Flavonoids from *Ficaria verna* Huds.

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A phytochemical investigation of the flowers and leaves of *Ficaria verna* Huds. (*Ranunculaceae*) yielded four additional known flavonoid compounds including: kaempferol 3-O-β-D-(6″-α-L-rhamnopyranosyl)-glucopyranoside (nicotiflorin), apigenin 8-C-β-D-glucopyranoside (vitexin), luteolin 8-C-β-D-glucopyranoside (orientin) and apigenin 8-C-β-D-(2″-O-β-D-glucopyranosyl)-glucopyranoside (flavosativaside). The characterisation of these compounds was achieved by various chromatographic and spectroscopic methods (UV, ^{1}H NMR, ^{13}C NMR and MS).

Introduction

The buttercup family, Ranunculaceae comprises 59 genera and about 1900 species (Evans, 1996). Members of the family are distributed throughout the world. Most of them are well represented as perennial plants widely occurring in all of Europe. The genus *Ficaria* (Ranunculaceae), often included in the genus *Ranunculus*, is represented in Poland only by two subspecies: *Ficaria verna* HUDS. (syn. *Ranunculus ficaria* L. subsp. *bulbifer* LAMBINON, *Ficaria ranunculoides* ROTH) and *Ficaria nudicaulis* A. KERN. (syn. *Ranunculus ficaria* L. subsp. *calthifolius* (RCHB.) ARCANG., *Ficaria calthifolia* (RCHB.) (Mirek *et al*., 1995; Tutin *et al*., 1964). *F. verna* known as pilewort is a common early-spring flowering perennial herb. It is used in folk medicine and homeopathy as an anti-inflammatory, astringent, antibiotic and anti-haemorrhagic treatment (Evans, 1996; Delacroix, 1969; Palliez *et al*., 1968; Docheva-Popova and Popov, 1955). The extracts of the plant are applied to haemorrhoids by topical application as ointment or suppository. Previous chemical studies of pilewort tubers proved the presence of triterpenoid saponins (Pourrat *et al*., 1979; Pourrat *et al*., 1982; Texier *et al*., 1984; Brisse-Le Menn *et al*., 1990). In the above-ground parts of the plant amino acids, mineral elements as well as vitamin C were detected (Perseca and Parvu, 1986; Rams, 1978; Franke and Kensbock, 1981; Istrătescu-Guti and Forstner, 1974). Pilewort is the first plant outside of the *Gentianaceae* family to be reported as containing the disaccharide–gentiobiose (Barthomeuf *et al*., 1987). In the fresh parts of the plant, ranunculin and products of its decomposition have been observed (Ruijgrok, 1966; Bonora *et al*., 1988).

According to reviewed literature, little is known about the presence of undefinable flavonoid compounds in *F. verna* (Cameroni and Bernabei, 1957; Lebreton, 1986). This has encouraged us to investigate the polyphenolic compounds of this plant growing in Poland. In the previous work we have initiated a phytochemical analysis of flavonoids and phenolic acids and reported the presence of derivatives of quercetin and kaempferol (Gudej and Tomczyk, 1999; Tomczyk and Gudej, 2000). The present paper describes isolation and structure elucidation of additional flavonoids from flowers and leaves of *F. verna*.

Materials and Methods

Plant material

F. verna flowers and leaves were collected in the Białystok area between March and April of 1997. A voucher specimen of plant has been identified by Dr. Jan Gudej and has been deposited in the herbarium of the Department of Pharmacognosy

Medical Academy of Białystok, Poland (No. FV 97004).

Equipment, reagents, solvents

All melting points (m.p.) were determined on BÜCHI 535 melting point apparatus and are uncorrected. All solvents of analytical grade were purchased from POCH (Gliwice, Poland). PC analysis was carried out using Whatman 3MM paper. TLC analysis was carried out using cellulose (TLCc) and Silica gel 60 F254 (TLCg) plates (MERCK, Darmstadt, Germany). Polyamide (ROTH, Karlsruhe, Germany) and Sephadex LH-20 (FLUKA, Buchs, Switzerland) were used for column chromatography (CC). Chromatographic systems: PPC: 5% MeOH (system S1); TLCc: *n*-BuOH/HOAc/H_2O (4:1:5 v/v/v) upper phase (system S2), 15% HOAc (system S3), HOAc/HCl conc./H_2O (30:3:10 v/v/v) (system S4), *n*-BuOH/pyridine/H_2O (6:4:3 v/v/v) (system S5); TLCg: EtOAc/HCOOH/H_2O (18:1:1 v/v/v) (system S6). Visualisation of plates was performed using visible light, UV fluorescence and/or spraying with the following reagents: R1: 2% $AlCl_3$, R2: Naturstoffreagenz A (ROTH, Karlsruhe, Germany), R3: aniline phthalate by heating at 110 °C for 5–10 min. CC: H_2O/MeOH increasing gradient (system S7), C_6H_6/MeOH increasing gradient (system S8), EtOAc/MeOH increasing gradient (system S9). Acid hydrolysis: the pure compounds were treated with 5% HCl for compound **I** and with 10% HCl for compounds **II, III, IV** at 100 °C for 4 hrs. UV spectra were recorded on a SPECORD 40 UV-VIS Spectrophotometer (Jena Analytik AG, Germany) according to Mabry *et al.* (1970). ^{1}H NMR and ^{13}C NMR were taken on a BRUKER instrument (200 and 50 MHz, respectively). MS were obtained with Finnigan MAT 95 mass spectrometer. All solvents from the extracts were evaporated to dryness using a BÜCHI Rotavapor R-200.

Extraction and isolation

Preparation of extracts from flowers has been described previously (Gudej and Tomczyk, 1999). Compound **I** was obtained from an EtOAc extract using a polyamide column (CC, system S7). The fractions eluted with 10% MeOH containing two compounds (mixture A) were repeatedly chromatographed on a polyamide column (CC, system S8). Pure **I** (9 mg) was obtained from fractions eluted with C_6H_6/MeOH (8:2 v/v). Further fractions eluted with 15% MeOH from the same EtOAc extract on a polyamide column (CC, system S7) containing the mixture of compounds **II** and **III** (mixture B). Mixture B was further chromatographed on a polyamide column and eluted with system S9. This procedure led to the isolation of **II** and a mixture of **II** and **III** (mixture C). Compound **II** (35 mg) was obtained from the fraction eluting with EtOAc/MeOH (7.5:2.5 v/v). Using preparative PC, which was twice developed with system S1 from the mixture C, additional amounts of compounds **II** (8 mg) and **III** (4 mg) were separated. Both compounds were purified by column chromatography on Sephadex LH-20 using MeOH as eluent.

The air-dried and powdered leaves of *F. verna* (2 kg) were extracted with petrol and $CHCl_3$ in a Soxhlet apparatus. Plant material purified in this way was successively extracted with MeOH. After solvent evaporation under reduced pressure, the MeOH extract was diluted with H_2O and successively partitioned between Et_2O, EtOAc and *n*-BuOH, affording 6.0, 20.0 and 119.0 g of each dried fraction, respectively. The EtOAc and *n*-BuOH extracts were chosen for further study. Initial isolation of compounds from the EtOAc extract was carried out by chromatography on polyamide eluting with solvents mixtures of increasing polarity (CC, system S7). Elution with 10% MeOH gave a mixture of two compounds (mixture D). Repeated chromatography of mixture D on a polyamide column (CC, system S8) yielded compound **IV** (72 mg). It was obtained from the fraction eluted with C_6H_6/MeOH (7:3 v/v) and was further purified by CC on Sephadex LH-20 using MeOH.

Identification of isolated flavonoid compounds

Compound **I**: Pale-yellow needles; m.p. 187–189 °C; R_f TLCc: S2-0.51; S3-0.56; TLCg: S6-0.35; UV λ_{max} MeOH 267, 297sh, 301, 350; +NaOMe 277, 326, 405; +$AlCl_3$ 274, 306, 352, 398; +$AlCl_3$/HCl 276, 303sh, 348, 397; +NaOAc 275, 326, 362; +NaOAc/H_3BO_3 267, 304sh, 351 nm. ^{1}H NMR (DMSO-d_6): 12.56 (1H, brs, H–HO-5), 7.98 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.87 (2H, d, J = 8.8

Hz, H-3′ and H-5′), 6.40 (1H, d, J = 2.0 Hz, H-8), 6.19 (1H, d, J = 2.0 Hz, H-6), 5.30 (1H, d, J = 7.1 Hz, H-1″), 5.11–4.44 (6H, m, H–OH-sugars), 4.37 (1H, s, H-1‴), 4.12–3.49 (10H, m, H-sugars), 0.79 (3H, d, J = 6.0 Hz, H-6‴, CH_3-rhamnose) ppm; ^{13}C NMR (DMSO-d_6): 177.34 (C-4), 164.29 (C-7), 161.17 (C-5), 159.88 (C-4′), 156.80 (C-9), 156.49 (C-2), 133.18 (C-3), 130.86 (C-2′ and C-6′), 120.87 (C-1′), 115.08 (C-3′ and C-5′), 103.90 (C-10), 101.32 (C-1″), 100.76 (C-1‴), 98.76 (C-6), 93.76 (C-8), 76.31 (C-3″), 75.70 (C-5″), 74.13 (C-2″), 71.77 (C-4‴), 70.55 (C-3‴), 70.30 (C-2‴), 69.88 (C-4″), 68.21 (C-5‴), 67.38 (C-6″), 17.72 (C-6‴) ppm. R_f value of the aglycone (coTLCc, S4, R1) after acid hydrolysis of **I** it was identical with kaempferol. R_f values of sugars (coTLCc, S5, R3) after hydrolysis were identical with the standards, glucose and rhamnose.

Compound **II**: Yellow needles; m.p. 275–276 °C; R_f TLCc: S2-0.41; S3-0.23; TLCg: S6-0.38; UV λ_{max} MeOH 269, 302sh, 332; +NaOMe 277, 329, 393; +$AlCl_3$ 276, 304, 346, 383; +$AlCl_3$/HCl 276, 303, 342, 382; +NaOAc 275, 302, 374; +NaOAc/H_3BO_3 270, 326sh, 344 nm. ^{1}H NMR (DMSO-d_6): 13.17 (1H, s, H–OH-5), 8.03 (2H, d, J = 8.6 Hz, H-2′ and H-6′), 6.89 (2H, d, J = 8.6 Hz, H-3′ and H-5′), 6.79 (1H, s, H-3), 6.27 (1H, s, H-6), 4.68 (1H, d, J = 10 Hz, H-1″) ppm; ^{13}C NMR (DMSO-d_6): 182.10 (C-4), 163.94 (C-2), 162.55 (C-7), 161.12 (C-4′), 160.38 (C-9), 155.98 (C-5), 128.96 (C-2′ and C-6′), 121.60 (C-1′), 115.80 (C-3′ and C-5′), 104.59 and 104.03 (C-8 and C-10), 102.45 (C-3), 98.12 (C-6), 81.84 (C-5″), 78.64 (C-1″) 73.37 (C-2″), 70.82 (C-3″), 70.51 (C-4″), 61.27 (C-6″) ppm. LSIMS calcd for $C_{21}H_{20}O_{10}$ 432.379; negative-ion 431 $[M-H]^-$; positive-ion 433 $[M+H]^+$, 313 $[M+H-120]^+$, 271 $[M+H-162]^+$. Total acid hydrolysis of **II** yielded vitexin accompanied by its Wessely-Moser isomer isovitexin (R_f, coTLC, S2, S3, R1, R2).

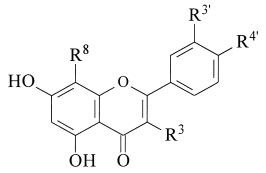
Compound **III**: Amorphous powder; m.p. 255–257 °C; R_f TLCc: S2-0.29; S3-0.15; TLCg: S6-0.28; UV λ_{max} MeOH 256, 267, 293sh, 346; +NaOMe 265, 305sh, 334sh, 405; +$AlCl_3$ 272, 303sh, 333, 426; +$AlCl_3$/HCl 262sh, 274, 356, 384; +NaOAc 271, 325, 386; +NaOAc/H_3BO_3 261, 302, 373 nm. ^{1}H NMR (DMSO-d_6): 13.17 (1H, s, H-OH-5), 7.52 (2H, d, J = 8.6 Hz, H-2′ and H-6′), 6.86 (1H, d, J = 8.2 Hz, H-5′), 6.64 (1H, s, H-6), 6.26 (1H, s, H-3), 4.68 (1H, d, J = 9.7 Hz, H-1″) ppm. Total acid hydrolysis of **III** yielded orientin accompanied by its Wessely-Moser isomer isoorientin (R_f, coTLC, S2, S3, R1, R2).

Compound **IV**: Pale-beige needles; m.p. 215–217 °C; R_f TLCc: S2-0.13; S3-0.79; TLCg: S6-0.09; UV λ_{max} MeOH 270, 302sh, 334; +NaOMe 280, 330, 396; +$AlCl_3$ 276, 304, 349, 386; +$AlCl_3$/HCl 278, 303, 343, 383; +NaOAc 280, 301, 356; +NaOAc/H_3BO_3 271, 301sh, 332 nm. ^{1}H NMR (DMSO-d_6): 11.01 (1H, s, H–OH-5), 8.01 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 6.89 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 6.74 (1H, s, H-3), 6.23 (1H, s, H-6), 4.79 (1H, d, J = 10 Hz, H-1 of 8-C-glc), 4.09 (1H, d, J = 8.5 Hz, H-1 of 2″-O-glc) ppm. ^{13}C NMR (DMSO-d_6): 182.04 (C-4), 163.80 (C-2), 162.57 (C-7), 161.08 (C-4′), 160.60 (C-5), 156.21 (C-9), 128.90 (C-2′ and C-6′), 121.74 (C-1′), 115.86 (C-3′ and C-5′), 105.15 (C-8), 103.89 (C-1‴), 103.65 (C-10), 102.66 (C-3), 98.15 (C-6), 81.77 (C-2″), 81.17 (C-5″), 78.43 (C-3″), 76.25 (C-3‴), 76.01 (C-5‴), 74.36 (C-2‴), 71.51 (C-1″), 70.11 (C-4″), 69.46 (C-4‴), 60.96 (C-6″), 60.39 (C-6‴) ppm. LSIMS calcd for $C_{27}H_{30}O_{15}$ 594.519; negative ion 593 $[M-H]^-$; positive ion 595 $[M+H]^+$, 475 $[M+H-120]^+$, 433 $[M+H-162]^+$, 313 $[M+H-162-120]^+$, 271 $[M+H-162-162]^+$. Total acid hydrolysis of **IV** yielded vitexin accompanied by its Wessely-Moser isomer isovitexin (R_f, coTLCc, S2, S3, R1, R2) and sugar–glucose (R_f, coTLCc, S5, R3).

Results and Discussion

The EtOAc extracts of *F. verna* flowers and leaves were fractionated on polyamide columns. Further separation and purification was achieved by combining PPC, polyamide columns and Sephadex LH-20 and gave pure flavonoid compounds **I–IV**. The identification of those compounds was carried out on the basis of R_f values, products of acid hydrolysis and spectroscopic methods (UV, ^{1}H NMR, ^{13}C NMR, MS). The flavonoids isolated from *F. verna* are listed in Table I. Acid hydrolysis of **I** released glucose and rhamnose in sugar's residue and kaempferol as an aglycone. The UV spectral data of **I** with diagnostic shift reagents indicated a flavonol substituted at position C-3, free C-7 and absence of an *ortho*-dihydroxyl pattern at B ring. The ^{1}H NMR spectrum suggested that **I** is a disaccharide of kaempferol on the basis of two signals in the sugar region at 5.30 (d, J = 7.1 Hz)

Table I. Flavonoids from *Ficaria verna* Huds.



Compound **I**	R^3–O-rut	$R^{3'}$–H	$R^{4'}$–OH	R^8–H
Compound **II**	R^3–H	$R^{3'}$–H	$R^{4'}$–OH	R^8–glc
Compound **III**	R^3–H	$R^{3'}$–OH	$R^{4'}$–OH	R^8–glc
Compound **IV**	R^3–H	$R^{3'}$–H	$R^{4'}$–OH	R^8–glc (1‴→ 2″) glc

rut – rutinose.
glc – glucose.

and 4.37 (s), corresponding to the anomeric protons of β-glucose and to the anomeric proton of the α-linked rhamnose, respectively. That fact indicated that α-rhamnose was attached to the C-6 at β-glucose moiety which was confirmed by its ^{13}C NMR spectrum and this also defined the disaccharide as a 3-O-rutinoside. The structure of compound **I** is therefore established as a kaempferol 3-O-β-D-(6″-O-α-L-rhamnopyranosyl)-glucopyranoside (nicotiflorin). Compounds **II** and **IV** showed the same UV spectra and diagnostic shifts as apigenin with free 5, 7 and 4′-hydroxyl groups. With the two former compounds acid hydrolysis yielded vitexin (identified by co-TLC with an authentic standards) accompanied by small amounts of its Wessely-Moser isomer. The ^1H NMR spectrum confirmed that **II** is a derivative of apigenin substituted by glucose at C-8. The presence of a C-glucosyl bond at C-8 of apigenin was shown by the ^{13}C NMR spectrum. In addition, the structure of **II** was confirmed by LSIMS spectrum. The spectrum of **II** gave a molecular ion peak $[M+H]^+$ at *m*/*z* 433 indicating a molecular mass of 432. A second prominent ion was obtained at *m*/*z* 271 $[M+H-162]^+$ resulting from the loss of a sugar chain consisting of one hexose. The presence of a peak at *m*/*z* 313 $[M+H-120]^+$ suggests that hexose is linked to the aglycone by a C-8 linkage, similarly as in **IV**. Compound **II** was identified as apigenin 8-C-β-D-glucopyranoside (vitexin). The UV spectral data of **III** with diagnostic shift reagent indicated a luteolin. The bathochromic shift observed in band II in the presence of NaOAc indicated a free 7-hydroxyl group. The presence of an *ortho*-dihydroxyl group in the B-ring of **III** was detected by comparing the spectrum in the presence of $AlCl_3$ with that obtained in $AlCl_3$/HCl and additionally confirmed by a bathochromic shift observed in band I in the presence of NaOAc/H_3BO_3. Structure of **III** was additionally recognized as luteolin 8-C-β-D-glucopyranoside (orientin) by analysing the mixture isomers after acid hydrolysis (Wessely-Moser rearrangement) and analysis of the ^1H NMR spectrum. The chromatographic behaviour of **IV** suggested a diglycosidic structure. Acid hydrolysis of **IV** gave compound **II** (co-TLC with authentic standards) and the sugar was identified as glucose. The ^1H NMR spectrum of **IV** exhibited two sugar anomeric protons at 4.79 (d, J = 10 Hz) and 4.09 (d, J = 8.5 Hz) with coupling constants appropriate for β-glucopyranose. The presence of the C-glycosidic bond in C-8 position in **IV** was confirmed by ^{13}C NMR. In this case the signal at 105.15 ppm indicates substitution at C-8 position of the aglycone. In addition, in the LSIMS spectra of **IV** peaks at *m*/*z* 595 (positive mode) and *m*/*z* 593 (negative mode) were observed. In order to obtain information about the structure of the sugar moiety, collisionally induced dissociation of $[M+H]^+$ ion was performed and linked scan spectra at constant B/E were recorded. The presence of peaks at *m*/*z* 433 $[M+H-162]^+$ and *m*/*z* 271 $[M+H-161-162]^+$ confirmed the presence of two hexose units. The formation of relative intense ions at *m*/*z* 475 $[M+H-120]^+$ and *m*/*z* 313 $[M+H-162-120]^+$ suggests that sugar chain is linked by a C-8 linkage (Gluchoff-Fiasson *et al.*, 1989; Qimin *et al.*, 1991). Compound **IV** was, based on products of acid hydrolysis and of its spectroscopic data, identified as apigenin 8-C-β-D-(2″-O-β-D-glucopyranosyl)-glucopyranoside (flavosativaside). The spectral properties of compounds **I–IV**, in-

cluding UV, ^{1}H NMR, ^{13}C NMR, were verified by comparison of its spectral data with those previously described in the literature (Mabry *et al.*, 1970; Markham *et al.*, 1978; Agrawal, 1989; Kartnig *et al.* 1991; Harborne, 1996). This is the first report of these compounds from *F. verna*.

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