ORIGINAL ARTICLE

PHARMACEUTICAL ASSESMENT OF ROMANIAN CROPS OF *ANTHRISCUS SYLVESTRIS* (*APIACEAE*)

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Manuscript received: October 2017

Abstract

Anthriscus sylvestris is a traditional remedy for various diseases due to the lignans contained by the root. The aerial parts of the plant are also used in the traditional medicine, but the pharmacological studies are limited. The objective of the study was to evaluate the anti-inflammatory activity of *A. sylvestris* aerial parts in order to capitalize the plants highly adaptability and ability to grow rapidly in almost any type of soil. The obtained results proved that the obtained and characterized *A. sylvestris* aerial parts extract exerted anti-inflammatory effects in rat induced inflammation models.

Rezumat

Anthriscus sylvestris este un remediu tradiţional pentru diferite afecţiuni datorita lignanilor conţinuţi în rădăcină. Părţile aeriene al plantei sunt de asemenea folosite în medicina tradiţională, dar studiile farmacologice realizate sunt puţine. Scopul acestui studiu a fost evaluarea activităţii antiinflamatoare a extractului din părţile aeriene pentru a valorifica adaptabilitatea şi capacitatea plantei de a creşte rapid în aproape orice tip de sol. Rezultatele obţinute au demonstrat că extractul obţinut şi caracterizat, din părţile aeriene ale *A. sylvestris* a exercitat efecte antiinflamatorii în modele de inflamaţie indusă la şobolani.

Keywords: *Anthriscus sylvestris*, hydroalcoholic extract, luteolin-7-O-glucoside, HPLC, chlorogenic acid, anti-inflammatory effect

Introduction

Anthriscus sylvestris, known as wild chervil or cow parsley, is a wild herbaceous plant common in most of the temperate regions, related to garden chervil (*Anthriscus cerefolium*), and belongs to *Apiaceae* (syn. *Umbelliferae*) family [6]. Wild chervil was traditionally used as herbal remedy to treat headaches, as antitussive, antipyretic, or analgesic in various diseases [8]. The pharmacological studies revealed high anti-proliferative effects against several human cancer cell lines growth [14, 16, 24, 25]. Several lignans, of which deoxypodophyllotoxin is the most active, were identified in the root and ground parts of the plant as the antitumor agents [19]. Like its congener podophyllotoxin, deoxypodophyllotoxin binds directly to tubulin, resulting in the inhibition of tubulin polymerization, cell cycle arrest, followed by apoptosis [6, 19]. The analgesic and anti-inflammatory activity of *A. sylvestris* dried roots extracts is also induced by deoxypodophyllotoxin [12, 17]. The root of the plant was extensively studied because deoxypodophyllotoxin is concentrated mainly in this organ, while the aerial parts contain only small amounts of lignans [8].

Even if the aerial parts of the plant are used in the traditional medicine, the pharmacological studies are limited. The crude methanol extract from the *A. sylvestris* aerial parts was found to possess *in vitro*

antioxidant properties, with the active fractions containing mainly chlorogenic acid and luteolin-7-O-glucoside [7]. Other studies also identified in the aerial parts quercetin, rutin, apigenin, quercetin-3-O-glucoside (isoquercetin), several isoflavones [1], as well as a number of volatile compounds [4]. However, no further biological activity tests were performed.

The objective of this study was to evaluate the anti-inflammatory activity of *A. sylvestris* aerial parts in order to capitalize the plants highly adaptability and ability to grow rapidly in almost any type of soil [19].

Materials and Methods

Plant material

Fresh aerial parts of *A. sylvestris* were harvested and subjected to sorting, the resulting material, containing less than 0.5% impurities from other plant species and less than 1.5% impurities from the same plant. The plant material was dried at room temperature to a humidity of 12 - 14% and then in a drying oven at 40 - 50°C to a humidity of 8 - 9%. The dry plant material was grounded and passed through a 2 mm sieve. The material retained by the 1 mm sieve was used for extraction.

Extraction process

The extraction procedure was carried out for 6 h, using a Soxhlet extractor, 60% (v/v) ethanol as a solvent and a solvent: plant material ratio of 12.5:1 (v/v). Three batches of 50 g each of dry plant material were used for the extraction.

After extraction, the extractive solutions were concentrated under vacuum using an Ingos RVO 004 rotary evaporator working at 40°C until an aqueous slurry with a volume of about 10 times lower than the initial volume was obtained. Drying of the concentrated extractive solution was performed by lyophilization on a ScanVac Coolsafe freeze-drying apparatus working at -55°C for 48 h.

Reproducibility of the production process

The reproducibility of the obtaining process was monitored by assessing the quality control of the three plant extracts batches (B1, B2 and B3). The quality control was performed by chromatographic and spectroscopic profiling, and by measuring the total phenolic content of each batch [10, 11].

The fingerprint of the three batches were compared using high-performance thin-layer chromatography (HPTLC) to verify the reproducibility of the extraction process [21]. The samples were spotted in the form of bands of 10 mm with Camag microlitre syringe on pre-coated silica gel aluminium plate 60F-254 of 20 × 20 cm (E. Merck, Germany). The mobile phase used to identify the flavonoid glycosides was ethyl acetate: acetic acid: formic acid: water (72:7:7:14), and for the identification of flavonoid aglycons toluene: ethyl acetate: formic acid (5:3:1) was used. Camag TLC scanner III was used for spectrodensitometric scanning and analysis at λ = 254 nm. For flavonoid glycosides analysis, samples from each plant extract batch were dissolved in methanol, centrifuged at 6000 rpm for 10 min (Centurion Scientific C2 centrifuge) and the supernatant concentrated and stored at -18°C until use. Kaempferol-7-neoglucoside, troxerutin, isoquercitrozide, rutoside, hyperiside, and vitexin (Roth) were used as reference substances.

The identification of flavonoid aglycons was performed using samples prepared from each plant extract batch by suspending it in distilled water and 2M HCl (1:1, v/v) [21]. The mixture was maintained under reflux for 30 min, and then extracted with ethyl ether 10 times, concentrated and stored at -18°C until further use. Chrysin, myricetin, quercetin, kaempferol, luteolin, umbelliferone, caffeic acid, fisetin, and 5-hydroxy-flavone were used as reference substances.

Fourier transform infrared spectroscopy (FT-IR) associated with two-dimensional correlation infrared spectroscopy methods were applied. The spectra were recorded on a JASCO FT/IR-4200 spectrometer (JASCO, Japan) with an ATR PRO450-S accessory in the scan range of 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} [21].

Total phenolic compounds

The total polyphenol content of the extract was determined using the Folin-Ciocalteu method [20, 22]. Samples were prepared from each batch by dissolving the plant extract from each batch in ethanol 50% for a final concentration of 2 mg/mL. The samples were centrifuged at 6000 rpm for 10 min (Centurion Scientific C2 centrifuge) and the supernatant was further used. Appropriate volumes were oxidized with Folin-Ciocalteu's reagent and neutralized with a 10% sodium carbonate solution for 15 min at 50°C in the dark using a water bath (Memmert WNB10). After cooling, the samples were centrifuged at 6000 rpm for 10 min (Centurion Scientific C2 centrifuge) and the absorbance of the clear supernatant was measured at 750 nm. The total polyphenol content in each sample was performed in triplicate and the results were calculated by interpolation on a standard curve, previously prepared using gallic acid (Sigma, St. Louis, MO, USA). The results were expressed as mg of gallic acid equivalent (GAE) *per* gram of dry plant extract (DM).

HPLC analysis

The main polyphenolic derivatives of the *A. sylvestris* aerial parts, i.e. chlorogenic acid and luteolin-7-O-glucoside, were determined quantitatively by using a HPLC method. The analytical system employed consisted of a 1200 series Agilent (Agilent Tech., Darmstadt, Germany), containing of a binary pump (G1312B), degasser (G1379B), thermostated automated sample processor (G1329A), column thermostat (G1316B) and diode array detector (G1315B). Data acquisition and processing was performed using

Chemstation software (Agilent Tech., Darmstadt, Germany). The chromatographic separation was performed using a Phenomenex Kinetex C18 core shell column (150 x 4.6 mm i.d., 5 µm particle size), with a C18 guard column. Temperature of the column oven was set at 30°C. The mobile phase consisted of a mixture of 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B), delivered in a binary gradient mode, as follows: from 0 to 2 minutes, the mobile phase composition was 97% A: 3% B, from 3 to 18 minutes, the acetonitrile percent was increased linearly to 22%, from 18 to 25 minutes, % B was increased to 60% in order to elute the more lipophilic compounds from the column, and at 25.1 minutes the mobile phase composition returned to the initial conditions. A post-time of 8 minutes was introduced before the next injection, in order to allow column equilibration. The flow rate of the mobile phase was 1.0 mL/min, and the injection volume was set to 5 µL. All solutions were filtered through a 0.45 µm pore size filter and degassed by sonication. The detection was set at 320 nm for chlorogenic acid and 346 nm for luteolin-7-O-glucoside. Spectra were recorded for each compound in the range of 190 - 400 nm.

The analytes were identified on the basis of both their retention times and UV spectra, by comparison with authentic standards. Both reference standards of chlorogenic acid and luteolin-7-O-glucoside were acquired from Sigma-Aldrich (St Louis, MO, USA). Standard stock solution of each compound, with a concentration of 1 mg/mL, were prepared in methanol. The stock solutions were further diluted to appropriate concentration in the range of 0.25 - 100 µg/mL for chlorogenic acid and 0.125 - 25 µg/mL for luteolin-7-O-glucoside. The calibration curves were obtained by plotting the peak areas *versus* the nominal concentration of each analyte. All analysis were performed in triplicate. The limits of detection (LOD) and quantification (LOQ) of the method were evaluated on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

The *A. Sylvestris* samples were obtained by diluting 10.0 mg of the dried extract with methanol, in a 5 mL volumetric flask. The obtained solution was filtered through a 0.22 µm membrane filter, and 5 µL were injected into the chromatographic system.

Animals

Male Wistar rats (n = 48, 212 ± 45 g) from the "Cantacuzino" Istitute, Bucharest animal facility were used for the experiments. The rats were housed in Individually Ventilated Cage - system (Tecniplast, Italy) with free access to granulated food and water.

Ethics

The study involving animal experiments was conducted in accordance with the European Community guidelines (2010/63/EU) and had the approval of the Institutional Ethics Committee.

Anti-inflammatory assay

The anti-inflammatory activity of *A. sylvestris* aerial parts extract was evaluated by using the plethysmometric method (Ugo Basile 7140 Plethysmometer) in rat induced inflammation models. The inflammatory agents used for inducing the rat's hind pow oedema were dextran (Dextran 6000, Sigma®); and kaolin (Kaolin, Health Chemicals Co. Ltd., China) [2] in order to obtain two types of inflammatory process mediated by serotonin and histamine release and proinflammatory cytokines [3, 13] respectively.

Study design

The animals were randomly distributed into 6 groups (n = 8), and were orally treaded with: distilled water, 1 mL/100 g body weight (b.w.), control groups 1-dex (dextran) and 1-cao (kaolin); diclofenac 100 mg/kg b.w., aqueous solution 1%, reference groups 2-dex and 2-cao; *A. sylvestris* aerial parts extract, 1 mL/ 100 g b.w., groups 3-dex and 3-cao.

Treatment regimen and inflammation induction

The animals from the groups 1 and 2 were pre-treated with distilled water 1 mL/100 g b.w., twice a day, for 5 days. The animals from the groups 3 were pre-treated with *A. sylvestris* aerial parts extract 1 mL/ 100 g b.w., twice a day, for 5 days. In the 6th day of the experiment the animals from groups 1 received distilled water, from groups 2 received diclofenac and from groups 3 received the extract. After the treatment administration, the animals were anesthetized with urethane (Sigma®) and the initial pow volume was measured. The inflammation was induced by the intra-plantar administration of 0.2 mL of 0.6% dextran solution for the dex-groups and 0.2 mL of 10% kaolin suspension for the cao-groups, respectively. Pow oedema was evaluated at 1, 2, 3, 4, 5 and 24 hours.

Statistical analysis

Results are expressed as Mean ± Standard Deviation. The pow oedema was calculated using the formula

$$\% = [(V_{xh} - V_0)/V_0] \times 100,$$

where V_0 is the initial paw volume and V_{xh} is the paw volume corresponding to the time measurement. The anti-inflammatory effect was calculated as the difference between the evolutions of paw oedema of the treated groups and the control groups.

The statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA. The parametrical tests (t test, ANOVA) were performed after the identification and exclusion of the outliers. Statistical differences were considered for a p value < 0.05.

Results and Discussion

The yield of the dried extract was 22.30 ± 1.68%.

The reproducibility of the extraction procedure was demonstrated on three batches using two HPTLC fingerprints. In both methods, all the spots correspond, sharing the same Rf values and UV/VIS spectra

(Figure 2). Isoquercitrin was identified in all batches at Rf = 0.68. The flavonoids identified were: quercetin at Rf = 0.59, myricetin at Rf = 0.49, and luteolin at Rf = 0.53. In this method, caffeic acid was also identified at Rf = 0.56.

The FT-IR spectroscopy results revealed identity in numbers, positions, and relative intensity of all the peaks and a Pearson correlation coefficient over 0.95. The FT-IR profile can be used for the quality evaluation of future samples.

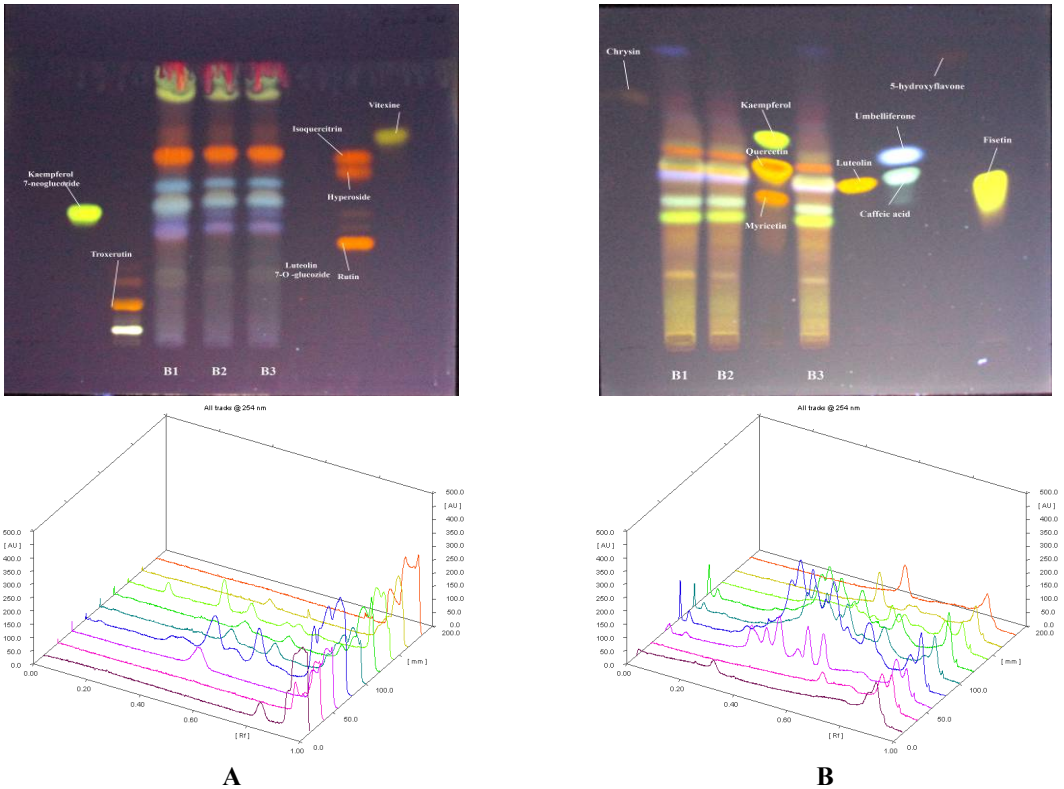
The results of phenolic content are presented in Table I. The standardized extract contains 4.44 - 6.56 g total polyphenols expressed in gallic acid equivalents/ 100 g dry extract.

The results of quantitative determinations were statistically analysed by applying the ANOVA and Tukey tests. There were no statistical differences between the total polyphenols in the three batches of *Anthriscus sylvestris* extract ($p = 0.4006$, $R^2 = 0.2268$).

Table I

Total phenolic content of *Anthriscus sylvestris* extract

No.	Batch	Means of total phenolic content (mg GAE/DM)	95% confidence interval of the mean (mg GAE/DM)
1	B1	5.06	3.20 - 6.92
2	B2	5.54	3.49 - 7.59
3	B3	5.91	4.56 - 7.26



A **B**

Figure 1.

HPTLC fingerprint in UV at 366 nm and density at λ = 254 nm for three batches of standardized extract of *Anthriscus sylvestris*: A. identification of heterosides; B. Identification of flavonoid aglycones

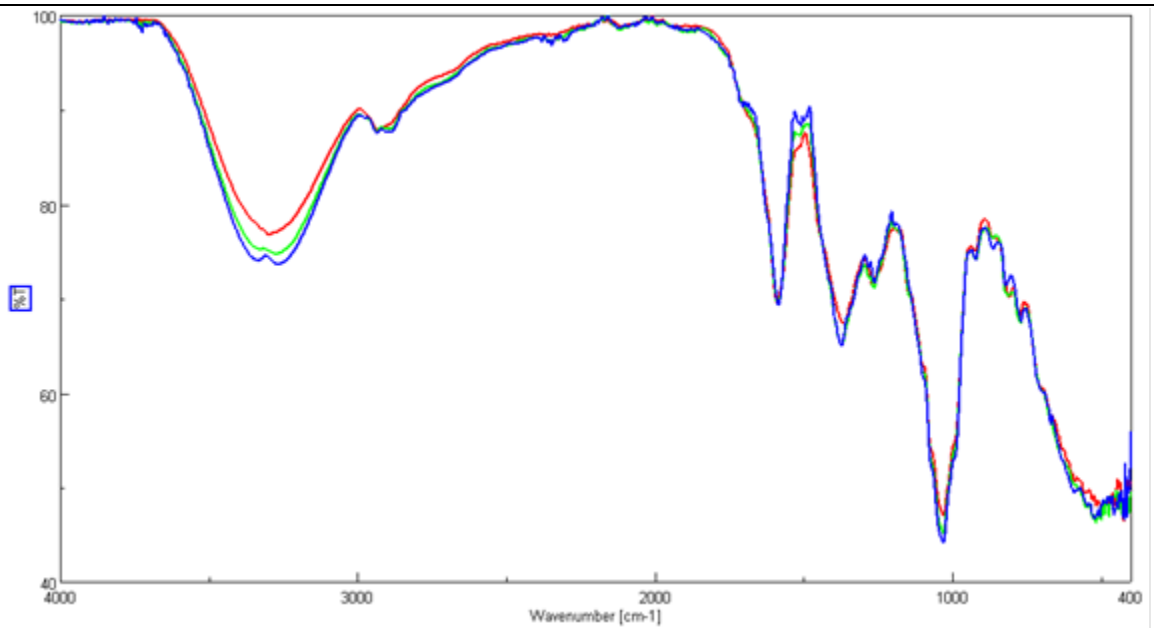


Figure 2.
The IR fingerprint of the standardized extracts of *Anthriscus sylvestris* on the 400 - 4000 cm^{-1} range

HPLC quantification of chlorogenic acid and luteolin-7-O-glucoside

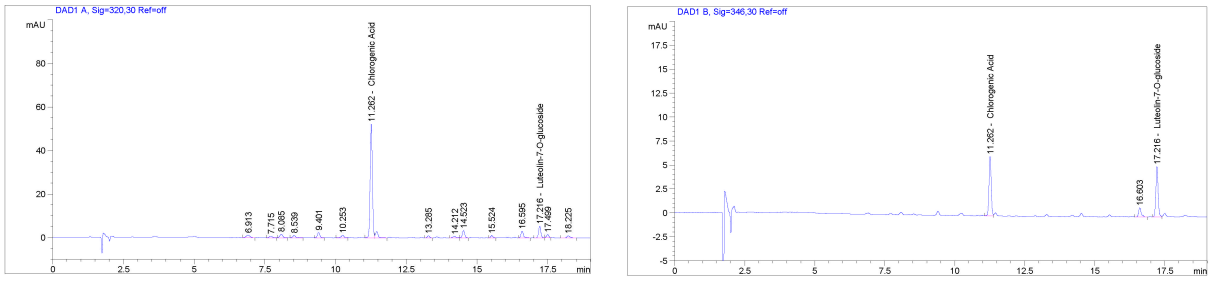
The HPLC method was adequate for the quantitative determination of both chlorogenic acid and luteolin-7-O-glucoside. The linearity of the method was established in the range of 0.125 - 25 µg/mL for luteolin-7-O-glucoside (r^2 = 0.9997), and 0.25 - 100 µg/mL for chlorogenic acid (r^2 = 0.9999) (Table II).

Table II

Calibration characteristics of the HPLC method for quantitative determination of chlorogenic acid and luteolin-7-O-glucoside

Analyte	Wavelength (nm)	Calibration range (µg/mL)	LOQ (µg/mL)	Regression equation	Correlation coefficient (r^2)
Chlorogenic acid	320	0.25 - 100	0.087	y = 12.33x - 0.263	0.9999
Luteolin-7-O-glucoside	346	0.125 - 25	0.106	y = 11.62x - 1.040	0.9997

A typical chromatogram obtained for the *A. sylvestris* extract is presented in Figure 3. The analysed hydroalcoholic *A. sylvestris* aerial parts extract contained 23.11 ± 0.93 mg/g dry extract chlorogenic acid and 3.38 ± 0.21 mg/g dry extract luteolin-7-O-glucoside.



(A) (B)

Figure 3.
A typical chromatogram obtained after the analysis of the *A. sylvestris* aerial parts extract. The signal was simultaneously monitored at λ = 320 nm for the quantification of chlorogenic acid (A) and at λ = 346 nm for the quantification of luteolin-7-O-glucoside (B)

The quantitative analysis of *A. sylvestris* main poliphenolic components is essential, taking into account the different antiinflamatory potency and efficacy of different poliphenol derivatives. Thus, luteolin and its glycosides have been reported to exert anti-inflamatory effects both *in vitro* and *in vivo* [5, 9, 23, 27. In fact, luteolin was identified as being the most potent flavonoid in inhibiting TNF-α release in macrophage, as well as in blocking lipopolysacharide (LPS) induced activation of the nuclear

factor-kappa B (NF-kB), which is responsible for increasing the expression of pro-inflammatory cytokines, chemokines and enzymes [26]. On the other hand, chlorogenic acid was also proved to present intrinsic anti-inflammatory activity, by multiple mechanisms, such as: activation of NF-kB signalling pathway, inhibition of the NO production and of the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [15, 18]. Since chlorogenic acid and luteolin-7-O-glucoside are the major polyphenols in the *A. sylvestris* aerial parts, and both have intrinsic anti-inflammatory effect, it is to be expected that the overall anti-inflammatory activity of the extracts to be proportional with their concentration in the respective extract.

Anti-inflammatory assay

The plethysmometric measurements of the rat's hind paw and the statistical interpretation of the results against the baseline values before administration of inflammatory agents are presented in the Tables III and IV.

Table III

Plethysmometric results and the statistical interpretation against the baseline values before administration of dextran

	V0 (mL)	V1h (mL)	V2h (mL)	V3h (mL)	V4h (mL)	V5h (mL)	V24h (mL)
Group 1-dex	1.27 ± 0.06	1.97 ± 0.21	2.08 ± 0.27	2.13 ± 0.25	2.14 ± 0.20	2.18 ± 0.18	1.40 ± 0.09
t test, p		p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Group 2-dex	1.09 ± 0.12	1.80 ± 0.23	1.85 ± 0.23	1.79 ± 0.17	1.79 ± 0.14	1.85 ± 0.23	1.30 ± 0.15
t test, p		p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Group 3-dex	1.07 ± 0.06	1.39 ± 0.11	1.56 ± 0.16	1.47 ± 0.23	1.52 ± 0.16	1.53 ± 0.14	1.22 ± 0.12
t test, p		p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05

V – the paw volume

Table IV

Plethysmometric results and the statistical interpretation against the baseline values before administration of kaolin

	V0 (mL)	V1h (mL)	V2h (mL)	V3h (mL)	V4h (mL)	V5h (mL)	V24h (mL)
Group 1-cao	1.24 ± 0.09	1.65 ± 0.05	1.74 ± 0.17	1.77 ± 0.09	1.80 ± 0.17	1.84 ± 0.12	1.94 ± 0.18
t test, p		p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Group 2-cao	1.13 ± 0.17	1.39 ± 0.13	1.46 ± 0.11	1.47 ± 0.14	1.59 ± 0.14	1.65 ± 0.17	1.80 ± 0.26
t test, p		p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05
Group 3-cao	1.08 ± 0.05	1.32 ± 0.13	1.31 ± 0.13	1.31 ± 0.12	1.36 ± 0.11	1.37 ± 0.13	1.64 ± 0.23
t test, p		p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05	p < 0.05

V – the paw volume

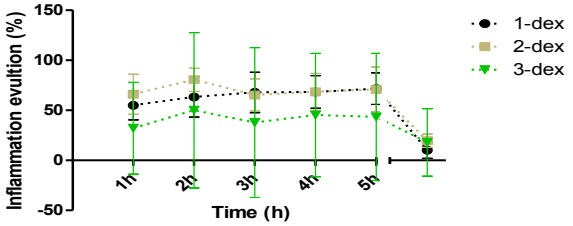


Figure 4.

The evolution of the inflammatory process (%) generated by dextran

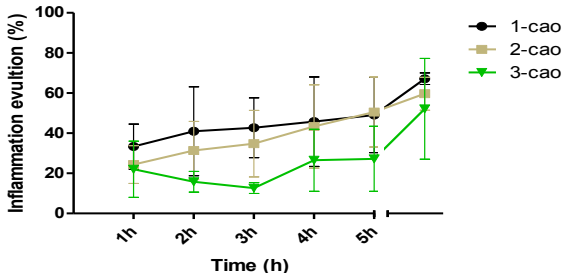


Figure 5.

The evolution of the inflammatory process (%) generated by kaolin

The hind paw oedema during the experiment did not returned to the initial volume, prior to the inflammatory agents' administration, for all the groups.

The mean hind paw oedema evolution is presented in Figures 4 and 5.

The global inflammatory processes were different for groups 2 and 3 when compared to the control groups (ANOVA, $p < 0.05$), suggesting different mechanisms for diclofenac and *A. sylvestris* extract. The analysis of the comparative dynamics of the inflammatory process for both inflammatory processes indicated an anti-inflammatory effect during the experiment, for the animals treated with *A. sylvestris* extract. Statistical significance was encountered for the dextran model when compared to the control group and the reference group at 1, 3, 4, 5 hours and 1, 2, 3, 4, 5 hours, respectively. Statistical significance was encountered for the kaolin model when compared to the control group and the reference group at 2, 3, 4, 5 hours and 2, 3, 5 hours respectively.

Conclusions

A reproductible extraction process for the polyphenols contained by the Romanian crops of *A. sylvestris* aerial parts was developed. The extract polyphenolic concentration was determined using a validated HPLC. The extract proved anti-inflammatory action in the murine model used.

Acknowledgement

This work received financial support from UEFISCDI through the project PN-II-PT-PCCA-2013-4-0953 No. 176/2014.

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