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Fitoterapia 92 (2014) 23–33

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

St. John's Wort seed and feverfew flower extracts relieve painful diabetic neuropathy in a rat model of diabetes

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ARTICLE INFO

Article history:
Received 29 July 2013
Accepted in revised form 28 September 2013
Available online 12 October 2013

Keywords:
St. John's Wort
Feverfew
Painful diabetic neuropathy
Hypericin
Hyperforin
Parthenolide

ABSTRACT

Painful diabetic peripheral neuropathy (DPN) is a common complication of diabetes and the few approved therapies for the management of pain have limited efficacy and side effects. With the aim to explore and develop new pharmacological treatments, we investigated the antihyperalgesic properties of St. John's Wort (SJW) and feverfew in streptozotocin (STZ)-diabetic rats. Acute administration of a SJW seed extract reversed mechanical hyperalgesia with a prolonged effect. A SJW extract obtained from the aerial portion of the plant and a feverfew flower extract partially relieved neuropathic pain whereas a feverfew leaf extract was ineffective. The antihyperalgesic efficacy of these herbal drugs was comparable to that of clinically used antihyperalgesic drugs (carbamazepine, lamotrigine, L-acetyl-levocarnitine). Further examinations of SJW and feverfew composition revealed that hyperforin and hypericin might be responsible for the antihyperalgesic properties of SJW whereas the efficacy of feverfew seems to be related to the presence of parthenolide. Rats undergoing treatment with SJW and feverfew did not show any behavioral side effect or sign of altered locomotor activity. Our results suggest that SJW and feverfew extracts may become new therapeutic perspectives for painful DPN.

1. Introduction

Diabetic peripheral neuropathy (DPN) is the most prevalent complication of diabetes. A population-based study reported that more than half of diabetic patients develop DPN [1]. Of these patients, 30 to 50% suffer from painful DPN, and chronic pain associated with diabetes is represented by hyperalgesia, allodynia, paresthesias, and spontaneous pain [2,3]. Although neuropathy is a common symptom among diabetic patients, its mechanisms remain unclear, and as a consequence, treatment is unsatisfactory in many cases. Glycemic control has been shown to be effective in slowing the progression of DPN [4], but patients with painful DPN often need other agents to palliate their symptoms. Agents used include tricyclic antidepressants, anticonvulsants, serotonin–norepinephrine reuptake inhibitors, opiates, opiate-like substances and topical medications. However, these medications usually at best provide only partial pain relief [5–7] and have severe side effects. Therefore, for an effective treatment of neuropathic pain, there is still a need to obtain therapeutics, which possess higher efficacy and a greater level of tolerability and safety.

Hypericum perforatum L., commonly known as St. John's Wort (SJW), is an herbal medicine known to have diverse medicinal uses for centuries, including psychiatric disorders, skin wounds, and inflammation. Numerous studies proved that SJW is endowed with many bioactivities with a favorable side effect profile. In addition to the well-documented antidepressant efficacy [8], SJW also produced anti-inflammatory [9,10] and analgesic effects [11,12].

Feverfew (*Tanacetum parthenium* L.), also known as "medieval aspirin", is a medicinal plant traditionally used as

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http://dx.doi.org/10.1016/j.fitote.2013.10.003

antipyretic and for the treatment of headaches, rheumatoid arthritis, stomach aches, toothaches, insect bites, infertility, and menstrual disorders [13]. At the beginning of the 1980s, feverfew gained attention for its apparent effectiveness in the prophylaxis of migraine headaches. Several placebo controlled clinical trials have shown feverfew to be an affective oral agent in reducing the frequency and severity of migraine headaches [14–17].

Due to the limited efficacy and tolerability of the therapeutic options for painful DPN, we aimed to identify a safer and effective treatment to manage pain in DPN. Based on the analgesic activity of SJW and feverfew in pain of various etiologies and on their elevated tolerability, we investigated the efficacy of both herbal medicines in a rat model of painful DPN. The most common SJW preparations used are hydroalcoholic extracts of the aerial portion of the plant that contain at least ten different kinds of biochemical compounds, including hypericin and hyperforin [18]. The dried leaves of feverfew contain a large number of natural products, but the active principles probably include one or more of the sesquiterpene lactones known to be present, including parthenolide [19]. To better elucidate the pharmacological profile of SJW and feverfew and the role of their main components, we compared the efficacy and phytochemical composition of different dried extracts obtained from SJW and feverfew.

2. Materials and methods

2.1. Feverfew and SJW extract preparation

Feverfew flowers and leaves, and SJW seed A were collected from Tuscany cultivations. SJW seed B was obtained from German cultivations. SJW seed, feverfew flower and leaf extracts were made by Aboca SpA according to the condition reported in Table 1. After 6–8 h, the EtOH/H_2O herb/seed mixture was dropped for 1 h and filtered to remove the exhausted herb/seed. The corresponding hydroalcoholic extract was concentrated under vacuum to evaporate ethanol. The water concentrate obtained underwent freeze-drying for 72 h and the resulting extracts were stored at 4 °C, protected from light and humidity. Peculiar extraction conditions (ethanol 70%) were chosen to extract the already known metabolite of SJW, avoiding extraction of fat and waxes.

2.2. Feverfew and SJW extracts' fingerprint analysis

2.2.1. Sample preparation

2.2.1.1. Feverfew. Ethanol 50% (25 ml) was added to a sample of the freeze-dried leaf, flower or seed extract (0.1 g). The resulting mixture was extracted in ultrasonic bath (20 min) and then centrifuged (5 min at 4000 rpm). The supernatant was then collected and the remaining solid material was further extracted with ethanol 50% (25 ml) under the same condition. After centrifugation the supernatant was added to the supernatant previously collected, to achieve the final volume of 50 ml. After filtration on cellulose acetate (0.45 μm) the samples were used for the LC–MS analysis.

2.2.1.2. SJW. 0.25 g of the freeze-dried SJW seed extract was weighed accurately and placed in a 50 ml volumetric tube. Twenty milliliters of methanol was added and the tube was sonicated for 30 min. The extract was centrifuged (5 min at 4000 rpm). The supernatant transferred into a 50 ml amber volumetric flask. The residue re-extracted with 20 ml of the same solvent. After centrifugation the supernatant was added to the supernatant previously collected to the 50 ml amber volumetric flask at 20 °C temperature and made up to volume. Finally the extract was filtered on cellulose acetate (0.45 μm). The samples were used for the LC/DAD analysis.

2.2.2. Instrumental conditions

The measurements were carried out by an Agilent 1100 LC–MSD trap system equipped with an ESI source and consisting of a vacuum degasser, a binary pump, a Peltier autosampler thermostated at 20 °C, and a Peltier column compartment. The ESI parameters were the following:

Feverfew. [ESI(−)]: Dry temp 350 °C, nebulizer 40.0 psi, dry gas 10.00 l/min, HV capillary 3500 V, skimmer −55.4 V, capillary exit −140.2 V, lens 1 4.2 V, lens 2 46.9 V; [ESI(+)] dry temp: 350 °C, nebulizer: 40.0 psi, dry gas: 10.00 l/min, HV capillary: −3500 V, skimmer 55.4 V, capillary exit: −140.2 V, lens 1: −4.2 V, lens 2: −46.9 V. The column used was from Agilent Technologies Inc. (Palo Alto, CA; poroshell RP-18, 3.0 mm × 100 mm, 100 Å, 2.7 μm). The elution was performed with H_20 0.1% HCOOH (solvent A) and MeOH (solvent B). The gradient program used was: 0 min 90% A, 14 min 50% A, and 30 min 5% A. The flow rate was 0.4 ml/min.

SJW. [ESI(−)]: Dry temp 350 °C, nebulizer 50.0 psi, dry gas 10.00 l/min, HV capillary 3516 V, skimmer −33.1 V, capillary exit −205.7 V, lens 1 3.8 V, lens 2 52.8 V. The column used was from Phenomenex (Torrance, CA; Luna RP-8, 4.60 mm × 150 mm, 100 Å, 5 μm). The elution was performed with H_20 0.1% HCOOH (solvent A) and CH_3CN (solvent B). The gradient program used was: 0 min 82% A, 8 min 82% A, 18 min 53% A, 18.10 min 97%, 38 min 97% and 40 min 82%. The flow rate was 0.8 ml/min until

Table 1
Feverfew and St. John's Wort extraction conditions.

Extract	Plant origin	Extraction solvent	Drug/solvent ratio	Extraction temperature
Feverfew flowers	Collected from Tuscany cultivation	EtOH 50°	1/10	50 °C
Feverfew leaves	Collected from Tuscany cultivation	EtOH 50°	1/13	50 °C
St. John's Wort seeds A	Collected from Tuscany cultivation	EtOH 70°	1/10	50 °C
St. John's Wort seeds B	Purchased from the market — Germany	EtOH 70°	1/10	50 °C

18.10 min and 1.2 ml/min until 40 min. The injector volume is 10.00 μl.

2.3. Parthenolide analysis

2.3.1. Sample preparation

The HPLC analysis in leaf or flower feverfew extract was performed according to the European Pharmacopoeia monograph (VI Ed) reported for the feverfew aerial part. Methanol (40 ml) was added to a sample of freeze-dried extract (0.5 g) and the resulting mixture was extracted in a water-bath at 60 °C for 10 min and then filtered. The filter was rinsed with further 40 ml of methanol in a water-bath at 60 °C for 10 min. After filtration the collected solution was evaporated to dryness under reduced pressure. The residue was taken up with methanol and diluted to 20 ml with the same solvent. The resulting solution was diluted 1:2 before filtration on a cellulose acetate filter (0.45 μm).

2.3.2. Instrumental conditions

The measures were carried out by an Agilent 1100 Series LC/UV system consisting of a vacuum degasser, a quaternary pump, a Peltier autosampler thermostated at 20 °C, a Peltier column compartment thermostated at 20 °C, and a UV detector. The column used was from Phenomenex (Torrance, CA; Prodigy RP-18, 250 mm × 4.6 mm, 5 μm) and the elution was performed with H_2O (solvent A) and CH_3CN (solvent B). The gradient program used was: 0 min 60% A: 40% B, 25 min 60% A: 40% B, 25.1 min 40% A: 60% B, and 30 min 40% A: 60% B. The flow rate was 1.0 ml/min. The detector UV/Vis was set at 220 nm. Parthenolide was purchased from Sigma (P0667) and dissolved in methanol 80%. The linearity of the method was found between 0.05 and 0.5 mg/ml. The working solutions were 0.066, 0.13, 0.26, and 0.52 mg/ml. The correlation found was $r = 0.999987$. Each solution was injected three times, the CV was inferior to 1%.

2.4. Hypericin and hyperforin analysis

2.4.1. Sample preparation

The HPLC analysis in seed SJW extract was performed according to the following method. Methanol (25 ml) was added to a sample of freeze-dried extract (0.5 g) and the resulting mixture was extracted protected from the light in an ultrasound bath at 35 °C for 30 min and then filtered in a volumetric flask. The filter was rinsed with further 25 ml of methanol and extracted again in the same conditions. After filtration the collected solutions were brought to 50 ml. The resulting solution was filtrated on a cellulose acetate filter (0.45 μm) and used to perform HPLC analysis.

2.4.2. Hypericin assay instrumental conditions

The measures were carried out by an Agilent 1100 Series LC/UV system consisting of a vacuum degasser, a quaternary pump, a Peltier autosampler thermostated at 20 °C, a Peltier column compartment thermostated at 20 °C, and a UV detector. The column used was from Waters (Milford, MA; Spherisorb ODS2, 250 mm × 4.6 mm, 5 μm) and the elution was performed with SDS 10 mM solution, pH 2.5 controlled with phosphoric acid (solvent A), and CH_3CN (solvent B). The gradient program used was: 0 min 25% A: 75% B, and 15 min 5% A: 95% B. The flow rate was 1.2 ml/min. The detector UV/Vis was set at 590 nm. Hypericin (Sigma-Aldrich, Milan, Italy) was dissolved in methanol/pyridine 18/2. The linearity of the method was found between 0.06 and 0.3 mg/ml. The working solutions were 0.061, 0.122, and 0.244 mg/ml. The correlation found was $r = 0.999966$. Each solution was injected three times; the CV was inferior to 1%.

2.4.3. Hyperforin assay instrumental conditions

The measures were carried out by an Agilent 1100 Series LC/UV system consisting of a vacuum degasser, a quaternary pump, a Peltier autosampler thermostated at 20 °C, a Peltier column compartment thermostated at 20 °C, and a UV detector. The column used was from Waters (Milford, MA; Spherisorb ODS2, 250 mm × 4.6 mm, 5 μm) and the elution was performed with H_2O 0.2% phosphoric acid (solvent A), CH_3CN (solvent B), and CH_3OH (solvent C). The gradient program used was: 0 min 85% A: 15% B: 0% C, 10 min 70% A: 20% B: 10% C, and 20 min 15% A: 75% B: 10% C in isocratic conditions till 45 min. The flow rate was 1.2 ml/min. The detector UV/Vis was set at 270 nm. Hyperforin (Phytolab, Italy) was dissolved in methanol/acetone 99.8/0.2. The linearity of the method was found between 0.125 and 0.5 mg/ml. The working solutions were 0.125, 0.25, and 0.5 mg/ml. The correlation found was $r = 0.999991$. Each solution was injected three times; the CV was inferior to 1%.

2.5. Behavioral testing

Male Sprague–Dawley albino rats (180–200 g) from Harlan (S. Piero al Natisone, Italy) were used. Four rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C. The rats had a 12-h light/dark cycle, light at 7 AM. Animals were habituated to the experimental room and were investigated by observers blinded for treatment of the animals. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.6. Production of diabetic neuropathy

Diabetes was induced by i.p. injection of streptozotocin (STZ; Sigma-Aldrich, Milan, Italy) freshly dissolved in normal saline at a dose of 50 mg/kg, administered the morning after an overnight fast. Three days later glucose was measured by reflectance photometry in tail vein blood and any STZ-treated rats with blood glucose less than 270 mg/dl were rejected from the study. Animals were then group-housed with full access to food and water for 3 weeks and treatments were given as defined below. All the animals were weekly weighted and daily observed during the study. The experiments were carried out on day 21.

2.7. Paw-pressure test

The instrument has a cone-shaped pusher and exerts a force which is applied at a constant rate (32 g per second) on the upper surface of the rat hind paw. The force is continuously monitored by a pointer which moves along a linear scale. The pain threshold is given by the force which first induces struggling from the rat. An arbitrary cut off value of 250 g was adopted. Those rats scoring less than 40 g or over 75 g in the pretest, performed before STZ administration, were rejected (25%). The pain threshold of diabetic animals was measured on day 21 and only hyperalgesic animals were included in the study (50%).

2.8. Rotarod test

The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod was placed at a height of 29 cm from the base and the rod-rotation speed was 4 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s, before and 15, 30, 45 and 60 min after the beginning of the test.

2.9. Drug administration

The nociceptive threshold was evaluated 15, 30, 45 and 60 min after the beginning of the test. SJW and feverfew extracts, and Remotive (standardized SJW dried extract containing 3% hyperforin and 0.32% hypericins) (Aboca SpA, Sansepolcro, Italy) were p.o. administered 30 min before the beginning of behavioral tests, in correspondence to their maximum effect as determined by time-course experiments.

Carbamazepine (20 mg/kg p.o.), lamotrigine (200 mg/kg p.o.), phenobarbital (20 mg/kg p.o.) (Sigma, Milan, Italy) and L-acetyl-levocarnitine (ALCAR, Sigma-Tau, Pomezia, Italy) were used as antinociceptive reference drugs and employed at the maximal doses unable to reduce spontaneous mobility. ALCAR was administered at the dose of 30 or 100 mg/kg i.p. twice daily for 14 days. Following reference drug administration, the pain threshold was evaluated 30, 60, and 90 min after injection.

2.10. Statistical analysis

The data used in statistical analysis was acquired from eight animals for each group. All experimental results are given as the mean ± S.E.M. One-way and two-way analyses of variance, followed by Tukey and Bonferroni post hoc test, respectively, were used for statistical analysis.

3. Results and discussion

3.1. Phytochemical analysis

3.1.1. Characterization of the SJW extracts: hypericin, hyperforin content and fingerprint profile

The SJW seed extracts were evaluated by means of LC–UV and LC–MS analyses. In Fig. 1 the relative amounts of hypericins and hyperforins of the two seed extracts are reported. The SJW seed A sample contains much more hypericins and hyperforins than the SJW seed B sample. The hypericin quantitative data was referred to the sum of hypericin and pseudohypericin, the hyperforin quantitative data was the sum of hyperforin and adhyperforin.

The fingerprint profile of SJW seed extracts was analyzed using a RP-HPLC–MS method and TIC chromatograms were recorded (Fig. 1). This analysis revealed quite similar metabolic patterns for the SJW seed A (Fig. 1A) and SJW seed B (Fig. 1B) extracts, with differences likely caused by the abundance of the different phytochemical species. The chromatogram reported in Fig. 1 also confirms the hypothesis that SJW seed extract A is quite similar to SJW flowerhead extracts (Fig. 1C), being present most of the chemical species characteristic of SJW flowerhead extracts. An additional fingerprint profile in Fig. 1D is reported to capture visible profile at 590 nm characteristic of hypericins, as it was not possible to see them on the TIC chromatogram. The chromatographic profile confirms the quantitative data.

The use of a SJW seed extract seems intriguing, as it should contain in potency all the metabolites expressed by the mature plant individuals.

3.1.2. Characterization of the feverfew extracts: parthenolide content and fingerprint profile

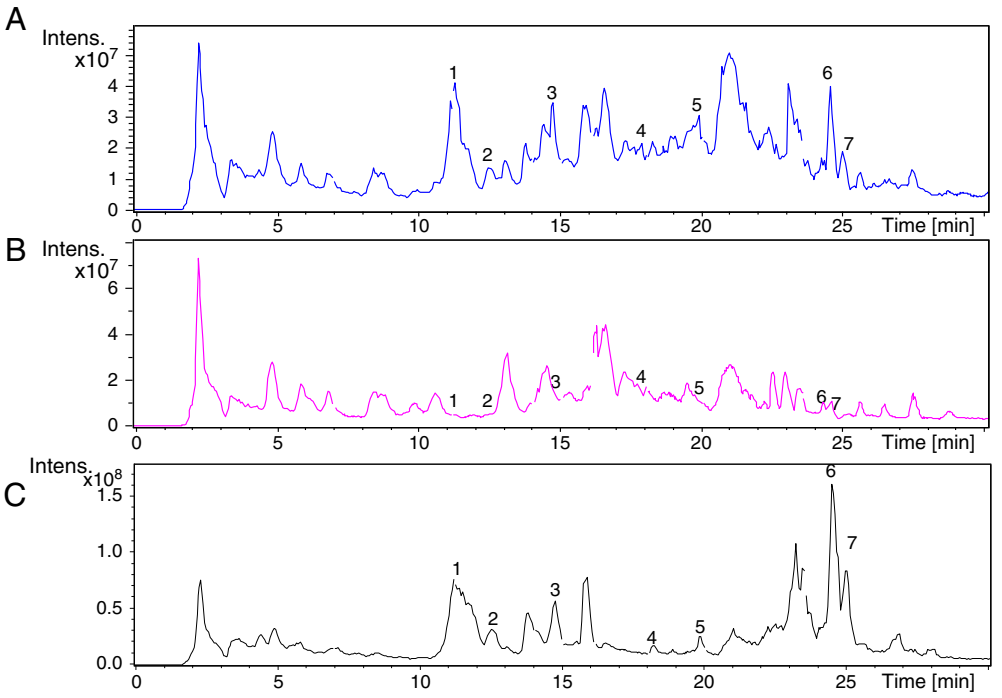
The parthenolide content and molecular fingerprint profile of our feverfew extracts were evaluated by means of LC–UV and LC–MS analyses. In Fig. 2 the relative amounts of parthenolide of the two feverfew extracts are reported. The flower extract contained much more parthenolide than the leaf extract.

The fingerprint profile of feverfew extracts was analyzed using the RP-HPLC–MS method and recording TIC chromatograms (Fig. 2). The compound identification was done by means of MS/MS studies, comparison with internal MS library and data reported in literature [13]. The analysis revealed quite similar metabolic patterns for the two extracts, with differences likely caused by the abundance of the different phytochemical species. All the chromatograms reported confirm that feverfew flower (Fig. 2A) and feverfew leaf (Fig. 2B) extracts differ not only for the parthenolide content but also for the amount of the compounds 3 and 4. Feverfew flower extract, even if endowed with a similar profile to the leaf extract, differs in the abundance of important constituents being enriched of the active constituent parthenolide and of compound 3 with retention time of 14.5 min (tri-caffeoylquinic acid).

The chemical structure of SJW and feverfew main constituents is reported in Fig. 3.

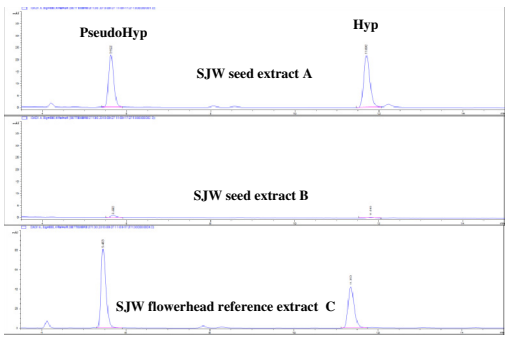
3.2. Antihyperalgesic activity of SJW dried extract

Peripheral administration of STZ was used as a rat model of painful diabetic neuropathy. In the paw pressure test, obtained data clearly demonstrated a significant decrease in the mechanical pain threshold of STZ-diabetic rats indicating the occurrence of hyperalgesia. These findings are in agreement with previous papers reporting on the mechanical hyperalgesia of animals at early stages of experimental diabetes [20,21]. A single oral administration of SJW seed extract A dose-dependently (20–200 mg/kg) reversed the mechanical hyperalgesia (Fig. 4A). The dose of 20 mg/kg was



Number	RT		MS[1]	MS[2]			Compound	Comparison of relative abundance		
		Ion Polarity	Parention	BasePeack	Secondary peack			SJW seed extract A	SJW seed extract B	SJW flowerhead reference extract C
	min		m/z	m/z	m/z	m/z				
1	11,2	neg	462,8 [M-H]$^-$	300,7			Hyperoside	+++	+	+++
2	12,6	neg	476,8 [M-H]$^-$	300,8			Quercetinglucoronide	+++	+	+++
3	14,7	neg	446,8 [M-H]$^-$	300,8			Quercitroside	+++	+	+++
4	18,3	neg	300,9 [M-H]$^-$	178,6	150,7		Quercetin	++	+	+++
5	19,8	neg	536,9 [M-H]$^-$	442,8	384,8	299,6	Biapigenin	++	+	+++
6	24,5	neg	535,6 [M-H]$^-$	466,0			Hyperforin	+++	+	+++
7	24,9	neg	549,6 [M-H]$^-$	480,1			Adhyperforin	+++	+	+++

D

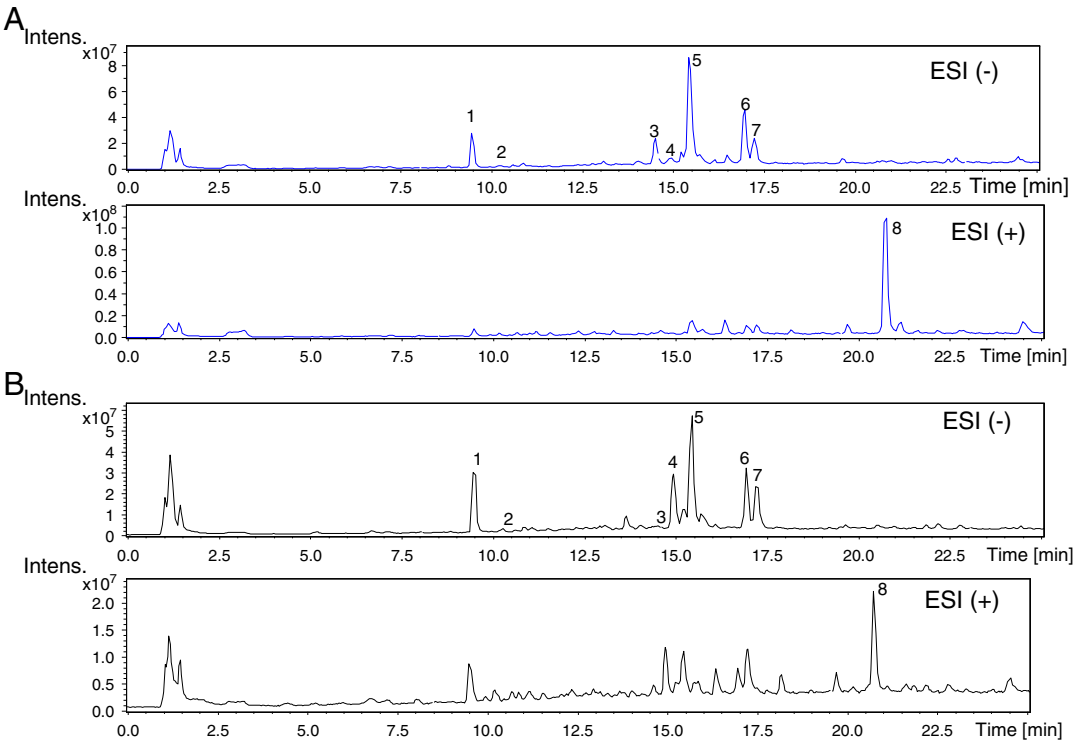


RT (min)	Compound
5,6 (±0,2)	PseudoHypericin
11,6 (±0,3)	Hypericin

Sample	% Hypericins (p/p)	% Hyperforins (p/p)
SJW seed extract A	**0.11**	**0.6**
SJW seed extract B	**0.001**	**<LDQ (0,01)**

Fig. 1. LC–MS fingerprint chromatograms (TIC) for SJW seed extracts A (A) and B (B); Main constituents and corresponding MS data are shown. Hypericin and hyperforin contents were reported.

devoid of any effect; at 60 mg/kg SJW significantly increased the rat pain threshold peaking at 120 mg/kg. At higher concentrations, the antihyperalgesic effect diminished showing a bell-shaped trend (Fig. 4A). Time course experiments showed the prolonged effect of SJW, being effective for 60 min (Fig. 4B). Conversely, SJW seed extract B was ineffective



Number	Rt.	Ion Polarity	MS[1]	MS[2]		Compound		
			Parent ion	Base Peack	Secondary Peack		TP flower extract	TP leaf extract
	min		m/z	m/z	m/z			
1	9,5	neg	352,9 $[M-H]^-$	190,7	178,7	Chlorogenic Acid isomer	+++	+++
2	10,3	neg	352,9 $[M-H]^-$	190,8	178,7	Chlorogenic Acid isomer	+++	+++
3	14,5	neg	677,0 $[M-H]^-$	514,9	352,9	tri-CaffeoylQuiniq Acid	+++	+
4	14,8	neg	621,1 $[M-H]^-$	351,1	269,2	Apigenin diglucuronide isomer	+	+++
5	15,4	neg	514,9 $[M-H]^-$	352,9		di-CaffeoylQuinic Acid isomer	+++	+++
6	17,0	neg	514,9 $[M-H]^-$	352,9		di-CaffeoylQuinic Acid isomer	+++	+++
7	17,1	neg	444,8 $[M-H]^-$	268,8	174,7	Apigenin glucuronide isomer	+++	+++
8	21,8	pos	519,0 $[2M+Na]^+$	271,3 $[M+Na]^+$		Parthenolide	+++	+

Sample	**% parthenolide (p/p)**
Feverfew flower extract	**2.5**
Feverfew leaf extract	**0.38**

Fig. 2. LC–MS fingerprint chromatograms (TIC) for feverfew flower (A) and leaf (B) extracts. Main constituents and corresponding MS data are shown. Parthenolide content was indicated.

(Fig. 4C, D). Our results show the ability of SJW to relieve neuropathic pain confirming our previous findings indicating the improving effect of SJW on nerve injury- and chemotherapy-induced neuropathic pain models [12] and extending the SJW antihyperalgesic efficacy to the diabetic neuropathy.

The pharmacological efficacy of SJW in humans and laboratory animals has been demonstrated by using SJW dried extracts obtained from the aerial portion of the plant. The activity of the seed extract A was, therefore, compared to that exerted by a SJW aerial portion extract, Remotive, that showed a partial reversal of mechanical hyperalgesia (Fig. 4E) with a short-lasting effect (Fig. 4F). Our data indicate that SJW seed extract A is a more effective than seed extract B and Remotive in our experimental model, which clarifies that not all extracts from the same plant species exert the same pharmacological profile.

Antihyperalgesic effects of SJW on diabetic animals may be related to the antidiabetic activity of the herbal drug. SJW has been reported for its traditional use by diabetic patients

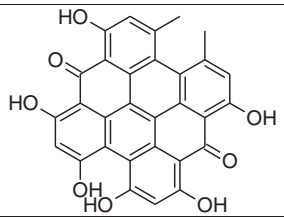
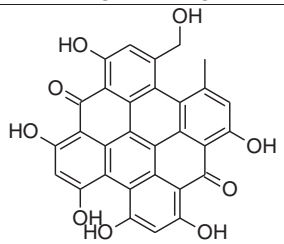
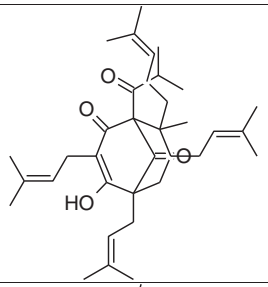
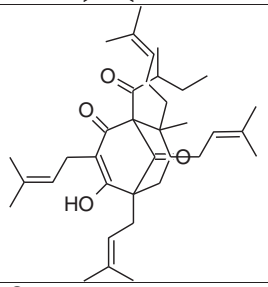
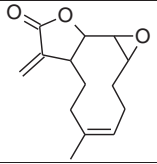
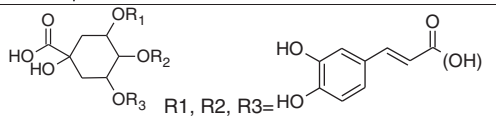
Compound	Chemical structure
Hypericin	
Pseudohypericin	
Hyperforin	
Adhyperforin	
Parthenolide	
Caffeoyl Quinic Acid Derivatives Chlorogenic acid: R1 = caffeoyl, R2 = R3 = H 4,5-dicaffeoylquinic acid: R1 = H, R2 = R3 = caffeoyl 3,5-dicaffeoylquinic acid: R1 = R2 = caffeoyl, R3 = H 3,4,5-tricaffeoylquinic acid: R1 = R2 = R3 = caffeoyl	

Fig. 3. Chemical structure of SJW and feverfew main components.

and animal studies showed that repeated administration of SJW for fourteen days induced antihyperglycemic effects on STZ-diabetic rats [22,23]. On the other hand, restoration of mechanical hyperalgesia observed in the present study was obtained after a single administration of the extract, suggesting that the improvement of painful DPN may probably be related to a direct antinociceptive activity of SJW.

The phytochemical composition of each extract was analyzed in order to identify the compounds involved in the antinociceptive activity. The SJW seed extracts investigated

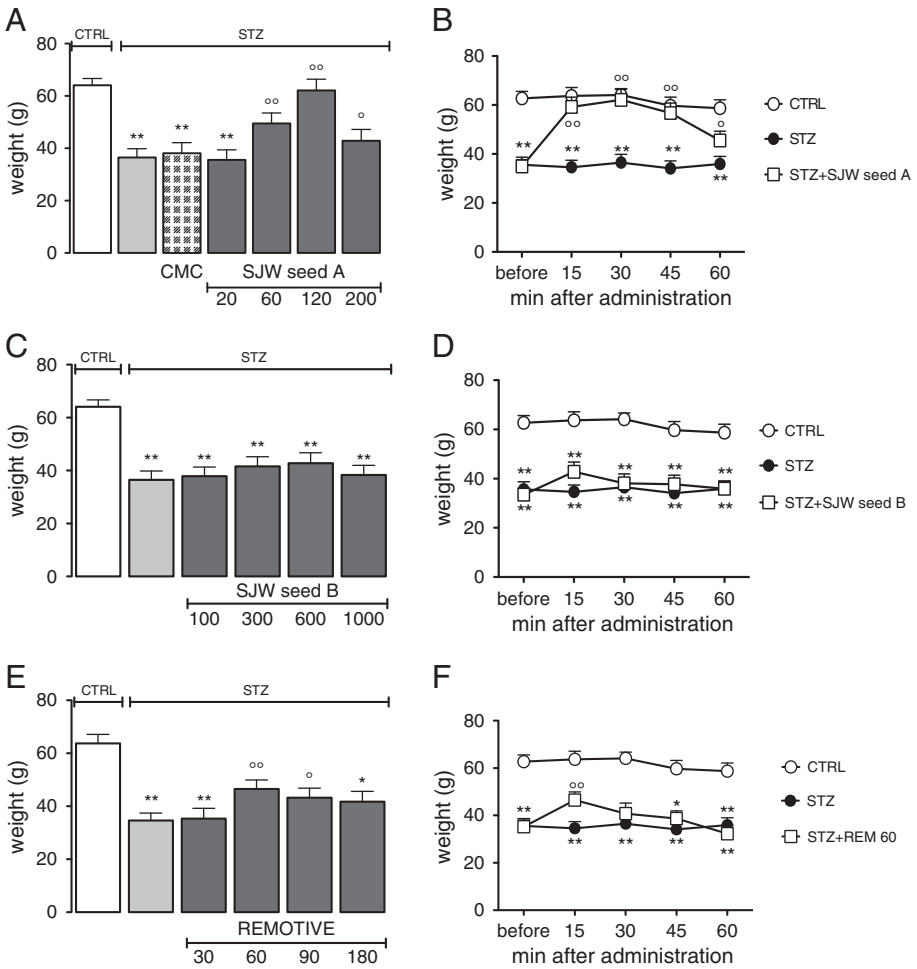


Fig. 4. Effect of SJW seed extracts on mechanical hyperalgesia in STZ-diabetic rats. (A) Dose–response (20–200 mg/kg) and (B) time-course (30 mg/kg) curves for the antihyperalgesic effect of SJW dried extract A. (C) Dose–response (100–1000 mg/kg) and (D) time-course (600 mg/kg) curves for SJW dried extract B. (E) Dose–response (30–180 mg/kg) and (F) time-course (60 mg/kg) curves for the antihyperalgesic effect of Remotive A. *P < 0.05, **P < 0.01 vs CTRL group; °P < 0.05, °°P < 0.01 versus STZ-treated rats.

differed for the pharmacological profile being the extract A antihyperalgesic and the extract B ineffective. Both extracts showed a similar phytochemical profile, even if the active constituents were more abundant in the extract A. In particular, hyperforin was absent in the extract B and hypericin was present at a concentration 100 times lower than that found in the extract A. We have previously demonstrated that both hyperforin and hypericin induced antinociception [11] and antihypernociception [12]. We can, hence, hypothesize that the antihyperalgesic activity of the extract A might be related to the presence of both phytochemicals. However Remotive only partially reduced mechanical hyperalgesia. By comparing the phytochemical compositions at the highest effective doses, Remotive showed a slightly lower concentration of hypericin than extract A and a 2 times higher hyperforin concentration. On these bases we might propose a prominent role of hypericin in the antihyperalgesic activity of the SJW seed extract.

Administration of drugs by oral gavage can be a stressful procedure for the animal and currently it is not possible to critically evaluate the extent to which some of the drug effects observed might be due to stress-induced analgesia associated with handling and injection of rats. An orally administered vehicle group (CMC) was, therefore, included in the study. A single p.o. administration of CMC did not alter the pain threshold in comparison with the pre-treatment values and did not show any antihyperalgesic activity in the STZ-treated group (Fig. 4A).

3.3. Antihyperalgesic activity of feverfew dried extract

A single oral administration of the feverfew flower extract prolonged the reduced response of diabetic animals, meaning partial restoration of mechanical hyperalgesia. Feverfew flower extract showed antihyperalgesic activity in the STZ model, even if to a lesser extent to the SJW seed extract A. At the dose of 1000 mg/kg partially reversed the mechanical

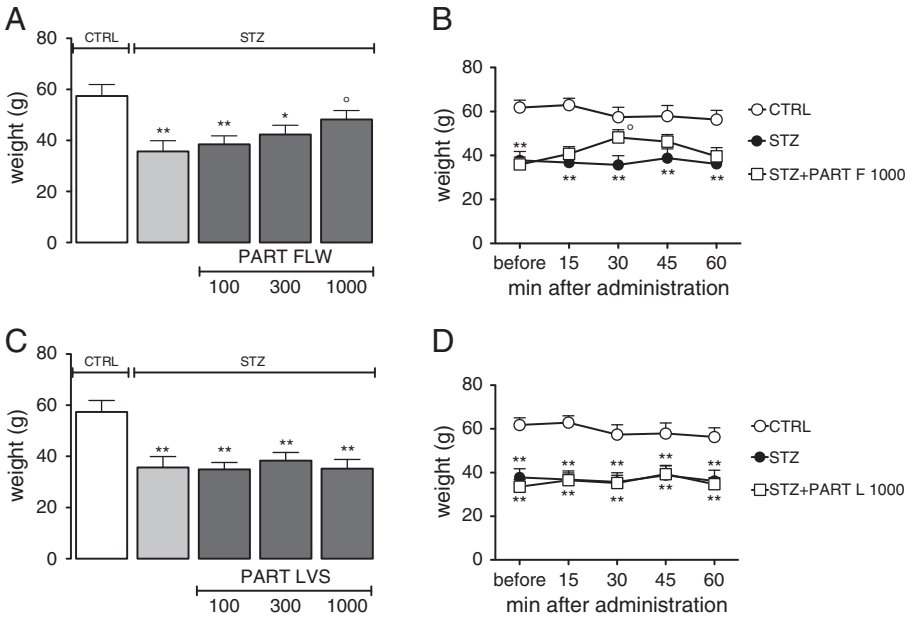


Fig. 5. Effect of feverfew extracts on mechanical hyperalgesia in STZ-diabetic rats. (A) Dose–response (100–1000 mg/kg) and (B) time-course (1000 mg/kg) curves for the antihyperalgesic effect of feverfew flower extract. (C) Dose–response (100–1000 mg/kg) and (D) time-course (1000 mg/kg) curves for feverfew leaf extract. *P < 0.05, **P < 0.01 versus control group; °P < 0.05 versus STZ-treated rats.

hyperalgesia in STZ-treated rats, whereas at lower concentration was devoid of any effect (Fig. 5A). Time course studies showed a short-lasting antihyperalgesic activity of the extract (Fig. 5B). Conversely, the feverfew leave dried extract was ineffective (Fig. 5C, D). Feverfew has been widely used in indigenous medical practices for curing high fever, headache, stomachache, toothache, rheumatoid arthritis, menstrual irregularities, and other inflammatory diseases. The anti-inflammatory properties of this herbal plant are related to the NF-κB inhibiting activity of its main component, parthenolide [24]. Feverfew also showed benefits in migraine prophylaxis [17]. Here we extended the therapeutic applications of this herbal drug providing the first evidence for a neuropathic pain relieving activity.

Similarly to the SJW seed extracts, also feverfew flower and leaf extracts showed a similar phytochemical profile, but the flower extract differed in the abundance of the main constituent, being enriched of parthenolide. Since the leaf extract was ineffective, it appears that the presence of higher concentrations of parthenolide is necessary to improve nociception in STZ-diabetic rats.

3.4. Effect of anticonvulsant and neuroprotective drugs on mechanical nociception

Anticonvulsant drugs (lamotrigine, carbamazepine) and neuroprotective agents (ALCAR) are clinically used in the diabetic neuropathy [25,26]. Carbamazepine showed the highest efficacy since it was able to completely reverse the mechanical hypernociception induced by STZ administration. Lamotrigine partially antagonized nociceptive behavior. Phenobarbital, a non-analgesic anticonvulsant drug used as negative

Table 2
Effect of carbamazepine, lamotrigine, and phenobarbital on hyperalgesia induced by STZ in the rat paw-pressure test.

Pre-treatment	Treatment (mg/kg p.o.)	Mechanical threshold (g)			
		Before treatment	After treatment		
			30 min	60 min	90 min
Saline	Saline	62.3 ± 4.0	65.4 ± 5.5	62.2 ± 4.4	64.9 ± 5.1
STZ	Saline	35.7 ± 4.3	33.6 ± 4.7	34.1 ± 5.1	30.8 ± 4.1
Saline	Carbamazepine 20	61.6 ± 3.7	60.5 ± 4.9	60.8 ± 4.3	62.6 ± 4.9
STZ	Carbamazepine 20	32.9 ± 4.2	58.5 ± 5.3*	54.6 ± 6.4*	39.4 ± 4.4
Saline	Lamotrigine 200	63.8 ± 4.5	60.6 ± 4.3	63.3 ± 6.0	58.0 ± 5.6
STZ	Lamotrigine 200	31.5 ± 5.0	50.3 ± 4.5*	49.3 ± 5.2*	38.4 ± 4.0
Saline	Phenobarbital 20	59.7 ± 4.3	58.4 ± 6.3	66.2 ± 6.1	62.5 ± 5.0
STZ	Phenobarbital 20	31.7 ± 3.5	30.7 ± 4.7	35.7 ± 4.5	37.3 ± 5.3

* P < 0.05 versus STZ-treated rats.

Table 3
Effect of ALCAR on hyperalgesia induced by STZ in the rat paw-pressure test.

Pre-treatment	Treatment (mg/kg i.p.)	Mechanical threshold (g)			
		Before the last injection of ALCAR	After the last injection of ALCAR		
			30 min	45 min	60 min
Saline	Saline	60.4 ± 4.4	65.5 ± 5.7	61.3 ± 4.9	64.5 ± 5.1
STZ	Saline	41.1 ± 4.8	39.6 ± 6.2	36.4 ± 4.3	37.6 ± 4.9
Saline	ALCAR 30	69.3 ± 5.0	72.8 ± 4.5	70.5 ± 3.7	68.9 ± 5.0
STZ	ALCAR 30	42.4 ± 4.3	43.6 ± 5.4	39.3 ± 4.6	38.7 ± 5.5
Saline	ALCAR 100	147.3 ± 6.2§	151.8 ± 6.6§	155.0 ± 7.4§	148.7 ± 7.1§
STZ	ALCAR 100	69.4 ± 4.5*	72.9 ± 5.5*	77.5 ± 6.3*	73.9 ± 6.4*

ALCAR was administered twice daily for 14 days.
* $P < 0.05$ versus STZ-treated rats.
§ $P < 0.05$ versus saline-treated rats.

control, was ineffective. Anticonvulsants were all unable to increase the pain threshold in non-diabetic control mice (Table 2).

ALCAR showed antihyperalgesic efficacy at a dose (100 mg/kg) able to increase the pain threshold in saline-treated control animals. Conversely, at a non-analgesic lower dose (30 mg/kg) was devoid of any effect (Table 3).

The intensity of the antihyperalgesic effect of the seed extract A was comparable to that produced by drugs clinically used in the treatment of painful DPN, such as the anticonvulsant drug carbamazepine and the neuroprotective agent ALCAR. While the flower extract showed a lower efficacy than the SJW seed extract, the intensity of the feverfew effect was comparable to that produced by lamotrigine.

3.5. Lack of effect of SJW and feverfew extracts on rat locomotor behavior

An important drawback of the current analgesic therapy of DPN is the high occurrence of side effects. The potential induction of side effects by SJW and feverfew extracts was, therefore, investigated. Both herbal drugs did not show any alteration of the rats' gross behavior. Furthermore, the SJW and feverfew extracts were tested, at the highest effective concentrations, to assess their effect on motor coordination by use of the rotarod test. The number of falls from the rotating rod of rats orally pretreated with the above-mentioned extracts was comparable to the control group, showing the lack of any motor impairment. Each group progressively reduced the number of falls because mice learned how to balance on the rotating rod (Table 4). Present results are in accordance with literature data that indicate SJW [27] and feverfew [28] as medicinal plants with a favorable tolerability and safety profile.

Table 4
Lack of locomotor impairment by SJW seed A and feverfew flower extracts in the rotarod test.

Treatment	Number of falls in 30 s				
	Before treatment	After treatment			
		15 min	30 min	45 min	60 min
Saline	5.9 ± 0.6	4.5 ± 0.5	2.1 ± 0.5	2.0 ± 0.5	1.3 ± 0.6
SJW seed A	5.5 ± 0.3	4.2 ± 0.7	2.7 ± 0.5	2.2 ± 0.5	1.5 ± 0.3
Feverfew FLW	6.0 ± 0.5	4.7 ± 0.7	2.5 ± 0.5	2.2 ± 0.5	1.9 ± 0.4

4. Conclusion

Present results exhibited for the first time the capability of a single oral administration of a SJW seed extract and feverfew flower extract for improvement of the mechanical hyperalgesia developed in STZ-diabetic rats. These herbal drugs showed a good efficacy along with a favorable safety profile. These findings suggest these plants as a new drug candidate/source for the treatment of diabetic pain.

Conflicts of interest

All authors have no conflict of interest to disclose.

Acknowledgment

This work was supported by grants from MIUR and Aboca S.p.A.

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