray whereas soyasaponin III and DDMP S-III were found as minor components. Components corresponding in molecular mass to C-22 glycosides of soyasaponin I and soyasaponin III were also detected in saponin-enriched extracts. Neither species was a good source of alkaloids.

Keywords: *Desmodium canadense*, *Desmodium illinoense*, soyasaponins, electrospray mass spectrometry.

INTRODUCTION

Desmodium canadense (L.) DC., commonly known as Canada tick trefoil, showy tick trefoil or beggar's lice, is a native perennial legume flower of central Canada and north-eastern United States. Although 275 species of *Desmodium* (Fabaceae) have been identified throughout the world [1] and many of these species have demonstrated diverse pharmacological effects [2], phytochemical and ethnomedical information is sparse on *D. canadense*. In 1973, three flavonoids were isolated from the aerial parts of *D. canadense* grown in Russia [3]. A flavonoid from *D. canadense* was subsequently reported to have pronounced analgesic activity in mice [4]. In other animal models, antiinflammatory activity attributed to flavonoids [5] and a nephroprotective effect attributed to antioxidative phenolic substances [6] have also been described for dry extracts of *D. canadense*. Diak [7] tested alcoholic extracts of *D. canadense* for alkaloids and amines, but none were identified.

A few species of *Desmodium* are known to be rich sources of triterpenoid saponins of the soyasaponin type (aglycones of soyasapogenols B and E), like many other legume plants. Leaves of *D. adscendens*, a tropical shrub of medicinal interest [8], furnished soyasaponin I (S-I), dehydrosoyasaponin I (D-I or DHS-I) and soyasaponin III (S-III) by Soxhlet extraction with water and countercurrent chromatography [9]. S-I and D-I (Fig. 1) were also isolated from whole plants of *D. styracifolium* [10, 11]. In addition, soyas-

aponin VI (S-VI), the unstable precursor of S-I in soybeans and peas [12, 13], has been detected by thin layer chromatography (TLC) in extracts of *D. adscendens* from different geographical origins of Africa [14]. Phytochemical tests on the aerial parts of *D. triangulare* (= *cephalotes*) and *D. scorpiurus* revealed the presence of saponins [15, 16] whereas foliage of *D. uncinatum* (silverleaf desmodium) grown in Zimbabwe was reported to be free of these compounds [17]. Phytochemicals of *D. uncinatum* and of other species such as *D. canum*, *D. gangeticum*, *D. heterocarpon*, *D. sequax* and *D. tortuosum* have been studied in some detail but the occurrence of soyasaponins has not been reported. Antimicrobial activity of aqueous ethanolic extracts of *D. illinoense* and *D. canadense* has recently been described [18].

The objectives of the present work were to study seed and foliage extracts of *D. canadense* for saponin components and to identify the main saponins by electrospray mass spectrometry (LC/MS). Aqueous methanolic extracts from ground seeds of *D. illinoense* (Illinois tick clover or prairie tick trefoil) were also examined for soyasaponins by HPLC and LC/MS.

MATERIALS AND METHODS

Materials

Reference samples of S-I and S-III were obtained from ChromaDex (Irvine, CA). Seeds of *D. canadense* and *D. illinoense* were purchased from Ion Exchange (Harpers Ferry, IA). Voucher samples were deposited in Plant Gene Resources of Canada. Flours were obtained with a Wiley mill by sequentially grinding through 20 and 40 mesh screens. Foliage of *D. canadense* was obtained by potting the seeds and growing the plants in a greenhouse (Saskatoon,

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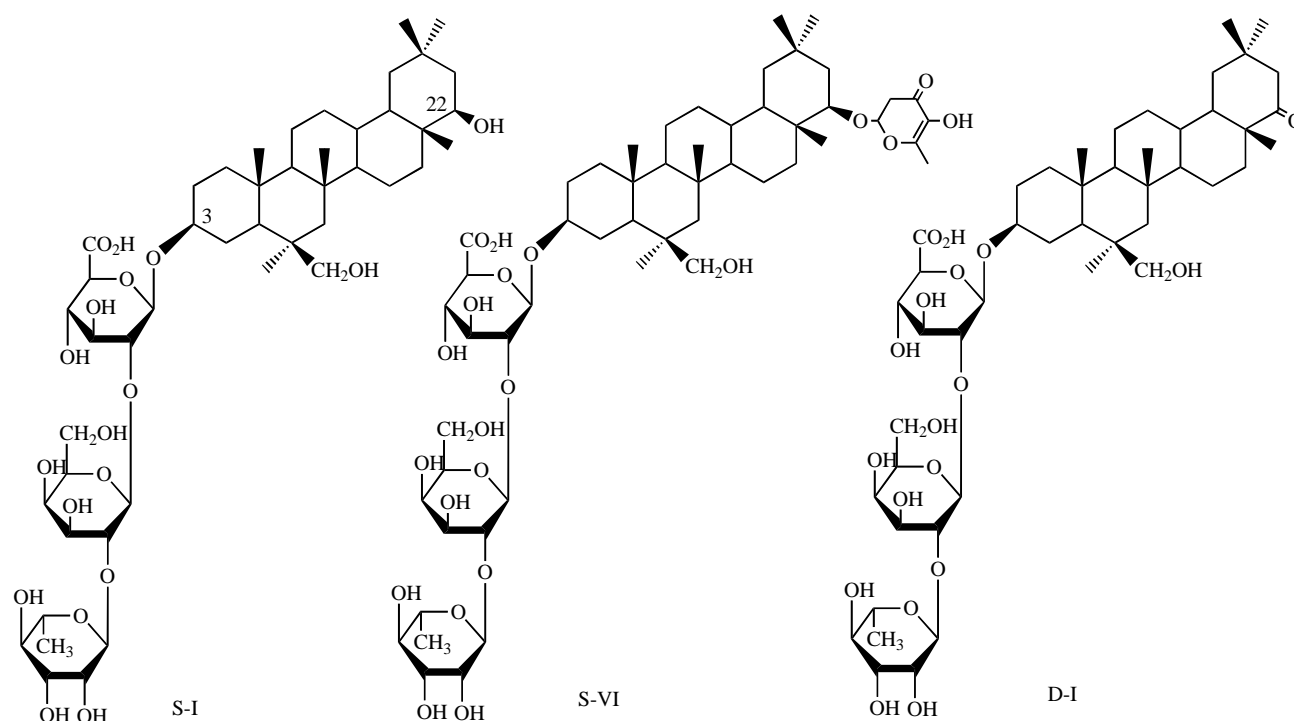


Fig. (1). Structures of S-I, S-VI and D-I.

SK). Leaves from the first growth (in 2003) were collected at the flowering stage and processed by methods A, B and C (see below). During 2004, an additional collection of leaves from the same plants was obtained for processing by method B. Leaves from additional new plants were collected in 2004 for processing (method B). About forty new plants started in the greenhouse in 2004 were transferred in the spring of 2005 to a field plot located at the Agriculture and Agri-Food Canada Research Farm (Saskatoon, SK). The field-grown leaves were collected in September 2005 and processed, again by method B. The plants survived during the winter of 2005-06 and additional leaves from the field were collected in September 2006. The leaves, frozen at $-70\text{ }^{\circ}\text{C}$, were prepared for extraction by freeze-drying followed by grinding with a Wiley mill equipped with a 40 mesh screen.

Additional seed samples of *D. canadense* (also obtained from Ion Exchange) and *D. illinoense* (obtained from Prairie Moon Nursery, Winona, MN) were ground with a Retsch centrifugal mill (model ZM 200) equipped with a 0.5 mm sieve. These flours, defatted with hexane, were processed by method A.

Extraction Methods

(A). Defatted flour and leaf materials (50-100 g) were extracted with hot 80% MeOH according to a previous method [19]. After rotary evaporation and dilution with water, the saponins were isolated with Diaion HP-20 beads (particle size 250-600 μm ; porosity 300-600D) from a cartridge (Bio-tage Inc., Charlottesville, VA). After collecting the beads by filtration (the filtrate was designated as the 15% MeOH extract), the beads were washed with 30% MeOH (500 mL). The beads were additionally washed with MeOH (500 mL). The methanol filtrate was removed by rotary evaporation and the residue remaining in the flask transferred to a test tube. Evaporation was completed at $43\text{ }^{\circ}\text{C}$ with a centrifugal

evaporator (model SC 110A Savant SpeedVac Plus) and the brown powder that remained, designated as an HP-20 MeOH fraction, was dried under vacuum in a desiccator before analysis. The 15% MeOH extract and 30% MeOH extract were evaporated and dried by the same techniques.

(B). Using procedures from McManus *et al.* [9], the dried, ground seed or leaf material (25 g) was transferred to a cellulose extraction thimble (Whatman 43 x 123 mm) and extracted for 2 d with water (300 mL). The mixture was concentrated by rotary evaporation (or by freeze drying of foaming samples followed by addition of 50 mL of water) and extracted three times with *n*-butanol (50 mL). Combined *n*-butanol extracts were concentrated to near dryness on a rotary evaporator. Methanol was added, followed by rotary evaporation. Solvent evaporation was completed in a test tube with a Savant apparatus.

(C). With procedures adopted from Silva *et al.* [20], the ground seed or leaf material (25 g) was transferred to a cellulose extraction thimble (Whatman 43 x 123 mm) and extracted for 2 h with hexane (150 mL). The solvent was decanted and the hexane fraction (expected to contain nonpolar lipids) was obtained by rotary and Savant evaporation. After air-drying the hexane-insoluble material, 80% MeOH (350 mL) was added and Soxhlet extraction was continued for 18 h. The alcohol was removed by rotary evaporation, lowering as necessary the bath temperature to about $15\text{ }^{\circ}\text{C}$ to reduce foaming and suppress bumping. The mixture that remained (ca. 50 mL) was cooled (ice bath), acidified with 1M HCl (50 mL) and, after stirring for 15 min, the acidified extract was extracted with methyl *t*-butyl ether (MTBE, 3 x 30 mL). The combined MTBE extracts were washed with water (30 mL) and the lower aqueous wash was combined with the acidic aqueous phase. An MTBE extract (expected to contain neutral and acidic compounds) was obtained by rotary and

Savant evaporation. The aqueous phase, adjusted with stirring to pH 10.5 by addition of concentrated ammonium hydroxide, was extracted with chloroform (4 x 35 mL). The combined chloroform extracts (expected to contain alkaloids) were evaporated to dryness. The aqueous phase that remained (expected to contain quaternary alkaloids and other water-soluble components) after neutralization to pH 7 with HCl was freeze-dried. Masses of the four isolated fractions were obtained before subsamples were removed for HPLC/ELSD, LC/MS and TLC analyses.

Alkaline Hydrolysis

A 504 mg sample of the Diaion HP-20 MeOH extract from seed flour of *D. canadense* was dissolved in 25 mM NaOH solution (25 mL) and stored for 18 h at 4 °C [13]. The mixture was neutralized with 10% HCl and taken to dryness with a rotary evaporator and Savant apparatus. A brown powder was obtained for analyses by HPLC and LC/MS. A sample of the Diaion HP-20 MeOH extract from *D. illinoense* was hydrolysed by the same techniques.

HPLC

The instrument consisted of an Alliance 2695 separations module (Waters Canada, Mississauga, ON) equipped with a Waters 996 photodiode array detector (PDA) and a PL-EMD-960 evaporative light scattering detector (ELSD) (Polymer Laboratories, Amherst, MA) controlled by Waters Millennium or Empower software. Samples were prepared in 80% MeOH at 4 or 8 mg/mL and syringe filtered (0.45 µm) before injection (5 or 10 µL) onto a Waters reversed phase C18 Symmetry™ column (3.0 x 150 mm, 5 µm particle size) maintained at 30 °C. The mobile phase (0.4 mL/min) consisted of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (solvent B). The gradient elution program consisted of 95% A and 5% B at time 0. The composition was 65% A and 35% B after 10 min. The linear gradient progressed to 50% A and 50% B over 15 min and to 5% A - 95% B over 5 min. The column was held at 5% A - 95% B for 5 min before reverting to 95% A and 5% B from 35 to 43 min.

LC/MS Techniques

Positive ion electrospray ionization (ESI) mass spectra were obtained with a bench top tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Limited) equipped with an atmospheric pressure ESI source interfaced directly to a Waters Alliance 2695 separations module. Nitrogen gas was used for nebulization and desolvation. The instrument was controlled by Micromass MassLynx software (version 3.3 and higher). A C18 Symmetry™ column (2.1 x 150 mm, 5 µm) held at 30 °C was used for LC/MS. The mobile phase (0.2 mL/min) consisted of 0.1% formic acid in water (solvent A) or acetonitrile (solvent B). The starting mixture was 95% solvent A - 5% solvent B (65% A and 35% B after 10 min, 50% A and 50% B over 15 min and 5% A - 95% B over 5 min, then held at 5% A - 95% B for 5 min). Collision induced dissociation (CID) experiments were conducted in the positive ion mode with argon and collision energies of 30-35 eV. Quasimolecular ions (MH⁺) were used to generate product ion spectra.

Thin Layer Chromatography

Precoated silica gel 60 F₂₅₄ plastic sheets (Merck) of 0.2 mm layer thickness were developed with solvent mixtures of the lower layer of chloroform-methanol-water (65-35-10), with *n*-butanol-ethanol-ammonia (7-2-5) and occasionally with *n*-butanol-acetic acid-water (4-1-2). Samples for spotting were prepared at 4 mg/mL in 80% MeOH. After development, the plates were examined under UV light and then sprayed with ninhydrin, Liebermann-Burchard, molybdenum blue or phosphomolybdic acid.

RESULTS AND DISCUSSION

Starting with defatted flour prepared from commercial seeds of *D. canadense* followed by extraction with hot 80% MeOH, three fractions were isolated by Diaion HP-20 treatment of the crude methanol extract, designated as HP-20 15% MeOH, HP-20 30% MeOH and HP-20 MeOH. It was anticipated that saponins would be enriched in the HP-20 MeOH fraction, based on experience with other legume species [19]. Indeed, TLC examination of this fraction, isolated in relatively high yield (1.9%), revealed the presence of at least three soyasaponins, detected as purple-grey spots following spraying with the Liebermann-Burchard reagent. It appeared that S-I and S-VI, common saponins of legume seeds, were minor components. TLC spots attributed to soyasaponins were undetectable in the other fractions including the extract from defatting with chloroform. The 15% MeOH fraction contained the most material (16.7% yield) and some of the components gave ninhydrin-positive (pink) spots indicative of amino acids or proteins. The yield of the Diaion HP-20 MeOH fraction from a second batch of *D. canadense*, purchased from the same source but five years later, was 3.3%.

Examination of the HP-20 MeOH fraction by HPLC/ELSD gave the chromatogram shown (Fig. 2). Retention time comparisons supported the view that S-I, S-VI and D-I were present in relatively low concentrations. Additional

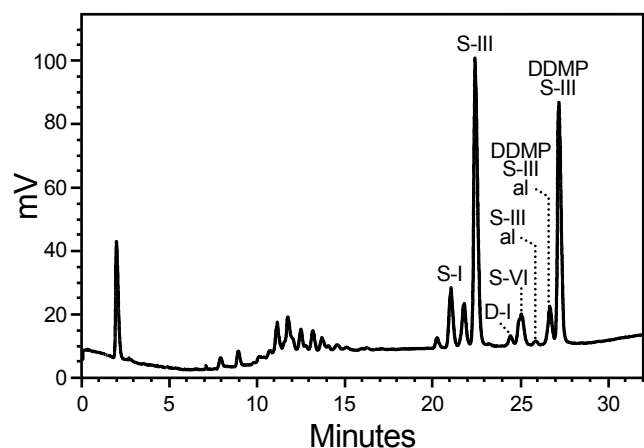


Fig. (2). HPLC/ELSD trace of a Diaion HP-20 MeOH extract from a chloroform-defatted flour of *D. canadense* seeds. Labelled peaks are as follows: S-I, soyasaponin I; S-III, soyasaponin III; D-I, dehydrosoyasaponin I; S-VI, soyasaponin VI; S-III al, C-24 aldehyde of S-III; DDMP S-III al, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one conjugate of S-III al; DDMP S-III, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one conjugate of S-III.

Table 1. Product Ions Observed during CID Experiments on Soyasaponin I, Dehydrosoyasaponin I and Soyasaponin VI^a

Sample	MH ⁺	(M - rha + H) ⁺	(M - rha - gal + H) ⁺	(M - rha - gal - H ₂ O + H) ⁺	(M - rha - gal - 2H ₂ O + H) ⁺	(aglycone ^b + H) ⁺	(aglycone + H - H ₂ O) ⁺	(aglycone + H - 2H ₂ O) ⁺	(aglycone + H - 3H ₂ O) ⁺
S-I ^c	943 (100)	797 (30)	635 (20)	617 (10)	599 (25)	459 (5)	441 (55)	423 (25)	405 (5)
S-I ^d	943 (100)	797 (40)	635 (20)	617 (15)	599 (30)	459 (15)	441 (55)	423 (40)	405 (5)
D-I ^d	941 (100)	795 (15)	633 (25)	615 (30)	597 (15)	457 (25)	439 (100)	421 (90)	403 (<2)
S-VI ^d	1069 (80)	923 (55)	761 (20)	743 (10)	725 (20)	459 ^e (<2)	441 ^f (30)	423 ^g (50)	405 ^h (30)

^aThe collision energy was set at 30 eV. Numbers shown are *m/z* values, with the relative intensities of the ions in brackets.

^bThe aglycone of S-I (and S-VI) is soyasapogenol B (molecular mass of 458). The aglycone of D-I is soyasapogenol E (456).

^cReference sample purchased from ChromaDex (Irvine, California).

^dData obtained during LC/MS on a HP-20 MeOH extract from seeds of *D. canadense*. Identification by LC/MS does not exclude closely-related isomers.

^{e-h}The pseudo aglycone of S-VI with DDMP group at C-22 gave corresponding ions at *m/z* 585 (15%), 567 (100), 549 (15) and 531 (<2).

evidence for the identity of these minor saponins in extracts of *D. canadense* was obtained from comparisons to authentic samples by LC/MS. These saponins gave prominent quasi-molecular ions under scanning conditions whereas characteristic product ions were observed in CID experiments (Table 1). But the major components required further study.

A major component eluting at 22.5 minutes gave a quasi-molecular ion at *m/z* 797 corresponding to the molecular mass of S-III (Fig. 3). This saponin occurred as a minor component of soybeans [21] and certain other legume plants, including *D. adscendens* [9]. Evidence from CID experiments (Table 2) indicated the presence of S-III in the HP-20

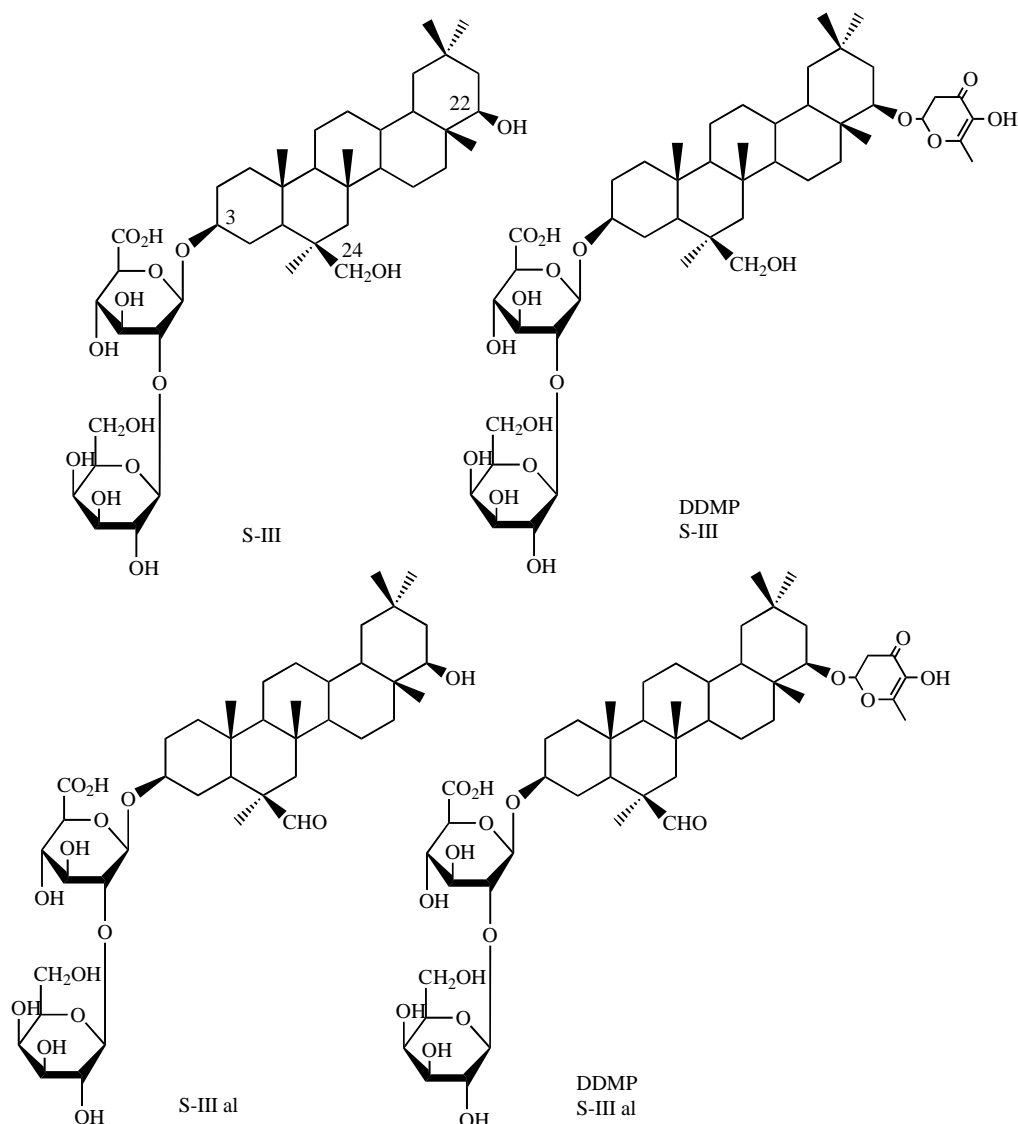
**Fig. (3).** Structures of S-III, DDMP S-III, S-III al and DDMP S-III al.

Table 2. Product Ions Observed during CID Experiments on Soyasaponin III and the DDMP Conjugate of Soyasaponin III^a

Sample	MH ⁺	(M - gal + H) ⁺	(M - gal - H ₂ O + H) ⁺	(M - gal - 2H ₂ O + H) ⁺	(aglycone ^b + H) ⁺	(aglycone + H - H ₂ O) ⁺	(aglycone + H - 2H ₂ O) ⁺	(aglycone + H - 3H ₂ O) ⁺
S-III ^c	797 (100)	635 (5)	617 (8)	599 (25)	459 (5)	441 (70)	423 (40)	405 (5)
S-III ^d	797 (100)	635 (15)	617 (8)	599 (30)	459 (5)	441 (65)	423 (35)	405 (<5)
DDMP S-III ^d	923 (100)	761 (10)	743 (15)	725 (10)	459 ^e (<2)	441 ^f (15)	423 ^g (50)	405 ^h (15)

^aThe collision energy was set at 30 eV. Numbers shown are *m/z* values, with the relative intensities of the ions in brackets.

^bThe aglycone of S-III (and DDMP S-III) is soyasapogenol B (molecular mass of 458).

^cReference sample purchased from ChromaDex (Irvine, California).

^dData obtained during LC/MS on a HP-20 MeOH extract from seeds of *D. canadense*. Identification by LC/MS does not exclude closely-related isomers.

^{e-h}The pseudo aglycone of DDMP S-III with the DDMP group at C-22 gave corresponding ions at *m/z* 585 (5%), 567 (40), 549 (10) and 531 (<2).

MeOH fraction of *D. canadense*. A second major component, eluting at about 27.3 minutes in Fig. (2), corresponded in molecular mass to DDMP S-III (MH⁺ 923 Da). This assignment was supported by CID experiments (Table 2), including the appearance of ions for the expected pseudo aglycone, and by alkaline hydrolysis of DDMP S-III in the mixture to S-III.

Two other small components, tentatively identified as S-III al (MH⁺ 795 Da) and DDMP S-III al (921 Da), were found in the saponin seed extract (Table 3). The aglycone of this aldehyde (3,22-dihydroxy-12-oleanen-24-al or sandosapogenol) has been reported as a sapogenin of French bean (*Phaseolus vulgaris*) seed [22]. In theory, the component with MH⁺ 795 Da could correspond to the C-22 ketone of S-III but this was considered an unlikely possibility. Further, ions were found for the expected pseudo aglycone (with a DDMP group at C-22) and the concentration of S-III al appeared to increase following alkaline hydrolysis.

The seven saponins of *D. canadense* were readily detected in the Diaion HP-20 MeOH extract. In contrast, none of these saponins were found in the Diaion HP-20 30% MeOH fraction. All three Diaion HP-20 fractions showed some earlier eluting saponins in low concentrations that were tentatively attributed to higher glycosides of S-III, probably 22-O-glycosides of S-III (Table 4). For example, glycosides with MH⁺ 1091 and 1121 were found during LC/MS (eluting at 15.7 and 17.5 minutes) and their mass spectra showed an ion at *m/z* 797 corresponding to the possible loss of a disaccharide moiety (- 294 or 380 Da) by cone voltage fragmentation. Although glycosides of S-III with this mass were not found in the literature, the corresponding 22-O-diglycosides of S-I (with masses of 1237 and 1267) have been described and corresponded respectively to 22-O-[β-D-glucopyr-

anosyl-β-D-xylopyranoside] (subproside VII) and 22-O-[β-D-glucopyranosyl-β-D-glucopyranoside] (bersimoside I) [23, 24]. In the Diaion HP-20 MeOH extract, another small peak eluting at 16.5 minutes during LC/MS with MH⁺ 959 (and again with an accompanying ion at *m/z* 797) could represent a 22 - O - monoglucoside of S-III but a 24 - O - monoglucoside of S-III or soyasaponin V, with a glcUA - gal - glc moiety at C-3 and a C-22 OH, were also possible. Very small peaks with MH⁺ of 1177 and 1207 were also found.

We compared the distribution of saponins in extracts prepared by different techniques. Saponins are often isolated initially by water extraction then by extraction of the aqueous phase with *n*-butanol (method B). Following these techniques, a fraction was isolated that showed by LC/MS a similar saponin profile to the Diaion HP-20 MeOH fraction.

Alkaloids occur in several *Desmodium* species [see for example, 25-29] so it was of interest to examine *D. canadense* using a conventional extraction procedure for alkaloids (method C) followed by analysis of the extracts by TLC, HPLC and LC/MS. After extraction of the defatted flour with aqueous methanol, the acidified aqueous extract on extraction with MTBE gave a black semi-solid containing most of the saponin components detected previously (Table 4). Following adjustment to alkaline pH and extraction with chloroform, all of the previously detected saponins were readily found in the chloroform extract as well. Examination of this extract by LC/MS showed low concentrations of four unidentified components (possibly alkaloids) but the main components by far were saponins. Saponins were also detected as the main components in the remaining aqueous extract, after lyophilization. The procedures of method C illustrated the complications that can occur with saponins that are partially soluble in organic solvents.

Table 3. Product Ions Observed during CID Experiments on an Extract of *D. canadense* containing the 24-aldehyde of Soyasaponin III (S-III al) and DDMP S-III al^a

Sample	MH ⁺	(M - gal + H) ⁺	(M - gal - H ₂ O + H) ⁺	(M - gal - 2H ₂ O + H) ⁺	(aglycone ^b + H) ⁺	(aglycone + H - H ₂ O) ⁺	(aglycone + H - 2H ₂ O) ⁺	(aglycone + H - 3H ₂ O) ⁺
S-III al ^c	795 (100)	633 (10)	615 (95)	597 (40)	457 (5)	439 (90)	421 (40)	403 (10)
DDMP S-III al ^c	921 (40)	759 (30)	741 (5)	723 (<2)	457 ^d (<2)	439 ^e (40)	421 ^f (20)	403 ^g (<2)

^aThe collision energy was set at 30 eV. Numbers shown are *m/z* values, with the relative intensities of the ions in brackets.

^bThe aglycone of S-III al (and DDMP S-III al) is 3,22-dihydroxy-12-oleanen-24-al (sandosapogenol, molecular mass of 456).

^cData obtained during LC/MS on a HP-20 MeOH seed extract. Identification by LC/MS does not exclude closely-related isomers.

^{d-g}The pseudo aglycone of DDMP S-III al with the DDMP group at C-22 gave corresponding ions at *m/z* 583 (100%), 565 (15), 547 (5) and 529 (<5).

Table 4. Soyasaponins from Seed of *D. canadense* Detected by Electrospray LC/MS

Extraction procedure	Flour (g)	Fraction	Extract (g)	Color	Soyasaponins (MH ⁺)							22 - O - glycosides of S-III	22 - O - glycosides of S-I
					S-III al (795)	S-III (797)	DDMP S-III al (921)	DDMP S-III (923)	D-I (941)	S-I (943)	S-VI (1069)		
(A) ^a	91.5	15% MeOH	15.258	brown semi-solid	- ^b	+	-	-	-	±	-	+ ^c	- ^d
		30% MeOH	0.078	brown semi-solid	-	-	-	-	-	-	-	+	-
		100% MeOH	1.734 ^e	brown powder	+	+	+	+	+	+	+	+	±
(B)	25.0	H ₂ O/ <i>n</i> -BuOH	0.207 ^e	brown solid	+	+	-	+	+	+	+	+	±
(C)	50.7	hexane	5.458	light green oil	-	-	-	-	-	-	-	-	-
		MTBE	1.630 ^e	black semi-solid	±	+	+	+	±	+	+	-	-
		CHCl ₃	0.151 ^{e,f}	brown solid	+	+	+	+	+	+	+	+	±
		aqueous	11.466 ^e	brown powder	+	+	+	+	±	+	+	+	±

^aA brown oil obtained from chloroform defatting of the flour did not show any soyasaponins.

^bThe indicated saponins were detected (+), were probably detected at trace concentrations (±) or were undetectable (-) as determined by LC/MS.

^cA + symbol in this column indicates detection of at least two MH⁺ ions of *m/z* 929, 943, 959, 1075, 1105, 1091, 1121, 1177, 1207 and 1253.

^dMH⁺ ions of *m/z* 1075, 1089, 1105, 1221, 1237, 1251, 1267 and 1399 were not found (-) or not consistently found (±).

^eIn these fractions, a component eluting just before S-I (at 23.3 min) and with a MH⁺ ion of *m/z* 963 might also be a saponin.

^fFour unidentified components of the chloroform fraction showed MH⁺ ions of *m/z* 127, 219, 279 and 355.

It was of interest to examine the foliage of *D. canadense* for saponins found in the seed. Plants were therefore grown, initially in a greenhouse (for 3 harvests of leaves) and then for two years in a field plot (2 harvests). Leaves collected at the flowering stage or after flowering were processed by methods A, B or C (Table 5). Using methods A and C, none of the leaf extracts gave detectable levels of saponins on examination by LC/MS. Extracts obtained by method B gave detectable or probably detectable levels of S-I and S-III in leaf extracts from greenhouse-grown leaves. No DDMP or other C-22 conjugated forms were found at all. Levels of S-I and S-III were extremely low compared to seed levels. Leaves from field-grown plants did not show any known saponins in their extracts. Under the greenhouse and field conditions that were studied, the foliage of *D. canadense* was an extremely poor source of saponins, like *D. uncinatum* [17] but unlike *D. adscendens* [9, 14], *D. scorpiurus* [16] and *D. triangulare* [15]. Of course, control of glycoside levels in the leaves of legume plants could be dependent on additional morphological, environmental or genetic factors, see for example Moriyama et al. [30] and Kim et al. [31]. The influence of such factors on saponin levels is an area requiring further study.

The literature revealed that S-III was more active than S-I as a hepatoprotective agent in a rat hepatocyte model [32, 33]. A similar trend was noted with human liver cells

(HepG2), although the monoglucuronide showed the most potent hepatoprotective activity, followed by soyasapogenol B [34]. Concentrated extracts of soyasapogenol A and soyasapogenol B were recently shown to effectively inhibit the proliferation of cultured HepG2 cells [35]. S-III and soyasapogenol B monoglucuronide were marginally bioactive against colon cancer cells whereas S-I was not [36]. On the other hand, S-I may be useful in treatment of polycystic kidney disease [37]. Soyasaponin-enriched extracts or purified isolates of *D. canadense* might therefore prove to be valuable for management of various disorders.

Lastly, we studied the triterpenoid saponins from seeds of *D. illinoense*, a species that overlaps in habitat with *D. canadense*. Table 6 gives the soyasaponins detected in flour extracts from methods A-C. With the exception of S-III al and DDMP S-III al, the distribution of saponins was similar in extracts from these species, but the main saponin component of *D. illinoense* was S-VI (Fig. 4). Relative concentrations of S-I and D-I appeared to be higher whereas S-III and DDMP S-III were lower than in *D. canadense*. In two experiments with seeds of different origins, yields of the saponin-enriched HP-20 MeOH fractions were 3.4% (Ion Exchange) and 2.6% (Prairie Moon Nursery), approximately a six-fold increase compared to lentils [19]. Apart from elevated yields, the saponin profile of *D. illinoense* was reminiscent of the profile of common legume crops such as

Table 5. Properties of Leaf Extracts from *D. canadense*

Year	Location	Stage of leaf collection	Extraction procedure	Amount extracted (g)	Fraction	Extract (g)	Color	Saponins detected by LC/MS
2003	greenhouse	flowering	(A)	53.2 ^a	15% MeOH	5.626	light green powder ^b	-
					30% MeOH	1.479	red brown semi-solid	-
					100% MeOH	4.331	red brown solid	-
2003	greenhouse	flowering	(B)	25.0	H ₂ O/ <i>n</i> -BuOH	0.380	red brown solid	+ S-I; ± S-III
2003	greenhouse	flowering	(C)	55.0	hexane	1.041	dark green solid	-
					MTBE	1.759	black solid	-
					CHCl ₃ ^c	0.381	black semi-solid	-
					aqueous	7.465	light brown powder	-
2004	greenhouse	flowering	(B)	25.3	H ₂ O/ <i>n</i> -BuOH	0.767	red brown semi-solid	± S-I; ± S-III
2004 ^d	greenhouse	post-flowering	(B)	25.0	H ₂ O/ <i>n</i> -BuOH	0.952	red brown solid	+ S-I; + S-III
2005	field plot	post-flowering	(B)	25.0	H ₂ O/ <i>n</i> -BuOH	1.361	red brown semi-solid	-
2006 ^e	field plot	post-flowering	(B)	25.0	H ₂ O/ <i>n</i> -BuOH	1.196	red brown semi-solid	-

^aDefatted material. A fraction obtained from chloroform extraction of the freeze-dried ground leaf material did not show any soyasaponins.

^bThis powder was obtained by freeze-drying.

^cThe four unidentified components of the chloroform fraction from seeds (Table 4) were not detected here.

^dNew plantings from the same seed source as previous entries.

^eLeaf material collected near the end of the second year of outside growth, after over-wintering of plants in the field plot.

Table 6. Soyasaponins from Seed of *D. illinoense* Detected by Electrospray LC/MS

Extraction procedure	Flour (g)	Fraction	Extract (g)	Color	Soyasaponins (MH ⁺)							22 - O - glycosides of S-III	22 - O - glycosides of S-I
					S-III al (795)	S-III (797)	DDMP S-III al (921)	DDMP S-III (923)	D-I (941)	S-I (943)	S-VI (1069)		
(A) ^a	100.2	15% MeOH	15.578	brown semi-solid	- ^b	-	-	-	-	+	±	- ^c	- ^d
		30% MeOH	2.153	brown semi-solid	-	±	-	-	-	+	+	-	-
		100% MeOH	3.444 ^e	brown solid	±	+	-	+	+	+	+	±	+
(B)	25.1	H ₂ O/ <i>n</i> -BuOH	0.599 ^e	brown solid	±	+	-	±	+	+	+	±	+
(C)	50.0	hexane	4.151	light brown oil	-	-	-	-	-	-	-	-	-
		MTBE	2.010 ^e	black solid	±	+	-	+	+	+	+	-	-
		CHCl ₃	0.112 ^{e,f}	brown semi-solid	-	+	-	±	+	+	+	-	±
		aqueous	11.988 ^e	brown solid	-	+	-	+	+	+	+	-	+

^aA light green oil obtained from chloroform defatting of the flour did not show any soyasaponins.

^bThe indicated saponins were detected (+), were probably detected at trace concentrations (±) or were undetectable (-) as determined by LC/MS.

^cMH⁺ ions of *m/z* 929, 943, 959, 1075, 1105, 1091, 1121, 1177, 1207 and 1253 were not found (-) or not consistently found (±).

^dA + symbol in this column indicates detection of at least two MH⁺ ions of *m/z* 1075, 1089, 1105, 1221, 1237, 1251, 1267 and 1399.

^eIn these fractions, a component eluting just before S-I (at 23.3 min) and with a MH⁺ ion of *m/z* 963 might also be a saponin.

^fFour unidentified components of the chloroform fraction showed MH⁺ ions at *m/z* 127, 219, 279 and 355.

lentils and field peas. But the profile of *D. canadense* differed, especially with regard to S-III.

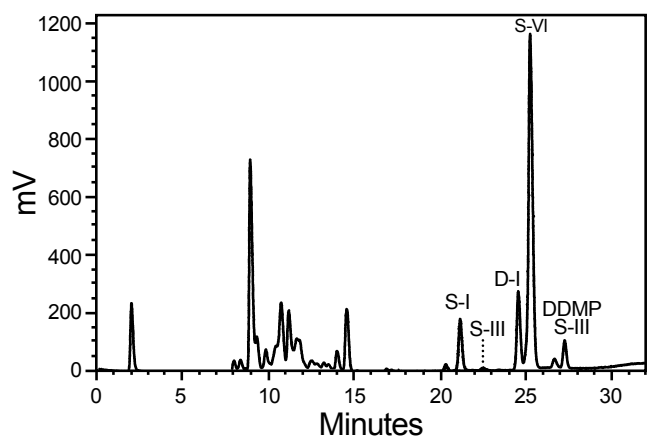


Fig. (4). HPLC/ELSD trace of a Diaion HP-20 MeOH extract from a defatted flour of *D. illinoense* seeds. Labelled peaks are as follows: S-I, soyasaponin I; S-III, soyasaponin III; D-I, dehydrosoyasaponin I; S-VI, soyasaponin VI; DDMP S-III, 2,3-dihydroxy-2,5-dihydroxy-6-methyl-4H-pyran-4-one conjugate of S-III.

CONCLUSIONS

In the present study, we found that extracts from seeds of *D. canadense* and *D. illinoense* were promising sources of soyasaponins, complex natural products of current medicinal interest. These species, both native to northern climates, could be exploited for their medicinal properties, perhaps similar to *D. adscendens* of tropical origin.

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