Withania somnifera (L.) Dunal root extract alleviates formalin-induced nociception in mice: involvement of the opioidergic system

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Withania somnifera **(L.) Dunal extracts (WSEs) may possess therapeutic perspectives in the treatment of inflammation and pain. We aimed to evaluate the antinociceptive property of a WSE in the formalin test and to investigate the involvement of several neurotransmitter systems in this effect. The time spent licking the formalin-injected paw was recorded in CD1 mice after pretreatment with increasing doses of WSE. Also, c-Fos spinal cord expression and the effects of different compounds were investigated under these experimental conditions. Finally, the efficacy of WSE was analyzed following an injection of glutamate. WSE reduced the antinociceptive response during the tonic but not the acute phase of the formalin test and decreased formalin-induced c-Fos expression in spinal neurons. These effects were antagonized by the opioid antagonist naltrexone, whereas GABA, cannabinoid, δ-opioid, and nitric oxide compounds were ineffective. The administration of WSE also reduced nociception and c-Fos expression induced by glutamate injection. These results showed that WSE is effective in assays of chemical-induced nociception, indicating that this plant has potential valuable properties for the treatment of specific painful conditions. The antinocicetive effects of WSE in the formalin test appeared to be specifically mediated by the opioidergic system, although the involvement of the glutamatergic system cannot be excluded.** ***Behavioural Pharmacology*** **27:57–68**

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Introduction

The use of medicinal plants can represent a valid therapeutic support to conventional medicine in the treatment of a wide spectrum of diseases. Besides their direct benefits, medicinal plants are a potential source of pharmacologically active ingredients and lead compounds for rational drug design. Notably, natural or natural-derived products represented 39% of the pharmacological agents approved between 1981 and 2010 (Newman and Cragg, 2012).

Among medicinal plants, *Withania somnifera* (L.) Dunal is attracting a growing interest for its broad spectrum of pharmacological activity. *W. somnifera* (L.) Dunal (WS, family: Solanaceae) is a plant used in Ayurvedic medicine (India's traditional medical system) to treat several diseases (Alam *et al.*, 2012). Preclinical studies have confirmed that *W. somnifera* (L.) Dunal extracts (WSEs) or its constituents have anticancer, anti-inflammatory, immunomodulatory, adaptogenic, neuroprotective, and anti-addictive properties (Alam *et al.*, 2012; Ruiu *et al.*, 2013), suggesting the potential role of this plant as a therapeutic agent. Preliminary clinical studies have recently shown that WSE can improve cognitive performance in healthy individuals (Pingali *et al.*, 2014) and in patients with bipolar disorder (Chengappa *et al.*, 2013), as well as decrease anxiety levels (Cooley *et al.*, 2009) and promote general well-being in breast cancer patients (Biswal *et al.*, 2013).

A new and intriguing therapeutic perspective of WSE is based on preclinical studies that suggest its ability to promote analgesia and alleviate inflammatory states. The administration of WSE alleviates inflammatory nociception in collagen-induced arthritis in rats (Gupta and Singh, 2014), but is devoid of antinociceptive activity in animal models of acute nociception (Kulkarni and Ninan, 1997; Orrù *et al.*, 2014), although its coadministration with morphine prolonged morphine analgesia (Orrù *et al.*, 2014) and prevented the emergence of morphine-induced hyperalgesia (Orrù *et al.*, 2014) and analgesic tolerance (Kulkarni and Ninan, 1997). It has been suggested that some of these effects might be induced, at least in part, by the ability of WSE to counteract morphine-induced sensitization, in which the glutamatergic system plays a prominent role (Orrù *et al.*, 2014). Finally, evidence exists supporting the anti-inflammatory properties of WSE. In fact, the administration of WSE

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reduces levels of inflammation markers in several animal models of inflammatory-based diseases such as lupus erythematosus (Minhas *et al.*, 2011), arthritis (Rasool and Varalakshmi, 2006), and inflammatory bowel disease (Pawar *et al.*, 2011). Overall, this experimental evidence suggests that WSE can exert a dual beneficial effect in the treatment of pain conditions characterized by a strong inflammatory component (Rasool and Varalakshmi, 2006; Minhas *et al.*, 2011; Pawar *et al.*, 2011) and it can promote analgesia when the nociceptive system is dysregulated (Kulkarni and Ninan, 1997; Orrù *et al.*, 2014).

Keeping in mind this possibility, the first aim of this study was to evaluate the ability of WSE to modulate nociceptive responses in the formalin test, a well-established animal model for assessing tonic pain and for identifying compounds with analgesic activity. The intraplantar injection of formalin elicits a biphasic nociceptive response: an early phase promoted by the direct activation of nociceptors and a late response induced by a complex interplay between afferent activation, inflammation, and *N*-methyl-D-aspartate (NMDA) receptor-mediated sensitization (Sawynok and Liu, 2004). These behavioral responses are associated with an increase in c-Fos expression in the dorsal horn of the spinal cord, a marker of neuronal activation (Harris, 1998). Our study hypothesis was that WSE could alleviate the nociceptive response during the second phase of the formalin test as it has anti-inflammatory activity and it can counteract spinal sensitization; a corresponding decrease in c-Fos expression was also expected.

The second aim of this study was to identify the neurotransmitter systems potentially involved in the antinociceptive activity of WSE as no information exists on this topic. To achieve this goal, we evaluated the ability of several compounds to antagonize the effects of WSE in the formalin test. These compounds were chosen on the basis of the pharmacodynamic properties of WSE. We showed previously, through radioligand-binding studies, that WSE shows affinity for the $GABA_A$, $GABA_B$, NMDA, and opioid receptors (Orrù *et al.*, 2014); moreover, evidence exists suggesting the ability of WSE to modulate the production of nitric oxide (NO) (Iuvone *et al.*, 2003). This evidence led us to hypothesize the involvement of these receptors in the pharmacological effects of WSE in the formalin test.

Methods

Subjects

Male CD1 mice (Charles River, Calco, Italy), 20–25 g, were used. Animals were housed in an animal facility on a 12 h light/dark cycle (lights on from 07:00 h) at a constant room temperature of 21 ± 1°C (relative humidity $\sim 60\%$). Standard rodent chow and water were freely available. Animals were allowed to adapt to the animal facility conditions for at least 2 weeks after arrival. Procedures involving animals and their care were performed in accordance with institutional guidelines that are in compliance with national (DL 116/1992) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, 12 December 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Every effort was made to minimize pain and discomfort and to reduce the number of experimental subjects.

Plant material

The scientific name of WS was checked for accuracy according to the Plant List (*http://www.theplantlist.org*). The standardized methanolic root extract of *W. somnifera* (L.) Dunal (WSE), prepared according to a standardized procedure, was kindly provided by Natural Remedies Pvt. Ltd (Bangalore, India). Briefly, the WS root was authenticated at the National Institute of Science Communication and Information Resources (NISCAIR) (New Delhi, India), and was extracted with methyl alcohol by refluxing at 60–65°C in the manufacturing facility of M/s Natural Remedies Pvt. Ltd. The liquid extract was combined and concentrated by distillation under vacuum to a thick paste (I). The marc left after methyl alcohol extraction was further refluxed with water at 85–90°C. The liquid extract was combined and concentrated by distillation under vacuum to a thick paste (II). The concentrated extracts (I and II) were mixed and dried in a vacuum tray dyer at less than 70°C to obtain the final powdered extract.

WSE was dissolved in saline and administered intraperitoneally in a volume of 5 ml/kg. The dose of WSE and the route of administration for analgesia experiments were selected on the basis of previous studies (Ruiu *et al.*, 2013; Orrù *et al.*, 2014).

High-performance liquid chromatographic analysis

The methanolic WS root extract (batch number: WS/07030) has been characterized by a high-performance liquid chromatographic (HPLC)-fingerprint analysis, as certified by Natural Remedies Pvt. Ltd, with identification of the main withanolides (Kasture *et al.*, 2009). An HPLC system (Shimadzu, LC2010A; Kyoto, Japan) equipped with a UV detector, an autoinjector, and a column oven with class VP software was used. The stationary phase was an octadecylsilane column [Luna; Phenomenex (Torrance, California, USA); C18, 5 µm, 250×4.6 mm]. The mobile phase was a mixture of phosphate buffer (solvent A) (prepared by dissolving 0.136 g of KH_2PO_4 in 900 ml of HPLC grade water and by adding 10% dilute aqueous H_3PO_4, adjusting the pH to 2.8 ± 0.05 and making the volume of 1000 ml with water) and acetonitrile (solvent B). The following withanolides were identified: withanoside-IV, 0.49% w/w; physagulin D, 0.11% w/w; 27-hydroxywithanone, 0.01% w/w; withanoside-V, 0.33% w/w; withaferin-A, 0.11% w/w; 12-deoxy withastramonolide, 0.16% w/w; withanolide-A,

0.19% w/w; withanone 0.004% w/w; and withanolide-B, 0.03% w/w.

Formalin test

The formalin test was performed according to previously described procedures (Bannon and Malmberg, 2007). Mice were placed in a Plexiglass observation cylinder for 30 min to familiarize them to the experimental conditions. Mice were then pretreated with 0, 100, 150, or 200 mg/kg, intraperitoneal, of WSE. Formalin (20 µl of 5% formalin diluted in saline) was then injected into the hind paw 30 min after the administration of WSE and mice were placed back in the cylinder, where the time spent licking the formalin-injected paw was recorded for 45 min. Phases were defined as follows: first phase (0–10 min) and second phase (15–45 min). To investigate the potential mechanisms involved in the antinociceptive properties of WSE, antagonism studies were carried out. Mice were pretreated with AM630 (0.8 mg/kg), bicuculline (2 mg/kg), CGP35348 (100 mg/kg), flumazenil (10 mg/kg), naltrexone (3 mg/kg), naltrindole (1.5 mg/kg), or SR141716A (1 mg/kg) 10 min before the administration of WSE (150 mg/kg) or with glibenclamide (5 mg/kg), L-NAME (1 mg/kg) or L-arginine (600 mg/kg) 15 min before the administration of WSE (150 mg/kg). These dosages were selected as 'ineffective maximal doses' on the basis of previous preliminary studies carried out in our laboratory, by which we mean a dose devoid of the ability to modify the algesic threshold or to negatively impact on the mouse's general behavior. It should also be noted that the dosages used are consistent with those used in other studies (Moore *et al.*, 1991; Sabetkasai *et al.*, 1999; Choi *et al.*, 2003; Yano *et al.*, 2006; Maione *et al.*, 2008; Burgos *et al.*, 2010; Marinho *et al.*, 2013; Montiel-Ruiz *et al.*, 2013).

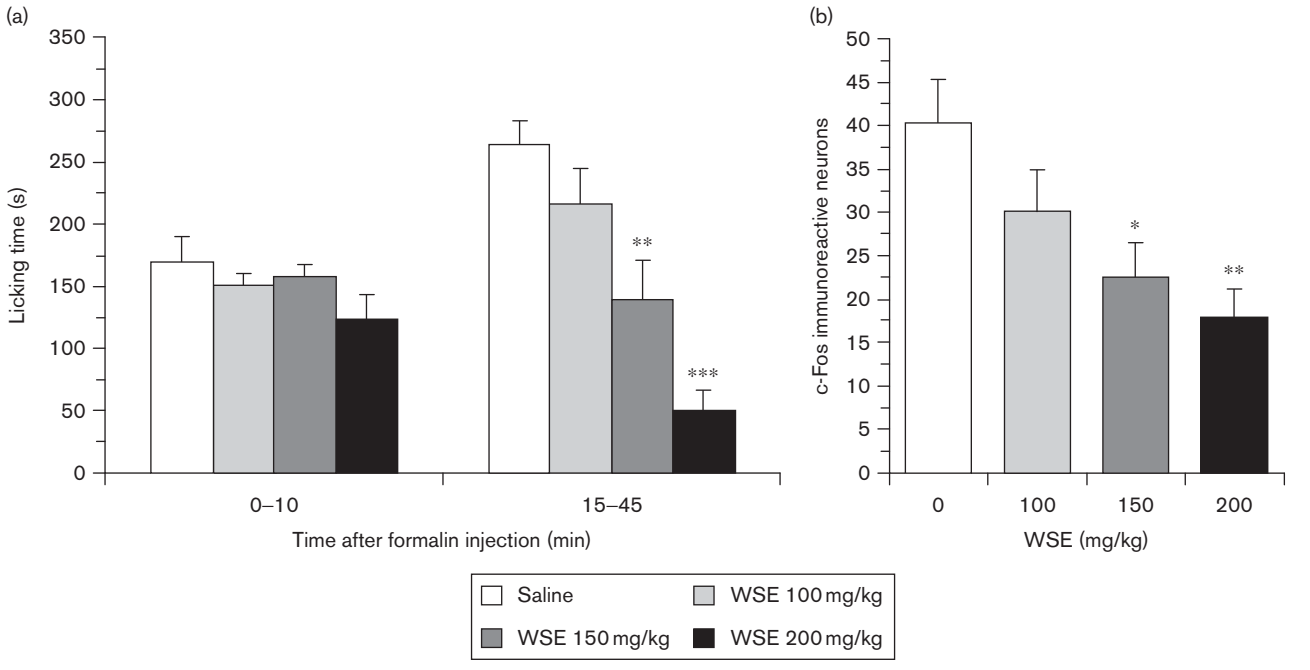
Glutamate-induced nociceptive test

The glutamate-induced nociceptive test was performed according to previously described procedures (Beirith *et al.*, 2002). Mice were placed in a Plexiglass observation cylinder for 30 min to familiarize them with the experimental conditions. Mice were then pretreated with 0, 100, or 150 mg/kg of WSE. Glutamate (2 µmol–20 µl diluted in saline) was then injected into the hind paw 30 min after the administration of WSE and mice were placed back in the cylinder, where the time spent licking the glutamate-injected paw was recorded for 15 min.

c-Fos immunohistochemistry

Thirty minute after completion of the test, mice were killed and lumbar spinal cord segments L4–L5 were immersed in 4% paraformaldehyde. After fixation, samples were cryoprotected overnight in a solution of 30% sucrose. Cross-sections of 40 µm were cut using a freezing

Fig. 1



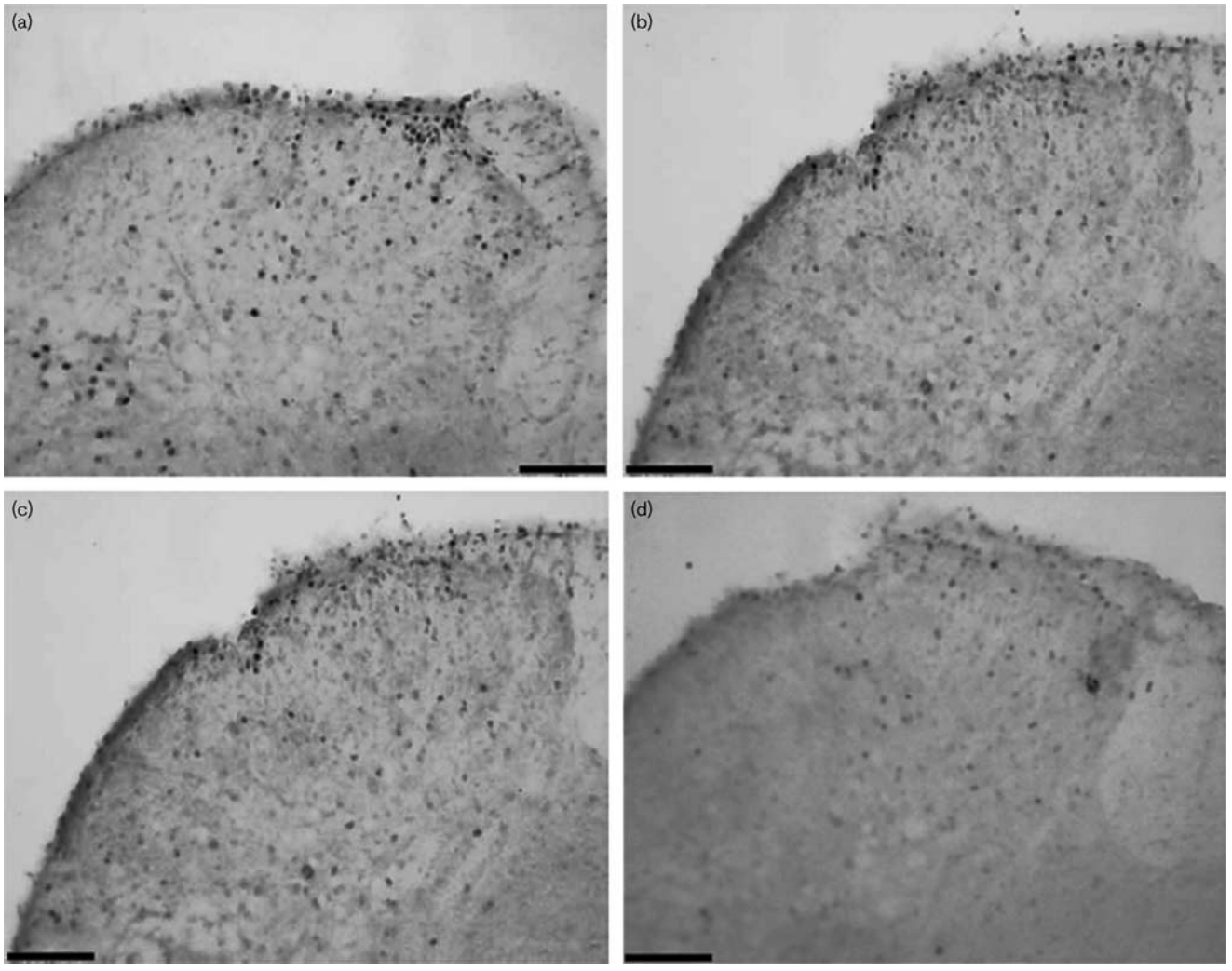
Effects of administration of *Withania somnifera* (L.) Dunal extract (WSE) on the formalin-induced nociceptive response (a) and formalin-induced c-Fos expression in the dorsal horn of the spinal cord (b). Saline or WSE (100, 150, and 200 mg/kg, intraperitoneal) was administered 30 min before a 5% formalin (20 µl of 5% formalin diluted in saline) injection into the mouse hind paw. The time spent licking the injected paw was evaluated during the acute (0–10 min) and tonic (15–45 min) phases after formalin injection. c-Fos protein immunoreactivity was evaluated 90 min after formalin injection. Results are expressed as mean ± SEM of 7–11 (behavioral analysis) mice per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus saline-treated mice.

microtome. After rinsing in PBS with 0.2% Triton X-100 (PBS + T), sections were incubated with 0.3% of H_2O_2 in PBS and, after extensive washing, with a blocking solution containing 1% BSA and 20% normal goat serum in PBS + T to reduce background. Sections were incubated overnight at 4°C with a rabbit anti-c-Fos polyclonal antibody [1 : 4000; Santa Cruz Biotechnology (Dallas, Texas, USA)]. After rinsing, sections were incubated with an anti-rabbit biotinylated IgG [1 : 200; Vectastain ABC Kit; Vector (Burlingame, California, USA)] for 1 h, followed by an avidin–biotin complex (1 : 400; Vectastain ABC Kit; Vector) for an additional hour. After washing, sections were exposed to 3,3-diaminobenzidine containing 1% cobalt chloride and 1% nickel ammonium sulfate for 15 min. Immunostaining was developed by adding H_2O_2. After washing in PBS, all sections were mounted on gelatin-coated glass slides, air dried, dehydrated in ascending concentrations of ethanol, cleared with xylene, and coverslipped with Entellan (Merck; Darmstadt, Germany). Six alternated sections per mouse were analyzed under light microscopy by an image analysis system and using an objective lens of ×40. The number of c-Fos immunoreactive neurons (Fos-IR), visualized as black nuclei, was counted in laminae I and II of the spinal gray matter.

Drugs

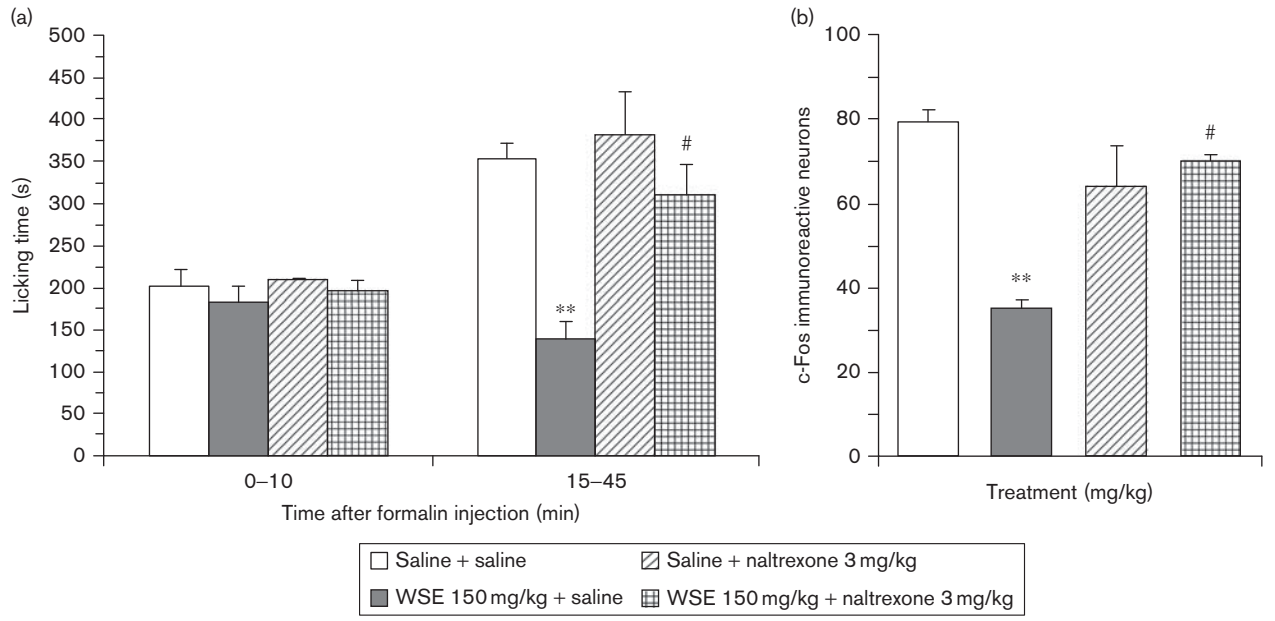
The compounds used in the study were AM630 (CB_2 antagonist), bicuculline ($GABA_A$ antagonist), CGP35348 ($GABA_B$ antagonist), flumazenil (benzodiazepine antagonist), glibenclamide (ATP-sensible potassium channel inhibitor), glutamate, L-arginine (substrate for

Fig. 2



Micrographs showing the dose-dependent inhibitory effect of *Withania somnifera* (L.) Dunal extract (WSE) on formalin-induced c-Fos protein immunoreactivity in the spinal cord dorsal horn (ipsilateral side to the formalin injection). (a) Saline, (b) WSE 100 mg/kg, (c) WSE 150 mg/kg, (d) WSE 200 mg/kg. Scale bars = 100 μm.

Fig. 3



Antagonism of *Withania somnifera* (L.) Dunal extract (WSE) effects (150 mg/kg, intraperitoneal, 30 min before a 5% formalin injection) on the formalin test by naltrexone pretreatment (3 mg/kg, intraperitoneal, 10 min before WSE). The time spent licking the injected paw was evaluated during the acute (0–10 min) and tonic (15–45 min) phases after formalin injection (a). c-Fos protein immunoreactivity was evaluated 90 min after formalin injection (b). Results are expressed as mean ± SEM of 5–8 mice per group. $^{**}P < 0.01$ versus saline + saline-treated mice; $^{\#}P < 0.05$ versus WSE + saline-treated mice.

NO synthase), L-NAME (oxide-synthase inhibitor), naltrexone (opioid antagonist), naltrindole (δ-opioid antagonist), and SR141716A (CB_1 antagonist). All compounds, purchased from Tocris (Bristol, UK), were dissolved in saline, except AM630, suspended in saline with DMSO (10%) and Tween 80 (5%); bicuculline, dissolved in 0.1 N HCl and the pH adjusted to pH 6 with 0.1 N NaOH; and flumazenil, glibenclamide, and SR141716A, suspended in saline with Tween 80 (5%). All compounds were administered intraperitoneally in a volume of 5 ml/kg.

Data analysis

The results of behavioral experiments were expressed as mean ± SEM of the time spent licking (s) the injected paw. The results of immunohistochemistry experiments were expressed as mean ± SEM of the c-Fos-immunolabeled neurons. The effects of WSE treatment on the time spent licking the injected paw and the number of c-Fos-immunolabeled neurons after an injection of formalin or glutamate were analyzed by one-way analysis of variance (ANOVA). The results from antagonism studies were analyzed by two-way ANOVA with pretreatment ('antagonist' administration) and treatment (WSE administration) as between-subject factors. Post-hoc comparisons were performed using Dunnett's test (one-way ANOVA) and Tukey's test for multiple comparison (two-way ANOVA). A *P*-value of less than 0.05 was considered statistically significant.

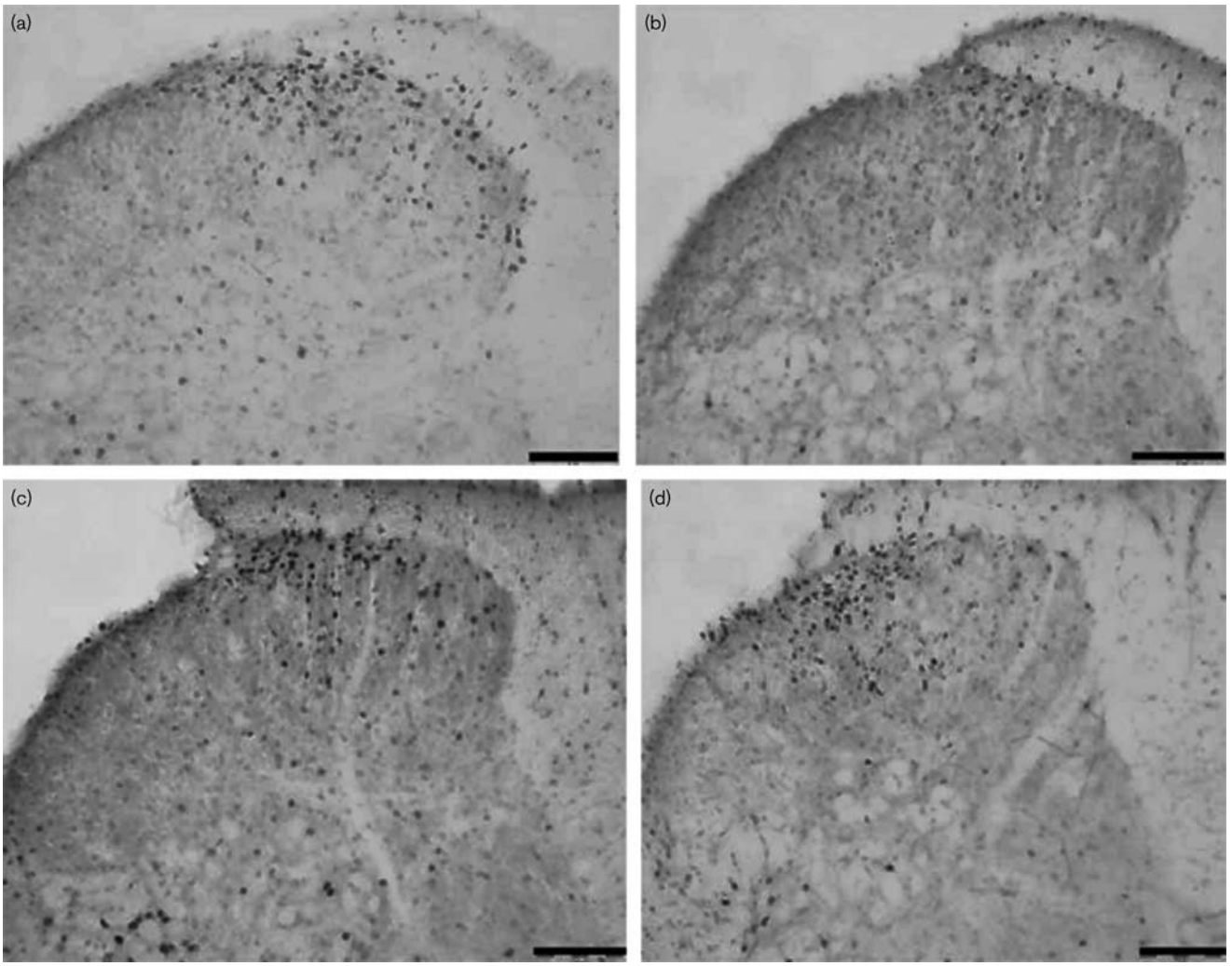
Results

Effects of *W. somnifera* (L.) Dunal extract in the formalin test

Figure 1a shows the effects of administration of WSE in the two phases of the formalin test. In saline-treated mice, intraplantar formalin injection induced the characteristic biphasic nociceptive response consisting of an early and short-lasting acute phase (0–10 min), followed by a late tonic phase (15–45 min), separated by a 5-min interphase.

In the acute phase, WSE was devoid of antinociceptive activity [$F(3,32) = 1.2$, NS]. However, in the tonic phase, the administration of WSE induced a dose-dependent reduction in the time spent licking the formalin-injected paw [$F(3,32) = 13.6$, $P < 0.001$], with significant effects at 150 and 200 mg/kg (Dunnett's test: $P < 0.01$ and < 0.001, respectively, vs. saline-treated mice). Figure 1b shows the effects of administration of WSE on c-Fos expression in layer I–II of the lumbar spinal cord after intraplantar formalin injection. The intraplantar injection of formalin dramatically increased c-Fos immunolabeling on the ipsilateral side of laminae I–II [$F(3,26) = 4.7$, $P < 0.01$]. This increase was prevented by the administration of WSE in a dose-dependent manner: the decrease in the number of immunoreactive neurons was statistically significant at 150 and 200 mg/kg WSE (Dunnett's test: $P < 0.05$ vs. saline + formalin-treated mice). Figure 2 shows c-Fos

Fig. 4



Micrographs showing the combination of 150 mg/kg of *Withania somnifera* (L.) Dunal extract (WSE) with naltrexone. Naltrexone antagonized the reduction of c-Fos expression induced by WSE in the neurons of the spinal cord dorsal horn. (a) Saline + saline, (b) saline + WSE 150 mg/kg, (c) naltrexone 3 mg/kg + saline, (d) naltrexone 3 mg/kg + WSE 200 mg/kg. Scale bars = 100 μm.

immunolabeling in the ipsilateral side of the spinal cord in each experimental group.

Antagonism studies were carried out to elucidate the potential mechanism contributing toward the anti-nociceptive properties of WSE in the formalin test. Among the compounds tested, only the opioid antagonist naltrexone completely abolished the ability of WSE to alleviate the nociceptive response during the second phase of the formalin test. As shown in Fig. 3a, 150 mg/kg WSE reduced the time spent licking the formalin-injected paw during the tonic phase [$F_{\text{treatment}}(1,19) = 16.0$, $P < 0.001$; Tukey's test, $P < 0.05$ vs. saline + saline-treated mice]. Naltrexone (3 mg/kg) pretreatment did not alter the nociceptive response in either the acute [$F_{\text{pretreatment}}(1,19) = 0.5$, NS; $F_{\text{interaction}}(1,19) = 0.1$, NS] or the tonic phase, but its administration reversed the reduction of licking time induced by WSE during the tonic phase of the formalin test [$F_{\text{pretreatment}}(1,19) = 8.9$, $P < 0.01$; $F_{\text{interaction}}(1,19) = 4.6$, $P < 0.05$; Tukey's test, $P < 0.05$ vs. WSE + saline-treated mice]. Changes in c-Fos expression analysis in layer I–II of the dorsal horn of the spinal cord matched those observed in the behavioral study (Fig. 3b). In fact, the WSE-induced decrease in the nociceptive response after formalin injection was followed by a decrease in c-Fos immunoreactivity (Tukey's test, $P < 0.05$ vs. saline + saline-treated mice), and this effect was completely abolished by the administration of 3 mg/kg naltrexone [$F_{\text{pretreatment}}(1,16) = 2.9$, NS; $F_{\text{treatment}}(1,16) = 11.2$, $P < 0.005$; $F_{\text{interaction}}(1,16) = 19.3$, $P < 0.001$; Tukey's test, $P < 0.05$ vs. saline + WSE-treated mice]. Figure 4 shows c-Fos immunolabeling in the ipsilateral side of the spinal cord in each experimental group.

Table 1 Effects of pretreatment with bicuculline, flumazenil, CGP35348, naltrindole, SR141716A, AM630, L-arginine, glibenclamide, and L-NAME on the antinociceptive activity of *Withania somnifera* (L.) Dunal extract during the tonic phase of the formalin test

Compounds	Experimental group	Licking time (s)	*F* values
Bicuculline	Vehicle + saline	375 ± 24	$F_{pretreatment}(1,16) = 0.7$, $P = 0.41$
2 mg/kg	Vehicle + WSE 150	174 ± 19*	$F_{treatment}(1,16) = 86.2$, $P < 0.0001$
	Bicuculline 2 + saline	363 ± 21	$F_{interaction}(1,16) = 0.1$, $P = 0.78$
	Bicuculline 2 + WSE 150	149 ± 23*	
Flumazenil	Vehicle + saline	382 ± 19	$F_{pretreatment}(1,14) = 2.1$, $P = 0.26$
10 mg/kg	Vehicle + WSE 150	175 ± 19*	$F_{treatment}(1,14) = 91.3$, $P < 0.0001$
	Flumazenil 10 + saline	358 ± 20	$F_{interaction}(1,14) = 0.1$, $P = 0.71$
	Flumazenil 10 + WSE 150	133 ± 22*	
CGP35348	Vehicle + saline	408 ± 17	$F_{pretreatment}(1,22) = 0.0$, $P = 1.0$
100 mg/kg	Vehicle + WSE 150	159 ± 20*	$F_{treatment}(1,22) = 24.2$, $P < 0.0001$
	CGP35348 100 + saline	333 ± 17	$F_{interaction}(1,22) = 4.5$, $P < 0.05$
	CGP35348 100 + WSE 150	234 ± 43*	
Naltrindole	Vehicle + saline	411 ± 24	$F_{pretreatment}(1,16) = 0.2$, $P = 0.66$
1.5 mg/kg	Vehicle + WSE 150	143 ± 21*	$F_{treatment}(1,16) = 67.6$, $P < 0.0001$
	Naltrindole 1.5 + saline	401 ± 48	$F_{interaction}(1,16) = 0.6$, $P = 0.44$
	Naltrindole 1.5 + WSE 150	180 ± 28*	
SR141716A	Vehicle + saline	401 ± 20	$F_{pretreatment}(1,16) = 0.0$, $P = 0.93$
1 mg/kg	Vehicle + WSE 150	148 ± 18*	$F_{treatment}(1,16) = 29.2$, $P < 0.0001$
	SR141716A 1 + saline	371 ± 92	$F_{interaction}(1,16) = 0.4$, $P = 0.52$
	SR141716A 1 + WSE 150	172 ± 30*	
AM630	Vehicle + saline	428 ± 26	$F_{pretreatment}(1,22) = 1.1$, $P = 0.30$
0.8 mg/kg	Vehicle + WSE 150	149 ± 17*	$F_{treatment}(1,22) = 101.9$, $P < 0.0001$
	AM630 0.8 + saline	433 ± 46	$F_{interaction}(1,22) = 0.8$, $P = 0.37$
	AM630 0.8 + WSE 150	198 ± 20*	
L-Arginine	Vehicle + saline	374 ± 19	$F_{pretreatment}(1,21) = 0.2$, $P = 0.68$
600 mg/kg	Vehicle + WSE 150	111 ± 24*	$F_{treatment}(1,21) = 20.4$, $P < 0.0005$
	L-Arginine 600 + saline	325 ± 63	$F_{interaction}(1,21) = 2.4$, $P = 0.14$
	L-Arginine 600 + WSE 150	196 ± 42*	
Glibenclamide	Vehicle + saline	400 ± 11	$F_{pretreatment}(1,16) = 2.8$, $P = 0.11$
5 mg/kg	Vehicle + WSE 150	173 ± 19*	$F_{treatment}(1,16) = 24.5$, $P < 0.0001$
	Glibenclamide 5 + saline	290 ± 49	$F_{interaction}(1,16) = 1.9$, $P = 0.18$
	Glibenclamide 5 + WSE 150	163 ± 38*	
L-NAME	Vehicle + saline	430 ± 35	$F_{pretreatment}(1,16) = 1.5$, $P = 0.23$
1 mg/kg	Vehicle + WSE 150	134 ± 24*	$F_{treatment}(1,16) = 39.2$, $P < 0.0001$
	L-NAME 1 + saline	304 ± 41	$F_{interaction}(1,16) = 6.2$, $P < 0.05$
	L-NAME 1 + WSE 150	176 ± 33*	

WSE, *Withania somnifera* (L.) Dunal extract.
*$P < 0.05$ versus vehicle + saline-treated mice (two-way analysis of variance, followed by Tukey's post-hoc test).

We also evaluated whether other systems were involved in the antinociceptive effects of WSE. The effects of the combination of WSE with bicuculline, flumazenil, CGP35348, naltrindole, SR141716A, AM630, L-arginine, glibenclamide, and L-NAME on licking time during the tonic phase of the formalin test and c-Fos expression on layer I–II of the dorsal horn of the lumbar spinal cord are shown, respectively, in Tables 1 and 2. As we did not observe any effect on the nociceptive response in the acute phase of the formalin test, only the time spent licking the injected paw during the tonic phase is shown. Two-way ANOVA showed a statistically significant interaction effect when WSE was combined with CGP39348 and L-NAME; this was probably because of a trend toward reduction of licking time induced by CGP39348 and L-NAME and their ability to partially reverse the antinociceptive effect of WSE. However, post-hoc analysis failed to show a statistically significant difference between saline + saline-treated and CGP39348 (or L-NAME)-treated mice or between saline + WSE-treated and CGP39348 (or L-NAME)-treated mice. Moreover, both CGP39348 and L-NAME failed to reverse the effect of WSE on formalin-induced spinal c-Fos expression.

The other compounds used in association with WSE were devoid of antinociceptive activity and failed to reverse the effects of WSE on both nociceptive behavior and spinal c-Fos expression.

Effects of *W. somnifera* (L.) Dunal extract on the glutamate-induced nociception

Figure 5a shows the effects of administration of WSE on glutamate-induced nociception. An intraplantar injection of glutamate induced an acute and short-lasting (15 min) nociceptive response characterized by licking the injected paw. The administration of WSE reduced the time spent licking the injected paw in a dose-dependent manner [$F(2,20) = 7.6$, $P < 0.005$], which was statistically significant at 150 mg/kg (51% decrease: Dunnett's test, $P < 0.05$ vs. saline-treated mice). A similar pattern was observed for c-Fos expression in layer I–II of the lumbar spinal cord after an intraplantar glutamate injection (Fig. 5b). The nociceptive response induced by glutamate injection was associated with an increase in c-Fos

Table 2 Effects of pretreatment with bicuculline, flumazenil, CGP35348, naltrindole, SR141716A, AM630, L-arginine, glibenclamide, and L-NAME on the ability of *Withania somnifera* (L.) Dunal extract to counteract formalin-induced increase of spinal c-Fos expression

Compounds	Experimental group	C-Fos Immunoreactive neurons	*F* values
Bicuculline	Vehicle + saline	72.2 ± 3.0	$F_{pretreatment}(1,15) = 1.2$, $P = 0.30$
2 mg/kg	Vehicle + WSE 150	32.0 ± 2.5*	$F_{treatment}(1,15) = 43.5$, $P < 0.0001$
	Bicuculline 2 + saline	68.7 ± 4.8	$F_{interaction}(1,15) = 3.3$, $P = 0.09$
	Bicuculline 2 + WSE 150	45.8 ± 5.1*	
Flumazenil	Vehicle + saline	73.8 ± 3.3	$F_{pretreatment}(1,12) = 1.5$, $P = 0.25$
10 mg/kg	Vehicle + WSE 150	29.7 ± 0.8*	$F_{treatment}(1,12) = 58.3$, $P < 0.0001$
	Flumazenil 10 + saline	61.8 ± 8.8	$F_{interaction}(1,12) = 1.4$, $P = 0.26$
	Flumazenil 10 + WSE 150	29.5 ± 3.2*	
CGP35348	Vehicle + saline	86.0 ± 4.9	$F_{pretreatment}(1,11) = 0.2$, $P = 0.63$
100 mg/kg	Vehicle + WSE 150	40.7 ± 3.4*	$F_{treatment}(1,11) = 68.4$, $P < 0.0001$
	CGP35348 100 + saline	77.1 ± 2.7	$F_{interaction}(1,11) = 1.9$, $P = 0.19$
	CGP35348 100 + WSE 150	44.9 ± 4.3*	
Naltrindole	Vehicle + saline	76.2 ± 3.0	$F_{pretreatment}(1,11) = 0.9$, $P = 0.36$
1.5 mg/kg	Vehicle + WSE 150	43.6 ± 1.5*	$F_{treatment}(1,11) = 68.7$, $P < 0.0001$
	Naltrindole 1.5 + saline	69.2 ± 4.4	$F_{interaction}(1,11) = 1.1$, $P = 0.31$
	Naltrindole 1.5 + WSE 150	44.0 ± 4.4*	
SR141716A	Vehicle + saline	78.1 ± 3.3	$F_{pretreatment}(1,12) = 1.2$, $P = 0.29$
1 mg/kg	Vehicle + WSE 150	37.2 ± 3.3*	$F_{treatment}(1,12) = 34.8$, $P < 0.0001$
	SR141716A 1 + saline	65.9 ± 10.6	$F_{interaction}(1,12) = 0.9$, $P = 0.37$
	SR141716A 1 + WSE 150	36.2 ± 2.9*	
AM630	Vehicle + saline	85.8 ± 4.7	$F_{pretreatment}(1,16) = 2.5$, $P = 0.13$
0.8 mg/kg	Vehicle + WSE 150	44.9 ± 1.4*	$F_{treatment}(1,16) = 46.0$, $P < 0.0001$
	AM630 0.8 + saline	69.3 ± 2.2	$F_{interaction}(1,16) = 3.8$, $P = 0.07$
	AM630 0.8 + WSE 150	46.6 ± 5.5*	
L-Arginine	Vehicle + saline	70.1 ± 0.9	$F_{pretreatment}(1,14) = 1.0$, $P = 0.32$
600 mg/kg	Vehicle + WSE 150	19.6 ± 3.8*	$F_{treatment}(1,14) = 105.0$, $P < 0.0001$
	L-Arginine 600 + saline	61.5 ± 5.4	$F_{interaction}(1,14) = 0.7$, $P = 0.40$
	L-Arginine 600 + WSE 150	18.8 ± 5.7*	
Glibenclamide	Vehicle + saline	78.0 ± 3.3	$F_{pretreatment}(1,14) = 0.3$, $P = 0.58$
5 mg/kg	Vehicle + WSE 150	29.7 ± 0.8*	$F_{treatment}(1,14) = 269.5$, $P < 0.0001$
	Glibenclamide 5 + saline	75.9 ± 3.3	$F_{interaction}(1,14) = 0.0$, $P = 0.86$
	Glibenclamide 5 + WSE 150	28.5 ± 2.0*	
L-NAME	Vehicle + saline	78.5 ± 2.5	$F_{pretreatment}(1,11) = 0.2$, $P = 0.64$
1 mg/kg	Vehicle + WSE 150	43.0 ± 1.8*	$F_{treatment}(1,11) = 34.3$, $P < 0.0005$
	L-NAME 1 + saline	74.2 ± 3.0	$F_{interaction}(1,11) = 1.8$, $P < 0.21$
	L-NAME 1 + WSE 150	51.9 ± 11.2*	

WSE, *Withania somnifera* (L.) Dunal extract.
*$P < 0.05$ versus vehicle + saline-treated mice (two-way analysis of variance, followed by Tukey's post-hoc test).

expression and the administration of WSE caused a dose-dependent reduction in the number of c-Fos immunoreactive neurons [$F(2,15) = 66.2$, $P < 0.001$]; post-hoc analysis showed that the active dose of WSE in reducing glutamate-induced nociceptive response also decreased c-Fos immunoreactivity by 59% (Dunnett's test, $P < 0.05$ vs. saline-treated mice). Figure 6 shows c-Fos immunolabeling in the ipsilateral side of the spinal cord in each experimental group.

Discussion

The main finding of this study is the ability of WSE to exert a significant antinociceptive activity in the tonic phase of the formalin test through the involvement of the opioidergic system. Our preliminary evidence cannot also rule out the potential involvement of the glutamatergic system in the antinociceptive properties of WSE; however, further studies are needed to confirm this hypothesis.

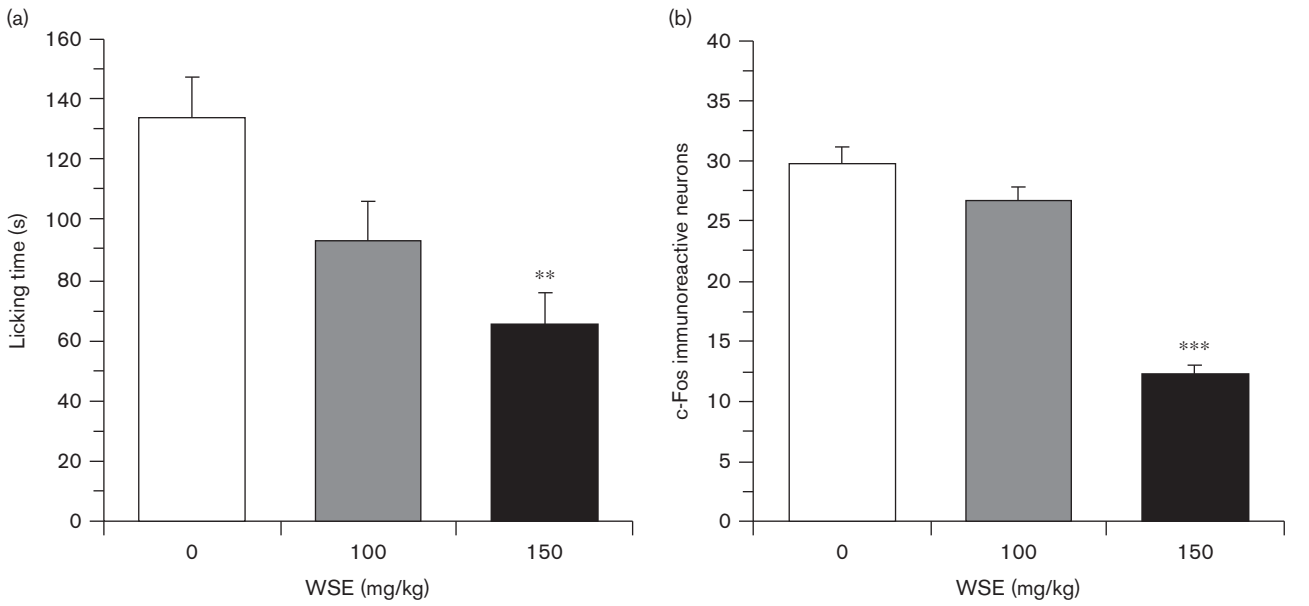
The formalin test closely resembles clinical pain conditions, and for this reason, it represents a well-established model for assessing tonic pain and for identifying compounds with analgesic activity (Sawynok and Liu, 2004). By using the formalin test, we expected to better characterize the potential antinociceptive properties of WSE. The two phases characterizing this test are sensitive to different classes of analgesics, enabling discrimination between centrally versus peripherally acting compounds (Shibata *et al.*, 1989). Consistently, centrally acting analgesics and NSAIDs with central actions attenuate nociception during both the acute and the tonic phase of the test; however, NSAIDs devoid of central actions and compounds preventing spinal sensitization alleviate nociception mainly during the tonic phase (Hunskaar and Hole, 1987; Berrino *et al.*, 2003). The antinociceptive activity of WSE is similar to this latter class of compounds, suggesting a peripheral or a spinal action. The lack of activity of WSE during the acute phase of the formalin test was not surprising as previous studies showed that the administration of WSE failed to modify the nociceptive response in animal models of acute pain such as the tail-flick test (Kulkarni and Ninan, 1997; Orrù *et al.*, 2014) and the hot-plate test (Orrù *et al.*, 2014).

The antinociceptive activity of WSE is also confirmed by analysis of c-Fos expression. Studies have shown that the proto-oncogene c-Fos is activated in the dorsal horn of the spinal cord following various types of noxious stimulation (Harris, 1998), and consequently, c-Fos protein expression has been used widely as a functional marker to identify the activation of spinal neurons by noxious stimulation (Harris, 1998). The usefulness of c-Fos as a marker of nociception is also supported by the inverse relationship between analgesic efficacy and c-Fos expression (Harris, 1998). Consistently, we found that the antinociceptive effect of WSE was followed by a decrease in formalin-induced c-Fos protein expression in the dorsal horn (laminae I–II) of the lumbar (L4–L5) spinal cord.

Our study provides the first direct evidence showing that the antinociceptive effect of WSE may be mediated specifically by the opioidergic system. In the presence of the opioid receptor antagonist naltrexone, WSE failed to alleviate formalin-induced nociception during the tonic phase or to counteract the consequent increase in spinal c-Fos expression. These results are consistent with previous indirect evidence showing that WSE has affinity for the μ-opioid receptor (Orrù *et al.*, 2014). Interestingly, the interaction of WSE with the opioidergic system has also been found to play a prominent role in other pharmacological effects of WSE: the antitussive effect of WSE has been found to be mediated by μ-opioid receptors (Nosálová *et al.*, 2015). Taken together, this evidence suggests that WSE elicits some of its pharmacological effects by modulating μ-opioid receptors. The effects of WSE in the formalin test resemble those of opioid agonists selective for peripheral opioid receptors. Loperamide and *N*-methylmorphine, similar to WSE, exert a selective inhibition of the nociceptive response during the tonic phase of the formalin test (Oluyomi *et al.*, 1992; DeHaven-Hudkins *et al.*, 1999). This local effect of peripherally acting opioids is supported by studies showing the existence of opioid receptors in the peripheral nervous system (Vadivelu *et al.*, 2011). It is also noteworthy that ongoing inflammatory processes are pivotal for the full expression of the antinociceptive effects induced by peripherally acting opioids. Inflammation gives rise to the disruption of the perineurial sheath, increasing the availability of opioid receptors to the drugs (Antonijevic *et al.*, 1995). Overall, it might be possible that WSE alleviated formalin-induced nociception by acting as a peripheral opioid agonist; the development of inflammatory processes that occurs during the tonic phase of the formalin test might have promoted this effect.

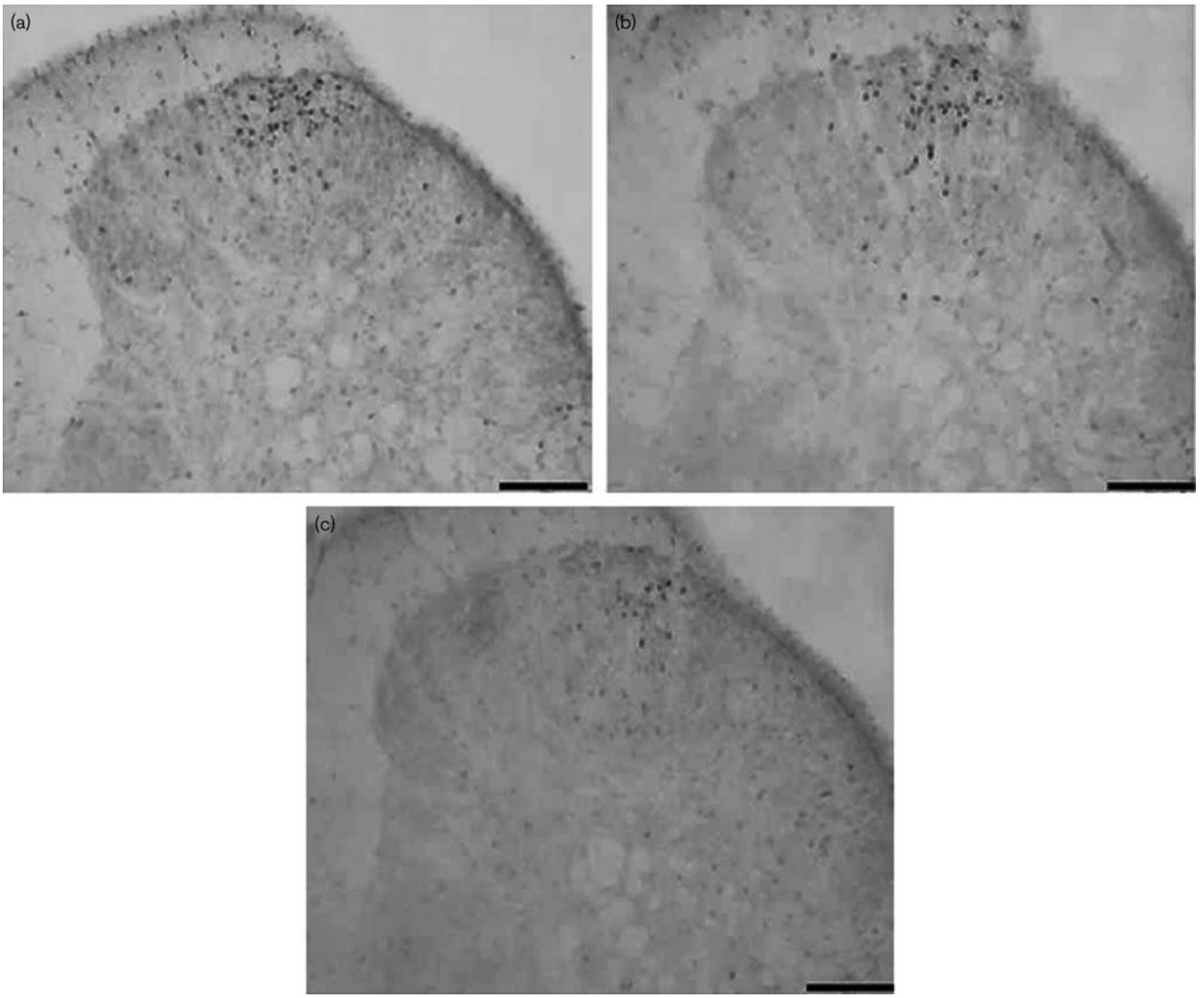
The concurrent reduction of c-Fos expression observed in our study should come as no surprise. The activation of primary afferents during the second phase of the formalin test strongly contributes toward induction of a painful state and toward increased c-Fos expression in the spinal

Fig. 5



Effects of administration of *Withania somnifera* (L.) Dunal extract (WSE) on glutamate-induced nociceptive response (a) and glutamate-induced c-Fos expression in the dorsal horn of the spinal cord (b). Saline or WSE (100 and 150 mg/kg, intraperitoneal) was administered 30 min before a glutamate (2 μmol–20 μl diluted in saline) injection in the mouse hind paw. The time spent licking the injected paw was recorded for 15 min. c-Fos protein immunoreactivity was evaluated 90 min after glutamate injection. Results are expressed as mean ± SEM of 6–9 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$ versus saline-treated mice.

Fig. 6



Micrographs showing the dose-dependent inhibitory effect of *Withania somnifera* (L.) Dunal extract (WSE) on glutamate-induced c-Fos protein immunoreactivity in the spinal cord dorsal horn (ipsilateral side to the formalin injection). (a) Saline, (b) WSE 100 mg/kg, (c) WSE 150 mg/kg. Scale bars = 100 μm.

cord (Abbadie *et al.*, 1997); hence, the local blockade of primary afferents activity may be sufficient to promote analgesia and to counteract the increase of spinal c-Fos. Consistently, it has been found that peripherally acting agents (such as NSAIDs and QX-314, a quaternary lidocaine derivative that cannot penetrate the blood–brain barrier) exert an antinociceptive effect during the tonic phase of the formalin test and counteract formalin-induced increase of c-Fos immunoreactivity in the lumbar spinal cord (Abbadie *et al.*, 1997; Coggeshall, 2005).

However, an indirect modulation of the opioid system on the antinociceptive effects of WSE could be possible. WSE and its main bioactive ingredients (withanolides) have anti-inflammatory properties (Misico *et al.*, 2011; Alam *et al.*, 2012; Sivamani *et al.*, 2014) by inhibition of the enzyme cyclooxygenase-2 (COX-2) (Min *et al.*, 2011). It has been found that COX-2 inhibitors increase the release of endogenous opioid peptides (Michel *et al.*, 1996); consistent with this evidence, the μ-opioid antagonist CTOP reversed the antinociceptive effects of the COX-2 inhibitor DU-697 during the tonic phase of the formalin test (Choi *et al.*, 2010). Thus, similar to other COX-2 inhibitors, WSE might have increased the release of endogenous peptides that mediated its antinociceptive activity.

None of the other neurotransmitter systems investigated could counteract the antinociceptive effects of WSE. In

fact, albeit that we recently found that WSE shows high affinity for the $GABA_A$, and $GABA_B$ receptor (Orrù *et al.*, 2014), pretreatment with bicuculline, flumazenil, and CGP35348 did not block the effects of WSE on formalin-induced nociception and c-Fos expression. We also evaluated whether the NO system might have contributed toward the effects of WSE. The NO system has been implicated in the development of inflammatory disorders (Sharma *et al.*, 2007) and painful conditions in general (Miclescu and Gordh, 2009); consistently, the antinociceptive effects of several classes of analgesics in the formalin test were found to be partially mediated by the modulation of NO production (Granados-Soto *et al.*, 1997; Ortiz *et al.*, 2002). Evidence exists that WSE modulates NO production (Iuvone *et al.*, 2003); however, neither inhibition (L-NAME) nor activation (L-arginine) of NO production, or inhibition of ATP-sensible potassium channels (a downstream target of NO) by glibenclamide antagonized the effects of WSE in the formalin test. Finally, CB_1, CB_2, and δ-opioid receptor antagonists also failed to modulate the effects of WSE on formalin-induced nociception and c-Fos expression.

In this study, we also aimed to verify whether the glutamatergic system participates in the antinociceptive properties of WSE. On the basis of previous findings, our working hypothesis was that WSE may act by blocking glutamate-mediated neurotransmission (Orrù *et al.*, 2014). We indirectly tested this hypothesis by evaluating the ability of WSE to counteract glutamate-induced nociception. We present evidence that the administration of WSE attenuates nociception and c-Fos expression induced by the intraplantar injection of glutamate, as observed previously with MK-801, a synthetic NMDA receptor antagonist (Beirith *et al.*, 2002), and with honokiol and magnolol, natural NMDA receptor antagonists extracted from *Magnolia officinalis* (Lin *et al.*, 2009). Therefore, it is not beyond the bounds of possibility that the blockade of neurotransmission mediated by NMDA receptors may contribute toward the antinociceptive effect of WSE in the tonic phase of the formalin test. The affinity of WSE for NMDA receptors seems to support this hypothesis, although further studies are needed to rule out the involvement of other neurotransmission pathways (Orrù *et al.*, 2014).

Another possibility is that WSE might alleviate nociception during the tonic phase of the formalin test by counteracting the development of spinal sensitization. Spinal sensitization plays a prominent role in the nociceptive response during the second phase of the formalin test (Coderre *et al.*, 1990; Coderre, 2001), and NMDA receptor-mediated neurotransmission is strongly involved in its development (Skilling *et al.*, 1988; Coderre and Melzack, 1992; Leong *et al.*, 2000; Coderre, 2001). Consistently, the administration of NMDA receptor antagonists has been found to reduce formalin-induced pain-related behaviors during the tonic but not the acute phase, and the consequent increase in spinal c-Fos expression (Yamamoto and Yaksh, 1992; Berrino *et al.*, 2003).

Overall, we found that WSE has antinociceptive activity during the tonic phase of the formalin test. This effect appears to have a peripheral origin and to be specifically mediated by the opioidergic system. Further studies are necessary to verify whether a central mechanism, mediated by the glutamatergic system, can contribute toward the antinociceptive effects of WSE. In conclusion, the results make WSE worthy of further investigation as a potentially valuable agent for the management of specific painful conditions in which inflammation and sensitization participate, alone or in combination, in their etiology.

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The product used in the present study is still investigational.

Conflicts of interest

There are no conflicts of interest.

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