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HPLC ANALYSIS OF POLYPHENOLIC COMPOUNDS, PHYTOESTROGENS AND STEROLS FROM *GLYCYRRHIZA GLABRA* L. TINCTURE

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ABSTRACT. The presence and quantification of polyphenolic compounds, phytoestrogens and sterols from *Glycyrrhiza glabra* tincture, was assessed through HPLC methods. Phenol carboxylic acids were present in larger quantities than flavonoids in tincture. p-Coumaric acid, ferulic acid, luteolin and apigenin were found and quantified in both hydrolyzed and unhydrolyzed samples. Kaempferol, fisetin, myricetin, hyperoside, quercitrin, isoquercitrin and rutoside could not be found in the analyzed samples. Regarding the phytoestrogens content, significant quantities of ononin and its aglycone formononetin were detected. Among sterols, the largest amount recovered in the tincture was beta-sitosterol and the smallest ergosterol.

Keywords: *polyphenolic compounds, phytoestrogens, sterols, HPLC-DAD, HPLC-MS, Glycyrrhiza glabra*

INTRODUCTION

The roots and rhizomes of *Glycyrrhiza glabra* L., licorice, have been used as an herbal medicine for more than 4000 years. Licorice contains a biologically active complex composed of saponins, mostly triterpenoid saponins - glycyrrhizin, a mixture of potassium and calcium salts of glycyrrhizic acid, liquiritic acid, glycyrretol, glabrolide, isoglabrolide and licorice acid, flavonoids, chalcones and coumarins. Other compounds are stilbenoids, fatty acids, phenols, lactones, asparagines, glucose, sucrose, starch, polysaccharides, and sterols. Licorice root exhibits a variety of useful pharmacological activities such as anti-inflammatory, antimicrobial, antioxidant, anticancer, immunomodulatory, hepatoprotective, antiviral, antispasmodic, demulcent, diuretic, emollient, expectorant, mild estrogenic, tonic and cardioprotective [1-8].

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Glycyrrhiza glabra L. is widespread in Syrian spontaneous flora and it is widely used in therapy for its pharmacological actions and also as an ingredient of soft drinks. We continue our phytochemical studies on licorice by analyzing the tincture which is a widely marketed commercial preparation. In the present study, we employed three methods of analysis previously developed in our work [9-11] in order to characterize polyphenolic compounds, phytoestrogens and sterols from a pharmaceutical form of drug administration.

For all analyses we have used high performance liquid chromatography coupled with DAD and mass spectrometry [12-15]. Eighteen polyphenolic compounds have been researched: one hydroxybenzoic acid, six cynnamic acid derivatives, four quercetin glycosides, and seven aglycones of flavonol and flavone type. Eight compounds with phytoestrogenic activity (seven isoflavones and one coumestan) and four sterols were also determined.

RESULTS AND DISCUSSION

Considering the conditions of the methods described in our previous papers (retention time, parameters of calibration curves, characteristic ions) we obtained the following results for the tincture sample. The amounts of polyphenolic compounds found in *Glycyrrhiza glabra* tincture are presented in Table 1, expressed in µg/ml tincture.

Table 1. The content (µg/mL) of polyphenolic compounds in *Glycyrrhiza glabra* tincture

Compound	MS identified		Content (µg/ml)	
	NH	H	NH	H
Gentisic acid	Yes	Yes	-	-
Caffeic acid	Yes	Yes	-	-
p-coumaric acid	Yes	Yes	1.270	13.859
Ferulic acid	Yes	Yes	1.560	25.912
Luteolin	Yes	Yes	0.620	0.966
Apigenin	Yes	Yes	1.708	2.361
Sinapic acid	Yes	Yes	-	9.534

NH non hydrolyzed sample; H hydrolyzed sample

Results show that phenol carboxylic acids were primarily extracted in tincture. Ferulic acid and p-coumaric acid are found in appreciable quantities, especially as esters. Sinapic acid was determined only in the hydrolyzed sample. Gentisic and caffeic acid could not be determined quantitatively in any of the samples, the amount was below the limit of quantification. As for flavonoids, in none of the samples were found kaempferol, fisetin, myricetin,

hyperoside, quercitrin, isoquercitrin or rutoside. Luteolin and apigenin are present both as free aglycones and in glycosides form, being found in larger quantities after hydrolysis.

Results obtained for phytoestrogens compounds from *Glycyrrhiza glabra* are presented in Table 2, expressed in ng/ml tincture.

Table 2. The content (ng/mL) of phytoestrogen compounds in *Glycyrrhiza glabra* tincture

Sample Compound	Tincture unhydrolyzed sample	Tincture hydrolyzed sample
Daidzin	884.3	286.8
Genistin	0.0	251.2
Ononin	13744.6	11620.5
Daidzein	2672.3	2682.1
Glycitein	1237.7	1907.6
Genistein	513.2	531.3
Coumestrol	948.8	211.1
Formononetin	12749.0	10030.7

Ononin and its aglycon formononetin are found in large quantities in tincture, followed by daidzein and glycitein. There are also smaller amounts of daidzin, genistein and coumestrol. Genistin is present only in the hydrolyzed sample.

Sterols content from *Glycyrrhiza glabra* tincture are presented in Table 3. The tincture contains all four analysed sterols: beta-sitosterol, ergosterol, stigmasterol, campesterol. Beta-sitosterol and stigmasterol are predominant. Much lower amounts of campesterol and ergosterol were detected.

Table 3. The sterols content (ng/mL) in *Glycyrrhiza glabra* tincture

Compound	Content (ng/ml)
Ergosterol	1856.3
Stigmasterol	14847.5
Campesterol	4704.0
Sitosterol	27549.2

CONCLUSIONS

It is a well known fact that plant extracts contain a great number of pharmacologically active compounds which together form the phytocomplex. Each substance in the phytocomplex contributes to the overall therapeutic effect of the extract. Few commercial preparations with plant extracts that are in use nowadays are characterised and their chemical composition is known.

In this context, the current research tried to determine three groups of phytochemicals present in licorice tincture: polyphenolic compounds, phytoestrogens, and sterols. Among the polyphenolic compounds, the phenol carboxylic acids were found in significant quantities in the preparations, especially ferulic acid and p-coumaric acid. As for phytoestrogens, ononin and its aglycon formononetin were detected in appreciable amounts in tincture. Regarding the sterols content, the largest quantity recovered in licorice tincture was beta-sitosterol.

Although in minor quantities, the compounds identified in *Glycyrrhiza glabra* tincture contribute to the therapeutic effect of the biologically active complex. Our results allow a better characterization of the active principles from an herbal drug preparation of *Glycyrrhiza glabra.*

EXPERIMENTAL SECTION

Plant material: The root parts of *Glycyrrhiza glabra* were collected from north of Syria and air-dried at room temperature. Plants were identified by one of the authors and a voucher specimen (no. GG-0509) was deposited at the Pharmaceutical Botany Department, Faculty of Pharmacy, University of Medicine and Pharmacy, Iasi.

Sample preparation: Over 2 g of finely pulverized plant material was added to 20 g 96 % ethanol in a tightly closed container. The vessel was kept at room temperature for ten days, stirring 3-4 times daily. The liquid extract obtained was decanted and the residue was pressed. Extraction fluids were collected and homogenized; they are left to settle out for 6 days at 5-10 ^{0}C and then filtrated. In order to study the aglycones that can be obtained by hydrolysis, 0.5 ml of the tincture were treated with 0.5 ml 2 N hydrochloric acid (to determine polyphenols) or with 0.5 ml 6 N hydrochloric acid (to determine phytoestrogens) in a centrifuge tube. In parallel, extracts were diluted with equal quantities of water. The samples obtained were heated 40 minute at 80^{0}C on a water bath, and then centrifuged at 10,000 rpm. Supernatants were diluted and centrifuged again at 8000 rpm, filtered through a filter with 0.45 mm diameter pores and then injected into the chromatographic system.

Chemicals*:* Methanol of HPLC analytical-grade, chloroform, ethanol, acetic acid and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Standards: caffeic acid, chlorogenic acid, p-coumaric acid, kaempferol, apigenin, rutoside, quercetin, quercitrin, isoquercitrin, fisetin, hyperoside, myricetin, daidzin, genistin, ononin, daidzein, genistein, formononetin, glycitein, coumestrol, beta-sitosterol, stigmasterol, campesterol and ergosterol from Sigma (Germany), ferulic acid, gentisic acid, sinapic acid, patuletin, luteolin from Roth (Germany) and caftaric acid from Dalton (SUA) were used.

Apparatus and chromatographic conditions: The analyses were carried out using an Agilent 1100 HPLC Series system equipped with a degasser, binary pump, autosampler, column thermostat, UV detector. The

HPLC was coupled with an Agilent Ion Trap 1100 VL mass detector. For the separation we used a reversed-phased Zorbax SB-C18 analytical column (100 mm x 3.0 mm i.d., 3.5 μm particle – for polyphenols and 100 mm x 3.0 mm i.d., 5μm particle – for sterols and phytoestrogens) fitted with precolumn Zorbax SB-C18, both operated at 48^{0}C (polyphenols), 50^{0}C (phytoestrogens), 40^{0}C (sterols).

The mobile phase was prepared as follows:

- ***polyphenols analysis***: methanol:acetic acid 0.1% (v/v), the elution began with a linear gradient (started at 5% to 42% methanol for the first 35 minutes), followed by isocratic elution (with 42% methanol for the next 3 minutes).

- ***phytoestrogens analysis:*** methanol:acetic acid 0.1% (v/v), gradient elution, as shown in Table 4.

Table 4. Composition of the mobile phase employed in the HPLC analysis of phytoestrogens

TIME (MIN)	COMPOSITION OF MOBILE PHASE (%)	
	methanol	acetic acid 0.1%
0	20	80
2	20	80
10	40	60
10.5	40	60
11.5	45	55
12.5	45	55

- sterols analysis: methanol:acetonitril 30:70 (v/v), isocratic elution.

The flow rate was 1 mL/min and the injection volume was 5 μL (for polyphenols and phytoestrogens analysis) and 4 μL (for sterols analysis).

The detection was performed in the following manner:

Polyphenols analysis was carried out by MS, using a mass spectrometer equipped with a Turbo-Ionspray (ESI - electrospray ionization) interface, working in negative ion mode. ESI settings were: negative ionization, ion source temperature 360^{0}C, gas: nitrogen, flow rate 12 L/min, nebuliser: nitrogen at 70 psi pressure, capillary voltage 3000 V. The analysis mode was multiple reaction monitoring (MRM) and single ion monitoring (SIM). UV detection and quantification was performed at 330 nm for phenol carboxylic acids and at 370 nm for flavonoids using a G1315A diode array detector system.

Phytoestrogen were analysed by MS, ESI settings were: negative ionization, ion source temperature 360^{0}C, gas: nitrogen, flow rate 12 L/min, nebuliser: nitrogen at 65 psi pressure, capillary voltage 4500 V. For aglycones was used the Single Ion Monitoring (SIM) analysis mode and for glycosides the Single Reaction Monitoring (SRM) mode.

Sterols were analysed by MS, using an APCI interface, positive ion mode, gas – nitrogen, flow rate 7 L/min, ion source temperature 250^{0}C, nebuliser: nitrogen at 50 psi pressure, capillary voltage -4000 V.

All chromatographic data were processed using ChemStation (vA09.03) software and Data Analysis (v 5.3) from Agilent, USA.

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