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Analgesic and anti-inflammatory activity of Artemisia extracts on animal models of nociception

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Abstract

Introduction. The study aims to assess the antinociceptive and anti-inflammatory activity of extracts obtained from indigenous species of wormwood: *Artemisia absinthium, A. annua, A. vulgaris* and *A. pontica*.

Materials and methods. For these experiments, we employed nociception models using thermal stimulus (hot plate and tail immersion tests, t = 52.5 ^{0}C; 30, 60 and 90 minutes testing), chemical stimulus (Zymosan-induced abdominal constriction response test, using distinct lots and testing at 60, 90 and 120 minutes after administration of samples) and pressure stimulus (Randall Selitto test) and an inflammation model for the evaluation of inflammatory edema by Plethysmometer test. Groups of 6 Swiss mice / lot were used, receiving by oral administration the plant extracts suspended in 0.1% CMC-Na. The doses were administered in geometric progression. Chemical analyses were performed by HPLC-MS in order to identify bioactive substances present in extracts: methoxylated flavonoids, sesquiterpene lactones, phytosterols and hydroxycinnamic acids.

Results and discussion. All plant extracts showed antinociceptive action on the models with thermal stimulus, as demonstrated by the ED_{50} values obtained at different test times. In the models of nociception with chemical and mechanical stimulus, models based on inflammatory mediation, the studied fractions have partially proved their antinociceptive action. Regarding the degree of inhibition of inflammatory edema, the highest potency was exhibited by *Artemisia pontica* extract (86.5% inhibition for the dose of 100 mg/kg). Hispidulin and eupatorin, known anti-inflammatory compounds, were identified in all extracts, along with caffeic and chlorogenic acids, stigmasterol, campesterol and β-sitosterol.

Conclusions. The obtained results support the use of these plant extracts in moderate intensity pain, triggered by both central and peripheral mechanisms.

Keywords: *hot-plate test, tail immersion test, abdominal constrictive response, Randall-Selitto test, inflammatory edema,*

Introduction

The *Artemisia* genus - wormwood, includes over 500 species, widespread around the globe, especially in the northern hemisphere. These are aromatic, medicinal and food plants, many being used in the folk medicine of different countries in various diseases (1). The Romanian Pharmacopoeia includes the monograph *Absinthii herba* (the aerial parts of *Artemisia absinthium*) with the indication bitter tonic, but it is also recommended as cholagogue and anthelmintic (2). Similarly, *Artemisia pontica* is used as tonic and anthelmintic, sometimes as a substitute for *A. absinthium*, although its properties are milder than those of common wormwood (3). *Artemisia vulgaris* is a cosmopolite species, known as tonic, hypoglycemic, emmenagogue, anti-septic, analgesic, and anti-helminthic, frequently utilized in Chinese medicine in combination with acupuncture treatment (4).

Research on *Artemisia* species intensified with the discovery of artemisinin, an antimalarial compound from *Artemisia annua*, which also exhibits antiphlogistic, immunomodulatory, anticancer and antimicrobial effects (5). The analgesic and anti-inflammatory actions of *Artemisia annua* extracts are attributable to the sesquiterpene lactones, such as artemisinin, but also to flavonoids, phenolic acids and coumarins (6, 7).

In the present study we aimed to evaluate the antinociceptive and anti-inflammatory action of crude methanol extracts obtained from wormwood species widespread in the Romanian flora: *A. absinthium* - wormwood, *A. vulgaris* – common mugwort, *A. annua* – sweet wormwood and *A. pontica* - Roman wormwood. The plant extracts were then chemically characterized by liquid chromatography-mass spectrometry analysis in order to identify biologically active compounds, such as sesquiterpene lactones, methoxylated flavones, phytosterols and polyphenols.

Materials and methods

Plant material and extraction procedure

The aerial parts of *Artemisia annua* L., *A. vulgaris* L., *A. absinthium* L. and *A. pontica* L. were harvested at the flowering stage (July-September) from the countryside around Iasi, Romania. The species were identified by a specialist and voucher specimens were deposited in the Herbarium of Pharmaceutical Botany Department from "Grigore T. Popa" University of Medicine and Pharmacy. The plants were air-dried at room temperature and grounded to a fine powder. The extracts were obtained by maceration of dry plant material (300 g) with methanol (1:10 ratio m/v) for 24 hours with continuous agitation. Extract solutions were filtered and evaporated to dryness to afford the crude methanol extracts, labeled A_1 - *A. annua* (41,23 g), A_2 - *A. vulgaris* (25,64 g), A_3 - *A. absinthium* (34,63 g) and A_4 - *A. pontica* (43,85g). The extracts were appropriately diluted before injection in HPLC.

Pharmacological study

Animals. In this study we used male Swiss mice provided by the "Cantacuzino" Institute Bucharest, Romania, weighing 20-30 g. All animals were housed at 21 ± 2°C under a 12-h light/dark cycle with access to standard food and water *ad libitum*. Prior to each experiment, animals were habituated to the testing room and the equipment for five consecutive days. Animals received orally, dose sequences in geometric progression (ratio 2) of the studied extracts. The extracts were suspended in 0.1% sodium carboxy-methylcellulose (CMC-Na) (Sigma), while the control group received only the vehicle, CMC-Na. Habitation conditions were set inside the laboratory of experimental pharmacodynamics in the department of Pharmacodynamics and Clinical Pharmacy, at "Grigore T. Popa" University.

Ethics statement. All experiments were conducted in strict conformity with the specific regulations approved by "Grigore T. Popa" University of Medicine and Pharmacy Iași, European bioethical regulations (Directive 2010/63/EU) and International Association for the Study of Pain regulations.

The hot plate test was performed according to the method described by Woolfe and MacDonald with some minor modifications (8). The mice were individually placed in the cylindrical chamber of the hot plate (Model 7280 UGO Basile) on the heated surface at 52.5°C ± 0.1°C. The pain latency period was measured, with a 30 seconds cut-off. The response for testing the pain threshold at the thermal stimulus consists in licking and/or shaking the hind paw or the tendency to jump in order to leave the enclosure. The animals were tested for the thermal stimulus 1 hour before being treated, eliminating from the experiment the animals that did not respond in 15 seconds. After treatment, retesting was performed at 30, 60, 90 minutes, and the latency period of the pain reaction was measured. Percentage inhibition of the response to the nociceptive stimulus was expressed according to the formula:

% inhibition = $(T_x - T_0)/(T_m - T_0) \times 100$, where T_0 - latency of the response measured prior to administration of the extract, T_x – latency at different time intervals following administration of the test extract, T_m - cut-off time.

The tail immersion test measures the latency period of the mouse pain reaction when the tail is fully immersed in hot water at 52.5 ± 0.1 °C for 15 seconds (cut-off). The time elapsed between immersion and purposeful withdrawal of the tail from the bath is recorded. Animals were tested for thermal stimulation 45 minutes before treatment, and animals that did not respond within 15 seconds were removed from the experiment. The experimental model was evaluated quantitatively as a quantal type. By successive determinations, the graded effect was transformed into a quantal one, by setting the 7-second threshold for non-responsive animals when applying the stimulus after treatment.

Zymosan A-induced writhing test. The principle of the method relies on the fact that through the intraperitoneal injection of a suspension of Zymosan A, a characteristic response is induced, represented by the stretching followed by the torsion of the body, abdominal retraction and opisthotonus. The test allows for the evaluation of the central and peripheral analgesia, and by using Zymosan, the test is more relevant for the pathogenesis of inflammatory pain. The test consists of the intraperitoneal administration of a suspension of Zymosan A, 40 mg/kg body weight and the recording of the abdominal constrictive responses for 12 minutes after that. The evaluation of the response was of the quantal type, characterized by the presence or the absence of the constrictive response. It was considered as antinociceptive effect of the extracts taken into study, the inhibition percentage obtained through the absence of the response of the total number of animals tested: % inhibition = (number of non-responders / total number of animals) x 100 (9).

The Randall-Selitto assay (10) allows the assessment of pain in inflammatory conditions. The test consists in applying a mechanical stimulus (Ugo Basile Analgesimeter 37215) on the inflamed paw of the animal (cut-off pressure 250 g). The edema is obtained by the subcutaneous injection into the plantar region of 3% saline suspension of lambda-carrageenan (Sigma) in mice. The stimulus is applied up to the cut-off value. The increase of the latency time of the pain reaction is recorded after 4 hours from the development of edema. The evaluation is made in comparison with the contralateral paw where only simple saline was injected (Zentiva). The antinociceptive effect was calculated according to the formula: % inhibition = $(g_x + g_0)/(g_m -$

g_0) x 100, where: g_0 – measured response latency before the administration of the tested extract, g_x - latency at different times following the administration of the extract, g_m - the maximum permissible weight (cut-off).

Carrageenan-induced paw edema is an experimental model of acute inflammation that allows the evaluation of the degree of inhibition of inflammatory edema induced by chemical stimulus. The test is performed by measuring the volume of the inflamed paw by subcutaneous administration in the plantar region of a 3% saline suspension of λ-carrageenan. The 7140 Ugo Basile plethysmometer was used to measure the volume of the inflamed paw 4 hours after the development of inflammatory edema compared to the contralateral paw of the treated groups compared to the control group. The assessment of the degree of inflammation has indicative value in the present study and was performed in parallel with the Randall-Selitto test for the sequence of doses that showed antinociceptive action. The results were expressed as percentage relative to the maximum possible effect level (MPE%) for each extract, using the formula: % inhibition = (M-T) / M x 100, where M - the value of the degree of inhibition of the control group and T - the value of the degree of inhibition of the treated group.

Chemical analysis of plant extracts

The analysis of sesquiterpene lactones was carried out by high-performance liquid chromatography coupled with mass spectrometry (LC-MS), using six standards: vulgarin, α-santonin, dehidroleucodine, artemisinin, costunolide and alantolactone (Sigma, Germany). Calibration curves in the range 0.02 - 3 µg/mL showed a linear correlation coefficient ($R^2 > 0.99$) and were employed to quantify the lactones in the extracts (11).

Methoxylated flavonoids were identified through a LC-MS method described before (12). Six standards were used: jaceosidin, eupatilin (ALB Technology, China), casticin, acacetin, eupatorin, hispidulin (Sigma, Germany). Calibration curves in the 0.02 - 6 µg/mL range with good linearity ($R^2 > 0.99$) were used to determine the concentration of methoxylated flavones.

Caffeic and chlorogenic acids were determined quantitatively by a LC-MS method using standards acquired from Sigma, Germany. Calibration curves in the range of selected concentrations (0.06 - 4 µg/mL) had a good linear correlation coefficient ($R^2 > 0.99$) and were used to calculate the concentrations of the hydroxycynnamic acids (11).

Phytosterols analysis was performed by a previously reported LC-MS method (12) using five standards: β-sitosterol, stigmasterol, campesterol, brassicasterol and ergosterol, acquired from Sigma, Germany. Calibration curves of the sterols in the range of selected concentrations (0.06 - 6 µg/mL) displayed a good linearity ($R^2 > 0.99$).

Results and discussions

Pharmacological study

The hot plate test. A_1 extract was tested at doses of 25-200 mg / kg body weight p.o. in CMC-Na 0.1% and an MPE of 90.00% was obtained at 90 minutes. Extracts A_2, A_3, A_4 were tested at doses of 100-400 mg / kg body weight p.o. in CMC-Na 0.1%, obtaining the following values: MPE at 30 minutes (61.7%) for A_2 extract, MPE at 60 minutes (64.6%) for A_3 extract, and MPE at 30 minutes (62.6%) for the A_4 extract. By the administration of the mentioned dose sequences, in geometric progression, ratio 2, we could establish the median effective dose (ED_{50}) value of each extract (Table 1).

Table 1. ED_{50} values of extracts in the hot-plate test

ED_{50} mg/kg body weight/p.o.			
A_1	**A_2**	**A_3**	**A_4**
58.44 ± 4.784 Y = -87.238 + 77.680*X R =0.970	263.62 ± 18.268 Y = -124.92 + 72.253*X R = 0.994	172.37 ± 33.762 Y = -56.24 +47.50*X R = 0.948	202.10 ± 3.72 Y = -51.863 + 44.182*X R=0.998

The tail immersion test. A_1 extract at doses of 25-200 mg / kg body weight p.o. shows maximum possible effect at 90 minutes (83.33%). A_2 extract, tested at doses of 50-400 mg / kg body weight p.o. shows maximum possible effect at 60 minutes (100.00%). A_3 extract at doses of 100-400 mg / kg body weight p.o. exerts maximum possible effect at 90 minutes (83.33%). A_4 extract was tested at 50-400 mg / kg body weight and manifested maximum possible effect at 90 minutes (66.67%). The ED_{50} values of tested extracts are shown in Table 2.

Table 2. ED_{50} values of plant extracts in the tail immersion test

ED_{50} mg/kg body weight/p.o.			
A_1	**A_2**	**A_3**	**A_4**
59.41 ± 22.608 Y = 2.044 + 1.666*X R =0.942	78.939 ± 27.469 Y = 0.845 + 2.190*X R = 0.891	236.55 ± 76,930 Y = 0.416 + 2.281*X R = 0.973	230.80 ± 94.29 Y = 0.858 + 1,753*X R = 0.950

Zymosan A-induced writhing test. The animals were treated orally with suspensions from dried plant extracts, in the doses sequence of 25-400 mg / kg body weight for A_1 and A_2, 50-200 mg / kg body weight for A_3 and 100-800 mg / kg body weight for A_4. The maximum possible effects of extracts were: 66.67% for A_1, 50% for A_2, 83.33% for A_3 and 66.66% for A_4 extract. The ED_{50} values of tested extracts were calculated and presented in Table 3.

Table 3. ED_{50} values of extracts in the writhing test

ED_{50} mg/kg body weight/p.o.			
A_1	**A_2**	**A_3**	**A_4**
101.44 ± 44.40 Y = 1,935 + 1,528*X R = 0.998	177.72 ± 79.14 Y = 0.198 + 2.134*X R = 0.953	72.49 ± 24.79 Y = 0.624 + 2.352*X R = 0.990	346.64 ± 162.81 Y = 1.607 + 1.336*X R = 0.906

The Randall-Selitto test. A_1-A_4 extracts were analyzed at doses of 100-400 mg / kg body weight p.o. in 0.1% CMC-Na. A_1-A_3 extracts showed a hyperalgesia tendency for the studied dose sequence. A4 extract showed a maximum possible effect of 49% at a dose of 100 mg. The value obtained could allow the study to be continued for another doses sequence.

Inflammatory edema test. A_1-A_4 extracts were tested at doses of 100-400 mg / kg body weight p.o. in 0.1% CMC-Na. Regarding the degree of inhibition of inflammatory edema, the following data were obtained: A_1 extract shows MPE = 50% at a dose of 400 mg / kg body weight, A_2 extract exerts MPE = 56.3% at a dose of 200 mg / kg body weight, A_3 extract manifest MPE = 49% at a dose of 100 mg / kg body weight and A_4 extract presents MPE = 86.5% at a dose of 100 mg / kg body weight. These values support the validity of the working hypothesis, which encourages further study.

Chemical analysis of plant extracts

Phytochemical analyzes identified in all plant extracts variable concentrations of caffeic and chlorogenic acid, these compounds being predominant in the A_2 extract (Table 4). The anti-inflammatory activity of chlorogenic acids, esters formed between quinic acid and trans-cinnamic acids, is well documented and traceable to their ability to relieve intracellular oxidative stress and to inhibit pro-inflammatory cytokines by regulation of key transcription factors (13).

Table 4. Concentration of the hydroxycynnamic acids in the tested extracts (µg/g dry extract)

	Caffeic acid	**Chlorogenic acid**
A_1	211.66	11786.66
A_2	235.71	19112.5
A_3	126.66	9750
A_4	203.12	14718.75

LC-MS analysis of phytosterols revealed in all extracts the presence of stigmasterol, sitosterol and campesterol (Table 5). Sitosterol predominates in all four extracts, while ergosterol is found only in small amounts in samples A_2 and A_3. Besides their cholesterol-lowering effect, plant sterols have been shown to reduce plasma levels of C-reactive protein (CRP), interleukin 6 (IL-6), tumor necrosis factor (TNF-α), phospholipase A1, and fibrinogen, thus manifesting anti-inflammatory activity (14).

Table 5. Concentration of phytosterols in the tested extracts (µg/g dry extract)

	ergo-sterol	**stigma-sterol**	**β-sito-sterol**	**campe-sterol**	**brassica-sterol**
A_1	-	1981.66	5367.83	192.5	-
A_2	25.53	1406.96	9841.78	147.32	15.53
A_3	3.16	666.16	3545.16	67.83	-
A_4	-	448.59	7581.71	52.96	-

In the A_3 extract, the sesquiterpene lactones costunolide and dehydroleucodine were identified. They are cited in the literature for their antinociceptive and anti-inflammatory action (15, 16).

As expected from a previous study (17), the A_1 extract contains high amounts of artemisinin. Analgesic and anti-inflammatory activity of artemisinin is well documented in the specialty literature (18, 19). Numerous studies have shown that artemisinin is able to decrease neutrophils count, suppress the secretion of cytokines, inhibit macrophage activation and their responses, and block lymphocytes proliferation. The anti-inflammatory effects of artemisinin is owed to the inhibition of different signaling pathways (19). Additional research demonstrated that artemisinin modulates neuropathic pain and its antinociceptive activity is mediated by γ-aminobutyric acid A ($GABA_A$) receptors (18).

Costunolide, was also identified in A_1 and A_2 extracts. Costunolide modulates various intracellular signaling pathways involved in tissue inflammation, including intracellular kinases and redox-regulated transcription factors, and also reduces the production and expression of pro-inflammatory mediators, such as cyclooxygenase-2, inducible nitric oxide synthase, nitric oxide, prostaglandins, and cytokines (15).

Allantolactone, vulgarin and santonin were not identified in the tested extracts and none of the standards lactones used were found in the A_4 extract, as seen in Table 6.

Table 6. Concentration of sesquiterpene lactones in the tested extracts (µg/g dry extract)

	artemisinin	**costunolide**	**dehydroleucodine**
A_1	6790.35	361.28	-
A_2	-	866.79	-
A_3	-	10.71	454.7

Among the methoxylated flavones with anti-inflammatory action (20-22), specific to the genus *Artemisia*, hispidulin and eupatorin were found in all extracts, while jaceosidine was not identified in the analyzed samples. Casticin was found in extracts A_1-A_3, notably in A_1 extract. Eupatilin was identified in small

quantities only in A_1 extract, while acacetin only in A_4 extract.

Flavonoids usually manifest antiphlogistic activity by modulating pro-inflammatory gene expression and intervening in multiple signaling pathways, chiefly nuclear factor – kappa B (NF-κB) and mitogen-activated protein kinase (MAPK). The anti-inflammatory response of flavonoids seems to be signal specific and dependent on the cell type. In addition, flavonoids have the ability to scavenge free radicals and manifest antioxidant activity, thus reducing intracellular oxidative stress and inflammation. However, they can act as a pro-oxidant at high concentrations and caution is necessary when administering flavonoids (23).

Table 7. Concentration of methoxylated flavonoids in the tested extracts (μg/g dry extract)

	eupatorin	**eupa-tilin**	**aca-cetin**	**casticin**	**hispidulin**
A_1	67.30	7.86	-	5237.14	102.79
A_2	6.58	-	-	446.65	14.69
A_3	121.73	-	-	688.33	32.71
A_4	4.47	-	41.53	-	368.85

It seems that high amounts of sesquiterpene lactones and methoxylated flavonoids, as found in A_1 extract, could be correlated with potent analgesic effects manifested in the thermal stimulus model of nociception.

Conclusions

The present study carried out the evaluation of the antinociceptive activity of some *Artemisia* species from the spontaneous flora of Romania. In addition to pharmacological testing, the chemical characterization of the extracts in terms of biologically active compounds was performed. On the pharmacological models with thermal stimulus, the studied extracts demonstrated antinociceptive action through the ED_{50} values obtained, with *A. annua* extract being the most efficient. On the nociception models with chemical and mechanical stimulus, models based on inflammatory mediation, the studied fractions partially proved their antinociceptive action. *A. pontica* extract exhibited good anti-inflammatory activity against carrageenan induced paw edema. The results obtained support the use of these extracts in pain of moderate intensity, triggered by both central and peripheral mechanism. Further studies are needed to isolate the bioactive fractions and compounds and to evaluate their antinociceptive and anti-inflammatory actions.

Authors' contributions. All authors have contributed equally to this work.

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