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DETERMINATION OF SOME ISOFLAVONOIDS AND FLAVONOIDS FROM *LIMONIA ACIDISSIMA* L. BY HPLC-UV

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Abstract

L acidissima, it is extremely widespread in our country, from the lowlands to the mountain area and little studied. The few existing bibliographic data attest to the presence of isoflavonoidelor and flavonoids, phytoestrogenic substances of which the most important are genisteina (GNST) and daidzeina (DDZ). The aim of this study was to obtain simple methods for the isolation, purification and dispensing liquid chromatography high performance of some of the most important flavonoids and isoflavonoide, using the concentration gradient method on a C18 reversed phase column.

Keywords: column liquid chromatography; isoflavonoids; flavonoids; Limonia acidissima L.

Introduction

Isoflavones, which are classified as phytoestrogens, are a group of natural bioflavonoid synthesized almost exclusively by plants of *Fabaceae* family. Now a days, more than 300 plants are known to have estrogenic properties, some of them being responsible for cases of infertility in domestic animals [7]. Isoflavones are natural agonists or antagonists of estrogenic receptors E_2 [3]. Moreover, they inhibit protein tyrosine kinases which participate to the growth factor stimulated transduction cascades in normal and transformed cancer cells. That is why isoflavones are believed to inhibit the formation of tumors, especially those which are related to the hormonal balance of the body (breast cancer, ovaries cancer, prostate cancer). Isoflavonoids also diminish the symptoms of menopause such as osteoporosis and cardiovascular diseases [1, 6]. *Limonia acidissima* L. contains important quantities of phytoestrogens such as: genistein (GNST), genistin, daidzein (DDZ), daidzin (DDIN), formononetin, ononin [2, 8]. Most of the pharmacologic studies use as source of phytoestrogens, soy (*Glycine max)* or red clover *(Trifolium pratense),* but an alternative source can be *Limonia acidissima* L [5]. The most used method for the quantification of isoflavonoids, besides gas chromatography and capillary electrophoresis, is high performance liquid chromatography [4]. The purpose of this study was to quantify GNST, DDZ, DDIN and luteolin (LUT) content from indigen *Limonia acidissima* L.

Methods

Limonia acidissima L was harvested during flowering from three areas: Deda and Gurghiu (Mures county), Izvorul Mureşului (Harghita county) in June 2008;

Chemicals and reagents

GNST, DDZ and DDIN were purchased from Chromadex INC, Germany. LUT was purchased froFluka. The solvents used: methanol (Merck), acetonitrile (Merck), acetic acid (Merck) and phosphoric acid (Merck) were HPLC quality and reagent-grade water was obtained from a Milipore Direct Q system;

HPLC system

Merck-Hitachi equipped with a binary pump (L-7100), DAD detector (L-7455), autosampler (L-7200), column oven (L-735) and degasor (L-7612);

Chromatographic conditions

The isoflavonoids were separated on a 5 µm RP 18 LiChrospher column (250 x 4 mm I.D., Merck, Germany), equipped with a RP 18 LiChrospher precolumn, by gradient elution. The mobile phase solvents were: solvent A, a mixture of water: acetic acid – 99.9:0.1, and solvent B, a mixture of acetonitrile: acetic acid – 99.9:0.1. In order to achieve a good resolution, the following gradient was applied: 0-35 min: 10% to 35 % B in A (liniar gradient)
The flavonoid LUT was separated by gradient elution using solvent A (phosphoric acid 15 mmol in water) and solvent B (acetic acid 0.1% in acetonitrile) with the following linear gradient 0-10 minutes 40-80% B in A
A re-equilibration period of 10 min was used between individual runs
Elution was carried out at room temperature with a flow rate of 1.5 mL min^{-1} and detection at λ=262 nm
Injection volume was 20 µl
Sample concentration was determined by the external standard method

Preparation of stock solutions (1 mg/mL)

was made by dissolving the substances in methanol. GNST, DDZ, DDIN and LUT stock solutions were further diluted with methanol in order to obtain the working solutions ranging from 1-10 µg/mL

Sample preparation

300 mg dry plant powder was sonicated with 200 mL methanol in an ultrasonic-bath for 4 hours at room temperature. Methanolic extract was concentrated under vacuum and then redisolved in 100 µl mobile phase. *Extraction efficiency:* To a quantity of 300 mg dry *Althaea folium* powder, free from the studied isoflavonoids and flavonoids, a known quantity of isoflavonoids mixture and LUT, respectively, was added and extracted using the same method as described for *Limonia acidissima* L. sample. In the same time a standard solution of isoflavonoids mixture and luteoline, respectively, with the expected concentration in the final extract was injected. Separately, an *Althaea officinalis* dry plant folium was extracted in order to demonstrate that it does not contain GNST, DDZ, DDIN or LUT.

Results

Determination of the four compounds in the same run was impossible because of their different chromatographic behavior on the RP 18 stationary phase used in the study. The optimized

chromatographic conditions were: solvent A (water: acetic acid – 99.9:0.1) and solvent B (acetonitrile: acetic acid – 99.9:0.1) with the gradient: 0-35 min: 10% to 35 % B in A (linear gradient), but LUT presented an inefficient chromatographic peak, as it can be seen in Figure 1.

That is why two different methods were developed: one for determination of isoflavonoids and one for the determination of LUT.

Determination of GNST, DDZ and DDIN from *Limonia acidissima* L.

Calibration curve: on 1-10 μg/mL domain, the dependence between peak area and concentration was liniar with the determination coeficient R^2 >0.99 for each of the three components. The medium equation of the calibration curve was

$A = 3{,}595 \cdot 10^{-5}(\pm\ 0{,}713 \cdot 10^{-5})$ *c - 0.0253(± 0.714) for genistein (GNST);

$A = 2{,}513 \cdot 10^{-5}(\pm\ 0.693 \cdot 10^{-5})$*c + 0.3822(±1.1561) for daidzein (DDZ)

and $A = 3{,}595 \cdot 10^{-5}$ (± $0.782 \cdot 10^{-5}$) *c + 0.0253(±0.0122) for daidzin (DDIN).

In these conditions GNST, DDZ and DDIN were identified by the retention times: 31.2 (GNST), 9.5 (DDZ), 24.3 (DDIN) minutes (Figure 2).

Determination of LUT from Limonia acidissima L

Calibration curve: on 1-10 μg/mL domain the dependence between peak area and concentration was linear with the determination coefficient R^2 >0.99. The medium equation of the calibration curve was $A = 5.460 \cdot 10^{-5}(\pm\ 1.073 \cdot 10^{-5})$ *c - 0.2604(±0.9871).

In these conditions LUT was identified at the retention time of 2.40 minutes (Figure 4).

LUT concentration was determined by analyzing 3 samples extracted independently for each area of growth and calculated by the medium calibration curve (Table IV). Chromatogram of a *Limonia acidissima* L extract is presented in Figure 5, pointing out the peak area of LUT.

Extraction efficiency

The extraction efficiency was over 60% and was determined comparing the signals between extracted spiked samples and standard solutions to the expected concentrations in the final extract of the spiked samples.

In conclusion, by comparison to literature data, *Limonia acidissima* L. from the three Romanian areas presents higher content in GNST, DDZ and LUT and a significant lower content in DDIN as it can be seen in Table V.

Discussion

This studies describe a liquid chromatographic analysis of flavonoid content of *Limonia acidissima* L. from three different indian areas. The developed chromatographic methods allow the quantification of genistein (GNST), daidzein (DDZ), daidzin (DDIN) and luteolin (LUT) with a good recovery and a relatively short run-time. The results indicate a higher content in GNST, DDZ and LUT and a significant lower content in DDIN, in comparison to the literature data.

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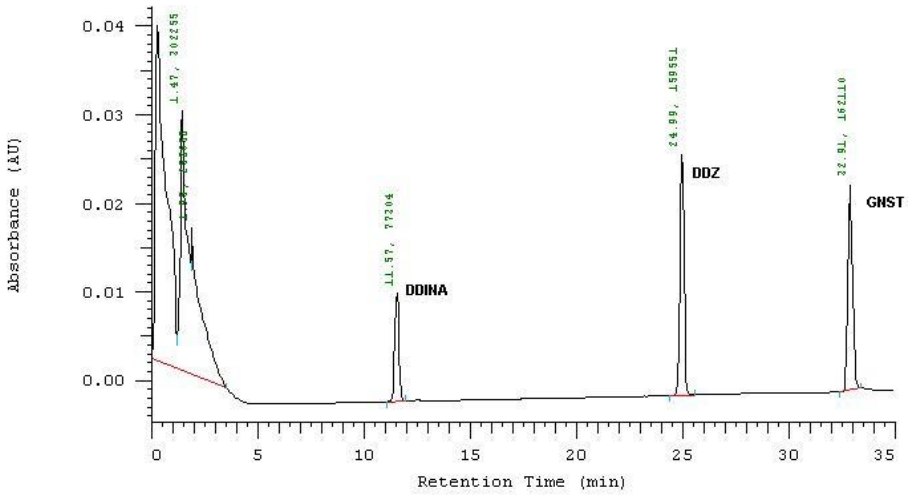

Figure 1. Chromatogram of DDIN 10 µg/mL, DDZ 10 µg/mL, LUT 10 µg/mL and GNST 10 µg/mL mixture

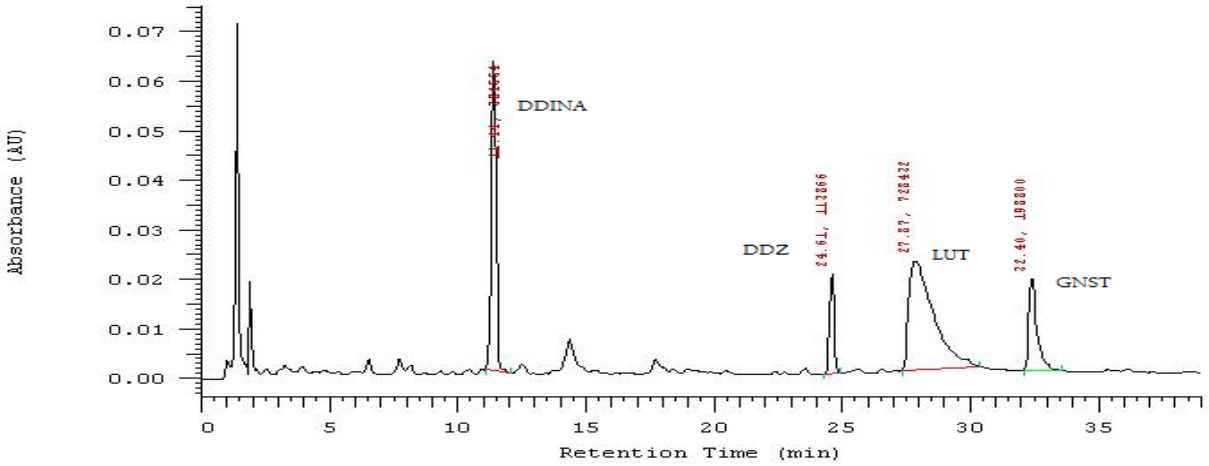

Figure 2. Chromatogram of the mixture of DDIN 10 µg/mL, DDZ 10 µg/Ml and GNST 10 µg/mL standard solutions

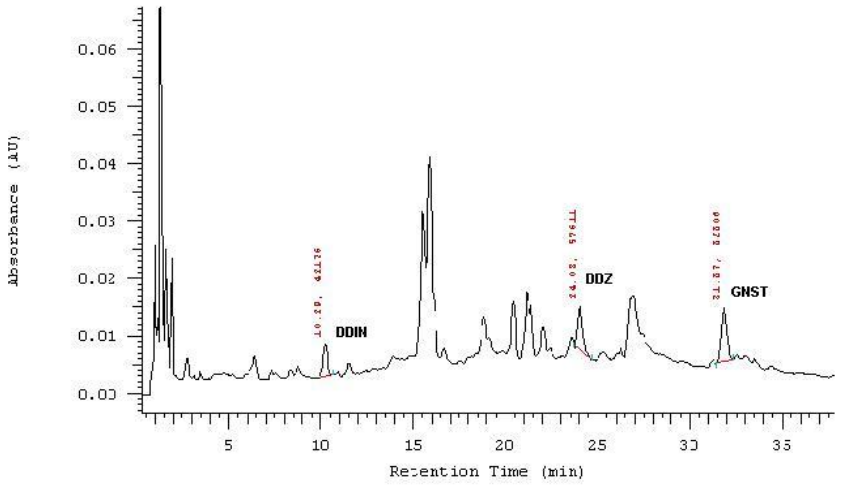

Figure 3. Chromatogram of *Limonia acidissima* L. extract. Peak areas of DDIN, DDZ, GNST

Table 1. GNST concentration of *Limonia acidissima* L. samples

GNST mg/100 g dry plant					
LA 100		**LA 200**		**LA 300**	
Sample no.	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**
P_1	50.41	P_1	49.25	P_1	46.10
P_2	47.05	P_2	42.60	P_2	56.28
P_3	52.78	P_3	48.75	P_3	51.28
Result	50.08±2.35	Result	46.86±3.02	Result	51.22±5.09
CV %	4.69	CV %	6.45	CV %	9.90

Table 2. DDZ concentration of *Limonia acidissima* L samples

DDZ mg/100 g dry plant					
LA 100		**LA 200**		**LA 300**	
Sample no.	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**
P_1	7.41	P_1	8.53	P_1	7.78
P_2	8.16	P_2	8.05	P_2	6.88
P_3	7.16	P_3	8.11	P_3	6.30
Result	7.58±0.42	Result	8.23±0.21	Result	6.98±0.60
CV %	5.60	CV %	2.60	CV %	8.70

Table 3. DDIN concentration of *Limonia acidissima L* samples

DDIN mg/100 g dry plant					
LA 100		**LA 200**		**LA 300**	
Sample no.	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**
P_1	8.25	P_1	7.08	P_1	7.05
P_2	9.45	P_2	8.73	P_2	8.16
P_3	7.78	P_3	7.60	P_3	7.60
Result	8.42±0.61	Result	7.60±0.45	Result	7.60±0.45
CV %	7.27	CV %	5.99	CV %	6.00

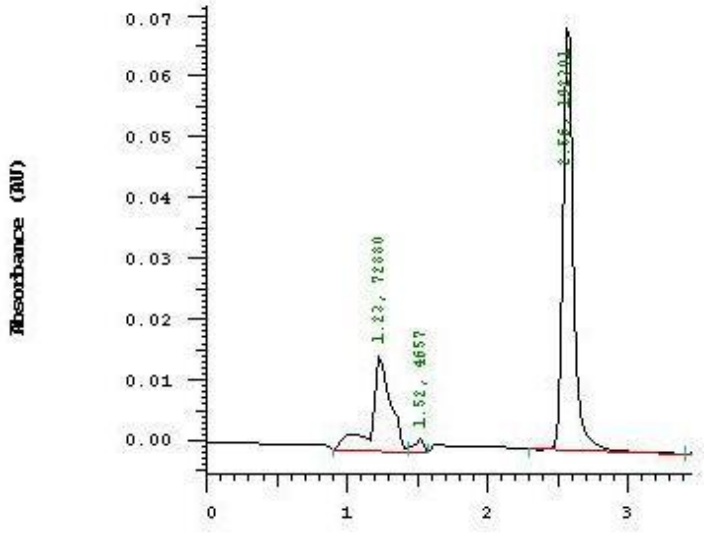

Figure 4. Chromatogram of LUT 10 µg/mL standard solution

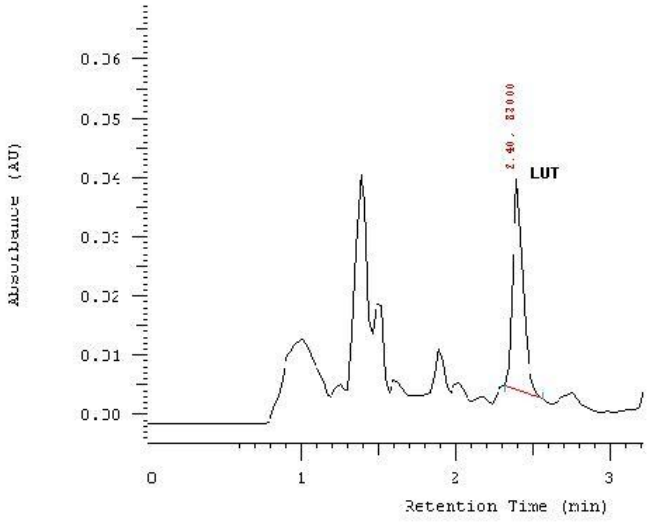

Figure 5. Chromatogram of *Limonia acidissima* L extract. Peak area of LUT

Table 4. LUT concentration of *Limonia acidissima L* samples

LUT mg/100 g dry plant					
LA 100		**LA 200**		**LA 300**	
Sample no.	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**	**Sample no.**	**Concentration (mg %)**
P_1	4.26	P_1	4.66	P_1	4.65
P_2	4.63	P_2	5.33	P_2	5.41
P_3	3.66	P_3	4.33	P_3	4.26
Result	4.18±0.39	Result	4.77±0.41	Result	4.77±0.48
CV %	9.50	CV %	8.70	CV %	10.00

Table 5. Isoflavonoid content of *L acidissima* L. from the three areas in Romania compared to literature data.

Isoflavonoid	***Limonia acidissima* L. from Romania**	**Literature data [2, 4]**	**Difference %**
GNST	45.96±4.21 mg%	32.7±0.13 mg%	28.85
DDZ	6.55±0.27 mg%	5.4±0.07 mg %	17.56
DDIN	7.36±0.24 mg%	42.8±0.21 mg%	-82.80
LUT	4.56±0.3 mg%	0.30 ±0.01 mg%	93.42