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LC-MS ANALYSIS AND ANTI-INFLAMMATORY ACTIVITY OF A TINCTURE FROM *ERYNGIUM PLANUM* IN A RAT MODEL OF ACUTE INFLAMMATION

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The aim of this study was to conduct a LC-MS analysis on the polyphenols, the main chemical constituents from an ethanolic tincture from the aerial part of *Eryngium planum* (plain eryngo), and also to test the anti-inflammatory potential of the tincture by the rat paw oedema test. For the phytochemical analysis of polyphenols, a reversed phase high-performance liquid chromatography (RPHPLC) with UV detection coupled with mass spectrometric detection (MS) was used. Samples were analyzed before and after hydrolysis in order to identify the flavonoidic glycosides and free aglycons. The results confirmed the presence of the following compounds: isoquercitrin, quercitrin, kaempferol, quercetol, chlorogenic acid, caffeic acid, p-coumaric acid and ferulic acid. The anti-inflammatory potential of the tincture from *Eryngium planum* was evaluated in two different doses (100 mg/kg b.w. and 500 mg/kg b.w.). The results showed that the tincture from *E. planum* significantly inhibited the kaolin-induced rat paw oedema in all the three time intervals of the determination (2h, 4h and 24h), reducing the oedema volume by 45% ($p<0,01$), 4 hours after the oedema was evoked, the effect being dose-dependent.

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1. Introduction

Romanian traditional medicine recommends a wide range of indigenous medicinal plants in the empirical treatment of several inflammatory disorders. Some of these species were included recently in the modern phytotherapy, while others are still not valued as phytomedicines due to the lack of thorough phytochemical and pharmacological studies [1].

Several plant-derived compounds showing anti-inflammatory activities have already been identified: flavonoids, carotenoids, alkaloids, triterpenoids, phytosterols, coumarins, monophenolic alcohols, monoterpenes, phenolic acids, tannins, saponins and others. The majority of biocompounds identified so far have antioxidative and antiinflammatory activities and belong to the vast family of polyphenols [2].

The molecular mechanism of anti-inflammatory phytomedicines share common molecular targets with nonsteroidal anti-inflammatory drugs, as well as with steroidal drugs [3].

The genus *Eryngium* belonging to *Apiaceae* family is well-represented in Central Asia and Central and Eastern Europe. Previous papers revealed the anti-inflammatory effects of several

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Eryngium species extracts, suggesting their involvement in the vascular response associated to the acute stage of inflammation [4, 5, 6].

The relatively few papers on the chemical composition of *Eryngium planum* and also on its pharmacological, prompted us to conduct a study on the polyphenolic compounds from an ethanolic tincture prepared from the aerial parts of *Eryngium planum* by using a RPHPLC-UV-MS method and also on its anti-inflammatory potential.

2. Experimental

2.1 Plant material

The aerial parts of *Eryngium planum* were collected in July from Jucu (Cluj County, Romania). Plant materials were identified by PhD Professor Mircea Tămaş, a voucher specimen being deposited at the Department of Pharmaceutical Botany, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

Sample preparation: 100g air-dried plant material was macerated at room temperature in 500 mL 70^0ethanol for 10 days. After filtering, 70^0ethanol was added to obtain 500 mL of tincture of *Eryngium planum* [7].

2.2 Study of the polyphenols (glycosides, aglycons and polyphenolic acids)

Polyphenols from the 20% ethanolic tincture from the aerial part of *Eryngium planum* were identified and quantified using a reversed phase high-performance liquid chromatography (RPHPLC) with UV detection coupled with mass spectrometric detection (MS). 18 polyphenolic standards were used. Samples were analyzed before and after hydrolysis in order to identify the flavonoidic glycosides and also flavonoid aglycons released after hydrolysis.

The standards were: caffeic acid, chlorogenic acid, p-coumaric acid, kaempferol, apigenin, rutoside, quercetin, quercitrin, isoquercitrin, fisetin, hyperoside, myricetin (Sigma, Germany), ferulic acid, gentisic acid, sinapic acid, patuletin, luteolin (Roth, Germany), caftaric acid (Dalton, USA).

In order to study the flavonoid aglycons released after hydrolysis, *Eryngium planum*`s tincture was treated with an equal volume of 2N hydrochloric acid and heated for 40 minutes at 80^oC on a water bath. The resulting samples were properly diluted with distilled water [8-10].

An Agilent 1100 HPLC Series system (Agilent, USA) equipped with a degasser, a binary pump, an autosampler, a column thermostat, and a UV detector was used. The HPLC system was coupled with an Agilent 1100 MSD Ion Trap VL mass detector.

Compounds were separated on a reversed-phased 100 mm x 3.0 mm i.d., 3.5 μm particle, Zorbax SB-C18 analytical column, with methanol: acetic acid 0.1% (v/v) as mobile phase. The elution begun 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). The flow rate was 1 mL/min and the injection volume was 5 μL. Detection was performed at 330 nm and 370 nm. The time required for chromatography of one sample was 35 min.

The MS was equipped with a Turbo-Ion spray (ESI) interface, negative ion mode. ESI settings were: negative ionization, ion source temperature 360^oC, gas: nitrogen, flow rate 12 L/min, nebulizer: nitrogen at 70 psi pressure, capillary voltage 3000 V. The analysis mode was multiple reaction monitoring (MRM) and single ion monitoring (SIM) [11-14].

2.3 Evaluation of the anti-inflammatory effect

In order to study the anti-inflammatory effect of the tincture from *Eryngium planum*, the kaolin-induced rat paw oedema model was used, followed by the plethysmometric evaluation of the oedema`s volume. 4 groups of 8 Wistar rats (120-130 g) were used for this experimental model. The animals were kept in standard conditions with food and water access being prohibited 24 hours before the experiment.

Eryngium planum`s tincture was diluted in sterile saline in order to test two different doses, 100 mg/Kg b.w. and 500 mg/Kg b.w., expressed as dried powdered plant material.

Rats were administered intraperitoneally the following substances:

Group I (negative control): 1 mL i.p. sterile saline;
Group II: 50 mg/Kg i.p. phenylbutazone, a reference anti-inflammatory drug;
Group III: 100 mg/Kg i.p. *Eryngium planum`s* tincture;
Group IV: 500 mg/ Kg i.p. *Eryngium planum`s* tincture.

One hour after the administration of the substances, the initial volume of the left hind paw of the rats was measured with a digital plethysmometer (model 7140 Ugo Basile, Italy), followed by intraplantar injection of 0.1 mL of 10% kaolin suspension in the same paw. Oedema`s volume was measured every 2 hrs, 4hrs and 24 hrs after injection of the oedemogen agent (kaolin) [15].

The inflammatory oedema expressed in mL represents the difference between the volume of the injected paw (after 2 hrs., 4 hrs. and 24 hrs) and the initial volume.

The statistical analysis of data was performed by Student's *t*-test. Results were expressed as mean values ± standard error (M ± S.E.). All the biological experiments were approved by the Ethics Commission of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca and were conducted according to the EC directive 86/609/EEC, which regulates the use of laboratory animals.

3. Results and discussions

3.1 Polyphenols analysis by UV detection

A new high performance liquid chromatography method was employed for the separation of 18 polyphenolic compounds (Fig. 1).

Each class of polyphenols was detected according to the wavelength corresponding to the maximum absorption of the UV spectrum. Therefore, polyphenolic acids were detected at 300nm, while flavonoids (glycosides and aglycons) were detected at 370nm.

Calibration plots were obtained by using 18 calibration standards in the 0.5-50 μg/mL range; R^2 values were 0.9999. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5-50 mg mL^{-1} range with good linearity (R2>0.999) for a five point plot were used to determine the concentration of polyphenols in plant samples.

Figures 1 and 2 show the HPLC chromatogram of the 18 standards of polyphenols and the HPLC chromatogram of the unhydrolized/hydrolyzed tincture from *Eryngium planum*.

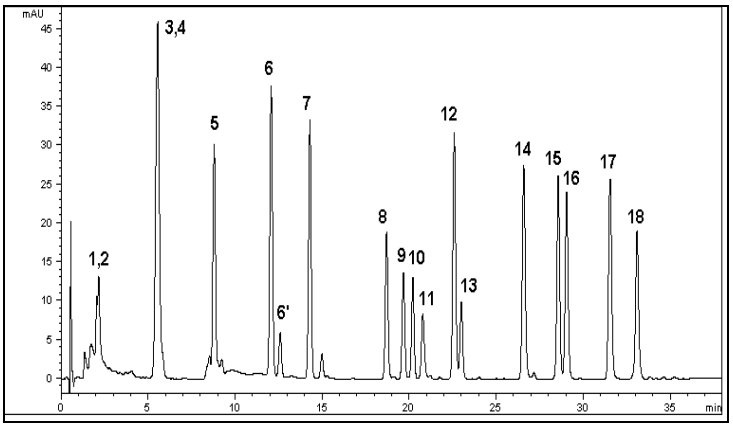

Fig. 1. HPLC chromatogram of 18 polyphenol standards (UV detection at 330 nm and 370 nm) and of the standards mixture

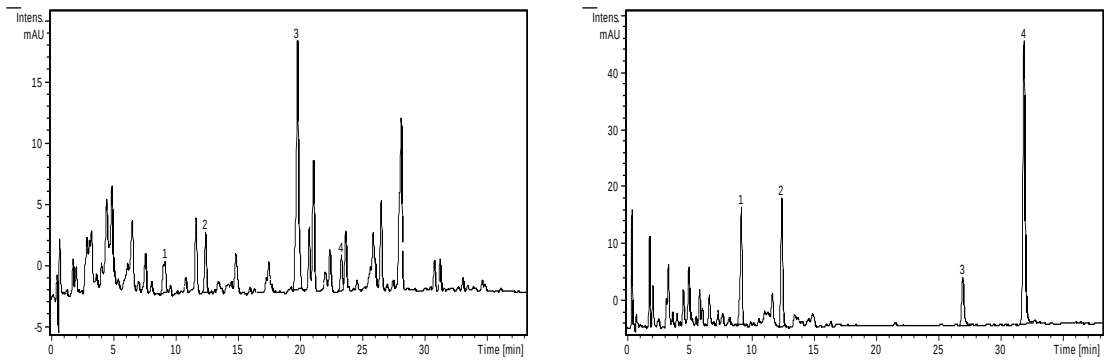

Fig. 2. HPLC chromatogram of the Eryngium planum`s tincture (unhydrolized and hydrolyzed)

Considering the incomplete separation of the two pairs of phenolic acids (caftaric acid-gentisic acid, caffeic acid-chlorogenic acid), only a qualitative analysis based on the MS information was performed for these standard compounds, using UV detection. The results are presented in Table 1.

Table 1. Retention time values and the parameters of the calibration line equation for the standards with UV detection (A = peak area (mAU×s), x = concentration ($\mu g\ mL^{-1}$)

No.	Phenolic compund	Retention time (min)	Calibration line equation
1	Caftaric acid	2.10	qualitatively
2	Gentisic acid	2.15	qualitatively
3	Caffeic acid	5.6	qualitatively
4	Chlorogenic acid	5.6	qualitatively
5	p-coumaric acid	8.7	A = - 0.325 + 33.23 x
6	Ferulic acid	12.2	A = - 1.016 + 39.55 x
7	Sinapic acid	14.3	A = - 0.236 + 37.10 x
8	Hyperoside	18.6	A = 0.107 + 19.29 x
9	Isoquercitrin	19.6	A = - 0.273 + 12.97 x
10	Rutoside	20.2	A = 0.226 + 13.47 x
11	Myricetin	20.7	A = -0.544 + 26.45 x
12	Fisetin	22.6	A = 0.241 + 19.19 x
13	Quercitrin	23.0	A = 0.047 + 10.69 x
14	Quercetin	26.8	A = -1.152 + 36.32 x
15	Patuletin	28.7	A= - 0.429 + 31.44 x
16	Luteolin	29.1	A = - 0.760 + 28.97 x
17	Kaempferol	31.6	A = - 1.270 + 30.15 x
18	Apigenin	33.1	A = - 0.908 + 20.40 x

3.2 Polyphenols analysis by MS detection

The polyphenolic compounds contain in their molecule at least one phenolic function (and one carboxyl for polyphenolic acids). Thus, they can be transformed into negative ions (M-H) and they can be analyzed by negative ionization. The mass spectrometer is set to isolate the ions of interest and then to fragment them, finally recording the corresponding mass spectrum.

If the molecular weight of ions from the tested samples matches the molecular weight of the ions from a particular standard, then this compound is considered to be identified in the sample.

The results confirmed the presence of isoquercitrin, quercitrin, kaempferol, quercetol, chlorogenic acid, caffeic acid, p-coumaric acid and ferulic acid in the tested samples. Isoquercitrin was identified as the major flavonoid, its content being 20.979 mg% in the unhydrolized tincture.

Polyphenolic acids like caffeic acid, chlorogenic acid, p-coumaric acid and ferulic acid were also identified in small amounts.

The detailed results are presented in Tables 2, 3 and 4:

Table 2. MS analysis mode and the specific ions from the mass spectra of the 18 polyphenols used as standards.

No.	Phenolic compound	MS analysis mode	Specific ions for identification Ion [M-H] > Ions from spectrum
1	Caftaric acid	MRM	311>148.6, 178.6
2	Gentisic acid	MRM	153>108.7
3	Caffeic acid	MRM	179.4>134.7
4	Chlorogenic acid	MRM	353.5>178.7, 190.7
5	p-coumaric acid	MRM	163> 118.7
6	Ferulic acid	MRM	193.2> 133.7, 148.7, 177.6
7	Sinapic acid	MRM	223.4>148.6, 163.6, 178.7, 207.7
8	Hyperoside	SIM	463.1
9	Isoquercitrin	SIM	463.1
10	Rutoside	SIM	609.1
11	Myricetin	SIM	317.1
12	Fisetin	SIM	285.1
13	Quercitrin	SIM	447.1
14	Quercetin	SIM	301.1
15	Patuletin	SIM	331.1
16	Luteolin	SIM	285.1
17	Kaempferol	SIM	285.1
18	Apigenin	SIM	269.2

Table 3. Compounds identified in Eryngium planum`s tincture (unhydrolyzed)

No. on chromatogram	Compound	No. of standard	UV identified	MS identified	Content in the sample (mg/100 g plant material)
-	chlorogenic acid	4	no	yes	qualitatively
-	p-coumaric acid	5	yes	yes	1.304
1	ferulic acid	6	yes	yes	1.315
2	isoquercitrin	9	yes	yes	20.979
3	quercitrin	13	yes	yes	2.893

Table 4. Compounds identified in Eryngium planum`s tincture (hydrolyzed)

No. on chromatogram	Compound	No. of standard	UV Identified	MS identified	Content in the sample (mg/100 g plant material)
-	caffeic acid	3	no	yes	qualitatively
-	chlorogenic acid	4	no	yes	qualitatively
1	p-coumaric acid	5	yes	yes	6.570
2	ferulic acid	6	yes	yes	5.966
	sinapic acid	7	yes	yes	qualitatively
3	quercetol	14	yes	yes	3.005
4	kaempferol	17	yes	yes	20.969

3.3 Study of the anti-inflammatory effect

The anti-inflammatory effect of the tested groups is shown in figure 3 and tables 5 and 6.

In the rat paw oedema test, both tested doses of *E. planum`s* tincture (100 mg/Kg and 500 mg/Kg) inhibited the kaolin-induced oedema within the time range of 4-24 h, the results being statistically significant ($p<0.05$).

Table 5. Anti-inflammatory effect for the tested groups in the rat paw oedema test.

Group	**Oedema volume (mL) M ± S.E.**		
	2 h	**4 h**	**24 h**
Control	1,27 ± 0,49	1,67 ± 0,428	1,91 ± 0,575
E. planum tincture (100 mg/Kg)	1 ± 0,19 (p>0,05)	1,3 ± 0,189 (p<0,05)	1,26 ± 0,42 (p<0,05)
E. planum tincture (500 mg/Kg)	0,79 ± 0,224 (p<0,05)	0,92 ± 0,286 (p<0,01)	1,42 ± 0,179 (p<0,05)
Phenylbutazone (50 mg/Kg)	0,56 ± 0,152 (p<0,001)	1,07 ± 0,14 (p<0,001)	0,96 ± 0,153 (p<0,001)

The anti-inflammatory effect was dose-dependent, the higher dose (500 mg/Kg) having a significantly superior effect, lowering oedema volume by 25% - 45%, the effect being maximal 4 h after the oedema was evoked ($p <0.01$).

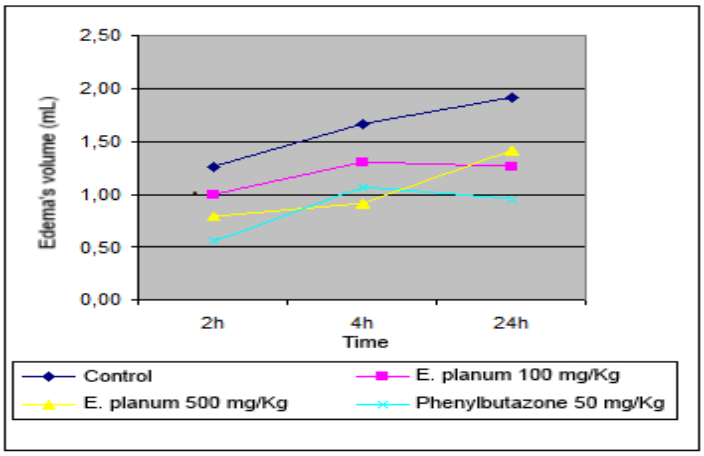

Fig. 3. The graphical representation of the oedema`s volume (mL) 2h, 4h and 24h after oedema was evoked in the rat paw oedema test.

Table 6. The percentages of oedema`s inhibition for the tested groups in the rat paw oedema test

% of oedema`s inhibition	**Time (h)**		
	2 h	**4 h**	**24 h**
E. planum tincture (100 mg/Kg i.p.)	21,26	22,15	34,03
E. planum tincture (500 mg/Kg i.p.)	37,8	45	25
Phenylbutazone (50 mg/Kg i.p.)	40,26	35,7	49,35

In the rat paw oedema induced by kaolin, the kinins and prostaglandins are involved, thus, the experimental results further confirmed the anti-inflammatory activity of the tincture from *Eryngium planum*, which should be at least partially due to an inhibition of prostaglandins.

4. Conclusions

The results of the LC-MS analysis confirmed the presence of isoquercitrin, quercitrin, chlorogenic acid, p-coumaric acid and ferulic acid, in the tincture from *Eryngium planum*. Isoquercitrin was identified as the major flavonoid. After hydrolysis, quercetol and kaempferol were identified and quantified as the major flavonoidic aglycons.

The experimental data showed that the tincture from *Eryngium planum* had significant and dose-dependent anti-inflammatory properties in the rat paw-oedema test, probably due to the content in polyphenolic compounds.

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