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NATURAL DRUGS

FLAVONOIDS AND FREE PHENOLIC ACIDS FROM *PHYTOLACCA AMERICANA* L. LEAVES

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Abstract: In the leaves of *Phytolacca americana* L. flavonoid compounds: kaempferol 3–O–β–D–glucopyranoside, kaempferol 3–O–β–D–xylopyranosyl (1→2)–β–D–glucopyranoside, kaempferol 3–O–α–L–rhamnopyranosyl (1→2)–β–D–glucopyranoside, kaempferol 3–O–diglucoside and quercetin 3–O–glucoside were identified by chemical and spectroscopic methods and using co–chromatography, phenolic acids: p–hydroxybenzoic, vanillic, synapic, p–coumaric ferulic and caffeic were also confirmed.

Keywords: *Phytolacca americana* L., leaves flavonoids and phenolic acids, isolation, identification.

Phytolacca americana L. = *P. decandra* L. (pokeweed) (*Phytolaccaceae*) is a perennial plant occuring throughout the world, mostly in the southern USA, Mexico, India and the Mediterranean region. It is cultivated in Poland as ornament plant.

The family *Phytolaccaceae* is known to be rich in saponins. Their presence has been investigated in different species from *Phytolacca* genus: *P. americana* L. (1), *P. esculenta* Van Houtte (2), *P. acinosa* Roxb. (3), *P. rivinoides, P. bogotensis* H.B.K. (4), *P. polyandra* Bat. (5), *P. thyrsiflora* Fenxl ex Schmidt (6) and *P. dodecandra* L'Herit (7, 8).

The seeds of *P. americana* L. and *P. esculenta* Van Houtte abound in triterpene acids (9, 10). Pentacyclic triterpenoids, derivatives of oleanane, have been isolated from the leaves (11) and fruits (12) of *P. acinosa* Roxb.

Betacyanins are pigments of *P. americana* L.. The black–violet berries contain mainly betanin 6'–O–sulphate and its isoform, while feruloylated derivatives occur in stems and cell cultures as a major component (13).

Flavonoids were analysed in three species of *Phytolacca* L. genus. In 1956 isoquercitrin and astragalin were isolated from the leaves of *P. americana* L. (14) and rutoside together with 3–rutinoside of 7,4'–dimethylquercetin from *P. dioica* l. (14). Moreover, kaempferol 3–O–β–D–glucopyranosyl (1→2)–β–D–galactopyranoside, kaempferol 7–O–methyl–3–O–[β–D–glucopyranosyl (1→2)–β–D–galactopyranoside] and kaempferol 7–O–methyl–3–O–[β–D–xylopyranosyl (1→2)–β–D–galactopyranoside] were found in the leaves of *P. thyrsiflora* Fenzl ex Schmidt (6).

P. americana L. has been reported to possess a variety of biological properties:antiviral effect and induce γ–interferon formation, a purgative and emetic (14, 15) as well as in the treatment of chronic rheumatism (14).

Other species from the genus *Phytolacca* L., e.g. *P. acinosa* Roxb., *P. bogotensis* H.B.K., *P. dodecandra* l'Herit and *P. insularis, P. octandra, P. rivinoides*, are used against ailments of joints, in rheumatism and has different biological properties like molluscicidal and antiinflammatory activity as well as considerable stimulation of immune interferon (IFN–r) and tumor necrosis factor (TNF) (1, 2, 4, 6, 7, 8).

P. americana L. produces an antiviral protein (PAP) and other ribosom–inactivating proteins (RIP's); which were analysed also in *P. dodecandra* L'Herit (16, 17). Cysteine protease, called phytolacain R, was isolated from the reddish fruits of pokeweed and its activity was compared with papaine (18).

Glycoproteins with high content of cysteine (ca. 29%) and 4–5% of carbohydrates are regarded as lymphocyte stimulating agents (15).

Pokeweed is an important homeopathic drug used in acute colds and rheumatism as immunomodulator (15). The juice from the berries was used for colouring of wine and food; however, the whole berries are toxic.

The aim of our work was the isolation and identification of flavonoids and analysis of phenolic acids occuring in the leaves of *P. americana* L.

EXPERIMENTAL

Material and methods

The leaves of *P. americana* L. were collected

in autumn 1994 at the Botanical Garden of A. Mickiewicz University (Poznań).

Chromatography

Paper chromatography, Whatman No 1:
PC–1 HOAc–H_2O (15:85);
PC–2 n–BuOH–HOAc–H_2O (4:1:2);
PC–3 C_6H_6–HOAc–H_2O (125:72:3);
PC–4 EtOAc–HCOOH–H_2O (10:2:3) organic phase;

Thin layer chromatography, silica gel G Merck:
TLC–5 nPrOH–EtOAc–H_2O (7:2:1)
cellulose 2D TLC: (I) C_6H_6–HOAc–H_2O (6:7:3) organic phase;
II HOAc–H_2O (15:85)

Column chromatography:
CC–6 polyamide Woelm H_2O and H_2O–MeOH with increasing percentage of MeOH;
CC–7 cellulose Whatman CF–11 EtOAc–MeOH–H_2O (100:5:5);
CC–8 sephadex LH–20 MeOH

Visualization reagents

Flavonoids: 1% methanolic $AlCl_3$; 0.5% methanolic NA reagent, UV_{366nm} phenolic acids: UV_{366nm}, diazotized sulphanilic acid and 20% Na_2CO_3 (1:1), VIS

Isolation

The dried leaves (150 g) were extracted exhaustively with boiling methanol. The extract was concentrated and, after addition of hot water, the resulted precipitate was filtered off. The filtrate was extracted successively with chloroform, diethyl, ether, ethyl acetate and a mixture of ethyl acetate and methanol (9:1). The etheral fraction was analysed for the presence of phenolic acids. The ethyl acetate and ethyl acetate – methanol fractions were combined and concentrated. The extract was chromatographed over a short large diameter polyamide column (CC–6) and eluted first, with H_2O followed by H_2O–MeOH with increasing percentage of MeOH. Fractions containing flavonoids were evaporated, and for separation were subjected to CC–7. The final purification was done by CC–8 to yield compounds **III, IV, V, VI** and **VII**.

Identification

Phenolic acids

The etheral fraction was subjected to 2D TLC co–chromatography for the presence of phenolic acids by comparison of R_f, fluorescence under UV_{366} and colour of the spots after spraying with diazotized sulphanilic acid and Na_2CO_3 with authentic samples (Table 1).

Flavonoids

Spectral analysis

The UV spectra (Specord UV–VIS) wee recorded in methanol, also after addition of the reagents according to Mabry (19).

The ^1H NMR (300 MHz), ^{13}C NMR (75 MHz) spectra were run on the Varian Unity instrument in DMSO–d_6 with TMS as internal standard. The ^1H NMR spectra were also recorded with the addition of D_2O. The chemical shifts were given in ppm.

Hydrolysis

Total acid hydrolysis: 1 mg of compound **III, V, VII, VI** and **IV** was heated in 1 ml of 2% HCl for 2 hours. The hydrolyzates were extracted with ethyl acetate. The aglycones in organic phase were identified by co–chromatography (PC–1,3), the water phase was analyzed for sugars (TLC–5).

Partial acid hydrolysis: 1 mg of compounds **V, VII** and **VI** was heated for 5 minutes in 1 ml of 0.5% HCl. The hydrolyzates were extracted with ethyl acetate and the organic phase was separated by preparative chromatography on Whatman 3 (PC–1). The bands corresponding to the intermediate products were eluted and subjected to UV spectral analysis and complete hydrolysis.

Kaempferol 3–O–β–D–glucopyranoside [III]

PC–1 Rf=0.56 yellow needles.
UV (MeOH) λ max nm: 268, 353; +NaOAc 277, 381; +NaOAc–H_3BO_3 269, 358; +NaOMe 270, 410; +$AlCl_3$ 278, 305, 352, 402; +$AlCl_3$–HCl 278, 305, 352, 402.
Acid hydrolysis: kaempferol and glucose.
^{13}C NMR δ ppm: 177.8 (C–4); 163.5 (C–7); 160.9 (C–5); 159.0 (C–4'); 156.3 (C–9); 156.3 (C–2); 133.0 (C–3); 130.7 (C–2',6'); 121.9 (C–1'); 115.0 (C–3',5'); 104.4 (C–10); 101.0 (C–1''), 98.8 (C–6); 94.6 (C–8); 77.5 (C–3''); 76.3 (C–5''); 74.0 (C–2''); 70.5 (C–4''); 61.0 (C–6''). ^1H NMR δ ppm: 8.04 (2 H, d, J–8.8 Hz, H–2', 6'); 6.86 (2 H, d, J–8.8 Hz, H–3',5'); 6.40 (1 H, d, J–2.0 Hz, H–8); 6.21 (1 H, d, J–2.0 Hz, H–6); 5.44 (1 H, d, J–7.5 Hz, H–1''), 3.75–3.10 (m, the remaining protons of glucose).

Kaempferol 3–O–β–D–xylopyranosyl (1→2)–β–D–glucopyranoside [V]

PC–1 Rf–0.74, yellow amorphous powder.
UV (MeOH) λ max nm: 267, 352; +NaOAc 277, 378; +NaoAc–H_3BO_3 270, 250; +NaOMe 269, 400; +$AlCl_3$ 277, 303, 348, 400; +$AlCl_3$–HCl 277, 300, 340, 400.

Table 1. Chromatographic analysis of free phenolic acids isolated from leaves P. americana

Phenolic acid	Rf values in solvent system		UV_{366}	Diazotized sulphanilic
	I	II	nm	acid + 20% Na_2CO_3
p-hydroxybenzoic	0.40	0.66	br	y
vanillic	0.55	0.60	br	o
sinapic	0.63	0.40 (0.60)	dbl	dv
p-coumaric	0.41	0.54 (0.78)	bl	vr
ferrulic	0.69	0.48 (0.70)	bl	v
caffeic	0.04	0.40 (0.68)	bl	o

Notes:
(I) C_6H_6 – HOAc – H_2O (6:7:3) organic phase
(II) HOAc – H_2O (15:85) Fluorescence / colouration:
V – violet; y – yellow; o – orange; bl — blue; d – dark; r – red; br – brown; o – olive
Rf values of cis-isomers of the corresponding acids are given in brackets.

Total acid hydrolysis: glucose, xylose and kaempferol; partial hydrolysis kaempferol 3–O–glucoside.
^{13}C NMR δ ppm: 178.0 (C–4); 163.0 (C–7); 160.7 (C–5); 159.0 (C–4'); 157.0 (C–9); 156.9 (C–2); 134.5 (C–3); 130.9 (C–2',6'); 122.0 (C–1'); 115.8 (C–3',5'); 105.2 (C–10); 103.3 (C–1'''); 100.1 (C–1''); 99.5 (C–6); 95.8 (C–8); 79.8 (C–2''); 76.4 (C–3'',5'',3'''); 74.8 (C–2'''); 70.1 (C–4'',4'''); 64.8 (C–5'''); 60.0 (C–6'').
^1H NMR δ ppm: 8.01 (2 H, d, J–8.5 Hz, H–2',6'); 6.84 (2 H, d, J–8.5 Hz, H–3',5'); 6.38 (1 H, d, J=2.0 Hz, H–8); 6.19 (1 H, d, J=2.0 Hz, H–6); 5.69 (1 H, d, J–7.8 Hz, H–1''); 4.60 (1 H, d, J=7.8 Hz, H–1'''); 3.75–3.18 (m, the remaining protons of sugars).

Kaempferol 3–O–α–L–rhamnopyranosyl (1→2)–β–D–glucopyranoside [VII]

PC–1 Rf=0.86, yellow amorphous powder.
UV (MeOH) λ max nm: 268, 350; +NaOAc 278, 390; +NaOAc–H_3BO_3 267, 358; +NaOMe 268, 399; +$AlCl_3$ 275, 300, 353, 395; +$AlCl_3$–HCl 278, 305, 350, 393.
Total acid hydrolysis: rhamnose, glucose and kaempferol; partial acid hydrolysis: kaempferol 3–O–glucoside as secondary heteroside.
^{13}C NMR δ ppm: 177.1 (C–4); 162.3 (C–7); 160.3 (C–5); 159.3 (C–4'); 156.8 (C–2); 155.0 (C–9); 134.5 (C–3); 129.9 (C–2',6'); 121.9 (C–1'); 115.1 (C–3',5'); 104.9 (C–10); 100.3 (C–1'''); 99.1 (C–6); 98.5 (C–1''); 95.9 (C–8); 76.5 (C–2'',5''); 75.9 (C–3''); 72.0 (C–4'''); 70.5 (C–2'''); 70.3 (C–3'''); 69.2 (C–4''); 68.5 (C–5'''); 60.3 (C–6''); 18.9 (C–6''').
^1H NMR δ ppm: 8.01 (2 H, d, J=8.5 Hz, H–2',6'); 6.85 (2 H, d, J=8.5 Hz, H–3',5'); 6.37 (1 H, d, J=2.0 Hz, H–8); 6.17 (1 H, d, J–2.0 Hz, H–6); 5.63 (1 H, d, J=7.8 Hz, H–1''); 5.08 (1 H, s, H–1'''); 3.15–3.70 (m, the remaining protons of glucose and rhamnose), 1,18 (3 H, d, J–6.0 Hz, Me of rhamnose).

Kaempferol 3–O–diglucoside [VI]

PC–1 Rf–0.64, yellow amorphous powder.
UV (MeOH) λ max nm: 266, 348; +NaOAc 269, 388; +NaOAc–H_3BO_3 266, 350; +NaOMe 266, 390; +$AlCl_3$ 277, 305, 350, 400; +$AlCl_3$–HCl 275, 305, 342, 400.
Total acid hydrolysis: glucose and kaempferol; partial acid hydrolysis: kaempferol 3–O glucoside as secondary heteroside.

Quercetin 3–O–glucoside [IV]

PC–1 Rf=0.48, yellow amorphous powder.
UV (MeOH) λ max nm: 259, 360; +NaOAc 262, 389; +NaOAc–H_3BO_3 268, 380; +NaOMe 272, 410; +$AlCl_3$ 270, 300, 340, 439; +$AlCl_3$–HCl 275, 298, 381, 409.
Acid hydrolysis: quercetin and glucose.

RESULTS AND DISCUSSION

The etheral, ethyl acetate and ethyl acetate–methanol fractions were obtained from the methanolic extract from the leaves of *P. americana* L. The etheral fraction was analysed for the presence of phenolic acids by co–chromatography against the reference samples. Comparison of the R_f values, fluorescence under UV_{366} and spot colours before and after visualization with a mixture of diazotized sulphanilic acid and Na_2CO_3 showed that the fraction contained at least eight phenolic

acids from which: synapic, p–hydroxybenzoic, vanillic, p–coumaric, ferulic and caffeic acid were identified. Column chromatography of the ethyl acetate and ethyl acetate–methanol fractions afforded five flavonoids. The flavonoids were identified by the analysis of their UV spectra (19) and product of partial and complete acid hydrolysis. Compounds **III, V** and **VII** were additionally characterized by ^{1}H and ^{13}C NMR spectra (20, 21, 22).

Compound **III**, a major component of the flavonoid fraction, gave kaempferol and glucose upon hydrolysis. The ^{1}H and ^{13}C NMR spectra revealed that this compound was a derivative of kaempferol substituted at C–3 by glucose. The presence of anomeric proton at 5,44 ppm with coupling constant J–7.5 Hz indicated β–configuration of glucose. Therefore compound **III** is kaempferol 3–O–β–D–glucopyranoside.

Complete hydrolysis of flavonoid **V** afforded xylose, glucose and kaempferol, while kaempferol 3–O–glucoside were obtained from partial hydrolysis. The sugar region of the ^{1}H NMR, spectrum revealed two signals of the anomeric protons of xylose and glucose at 4.60 and 5.60 ppm, respectively with coupling constants J=7.8 Hz characteristic for the β–configurations. Attachment of the xylose at C–2 glucose was determined by shift of C–2 (4.0 ppm) as compared to the corresponding signal spectra ^{13}C NMR of kaempferol 3–O–glucoside. Compound **V** is thus kaempferol 3–O–β–D–xylopyranosyl(1→2)–β–D–glucopyranoside.

Total acid hydrolysis of flavonoid **VII** gave glucose, rhamnose and kaempferol, whereas partial yielded kaempferol 3–O–glucoside. In the ^{1}H NMR spectrum of compound **VII** two signals were observed in the region characteristic for anomeric proton of sugars. Doublet at 5.63 ppm (J=7.8 Hz) was assigned to glucose β–linked to the aglycone and singlet at 5.8 ppm corresponded to the anomeric proton of the α–linked rhamnose. This chemical shift date indicated that α–rhamnose was attached to the C–2 of β–glucose moiety as well as in the neohesperidosides; in 3–rutinosides the glucose and rhamnose H–1 signals appear at ca. 5.3 and 4.4 ppm respectively. This sugestion was confirmed by ^{13}C NMR: a downfield shift of 2.5 ppm for the C–2 signal of glucose revealed that the interglycosidic linkage was in this position, like in neohesperidosides. Thus, compound **VII** is kaempferol 3–O–α–rhamnopyranosyl (1→2)–β–D–glucopyranoside.

Compounds **IV** and **VI** were identified by analysis of their UV spectra and products of hydrolysis as well as by co–chromatography as querectin 3–O–glucoside and kaempferol 3–O–diglucoside, respectively.

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