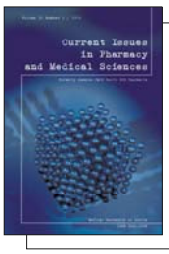

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Determination of flavonols and phenolic acids in *Puearia lobata* (Kudzu) root by HPLC-PDA method

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ABSTRACT

Phenolic acids and derivatives of quercetin in Kudzu root were determined by high-performance liquid chromatography with diode array detector. The calibration curves were constructed using phenolic compounds standards (the coefficient of determination (R^2) was 0.9990-0.9997 for phenolic acids and 0.9989–0.9994 for flavonols. respectively). Ethanol was the most efficient solvent in extracting flavonols and phenolic acids from dried Kudzu roots at the optimal extraction time 60 min. Due to acidic hydrolysis the content of free phenolic acids was increasing. The predominant phenolic acid in Kudzu root determined by HPLC-PDA method with hydrolysis digestion is p-hydrozybenzoic acid (39.57±1.44 mg/g d.m.), next one was: p-coumaric acid (6.20±0.11 mg/g d.m.), followed by ferulic acid (11.52±0.21 mg/g d.m.).On the other hand, the highest content of flavonols was found for routine (6.13±0.11 mg/g d.m.). The contents of the other flavonols in kudzu root are between 0.73-2.52 mg/g d.m. for Rh and Q.

Keywords: chromatography, Kudzu root, phenolic acids, flavonols

INTRODUCTION

Kudzu (*Pueraria lobata*) is native to southern Japan and southeast China. Kudzu root is a medicinal plant (called "Ge-gen" and in Japan as "Kakkon"), known in Traditional Chinese Medicine for thousands of years. In these regions, it was used mainly in the treatment of older people and women in the peri-and postmenopausal time [3, 6, 8, 12].

Recently, Kudzu root extracts have been extensively studied in many centers research, which revealed the presence of large amounts of isoflavones (an average of 1.77 to 12% dry matter), including daidzin (structurally related to genistein, an antileukemic agent), daidzein (an anti-inflammatory and antimicrobial agent), puerarin, and other compounds [1-3, 7, 9, 10]. Daidzin action is caused by its effect on the inhibition of aldehyde dehydrogenase metabolism [4, 5]. In vitro studies have shown that daidzin is a potent, selective and reversible inhibitor of aldehyde dehydrogenase present in the mitochondria of human, guinea pig and rat [4, 5]. Hence, some researchers suggest that using kudzu is a possible way to treat alcoholic cravings, but the mechanism for this is not yet established [3, 11, 13, 14]. Moreover, compounds isolated from Kudzu root can affect neurotransmitters (including serotonin, GABA, and glutamate) [3]. Kudzu is also used to treat osteoporosis, migraine, headaches, coronary insufficiency, hypertension, allergies, diarrhea [1, 8, 11]. It has also shown a potential in animal models of Alzheimer's disease [1, 11]. There are many other examples of Puerariae radix beneficial effects, but do not have the character of scientific reports, which highlight other authors [13].

In the present work, previously described HPLC with a photodiode array detector (HPLC-PDA) method for the determination of quercetin derivatives (Q – quercetin, Qc – quercitrin, H – hyperoside, R – rutin, Rh – rhamnetin, K – keampferol, and M – myricetin) and derivatives of benzoic acid and cinnamic acid (GA – gallic acid, CA – caffeic acid, ChA – chlorogenic acid, FA – ferulic acid, SA – sinapic acid, pCA – *p*-coumaric acid, hBA – *p*-hydroxybenzoic acids) in the Kudzu root, is applied. The impact of the extraction conditions, procedure (water-bath with ultrasonic extraction), solvent, time and hydrolysis digestion was tested.

MATERIALS AND METHODS

Materials. The phenolic compounds were isolated from Kudzu root purchased from the Pharmaceutical Companies (STANDARD, Lublin, Poland). Methanol

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(HPLC grade and 99.8%), quercetin derivatives, gallic, caffeic and chlorogenic acids, isopropanol, tetrahydrofuran (HPLC-grade purity), ethanol, glacial acetic acid were supplied from Sigma–Aldrich (Poznań. Poland). Ferulic, sinapic acids were from Fluka (USA). P-coumaric and p-hydroxybenzoic acids were purchased from Polskie Odczynniki Chemiczne (Poland). Hydrochloric acid (35–38 %) was supplied by Chempur (Poland). Redistilled water was used for preparation of solutions.

Apparatus. UV-Vis spectra were recorded on a UV-Vis HELIOS á spectrophotometer (Unicam. Cambridge. United Kingdom) in 1 cm quartz cell. HPLC SHIMADZU (Kyoto. Japan) system with auto sampler SIL-20AC HT and photodiode multi-wavelength detector (SPD-M20A diode array detector), was applied. The chromatographic data were recorded and processed by the *LCsolution version 1.23 SP*. The absorption was measured both as a spectrum (200-800 nm) and at the wavelengths of: 254 nm (for *p*-hydroxybenzoic and gallic acids), 325 nm (for caffeic, chlorogenic, sinapic, ferulic and *p*-coumaric acids) and 360 nm for quercetin derivatives.

Extraction procedure. The grounded dried samples (1.00±0.01 g. 40 mesh) of Kudzu roots were extracted three times with 10 ml of solvent. The different kind of solvents used was follows: water and ethanol. Extraction was carried out at room temperature (22°C) by extraction in ultrasonic water-bath. The extraction times were 20, 40 and 60 min. Next extracts were centrifuged (4500 rpm. 15 min) and filtered with filter paper and then with a syringe filter. The influence of hydrolysis digestion was also tested.

Chromatographic conditions. A reverse-phase column (Discovery C-18. 150 mm × 4.6 mm. 5 µm particle size; Supelco, USA) was used as a stationary phase. For phenolic acids, the mobile phase was acetic acid (2 %) (A) and methanol (B) at a total flow-rate of 1 ml/min. The analysis followed a nonlinear gradient program. Initial condition were 0% phase B, 0-11 min changed to 25% B, 11-15 min to 28.75% B, 15-25 min to 36% B, 25-35 min to 45% B, 35-38 min to 65% B and 38-41 min back to 0% phase B, kept to 45 min. The injection volume was 10 µL. Eluates were detected at 254 nm (GA. hBA) and 325 nm (CA, ChA, FA, SA, and pCA).

For flavonols analyses, mobile phase A was a mixture of water/isopropanol (19:1) and phase B was water/isopropanol/ THF (5:4:1). The non-linear gradient programme analysis for the separation of quercetin derivatives: 0-2 min changed to 30.00% B, 2-13 min to 45.00% B, 13-15 min 45.00% B, 15-25 min 55.00% B, 25-27 min 55.00% B, 27-32 min 60.00 % B, 32-35 min 65.00% B, 35-40 min 70.00% B, 40-42 min back to 20.00% B, 42-45 min 10.00% B, 45-46 back to initial 0.00 % B and finally kept 0% B for 3 min. The flow-rate of 1 ml/min and detection at 360 nm was used. The injection volume was 20 µL.

Peaks were identified by retention time and spiking of extracts with standards of phenolic acids or the quercetin derivatives.

HPLC – Calibration curves. The phenolic acids analysed were dissolved in a methanol/water mixture (8:2), whereas the flavonols were dissolved in ethanol and diluted to appropriate concentration ranges for the construction of calibration curves. The calibration curves were based on the analysis of 5 repeat determinations of standards at 10 concentration levels (Tables 1 and 2). The curves were constructed by plotting peak area as a function of concentration.

Table 1. Calibration curves for phenolic acids standards

Phenolic acid	Linear model $y = ax + b$		R^2	Concentration range	LOD	LOQ
	$a \pm S_a$	$b \pm S_b$		mg/L	mg/L	mg/L
GA	18480±188	5412±1186	0.9990	1.50–150	0.36	1.09
ChA	28406±264	136259±69405	0.9992	25.00–300	7.98	24.04
CA	54327±430	48342±4260	0.9994	25.00–300	7.55	22.87
FA	52474±292	11271±2973	0.9997	15.00-200	4.63	14.04
SA	52692±815	78950±27412	0.9990	10.00–100	3.30	10.00
pCA	30487±269	15126±2119	0.9992	25.00–200	7.39	22.40
hBA	5024±42	18527±4387	0.9994	25.00–200	6.66	20.19

x – Concentration; y – peak area; a – slope; b – intercept; $\pm S_a$ – standard deviation of slope; $\pm S_b$ – standard deviation of intercept; R^2 – the coefficient of determination. $S_{x/y}$ – standard deviation of y-residuals. LOD – detection limit ($(3.3S_{x/y})/a$); LOQ – quantification limit ($(10S_{x/y})/a$).

Table 2. Calibration curves for flavonols (standards)

Flavonols	Linear model $y = ax + b$		R^2	Concentration range	LOD	LOQ
	$a \pm S_a$	$b \pm S_b$		mg/L	mg/L	mg/L
R	22164±261	5104±734	0.9990	6.00–100	1.74	5.27
H	169792±1529	(–33514±7802)	0.9994	1.00–50	0.28	0.85
Qc	44009±734	(–80465±1317)	0.9989	5.50–100	1.82	5.51
M	21217±449	(–11759±2638)	0.9990	2.00–100	0.58	1.75
Q	30065±412	5131±973	0.9989	6.00–100	1.80	5.47
K	37672±3301	(–18017±2529)	0.9994	1.50–100	0.41	1.24
Rh	24676±651	(–3452±332)	0.9991	2.50–100	0.82	2.49

x – Concentration; y – peak area; a – slope; b – intercept; $\pm S_a$ – standard deviation of slope; $\pm S_b$ – standard deviation of intercept; $S_{x/y}$ – standard deviation of y-residuals. LOD – detection limit ($(3.3S_{x/y})/a$); LOQ – quantification limit ($(10S_{x/y})/a$).

RESULTS

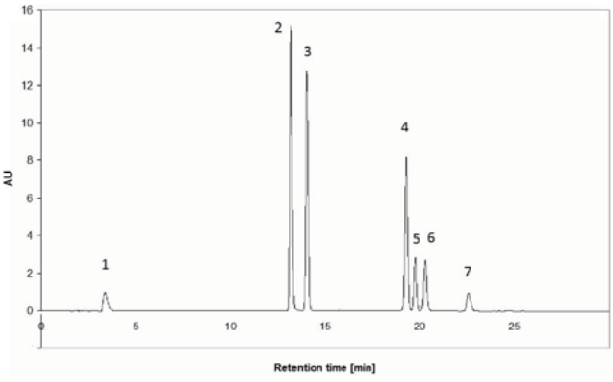

Fig. 1. HPLC chromatogram of phenolic acids standards (1 – GA, 2 – ChA, 3 – CA, 4 - FA, 5 – SA, 6- hBA, 7 – pCA)

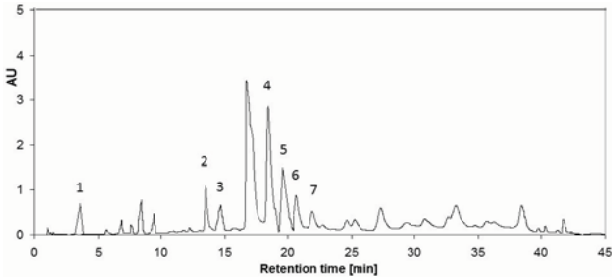

Fig. 2. HPLC chromatogram of Kudzu root ethanolic extract (1 – GA, 2 – ChA, 3 – CA, 4 - FA, 5 – SA, 6- hBA, 7 – pCA)

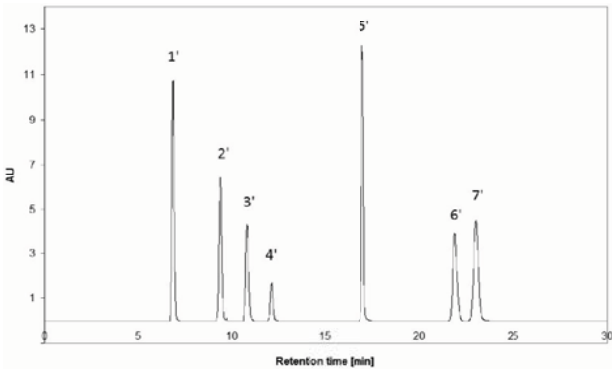

Fig. 3. HPLC chromatogram at 360 nm for quercetin derivative standard (1'- R, 2' – H, 3' – Qc, 4' – M, 5' – Q, 6' – K, 7' – Rh)

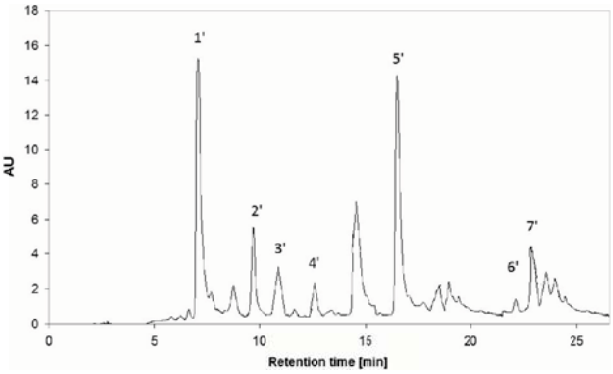

Fig. 4. HPLC chromatogram at 360 nm of Kudzu root (1'- R, 2' – H, 3' – Qc, 4' – M, 5' – Q, 6' – K, 7' – Rh)

mg/L for quercetin and chlorogenic acid, respectively. The quantification limits calculated were 0.85–24.04 mg/L for hyperoside and chlorogenic acid, respectively.

Typical chromatograms for separation of phenolic acids and flavonols standards and for Kudzu extracts are presented in Figure 1-4. Peaks on the chromatograms were identified by the retention times using the reference standards in the same chromatographic conditions or by spiking the extracts with the reference standards. The purity of each peak was checked by a PDA software. Due to the complexity of the natural samples extracts, lack of standards and applied detector, the precise identification of the every peak on chromatograms was not performed.

Table 3. Phenolic acids content in Kudzu root determined by HPLC-PDA method

Solvent	Extraction time [min]	CONTENT OF PHENOLIC ACIDS average ±μ [mg/g] (p=0.05, n=3)						
		GA	CA	CA	FA	SA	pCA	pHB
H_2O	20	2.28±0.01	0.14±0.01	0.91±0.08	9.15±0.26	-	2.61±0.04	28.86 ±4.05
	40	2.66±0.01	0.14±0.01	0.91±0.01	9.91±1.09	-	2.97±0.19	30.44 ±0.75
	60	3.58±0.01	0.23±0.02	1.05±0.03	11.01±0.72	-	4.34±0.62	35.93 ±0.40
	hydrolysis digestion	3.83±0.02	0.25±0.01	1.09±0.03	11.41±0.87	-	4.58±0.12	36.56 ±0.77
EtOH	20	2.63±0.07	0.17±0.01	1.02±0.02	9.88±0.27	2.04±0.27	3.09±0.12	24.51 ±2.90
	40	2.94±0.03	0.17±0.01	1.09±0.01	10.40±0.09	2.24±0.05	3.67±0.02	32.34± 2.21
	60	3.15±0.04	0.27±0.01	1.30±0.03	11.49±0.23	2.70±0.01	4.61±0.33	37.80± 2.08
	hydrolysis digestion	4.08±0.11	0.29±0.01	1.33±0.02	11.52±0.21	2.73±0.01	6.20±0.11	39.57 ±1.44

Table 4. Flavonols content in Kudzu root determined by HPLC-PDA method

Solvent	Extraction time [min]	CONTENT OF FLAVONOLS average ±μ [mg/g] (p=0.05. n=3)						
		R	H	Qc	M	Q	K	Rh
H_2O	20	2.87±0.12	0.45±0.03	0.98±0.09	0.26 ± 0.001	2.02±0.01	0.84±0.06	0.052±0.001
	40	3.89±0.18	0.47±0.01	1.01±0.06	0.26±0.001	2.03±0.02	0.90±0.05	0.069±0.002
	60	5.99±0.14	0.52±0.01	1.22±0.02	0.28±0.001	2.50±0.03	1.00±0.02	0.116±0.011
	hydrolysis digestion	6.01±0.02	0.59±0.01	1.64±0.04	0.33±0.002	2.71±0.03	1.23±0.01	0.142±0.016
EtOH	20	5.06±0.12	0.85±0.03	1.08±0.01	1.38±0.11	1.97±0.01	0.95±0.01	0.52±0.01
	40	5.36±0.16	0.87±0.02	1.18±0.11	1.47±0.05	2.06±0.02	0.82±0.02	0.66±0.05
	60	6.01±0.01	1.37±0.01	1.22±0.01	1.90±0.08	2.52±0.05	1.77±0.03	0.73±0.03
	hydrolysis digestion	6.13±0.01	1.42±0.03	1.83±0.10	2.21±0.03	2.74±0.11	1.88±0.02	0.81±0.02

DISCUSSION OF RESULTS

For the quantitative analysis the HPLC-PDA method with calibration curves was used. The statistical parameters for calibration curves are presented in Table 1 and Table 2. The mean recovery for every standard was higher than 98.90%±0.32 (± SD). The lowest detection limit was 0.28 mg/L and 0.36 mg/L for hyperoside and gallic acid, respectively, whereas the highest was 1.80 mg/L and 7.98

As we can see in Tables 3 and 4, ethanol was the most efficient solvent to extraction flavonols and phenolic acids from Kudzu root. The optimal extraction time was 60 min. Extraction was carried out at room temperature in water bath shaker with ultrasonic. Analysis of phenolic

acids revealed that the predominant are p-hydroxybenzoic acid (37.80±2.08 mg/g d.m.), ferulic (11.49±0.23 mg/g d.m.) and p-coumaric (4.61±0.33 mg/g d.m.). The highest concentration of flavonols obtained for rutin (6.01±0.01 mg/g d.m.). The contents of the other flavonols in kudzu root are between 0.73-2.52 mg/g d.m. for Rh and Q, respectively. After hydrolysis digestion the concentration of phenolic compounds was increasing (e.g. pHA 37.80±2.08 mg/g and 39.57±1.44 mg/g d.m., pCA 4.61±0.33 m/g and 6.20±0.11 mg/g d.m., R 6.01±0.01 mg/g and 6.13±0.01 mg/g d.m., before and after hydrolysis, respectively). The increase can be caused by the acidic hydrolysis, which was used for flavonoid glycosides hydrolysis to aglycones and phenolic acid esters to phenolic acids.

In the literature there is lack of information about content of flavonols and phenolic acids, which was described in this publication, hence the comparison is impossible. Daidzein, puerarin, daidzina, genistein, genistin, [2, 7, 9, 15] and isoflavonoids are the centre of interesting other researchers.

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REFERENCES

1. Choo M.K. et al.: Antitrombotic and antiallergic activities of daidzein, a metabolite of puerarin and daidzin produced by human intestinal microflora. *Biol. Pharmaceut. Biul.*, 25: 1328, 2002.
2. Delmonte P. et al.: Determination of isoflavones in dietary supplements containing soy,Red Clover and kudzu: Extraction followed by basic or acid hydrolysis, *Journal of Chromatography*, A, 1107, 59, 2006.
3. Keung W.M.: Anti-dipsotropic isoflavones: the potential theraupeutic agents for alkohol dependence. *Med. Res Rev.*, 23: 669, 2003.
4. Keung W.M., Klyosow B.L., Vallee B.L.: Daidzin inhibits mitochondria aldehyde dehydrogenase and suppresses etanol intake of Syrian golden hamsters. *Proc Natl Acad Sci USA*, 94:1675, 1997.
5. Keung W.M., Valle B.L.: Daidzin and daidzein suppress free-choice etanol intake by Syrian golden hamsters. *Proc Natl Acad Sci USA*, 90:10008, 1993.
6. Kohlműnzer S. (1985): *Farmakognozja podręcznik dla studentów farmacji*, Warszawa, PZWL, p. 124.
7. Kwun K.H., Kim G.J., Shin H.J.: Ultrasonication Assistance Increases the Efficiency of Isoflavones Extraction from Kudzu (Pueraria lobata Ohwi) Roots Waste. *Biotechnology and Bioprocess Engineering*, 14, 345, 2009.
8. Lamer-Zarawska D., Kowal-Gierczak B., Niedworok J. (eds) (2007). *Fitoterapia i leki roślinne*, Warszawa, PZWL, p. 415.
9. Lau C.S. et al.: A glicoside Flavonid in kudzu *(Pueraria lobata),* Identification, Quantification, and Determination of Antioxidant Activity. *Applied Biochemistry and Botechnology*, vol. 121, 2005.
10. Lee K.T. et al.: Tectorigenin, an isoflavonnne of puerariathunbergianaBenth; induced differentiation and apoptosis in human promyelocytic leukemia HL- 60 cells. *Biol. Pharm. Bull.*, 24, 1117, 2001.
11. Lukas S.E. et al.: An extract of the Chinese herbal root kudzu reduces alcohol drinking by heavy drinkers in a naturalistic setting. *Alcohol Clin Exp Res.*, 29(5):756-62, 2005.
12. Marinelli M. (2006). Wielka Encyklopedia Roślin, Warszawa, Świat książki, p. 466.
13. Rezvani A.H. et al.: Plant derivatives in the treatment of alkohol dependenci. *Pharmacol Biochem Behav.*, 75:593, 2003.
14. Shebeck J., Rindone J.P.: A pilot study exploring the effect of kudzu Root on the drinking habits of patiens with chronic alcoholism. *J Altern Complement Med.*, 6:45-8, 2000.
15. Zhang Y. et al.: High-performance iquid chromatography-tandem mass spectometry for identification of isoflavones and description of the biotransformation of kudzu root. *Anal Bioanal Chem.*, 383: 787, 2005.