ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 57, nr 4: 563-571
1988

Isolation and identification of flavonoids from *Medicago lupulina* L. flowers

STANISŁAW BURDA, MARIAN JURZYSTA

Department of Biochemistry and Physiology of Crop Plants, Insititute of Soil Science and Plant Cultivation, 24-100 Puławy, Poland

(Received: December 4, 1987, Accepted: June 27, 1988)

Abstract

Four dominant flavonoid compounds in crystal form were isolated from flowers of *Medicago lupulina* L. and identified as laricytrin (3'-metoxy-3,4',5,5'7-pentahydroxy-flavone), and its three glycosides: 5'-O-β-D-glucoside, 3,5'-O-β-D-diglucoside and 3,5',7-0-β-D-triglucoside. The described monoglucoside is a new compound, isolated for the first time from plant material. The remaining three flavonoid constituents appear only in trace amounts characterized as heterosides of kaempferol, quercetin and myricetin with O-glycoside bounded in the position 3.

Key words: Medicago lupulina, flavonoids, laricytrin

INTRODUCTION

The flavonoid constituents of black medic trefoil are not very well known. Four C-glycosides are known to appear in *M. lupulina* seeds: vitexin, isovitexin, orientin and isoorientin (Jurzysta 1975). The aim of the following work was to separate the flavonoid compounds from *M. lupulina* flowers and study their chemical structures.

MATERIAL AND METHODS

Plant material. Flowers of *M. lupulina* harvested in May from an experimental field in the second year of growth were used in the investigation.

Isolation of flavonoids. Ethanol extracts obtained after full extraction with alcohol were concentrated to a volume of 1 litre, filtered off and the filtrate,

after evaporation of the alcohol, was diluted with water. The water mixture, after defatting with petroleum ether and methylene chloride, was applied to a column packed with poliamide powder (Woelm). The column was then washed with water until a colourless liquid appeared. At this point the poliamide powder was removed from the column, placed under an UV lamp and separated into five layers numbered from 1 to 5. After drying, the flavonoids were extracted from each layer with ethyl alcohol. The extracts of layers 2-5 yielded the following precipitates:

layer 2 – compound 2 – 142 mg
layer 3 – compounds 2 and 3 – 273 mg
layer 4 – compound 3 – 420 mg
layer 5 – compound 4 – 27 mg

Compounds 2, 3 and 4 were recrystallized from the diluted ethanol.

The components of the ethanol extract from the first layer were separated in a column filled with cellulose powder MN 2100 (Macherey-Nagel) using a mixture of chloroform-methanol-water (100:10:1). Three fractions were obtained and found to contain:

fraction 1 – compound 2
fraction 2 – mixture of compounds 5, 6 and 7
fraction 3 – compound 1.

From fraction 3, 41 mg of compound 1 were obtained after recrystallization from ethanol while 560 mg of compound 4 were obtained from the water eluate from the polyamide column after concentration and crystallization.

Thin layer chromatography. Adsorbents: cellulose, silicagel and polyamide pre-coated plates (Merck). Solvent system: S_1 n-butanol-acetic acid-water (4:1:5) organic phase, S_2 acetic acid 15%, S_3 iso-butanol-pyridine-acetic acid-water (12:8:4:3), S_4 iso-butanol-pyridine-acetic acid-water (12:3:4:3), S_5 chloroform-acetic acid-water (50:45:5), S_6 phenol-water (4:1), S_7 iso-butanol-acetic acid-water (10:4:7), S_8 chloroform-acetic acid (2:3), S_9 acetic acid-hydrochloric acid-water (30:3:10), S_{10} chloroform-ethyl acetate (9:1), S_{11} toluene-chloroform-acetone (40:25:35), S_{12} n-butanol-pyridine-water-benzene (5:3:3:1). Detection reagents: R_1 – 2% alcohol solution of aluminium chloride, R_2 – aniline phtalate in butanol saturated with water, R_3 – Pauly reagent (diazotised sulphanilic acid).

Quantitative hydrolysis. A sample (10-40 mg) of each of the given glycosides was refluxed with a mixture of 2N HCl and ethanol (1:1) for 4 hrs. The precipitate was filtered, dried and weighed. The sugars, after deacidification on Amberlite IR 40, were identified by TLC (cellulose, S_{12}, R_2).

Partial hydrolysis. A sample of about 10 mg of glycoside was heated in a mixture 0.1% sulphuric acid and ethanol (1:1). The solution was analysed by TLC (cellulose, S_3, R_1) after 15, 30, 60 and 120 minutes.

Enzymic hydrolysis. A small amount of β-glycosidase was added to 1 cm^3 of solution containing about 1 mg of the examined heteroside and incubated

for 20 hrs at 37°C. Next, 1 cm^3 of methanol was added. The precipitate was centrifuged and the supernatant was analysed by TLC (cellulose S_1, S_2, R_1).

Acetylation of the aglycone. Compound 1 (50 mg) was heated with a mixture of 10 cm^3 acetic anhydride and 1 g anhydrous sodium acetate at 105°C for 1 hr. Next, the reaction mixture was poured into water. The acetate precipitate was filtered and crystallized two times from methanol.

Methylation of the aglycone. Compound 1 (84 mg) was methylated using diazomethane. The obtained permethyl ether was isolated by preparative thin layer chromatography on cellulose (S_{10}) and was crystallized from methanol.

Methylation of compound 4 and subsequent hydrolysis. A sample of 112 mg of compound 4 was methylated by adding diazomethane solution. The raw oily product was hydrolysed with 4N HCl for 6 hrs. The partial methyl ether was extracted from the reaction mixture of ethyl acetate and crystallized from methanol.

Alkaline clevage. A sample of 10 mg of compound 1 was melted with a small amount of sodium hydroxide in 205-210°C for 15 minutes. After cooling the melt was dissolved in dilute hydrochloric acid. Next the products of degradation were extracted into ethyl acetate and analysed by TLC (polyamide S_{13}, silicagel S_{11}, R_3) with the standard of the phenolic compounds and the products of degradation of myricetin obtained in the same way.

Demethylation of compound 1. A small amount of compound 1 was heated for 1 hr with 20 mg of aniline hydrochloride (Egger 1962) or 40 mg pyridine hydrochloride (Harborne 1967). After cooling and leaching, the alloy was dissolved in methanol and analysed by TLC (cellulose S_1, S_5, S_6,S_7, S_8, S_9, R_3).

Spectral analysis. The absorption spectra in UV was taken with a Beckman DB-T spectrophotometer according to Mabry et al. (1970) and Markham (1982). EIMS spectra was taken with an LKB 9000 spectrometer. The NMR spectrum of the TMS-derivative of compound 1 obtained according to Mabry et al. (1970), was taken with the Jeol 100 MHz apparatus in $CHCl_3$, using tetramethylsilane as an internal standard.

RESULTS

Two-dimensional TLC of the alcohol extract of black medic trefoil fresh flowers on cellulose in S_1 and S_2 showed that there were seven compounds with yellow or brown fluoroscence. This is characteristic of flavonoids. Compounds 1, 2, 3, and 4 were obtained in crystalline form and identified by chemical and spectral methods. A combination of compounds 5, 6 and 7 was hydrolysed and analyzed chromatographically for aglycones.

Compound 1 – yellow crystals of m.p. 318-319°C (decomp.), heated with 2N NCl did not hydrolyse; TLC see Table 1; MS (70 eV) m/z (rel. int.): 332

M^+ (100) $C_{16}H_{12}O_8$, 317 $[M - CH_3]^+$ (7), 304 (8), 167 B_2^+ (12), 153 $[A_1 + H]^+$ (11); PMR of TMS-derivative (100 MHz, CCl_4), (ppm): 3.97 (s, $-OCH_3$), 6.28 (d, H-6), 6.45 (d, H-8), 7.47 (s, H-2' and H-6'); V λ_{max}(nm) in CH_3OH 253, (265), 303, 377, + $AlCl_3$ 270, 308, 465, + $AlCl_3$-HCl 264, (273), 308, 371, 435, + CH_3COONa-H_3BO_3 257 (304), 392 (spectra with CH_3COONa and CH_3ONa unstable). Permethyl ether of compound 1 – colourless crystals of m.p. 148-151°C. MS (15 eV) m/z (rel. int): 402 M^+ (93) $C_{21}H_{22}O_8$, 387 $[M - CH_3]^+$ (100), 371 $[M - OCH_3]^+$ (19), 195 B_2^+ (3), 181 $[A_1 + H]^+$ (9). Acetyl derivative of compound 1 – pale yellow crystals of m.p. 184-184.5°C. Alkaline degradation of compound 1 – gallic acid and fluoroglucinol were found in the degradation products. Demethylation with aniline hydrochloride (1 hr, 180°C) did not take place. A compound chromatographically identical with standard myricetin was obtained by demethylation with pyridine hydrochloride (1hr, 130°C).

Table 1

Chromatographic characteristics of flavonoid compounds from *M. lupulina* flowers

Compound	Colour of the spots in UV		R_f value in solvent			
	–	$AlCl_3$	S_1	S_2	S_3	S_4
1	yellow	yellow-green	0.55	0.00	0.96	0.84
2	,,	,,	0.27	0.03	0.77	0.44
3	tawny	yellow	0.32	0.48	0.65	0.29
4	,,	,,	0.03	0.69	0.49	0.04
5	,,	,,	0.46	0.27	–	–
6	,,	,,	0.53	0.36	–	–
7	,,	,,	0.63	0.47	–	–

Compound 2 – yellow crystals of m.p. 239°C; TLC see Table 1; MS (70 eV) m/z (rel. int.): 332 aglycone $C_{16}H_{12}O_8$ (100), 317 (7), 167 (6), 153 (12); UV λ_{max}(nm) in CH_3OH 251, 266, (308), 373, + CH_3ONa 238, (264), 329, 433, + $AlCl_3$ 262, (273), 310, 370, + $AlCl_3$-HCl 261, (272), 307, 360, 430, + CH_3COONa (242), 333, + CH_3COONa-H_3BO_3 279, 323. Glucose (TLC) and compound 1 (TLC, UV, MS) were obtained through hydrolysis with 2N HCl. The molar ratio was found to be 1.23.

Compound 3 – yellow crystals of m.p. 219-224°C (decomp.); TLC see Table 1; UV λ_{max}(nm) in CH_3OH 251, 266, (310), 358, + CH_3ONa 265, 332, 418, + $AlCl_3$ 272, 308, (369), 405, + $AlCl_3$-HCl 274, 308, 361, 402, + CH_3COONa 264, 329, 416, + CH_3COONa-H_3BO_3 261, 302, 365. Glucose (TLC) and compound 1 (TLC, UV, MS) were obtained through hydrolysis with 2N HCl. The molar ratio was found to be 2.19.

Compound 4 – yellow crystals of m.p. 229-230.5°C; TLC see Table 1; UV λ_{max}(nm) in CH_3OH 251, (264), (300), 357, + CH_3ONa (251), 264, 408, + $AlCl_3$ 269, 301, 400, + AlCl-HCl 268, 302, 368, 398, CH_3COONa 260, 405, + CH_3COONa-H_3BO_3 261, 302, 365. Glucose (TLC) and compound 1 (TLC, UV, MS) were obtained through hydrolysis with 2N HCl. The molar ratio was found to be 3.03. Compound 1 and 2 were found after hydrolyzing compound 4 with 0.05% H_2SO_4. The acid hydrolysis product of permethyl ether of compound 4: MS (70 eV) m/z (rel. int.) 360 M^+ $C_{18}H_{16}O_8$ (100), 345 $[M - CH_3]^+$ (143), $[M - CO]^+$ (5), 181 B_2^+ (6), 167 $[A_1 + H]^+$ (5).

Compounds 5, 6 and 7. In the hydrolysis products of combined compounds 5, 6 and 7, three aglycones were found with yellow fluoroscence in ultraviolet light. Characteristic fluorescence and the same R_f as for standards permit the identification of these aglycones with high probability as kaempferol, quercetin and myricetin (Table 2).

Table 2

Chromatographic characteristics of the aglycones of compound 5, 6 and 7

Aglycone	Colour of the spots in UV		R_f value in solvent		
	–	$AlCl_3$	S_5	S_6	S_7
1	yellow	yellow-green	0.68	9.64	0.68
Kaempferol	,,	,,	0.69	0.66	0.68
2	,,	yellow	0.30	0.48	0.67
Quercetin	,,	,,	0.29	0.48	0.65
3	,,	yellow-green	0.08	0.08	0.32
Myricetin	,,	,,	0.07	0.08	0.32

DISCUSSION

Four flavonoid compounds were separated from black medic trefoil flowers in crystalline form. Analysis of the acid hydrolyzate showed that compounds 2, 3 and 4 were glycosides of compound 1. The structure of the aglycone was established upon interpretation of absorption spectra in UV, mass spectrum (MS) and proton magnetic resonance spectrum (NMR) of the TMS-ether derivative (Mabry et al. 1970, Markham 1982, Harborne et al. 1975). This data was supported by chemical studies and spectral analysis of its methyl ether and acetate. The structure of the glycosides was established on the basis of the results of quantitative hydrolysis along with chromatographic identification of sugars, enzymatic hydrolysis and spectral analysis.

Compound 1. 3'-methoxy-3,4',5,5',7-pentahydroxyflavone (laricytrin). The stability of the compound through four hours of heating with 2N HCl, yellow UV fluorescence and the location of band 1 in the UV spectrum (377 nm) indicate an unbounded glycoside flavonol with a free-OH group in the position 3. The mass spectrum showed a parent ion (m/z 332) which corresponds with the molecular structure $C_{16}H_{12}O_8$. The NMR spectrum of the TMS-derivative showed the presence of unsubstituted C-6 and C-8 positions (doublets 6.28 and 6.45 ppm), as well as C-2' and C-6' (singlet 7,47 ppm) and the presence of a methoxyl group in compound 1 (singlet 3.97 ppm). This data shows that compound 1 is single methyl ether of myricetin. The demethylation of compound 1 resulted in a product chromatographically identical with standard myricetine. In the mass spectrum of the methyl derivative of compound 1 a molecular ion of hexamethoxyflavone (m/z 402) and characteristic fragments for flavonols were found: $[A_1 + H]^+$ – ring A with two -OCH_3 groups (m/z 181), B_2^+ – ring B with three -OCH_3 groups (m/z 195). There were gallic acid (ring B) and fluoroglucinol (ring A) in the alkaline degradation products. Demethylation with pyridine hydrochloride proceeded relatively easily, which indicates that no hydroxyl group is in the position 7 (Harborne 1967). The specific demethylation reaction with aniline hydrochloride for 5-methyl derivatives took place very slowly (Egger 1962). In relation to its spectrum in methanol, the batochromic shift of band I in the UV spectrum with CH_3COONa and H_3BO_3 (16 nm) indicates an ortho-di-OH structure in ring B. The hipsochromic shift of band I of 30 nm with $AlCl_3$ after addition of HCl also supports this. As the substituents of ring B are in positions 3', 4' and 5', the existence of the ortho-dihydroxyl structure is possible if the methoxyl group is in the C-3' position.

Compound 2. Laricytrin 5'-O-β-D-glucoside. Hydrolysis with 2N HCl quickly yielded compounds identified as compound 1 (laricytrin) (UV, TLC, MS) and glucose (TLC) with a molar ratio of 1:1. It also easly hydrolyzed with β-glucosidase. The absorption spectrum in UV after the addition of sodium methoxide showed an intensive peak at 433 nm (in methanol 373 nm), which indicates the presence of a free hydroxyl group in the C-4' position. The spectrum with $AlCl_3$ and HCl is identical to the spectrum with $AlCl_3$ which excludes the presence of the ortho-di-OH structure. This data permits the location of glucose in compound 2 in the C-5' position.

Compound 3. Laricytrin 3,5'-O-β-D-diglucoside. The brown colour in UV light suggests lack of an unbonded hydroxyl in the position 3. 2N HCl hydrolysis yielded an aglycone, which was identified (UV, TLC, MS) as laricytrin (compound 1) and glucose with a molar ratio of 1:2. The aglycone (laricytrin), compound 2 (laricytrin 5'-glucoside) and unhydrolyzed compound 3 were found in the hydrolysate (0.05% H_2SO_4 at 100°C) following chromatography. Enzymatic hydrolysis with β-glucosidase slowly yielded the aglycone

and an intermediate compound with brown colouring in UV light, probably laricytrin 3-glucoside.

Compound 4. Laricytrin 3,5',7-O-β-D-triglucoside. Laricytrin and glucose at a molar ratio 1:3 were obtained after 2N HCl hydrolysis. In the MS spectrum of the compound obtained through hydrolysis of permethylether of compound 4, a trihydroxytrimethoxyflavone ion (m/z 360) was visible, which suggests the substitution of glucose in compound 4 of three hydroxyls, one of which is in ring A (fragment m/z 167), and the second in ring B (fragment m/z 181). In the hydrolysate with β-glucosidase, compound 3 (laricytrin 3,5'-di-glucoside) was found, and in the acid hydrolysate (0.05% H_2SO_4) compound 2 (laricytrin 5'-glucoside). This indicates the substitution of glucose in compound 4 in positions C-3 and C-5'. The UV absorption spectrum of compound 4 in the presence of sodium methoxide showed a high intensity peak shifted in the direction longer waves by 51 nm in band I. A similar peak was observed in the spectrum with sodium acetate. This proves the presence of a free -OH group in the position 4'. Band I was bathochromically shifted 41 nm in the UV spectrum with $AlCl_3$ and HCl, in relation to the spectrum in methanol, thus showing the presence of a free hydroxyl group in the C-5 position in the molecule (by the bounded glycoside hydroxyl in the C-3 position). This permits the identification of the third glucose molecule in compound 4 in position 7.

Compounds 5, 6 and 7 appeared in small amounts in the examined material. They have glycoside structures indicated by their location on the chromatogram. This is further supported by the changes occurring during heating of the compounds with hydrochloric acid. As the result of hydrolysis and chromatographic analysis, kaempferol, quercetin and myricetin were identified. Brown fluorescence in UV light of compounds 5, 6 and 7 and yellow fluorescence of their aglycones show the presence of glycoside bonds in heteroside molecules in the C-3 position.

Laricytrin is a seldom-found flavonoid in plants. The presence of its glycosides has been proved both in gymnosperms, in the genera *Larix* and *Cedrus* (*Pinaceae*) (Niemann 1972, 1977, 1980, Medvedeva et al. 1974) and in angiosperms. Geiger (1979) isolated laricytrin 3-rhamnoglucoside from the leaves of *Ginkgo biloba* L. Laricytrin glycosides have also been found in a few species of the genera *Sedum* (*Crassulaceae*) (Denton and Kerwin 1980), *Heuchera* (*Saxifragaceae*) (Well and Bohm 1980), *Chondropetalum* (*Restionaceae*) (Harborne et al. 1985) and in *Limnanthes douglasii* R. Brown (Parker and Bohm 1975).

In the *Fabaceae* family, the presence of laricytrin has been found only in the flowers of three species: *Tetragonolobus siliquosus* Roth. (Jay et al. 1978, 1980), *Medicago arborea* L. (Torck and Pinkas 1980) and *Lathyrus pratensis* (Ismaili et al. 1981). The structure of the isolated glycosides from *M. lupulina*

flowers is very interesting. Only 3-glycosides of laricytrin have been generally known until now. The substitution of flavonol in the position 5' is very rare. Torck et al. (1983) identified laricytrin 3,5'-diglucoside and 3,5',7-triglucoside in an extract of *M. arborea* flowers. Laricytrin 5'-glucoside is a new flavonoid which has been isolated from plant material for the first time.

Acknowledgement

We wish to thank Bruce Dean for his help in preparing this article.

REFERENCES

Denton M. F., Kerwin J. L., 1980. Survey of vegetative flavonoids of *Sedum* section *Gormania* (*Crassulaceae*). Can. J. Bot. 58: 902-905.

Egger K. 1962. Myricetin-5-methylether in *Rhododendron*-Bluten. Z. Naturforschg. 17b: 489-490.

Geiger H. 1979. 3'-O-Methylmyricetin-3-rhamnoglucosid, ein neves Flavonoid aus dem Herbstlaub von *Ginkgo biloba* L. Z. Naturforschg. 34c: 878-879.

Harborne J. B. 1967. Correlations between chemistry, pollen morphology and systematics in the family *Plumbaginaceae*. Phytochemistry 6: 1415-1419.

Harborne J. B., Mabry T. J., Mabry H. 1975. The flavonoids. Chapman and Hall, London, pp. 45-213.

Harborne J. B., Boardley M., Linder H. P. 1985. Variation in flavonoid patterns within the genus *Chondropetalum* (*Restionaceae*). Phytochemistry 24: 273-278.

Ismaili A., Reynaud J., Jay M., Reynaud J. 1981. Les flavonoides de *Lathyrus pratensis*. Plant. Med. Phytother. 15: 155-159.

Jay M., Hasan A., Voirin B., Favre-Bonvin J., Viricel M. R. 1978. Les flavonoides de *Dorycnium suffructicosum* et de *Tetragonolobus siliquosus*. Phytochemistry 17: 1196-1198.

Jay M., Voirin B., Hasan A., Gonnet J. F., Viricel M. R. 1980. Essai Chimiosystematique sur la Tribu des Lotees. Biochem. Syst. Ecol. 8: 127-132.

Jurzysta M. 1975. C-glikozydy flavonoidowe nasion lucerny chmielowej (*Medicago lupulina* L.). Streszczenia prac XIII Zjazdu PT Biochem., Toruń. pp. 21.

Mabry T. J., Markham K. R., Thomas M. B. 1970. The systematic identification of flavonoids. Springer-Verlag, New York.

Markham K. R. 1982. The techniques of flavonoid identification. Acad. Press, London.

Medvedeva S. A., Tjukavina N. A., Ivanova S. Z. 1974. Fenolnye ekhstraktivnye soedinenija khvoi listvennicy sibirskoj. Khim. Drev. 15: 144-152.

Niemann G. J. 1972. Fenolics from *Larix* needles. Part 4. Constituents of *L. laricina*. Acta Bot. Neerl. 21: 549-552.

Niemann G. J. 1977. Flavonoids and related compounds in leaves of *Pinaceae*. Part 2. *Cedrus atlantica* cv. Glauca. Z. Naturforschg. 32c: 1015-1017.

Niemann G. J. 1980. Phenolics from *Larix* needles. Part 15. High-performance liquid chromatography of *L. gmelinii* flavonoids. Z. Naturforschg. 35c: 514-515.

Parker W. H., Bohm B. A. 1975. Flavonol glycosides of *Limnanthes douglasii*. Phytochemistry 14: 553-555.

Torck M., Pinkas M. 1980. Sur quelques polyphenols de *Medicago arborea* L. (*Legumineuses*). Plant. Med. Phytother. 14: 20-25.

Torck M., Pinkas M., Jay M., Favre-Bonvin J. 1983. Heterosides flavoniques de *Medicago arborea* L. Pharmazie 38: 783.

Well E. F., Bohm B. A., 1980. Chemotaksonomic studies in the *Sacifragaceae* s.l. Part 15. The flavonoids of subsection Villosae section *Heuchera* in the genus *Heuchera*. Can. J. Bot. 58: 1459-1463.

Izolacja i identyfikacja flawonoidów z kwiatów Medicago lupulina L.

Streszczenie

Z kwiatów lucerny chmielowej wyodrębniono w postaci jednorodnych substancji krystalicznych cztery związki flawonoidowe, które zidentyfikowano na podstawie MS, UV i NMR oraz badań chemicznych jako larycytrynę (3'-metoksy-3,4',5,5',7-pentahydroksyflavon), 5'-O-β-D-glukozyd larycytryny, 3,5'-O-β-D-diglukozyd larycytryny i 3,5'7-O-β-D-triglukozyd larycytryny. Pozostałe trzy składniki flawonoidowe występujące w kwiatach *M. lupulina* w niewielkich ilościach scharakteryzowano jako heterozydy kempferolu, kwercetyny i myrycetyny zawierające wiązania O-glikozydowe w pozycji 3.