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# Studies on *Medicago lupulina* saponins. 2. Isolation, chemical characterization and biological activity of saponins from *M. lupulina* tops

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Abstract

Two pure saponin fractions from the tops of *M. lupulina* were isolated and characterized. The fractions named Ma and Ss varied in composition and biological activity. It was shown by TLC that fraction Ma contained 5 components. In the acid hydrolysates of that fraction only one aglycone (medicagenic acid) and 4 sugars: rhamnose, xylose, arabinose, glucuronic acid were found. The Ss fraction contained 11 components. In its acid hydrolysates, soyasapogenols B, C, D, E, F and two new aglycones N and An were found. The same sugars as above and additionally glucose and galactose were identified. It was found that the Ma fraction of saponins (medicagenic acid glycosides) was highly fungistatic, haemolytic and toxic to fishes. The Ss fraction of saponins (soyasapogenol glycosides) showed no such activity.

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

## INTRODUCTION

In our previous paper (Górski et al. 1984) we reported the isolation and identification of sapogenins from *M. lupulina* tops. It was found that *M. lupulina* saponins contained 8 aglycones identified as soyasapogenols B, C, D, E, F, medicagenic acid and two new aglycones.

Saponins with a similar sapogenin composition were isolated from another lucerne — alfalfa (*Medicago media* Pers.). It was found that the saponins containing medicagenic acid showed biological activity

while saponins containing soyasapogenols were not active (Gestetner et al. 1971a, b).

The saponins from *M. lupulina* have not been characterized yet. Hence the purification, chemical characterization and biological activity of saponins from *M. lupulina* is the subject of our present investigation.

## MATERIAL AND METHODS

**Plant material.** Field-grown *M. lupulina* was used. Tops were cut at the stage of flowering, oven-dried at 60° C and ground.

**Isolation of crude saponins.** Crude saponins (CS) were isolated according to Wall et al. (1952).

**Isolation of Ma fraction of saponins.** The fraction of saponins was isolated using cholesterol precipitation according to Jurzysta (1973). In that method one exception was made: because the obtained saponin-cholesterol precipitate was difficult to filter, instead of filtration, repeated centrifugation (1 h, 2500 g) was used.

**Isolation of Ss fraction of saponins.** In the first step, lead acetate precipitation of saponins was employed (Jurzysta 1973). Then the saponins were purified by column chromatography as follows: 3 g of saponins were dissolved in dioxane-ethyl acetate (8 : 2) and layered on a 12 × 6 cm silica gel column (Schuhard 50/100 mesh). The column was then eluted with the following solvent systems: 1) dioxane 0.5 $dm^3$; 2) dioxane-water (99 : 1) 0.5 $dm^3$; 3) dioxane-water (98 : 2) 0.5 $dm^3$; 4) dioxane-water (95 : 5) 1.0 $dm^3$; 5) methanol 1.0 $dm^3$; 6) methanol-water (1 : 1) 1.0 $dm^3$. The eluates 1, 2, 3 and 6 containing impurities (TLC) were discarded. Eluates 4 and 5 containing saponins were evaporated until dry. The precipitate was then dissolved in water, precipitated with acetone, filtered and dried.

**Acid hydrolysis of saponins.** 100 mg of Ma saponins and 100 mg of Ss saponins were hydrolysed in 50 $cm^3$ of 2N HCl (methanol-water, 1 : 1) for 12 h under reflux. The aglycones were precipitated with water, filtered off and washed with water. The filtrate containing carbohydrates was neutralized with $Ag_2CO_3$, evaporated until dry and dissolved in 0.5 $cm^3$ of pyridine.

**Thin layer chromatography (TLC) of saponins.** The saponins were chromatographed on silica gel precoated Merck plates in $S_1$ ethyl acetate-acetic acid-water (7 : 2 : 2) and $S_2$ n-butonal-acetic acid-water (4 : 1 : : 1) solvent systems. The plates were visualised as previously reported (Górski et al. 1984).

**Identification of aglycones and sugars.** The aglycones were identified by TLC on silica gel plates as previously reported (Górski et al. 1984). The carbohydrates from acid hydrolysates were identified using stan-

dards by TLC on cellulose Merck plates in n-butanol-pyridine-water--benzene (5 : 3 : 3 : 1) solvent system.

*Analyses of biological activity.* Fungistatic activtiy of saponins was analysed using the *Trichoderma viride* biotest (Zimmer et al. 1967) modified by Jurzysta (1979). Haemolytic activity (haemolytic index) of saponins was determined according to Borkowski (1959). Toxicity to fishes was analysed according to Jones and Elliot (1969) using *Lebistus reticulatus* as the test fish.

## RESULTS AND DISCUSSION

### ISOLATION AND PURIFICATION OF SAPONINS

The saponins were isolated from *M. lupulina* tops according to Wall et al. (1952) with a yield of 9% DM. The obtained saponins contained impurities and were recognised as crude saponins (CS). The CS were purified by cholesterol precipitation yielding 4 g of powder-like saponins (Ma fraction). That saponin fraction was practically pure.

However, we found (TLC) that only a part of the saponins was obtained by the cholesterol method. The other saponins which did not precipitate with cholesterol were isolated from the water filtrate using butanol extraction. Those saponins were purified with lead acetate precipitation yielding 20 g of saponins. The saponins still contained some impurities, although many of the impurities were discarded.

Because of the partial success of the last purification, the method employing silica gel filtration of saponins in the dioxane-water gradient was used. That method yielded 2 g of pale yellow powder-like saponins (Ss fraction). This fraction was also practically pure. There were no losses of saponosides in the process of purification as was shown by TLC (Fig. 1).

### CHEMICAL CHARACTERIZATION OF SAPONINS

The obtained Ma and Ss saponin fractions were analysed by TLC and then subjected to acid hydrolysis to analyse their aglycone and sugar composition. TLC of Ma saponins showed the presence of 5 saponosides, two saponosides of the lowest Rf values being predominant (Table 1). In the acid hydrolysates of Ma saponins only one aglycone — medicagenic acid was found. Among the carbohydrates the following were found: rhamnose, xylose, arabinose and glucuronic acid. TLC of Ss saponins revealed the presence of 11 saponosides. The Rf and colours of the spots are presented in Table 2. In acid hydrolysates of these saponins the following aglycones were identified: soyasapogenols B, C, D,

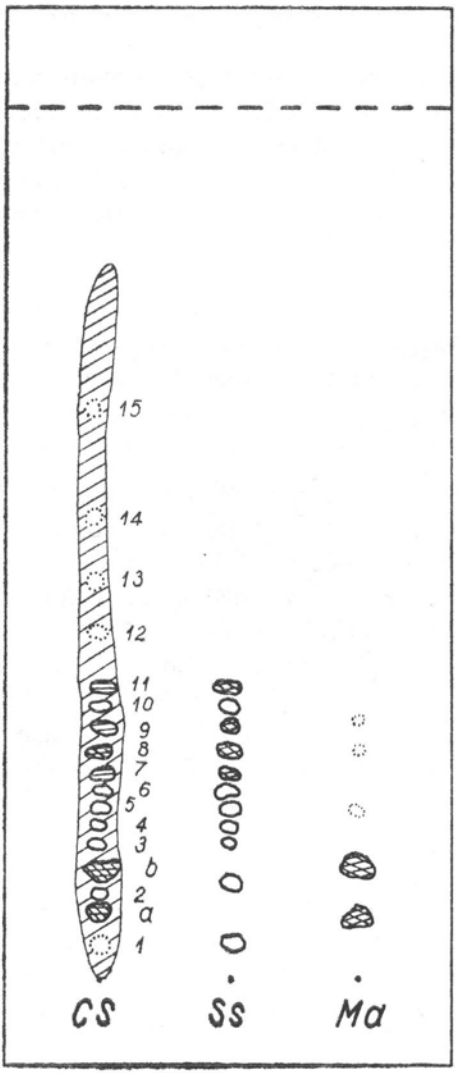


Fig. 1. Thin layer chromatogram of saponins. CS — crude saponin: 1-11 and a, b — saponosides, 12-15 — flavonoid impurities; Ss — soyasapogenol glycosides; Ma — medicagenic acid glycosides. Developed in $S_1$ solvent system

E, F and two previously found N and An. From among the sugars the same as above and additionally glucose and galactose were found.

On the basis of the presented data it was found that the two saponin fractions isolated from *M. lupulina* differed in chemical composition. The Ma fraction of saponins contained medicagenic acid glycosides and the Ss fraction contained soyasapogenol glycosides.

## BIOLOGICAL ACTIVITY OF SAPONINS

The fungistatic and haemolytic activities of medicagenic acid (Ma) glycosides and soyasapogenol (Ss) glycosides were investigated. It was found that these activities were characteristic of Ma glycosides while

Table 1

Rf values and colour of saponosides from the Ma fraction of saponins. Developed in $S_1$ and $S_2$ solvent systems, detected with Liebermann-Burchard reagent

| Colour of the spots | | Rf | |
|---|---|---|---|
| In day light | in UV | $S_1$ | $S_2$ |
| Green-grey | green | 0.06 | 0.21 |
| " | " | 0.13 | 0.32 |
| " | " | 0.19 | 0.34 |
| " | " | 0.26 | 0.40 |
| " | " | 0.30 | 0.55 |

Table 2

Rf values and colour of saponosides from the Ss fraction of saponins. Developed in $S_1$ and $S_2$ solvent systems, detected with Liebermann-Burchard reagent

| Colour of the spots | | Rf | |
|---|---|---|---|
| In day light | in UV | $S_1$ | $S_2$ |
| Grey-brown | brown | 0.04 | 0.17 |
| Grey | red | 0.11 | 0.23 |
| Brown-red | brown | 0.16 | 0.29 |
| Blue | red | 0.17 | 0.33 |
| Blue | red | 0.20 | 0.35 |
| Blue | red | 0.21 | 0.36 |
| Brown-red | brown | 0.24 | 0.38 |
| Brown | brown | 0.26 | 0.39 |
| Brown | red | 0.29 | 0.43 |
| Blue | red | 0.31 | 0.58 |
| Blue | red | 0.33 | 0.62 |

Ss glycosides lacked such biological activity (Table 3). These findings are in agreement with an earlier report of G e s t e t n e r et al. (1971a, b). They found that haemolytic and fungistatic properties of saponins were due to the presence of medicagenic acid.

Also J u r z y s t a (1982) isolated two saponin fractions from alfalfa: Ma glycosides and Ss glycosides. The first fraction showed haemolytic and fungistatic properties when the second was inactive. Both fractions of glycosides isolated from alfalfa contained the same aglycones and sugars as glycosides isolated from *M. lupulina*. Although in the Ss glycosides from *M. lupulina* two additional aglycones were found, no change in biological activity of the saponins was observed.

The chemical similarity of *M. lupulina* and alfalfa saponins resulted in their similar biological activity. In Table 3 it is shown that the toxi-

Table 3

Haemolytic index, toxicity to fishes and inhibition of *Trichoderma viride* growth by Ma and Ss saponin fractions from *M. lupulina* and *M. media*

| Biotests | *Medicago lupulina* | | *Medicago media* | |
|---|---|---|---|---|
| | Ma glycosides | Ss glycosides | Ma glycosides | Ss glycosides |
| Fish immobilization time, min* | 32 | not toxic | 45 | not toxic |
| Haemolytic index | 2960 | 0 | 3400 | 0 |
| *T. viride* growth inhibition, %* | 67 | 8 | 55 | not analysed |

* Saponis in the concentration of 0.01%.

city to fishes and haemolytic index of medicagenic acid glycosides from *M. lupulina* and alfalfa are very similar.

It seems that saponins isolated from *M. lupulina* and *M. media* are very similar in their chemical and biological nature although differences are observed. This is interesting from the chemotaxonomic point of view, because these two species are rather distantly related within the genus *Medicago* (Simon 1979).

Alfalfa hay is known to possess antinutritional properties. These properties are due to the presence of saponins (Cheeke 1971). The antinutritional properties of *M. lupulina* hay and saponins will be the subject of our next paper.

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## *Badania nad saponinami Medicago lupulina. 2. Izolacja i charakterystyka chemiczna oraz aktywność biologiczna saponin z części nadziemnych M. lupulina*

Streszczenie

Z obu części nadziemnych lucerny chmielowej (*Medicago lupulina*) wyizolowano i oczyszczono dwie frakcje saponinowe Ma i Ss. Saponiny obu frakcji różniły się składem chemicznym i aktywnością biologiczną. Przy użyciu chromatografii cienkowarstwowej wykazano, że saponiny frakcji Ma składają się z 5 saponozydów. W kwaśnych hydrolizatach saponin tej frakcji stwierdzono obecność tylko jednego aglikonu — kwasu medikagenowego oraz cukrów — ramnozy, ksylozy, arabinozy i kwasu glukuronowego. Frakcja saponinowa Ss zawierała 11 saponozydów. W kwaśnych hydrolizatach tej frakcji stwierdzono sojasapogenole B, C, D, E, F oraz dwa nowowyizolowane aglikony N i An. W części cukrowej występowały te same cukry co we frakcji Ma oraz dodatkowo glukoza i galaktoza. Stwierdzono, że saponiny frakcji Ma — glikozydy kwasu medikagenowego, wykazywały działanie fungistatyczne, hemolityczne i były toksyczne dla ryb. Saponiny frakcji Ss — glikozydy sojasapogenoli nie miały tych właściwości.