ISOFLAVONOIDS FROM *GLYCYRRHIZA SP*. AND *ONONIS SPINOSA*

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Abstract

In order to identify new sources of isoflavonoids, an analysis was carried out on three species of the *Fabaceae* family: *Glycyrrhiza glabra* L., *Glycyrrhiza echinata* L., *Ononis spinosa* L., harvested from the Romanian spontaneous flora. The HPLC-MS method was used to investigate the presence of isoflavonoids in the studied plants. In *Glycyrrhiza glabra* the isoflavonic glycosides like daidzin ($0.434x10^{-3}$%), genistin ($0.672x10^{-3}$%), ononin ($27.490\ x10^{-3}$ %), and the aglycon formononetin ($16.607\ x\ 10^{-3}$%) were found, while in *Glycyrrhiza echinata,* only formononetin ($0.864x10^{-3}$%) and ononin ($3.904x10^{-3}$%) were found. Formononetin was identified in both hydrolyzed solutions. *Ononis spinosa*, the richest species in isoflavonoids, contains daidzin ($0.944x10^{-3}$%), genistin ($1.173x10^{-3}$%), ononin ($175.7x10^{-3}$%), formononetin ($9.499x10^{-3}$%) and after hydrolysis, daidzein ($0.8196x10^{-3}$%), formononetin ($113.622x10^{-3}$%) and ononin ($18.939x10^{-3}$%) as residual glycosides.

Rezumat

În vederea identificării de noi surse de izoflavonoide, s-au analizat trei specii din familia *Fabaceae*: *Glycyrrhiza glabra* L., *Glycyrrhiza echinata* L., *Ononis spinosa* L., recoltate din flora spontană a României. A fost utilizată metoda HPLC-MS pentru a investiga prezenţa izoflavonoidelor în plantele studiate. *Glycyrrhiza glabra* conţine heterozidele daidzină ($0,434x10^{-3}$%), genistină ($0,672x10^{-3}$%) şi ononină ($27,490x10^{-3}$%) şi agliconul formonetină, iar în *Glycyrrhiza echinata* au fost identificate numai formononetina ($0,864x10^{-3}$%) şi ononina ($3,904x10^{-3}$%). În probele hidrolizate a fost identificată formononetina. Specia *Ononis spinosa*, cea mai bogată în aceste principii active, conţine daidzină ($0,944x10^{-3}$%), genisteină ($1,173x10^{-3}$%), ononină ($175,7x10^{-3}$%), formononetină ($9,499x10^{-3}$%); după hidroliză s-au identificat daidzeina ($0,8196x10^{-3}$%), formononetina ($113,622x10^{-3}$%) şi ononina ($18,939x10^{-3}$%).

Keywords: *Fabaceae*, isoflavonoids, HPLC-MS

Introduction

Isoflavonoids are plant secondary metabolites that have various biological functions and significant ecological impacts. It is known that they are frequently found in soybeans and other plants from *Fabaceae* family [1,2,12]. Isoflavones are a subgroup of phytoestrogens, natural plant substances with structures similar to 17-β-estradiol and capable of binding to estrogen receptors [2,3,9]. Recently, isoflavones have become of great of interest due to several reports on their positive effect on human health, in particular, in the prevention of some forms of hormone-dependent cancers, cardiovascular diseases, osteoporosis, adverse menopausal manifestations and age-related cognitive decline [2,9,12].

Glycyrrhiza glabra L. (licorice) contains not only triterpene saponins (glycyrrhizin), flavonoids, polysaccharides, but also various isoflavonoids: glabrone, glyzaglabrin, glyzarin, formononetin, glycyrrhizaisoflavones; *Glycyrrhiza echinata* was less studied, its aerial parts contain formononetin [1,5,6]. In the roots of *Ononis spinosa* L. (spiny restharrow) the folowing compounds are present: onocerin, sitosterol, isoflavones (ononin, formononetin, genistein, biochanin A 7-glucoside), as well as small amounts of the essential oil with trans-anethole, carvone and menthol [2,7].

The purpose of this study was to evaluate the isoflavone profile in the roots of some *Fabaceae* species from the Romanian spontaneous flora, by HPLC-MS analysis, in order to obtain new sources of phytoestrogens.

Materials and Methods

The roots of *Glycyrrhiza glabra* L. (voucher No. 579), the roots of *Glycyrrhiza echinata* L. (voucher No. 580) and the roots of *Ononis spinosa* L. (voucher No. 682) were collected in September-October 2009 (Cluj, Romania). Voucher specimens were deposited in the Herbarium of the Department of Pharmaceutical Botany of the Faculty of Pharmacy ("Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania). The hydroalcoholic extracts obtained from the roots (5% in 80% methanol, 60^0C) were analyzed by HPLC-MS, before and after acid hydrolysis (2M HCl) [4,5,11].

Reagents

Standards: daidzin (daidzein 7-glucoside), genistin (genistein 7-glucoside), ononin (formononetin 7-glucoside), daidzein, glycitein,

genistein, formononetin from Merck (Germany). Methanol and hydrochloric acid used for the HPLC analyses were purchased from Merck (Germany). Methanolic stock solutions (100 g/mL) of the above standards were prepared and stored at 4°C, protected from daylight. They were properly diluted with ultrapurified water in order to obtain the standard concentrations for the calibration curves [4,5,11].

Equipment and Chromatographic Conditions

The experiment was carried out using an Agilent 1100 HPLC system equipped with a degasser, binary pump, autosampler and column thermostat. For the separation of the compounds it was used a reversed-phase Zorbax SB-C18 analytical column (100x3.0 mm i.d., 5 μm). The column thermostat operated at 48°C. The mobile phase used for the separation of isoflavones was a mixture of 0.1% acetic acid (V/V) in water (A) and methanol (B), in linear gradient mode, as follows: until 2 min, 20% B, at 10 min 40% B, at 10.5 min 40% B, at 11.5 min 45% B, hold 45% B until 17 min. The flow rate was 1 mL/min. For detection and quantification, the HPLC system was coupled with an Agilent 1100 Ion Trap SL mass spectrometer, operated with an electrospray (ESI) ion source in negative ion mode. The nebulisation gas used by the mass spectrometer was nitrogen at 65 psi; the dry gas was also nitrogen at a flow rate of 12 L/min and heated at 360ºC. The capillary potential was set at +2500 V. The analysis mode of isoflavones was either in single ion monitoring mode (SIM) - for aglycons or in single reaction monitoring mode (SRM) – for glycosides [2, 5, 6, 8, 10,11]. The calibration curves for all isoflavones were built in the range of 40-4000 ng/mL. For fitting the calibration curves, a quadratic model and a 1/y weighing scheme were used. The accuracy of the calibration points, for each compound, was no more than ± 15% [4,5].

Results and Discussion

The retention time of isoflavones and their mass spectrometry detection parameters are presented in Table I. Generally, glycosides' ions lose the sugar group thus we can observe the aglycon ion, so all glycosides can be analyzed by the SRM mode. The aglycons ions were not efficiently fragmented, so for these compounds we applied a SIM mode analysis [4,11].

Table I
The retention time of isoflavones and their mass spectrometry detection parameters

Compounds	Retention time (min)	Detection mode*	Parent m/z ion $[M-H]^-$	Quantified m/z ion
Daidzin	3.7	SRM	415	253
Genistin	5.5	SRM	431	268, 269
Ononin	8.9	SRM	429	267
Daidzein	9.2	SIM	253	253
Glycitein	10.2	SIM	283	283
Genistein	11.0	SIM	269	269
Formononetin	14.4	SIM	267	267

*SRM= single reaction monitoring; SIM = single ion monitoring

The compounds (heterosides and aglycons of isoflavones) identified by HPLC and their levels are presented in Table II.

Table II
Content in isoflavones (mg/100g dry plant)

Isoflavones (standards)	*Glycyrrhiza glabra*		*Glycyrrhiza echinata*		*Ononis spinosa*	
	NH	H	NH	H	NH	H
daidzin	0.434	-	-	-	0.944	-
genistin	0.672	-	-	-	1.173	-
ononin	27.490	7.999	3.904	-	175.72	18.939
daidzein	-	-	-	-	-	0.819
glycitein	-	-	-	-	-	-
genistein	-	-	-	-	-	-
formononetin	16.607	27.856	0.864	5.218	9.499	113.622

NH – non hydrolyzed samples; H – hydrolyzed samples

The roots of *Glycyrrhiza glabra* contain daidzin ($0.434x10^{-3}$%), genistin ($0.672x10^{-3}$%), ononin ($27.490x10^{-3}$%) and formononetin ($16.607x10^{-3}$%). Only formononetin ($27.856x10^{-3}$%) and ononin ($7.999x10^{-3}$%) were identified after hydrolysis. The levels of isoflavones in our samples were smaller than in *Glycyrriza glabra* harvested from Syria [5]. Only ononin ($3.904x10^{-3}$%) and formononetin ($0.864x10^{-3}$%) were determined in the extract of *Glycyrrhiza echinata* roots. After acid hydrolysis the identified compound was formononetin ($5.218x10^{-3}$%).

The extract of *Ononis spinosa* roots contains daidzin ($0.944x10^{-3}$%), genistin ($1.173x10^{-3}$%), ononin ($175.7x10^{-3}$%) and formononetin ($9.499x10^{-3}$%). The hydrolysed solution contains two aglycons, daidzein ($0.819x10^{-3}$%) and formononetin ($113.622x10^{-3}$%) and ononin ($18.939x10^{-3}$%) as residual glycoside. *Ononis spinosa* was the richest species in isoflavonoids and it can be considered an important source of these active principles.

The isoflavonoids can be present in plants like glycosides or more complex combinations like esther-glycosides: acetyl-glucosides or malonyl-glucosides. The presence of ononin in the hydrolysed extracts could be explained by the fact that the acid hydrolysis was capable of cleaving ester bonds (malonyl- and acetyl-7-glucosides), but not sufficient for the quantitative cleavage of glycosidic bonds [8,10]. Increasing of formonetin levels after hydrolysis suggests the presence of its glycosides in the analyzed extracts.

The absence of daidzin and genistin from *Glycyrrhiza echinata* can be used for the differentiation of the two species, to avoid the substitution of *Glycyrrhiza glabra* with *Glycyrrhiza echinata.*

Conclusions

The extract of *Glycyrrhiza glabra* roots contain daidzin, genistin, ononin and formononetin, while the extract of *Glycyrrhiza echinata* roots contain small quantities of formononetin and ononin; that is why we cannot use both the roots of *Glycyrrhiza glabra* and *Glycyrrhiza echinata* as being the same therapeutical product.

The roots of *Ononis spinosa*, richer in isoflavonoids (daidzin, genistin, ononin, formononetin), represent an important natural source for oestrogenic therapy.

Ononin was the most abundant isoflavone glycoside and it was found in all samples; its aglycon, formononetin was present in all extracts, before and after hydrolysis.

Our results confirm the presence of isoflavones in the plants of *Fabaceae* family, compounds that belong to a class of substances known as non-steroidal phytoestrogens.

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Manuscript received: October 2nd 2010