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NEUROPHARMACOLOGY

St John's Wort reduces neuropathic pain through a hypericin-mediated inhibition of the protein kinase C γ and ε activity

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Running title: St John Wort's and neuropathic pain

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Abbreviations: CCI, chronic constriction injury; CHL, chloroformic fraction; i.c.v., intracerebroventricular; i.p., intraperitoneal; MET, methanolic fraction; OXA, oxaliplatin repeated administration; p.o.: per os; PAG, periaqueductal grey area; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; SJW, St. John's Wort; s.c., subcutaneous

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Abstract

Current pharmacological treatments for neuropathic pain have limited efficacy and severe side-effect limitations. St. John's Wort (SJW) is a medicinal plant, mainly used as antidepressant, with a favourable side-effect profile. We here demonstrate the ability of SJW to relieve neuropathic pain in rat models. The antihyperalgesic profile and mechanism of action of SJW and its main components was studied in two rat models of neuropathic pain: the chronic constriction injury and the repeated administration of oxaliplatin. SJW, acutely administered at low doses (30-60 mg kg^{-1} p.o.), reversed mechanical hyperalgesia with a prolonged effect, being effective up to 180 min after injection. Further examinations of the SJW main components revealed that hyperforin and hypericin were responsible for the antihyperalgesic properties whereas flavonoids were ineffective. The effect of SJW on the PKC expression and activation was investigated in the periaqueductal grey (PAG) area by immunoblotting experiments. Mechanistic studies showed a robust over-expression and hyperphosphorylation of the PKC-γ (227.0±15.0 % of control) and -ε (213.9±17.0) isoforms in the rat PAG area. A single oral administration of SJW produced a significant decrease of the PKC-γ (131.8±10.0) and -ε (105.2±12.0) phosphorylation in the PAG area due to the presence of hypericin. Furthermore, SJW showed a dual mechanism of action since hyperforin antinociception involves an opioid-dependent pathway. Rats undergoing treatment with SJW and purified components did not show any behavioural side effects or signs of altered locomotor activity. Our results indicate SJW as a prolonged antihyperalgesic treatment through inhibition of PKC isoforms and their phosphorylation.

Key words*:* *hypericum perforatum*; St. John's Wort; neuropathic pain; hypericin; hyperforin; PKC

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1. Introduction

Neuropathic pain can arise from a wide variety of injuries to peripheral or central nerves, including metabolic disorders, traumatic injury, inflammation, and neurotoxicity, and is characterized by spontaneous pain, hyperalgesia and allodynia which can persist long after the initial injury is resolved [1]. Common causes of neuropathy are diabetes, herpes zoster infections, chronic or acute trauma, and neurotoxins. Neuropathic pain occurs frequently in cancer as it may result from tumor invasion of nervous tissue, surgical nerve damage during tumor removal, radiation-induced nerve damage or as a side effect of many chemoterapeutic drugs [2,3] .

The underlying molecular mechanisms of neuropathic pain are still not completely understood, and as a consequence, treatment is unsatisfactory in many cases [3,4]. Despite the large number of approved analgesic drugs, effective treatment of chronic pain is still often unattainable due to the unsatisfactory performance of the available drugs and due to the negative side effects from the drugs [5,6]. Neuropathic pain is generally insensitive to non-steroidal anti-inflammatory drugs and relatively resistant to opioids, but can be treated by high opioid doses [7] with a high incidence of untoward side effects. The suggested treatment strategies for neuropathic pain management are represented by tricyclic antidepressants (TCAs) [8], that represent the first medication category proved to be effective for neuropathic pain in clinical trials [9] and still are a first choice treatment, anticonvulsants [10,11], antiarrythmics [12]. All these drugs have limited efficacy and have severe side effects. In particular, TCAs induce numerous untoward effects such as sedation, anticholinergic effects like dry mouth, constipation and postural hypotension, weight gain, greater risk to develop myocardial infarction [13,14]. Therefore, for an effective treatment of neuropathic pain, there is still a need to obtain therapeutics which possess a greater level of tolerability and safety.

Hypericum perforatum L., commonly known as St. John's Wort (SJW), has been proven to relieve mild-to-moderate forms of depression and tests have also shown SJW to have favourable side effects [15]. 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 [16]. SJW

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interacts with the monoaminergic system through different mechanisms: the MAO-inhibitory properties of SJW were mainly due to hypericin [17,18] whereas the inhibition of serotonin, dopamine and noradrenaline synaptosomal uptake is related to the presence of hyperforin [19-22]. It has therefore been suggested that SJW may induce antidepressant activity through a mechanism similar to TCAs [23]. This evidence may suggest that similar to TCAs, SJW can also relieve neuropathic pain. The aim of the present study was to investigate the antihyperalgesic properties of SJW in different animal models, which were suffering of neuropathic pain that was induced by chronic constriction injury of the sciatic nerve, or by the repeated administration of the chemotherapeutic agent oxaliplatin. The role of the SJW main constituents, hypericin, hyperforin and flavonoids, and their cellular and molecular effects in the modulation of the pain threshold was also investigated in comparison with the effects produced by the SJW dried extract in order to better elucidate the mechanism of action in this herbal plant.

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2. Materials and Methods

2.1 Animals

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. 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.

2.2 Production of neuropathy

2.2.1 Chronic constriction injury: A peripheral mono neuropathy was produced in adult rats by placing loosely constrictive ligatures around the common sciatic nerve according to the method described by Bennett and Xie [24]. Rats were anesthetized with chloral hydrate. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to sciatica's trifurcation, about 1 cm of the nerve was freed of adhering tissue and four ligatures (3/0 chromic tread) were tied loosely around it with about 1mm spacing. The length of the affected nerve was 4-5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was barely constricted when viewed with 40x magnification. In sham-operated animals, an identical dissection was performed, but the sciatic nerve was not ligated. The ligation was made on the right paws and left paws were untouched. Experiments were carried out 14 days after surgery.

2.2.2 Oxaliplatin repeated treatment: Oxaliplatin was dissolved in a 5% glucose solution at a concentration of 2.4 mg kg^{-1}. Oxaliplatin was administered i.p. for five consecutive days per week for three consecutive weeks according to a previously established protocol [25]. The control group

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received a 5% glucose solution. The experiments were carried out on day 21.

2.3 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 chronic constriction injury or oxaliplatin administration, were rejected (25%). The pain threshold of neuropathic animals was measured previously (pretest) and then 30, 60, 90, 120 and 180 min after administration of SJW and components.

2.4 Rota rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod, 30 cm in length, was placed at a height of 15 cm from the base. Up to five rats 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 s. Those rats scoring less than three and more than six falls in the pretest were rejected (20%). Their performance time was measured previously (pretest) and then 60, 90 and 120 min after SJW, CHL and MET fractions or purified components administration. A total of 4-5 rats per group were tested.

2.5 I.c.v. injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anesthesia with isotonic saline as a solvent which has been previously described [26].

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2.6 Preparation of membrane and cytosol fractions

Rat periaqueductal grey (PAG) area was dissected and homogenized in a homogenizing buffer. The homogenate was centrifuged at 9,000 x g for 15 min at 4°C and the low speed pellet was discarded. The supernatant (total proteins) was centrifugated at 100,000 x g for 60 min at 4°C. The resulting supernatant was the cytosol fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (wt/vol) Triton X-100. The homogenate was kept at 4°C for 60 min with occasional stirring and then centrifugated at 100,000 x g for 60 min at 4°C. The resulting supernatant was used as the membrane fraction. Protein concentration of the cytosol and membrane fractions was quantified using Bradford's method (protein assay kit, Bio Rad Laboratories, Milan, Italy).

2.7 Immunoblot analysis

Membrane homogenates (10-50 μg) of naïve, untreated CCI and SWJ treated CCI rats were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes (100 min at 100 V) using standard procedures. The membranes were blocked in PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 90 min. Following washings, blots were incubated overnight at 4° C with specific antibodies (Santa Cruz Biotechnology, CA, USA) against PKCγ, pPKCγ, PKCε, pPKCε or ß-actin (1:1000 dilution). After being washed with PBST, the nitrocellulose membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:5,000) and left for 90 min at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy). The exposition and developing times used were standardized for all the blots. Densitometric analysis of scanned images was performed on a Macintosh iMac computer using the public domain NIH Image program. Measurements in control samples were assigned a relative

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value of 100%. Measurements were normalised relative to ß-actin, used as loading control.

2.8 HPLC-DAD and HPLC-MS drug analyses

To evaluate the content of SJW main components, a HPLC analysis was performed using a method described in the [27], modified for our experimental requirements. The identification of the constituents was performed using combined HPLC-diode array detection (DAD) analysis and HPLC-thermospray mass spectrometry. The mobile phase was a five-step linear solvent gradient CH3CN/CH3OH/H2O (pH 3.2, HCOOH) over a 60 min period at a flow rate of 1 ml/min. 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 [27].

All the samples were analyzed in triplicate and a calibration graph with six data points of external standard was used.

2.9 Preparation of chloroform and methanol fractions

A solution of dried SJW 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.

2.10 Drugs

Indena Research Laboratories (Settala, Milan, Italy) offered a commercial sample of *Hypericum*

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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: oxaliplatin (Sequoia Research Product Ltd, USA), naloxone hydrochloride, hypericin, hyperforin, hyperoside, quercetin, amentoflavone, amitriptyline hydrochloride (Sigma, Milan, Italy); phorbol-12-myristate-13-acetate (PMA), (Calbiochem, Milan Italy); and carboxymethylcellulose sodium salt, (CMC) (Fluka Chemie GmbH, Steinheim, Germany). The other commercially available chemicals used were of the highest quality.

SJW dried extract, chloroform fraction (CHL), methanol fraction (MET), hypericin, hyperforin, quercetin, hyperoside and amentoflavone were dissolved in a 1% CMC solution immediately before use and administered by oral gavage. Naloxone and amitriptyline were dissolved in isotonic (NaCl 0.9 %) saline solution, PMA was dissolved in 0.1% DMSO immediately before use. Drug concentrations were prepared to ensure that the necessary dose could be administered in a volume of 10 ml kg^{-1} by intraperitoneal (i.p.), per os (p.o.) injection or in a volume of 5 μl by i.c.v. injection.

2.11 Drug administration

SJW dried extract, CHL and MET fractions, hypericin, hyperforin, quercetin, hyperoside and amentoflavone were administered 90 min before the Paw Pressure test, in correspondence to their maximum effect as determined by time-course experiments.

The SJW components were orally administered 90 min before the test at the following doses: hypericin 0.06 mg kg^{-1}, hyperforin 0.978 mg kg^{-1}, quercetin 0.249 mg kg^{-1}, hyperoside 1.905 mg kg^{-1} amentoflavone 0.016 mg kg^{-1}. These doses correspond to the amount of each component 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. The effect of amitriptyline was evaluated 30 min after admistration.

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Naloxone (1 mg kg^{-1} i.p.) was injected 70 min after SJW, CHL or MET administration. PMA (15 pmol per mouse i.c.v.) was injected 30 min after SJW, CHL or MET administration.

The doses and administration schedules of SJW and all other drugs used were chosen on the bases of time-course and dose-response experiments previously performed in our laboratory.

2.12 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 two means.

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3. **Results**

3.1 Composition of SJW dried extract, chloroform and methanol fractions.

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).

3.2 Antihyperalgesic/antinociceptive activity of SJW dried extract in rat models of neuropathic pain.

Two different rat models of neuropathic pain were used: chronic constriction injury of the sciatic nerve (CCI) and oxaliplatin repeated administration (OXA). CCI, 14 days after surgery, induced mechanical hyperalgesia (rat paw pressure test) as demonstrated by the reduced pain threshold in the hind paw affected by CCI in comparison with the controlateral uninjured leg (Figure 1a $F(1,19)44.631$ $p<0.001$). Pain threshold values comparable to those recorded in the controlateral uninjured leg were detected in the sham-operated before (65.0±5.5) and after (64.6±3.3) a single oral administration of CMC. Same results were obtained in naïve rats (Figure 1b). Similarly to CCI, oxaliplatin repeated administration induced the development of neuropathic pain as illustrated by nociceptive behaviour as compared with the saline-treated control group. A statistically significant hyperalgesia appeared on day 21, persisted unmodified up to day 27 and then it diminished (Figure 1c).

A single administration of SJW dried extract dose-dependently (1-100 mg kg^{-1} p.o.) reversed the mechanical hyperalgesia in both models of neuropathic pain: CCI (Figure 1a;) and OXA (Figure

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1d). The antihyperalgesic effect appeared at antinociceptive doses: the dose of 1 mg kg^{-1} p.o. was devoid of any effect, at 10 mg kg^{-1} p.o. SJW significantly increased the rat pain threshold peaking at 30 mg kg^{-1} p.o. At higher concentrations, the antihyperalgesic effect diminished showing a bell-shaped trend (Figure 1a,d). The SJW antihyperalgesic effect was of similar intensity to that produced by amitriptyline (10 mg kg^{-1} i.p.; 80.3±4.2), used as reference drug. Time-course experiments showed that the antinociceptive (Figure 2a,c) and the antihyperalgesic (Figure 2b,c) effects of SJW (30 mg kg^{-1} p.o.) share the same profile: they both became statistically significant 30 min after oral administration, peaked at 90 min, persisted up to 120 min and disappeared at 180 min in all of the rat models tested for neuropathic pain. In the sham-operated rats SJW (30 mg kg^{-1} p.o.) increased the pain threshold (controlateral 110.1±3.1: sham-operated 108.8±5.6) with similar intensity to that observed in naïve rats (Figure 1b) and in the controlateral uninjured leg of CCI rats (Figure 2a).

The effect produced by a single oral administration of SJW on rat pain threshold was investigated in the rat paw pressure test. 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 dried extract of SJW (30 mg kg^{-1} p.o.) showed antinociceptive activity against a mechanical stimulus in naïve rats in comparison with CMC-treated animals, used as control group (Figure 1b). A single p.o. administration of CMC did not alter the pain threshold in comparison with the pre-treatment values (Figure 1b).

A dried extract of SJW induced a dose-dependent (1-100 mg kg^{-1} p.o.) mechanical antinociception also in the uninjured non-hyperalgesic leg of CCI rats. The dose of 1 mg kg^{-1} p.o. was devoid of any effect ($F(1,14)2.714$ $p=0.121$). At 10 mg kg^{-1} p.o. SJW significantly increased the rat pain threshold. The antinociceptive effect peaked at 30 mg kg^{-1} p.o. and then it diminished (Figure 1a).

SJW was also able to increase the pain threshold in rats treated for 21 days with a daily injection of saline solution, animals that represented the control group of the oxaliplatin-treated rats (Figure 1d).

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Time course experiments confirmed the prolonged effect of SJW in the uninjured leg of CCI rats (Figure 2a) as well as in the saline-treated group (Figure 2c): the antinociception appeared 30 min after a single acute SJW p.o. administration, peaked at 90 min and persisted up to 120 min.

3.3 Analgesic and antihyperalgesic activity of SJW components.

The chloroform fraction (CHL), representing a hyperforin rich fraction, induced antinociception when administered in a concentration corresponding to the content of hyperforin present in a 30 mg kg^{-1} preparation of SJW dried extract. The CHL-induced increase of the pain threshold was observed in the uninjured leg of rat with CCI (Figure 3a) as well as in naïve rats (88.8±4.7). The CHL antinociceptive dose was also endowed with antihyperalgesic activity. CHL significantly reversed the hyperalgesia in CCI and OXA rat models. The effect produced by CHL administration in CCI rats, taken as example, was reported in Figure 3a. Conversely, the methanol fraction (MET), a hypericin/flavonoid rich fraction, selectively induced antihyperalgesia without changing the baseline mechanical threshold in control rats (lack of antinociceptive properties). MET, administered in a concentration corresponding to the content of hypericin present in a 30 mg kg^{-1} preparation of SJW dried extract, selectively reversed the mechanical hyperalgesia in the hind paw affected by CCI (Figure 3b) as well as in the OXA model (Figure 3c) by increasing the pain threshold up to values comparable to the control ones. Time-course experiments showed that CHL and MET were endowed with the same pharmacological profile of the SJW dried extract (Figure 3a,b,c). Purified hyperforin, when administered alone, produced an antinociceptive/antihyperalgesic effect of intensity comparable to CHL fraction (Figure 4a). Similarly, purified hypericin reversed the mechanical hyperalgesia up to a value comparable to that produced by MET fraction (Figure 4b). The purified flavonoids amentoflavone (AME) and hyperoside (HypS) neither showed antinociceptive/antihyperalgesic properties (Figure 4c) nor increased hypericin-induced antihyperalgesia (Figure 4d). Quercetin (QUER) produced a significant antihyperalgesic activity even if of lower intensity than hypericin (Figure 4c), but it was unable to potentate hypericin effect

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(Figure 4d).

3.4 Mechanism of antinociceptive/antihyperalgesic action of SJW dried extract and components.

SJW dried extract antinociception (30 mg kg^{-1} p.o.) was prevented by the opioid antagonist naloxone (1 mg kg^{-1} i.p.) whereas the antihyperalgesic activity was only slightly reduced (Figure 5a). The increase of pain threshold as well as the prevention of the hyperalgesia produced by CHL was completely antagonized by pre-treatment with the opioid antagonist naloxone (Figure 5b). Conversely, the antihyperalgesia induced by MET was naloxone insensitive (Figure 5c). The administration of the PKC activator PMA (15 pmol per mouse i.c.v.) significantly reduced the SJW dried extract (30 mg kg^{-1} p.o.) antihyperalgesic activity whereas the antinociception was only slightly reduced (Figure 6a). PMA selectively prevented the antihyperalgesic effect induced by MET (Figure 6b), but was devoid of any effect against the CHL-induced increase of pain threshold (Figure 6c). Similar results were obtained by administering purified hyperforin and hypericin (data not shown). The opioid antagonist naloxone as well as the PKC activator PMA, at the concentrations used, were devoid of any antinociceptive/hypernociceptive effect (71.7±4.7; 72.0±1.7, respectively). The dose of naloxone of 1 mg kg^{-1} has been demonstrated to prevent morphine analgesia without modifying the increase of the pain threshold induced by modification of other neurotransmission systems (i.e. GABAergic, muscarinic, serotoninergic, etc.). Higher naloxone doses were not employed since lacking of selectivity toward opioid analgesia.

Rats underwent CCI operation showed increased levels of PKCγ and PKCε (Figure 6d) isoforms in the PAG area. This effect was detected in the total protein preparation (TOT), in the membrane (MEM) and cytosol (CYT) fractions. A robust increase of the phosphorylation of PKCγ and PKCε was also observed in the total proteins (TOT) (PKCγ: $F(1,10)15.536$ $p<0.001$; PKCε: $F(1,10)18.196$ $p<0.001$) and in the membrane fraction (MEM) whereas in the cytosol fraction (CYT) the levels of p-PKCγ and p-PKCε were similar to the control group (Figure 6d). SJW treatment was able to

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reduce the phosphorilation of both PKC isoforms as demonstrated by the decrease of p-PKCγ and p-PKCε levels up to values corresponding to the control (Figure 6d). Similar results were obtained in the PAG of OXA rats (data not shown).

Immunoblots were re-probed for a protein considered to be not regulated as β-actin and no significant density difference was revealed for this protein among samples.

Cross-reactivity of the primary antibodies used was excluded.

3.5 Effects of SJW dried extract and fractions on rat locomotor behaviour.

The SJW dried extract, CHL and MET fractions were tested, at the highest effective doses, in order to assess their effect on rat locomotor behaviour. Rats pretreated with the above-mentioned extracts at the dose of 30 mg kg^{-1} p.o. were evaluated for motor coordination by use of the rota-rod test. The number of falls from the rotating rod, evaluated before and 60, 90 and 120 min after treatments, showed the lack of any impairment in the rat motor coordination by SJW dried extract and fractions administration in comparison with the control group (CMC) (Figure 7a). Each group progressively reduced its number of falls because mice learned how to balance on the rotating rod.

Amitriptyline, used as antinociceptive reference drug, at the dose of 10 mg kg^{-1} did not impair the motor coordination of rats. Conversely, at a 3 times higher dose (30 mg kg^{-1}), it produced an increase in the number of falls from the rotating rod indicating the presence of motor incoordination (Figure 7a). SJW, at a dose of 1000 mg kg^{-1} was still devoid of any side effect (Figure 7a). Similarly, purified hypericin, hyperforin, quercetin, hyperoside and amentoflavone, administered at a concentration corresponding to the content present in 30 mg kg^{-1} p.o. preparation of SJW dried extract, were devoid of any effect on the locomotor behaviour (Figure 7b).

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4. Discussion

We report here the first description of the capability of a SJW dried extract to reduce hyperalgesia in rat models of neuropathic pain after a single oral administration.

Painful neuropathy is a condition that may develop when nerve fibres are damaged or dysfunctional. Since it can arise from a wide variety of injuries, two different models of neuropathic pain were used. The chronic constriction injury of the sciatic nerve was used as a model of neuropathy being induced by nerve damage whereas the repeated administration of oxaliplatin represented a chemotherapy-induced neuropathic pain model. A single oral administration of SJW reversed mechanical hyperalgesia in both models of neuropathic pain producing a prolonged increase of the pain threshold that continued up to 120 min. The efficacy of SJW in the management of neuropathic pain, regardless of the kind of injury responsible for the painful condition, was suggested. A clinical study conducted in patients suffering from painful polyneuropathy reported a trend of lower pain scores when treated with SJW than did the patients who were treated with a placebo. A statistically significant relief of pain was not obtained [28], and this discrepancy might be explained by considering that the SJW-induced antinociception has a bell-shaped trend. The authors looked for an increase of the pain threshold at a concentration (2.7 mg/die total hypericins) similar to that which was employed in trials with depressed patients. This concentration was about 30 times higher than the analgesic concentration (0.096 mg total hypericins) and, as demonstrated by dose-response experiments, at this level the antinociceptive efficacy of SJW is strongly reduced.

At doses able to reverse hyperalgesia, SJW induced mechanical antinociception in untreated non-painful rats (control groups) as well as in the controlateral uninjured hind limbs of CCI rats. These reports confirm and support the hypothesis for the role that SJW has in the modulation of pain perception. This hypothesis has been suggested in a recent paper that indicates the antinociceptive properties of SJW in the mouse formalin test [29].

The main components of the SJW dried extract are phloroglucinols, naphtodiantrones and flavonoids. The effects produced by these constituents were investigated in order to discover the

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components responsible for the antinociceptive activity and to better elucidate the SJW mechanism of analgesic action. 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. CHL fraction reversed the mechanical hyperalgesia in all neuropathic pain models and increased the pain threshold in the control non-painful animals. The intensity of the CHL fraction activity was lower than that of the SJW dried extract, but a similar time-course profile was observed. The MET fraction was as effective as the CHL in relieving neuropathic pain. This indicates that the SJW main components present in both fractions are responsible for the antihyperalgesic properties of the plant. It is interesting to note that MET, conversely to CHL, was devoid of any analgesic activity in the non-painful rats. These results might suggest a selective role of hypericin/flavonoid in the relief of neuropathic pain, rather than being effective as a direct result of their antinociceptive properties.

The SJW antinociception underlies the activation of the opioid system as this analgesia is prevented by the opioid antagonist naloxone. Binding of [^{3}H]naloxone to the μ- and k-opioid receptor was inhibited in the presence of SJW extract [30], further supporting a SJW opioid mechanism. Phloroglucinols might be responsible for this opioid effect since hyperforin inhibited binding to the opioid receptors with a higher affinity than hypericin and flavonoids [30,31]. The prevention by naloxone of CHL-induced antinociception further supports this hypothesis. CHL fraction contains phloroglucinols together with a small amount of the flavonoid biapigenin that binds to the opioid receptor with a much lower affinity than hyperforin [31]. The administration of purified hyperforin produced an antinociceptive effect comparable to that induced by the CHL fraction, which further suggests the prominent role of hyperforin in the CHL activity.

The MET fraction contains hypericins and flavonoids, both of which might be potentially involved in the SJW mechanism of antinociceptive action. The total amount of hypericins add up to about 0.3% of the SJW dried extract and have long been known to be related to many pharmacological properties of SJW. Recent studies report antidepressive, antineoplastic, antitumor and antiviral activities of hypericin [18]. Flavonoids contribute to the antinflammatory effects of the

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plant [32] and antinociceptive properties have been reported for the flavonoid myricitrin [33]. Enzyme assays performed on rat brains demonstrated that hypericin and pseudohypericin are potent and selective inhibitors of the protein kinase C (PKC) [34,18]. In vitro studies reported that some flavonoids inhibit rat brain PKC activity. Among the flavonoids, quercetin is one of the most significant [35]. PKC is a family of enzymes involved in numerous important cellular events, including pain modulation. PKC integrates numerous receptor pathways into final effectors that increase excitatory signalling and decrease inhibitory signalling, thus inducing pain. 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 antagonized the MET-induced antinociception, whereas with the CHL-induced, the increase of the pain threshold remained unmodified. Conversely to the CHL fraction, the MET fractions mechanical antihyperalgesia remains unmodified by the naloxone pre-treatment. These results suggest that a selective blockade of the PKC-mediated intracellular pathway underlies the MET activity.

PKC is a family of serine/threonine kinases that are divided into three groups based on calcium and diacylglicerol dependence: conventional (α, ßI, ßII, γ), novel (δ,ε,η,θ), atypical (ζ, λ/ι) [37]. Among them, PKCε and PKCγ isoforms appear to play a prominent role in the modulation of pain perception [36,38,39]. In particular, the translocation of these enzymes from the cytosol to the synaptic membrane is thought to be necessary for their activation [40]. Increased translocation and activation of PKC in dorsal horn neurons has been shown in a number of pain models. This suggests the importance in the modulation of pain perception of spinally located PKC [36]. We have detected a specific upregulation of PKCγ and PKCε isoforms in the PAG area of neuropathic rats along with a robust increase of the PKCγ and PKCε phosphorilation. Furthermore, we discovered a lack of increase in any of the phosphorylation of PKCε and PKCγ in the cytosol in comparison with the high phosphorilated form levels observed in the membrane fraction of neuropathic rats. These results have highlighted the importance of supraspinally located PKC in the modulation of the sensation of pain. This suggests that in the presence of neuropathies, the activation of the PKC-mediated pathway is not only confined to the spinal cord. The SJW oral

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administration produced a complete reversal of the phosphorylation of both PKCε and PKCγ in the total protein preparation as well as in the membrane fraction. These data suggest the hypothesis of a PKC-inhibiting mechanism in SJW for antinociception and indicate the isoforms ε and γ of PKC as potential targets for this antinociceptive effect. Furthermore, the importance of the inhibition of the PKC activity in order to treat neuropathic pain is further confirmed.

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 the MET fraction. Conversely, hyperoside, which is the most abundant flavonoid present in the SJW dried extract, neither increased the pain threshold nor potentated the hypericin-induced antinociception. Anti-inflammatory properties were reported for the flavonoid amentoflavone [41], but, when administered alone in neuropathic rats, it was devoid of any antinociceptive effect. Quercetin was unable to potentate hypericin antinociception when co-administered, even if it resulted in being slightly effective in increasing the pain threshold. This is not surprising since quercetin, which is similar to hypericin, has PKC-blocking properties. A quercetin potency about 30 times lower [35] than hypericin [34] might explain the lower efficacy observed in behavioural tests. Hypericin appears, therefore, to be the most effective component of the MET fraction.

An important drawback of the current analgesic therapy of neuropathic pain is the high occurrence of side effects [42]. The potential induction of side effects by SJW and components at the doses able to increase the pain threshold was, therefore, investigated. In accordance with the literature data that indicate SJW as a medicinal plant with a favourable tolerability and safety profile [15], the SJW dried extract, CHL and MET fractions, at the highest active doses employed in the present study, caused neither detectable modification in the rats gross behaviour, nor did they alter their locomotive activity. Similar profile was also demonstrated by purified SJW main components.

In conclusion, our findings showed that a single oral administration of SJW, CHL and MET fractions, as well as purified hyperforin and hypericin, relieve neuropathic pain in rats with a very

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positive side effects profile. This increase of the pain threshold is related to the modulation of two different intracellular pathways: the activation of the opioid system, underling the presence of hyperforin, which is responsible for the antinociceptive properties, and a PKC-mediated mechanism through the PKC-blocking properties of hypericin, mainly related to the antihyperalgesic activity. Finally, these results further support the hypothesis that the inhibition of PKC specific isoforms might be a promising strategy for effective treatment of neuropathic pain.

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Figure 1. Dose-response curves for the antinociceptive and antihyperalgesic effect of SJW dried extract in the chronic constriction injury of the sciatic nerve (CCI) and repeated oxaliplatin treatment (OXA) rat model of neuropathic pain. ***(a)*** Dose-response curve of SJW dried extract (1-100 mg kg^{-1} p.o.) for the antinociceptive (uninjured leg; SJW 10 mg kg^{-1} p.o. ($F(1,22)6.730$ $p<0.05$; SJW 30 mg kg^{-1} p.o. ($F(1,17)35.317$ $p<0.001$) and antihyperalgesic (injured leg; SJW 30 mg kg^{-1} p.o. $F(4,44)10.690$ $p<0.001$) effect in the CCI rats. Data were recorded 90 min after SJW administration. Experiments were performed 14 days after surgery when a significant mechanical hyperalgesia was obtained ($F(1,19)44.631$ $p<0.001$) *P<0.001 in comparison with values recorded before treatment in the corresponding leg; ^P<0.001 in comparison with the uninjured leg. ***(b)*** Time-course curve of SJW dried extract (30 mg kg^{-1} p.o.) for the antinociceptive activity in naïve rats. *P<0.01 in comparison with CMC-treated group. ***(c)*** Time-course curve of the mechanical hyperalgesia induced by repeated administration of oxaliplatin in comparison with saline-treated rats. A statistically significant hyperalgesia appeared on day 21 ($F(1,14)22.345$ $p<0.001$); *P<0.001 compared to saline control group. ***(d)*** Dose-response curve of SJW dried extract (10-100 mg kg^{-1} p.o.) for the antinociceptive (saline-treated group; SAL) and antihyperalgesic (OXA; $F(4,42)12.718$ $p<0.001$) effect in the rat OXA model of neuropathic pain. Data were recorded 90 min after SJW administration. *P<0.001 in comparison with corresponding values recorded before treatment, ^P<0.001 compared to saline control group. The experiments were carried out on day 21.

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Figure 2. Time-course curves for the antinociceptive and antihyperalgesic effect of SJW dried extract in rat models of neuropathic pain induced by chronic constriction injury of the sciatic nerve (CCI) and repeated administration of oxaliplatin (OXA). Time-course curves of SJW dried extract (30 mg kg^{-1} p.o.) in the CCI model for the antinociceptive (uninjured leg) ***(a)*** and antihyperalgesic (injured leg) ***(b)*** effect. *P<0.05 in comparison with CMC-treated group. All data are presented as mean ± s.e.m. (***c)*** Time-course curves of SJW dried extract (30 mg kg^{-1} p.o.) in the OXA model for the antinociceptive (SAL) and antihyperalgesic (OXA) effect. *P<0.05 in comparison with SAL before treatment; ^P<0.05 in comparison with OXA before treatment. All data are presented as mean ± s.e.m. The experiments were carried out on day 21.

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Figure 3. Antinociceptive profile of SJW fractions containing the SJW major components in rat models of neuropathic pain. (***a)*** Antinociceptive (uninjured leg; F(3,35)5.422 p<0.001) and antihyperalgesic (injured leg) effect of the chloroform (CHL) fraction, administered in an amount containing 0.978 mg kg^{-1}hyperforin, in the CCI model. Data are presented as mean ± s.e.m.; *P<0.001 in comparison with the uninjured leg before treatment; ^P<0.01 in comparison with the injured leg before treatment. ***(b)*** Antihyperalgesic (injured leg; F(3,32) 6.002 p<0.001) effect of the methanol (MET) fraction (30 mg kg^{-1}), administered in an amount containing 0.06 mg kg^{-1} hypericin, in the CCI model. Data are presented as mean ± s.e.m.; *P<0.001 in comparison with uninjured leg before treatment; ^P<0.05 in comparison with injured leg before treatment. ***(c)*** MET prevented the mechanical hyperalgesia induced by oxaliplatin (OXA; F(3,33) 7.244 p<0.001) administration without inducing antinociception in the saline (SAL) group. Data are presented as mean ± s.e.m.; *P<0.001 in comparison with SAL; ^P<0.001 in comparison with OXA. The experiments were carried out on day 21.

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Figure 4. Antinociceptive profile of purified components of CHL and MET fractions in the CCI model of neuropathic pain. ***(a)*** Purified hyperforin (0.978 mg kg^{-1}) showed antinociceptive (uninjured leg) and antihyperalgesic (injured leg) properties with a similar profile to CHL ($F(3,32)4.871$ $p<0.001$). Data are presented as mean ± s.e.m.; *P<0.001 in comparison with uninjured leg before treatment; ^P<0.05 in comparison with injured leg before treatment. ***(b)*** Hypericin (0.06 mg kg^{-1}), similarly to MET, reversed the mechanical hyperalgesia (injured leg; $F(3,35)3.992$ $p<0.001$) without increasing the pain threshold in the non-hyperalgesic (uninjured) leg. Data are presented as mean ± s.e.m.; *P<0.001 in comparison with uninjured leg before treatment; ^P<0.001 in comparison with injured leg before treatment. ***(c)*** Lack of effect on pain threshold of the purified flavonoids amentoflavone (AME, 0.016 mg kg^{-1}) and hyperoside (HypS, 1.905 mg kg^{-1}). Quercetin (QUER, 0.249 mg kg^{-1}) showed a modest antihyperalgesic effect ($F(1,14)3.071$ $p<0.05$). Data are presented as mean ± s.e.m.; Data were recorded 90 min after administration of SJW constituents. *P<0.05 in comparison with uninjured leg before treatment; ^P<0.01 in comparison with injured leg before treatment. ***(d)*** Amentoflavone (AME), hyperoside (HypS) and quercetin (QUER) were unable to potentate the hypericin-induced antihyperalgesic effect. Data were recorded 90 min after administration of SJW constituents. Data are presented as mean ± s.e.m. *P<0.01 in comparison with uninjured leg before treatment; ^P<0.05 in comparison with injured leg before treatment. Each component was administered at a dose corresponding to the amount of each component present in a 30 mg kg^{-1} preparation of SJW dried extract.

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Figure 5. Involvement of the opioid system in the antinociceptive activity of SJW and CHL fraction in the CCI model of neuropathic pain. ***(a)*** Prevention by naloxone (1 mg kg^{-1} i.p.) of SJW dried extract (30 mg kg^{-1} p.o.) antinociception (naïve, sham-operated, uninjured leg; F(1,18)9.487 p<0.001). Lack of effect on the antihyperalgesic activity (injured leg; F(1,14)2.714 p=0.121). Data were recorded 90 min after administration of SJW. Data are presented as mean ± s.e.m.; *P<0.001 in comparison with uninjured leg before treatment; ^P<0.01 in comparison with injured leg before treatment. ***(b)*** Prevention by naloxone of CHL (administered in a dose containing 0.978 mg kg^{-1}hyperforin) antinociception (naïve, sham-operated, uninjured leg; F(1,16)8.422 p<0.001) and antihyperalgesia (injured leg; F(1,16)5.612 p<0.01). Data were recorded 90 min after administration of CHL. Data are presented as mean ± s.e.m.; *P<0.05 in comparison with uninjured leg before treatment; ^P<0.05 in comparison with injured leg before treatment. ***(c)*** Lack of effect of naloxone on MET (administered in a dose containing 0.06 mg kg^{-1} hypericin) antihyperalgesia (injured leg). Data were recorded 90 min after administration of MET. Data are presented as mean ± s.e.m.; *P<0.05 in comparison with uninjured leg before treatment; ^P<0.05 in comparison with injured leg before treatment.

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Figure 6. Blockade of the PKC activity as mechanism of the antihyperalgesic effect of SJW and MET fraction in the CCI model of neuropathic pain. ***(a)*** The administration of the PKC activator PMA (15 pmol per mouse i.c.v.) significantly reduced the SJW dried extract (30 mg kg^{-1} p.o.) antihyperalgesic activity (F(1,14)5.729 p<0.001) whereas the antinociception was only slightly reduced (F(1,14)2.714 p=0.0487). Data were recorded 90 min after administration of SJW. ***(b)*** Prevention by the PKC activator PMA of MET antihyperalgesic activity (injured leg; F(1,15)6.231 p<0.001). Data were recorded 90 min after administration of MET. ***(c)*** Lack of effect of PMA on the CHL-induced antinociception (uninjured leg) and antihyperalgesia (injured leg). Data were recorded 90 min after administration of CHL. Data are presented as mean ± s.e.m.; *P<0.001 in comparison with uninjured leg; ^P<0.01 in comparison with injured leg. ***(d)*** The CCI induced an increase in the PKCγ and PKCε isoforms levels observed in the total protein preparation (TOT; PKCγ: F(1,10)3.991 p<0.01; PKCε: F(1,10)4.961 p<0.01) and in the membrane (MEM) and cytosol (CYT) fractions from the periaqueductal grey (PAG) area of neuropathic rats. A dramatic increase of the phosphorylation of PKCγ and PKCε was observed in the total protein preparation (TOT) and membrane (MEM) fraction whereas in the cytosol (CYT) fraction no variation was detected. SJW decreased the phosphorylation of PKCγ and PKCε isoforms up to control values. Densitometric values are reported as percentage of control. *P<0.01 in comparison with control group; §P<0.05 in comparison with CCI.

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Figure 7. Lack of induction of locomotor side effects by SJW dried extract, fractions and purified components. ***(a)*** Lack of effect of SJW dried extract, CHL and MET fractions on rat motor coordination evaluated in the rat rota rod test. Effects produced by amitriptyline (AMI) at the doses of 10 and 30 mg kg^{-1}, used as reference antinociceptive drug. *$P < 0.05$ in comparison with CMC-treated rats. ***(b)*** Purified SJW main components showed the lack of any influence on locomotor behaviour. A total of 4-5 rats per group were tested.

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*Graphical Abstract

SJW relieves neuropathic pain in different rat models through a decrease of PKC γ and ε isoform expression and phosphorylation.

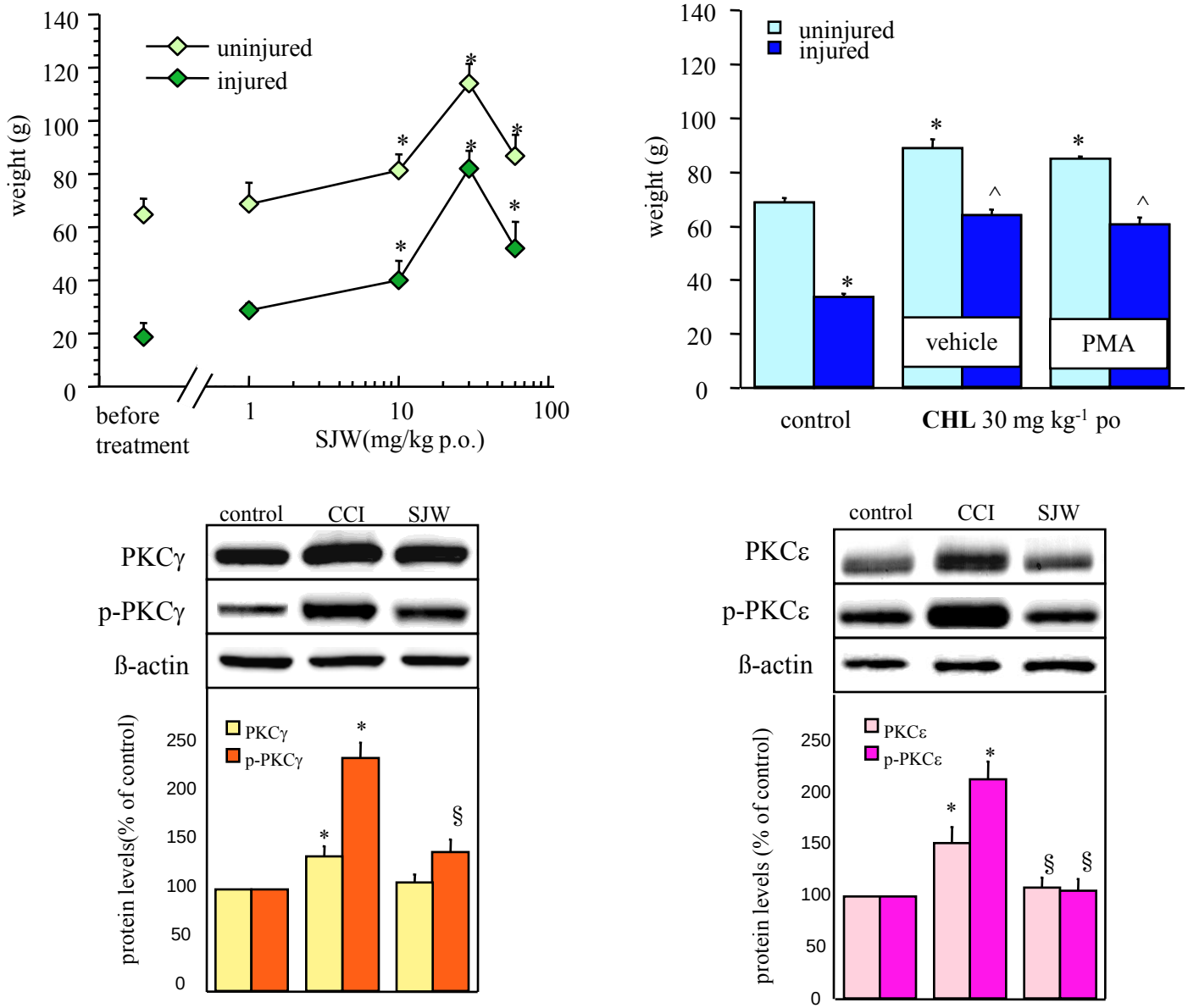

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Table 1. Composition of the SJW dried extract, CHL and MET fractions

		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		

CHL: chloroform fraction; MET: methanol fraction. The content of each constituent is expressed as percentage (mg/100 mg)

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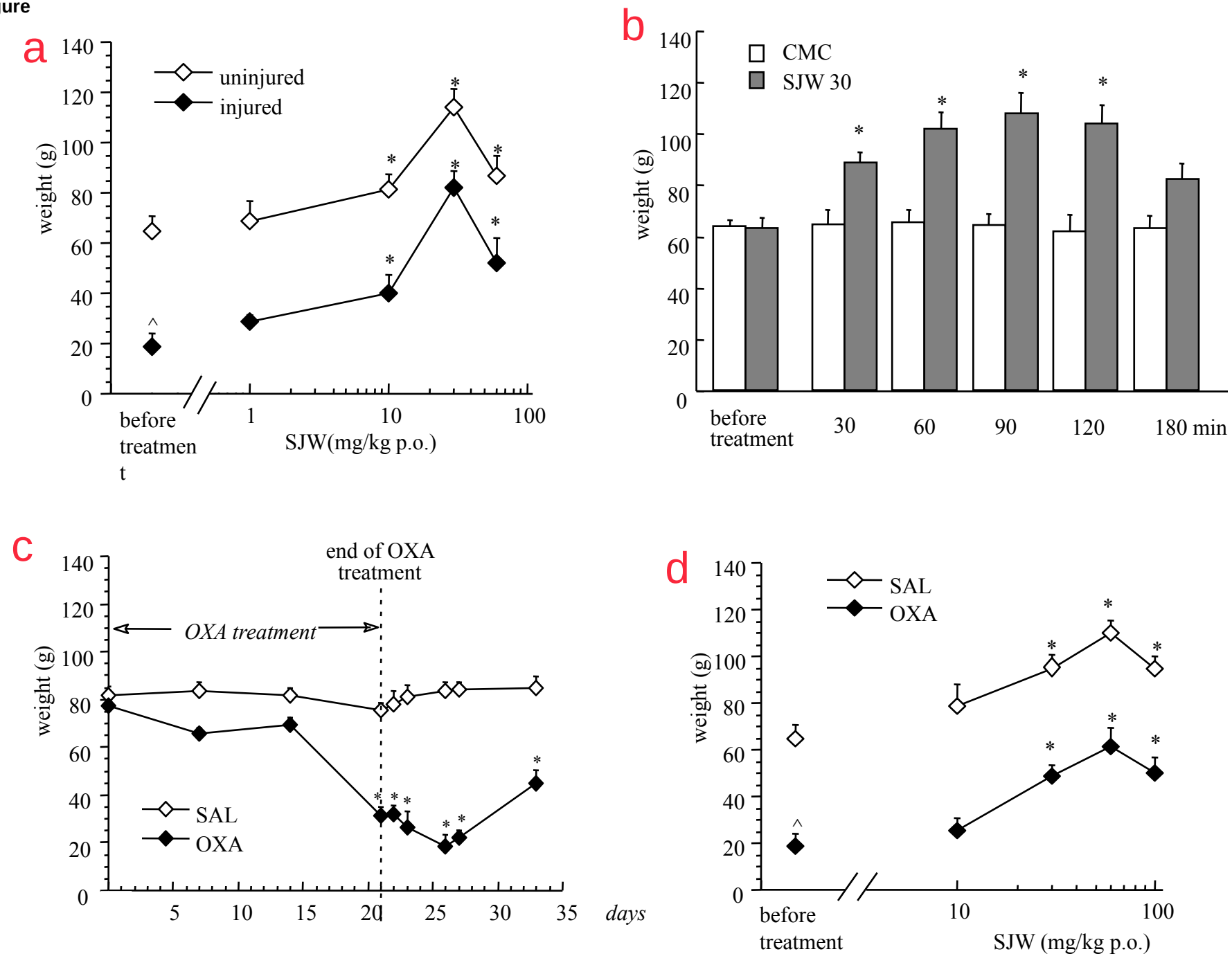
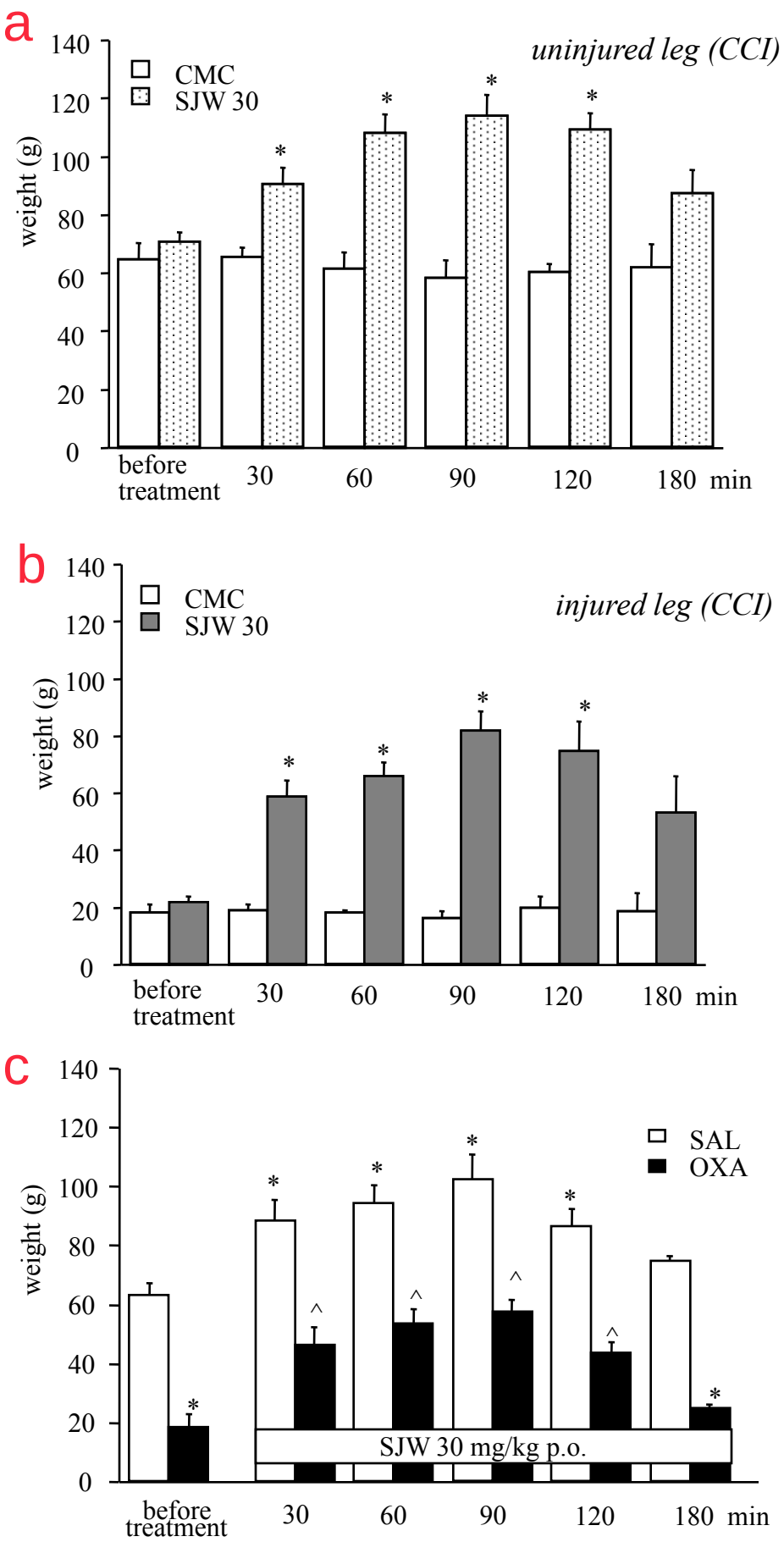
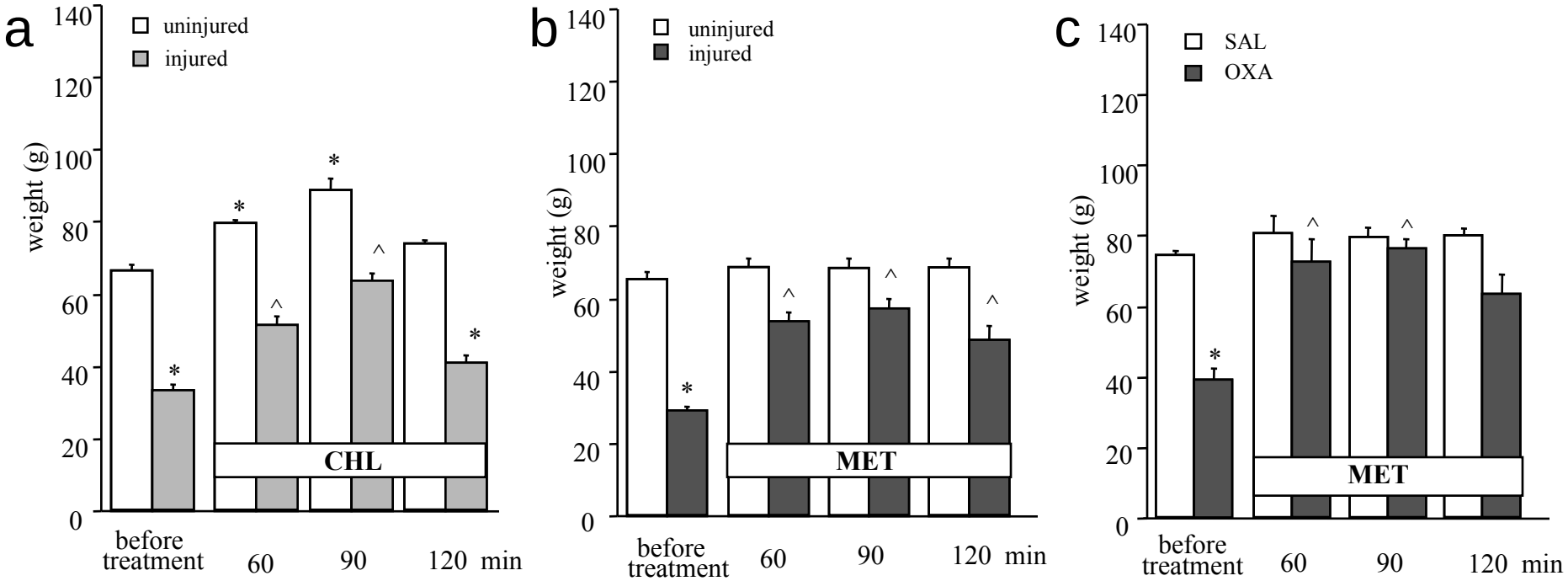
Figure


a
uninjured
injured
weight (g)
before treatment
1
10
100
SJW(mg/kg p.o.)
b
CMC
SJW 30
weight (g)
before treatment
30
60
90
120
180 min
c
end of OXA treatment
OXA treatment
weight (g)
SAL
OXA
days
d
SAL
OXA
weight (g)
before treatment
10
100
SJW (mg/kg p.o.)

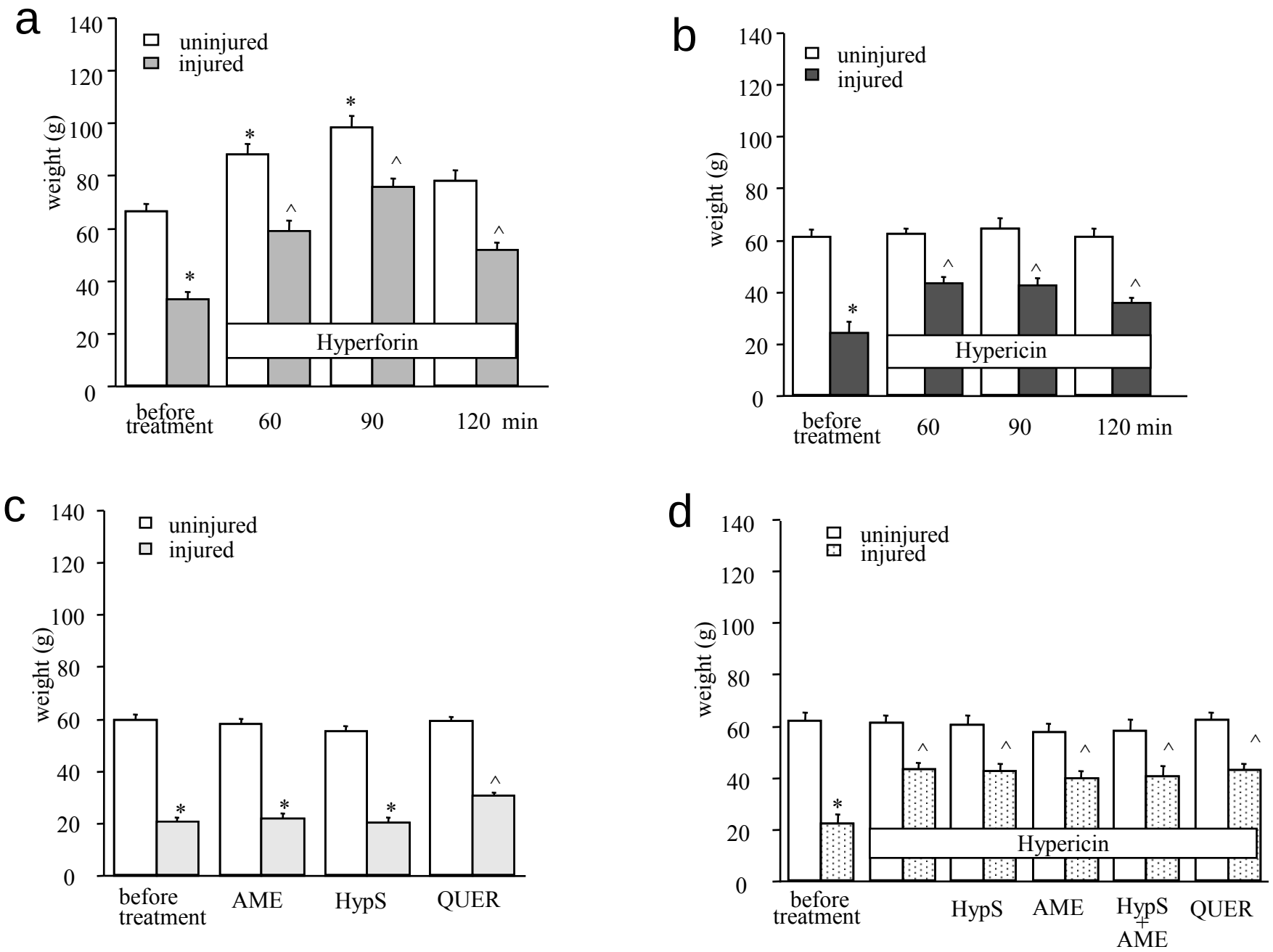
Figure


a
uninjured leg (CCI)
CMC
SJW 30
weight (g)
140
120
100
80
60
40
20
0
before treatment
30
60
90
120
180 min
b
injured leg (CCI)
CMC
SJW 30
weight (g)
before treatment
30
60
90
120
180 min
c
SAL
OXA
weight (g)
SJW 30 mg/kg p.o.
before treatment
30
60
90
120
180 min

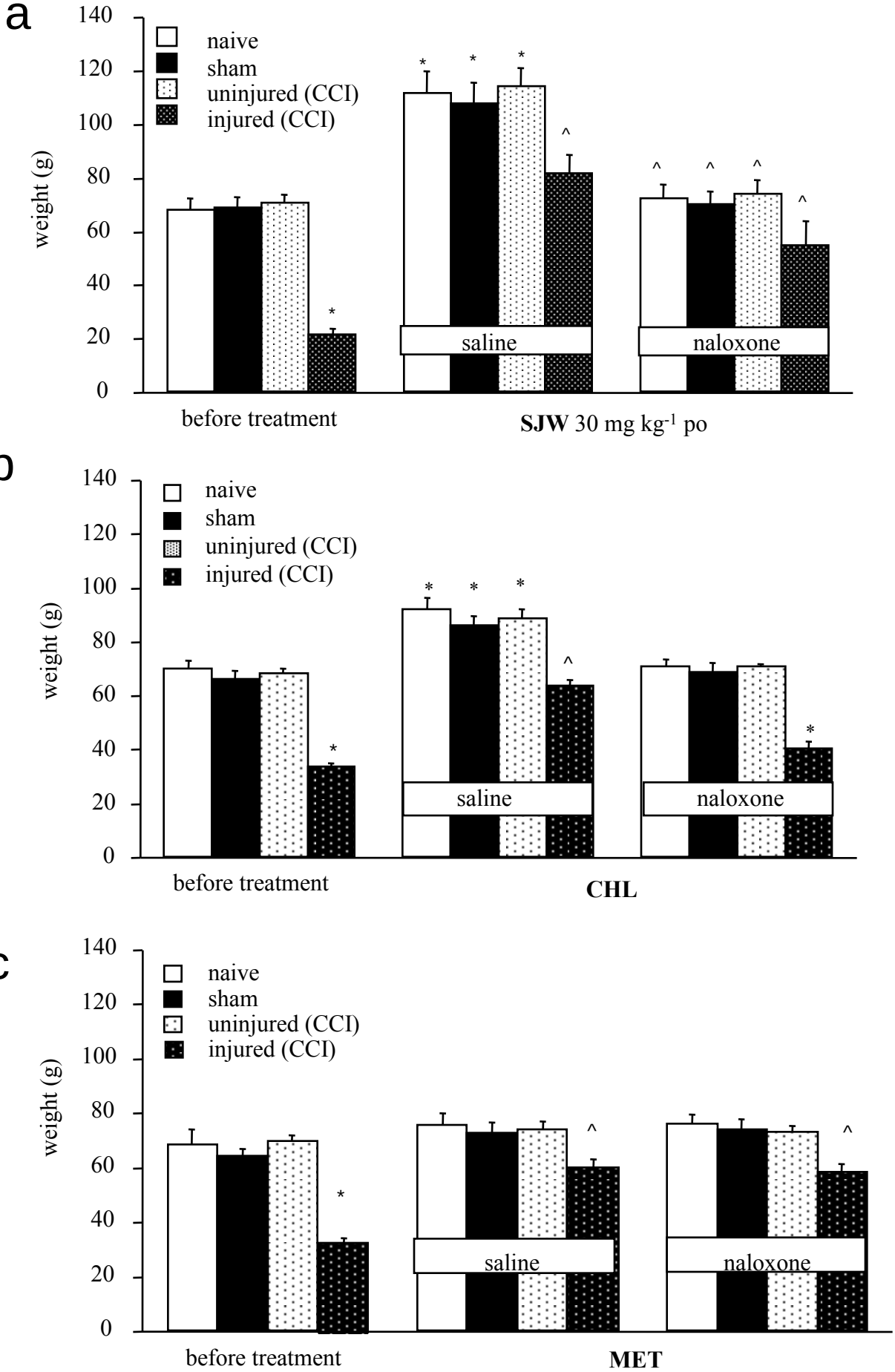
Figure


a
uninjured
injured
weight (g)
140
120
100
80
60
40
20
0
CHL
before treatment
60
90
120
min
b
uninjured
injured
weight (g)
MET
before treatment
60
90
120
min
c
SAL
OXA
weight (g)
MET
before treatment
60
90
120
min

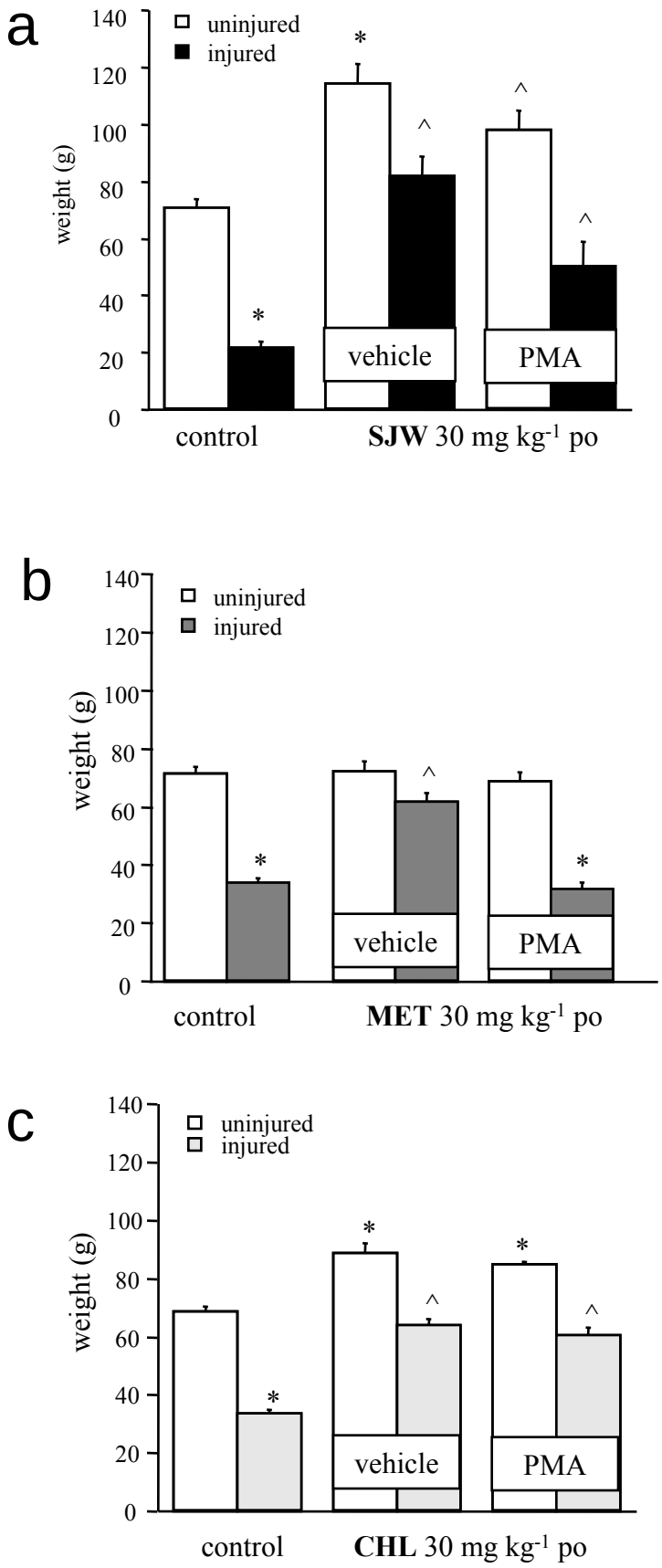
Figure


a
uninjured
injured
weight (g)
140
120
100
80
60
40
20
0
Hyperforin
before treatment
60
90
120 min
b
uninjured
injured
weight (g)
Hypericin
before treatment
60
90
120 min
c
uninjured
injured
weight (g)
before treatment
AME
HypS
QUER
d
uninjured
injured
weight (g)
Hypericin
before treatment
HypS
AME
HypS + AME
QUER

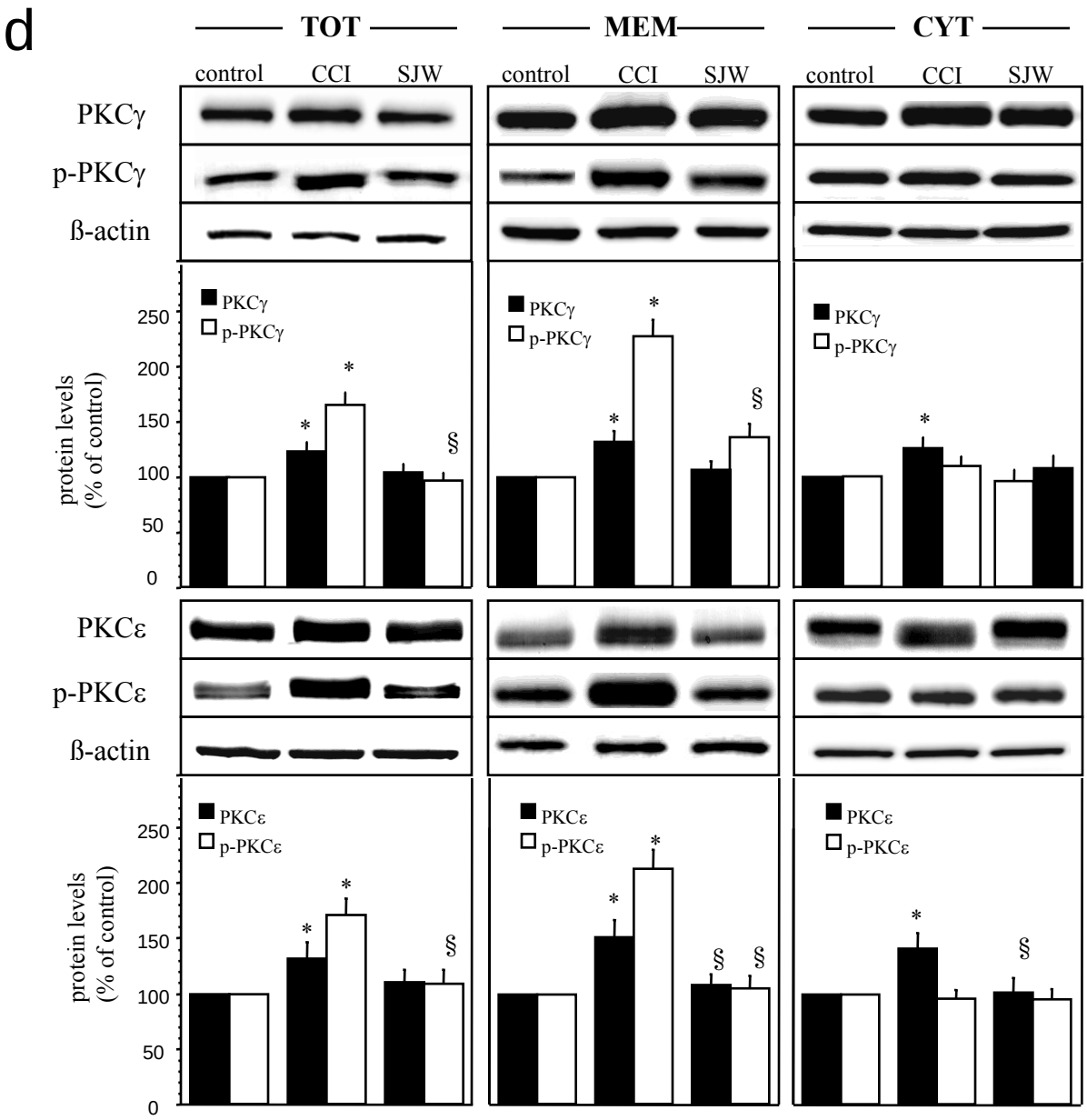
Figure


a
naive
sham
uninjured (CCI)
injured (CCI)
weight (g)
140
120
100
80
60
40
20
0
saline
naloxone
before treatment
SJW 30 mg kg-1 po
b
naive
sham
uninjured (CCI)
injured (CCI)
weight (g)
saline
naloxone
before treatment
CHL
c
naive
sham
uninjured (CCI)
injured (CCI)
weight (g)
saline
naloxone
before treatment
MET

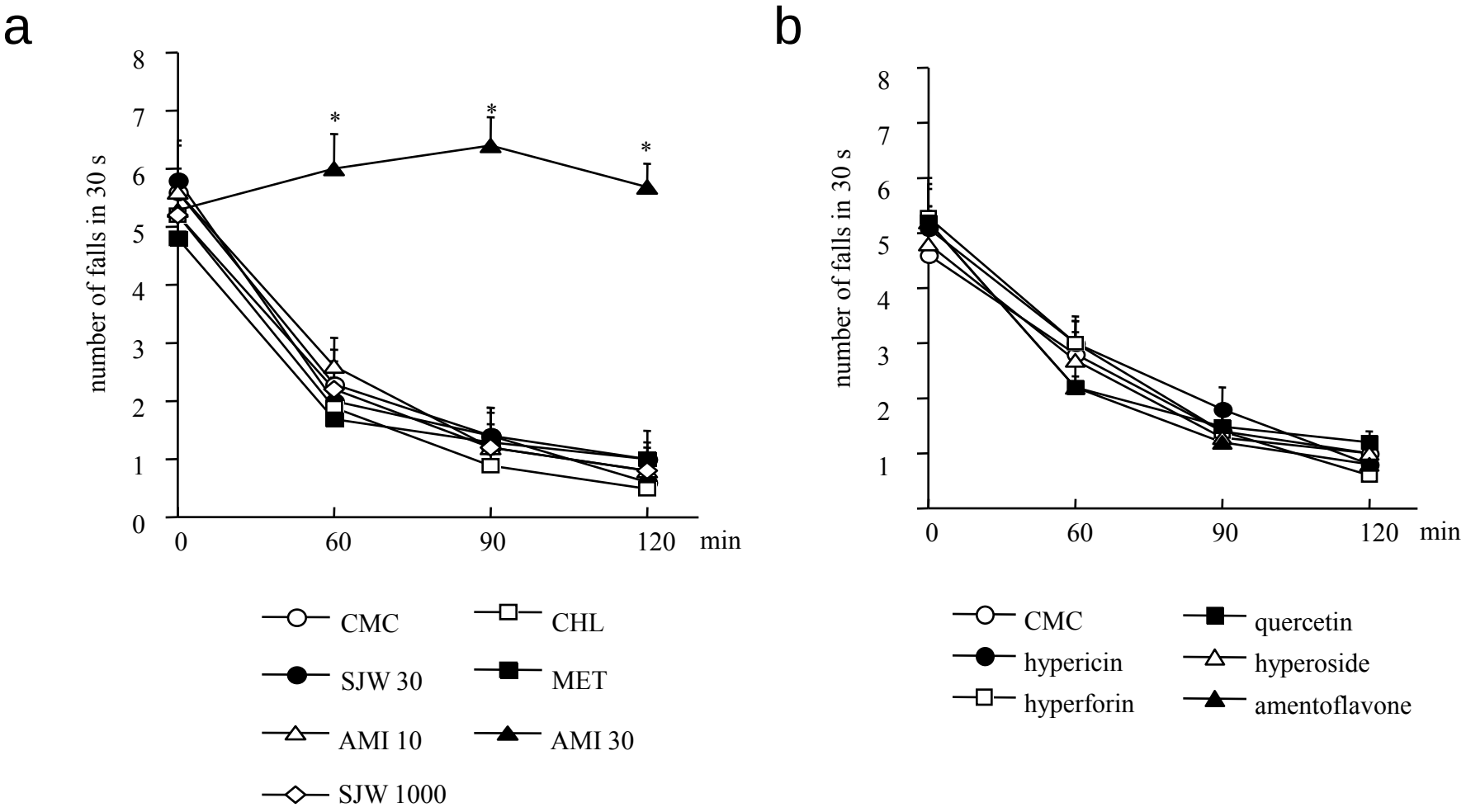
Figure


a
uninjured
injured
weight (g)
140
120
100
80
60
40
20
0
*
^
vehicle
PMA
control
SJW 30 mg kg-1 po
b
uninjured
injured
weight (g)
vehicle
PMA
control
MET 30 mg kg-1 po
c
uninjured
injured
weight (g)
vehicle
PMA
control
CHL 30 mg kg-1 po

Figure


d
TOT
MEM
CYT
control
CCI
SJW
PKCγ
p-PKCγ
ß-actin
PKCε
p-PKCε
protein levels
(% of control)
0
50
100
150
200
250
*
§

Figure


a
number of falls in 30 s
0
1
2
3
4
5
6
7
8
*
*
*
0
60
90
120
min
CMC
CHL
SJW 30
MET
AMI 10
AMI 30
SJW 1000
b
number of falls in 30 s
1
2
3
4
5
6
7
8
0
60
90
120
min
CMC
quercetin
hypericin
hyperoside
hyperforin
amentoflavone