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Research Paper

Antihyperalgesic activity of verbascoside in two models of neuropathic pain

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Abstract

Objectives This study reports on the rapid isolation of verbascoside from *Lippia citriodora* H.B.K. (Verbenaceae), an inexpensive and widespread source, and the evaluation of its antihyperalgesic activity.

Methods Isolation of verbascoside was achieved by size exclusion chromatography with Sephadex LH-20 eluting with 50% EtOH, which is proposed as a fast and efficient method of separation.

Key findings The antihyperalgesic activity of verbascoside was tested by in-vivo assay using the paw-pressure test in two animal models of neuropathic pain: a peripheral mononeuropathy produced either by a chronic constriction injury of the sciatic nerve (CCI) or by an intra-articular injection of sodium monoiodoacetate (MIA).

Conclusions Verbascoside administered intraperitoneally at a dose of 100 mg/kg reverted the mechanical hyperalgesia in both CCI and MIA treated rats, as evaluated in the paw-pressure test. Verbascoside was also effective against mechanical hyperalgesia after oral administration at doses of 300 and 600 mg/kg.

Keywords antihyperalgesic; *Lippia citriodora*; neuropathic pain; paw-pressure test; verbascoside

Introduction

Verbascoside (acteoside, Figure 1) is a phenylpropanoid glycoside isolated from several medicinal plants of different families such as Verbenaceae, Oleaceae, Buddlejaceae, Lamiaceae and Scrophulariaceae,[1,2] including plants used in Traditional Chinese Medicine.[3] The literature regarding the biological activity of this compound is extensive: anti-inflammatory, anti-ulcerogenic and antispasmodic activity,[2,4–8] antiproliferative properties and inhibition of telomerase,[9,10] immunomodulatory,[11] antioxidant, photoprotective[12–14] and analgesic activity[15–17] have all been reported. Nevertheless, to the best of our knowledge, no data are available on the activity of verbascoside against neuropathic pain, a widespread disorder induced by autoimmune diseases, drugs or toxin exposure, infections, metabolic insults or trauma. Typically, neuropathic pain is depicted by nerve damage, which causes muscle weakness, altered functionality and sensitivity, and a chronic pain syndrome characterized by allodynia (pain elicited by a non-noxious stimulus) and hyperalgesia (increased pain response to a noxious stimulus), which can persist for a long time after the initial injury is resolved.[18] The underlying molecular mechanisms of neuropathic pain are still not completely understood, and treatment is still unsatisfactory in most cases.[19] Thus, there is an urgent need to develop novel therapeutics for the effective treatment of neuropathic pain.

In this study, the rapid isolation of verbascoside from *Lippia citriodora* H.B.K. (Verbenaceae), a widespread and inexpensive source, is reported, and its antihyperalgesic activity was tested *in vivo*. The assays were carried out using the paw-pressure test[20] in two animal models of neuropathic pain where a peripheral mononeuropathy was produced either by a chronic constriction injury of the sciatic nerve (CCI)[21] or by an intra-articular injection of sodium monoiodoacetate (MIA).[22]

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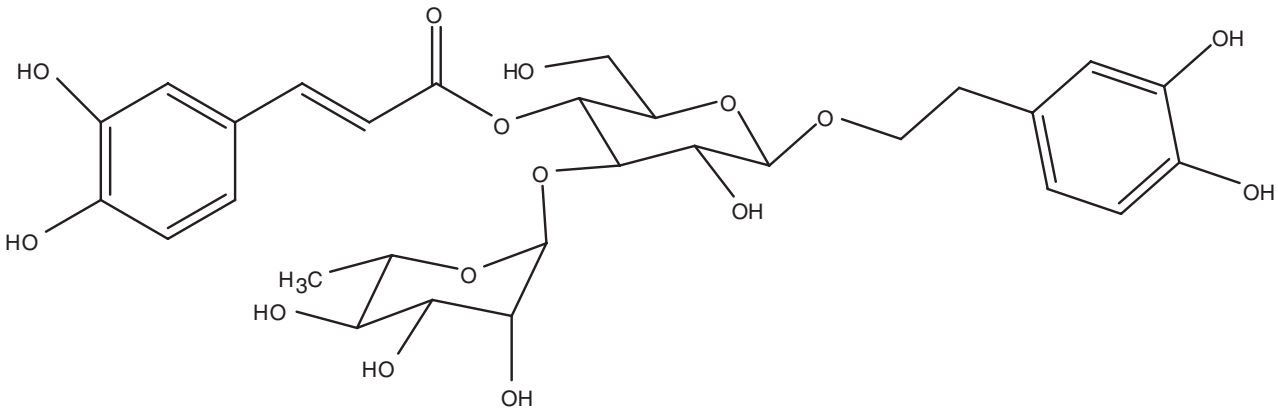

Figure 1 Chemical structure of verbascoside.

Materials and Methods

Chemicals

Ethanol, analytical reagent grade, was from Riedel-de Haen (Seelze, Germany). All solvents used were of high-performance liquid chromatography (HPLC) grade; CH_3CN and MeOH for HPLC were purchased from Merck (Darmstadt, Germany). HCOOH (85%) was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA). Carboxymethylcellulose sodium salt was from Fluka Chemie GmbH (Steinheim, Germany). NaCl 0.9% solution was from Fresenius Kabi Italia spa (Isola della Scala, Verona, Italy). Naloxone hydrochloride (>99%), tramadol (>99%) and ketorolac (≥99%) were from Sigma (St Louis, MA, USA). Morphine hydrochloride (>99%) was from S.A.L.A.R.S spa (Como, Italy). Pregabalin (>99%) was purchased from Chem Pacific (Baltimore, MD, USA).

Plant material

Commercial dried leaves of cultivated *L. citriodora* H.B.K. (Verbenaceae) harvested in 2004 were obtained from Aboca (Pistrino di Citerna, Perugia, Italy; lot no. 4L0466) and used for the isolation of verbascoside.

Isolation of verbascoside

A total of 500 g of herbal drug was exhaustively extracted with a hydroalcoholic solution (EtOH 80%) by maceration. This solution was concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (30°C), and then lyophilised, giving 34.75 g of dried extract (6.96% with respect to the herbal drug). A total of 5 g of the extract (containing ~12% of verbascoside as determined by HPLC/diode array detection (DAD)/electrospray ionization mass spectrometry (ESI MS)) was dissolved in 5 ml EtOH 50% and purified by size exclusion chromatography using Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) column chromatography (length 50 cm, diameter 4 cm) and EtOH 50% as the mobile phase. Fractions were analysed by thin layer chromatography (plates of silica gel 60 F254; Merck, Damstatt Germany; solvent $CHCl_3$/MeOH 7 : 3) using vanillin sulfuric reagent. Verbascoside (700 mg) was purified from the extract with a purity of 81.2% assessed by HPLC/DAD/ESI MS analysis using verbascoside reference standard (purity 98% by HPLC/DAD/ESI MS, gas chromatography and nuclear magnetic resonance). Verbascoside was re-crystallised from MeOH to obtain pure compound (510 mg, purity >98%). Purification of verbascoside from the extract was repeated six times to obtain enough pure verbascoside.

Analytical HPLC/DAD characterisation

The HPLC system consisted of a HP 1100 L instrument with a diode array detector and was managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The column was a Varian Polaris TM C18-E (250 mm × 4.6 mm i.d., 5 micron) maintained at 26°C with a pre-column of the same phase. The eluents were H_2O at pH 3.2 (by formic acid, A) and acetonitrile (B). The following multistep linear gradient was applied: from 87% A to 85% A in 10 min, to 75% B in 10 min and then a plateau for 3 min; 2 min to 95% CH_3CN and a final plateau of 3 min. The total time of analysis was 28 min, the equilibration time was 10 min, the flow rate was 0.8 ml/min, the injection volume was 10 µl and the oven temperature 26°C. The UV-Vis spectra were recorded between 220 and 500 nm, and the chromatographic profiles were registered at 240, 330 and 350 nm.

Analytical HPLC/ESI MS characterisation

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed use of analytical conditions similar to those used for HPLC/DAD analysis in order to achieve the maximum sensitivity of ESI values. The same column, time period and flow rate were used during the HPLC/ESI MS analyses. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionisation mode, scan spectra from m/Z 100 to 800, gas temperature of 350°C, nitrogen flow rate of 10 l/min, nebulizer pressure 30 psi, quadrupole temperature 30°C, and capillary voltage 3500 V. The applied fragmentors were over the range 80–180 V. Identification of constituents was carried out by HPLC/DAD and HPLC/ESI MS analysis, and/or by compari-

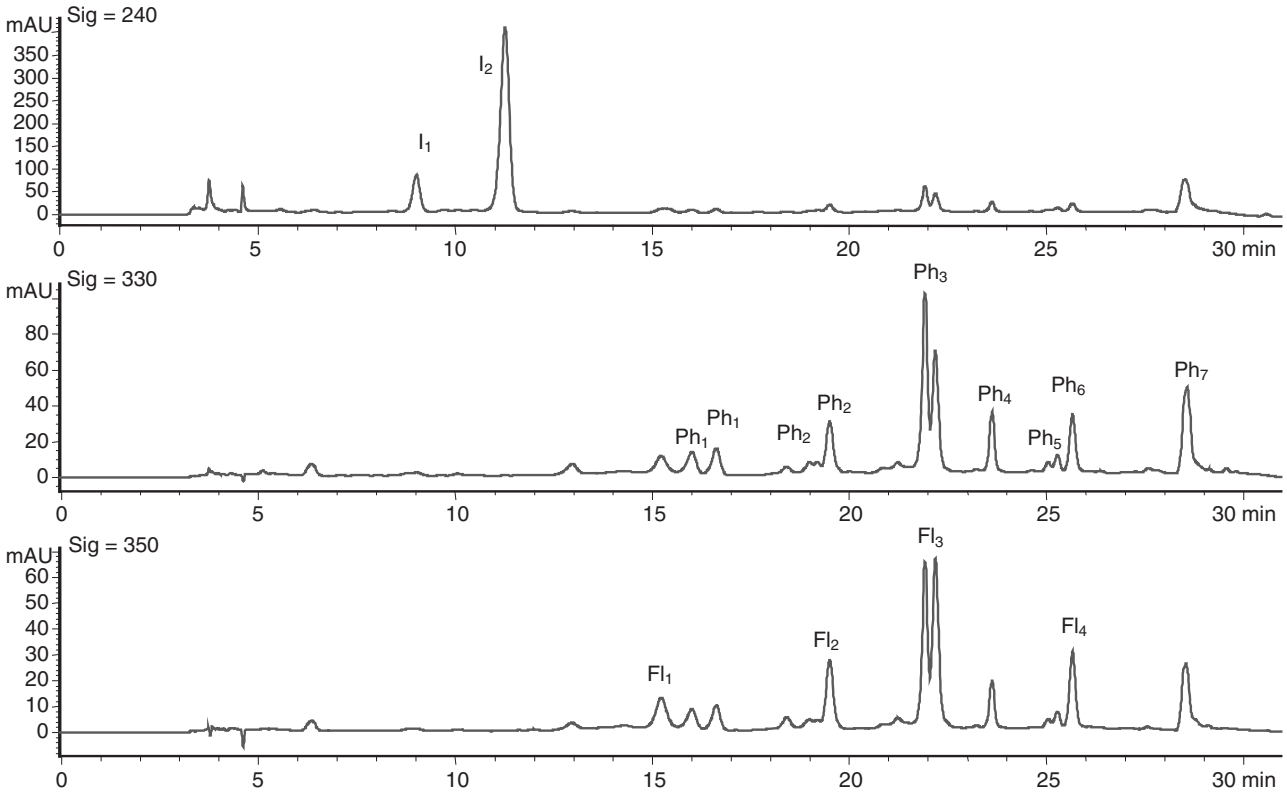

Figure 2 Chromatographic profiles at 240, 330 and 350 nm of the hydroalcoholic extract of *Lippia citriodora* H.B.K. I_1, hastatoside; I_2, verbenalin; Ph_1, β-OH verbascoside; Ph_2, β-OH isoverbascoside; Ph_3, verbascoside; Ph_4, isoverbascoside; Ph_5, eukovoside or isomer; Ph_6, eukovoside or isomer; Ph_7, eukovoside or isomer; Fl_1, luteolin-7-*O*-diglucuronide; Fl_2, apigenin-7-*O*-diglucuronide; Fl_3, luteolin-7-*O*-glucuronide; Fl_4, apigenin-7-*O*-glucuronide.

son and combination of their retention times, UV-Vis and mass spectra of the peaks with those of authentic standards when possible, isolated compounds or characterised extracts, as well as literature data.

In-vivo tests

Male Sprague-Dawley albino rats (180–200 g) from Harlan (S. Piero al Natisone, Italy) were used. Four rats were housed per cage and the cages were placed in the experimental room 24 h before the test for acclimatisation. The animals were fed a standard laboratory diet and tap water *ad libitum*, and were kept at 23 ± 1°C with a 12-h light–dark cycle (lights on at 0700 hours). The experiments were performed in accordance with the European Communities Council directive 86/609/EEC) and were approved by the Ethics Committee of our institution. All efforts were made to minimize the number and suffering of the animals used.

Application of test substances

Drugs were dissolved in isotonic (NaCl 0.9%) saline solution or dispersed in carboxymethylcellulose sodium 1% solution immediately before use. Doses ranged from 10 to 600 mg/kg and the dose–response curve allowed determination of the active doses for both intraperitoneal and oral administration. Drug concentrations were prepared and administered in a volume of 10 ml/kg for both administration routes.

Chronic constriction injury

A peripheral mononeuropathy was produced in adult rats by placing loosely constrictive ligatures around the common sciatic nerve according to the method described by Bennett and Xie.[21] Rats were anaesthetised with chloral hydrate. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatica trifurcation, about 1 cm of the nerve was freed of adhering tissue and four ligatures (3/0 silk tread) were tied loosely around it with about 1 mm spacing. The length of the nerve thus affected was 4–5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted when viewed with 40× magnification. In every animal, an identical dissection was performed on the opposite side except that the sciatic nerve was not ligated. The left paw was untouched.

Monoiodioacetate injection

Joint damage was induced by a single intra-articular injection of 2 mg of sodium monoiodioacetate into the left knee joint of anaesthetised rats in a total volume of 25 µl. The dose of monoiodoacetate was chosen based on a previous literature report[22] and in-house dose–response data using 0.5, 1 and 2 mg monoiodoacetate.

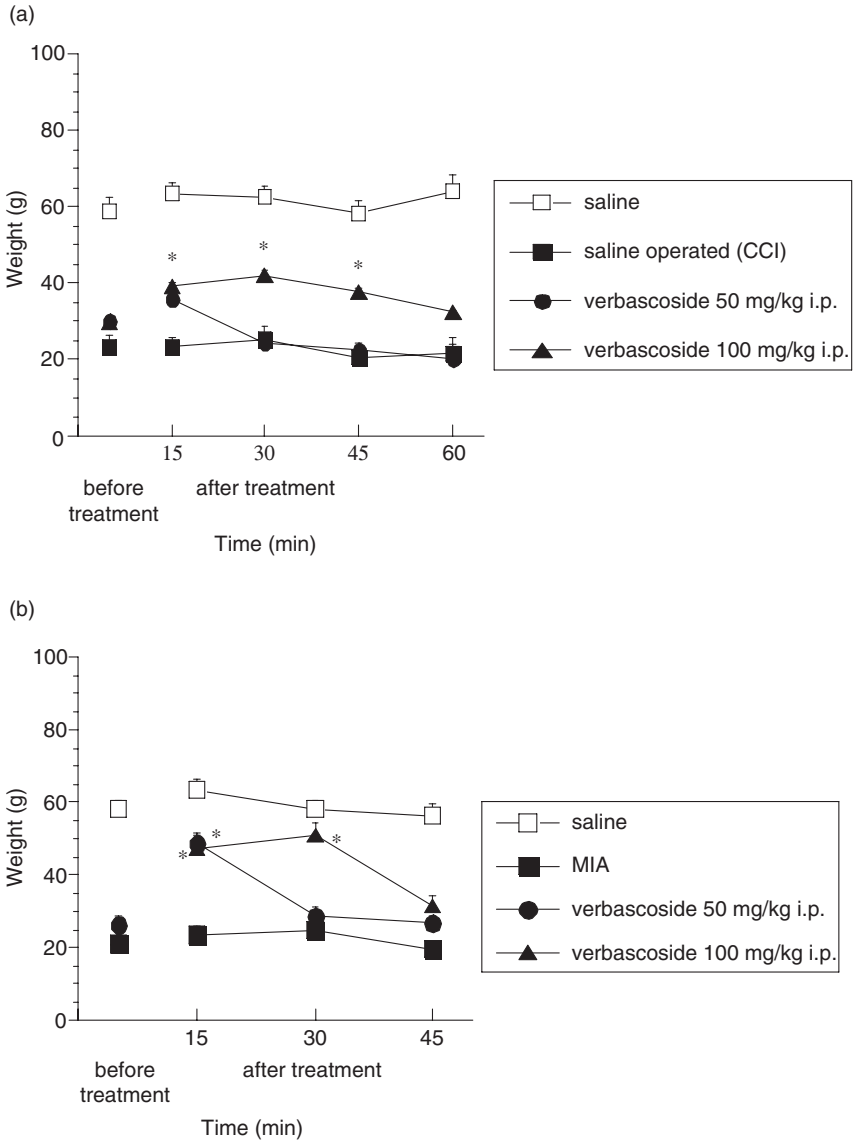

Figure 3 Effect of intraperitoneal administration of verbascoside in the rat paw-pressure test. Verbascoside was administered intraperitoneally in two animal models of neuropathic pain where a peripheral mononeuropathy was produced by either a chronic constriction injury of the sciatic nerve (CCI) (a) or by an intra-articular injection of sodium monoiodoacetate (MIA) (b).

Paw-pressure test

The Randall and Selitto instrument (Ugo Basile, Comerio, VA, Italy) exerts a force that is applied at a constant rate (32 g/s) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold was determined by the force that induced the first struggling by the rat. An arbitrary cut-off value of 250 g was adopted. Those mice scoring less than 40 g or over 75 g in the pretest were rejected (25%). The pain threshold was measured before (pretest) and at 15, 30 and 45 min after the beginning of the test.

Rota-rod test

The rota-rod apparatus (Ugo Basile, Comerio, VA, Italy) consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five rats were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rev/min. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. Those mice scoring less than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and at 15, 30 and 45 min after the beginning of the test. A total of 4–5 rats per group were tested.

Statistics

All experimental results are given as the mean ± SEM. Analysis of variance followed by Fisher's protected least significant difference procedure for post-hoc comparison were

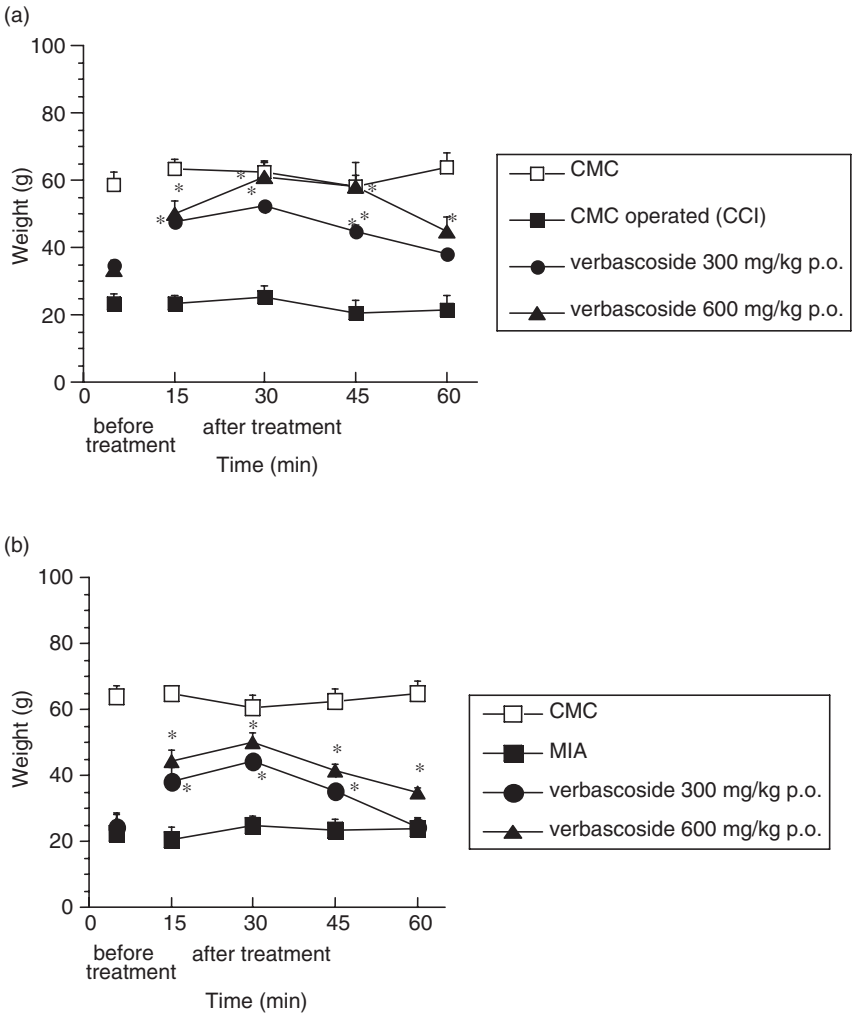

Figure 4 Effect of oral administration of verbascoside in the rat paw-pressure test. Verbascoside was administered orally in two animal models of neuropathic pain where a peripheral mononeuropathy was produced by either in a chronic constriction injury of the sciatic nerve (CCI) (a) or by an intra-articular injection of sodium monoiodoacetate (MIA) (b). CMC, carboxymethylcellulose sodium salt.

used to verify the significance of differences between two means. Data were analysed with StatView software (The MacIntosh Company, Hilliard, OH, USA) for the Macintosh. *P* values of less than 0.05 were considered significant.

Results and Discussion

Commercial dried plant material was exhaustively extracted with hydroalcoholic solution (EtOH 80%). The macerates were filtered, evaporated under reduced pressure and lyophilized to obtain a dried extract which was analysed by HPLC/DAD/ESI MS. The presence of verbascoside in the extract was assessed by comparison of its retention time (t_R) with the authentic sample verbascoside international standard and it was confirmed by UV and MS data (Figure 2). Verbascoside resulted in about 12% of the dried extract calculated using an international standard. Verbascoside was purified by size exclusion chromatography using Sephadex LH-20 and eluting with ethanol (50%). Verbascoside was crystallised with methanol to obtain a purity of >98%, determined by HPLC/DAD/ESI MS and confirmed by nuclear magnetic resonance. Size exclusion chromatography is proposed as a rapid and easily applied method at the industrial scale. This method enabled fast and efficient isolation and purification of verbascoside, recovering more than 85% of the constituent present in the crude dried extract. The advantages of this method include high recovery of the target product, short and well defined separation times, narrow bands which lead to good sensitivity, freedom from sample loss because solutes do not interact with stationary phase, and the possibility to use ecologically friendly solvents.

Neuropathic pain is characterised by nerve damage that causes a chronic pain syndrome characterized by allodynia and hyperalgesia. The antiallodynic effect was quantified by the von Frey filament test and has been reported elsewhere.[23] In this study, we measured the effect of verbascoside on hyperalgesia which is the component of neuropathic pain that can persist for a long time after the initial injury is resolved.[18] The antihyperalgesic activity of pure verbascoside was investigated in animal models of neuropathic pain

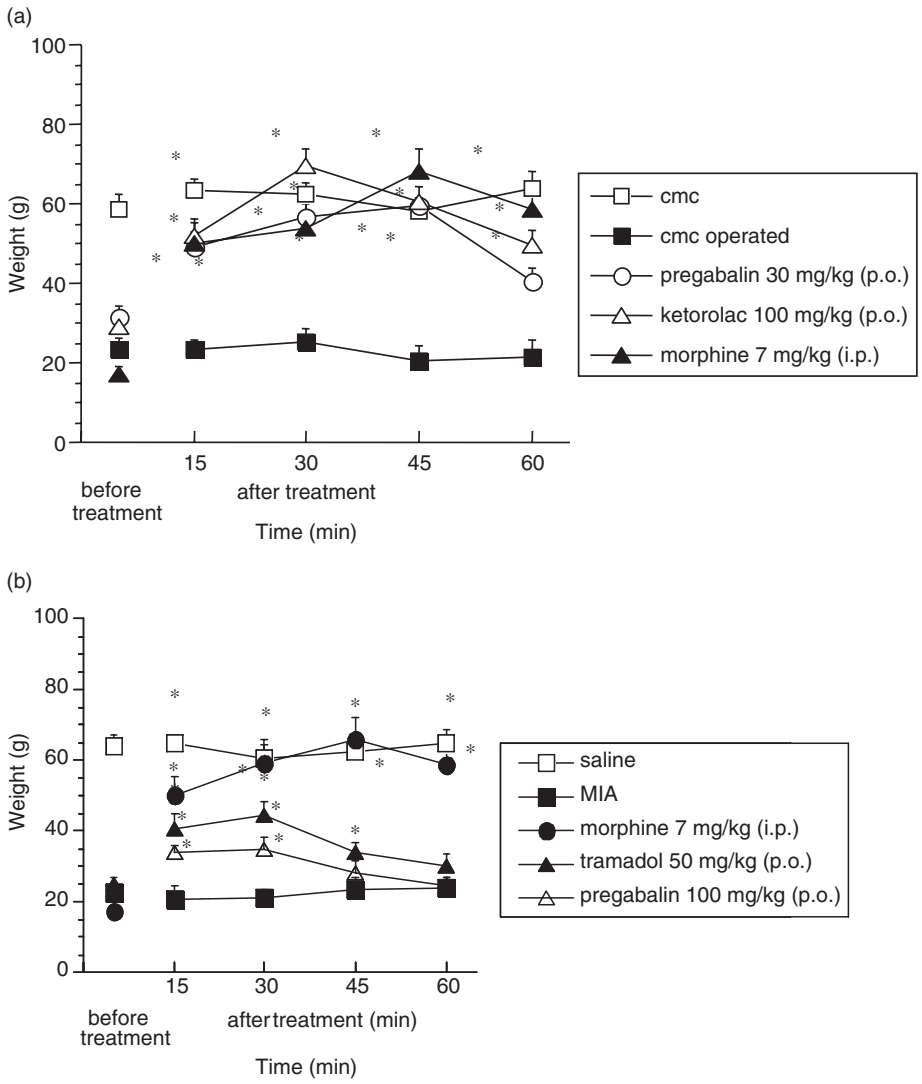

Figure 5 Positive controls used as reference compounds in two animal models of neuropathic pain. Peripheral mononeuropathy was produced by either a chronic constriction injury of the sciatic nerve (CCI) (a) or by an intra-articular injection of sodium monoiodoacetate (MIA) (b).

(CCI) and of peripheral neuropathic pain (MIA). The paw withdrawal threshold was measured using a Randall and Selitto apparatus exerting a force that increases at constant rate (32 g/s). The stimulus at which the rats withdrew the paw was recorded before treatment and after drug administration at different times (15, 30, 45 and 60 min). The results represent means ± SEM of the mechanical threshold expressed as grams. Tests were performed using increasing doses of verbascoside ranging from 10 to 600 mg/kg. A dose of 100 mg/kg was selected for the intraperitoneal injection and 300 mg/kg for the oral administration because these doses showed the best in-vivo performances to reduce hyperalgesia.

Verbascoside when administered intraperitoneally at a dose of 100 mg/kg reverted the mechanical hyperalgesia, as evaluated in the paw-pressure test, in CCI rats (Figure 3a). The antihyperalgesic effect started 15 min after administration and persisted up to 45 min. In the case of MIA treated rats (Figure 3b), verbascoside administered intraperitoneally at the same dose of 100 mg/kg was active against secondary hyperalgesia 15 min after administration and persisted up to 30 min.

Verbascoside was also effective against mechanical hyperalgesia after oral administration. At doses of 300 and 600 mg/kg it reverted the hyperalgesia induced by CCI (Figure 4a) and the secondary hyperalgesia induced by MIA injection with a long-lasting effect (Figure 4b): the antihyperalgesic effect started 15 after administration and it was still significant at 60 min. The positive controls used as reference compounds are reported in Figure 5.

Verbascoside was unable to increase the pain threshold in naïve animals, showing the lack of any analgesic activity at intraperitoneal doses of 50 and 100 mg/kg (Figure 6a) as well as oral doses of 300 and 600 mg/kg (Figure 6b). Therefore the antihyperalgesic activity of verbascoside as a consequence of its analgesic properties can be excluded. Analgesic activity of verbascoside has been reported when administered orally and

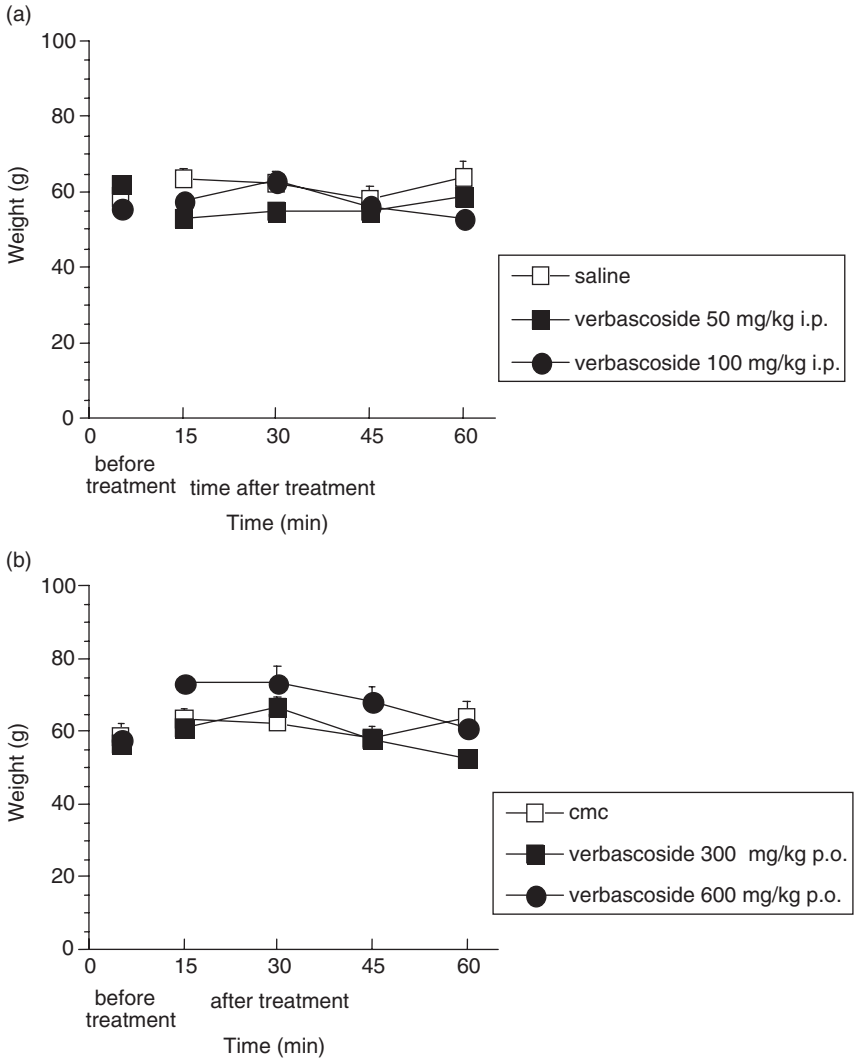

Figure 6 Lack of analgesic activity of verbascoside in the rat paw-pressure test. At interperitoneal doses of 50 and 100 mg/kg (a) as well as oral doses of 300 and 600 mg/kg (b) verbascoside showed a lack of any analgesic activity in the paw-pressure test.

topically in other tests such as writhing and tail-flick assays,[15] where different pathological mechanisms are involved.

The antihyperalgesic effect of verbascoside was not prevented by pretreatment with the opioid antagonist naloxone, suggesting that verbascoside does not require the activation of the opioid system to reverse hyperalgesia (data not shown).

The safety of verbascoside was also established by the rota-rod test: it did not modify gross behaviour of the animals at the highest effective doses. The rota-rod test was also used to reveal any alterations of motor coordination induced by the investigated compound. The number of falls from the rotating rod showed the lack of any impairment in the motor coordination of animals treated with verbascoside in comparison with the control group, ruling out the possibility that the results obtained were due to altered viability of the animals. During each session, the number of falls of mice with unaltered locomotor activity decreased as the animals learned how to balance on the rotating rod (Table 1).

Conclusions

Despite the large number of approved analgesics such as opioids and nonsteroidal anti-inflammatory drugs, the treatment of neuropathic pain or peripheral neuropathic pain is often hampered by poor activity of the available drugs and the occurrence of adverse drug reactions.[24] The current pharmacological treatment of neuropathic pain includes tricyclic antidepressants, anticonvulsants, serotonin-reuptake inhibitors and opioids. However, all of these drugs have limited efficacy combined with a number of side-effects, and the mechanism of how they relieve pain is not fully elucidated. The present findings illustrate the antihyperalgesic properties of the natural compound verbascoside and may help in the development of a new therapeutic strategy for neuropathic pain, particularly in view of its excellent safety profile. The relatively high doses used for both intraperitoneal and oral administration in this study could be related to low bioavailability of this

Table 1 Lack of effect of verbascoside in the rota-rod test

Treatment	Dose (mg/kg p.o.)	Before treatment	After treatment		
			30 min	45 min	60 min
Carboxymethylcellulose sodium salt	–	3.6 ± 0.3	2.2 ± 0.4	1.9 ± 0.3	1.2 ± 0.3
Verbascoside	1000	3.9 ± 0.4	2.5 ± 0.3	2.1 ± 0.3	0.7 ± 0.2

Each value represents the number of falls from the rod in 30 s, as mean of five mice (±SD).

molecule possibly due to very high water solubility and limited hydrophobic properties. Future investigation of its pharmacokinetic behaviour and the introduction of appropriate formulations could lead to a noticeable decrease in the active dose of verbascoside.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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