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FULL RESEARCH PAPER

Evaluation of nematicidal properties of saponins from *Medicago* spp.

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Abstract The nematicidal activity of saponins from *Medicago arborea* (tops), *M. arabica* (tops and roots) and *M. sativa* (tops and roots) against the plant-parasitic nematode *Xiphinema index* was investigated. Nematicidal activity of related prosapogenins and sapogenins on *X. index* is also described. Saponins from *Medicago* spp. at different concentrations were all nematicidal, those from *M. arborea* tops being the less effective. In general, saponins induced 100% mortality at 500 μg ml^{-1} between 8 and 48 h, while prosapogenins resulted in toxicity starting at 125 μg ml^{-1}. Differences in the effects on *X. index* induced by prosapogenins and sapogenins were less pronounced, although prosapogenins displayed a larger range of activity. Assays with purified sapogenins demonstrated the relationship of the observed nematicidal activity of *M. sativa* and *M. arborea* to the content of the main aglycones (medicagenic acid and hederagenin, respectively) in the saponin extracts. Hederagenin displayed the highest bioactivity, giving 38% mortality after 1 h at 125 μg ml^{-1}.

Keywords Saponins · *Medicago* · Nematodes · Nematicidal activity · *Xiphinema index*

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Introduction

Saponins are typical secondary metabolites widely present in the plant kingdom which include steroidal and triterpenoid glycosides classified according to the nature of their aglycones. Most saponins are hemolytic and display a range of various biological and pharmacological properties, such as molluscicidal, anti-inflammatory, antimicrobial and cytotoxic activities (Tava and Avato 2006). Commercial products containing plant saponins are available and used in the pharmaceutical, cosmetic and food industries (Tanaka et al. 1996). For example triterpene saponins from *Quillaja saponaria* have immunostimulatory properties and are included as adjuvants in vaccine formulations (Morein et al. 2004; Rönnberg et al.

1995). They are also used to control insect and nematode development (D'Addabbo et al. 2005; Pelah et al. 2002; San Martin 2004).

The chemical structure of saponins from several species within the genus *Medicago* (Fabaceae) has been studied (Bialy et al. 2004; Tava and Avato 2006; Tava et al. 2005). Generally they are complex mixtures of high-molecular weight triterpene glycosides with medicagenic acid, hederagenin, zahnic acid, bayogenin and soyasapogenol A and B as the dominant aglycones. Recently 2β,3β-dhydroxy-23-oxo-olean-12-en-28-oic acid was identified as a new aglycone moiety from *M. arborea* (Tava et al. 2005) and *M. hybrida* (Bialy et al. 2006). Structures of the main aglycones from the *Medicago* spp. used in this study are provided in Fig. 1. Purified saponins from *M. sativa* have been shown to inhibit *in vitro* the growth of human leukemic cells; depending on their structure, saponins from *Medicago* spp. also possess antimicrobial activity against plant pathogens and some yeasts and Gram-positive bacteria pathogenic to humans (Avato et al. 2005; Houghton et al. 2006; Tava and Avato 2006).

Plant-parasitic nematodes are responsible for substantial economic loss to agricultural crops. The dagger nematode, *Xiphinema index*, is considered the most harmful nematode species to grape (*Vitis* spp.), because it reduces plant growth and is the vector of grapevine fan leaf virus (GFLV; Raski 1996). Nematode management is generally based upon chemical treatments, but environmental con-

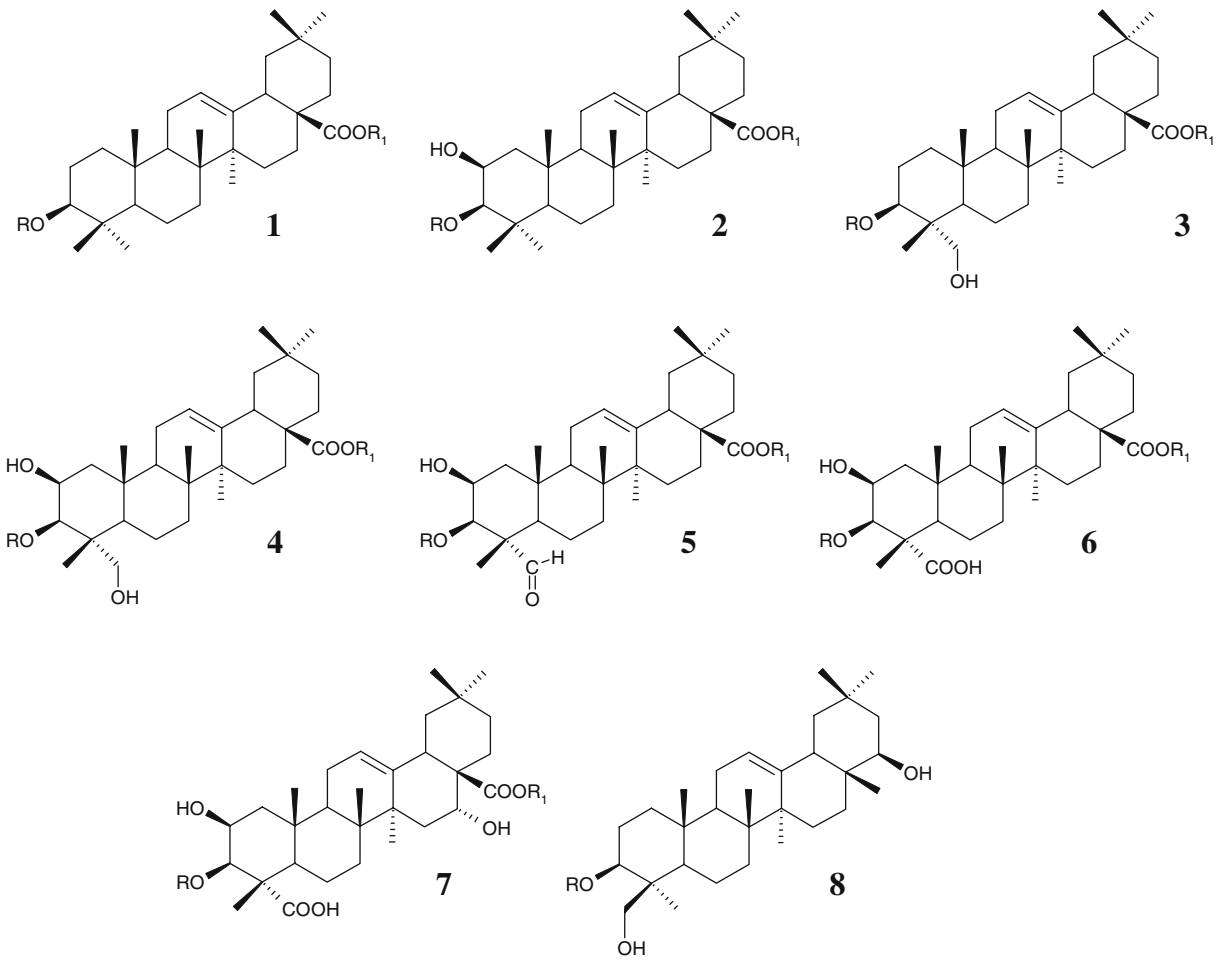

Fig. 1 Chemical structure of the most relevant triterpene compounds from the *Medicago* spp. used in this investigation. *R* sugar or sugar chain; R_1 H or sugar or sugar chain: *saponins*; *R* sugar or sugar chain; R_1 H: *prosapogenins*, obtained after basic hydrolysis of the corresponding saponins; *R* R_1 H: *sapogenins*, obtained after acid hydrolysis of the corresponding saponins; **1** oleanolic acid; **2** 2β-hydroxy oleanolic acid; **3** bayogenin; **4** hederagenin; **5** 2β,3β-dihydroxy-23oxo-olean-12-en-28-oic acid; **6** medicagenic acid; **7** zanhic acid: **8** soyasapogenol B

cerns and governmental regulations (UNEP 2000) are now resulting in a strong interest in nematicides of natural origin (Chitwood 2002). Few data are available on the activity of saponins against plant-parasitic nematodes (D'Addabbo et al. 2005; Chitwood 2002; Julier et al. 1996; Meher et al. 1988; Omar et al. 1994; Pedersen et al. 1976; San Martin and Magnunacelaya 2005), and there is no information on their mechanism of action. More specifically, the nematicidal activity of saponins from *Medicago* spp., to the best of our knowledge, has never been extensively studied.

The nematicidal effects of saponins from *M. arabica*, *M. arborea* and *M. sativa*, were studied in laboratory experiments on *X. index*. Nematicidal activity of related prosapogenins (obtained by basic hydrolysis of saponins; Fig. 1) and sapogenins (produced by acid hydrolysis of saponins; Fig. 1) is also described. As a comparison, soyasaponin I and purified aglycones from *Medicago* spp. (medicagenic acid, hederagenin and bayogenin) and a commercial mixture of saponins from *Q. saponaria*, were also included in the study.

Materials and methods

Plant material and saponin, prosapogenin and sapogenin preparations

The aerial parts (tops) and roots from *M. sativa* and *M. arabica*, and the tops from *M. arborea* were used for saponin extraction. *Medicago sativa* and *M. arborea* were grown at CRA., Italy; *M. arabica* was grown at the Institute of Soil Science and Plant Cultivation, Poland.

Saponins used in this study were newly extracted and purified following general procedures reported previously (Bialy et al. 2004; Tava et al. 2005). Purified mixtures of saponins were obtained as whitish powders in pure grade (90–95%). Reference pure soyasaponin I from *M. sativa* seeds, medicagenic acid and hederagenin from *M. sativa* roots, and bayogenin from *M. arabica* tops were obtained according to Bialy et al. (2004), Jurzysta (1982), Jurzysta et al. (1989). Saponins from *Q. saponaria* bark (90%) were purchased from Sigma-Aldrich (Milan, Italy).

Extracted and purified saponin mixtures were characterized for their quantitative aglycone composition by gas chromatography (GC) and GC/mass spectrometry (MS) analyses of derivatized sapogenins, obtained after acid hydrolysis as reported in Tava et al. (1993). Detailed structural elucidation was obtained by nuclear magnetic resonance (NMR) analysis (Bialy et al. 2004; Tava and Avato 2006; Tava et al. 2005)

Saponins of *M. sativa* and *M. arborea* tops were subjected to basic hydrolysis to obtain the related prosapogenins according to Oleszek et al. (1992). Acid hydrolysis of saponin mixtures from *Medicago* spp. was also conducted to obtain the related sapogenins according to Julier et al. (1996) and Avato et al. (2005). Reaction products were analyzed as described in Tava et al. (1993).

Nematicidal bioassays

Specimens of *X. index* were extracted from soil collected from a naturally infested vineyard using Cobb's decanting and sieving method (McSorley 1987). Nematode population were identified to species on the basis of morphological and biometrical characters of specimens according to reported descriptions for *X. index* (Thorne and Allen 1950). Batches of ten *X. index* adult females in 0.5 ml distilled water were placed in microtiter wells (Sigma-Aldrich, Milan, Italy). A 0.5 ml volume of each test solution was added to obtain final concentrations of 500, 250, 125, 62.5, 32.3 and 15.6 μg ml^{-1}. Distilled water was included as a control. The experimental design was randomized, each concentration and the controls were replicated three times and each experiment was repeated at least twice with separate controls.

Nematodes exposed to test solutions were observed under a light microscope at 1, 2, 4, 8, 24 and 48 h intervals and assessed as active or inactive by pricking the nematode body with a needle. Lack of mobility was evidence of nematicidal activity of saponins. Previous *in vitro* tests on *X. index* using the touch-response method demonstrated this method to be more accurate and to give less variable results than the use of dyes (Yamashita and Viglierchio 1987). Nematode mortality was confirmed after lack of movement to distilled water and observation after an additional 72 h (San Martin and Magnunacelaya 2005).

Statistical analysis

Rate of immobility (i) was calculated according to Abbott's formula (Finney 1978), where $i=100\times(1-n_t/n_c)$, in which; i=% nematode immobility; n_t=number

of active nematodes after the treatment; and n_c= number of active nematodes in the control. Data collected as percent of dead nematodes were arcsin-transformed and subjected to a one-way analysis of variance (ANOVA). Treatment means were compared using Fisher's LSD (least significant difference) pairwise procedure at $P<0.05$.

Results

Composition of purified saponins from this study was consistent with previously obtained results (Bialy et al. 2004; Tava et al. 2005; Tava and Avato 2006), that is, *M. arabica* were predominantly formed by hederagenin and bayogenin glycosides (35 and 30% of the total aglycones, respectively), together with oleanolic acid (5%) and 2-β-hydroxyoleanolic acid (7%). Saponins from *M. sativa* tops were characterized by a high concentration of medicagenic acid (52%), followed by zhanic acid (8%), hederagenin (4%) and bayogenin (2%), while saponins from *M. sativa* root were characterized by the presence of medicagenic acid (65%), hederagenin (3%) and zhanic acid (<1%). The tops of *M. arborea* had high concentrations of both medicagenic (34%) and zanhic acid (22%) and a low content of bayogenin (5%), 2β,3β-dihydroxy-23-oxo-olean-12-en-28-oic acid (2%), oleanolic acid (1%) and 2-β-hydroxyoleanolic acid (1%). Additionally, monodesmosidic saponins were most abundant in *M. arabica* and *M. sativa* root extracts, while saponins from *M. arborea* and *M. sativa* tops were predominantly constituted by bidesmosidic compounds.

Nematicidal activities of saponin extracts and related prosapogenins and sapogenins from *M. arabica*, *M. arborea* and *M. sativa* against *X. index* are reported in Tables 1, 2 and 3. While lower concentrations (15.6, 32.3 and 62.5 μg ml^{-1}) were tested, significant results were only obtained at 125, 250 and 500 μg ml^{-1}; data from assays using lower concentrations of saponins is not shown and only discussed in the text when relevant. All the saponins from *Medicago* spp. induced 100% mortality of *X. index* at the highest concentration (500 μg ml^{-1}) between 8 and 48 h of treatment (Table 1). At 250 μg ml^{-1} the crude saponins from *M. sativa* roots and *M. arabica* tops and roots resulted in significantly greater mortality after 48 h than *M. sativa* and *M. arborea* tops at the same concentration. Only *M. arabica* roots at 250 μg ml^{-1} resulted in similar mortality as the highest concentration tested. In general at all concentrations assayed, saponins from *M. arabica* roots were more active compared to the other samples. No significant differences among concentrations were found between most exposure times for *Q. saponaria* saponins and soyasaponin I from *M. sativa* seeds (Table 1). Treatment with soyasaponin I resulted in only 43% mortality, even at the maximum concentration. Treatment with *Q. saponaria* saponins showed nematicidal activity at the three highest concentrations 2 h after treatment, although mortality rates reached only 19–24%.

To compare the nematicidal potential of bidesmoside and monodesmoside saponins, prosapogenins from *M. arborea* and *M. sativa* tops were prepared. As reported above, these two species were particularly rich in bidesmoside compounds which, due to the cleavage of the estereal linkage, yielded the corresponding monodesmosides on basic hydrolysis. Prosapogenins were more nematicidal than the related saponins at the same dose, except for *M. sativa* tops at the maximal concentration. As shown, saponins activity was consistent only at 500 μg ml^{-1}, while prosapogenins resulted in toxicity starting at 125 μg ml^{-1}. At this concentration their activity almost overlapped that of the related sapogenins, progressively increasing at the other two assayed concentrations (Table 2).

In addition, compared to sapogenins, the nematicidal efficacy of both samples of prosapogenins (*M. sativa* and *M. arborea* tops) was evident after a shorter period of time. There was 57% mortality 8 h after treatment with prosapogenins at a concentration of 250 μg ml^{-1}. The rate of activity of sapogenins resulted in greater than 50% mortality after 24 h (*M. sativa* roots, *M. arabica* tops) or 48 h (*M. arabica* roots, *M. arborea* tops) of treatment at a concentration of 125 μg ml^{-1} (Table 3).

Among the three pure assayed aglycones, hederagenin was the most active (Table 3). *In vitro* treatment of *X. index* with hederagenin also gave positive results at lower concentrations; 57% mortality was observed after 24 h at 15.6 μg ml^{-1} (data not shown). Medicagenic acid appeared slightly more active than bayogenin, causing 52% mortality at 62.5 μg ml^{-1} after 48 h of treatment (data not shown). In general, activity of pure aglycones was however comparable with that of related *Medicago* spp.

Table 1 Effect of tested concentrations of saponins on *X. index* after different exposure times

Dose (μg ml^{-1})	Nematode mortality (%)											
	1 h		2 h		4 h		8 h		24 h		48 h	
M. sativa tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	0	a	0	a
250	0	a	0	a	0	a	0	a	0	a	0	a
500	0	a	38.1	e	61.9	e	100	e	100	g	100	g
M. sativa roots												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	9.5	abc	33.3	cd
250	0	a	0	a	0	a	0	a	47.6	e	61.9	ef
500	0	a	4.8	ab	33.3	cd	66.7	d	100	g	100	g
M. arabica tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	19.0	c	38.1	d
250	0	a	0	a	0	a	0	a	42.8	de	76.2	f
500	0	a	9.5	ab	23.8	bc	90.5	e	100	g	100	g
M. arabica roots												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	57.1	e	71.4	f
250	0	a	0	a	0	a	0	a	47.6	e	90.5	fg
500	0	a	0	a	0	a	0	a	80.9	f	100	g
M. arborea tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	4.8	ab	14.3	abc
250	0	a	0	a	0	a	0	a	4.8	ab	4.8	ab
500	0	a	0	a	4.8	ab	61.9	d	100	g	100	g
Q. saponaria bark												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	9.5	bc	9.5	abc	4.8	ab	14.3	bc	14.3	abc
250	0	a	14.3	cd	14.3	bc	14.3	c	19.0	c	23.8	cd
500	0	a	9.5	ab	14.3	abc	14.3	bc	19.0	c	19.0	bcd
Soyasaponin I												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	4.8	ab	9.5	bc	9.5	abc	14.3	abc
250	0	a	0	a	4.8	ab	4.8	ab	28.6	cd	42.8	de
500	0	a	0	a	0	a	0	a	14.3	bc	42.8	de

Means followed by the same letters on the same column are not significantly (P=0.05) different according to Fisher's LSD test.

Discussion

Described results indicate that saponins from *M. arborea*, *M. arabica* and *M. sativa* to different concentrations all possess nematicidal activity against the plant-parasitic nematode *X. index,* those from *M. arborea* tops being the less effective (Table 1). Use of saponins from *Medicago* spp. for the development of new nematicidal formulations appears therefore as a reasonable possibility.

Literature on the nematicidal activity of saponins is limited and data are mainly related to the assay of *Q. saponaria* extracts. Thus in previous *in vitro* and pot experiments it was shown that 260–280 ppm solutions of saponins reduced total populations, number of egg masses and viable juveniles of the root-knot nematode *Meloidogyne javanica* (Omar et al. 1994). Moreover, in another investigation an active *Q. saponaria* formulation of polyphenols (6%) and saponins (25%) resulted in the best nematode control

Table 2 Effect of tested concentrations of prosapogenins on *X. index* at different exposure times

Dose (μg ml^{-1})	Nematode mortality (%)											
	1 h		2 h		4 h		8 h		24 h		48 h	
M. sativa tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	4.8	a	4.8	a	0	a	9.5	ab	52.4	bc	52.4	b
250	4.8	a	19.0	b	28.6	b	57.1	c	71.4	bc	90.5	c
500	0	a	0	a	23.8	ab	42.8	bc	76.2	cd	95.2	c
M. arborea tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	9.5	a	33.3	b	80.9	b
250	0	a	14.3	b	47.6	bc	57.1	cd	71.4	c	95.2	c
500	0	a	0	a	90.5	c	100	e	100	d	100	c

Means followed by the same letters on the same column are not significantly (P=0.05) different according to Fisher's LSD test.

at low dosage (San Martin and Magnunacelaya 2005). Similarly, field trials with aqueous extracts of *Q. saponaria* significantly reduced the density of *M. incognita* in the soil and increased tomato or melon crop yield (D'Addabbo et al. 2005). The motility of *M. incognita* juveniles was also significantly reduced by exposure to eight different steroid and triterpenoid saponins from plants related to garden asparagus (Chitwood 2002).

Comparison of previous results with our data is not straight forward as we only carried out *in vitro* assays and used purified metabolites with a high content of active compounds. In general saponins from *Medicago* had a significatively higher bioactivity than saponins from *Q. saponaria* (Table 1). This lack of important nematicidal activity was consistent with results of San Martin and Magnunacelaya (2005), who showed a synergistic activity between saponin rich and polyphenol rich fractions from this plant.

None of the previous investigations have related the activity of saponins to the corresponding prosapogenins and sapogenins. A recent study on the antimicrobiotic potential of saponins from *Medicago* spp. (Avato et al. 2005) showed that the presence of sugars in the saponin molecules is not necessary for their activity against human pathogens and the antibiotic effect was higher from the saponin raw extracts and different sapogenins. Results from experiments on *X. index* presented in this work seem to confirm the same trend of activity. Comparison of data from *M. sativa* and *M. arborea* tops prosapogenins and related sapogenins from the same species suggests that further observations on the structure–activity relationship of these natural compounds are necessary. Although, they both were nematotoxic, prosapogenins had a larger range of activity compared to the related sapogenins, resulting in good nematicidal efficacy after a short exposure time and becoming dominant at higher doses. If results obtained by assaying sapogenins indicate that the presence of sugars in the molecules did not determine their activity, then the larger range of effects displayed by the monodesmoside type – prosapogenins suggests that the presence of some sugars in the molecules might enhance their bioactivity.

Biological effects of saponins are normally ascribed to their specific interaction with cell membranes (Tava and Avato 2006) causing changes in cell permeability. The implication in the process of cholesterol–saponin insoluble complexes is, however, still controversial (Francis et al. 2002). Moreover, it has been shown that the side sugar chains on the aglycones might contribute to saponin effects on cell membranes; that is, monodesmosides have generally a stronger haemolytic activity than bidesmosides (Tava and Avato 2006). In addition to the activity of saponins on biological systems, it has been demonstrated that they are also able to interact with proteins (Heng et al. 2004; Ikedo et al. 1996; Potter et al. 1993). Moreover, specific studies with *Q. saponaria* and soybean saponins (Heng et al. 2004; Ikedo et al. 1996; Potter et al. 1993) have shown that the interaction between the saponins and proteins is quite complex and also involves the aglycone moiety.

A critical structure for nematode viability is the protective cuticle, an extracellular matrix that forms

Table 3 Effect of tested concentrations of sapogenins on *X. index* at different exposure times

Dose (μg ml^{-1})	Nematode mortality (%)											
	1 h		2 h		4 h		8 h		24 h		48 h	
M. sativa tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	9.5	bc	19.0	bcd	47.6	bcd
250	0	a	0	a	0	a	0	a	14.3	abcd	61.9	cde
500	0	a	0	a	0	a	19.0	c	76.2	ijk	100	f
M. sativa roots												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	85.7	jkl	100	f
250	0	a	0	a	0	a	0	a	76.2	jkl	100	f
500	0	a	0	a	0	a	0	a	57.1	efghij	100	f
M. arabica tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	4.8	ab	4.8	ab	76.2	ijk	100	f
250	0	a	0	a	0	a	0	a	71.4	hij	100	f
500	0	a	0	a	0	a	0	a	95.2	kl	100	f
M. arabica roots												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	9.5	ab	100	f
250	0	a	0	a	0	a	0	a	47.6	defghi	100	f
500	0	a	0	a	0	a	0	a	66.6	ghij	100	f
M. arborea tops												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	4.8	a	4.8	ab	4.8	ab	42.8	cdefgh	66.7	de
250	0	a	0	a	0	a	0	a	14.3	abc	71.4	def
500	0	a	0	a	0	a	0	a	85.7	jkl	100	f
Soyasaponin I												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	9.5	ab	28.6	ab
250	0	a	0	a	14.3	bc	14.3	bc	19.0	abcd	52.4	bcd
500	0	a	0	a	0	a	0	a	23.8	bcd	80.9	def
Medicagenic acid												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	0	a	0	a	0	a	28.6	bcdef	85.7	ef
250	0	a	0	a	4.8	ab	4.8	ab	19.0	abcd	47.6	bcd
500	9.5	b	14.3	b	19.0	c	33.3	d	61.9	fghij	76.2	de
Hederagenin												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	38.1	cd	38.1	c	100	d	100	e	100	l	100	f
250	38.1	c	71.4	d	95.2	d	100	e	100	l	100	f
500	95.2	e	100.0	e	100	d	100	e	100	l	100	f
Bayogenin												
0	0	a	0	a	0	a	0	a	0	a	0	a
125	0	a	4.8	a	4.8	ab	14.3	c	33.3	cdefg	61.9	cde
250	0	a	4.8	a	4.8	ab	14.3	c	23.8	bcde	61.9	cde
500	0	a	0	a	4.8	ab	9.5	abc	38.1	cdefgh	71.4	de

Means followed by the same letters on the same column are not significantly (P=0.05) different according to Fisher's LSD test.

their exoskeleton. This structure is primarily composed of collagen proteins assembled into higher order complexes (Page and Winter 2003). It appears reasonable to speculate whether saponin interaction with collagen proteins from the cuticle might also be responsible for the observed nematotoxic effects. Furthermore, the rate of nematicidal activity induced by both prosapogenins and sapogenins also suggests the possible implication of the saponin aglycone. Natural saponins used in these *in vitro* tests were from different *Medicago* spp. and were chosen for their different saponin and sapogenin profiles. Combining previous compositional results with the new data we can conclude that the main aglycones present in the saponin extracts from *Medicago* spp. contributed to the bioactivity detected in the nematicidal bioassays.

In conclusion, data presented seem consistent with the potential use of saponins from *Medicago* spp. for new biotechnological applications, such as nematicidal formulations. The large biomass produced by *Medicago* species should make the industrial extraction of saponins economically viable, as demonstrated by the formulations of *Q. saponaria* extracts already commercially available.

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