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A Prolonged Protein Kinase C-Mediated, Opioid-Related Antinociceptive Effect of St John's Wort in Mice

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Abstract: **The antinociceptive profile of St. John's Wort (SJW) was investigated in mice in a condition of acute thermal and chemical pain, together with the mechanism that might underlie this effect. A dried extract of SJW induced a prolonged antinociception that persisted for 120 minutes after administration. The thermal antinociception was prevented by naloxone and by the protein kinase C (PKC) activator PMA, whereas the chemical antinociception was prevented by PMA, remaining naloxone insensitive. A chloroform (CHL) and a methanol (MET) fraction, obtained to investigate the involvement of the SJW main components, hyperforin and hypericin/flavonoid, respectively, increased pain threshold with a time course comparable to the dried extract. The CHL antinociception was prevented by naloxone, whereas the MET antinociception was antagonized by PMA. Purified hyperforin and hypericin showed an antinociceptive efficacy comparable to CHL and MET, respectively. Conversely, flavonoids were devoid of any effect. The administration of yohimbine and atropine did not modify SJW, CHL and MET antinociception. These results indicate that both CHL and MET fractions mediate the SJW-induced antinociception. In particular, the presence of hypericin was fundamental to induce both thermal and chemical antinociception through the inhibition of the PKC activity, whereas hyperforin selectively produced a thermal opioid antinociception.**

***Perspective:** This article presents evidence of a persistent thermal and chemical antinociception of SJW that is mainly mediated by PKC-inhibiting mechanisms. These findings identify important targets for a longer-acting activation of endogenous pain systems and should potentially help clinicians who seek safe, tolerable, and prolonged treatments for pain relief.*

Key words: *Hypericum perforatum, St. John's Wort, analgesia, antinociception, hypericin, hyperforin.*

Hypericum perforatum L., popularly called St. John's Wort (SJW), is a herbaceous perennial plant long known for its putative medicinal properties. It has been used, topically, in popular medicine since ancient times for the treatment of skin wounds, eczema, burns and inflammation.[2,6] In 1525, the Swiss physician Paracelsus established its use for the treatment of psychiatric disorders; since then, it has been used in traditional European medicine to treat neuralgia, anxiety, neurosis, and depression.[5] Although clinical trials have given contradictory results, alcoholic extracts of SJW are presently used mainly for the treatment of mild-to-moderate forms of depression as an alternative to classic antidepressants, with a favorable side-effect profile.[21]

The most common SJW preparations used are hydroalcoholic extracts of the aerial portion of the plant. These contain at least 10 different kinds of biochemical compounds: flavonoids (including rutin, hyperoside, quercetin, and quercitrin), naphtodianthrones (including hypericin and pseudohypericin), acylphloroglucinols (including hyperforin and adhyperforin), proanthocyanidins, procyanidines, tannins, essential oils, amino acids, phenylpropanes, xantones, and other hydrosoluble compounds (organic acids, peptides, and polysaccharides).[15] The first 3 groups are the most abundant ones and the agents mainly responsible for the described traditional use of the herbaceous plant. Hypericin and hyperforin have been isolated in only a few other plants. Total

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hypericins usually amount to about .3% of hydroalcoholic extracts and are the most potent natural photosensitizers so far described, with potent application for antitumoral photodynamic therapy.[1] Attention has recently increasingly been paid to hyperforin (1–5% in the SJW extract) as the main active component with antidepressant effect, but also endowed with other pharmacological effects including antinflammatory and proinflammatory effects, antibacterial, antitumoral, and antiangiogenetic properties.[23] Flavonoids account for about 2 to 4% of the hydroalcoholic extract with a wide distribution in many plants.

Pharmacological studies on SJW have focused mainly on its antidepressant activity. However, some studies have documented other bioactivities produced by this herbal plant. In particular, its anti-inflammatory activity following not only topical,[30] but also systemic, administration has been examined.[22] SJW inhibits some events involved in the inflammatory reaction such as the expression of cyclo-oxygenase-2,[22] prostaglandin E2,[17] inducible nitric-oxide synthase,[34] and interleukin 6.[12]

These reports hypothesize a role of SJW in the modulation of pain perception, supported by a recent paper that indicates the antinociceptive effect of high SJW concentrations in the formalin test.[35] Thus, the aim of this study was to characterize the antinociceptive profile of SJW with the mechanism that might underlie this effect in a condition of acute thermal (hot-plate test) and chemical (abdominal-constriction test) pain. The role of the SJW main components—hypericin, hyperforin, and flavonoids—in the modulation of the pain threshold was also investigated since, at present, very little is known on this aspect.

Methods

Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. Ten mice were housed per cage. The cages were placed in the experimental room 24 hours before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 $\pm$ 1°C with a 12-hour light/dark cycle, with light at 7 am. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Hot-Plate Test

The hot-plate test was performed as previously described.[13] Mice were placed inside a stainless-steel container, which was set thermostatically at 52.5 $\pm$ .1°C in a precision water bath (KW Mechanical Workshop, Siena, Italy). Reaction times (in seconds) were measured with a stopwatch before and 30, 60, 90, 120, 150 and 180 minutes after administration of the drug . The endpoint used was the licking of the fore or hind paws. Those mice scoring less than 12 and more than 18 seconds in the pretest were rejected (30%). An arbitrary cutoff time of 45 seconds was adopted. 12 mice per group were used.

Abdominal-Constriction Test

Mice were intraperitoneally (ip) injected with a .6% solution of acetic acid (10 mL kg^{-1}). The number of stretching movements was counted for 10 minutes, starting 5 minutes after acetic acid injection. 12 mice per group were used.

Rota-Rod Test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a nonslippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 seconds. Those mice scoring less than 3 and more than 6 falls in the pretest were rejected (20%). The number of falls was measured before (pretest) and 60, 90, and 120 min after administration of SJW, CHL and MET. 10 mice per group were used.

Hole-Board Test

The hole board test consisted of a 40-cm square plane with 16 flush-mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the center of the board 1 by 1 and allowed to move about freely for a period of 5 minutes each. Two photobeams, crossing the plane from midpoint to mid point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signaled the movement of the animal (counts in 5 minutes) on the surface of the plane (spontaneous motility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 minutes) the exploration of the holes (exploratory activity) by the mice. The test was performed 90 minutes after administration of SJW, CHL and MET. 10 mice per group were used.

Icv Injection Technique

Icv administration was performed under ether anaesthesia with isotonic saline as solvent as previously described.[13] During anaesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (.4 mm external diameter) attached to a 10-μL syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where a 5-μL solution was then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 5 μL of diluted 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of

the injection technique was evaluated with 95% of injections being correct.

HPLC-DAD and HPLC-MS Drug Analyses

To evaluate the constituents' content, an HPLC analysis was performed using a method described in the literature,[7] modified for our experimental necessities. The identification of the constituents was performed using combined HPLC-diode array detection (DAD) analysis and HPLC-thermospray mass spectrometry. The quantification of the constituents was performed using rutin as an external standard and consideration of each constituent and the relative response factor (RRF) with respect to the rutin, as previously reported.[7] All the samples were analyzed in triplicate and a calibration graph with 6 data points of external standard was used.

The detection limit (LoD) and the quantitatiton limit (LoQ) for rutin was determined by calculation of the signal-to-noise ratio. A 3:1 signal-to-noise ratio is generally considered acceptable for estimating the detection limit. The sample that produces a signal-to-noise ratio of approximately 10:1 corresponds to the concentration at which the analyte can be reliably quantified. The LoQ resulted in 15 ng and the LoD was 6 ng.

The HPLC system consisted of an HP 1100L instrument with a diode array detector and managed by an HP Chem workstation (Agilent Technologies, Palo Alto, CA). The reverse-phase column was a 201 TP 54 (5 ìm, 250 mm, .5 mm id, 300 A°; Vydac Separation Group, Hesperia, CA) maintained at 26°C. The mobile phase was a 5-step linear solvent gradient CH3CN/CH3OH/H2O (pH 3.2, HCOOH) over a 60-minute period at a flow rate of 1 mL/minute, previously reported in the literature.[3] The injected volume of sample was a 20-μL solution. UV–vis spectra were recorded in the range 200 to 590 nm, and chromatograms were acquired at 230, 254, 270, 350, and 590 nm.

The HPLC system was interfaced with an HP 1100 MSD APIelectrospray (Agilent Technologies). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of the HPLC-DAD analysis. The same column, mobile phase, time period, and flow rate were used.

Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values: gas temperature 350°C at a flow rate of 10 L/minute; nebulizer pressure 30 psi; quadrupole temperature 30°C; and capillary voltage 3,500 V. Full-scan spectra from m/z 100 to 800 in the positive ion mode were obtained (scan time 1 second).

Preparation of Chloroform and Methanol Fractions

A solution of dried extract (1 g) in 10 mL of chloroform was stirred for 3 hours in the dark. The mixture was filtered and the solution was evaporated to obtain a residue of 200 mg, containing the pholoroglucinols as confirmed by HPLC-DAD-ESI-MS analysis.

The residue present on the filter was washed with 10 mL of methanol. The methanol solution was evaporated and a residue of 700 mg was obtained. The HPLC-DAD-ESI-MS analysis showed in this fraction only the presence of flavonoids and naphtodianthrones.

Drugs

Indena Research Laboratories (Settala, Milan, Italy) offered a commercial sample of *Hypericum perforatum* dried extract –Lotto 28662/M1–B.A. 84204 and kindly provided the reference rutin trihydrate (batch no. K12408717, standard purity 96.25% considering the content of residual solvents, moisture and amount of impurities).

The following drugs were used: naloxone hydrochloride, atropine sulfate, yohimbine hydrochloride, benzyliden-naltrexone (BNTX), nor-binaltorphimine dihydrochloride (nor-BNI), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP), hypericin, hyperforin, hyperoside, quercetin (Sigma, Milan, Italy); phorbol-12-myristate-13-acetate (PMA), (Calbiochem, Milan, Italy); D- amphetamine (De Angeli, Rome, Italy), carboxymethylcellulose sodium salt, (CMC) (Fluka Chemie GmbH, Steinheim, Germany). Other chemicals were of the highest quality commercially available.

SJW dried extract, chloroform fraction (CHL), methanol fraction (MET), hypericin, hyperforin, quercetin, and hyperoside were dissolved in a 1% CMC solution immediately before use and administered by gavage. All other drugs were dissolved in isotonic (NaCl .9%) saline solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 10 mL kg^{-1} by intraperitoneal (ip), subcutaneous (sc), per os (po) injection, or in a volume of 5 μL by intracerebroventricular (icv) injection.

Drug Administration

SJW dried extract, CHL and MET fractions, hypericin, hyperforin, quercetin and hyperoside were administered 90 minutes before the hot-plate and abdominal-constriction tests, in correspondence to their maximum effect as determined by time-course experiments.

Hot plate hypericin .12 mg $kg,^{-1}$ hyperforin 1.956 mg $kg,^{-1}$ quercetin .498 mg $kg,^{-1}$ and hyperoside 3.81 mg kg^{-1} were orally administered 90 minutes before the test. These doses correspond to the percentages present in a 60 mg kg^{-1}-preparation of SJW dried extract. CHL and MET fractions were administered in a concentration containing the above-mentioned content of their main components, hyperforin and hypericin, respectively.

Abdominal constriction test: hypericin .06 mg $kg,^{-1}$ hyperforin .978 mg $kg,^{-1}$ quercetin .249 mg $kg,^{-1}$ and hyperoside 1.905 mg kg^{-1} were orally administered 90 minutes before the test. These doses correspond to the percentages present in a 30 mg kg^{-1}-preparation of SJW dried extract. CHL and MET fractions were administered in a concentration containing the above-mentioned content of their main components, hyperforin and hypericin, respectively.

Naloxone (1 mg kg^{-1} ip), atropine (5 mg k^{-1} ip), yohimbine (3 mg kg^{-1} ip), CTOP (.5 μg per mouse icv), naltriben (19 μg per mouse icv), BNTX (35 μg per mouse icv), nor-BNI (.735 μg per mouse icv) were injected 70 minutes after SJW, CHL or MET administration. PMA (15 pmol per mouse icv) was injected 30 minutes after SJW, CHL or MET administration. D-amphetamine (2 mg kg^{-1} sc), was administered 15 minutes before the hole-board test. Doses and administration schedule were chosen on the basis of time-course and dose-response experiments previously performed in our laboratory.

Statistical Analysis

All experimental results are given as the mean ±S.E.M. An analysis of variance ANOVA, followed by Fisher's Protected Least Significant Difference procedure for post hoc comparison, were used to verify significance between 2 means. *P* values of less than .05 were considered significant.

Results

The main components of SJW dried extract were flavonoids (12.72%), phloroglucinols (4.23%) and naphtodianthrones (0.32%), as reported in Table 1. In order to investigate the role of these components in the mechanism of action of SJW, a chloroform and a methanol fraction were obtained from the dried extract. The chloroform fraction (CHL) contained phloroglucinols whereas the methanol fraction (MET) showed the presence of naphtodiantrones and flavonoids representing a hypericin and flavonoid rich fraction (Table 1).

Table 1. Composition of the SJW Dried Extract, CHL, and MET Fractions

	Content % (mg/100 mg)		
Constituent	SJW	CHL	MET
Flavonoids			
Rutin	4.28		
Hyperoside	6.35		
Isoquercitrin	0.61		
Rutin, Hyperoside, Isoquercitrin		n.d.	9.29
Quercitrin	0.65	n.d.	0.69
Quercetin	0.83	n.d.	0.42
I3, II8-Biapigenin	0.62	0.29	0.25
Total flavonoids	12.72		
Phloroglucinols			
Oxihyperforin		1.16	n.d.
Furohyperforin		1.21	n.d.
Hyperforin		12.49	n.d.
Adhyperforin		1.34	n.d.
Total Phloroglucinols	4.23		
Naphtodianthrones			
Pseudohypericin		0.04	0.11
Hypericin		0.01	0.20
Total naphtodianthrones	0.32		

Abbreviations: SJW, St. John's Wort; CHL, chloroform fraction; MET, methanol fraction.
NOTE. The content of each constituent is expressed as percentage (mg/100 mg).

Fig 1 shows the HPLC profiles of *Hypericum perforatum* L. dried extract, at 270 nm (Fig 1A) and 590 nm (Fig 1B).

A dried extract of SJW showed a dose-dependent (1 to 100 mg kg^{-1} po) antinociceptive activity against a thermal stimulus in the mouse hot-plate test. The dose of 1 mg kg^{-1} po was devoid of any effect. At 10 mg kg^{-1} po, SJW significantly increased the mouse pain threshold. The antinociceptive effect peaked at 60 mg kg^{-1} po and then it diminished (Fig 2A). SJW 1000 mg kg^{-1} was ineffective. Time-course experiments showed that SJW antinociception (10 and 60 mg kg^{-1} po) became statistically significant 60 minutes after oral administration and peaked at 90 minutes. Then it slowly diminished, disappearing at 180 minutes (Fig 2B).

Both chloroform fraction (CHL) and methanol fraction (MET) showed antinociceptive properties when administered in a concentration corresponding to the content of their main antinociceptive components, hyperforin and hypericin, respectively, present in a 60 mg kg^{-1} preparation of SJW dried extract ($F_{3,44(5,177)}$ $P < .001$) (Fig 3A).

Time-course experiments showed that CHL and MET antinociception were endowed with the same pharmacological profile of SJW dried extract. Their antinociceptive effect became statistically significant 60 minutes after oral administration, peaked at 90 minutes, and slowly diminished, disappearing at 180 minutes (Fig 3B).

Hyperforin (HYF), when administered alone, produced an antinociceptive effect of intensity comparable to CHL fraction (Fig 3C). Similarly, hypericin (HYR) increased the pain threshold to a value comparable to that obtained in the MET group. Neither quercetin (QUER) or hyperoside (HYS) showed antinociceptive properties or increased hypericin-induced antinociception ($F_{8,99(6,948)}$ $P < .001$) (Fig 3C).

SJW dried extract antinociception (60 mg kg^{-1} po) was prevented by the opioid antagonist naloxone (1 mg kg^{-1} ip) and by the administration of the PKC activator PMA (15 pmol per mouse icv), as illustrated in Fig 4A. Pretreatment with atropine (5 mg kg^{-1} ip) or yohimbine (3 mg kg^{-1} ip) was devoid of any effect ($F_{5,66(6,560)}$ $P < .0001$) (Fig 4A).

The increase of pain threshold produced by CHL, administered at a concentration corresponding to the hyperforin content of a 60 mg kg^{-1} po SJW dried extract preparation, was selectively antagonized by naloxone pretreatment ($F_{5,66(5,903)}$ $P < .0001$) (Fig 4B), whereas MET (corresponding to the hypericin content present in 60 mg kg^{-1} preparation of SJW dried extract) was selectively prevented by PMA ($F_{5,66(5,479)}$ $P < .0001$) (Fig 4C). Pretreatment with atropine (5 mg kg^{-1} ip) or yohimbine (3 mg kg^{-1} ip) was devoid of any effect against both SJW fractions (Figs 4B, 4C).

The increase of pain threshold produced by SJW dried extract was prevented by the μ-opioid selective antagonist CTOP, the κ-opioid selective antagonist nor-BNI, the $\delta 1$ antagonist BNTX, and the $\delta 2$ antagonist naltriben (Fig 5A), indicating the lack of selectivity among the opioid receptor subtypes ($F_{5,66(5,915)}$ $P < .001$). CHL produced similar results ($F_{5,66(5,726)}$ $P < .001$) (Fig 5B).

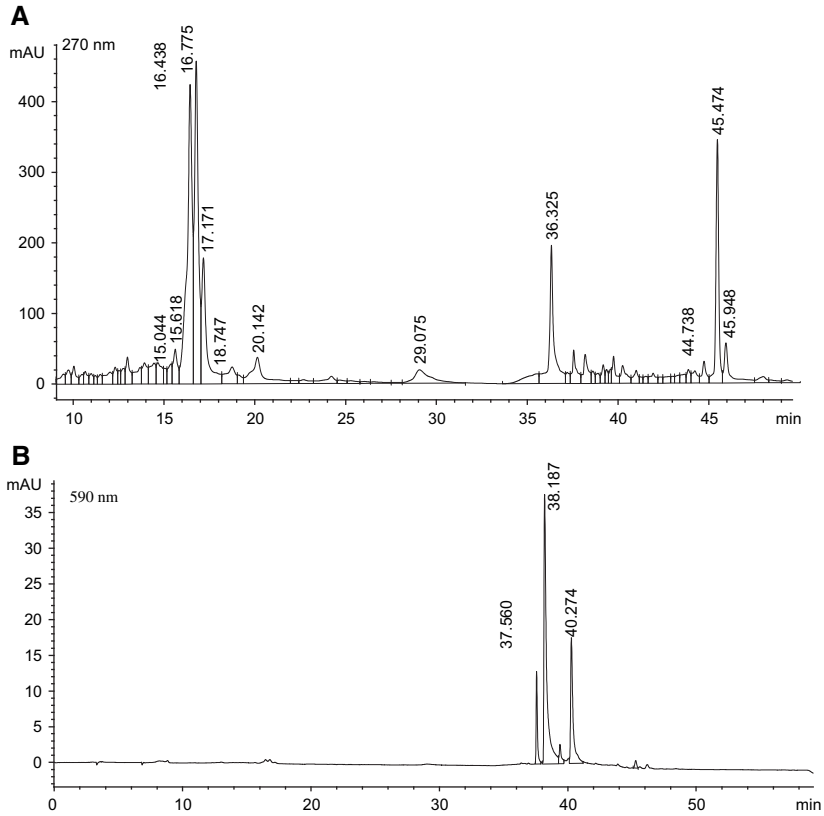

Figure 1. HPLC profiles of *Hypericum perforatum* L. extract. **(A):** HPLC profile at 270 nm. Rutin was linear from 4.5 to 100 μg/mL and all the curves had coefficient of linear correlation ≥.999 with *y*-intercepts which were close to zero. t_R = 16.43 minutes, rutin, t_R = 16.77 min., hyperoside, t_R = 17.17 minutes, isoquercitrin, t_R = 18.74 minutes, quercitrin, t_R = 29.07 minutes, quercetin, t_R = 36.32 minutes, I3, II8-biapigenin, t_R = 44.73 minutes, furohyperforin, t_R = 45.47 minutes, hyperforin, t_R = 45.94 minutes, adhyperforin. **(B):** HPLC profile at 590 nm. t_R = 38.18 minutes, pseudohypericin, t_R = 40.27 minutes, hypericin.

All the pharmacological antagonists as well as the PKC activator PMA, at the concentrations administered, were devoid of any antinociceptive/hypernociceptive effect, as previously demonstrated by us.[13]

Oral administration of SJW dried extract caused a significant inhibition on the writhing response induced by intraperitoneal injection of a .6% acetic acid solution when compared with CMC-treated mice used as control group. This effect became significant at 10 mg kg^{-1} po, peaked at 30 mg kg^{-1} po, and disappeared at higher concentration (F5,66(8,103) $P < .0001$) (Fig 6A). Time- course experiments showed that SJW antinociception became statistically significant 60 minutes after oral administration, peaked at 90 minutes, and was still significant at 120 minutes. Then it slowly diminished, disappearing at 180 minutes (data not shown).

Both MET and CHL reduced the number of abdominal constrictions showing antinociceptive properties. MET appeared to be the most effective fraction, being active at 10 and 30 mg kg-1 po. CHL was less effective than MET, reaching statistical significance only at 30 mg kg-1 po (F8,99(3,977) $P < .001$) (Fig 6B).

Hypericin (HYR) reduced the number of abdominal constrictions with the same intensity observed in the MET group. Neither Quercetin (QUER) nor hyperoside (HYS) showed antinociceptive properties or potentated hypericin-induced antinociception (F6,77(4,914) $P < .001$) (Fig. 6C).

The effect of naloxone and PMA on the SJW dried extract, CHL, and MET fraction antinociception in the abdominal-constriction test was examined. Naloxone did not modify the effect produced by SJW dried extract and MET fraction, whereas the antinociceptive effect induced by the CHL fraction was slightly attenuated by naloxone (F7,88(10,923) $P < .0001$) (Fig 7A). Conversely, PMA antagonized both SJW- and MET-induced antinociception. In the same experimental conditions, PMA did not influence CHL-induced antinociceptive effect (F7,88(7,713) $P < .0001$) (Fig 7B). Naloxone and PMA, when administered alone, did not modify the number of abdominal constrictions in comparison to the control group (Fig 7A,B).

The SJW dried extract, CHL, and MET fractions were tested at the highest effective doses in order to assess their effect on mouse locomotor behavior. Mice pretreated with the above-mentioned extracts at the dose of 60 mg kg^{-1} po were evaluated for motor coordination by use of the rota-rod test, and for spontaneous mobility and inspection activity by use of the hole-board test. The spontaneous mobility (Fig 8A) as well as the inspection activity (Fig 8B) of mice, expressed as counts in 5 minutes, were unmodified by pretreatment with SJW dried extract, CHL, and MET fractions in comparison with the control group (CMC). D-amphetamine (2 mg kg^{-1} sc), used as positive control, significantly increased both spontaneous mobility (F4 ,45(4 .343) $P < .05$) and exploratory activity (F4,45(3.998) $P < .05$) (Figs 8A, 8B). The

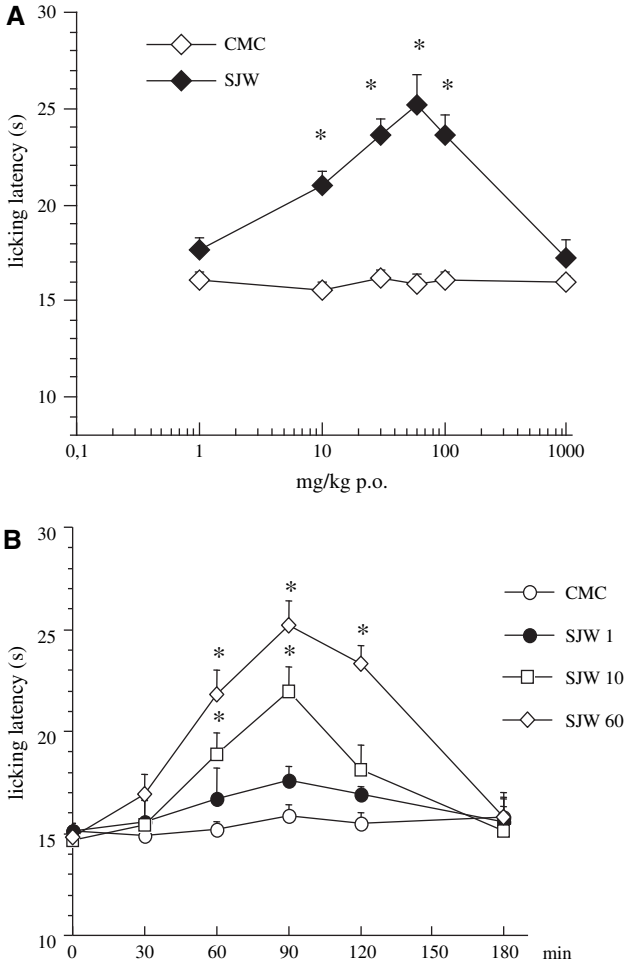

Figure 2. Antinociceptive effect of St. John's Wort (SJW) dried extract in the hot-plate test. **(A):** dose-response curve of SJW dried extract (1–100 mg/kg po). Vertical lines represent s.e.m.; *P < .05 in comparison with carboxymethylcellulose (CMC) group. **(B):** time-course curves of SJW-dried extract (1–10–60 mg/kg po). Vertical lines represent s.e.m.; *P < .05 in comparison with CMC group.

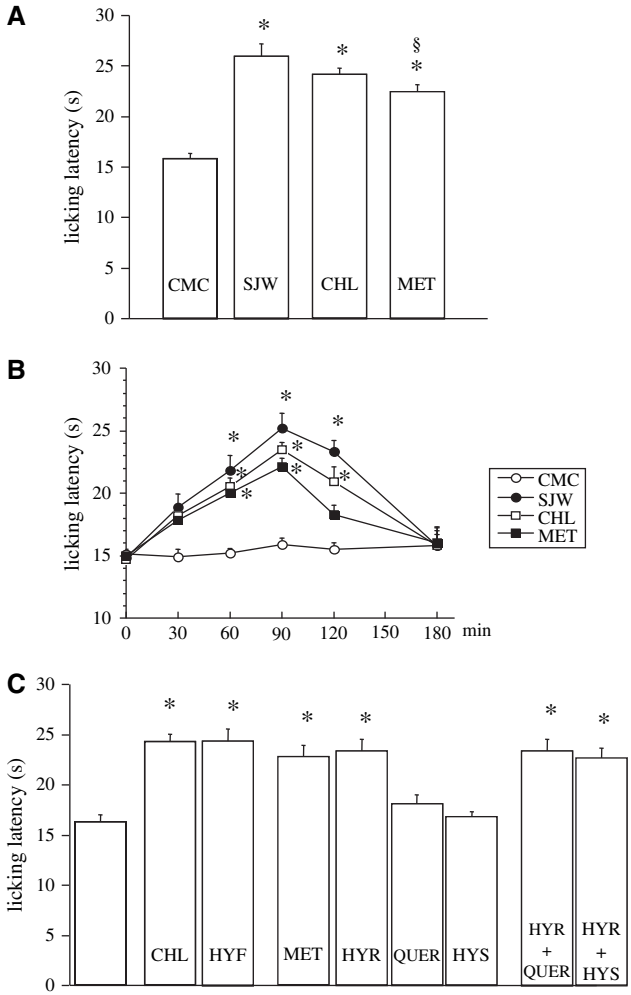

Figure 3. Antinociceptive profile of St. John's Wort (SJW) components in the hot-plate test. **(A):** antinociceptive effect of SJW dried extract (60 mg/kg po), chloroform (CHL) and methanol (MET) fractions. Vertical lines represent s.e.m.; *P < .05 in comparison with carboxymethylcellulose (CMC) group. §P < .05 in comparison with SJW-treated animals. **(B):** time-course curves of CHL and MET fractions (1–10–60 mg/kg po). Vertical lines represent s.e.m.; *P < .05 in comparison with CMC group. **(C):** comparison between CHL and MET fraction antinociception and their main components hyperforin (HYF), hypericin (HYR), quercetin (QUER), and hyperoside (HYS). Vertical lines represent s.e.m.; *P < .05 in comparison with CMC group.

number of falls from the rotating rod, evaluated before and 60, 90 and 120 minutes after treatment, showed the lack of any impairment in the mouse motor coordination by SJW dried extract and fractions administration in comparison with the CMC group (Fig 8C).

Discussion

An SJW dried extract, CHL, and MET fractions, given by oral route, induced thermal and chemical antinociception by increasing the licking latency values in the hot-plate test, and by inhibiting acetic acid-induced abdominal constrictions, respectively.

SJW dried extract produced a long-lasting increase of the thermal-pain threshold, which appeared 60 minutes after administration and was still detectable at 120 minutes. A recent study reported that an SJW extract was devoid of any antinociceptive effect in the mouse tail-flick test.[35] This discrepancy might be explained by considering that the SJW-induced antinociception is endowed with a bell-shaped trend. The authors looked for an increase in the pain threshold at a concentration about 15 times higher than the effective ones. As demonstrated by dose-response experiments, at this dose SJW is devoid of any antinociceptive activity.

SJW dried extract also attenuated the nociceptive response induced by intraperitoneal administration of an acetic acid solution, producing a long-lasting antinociception. A bell-shaped trend was observed also against chemical nociception. Acetic acid causes algesia by liberating endogenous mediators that excite pain nerve endings, and is a sensitive method to investigate peripheral and central antinociceptive efficacy of agents.[10] The SJW antinociceptive effect in the abdominal-constriction test peaked at a concentration lower than that needed to produce the maximal activity against a thermal stimulus. It was not surprising since it is widely considered that

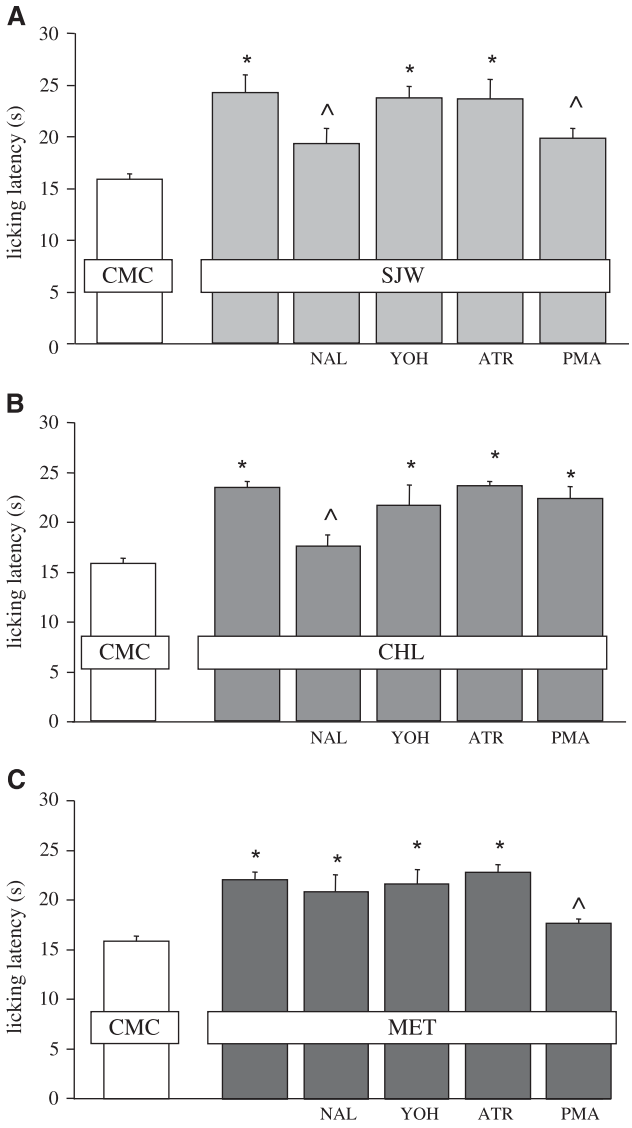

Figure 4. Mechanism of thermal antinociception of St. John's Wort (SJW) dried extract, chloroform (CHL), and methanol (MET) fractions. Effect produced by naloxone, yohimbine, atropine and PMA on SJW dried extract (60 mg kg^{-1} po) **(A),** CHL (60 mg kg^{-1} po) **(B),** and MET (60 mg kg^{-1} po) **(C)** fractions in the mouse hot-plate test. The licking latency values were recorded 90 minutes after SJW, CHL and MET administration. Vertical lines represent s.e.m.; *P < .01 in comparison with control group (CMC-treated mice); ^P < .05 in comparison with corresponding antinociceptive compound-treated mice.

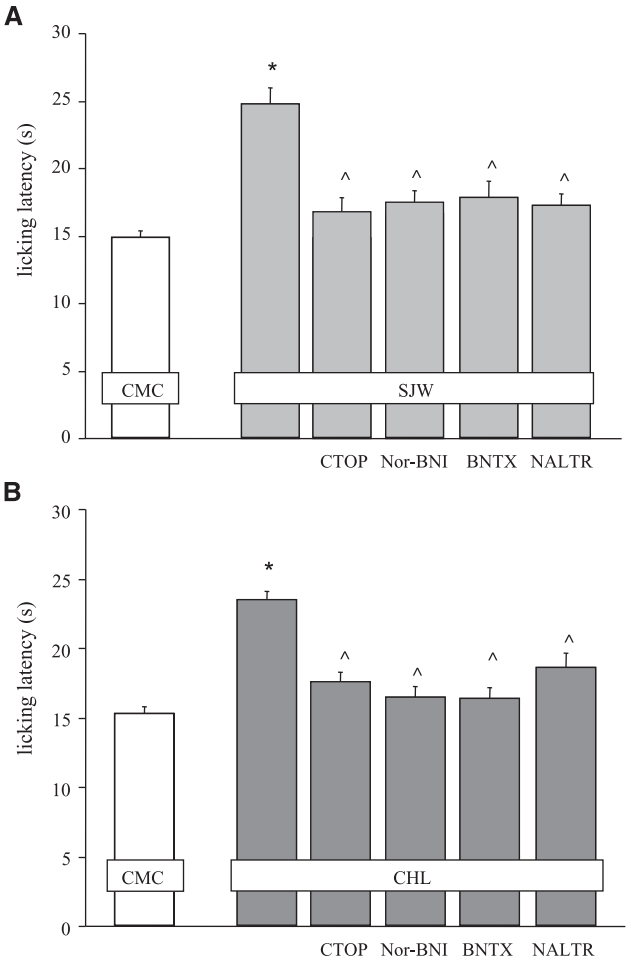

Figure 5. Prevention by CTOP, Nor-BNI, naltriben (NALTR) and BNTX of thermal antinociception of St. John's Wort (SJW) dried extract **(A)**, and chloroform (CHL) **(B)** fraction in the mouse hot-plate test. The licking latency values were recorded 90 minutes after SJW (60 mg kg^{-1} po) and CHL (60 mg kg^{-1} po) administration. Vertical lines represent s.e.m.; *P < .01 in comparison with control group (CMC-treated mice); ^P < .05 in comparison with corresponding antinociceptive compound-treated mice.

analgesic effectiveness depends on the nociceptive stimulus applied, and that the chemical stimulus is more sensitive than the thermal one.

SJW as well as CHL and MET fractions produced bell-shaped dose-response curves in both thermal and chemical painful conditions. Furthermore, CHL and MET fractions produced a bell- shaped dose-response curve not only in the abdominal-constriction test, but also in the hot-plate test. At present we do not have a clear explanation, but it appears to be a typical trait for SJW since even its antidepressant activity, one of the main properties of the plant, shows a bell-shaped trend.[19]

The main components of the SJW dried extract are phloroglucinols, naphtodiantrones, and flavonoids, and their role on the modulation of the pain threshold was investigated. We obtained a chloroform fraction (CHL) containing phloroglucinols, mainly represented by hyperforin, and a methanol fraction (MET) that was a hypericin- and flavonoid-rich fraction. Both CHL and MET fractions increased the licking latency values in the hot-plate test and inhibited the acetic acid-induced abdominal constrictions showing a time-course profile comparable to the dried extract. The intensity of the antinociceptive activity of both fractions against a thermal stimulus was comparable. Conversely, when a chemical nociceptive stimulus was applied, MET was more effective than CHL. These data indicate that all SJW main components are responsible for the antinociceptive properties of the SJW dried extract, suggesting a prominent role of hypericin/flavonoid against a chemical noxious stimulus.

The thermal antinociception induced by SJW dried extract underlies the activation of the opioid system as this analgesia is prevented by the opioid antagonist naloxone. Phloroglucinols might be responsible for this opioid effect since hyperforin inhibited binding to the

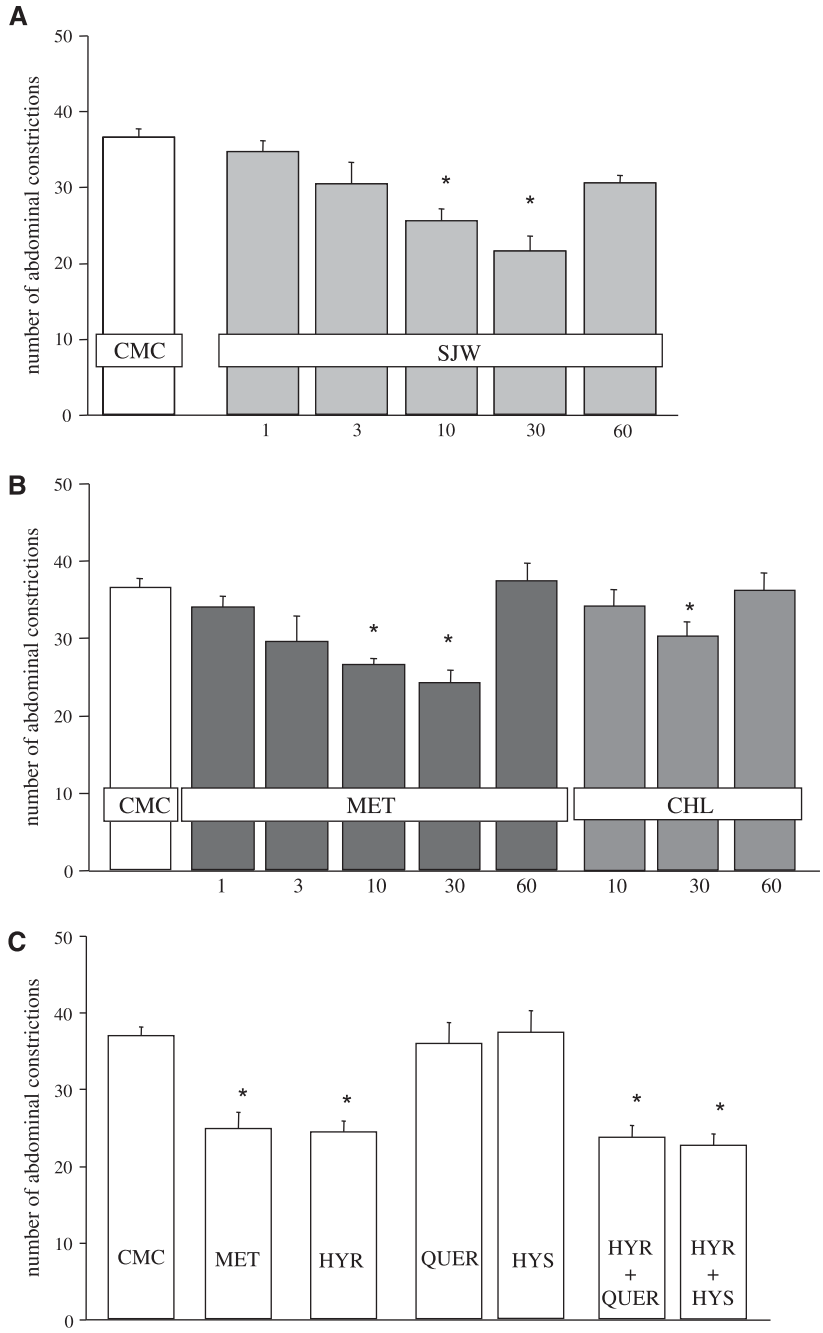

Figure 6. Antinociceptive profile of St. John's Wort (SJW) and chloroform (CHL), and methanol (MET) fractions in the acetic acid-induced abdominal constriction test. **(A)**: dose-response curve for the antinociceptive effect of SJW dried extract (1–60 mg/kg po). The number of abdominal constrictions was recorded 90 minutes after SJW administration. Vertical lines represent s.e.m.; *$P < .05$ in comparison with carboxymethylcellulose (CMC) group. **(B)**: dose-response curve of the CHL (1–60 mg/kg po) and MET (30–60 mg/kg po) fractions. The number of abdominal constrictions was recorded 90 minutes after CHL and MET administration. Vertical lines represent s.e.m.; *$P < .05$ in comparison with carboxymethylcellulose (CMC) group. **(C)**: comparison among MET fraction antinociception and its main components hypericin (HYR), quercetin (QUER), hyperoside (HYS). The number of abdominal constrictions was recorded 90 minutes after administration. Vertical lines represent s.e.m.; *$P < .05$ in comparison with carboxymethylcellulose (CMC) group.

opioid receptors with higher affinity than hypericin and flavonoids.[27,28] The prevention by naloxone of CHL antinociception further supports this hypothesis. The SJW and CHL opioid antinociception is mediated by the unselective stimulation of μ, δ and κ-opioid receptor subtypes since the κ-opioid antagonist nor-NBI,[18] the μ-opioid antagonist CTOP,[16] the δ1-opioid antagonist BNTX,[26] and the δ2-opioid antagonist naltriben[29] were all able to prevent SJW dried extract and CHL antinociception. Binding of [^{3}H]naloxone to the μ- and k-opioid receptor was inhibited in the presence of SJW extract,[28] supporting present results. CHL fraction contains phloroglucinols together with a small amount of the flavonoid biapigenin. The prevention by naloxone of the CHL antinociception indicates a major involvement of hyperforin since biapigenin binds to opioid receptors with a much lower affinity than hyperforin.[27] The administration of purified hyperforin produced an antinociceptive effect

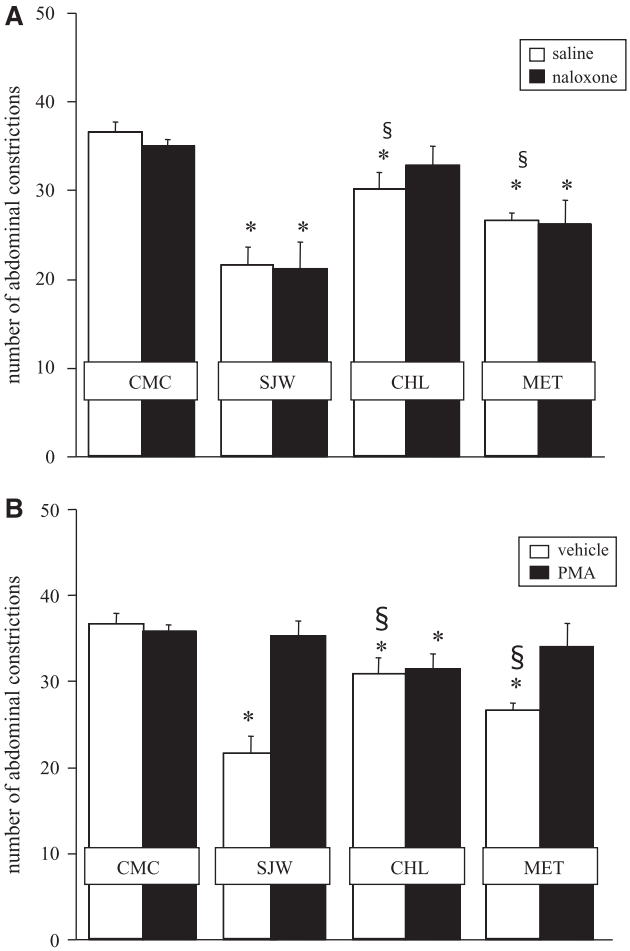

Figure 7. Mechanism of chemical antinociceptive activity of St. John's Wort (SJW) and fractions. **(A)**: Lack of effect by pretreatment with naloxone of SJW dried extract (30 mg kg^{-1} po) antinociception in the mouse acetic acid-induced abdominal constriction test. The number of abdominal constrictions was recorded 90 minutes after SJW, chloroform (CHL), and methanol (MET) administration. Vertical lines represent s.e.m.; $^{*}P < .05$ in comparison with control group (CMC-treated mice); $^{\S}P < 0.05$ in comparison with SJW-treated animals. **(B)**: Prevention by pretreatment with PMA of SJW dried extract (30 mg kg^{-1} po) and MET (30 mg kg^{-1} po) antinociception in the mouse acetic acid-induced abdominal constriction test. The number of abdominal constrictions was recorded 90 minutes after SJW, CHL and MET administration. Vertical lines represent s.e.m.; $^{*}P < .05$ in comparison with control group (CMC-treated mice); $^{\S}P < 0.05$ in comparison with SJW-treated animals.

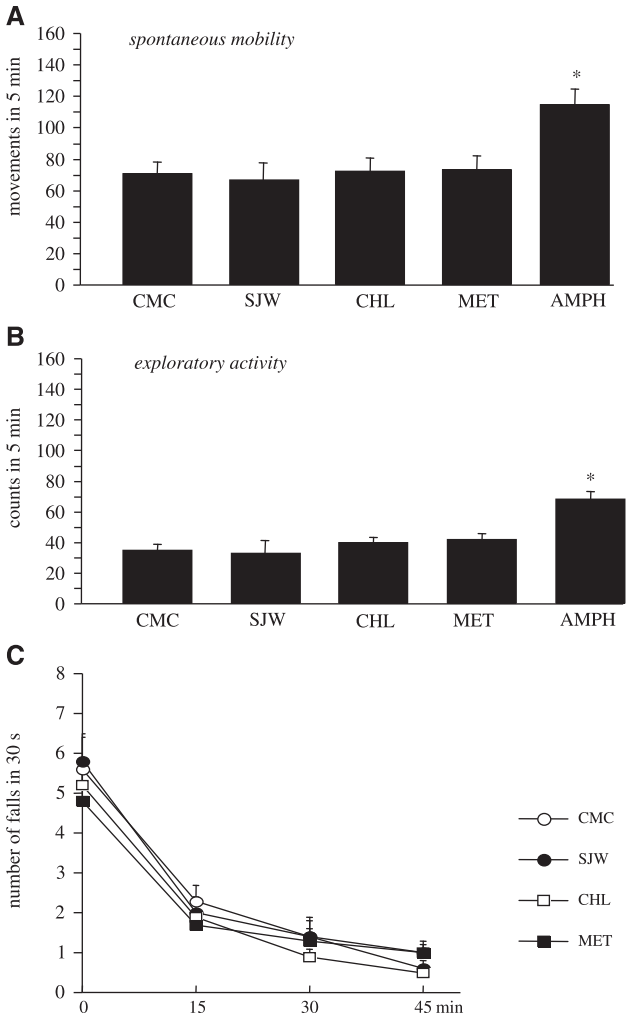

Figure 8. Lack of effect of St. John's Wort (SJW) dried extract, chloroform (CHL), and methanol (MET) fractions on mouse spontaneous mobility **(A)**, and inspection activity **(B)**, evaluated in the mouse hole-board test, and on mouse motor coordination evaluated in the mouse rota-rod test **(C)**. Amphetamine was administered at the dose of 2 mg kg^{-1} sc; Vertical lines represent s.e.m.

comparable to that induced by the CHL fraction, further suggesting the prominent role of hyperforin in the CHL activity.

Conversely to the CHL fraction, MET fraction thermal antinociception was unmodified by naloxone pretreatment. MET fraction contains hypericins and flavonoids, and both components might be potentially involved in the SJW mechanism of antinociceptive action. Total hypericins amount to about .3% of SJW dried extract, have been isolated only in few other plants, and have long been known to be related to many pharmacological properties of SJW such as antidepressive, antineoplastic, antitumor and antiviral activities.[20] Flavonoids account for about 12% of SJW dried extract. A role of flavonoids in the SJW-induced modulation of pain threshold might be hypothesized since it has been reported that flavonoids contribute to the anti-inflammatory effects of the plant,[34] and antinociceptive properties have been reported for the flavonoid myricitrin.[24] Enzyme assays performed on rat brain demonstrated that hypericin and pseudohypericin are potent and selective inhibitors of the protein kinase C (PKC).[20,33] In vitro studies reported that some flavonoids inhibit rat brain PKC activity. Among them, quercetin is one of the most potent.[11] PKC is a family of enzymes involved in numerous important cellular events, including pain modulation. The activation of PKC has been related to the induction of a painful condition whereas PKC blockers decreased nociception.[36] Pretreatment with the PKC activator PMA, administered at a concentration able to prevent PKC-mediated antinociception,[13] antagonized MET antinociception whereas CHL increase of pain threshold remained unmodified, indicating that the MET effect underlies the blockade of the PKC-mediated intracellular pathway. To identify the MET component responsible for the increase of pain threshold, the effect produced by administration of purified MET fraction components was investigated.

Hypericin showed antinociceptive properties of intensity comparable to that produced by MET whereas the flavonoids hyperoside and quercetin neither increased the pain threshold nor potentiated the hypericin-induced antinociception. Hypericin appears, therefore, to be the most effective component of the MET fraction.

Hypericin and hyperforin appear to be the most effective components of SJW in the induction of thermal antinociception. However, a cumulative effect of both components in the dried extract was not observed. We do not have a clear explanation for this effect, but it should be taken into account that, conversely to the purified active components, in a dried extract there are many constituents that, even if pharmacologically ineffective, might positively or negatively modulate the pharmacokinetic properties of the dried extract (ie, increasing/decreasing absorption, bioavailability, solubility, etc.), leading to an increased/decreased pharmacological activity.

Purified hypericin also reduced the number of abdominal constrictions, a result as effective as the MET fraction. The flavonoids hyperoside and quercetin were devoid of any antinociceptive and/or potentating effect. These data represent evidence for a prominent role of hypericin in the MET-induced antinociception and also in an acute chemical-pain condition. The PKC activator PMA prevented the inhibition of acetic acid-induced abdominal constrictions produced by SJW dried extract and MET that, conversely, remained unmodified by naloxone administration. SJW antinociceptive effect against a chemical stimulus appears to be mainly related to the PKC-blocking properties of hypericin and, conversely to the thermal antinociception, the opioid system was only marginally involved. This hypothesis was further supported by the higher efficacy showed by the MET fraction in comparison with the slight reduction of the number of abdominal constrictions produced by the CHL fraction. The contribution of CHL is so modest that it is probably unable to influence the effect produced by MET and, as a consequence, naloxone did not modify the SJW-induced chemical antinociception. In agreement with our findings, recent experiments performed in the mouse formalin test observed that the SJW antinociception was unaffected by naloxone.[35] It should also be noted that different mechanisms have been proposed for hyperforin,[23] including mechanisms that might be involved in the modulation of the pain threshold. For these reasons, mechanisms other than the opioid cannot be excluded.

SJW and its major components interact with numerous neuronal systems able to modulate the pain threshold. Receptor-binding and enzyme-inhibition assays carried out using hypericum extract demonstrated significant affinity for adenosine, $GABA_A$, $GABA_B$, benzodiapine, MAO_A, MAO_B, and inhibitory activity towards synaptosomal uptake of serotonin, dopamine, and noradrenaline.[2] The MAO-inhibitory properties of SJW were mainly due to hypericin[20,32] whereas the inhibition of monoamine synaptosomal uptake is related to the presence of hyperforin.[8,9,14,25] The involvement of the adrenergic system can be ruled out since the $á_2$-adrenoceptor antagonist yohimbine did not prevent SWJ-, CHL-, and MET-fractions-induced antinociception. A muscarinic-mediated mechanism can also be excluded due to the lack of antagonism by atropine.

SJW dried extract showed antinociceptive properties after oral administration indicating that it might potentially act through a central as well as a peripheral mechanism. Furthermore, the results from the abdominal-constriction test did not ascertain the SJW site of action. Acetic acid acts indirectly by inducing the release of peripherally—as well as centrally—acting endogenous mediators,[4] even if it is supposed to be mediated primarily via a peripheral mechanism.[31] However, the antagonism of SJW antinociception produced by the icv administration of PMA suggests that this effect is centrally mediated.

SJW dried extract, CHL, and MET fractions, at the highest active doses employed in the present study, did not cause any detectable modification in mouse gross behavior. An altered spontaneous mobility and locomotor activity induced by SJW administration could lead to a misinterpretation of the results obtained. Additional behavioral tests were performed to make sure that treatments did not alter the above-mentioned parameters.

In conclusion, our findings showed that SJW, CHL, and MET fractions produce a prolonged and tolerable antinociception in mice in a condition of acute thermal and chemical pain. In addition, the SJW major components, hypericin and hyperforin, increased the pain threshold through the selective modulation of 2 different intracellular pathways: the presence of hypericin was fundamental to induce both thermal and chemical antinociception through the inhibition of the PKC activity, whereas hyperforin selectively produced a thermal opioid antinociception.

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