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Studies on *Medicago lupulina* saponins. 6. Some chemical characteristics and biological activity of root saponins

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Abstract

The saponin fraction of black medic trefoil (*Medicago lupulina*) roots was isolated and purified. Its hydrolysis afforded several aglycones that have been identified by spectral and chemical properties as medicagenic acid, hederagenine and soyasapogenols: B, C, D, E and F. They made up, respectively, 46, 9 and 45% of total sapogenins isolated. Two-dimensional TLC of *M. lupulina* root saponins revealed fourteen compounds, two of which were medicagenic acid glycosides. The haemolytic, antifungal and allelopathic activities of *M. lupulina* and *M. media* roots are compared and discussed.

Key words: *Medicago lupulina roots, saponins, isolation, biological activity*

INTRODUCTION

A series of investigations on black medic trefoil (Medicago lupulina L.) saponins were undertaken. In previous papers of this series, saponins of *M. lupulina* tops (Górski et al. 1984a, b, c, d), blossom (Jurzysta et al. 1987) and partially seeds (Jurzysta 1973a, b) were described. As a continuation of the studies on this plant we present here some chemical characteristics and biological activities of root saponins.

MATERIAL AND METHODS

ISOLATION OF SAPONINS

Medicago lupulina L. cv. Renata roots were collected from field grown plants. Air dried and finely powdered roots (1.75 kg) were defatted in

a Soxhlet apparatus with chloroform and then extracted exhaustively with ethanol by boiling under reflux. The ethanol was removed in vacuo and the dry residue dissolved in distilled water, and saponins were extracted into n-butanol in a separatory funnel. Butanol was removed to give 160 g of brown solid crude saponins (CS). A 100 g of CS were subjected to the cholesterol precipitation procedure as previously described (Górski et al. 1984b).

ACID HYDROLYSIS

The CS (50 g) were hydrolysed in 1 dm^3 of 2N HCl in methanol and sapogenins were fractionated according to previously described methods (Oleszek and Jurzysta 1986a, b). This yielded crude fractions of soyasapogenols (4.5 g), hederagenine (2 g) and medicagenic acid (2.5 g).

ISOLATION AND IDENTIFICATION OF AGLYCONES

Crude medicagenic acid and hederagenine were purified by column chromatography (CC) over Kieselgel 60 (Merck, 70–230 mesh) and eluted with ethyl acetate. Then, the solvent was removed and medicagenic acid crystallized from dioxan-water yielding 1.5 g of crystalline compound mp. 351–353°C (lit. (Boar and Allen 1973) 349–350°C), MS m/z (rel. int.) 502 (1), 487 (1), 457 (3), 456 (4), 248 (100), 235 (6), 233 (10), 203 (76), 189 (18), 133 (28). Hederagenine was cristallizaed from ethanol affording 292 mg of crystalline compound mp. 330–332°C (lit. (Boar and Allen 1973, Jurzysta 1982) 330–332°C), MS m/z 472 (2), 458 (1), 248 (100), 224 (2), 235 (4), 233 (9), 206 (7), 203 (83), 175 (14), 133 (21). The crude sayasapogenol fraction (830 mg) was subjected to CC over Kieselgel 60 using as eluent 0.5–1.5% methanol in benzene, yielding soyasapogenol B (123 mg), crystalline mp. 255–257°C (lit. (Boar and Allen 1973, Jurzysta 1982) 257–259°C), MS m/z 458 (3), 443 (1), 440 (2), 234 (100), 224 (10), 206 (10), 219 (34), 216 (10), 175 (26), 133 (13), 119 (19), soyasapogenol C (10 mg), crystalline mp. 240–242°C (lit. (Boar and Allen 1973) 240–244°C) MS m/z 440 (8), 425 (2), 407 (2), 224 (13), 216 (100), 206 (14), 201 (24), 175 (27), 203 (16), 187 (13), 133 (25), soyasapogenol D (44 mg), crystalline, mp. 297–298°C (lit. (Cainelli et al. 1958) 295–297°C) MS m/z 472 (31), 457 (4), 440 (6), 248 (31), 235 (30), 234 (10), 224 (14), 221 (11), 219 (10), 203 (100), 175 (31) soyasapogenol E (50 mg), crystalline, mp. 249°C (lit. (Boar and Allen 1973) 250°C) MS m/z 456 (4), 441 (1), 438 (2), 232 (100), 224 (16), 217 (15), 206 (18), 175 (34), 139 (6), 119 (19), soyasapogenol F (19 mg), crystalline, mp. 312–314°C (lit. (Jurzysta 1982) 316–318°C) MS m/z 458 (36), 443 (7), 440 (10), 425 (7), 234 (52), 224 (23), 221 (31), 220 (30), 206 (33), 203 (100), 175 (82).

Melting points are uncorrected. MS spectra were taken with a LKB 9000 spectrometer (70 eV).

THIN LAYER CHROMATOGRAPHY

Glycosides were subjected to TLC on silica gel (DC-Fertigeplatten, Kieselgel 60 Merck, 0.25 mm). Two developing systems were used: S_1: ethyl acetate-acetic acid-water (7:2:2) or/and S_2: n-butanol-acetic acid-water (4:1:1). Spots were visualized by spraying plates with Liebermann-Burchard reagent followed by heating at 120°C. Sugars were identified by TLC on cellulose, with benzene-n-butanol-pyridine-water (1:5:3:3) as solvent system, visualized with silver nitrate solution.

BIOASSAYS

The haemolytic activities of individual glycosides were screened by covering the 2D-TLC plates with gelatine-blood suspension as previously reported (Jurzysta et al. 1987). The haemolytic indexes of *M. lupulina* and *M. media* roots were measured according to the method of Borkowski (1959).

For evaluation of the fungistatic activity the method of Zimmer et al. (1967) modified by Jurzysta (1979) was employed. In this test, finely ground roots were added to the fungus growth medium. *M. lupulina* root activity was compared to that of *M. media* roots. Similarly, sand culture tests were performed in order to evaluate the allelopathic potential of black medic trefoil and alfalfa roots. For that, alfalfa and black medic trefoil powdered roots were incorporated into silica sand at the rate of 0.25 and 9.5% (w/w), and this amended soil was placed in plastic pots (400 cm^3). Twenty winter wheat seeds of Grana cultivar were planted in each pot. The seedlings were allowed to grow 15 days in the greenhouse with an average day/night temperature of 20/15°C. The lengths of the roots and shoots were measured in four replicates.

RESULTS AND DISCUSSION

Extraction of *Medicago lupulina* root saponins by the standard procedure of Wall et al. (1952) furnished a fraction of crude saponins (CS) with a yield of 9% DM. The further mode of separation of CS into two saponin subfractions, similar to those obtained from the tops (Górski et al. 1984b) and blossoms (Jurzysta et al. 1987) failed. The cholesterol

precipitation method was unsuccessful and led only to further removal of impurities. However, the reason for this ineffectivness was not the absence of medicagenic acid glycosides in CS, as medicagenic acid was found in acid hydrolysates, but rather their chemical properties. Moreover, it was shown by two-dimentional chromatography (2D-TLC), that the CS fraction was composed of fourteen glycosides (Table 1), but only two of them were blue coloured in daylight and green under UV radiation, when the plates were visualised with Liebermann-Burchard reagent. These two glycosides slightly haemolysed red blood cells when 2D-TLC plates were covered with gelatine blood suspension. This suggests without doubt that those two compounds were medicagenic acid glycosides. They seem to be very similar or identical to the two alfalfa root medicagenic acid glycosides possesing the highest R_f values (Oleszek and Jurzysta 1986a). Those alfalfa saponins also hardly complexed with cholesterol and slightly haemolysed blood cells. We attributed this to their hydrophobic character. However, alfalfa roots contained beside those two glycosides, several green-coloured saponosides with R_f values very similar or even the same as those of alfalfa tops (Oleszek and Jurzysta 1986a). No such similarity in tops and roots was found for black medic trefoil. Its root medicagenic acid glycosides are completely different from those found in the tops (Górski et al. 1984b) and blossoms (Jurzysta et al. 1987). Beside medicagenic acid glycosides, the CS fraction consisted of twelve other saponosides haemolytically not active. The analysis of acid hydrolysis products permits the assertion that these compounds are the mixture of soyasapogenol B and hederagenine glycosides.

Acid hydrolysis of CS yielded medicagenic acid, hederagenine and soyasapogenol B and its artifacts C, D, E and F (Jurzysta 1984). On the basis of the isolation effeciency, it can be calculated that medicagenic acid was the predominant aglycone (46%), but soyasapogenols also made up a large percentage of the fraction (45%). Hederagenine made up 9% of total sapogenins.

This data demonstrates the qualitative similarity of sapogenins of *M. lupulina* and *M. media* roots since both species possess the same aglycones. However, distinct quantitative differences between them can be found. The *M. media* root saponins were much richer in medicagenic acid (92%), whereas soya sapogenols occurred only in trace amounts (2% of total) (Oleszek et al. 1985, Oleszek and Jurzysta 1986).

Some qualitative and quantitative variations among particular black medic trefoil plant parts can be pointed out. In contrast to aerial parts, the roots contain hederagenine, but the aglycones N and A_n reported by Górski et al. (1984a) for tops were not found in the roots and in any other plant organs. Comparison of particular aglycones percentage in total genins shows that the top saponins are richer in medicagenic

Table 1

2D-TLC characteristics of black medic trefoil root saponins

Spot number	R_f value in solvent		Relative quantity [1]	Colour after visualization with Liebermann-Burchard reagent		Haemolysis [2]
	S_1	S_2		in natural light	UV	
1	0.75	0.84	+	pink	pink	−
2	0.61	0.75	+ + + +	blue	green	+
3	0.54	0.79	+	bricky	bricky	−
4	0.47	0.70	+	violet-pink	pink	−
5	0.39	0.63	+ + +	blue	green	+
6	0.42	0.60	+	violet-pink	pink	−
7	0.41	0.48	+ +	violet-brown	pink	−
8	0.33	0.50	+ +	violet-brown	bricky	−
9	0.34	0.35	+ +	violet-brown	bricky	−
10	0.21	0.41	+ + +	violet-brown	bricky	−
11	0.29	0.34	+ +	violet-brown	bricky	−
12	0.26	0.28	+ + + +	brown	bricky	
13	0.22	0.27	+	brown	bricky	
14	0.19	0.17	+ +	brown	bricky	

[1] — Relative quantities were determined visually from spot colour intensities the highest quantities are represented by + + + + and the lowest by +

[2] — (−) no haemolysis; (+) haemolysis

S_1 — ethyl acetate-acetic acid-water (7: 2: 2), S_2 — n-butanol-acetic acid-water (4: 1: 1)

acid (78% of total) (Górski et al. 1984a) than are the roots (46.5%). The opposite medicagenic acid distribution pattern was found in alfalfa roots — 92% (Oleszek and Jurzysta 1986a) and tops — 52% (Jurzysta 1982). This is the most important comparison, considering it has been reported several times that from among the lucerne saponosides only medicagenic acid glycosides display a haemolytic or/and antifungal activity. The latter attribute might be of the greatest importance for plant roots since they are mostly exposed to fungal attack, and a high medicagenic acid glycosides content may give them satisfactory protection. Thus, the finding, that *M. lupulina* top saponins are richer in medicagenic acid than the roots may be to some degree surprising. But, in spite of the higher medicagenic acid content in tops than in roots, antifungal activity of black medic trefoil roots was ten times higher than that of the tops (Górski et al. 1984d). This data closely correlates with our latest findings (Oleszek and Jurzysta 1986a) showing that medicagenic acid glycosides of alfalfa roots inhibited *Trichoderma viride* growth four times more strongly than did a similar fraction of alfalfa (Jurzysta 1982) or black medic trefoil (Górski et al. 1984b) tops.

Since the separation of the medicagenic acid glycosides fraction from *M. lupulina* roots was unsuccessful, direct estimation of its antifungal, haemolytic and allelopathic activities was not possible. To evaluate these activities, a comparative method was employed. In this trial, finely powdered *M. lupulina* and *M. media* roots were used as substitutes for the medicagenic acid glycosides fraction. Such substitution in some cases is possible and can give quite valuable results (Oleszek and Jurzysta 1986b), and we deemed it of interest for study.

The results obtained from such comparative tests are presented in Table 2. They show, that the suppressive antifungal activity of black

Table 2

Antifungal, haemolytic and allelopathic activities of *M. lupulina* and *M. media* roots

Biotest		*Medicago lupulina*	*Medicago media*
Seedling root	a*	35	69
inhibition, % control	b	52	75
Seedling shoot	a	5	25
inhibition	b	24	42
Trichoderma viride	c	29	1
growth inhibition	d	54	45
% control	e	62	52
Haemolytic index		288	432

* a and b — rate of root incorporation 0.25 and 0.5%, respectively; c, d and e — amount of roots in fungus growth medium, 30, 50 and 100 mg 100 cm^{-3}, respectively.

medic trefoil roots is comparable or somewhat higher than that of alfalfa roots. We are unable to judge univocally from these results if the antifungal saponins concentrations in roots of booth species are equal, and thus antifungal root activities are similar, or if concentrations are different but chemical divergency make these activities even. It does seem, however, more likely, that in this case the chemical structure has a grater impact on the biological activity of saponins then their total quantity. The two medicagenic acid glycosides with the highest R_f values found in both species seem to be mostly responsible for this high antifungal activity. This may suggest that this activity is connected with the percentage share of aglycones in the glycoside moiety, but other than aglycone structural features cannot be excluded (Nonaka 1986). Full understanding of this phenomenon will be possible if each particular medicagenic acid glycoside is separated and its structure fully elucidated. It seems, however, to be very interesting that the high antifungal activity of *M. lupulina*

and *M. media* roots is not correlated with their haemolytic activities. While roots inhibited *T. viride* growth more strongly than the tops did, the haemolytic properties of tops and roots are very similar, and those of *M. lupulina* roots are lower than those of *M. media*. This suggests that different structural features of glycosides are responsible for the antifungal and haemolytic activities.

The same trend was found for the allelopathic potential of roots of the compared species (Table 2). *M. media* roots inhibited wheat seedling growth more strongly than those of *M. lupulina*. These suppressive activities seem to correlate more with haemolytic than with antifungal activities. Perhaps the same structural features are responsive both for allelopathic and haemolytic activities. This subject needs more fundamental research in order to provide full explanation of this phenomenon.

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Badania nad saponinami Medicago lupulina. 6. Niektóre dane chemiczne i aktywność biologiczna saponin korzeni

Streszczenie

Z korzeni *Medicago lupulina* wyizolowano frakcję saponinową. Próba rozdzielenia jej na saponiny wytrącalne i niewytrącalne cholesterolem nie powiodła się. Przy użyciu dwukierunkowej chromatografii cienkowarstwowej wykazano, że frakcja ta składała się z czternastu saponozydów. Dwa z nich zidentyfikowano jako glikozydy kwasu medikagenowego. Pozostałe saponozydy to glikozydy sojasapogenolu B i hederageniny. Z hydrolizatu frakcji wyizolowano w postaci krystalicznej kwas medikagenowy, hederageninę oraz sojasapogenole: B, C, D, E i F. Ich udział procentowy w wyizolowanych geninach wynosił odpowiednio 46, 9 i 45%.

Przedyskutowano aktywność biologiczną saponin korzeni lucerny chmielowej w porównaniu z lucerną mieszańcową.