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Pharmacological mechanism underlying the antinociceptive activity of vanillic acid

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ABSTRACT

Vanillic acid is found at high concentrations in many plants used in traditional medicine. It has been associated with a variety of pharmacologic activities such as carcinogenesis inhibition, apoptosis and inflammation; however, it has become most popular for its pleasant creamy odor. Since there are few reports concerning the antinociceptive activity of this phenolic compound, the aim of this work was to study this activity in in vivo animal models. Vanillic acid was administered by the intraperitoneal route producing a dose-dependent inhibition of the acetic acid-induced writhing response (ED_{50}: 9.3 mg/kg). The antinociceptive activity was inhibited by the pretreatment with ondansetron and yohimbine, indicating that the serotoninergic and adrenergic systems could participate in the mechanism underlying the analgesic activity of vanillic acid. This compound was also demonstrated to interact with ASICs (Acid-sensing Ion Channels) as well as with TPRV1, TRPA1, and TRPM8 receptors in vivo. Furthermore, vanillic acid did not interfere with the locomotor function or motor coordination. The plasmatic phenolic content, analyzed by HPLC, showed that its $t_{1/2}$ and AUC were 0.123 h and 1.38 μg·h/mL; respectively. In conclusion, vanillic acid might represent a potential therapeutic option for the treatment of pain.

1. Introduction

Pain is a transitory unpleasant sensation that follows a noxious or injurious stimulus, acting as a warning system. Particularly, the acute nociceptive pain is the consequence of the activation of primary afferent nociceptive fibers produced by mechanical, chemical or thermal stimuli. Both acute and chronic pains remain a significant health problem and although a considerable number of analgesic drugs are available, the development of novel substances that can effectively treat painful states remains as an important challenge. In this context, many plant-derived substances are attractive sources for developing new analgesic agents.

The role of secondary plant products such as phenolic acids, in the prevention of many human diseases has been extensively described. Particularly, vanillic acid (4-hydroxy-3 methoxybenzoic acid), a phenolic derivative found in several plants and fruits, has shown to possess an interesting pharmacological profile. Experimental studies have provided evidence of effectiveness on cardiovascular (StanelyMainzenPrince et al., 2011), gastrointestinal (Kim et al., 2010) and liver diseases (Itoh et al., 2009). The beneficial activity on acute inflammatory processes has also been described (Leal et al., 2011). Furthermore, vanillic acid has been demonstrated to inhibit the synthesis or release of tumor necrosis factor (TNF)-α, interleukin (IL)-6, cyclooxygenase-2 (COX-2) and nitric oxide (NO), which are mediators that are increased during inflammatory processes (Kim et al., 2011).

We have previously shown that *Lithraea molleoides* (Vell). Engl. (Anacardiaceae) was able to reduce the nociceptive effect induced by acetic acid and formalin tests and this effect was partly related to the presence of its main compound, vanillic acid (Morucci et al., 2012). This study suggested a predominant effect of vanillic acid in models with inflammatory components. However, research studies have not clearly demonstrated the underlying mechanisms involved in the antinociceptive effects of the phenolic compound. Therefore, the primary aim of this study was to investigate the mechanism of vanillic acid inducing antinociception. The pharmacokinetic profile of this compound was also evaluated.

2. Material and methods

2.1. Drugs

Amiloride, indomethacin, ketanserin, pindolol, diazepam, yohimbine, ondansetron, Evans blue, thiobarbituric acid (TBA), phosphotungstic acid, butylhydroxytoluene (BHT), capsaicin, cinnamaldehyde, menthol, ruthenium red, morphine, camphor and vanillic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ultrapure quality water (Milli-Q) was employed to prepare the mobile phase. Acetonitrile (HPLC) and butanol were purchased from J. T. Baker. Acetic acid and Sodium dodecyl sulfate (SDS) were purchased from Merck

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(Darmstadt, Germany). All reagents were of analytical grade. The purity of vanillic acid was checked by HPLC analysis and was 97% on the basis of peak area integration.

2.2. Animals

Female Swiss mice weighing 21–26 g were used. The experiments were carried out taking into account international guiding and local regulations concerning the care and use of laboratory animals for biomedical research. The experiments were approved by the local Ethics Committee (Exp-FyB: 0738658/2011). The animals had free access to a standard commercial diet and water ad libitum and were kept in a room maintained at 22 ± 1 °C with a 12-h light/dark cycle.

2.3. Antinociceptive activity

2.3.1. Acetic acid-induced abdominal writhing

The test was performed as described by Collier et al. (1968). Nociception was induced by intraperitoneal injection of 1.0% acetic acid, (0.1 mL/10 g body weight). Mice were treated with vanillic acid 30 min by the intraperitoneal route (i.p.) (1–100 mg/kg) or intracisternal route (i.c.) (10 and 50 μg/10 μL) before acetic acid injection. A group of mice was treated with indomethacin (10 mg/kg i.p.) as a reference drug. Control animals received a similar volume of saline solution (10 mL/kg, i.p.). The intracisternal route was performed during short anesthesia. Ten or 50 μg/10 μL of solution per mouse was injected slowly into the cisterna magna. Distribution of the injected substances was always checked by giving 0.4% methylene blue aqueous solution in the same manner as the drugs and the vehicle controls. Brains were dissected to verify the location and spread of each injection (Ueda et al., 1979). The animals were observed in experimental cages. Hand-operated counters and stopwatches were employed to score the number of abdominal writhes (full extension of both hind paws). The writhes were cumulatively counted over a period of 20 min immediately after the acetic acid injection. Doses of vanillic acid were selected based on pilot experiments. A significant reduction in the number of abdominal contractions between control and pre-treated animals was considered indicative of antinociceptive activity.

2.3.1.1. Pharmacological evaluation of the mechanism of action. To assess the possible participation of different systems on the antinociceptive effect of the phenolic compound, mice were pre-treated with yohimbine (1 mg/kg i.p.), an α_2 adrenoceptor antagonist; ondansetron (0.2 mg/kg i.p.), a 5-HT_3 receptor antagonist; ketanserin (0.3 mg/kg i.p.), a 5-HT_2 receptor antagonist; and pindolol (1 mg/kg i.p.), a β-adrenoceptor blocker/5-$HT_{1A/1B}$ antagonist, 30 min before the administration of vanillic acid (10 mg/kg i.p.). Doses and drug administration schedules were selected based on previous reports and on pilot experiments carried out in our laboratory (Salam, 2006; de Mattos et al., 2007; Spindola et al., 2011; Vidyalakshmi et al., 2012). The nociceptive response was evaluated in the acetic acid-induced abdominal writhing test.

2.3.2. Nociception induced by capsaicin, cinnamaldehyde, menthol and acidified saline

To test whether TRPV1, TRPA1, TRPM8 and ASIC (Acid-sensing Ion Channels) receptors are potential specific targets for the antinociceptive actions of vanillic acid, a single intraplantar (i.pl.) injection of either capsaicin (1.6 μg/paw), cinnamaldehyde (10 nmol/paw), menthol (1.2 μmol/paw), acidified saline (2% acetic acid in 0.9% saline) or the corresponding vehicle was delivered into the ventral surface of the right hind paw. Each animal was then placed, immediately and alone, into a glass cylinder of 20 cm of diameter positioned on a platform in front of a mirror to enable full view of hind paws. The time spent licking, biting or lifting the injected paw in seconds(s) was used as an index of nociceptive behavior intensity. This activity was recorded for 5 min (for capsaicin or cinnamaldehyde), 20 min (for menthol) or 15 min (for acid saline). Thirty minutes prior to i.pl. injection of the nociceptive agent, mice were treated with vanillic acid (1–100 mg/kg i.p.), morphine (5 mg/kg) ruthenium red (nonselective TRP antagonist, RR, 3 mg/kg i.p.), camphor (TRPA1 antagonist, 7.6 mg/kg i.p.) or amiloride (nonselective ASCI inhibitor, AML, 100 mg/kg i.p.). Control animals received a similar volume of saline solution (10 mL/kg) (Santos and Calixto, 1997; Rios et al., 2013).

2.3.3. Antinociceptive action of vanillic acid after local administration

To determine whether vanillic acid acted locally, 20 μl into the plantar surface of their right hind paw of either vehicles or doses of phenolic acid (10, 50 and 150 μg/paw) was administered 20 min before capsaicin injection into the ipsilateral paw. Also, vanillic acid was administered to the left (contralateral) paw 20 min before injection of capsaicin into the right paw.

2.4. Vascular permeability

The test was performed as described by Yu et al., 2012. Animals were pre-treated with vanillic acid (10–100 mg/kg i.p.) or vehicle; 30 min after the last administration, each mouse received an intravenous injection of 0.1 mL/10 g (0.5% W/V Evans blue solution in saline) and then injected with acetic acid 0.8% i.p. Twenty minutes after the administration of acetic acid, animals were sacrificed and the peritoneal cavity was washed with 6 mL of cold saline (divided into several washings), with a gentle manual massage, the exudates were collected and their volume was added up to 10 mL of saline, followed by centrifugation for 15 min at 3000 rpm. The optical density of the supernatant was measured at 590 nm in a spectrophotometer. The dye extravasation was quantified from the standard curve and the percentage of inhibition was calculated.

2.5. Measurement of TBARs

Thiobarbituric acid reactive substances (TBARs) are low molecular weight compounds formed via decomposition of certain primary and secondary lipid peroxidation products that at low pH and at high temperature participate in a nucleophilic addition reaction with thiobarbituric acid generating a red fluorescent complex (Fraga et al., 1987). At the end of the writhing test, mice were anesthetized with pentobarbital and blood samples were obtained. Samples were centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was collected and stored at −80 °C until analysis. One hundred microliters of the plasma was mixed with 4% BHT, 3% SDS, 10.0% phosphotungstic acid and 2 mL of a 0.7% TBA solution, boiled for 45 min and cooled at room temperature. The chromogen was then extracted with 2.0 mL of butanol by vigorous shaking for 1 min. After centrifuging, (10 min, 1500 g, 25 °C), MDA (malondialdehyde) was measured spectrofluorimetrically (excitation at 515 nm, emission at 555 nm).

2.6. Behavioral assessment

2.6.1. Rota-rod

To evaluate the possible occurrence of non-specific effects such as muscle-relaxation or sedation, the effect of vanillic acid on motor coordination was assessed in mice subjected to the rota-rod test (Dunham and Miya, 1957). Animals that were able to stay on the bar of the apparatus (2.5 cm diameter bar, 25 cm above the floor, turning at 14 rpm) for two consecutive periods of 120 s were selected to receive i.p. vanillic acid (10, 30, 100 mg/kg), diazepam (2 mg/kg i.p.) or saline solution (10 mL/kg). Thirty minutes after the treatment, animals were placed on the apparatus for up 120 s, and the time each animal remained on the bar during each trial was recorded.

2.6.2. Open field

To assess the possible effects of vanillic acid on locomotor activity, mice were evaluated individually in an open field paradigm (Menegatti et al., 2006). A wooden box (25 × 25 × 15 cm) with the floor divided into 9 squares was used. The number of squares crossed with the four paws was registered during a period of 5 min. Animals were treated with vanillic acid (10, 30, 100 mg/kg i.p.), diazepam (2 mg/kg i.p.) or saline solution (10 mL/kg) 30 min before the experiments.

2.6.3. Hole board

To perform the hole-board test a 40 × 40 cm, 2.2 cm thick Ugo Basile apparatus (model 6650), with 16 spaced holes with built-in infrared sensors was used. In brief, mice were randomly divided into 5 groups of 6 animals each. Three groups received the compound (10, 30 and 100 mg/kg, i.p.). One group received diazepam (DZP) (2 mg/kg, i.p.) and the remaining group (control) received saline. Thirty minutes later and over a period of 5 min, the number of head dipping into the holes was counted for each animal (Takeda et al., 1998).

2.7. Pharmacokinetic profile

The pharmacokinetic profile was determined in plasma by HPLC. The HPLC analysis was performed on a Varian Pro Star instrument equipped with a Rheodyne injection valve (20 μl) and a photodiode array detector set at 280 nm. A reverse-phase column Phenomenex-C18 Gemini (150 mm × 4.6 mm·5 μm) and guard column C18 were used. The flow rate was 0.8 mL/min and the separation was done at room temperature. The mobile phase consisted of aqueous acetic acid (1% v/v) and acetonitrile (85:15) pH 3. Chromatograms were obtained and processed using the Varian Star Chromatography Workstation version. A stock standard solution of vanillic acid (1 mg/mL) was prepared in methanol and stored at 4 °C. Working plasma standards from 0.1 to 25 μg/mL were prepared using blank rat plasma spiked with the stock standard of the phenolic compound and were kept at −20 °C. Blood samples were drawn into heparin-coated tubes at 5, 15, 30 and 45 min and centrifuged at 2500 rpm for 10 min. Plasma samples were stored at −20 °C in polypropylene vials. Prior to analysis, samples were thawed at room temperature. Plasma proteins were precipitated by the addition of acetonitrile and vortex mixing. Samples were then centrifuged at 13000 rpm for 10 min. The clear supernatant was injected into the loop of the HPLC device. The areas under the plasma concentration–time curve (AUC), systemic clearance (CL) as well as half-life ($t_{1/2}$) were determined by non-compartmental analysis, using the Tofit 2.1 software (Heizel et al., 1993). The absolute bioavailability (F) was calculated after i.p. or intravenous (i.v.) administration of vanillic acid, using the following equation: F: (i.p. $AUC_{0-\infty}$ / i.v. $AUC_{0-\infty}$) × (i.v. dose/i.p. dose) × 100%.

2.8. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences between groups was assessed by means of analysis of variance (ANOVA) followed by Dunnett's test. P values of 0.05 and 0.01 were considered significant. The statistical analysis was carried out using the Instant statistical package (Graph pad software, Inc., version 5 USA).

3. Results

3.1. Antinociceptive activity

Vanillic acid (3–100 mg/kg i.p.) produced a dose-dependent inhibition of the acetic acid-induced writhing response (ED_{50}: 9.3 mg/kg — 95% confidence interval: 11.2–8.1 mg/kg). The maximal antinociceptive effect (88.9%) was obtained at a dose of 100 mg/kg i.p., indicating that vanillic acid exerted a marked analgesic effect in this model of visceral pain. On the other hand, the pre-treatment of mice with indomethacin, reference group, showed a significant inhibition of the nociceptive effect exerted by the acetic acid (75%, Fig. 1A). Also, vanillic acid at a dose of 50 μg i.c. produced antinociceptive activity, showing an inhibition of 68.2% without any effect at lower dose (No. of writhes: saline group: 23 ± 2, 50 μg/10 μl: 5 ± 1, 10 μg/10 μl:20 ± 3). The mechanism of action of vanillic acid was investigated by pre-treating the animals with several drugs that interfere in different systems. The pre-treatment with ketanserin did not modify the antinociceptive response elicited by the compound while yohimbine, ondansetron and pindolol significantly prevented it (Fig. 1B). Furthermore, only the highest dose of vanillic acid inhibited the nociceptive response induced by acidified saline solution (inhibition of 48.3%). Moreover, the blocking of ASICs by amiloride (100 mg/kg, i.p.) also decreased the nociception mediated by acidified saline by 65.2% (Fig. 2A). In the capsaicin test, results showed the ability of vanillic acid to induce a significant and dose-dependent inhibition of the capsaicin-induced nociception (ED_{50}: 18 mg/kg, 95% confidence interval: 28–10 mg/kg). Moreover, morphine produced a significant antinociceptive activity in the same model (Fig. 2B). As shown in Fig. 2C, only the highest dose of vanillic acid inhibited the nociceptor behavior induced by cinnamaldehyde with an inhibition of 58.7%, meanwhile camphor, the reference drug, inhibited 61.0%. Finally, previous treatment with the phenolic acid (ED_{50}: 34 mg/kg, 95% confidence interval: 44–27 mg/kg) or ruthenium red reduced nociception evoked by menthol (Fig. 2D).

Local ipsilateral, but not contralateral administration of vanillic acid reduced in a dose dependent manner capsaicin induced nociceptive behavior. The nociception was significantly reduced when mice were treated with both higher doses of vanillic acid (time of licking: saline group: 95.0 ± 4.7 s, 10 μg/paw: 96.2 ± 9.1; 50 μg/paw: 64.2 ± 8.0 s; 150 μg/paw: 48.5 ± 3.6 s), suggesting that local mechanisms could be involved in the antinociception induced by vanillic acid.

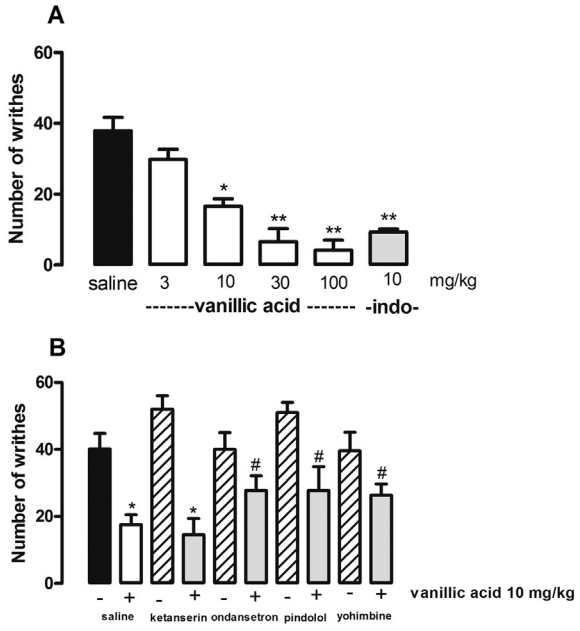

Fig. 1. Effects of intraperitoneal administration of vanillic acid in the writhing test. Results were obtained by intraperitoneal (i.p.) administration of vanillic acid and indomethacin (A). Pretreatment with different drugs that interfere in different systems (B). Each value represents mean ± SEM of results obtained from 8 mice. Statistical differences were determined by ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01 (compared with saline group), #P < 0.05 compared with vanillic group (10 mg/kg, i.p.).

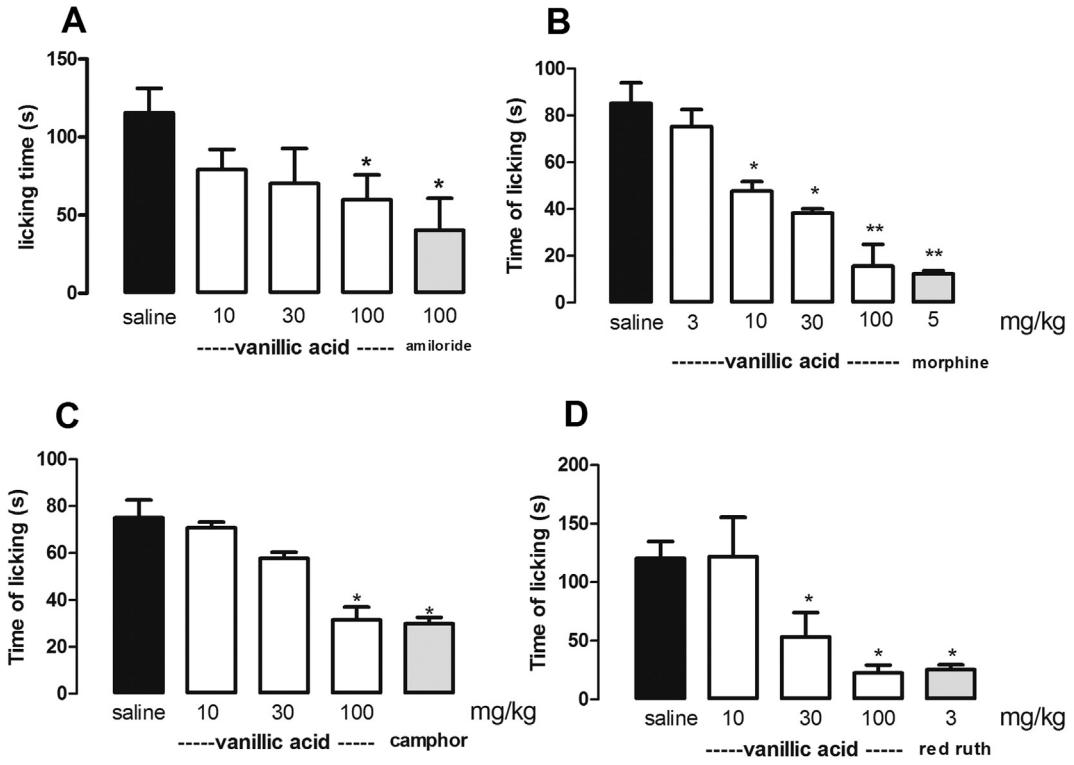

Fig. 2. Effects of intraperitoneal administration of vanillic acid on ASCIC receptor evaluation test (A), on capsaicin test (B), on cinamaldehyde test (C) and menthol test (D). Each value represents mean ± SEM of results obtained from 8 mice. Statistical differences were determined by ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01 (compared with saline group).

3.2. Vascular permeability

As shown in Fig. 3A, vanillic acid at 10, 30 and 100 mg/kg exhibited significant and dose-dependent inhibitory effects on the increased vascular permeability induced by acetic acid in mice, with an inhibition of 76.1% obtained with the highest dose of the compound. The positive control drug, indomethacin, also reduced the dye extravasation with an inhibition rate of 34.8%.

3.3. Measurement of TBARs

As observed in Fig. 3B, MDA levels in plasma were significantly reduced when mice were treated with vanillic acid, showing a maximum inhibition (64%) at 100 mg/kg.

3.4. Behavioral assessment

Behavioral assessment was carried out to determine if the antinociceptive effects of vanillic acid were caused by any disturbances on the central nervous system. The locomotor activity and neuromuscular coordination were not affected by different doses of vanillic acid. Diazepam, used as the reference drug, produced alteration in the locomotor activity and on the motor performance (Fig. 4A and B). Besides, in the hole board test, a well-established method to evaluate potential anxiolytic and/or sedative effects induced by drugs, vanillic acid did not cause any change in the head-dipping response while DZP (2 mg/kg), the reference drug, caused a marked reduction of such behavior (Fig. 4C). The locomotor activity was not affected by vanillic acid i.c. at any doses (No. of crossed lines/5 min: 585 ± 70, 50 μg/10 μl: 550 ± 85, 10 μg/10 μl: 532 ± 58).

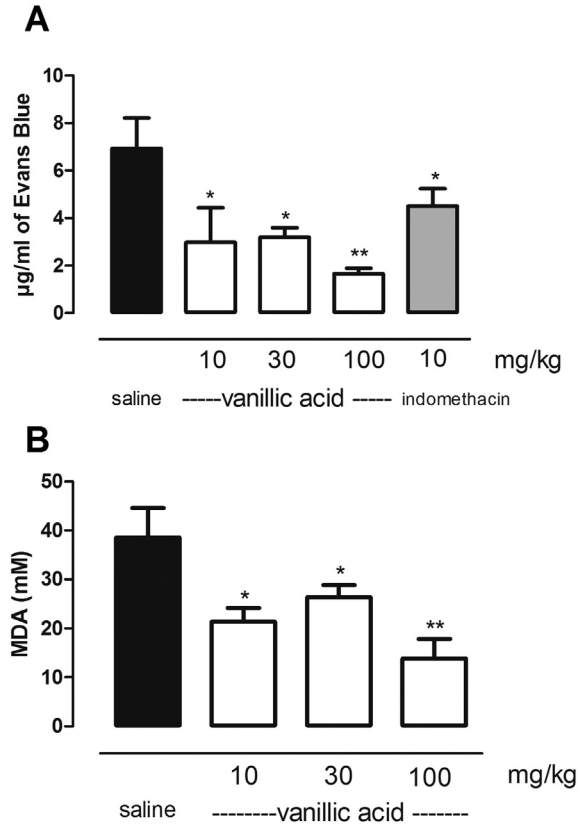

Fig. 3. Effect of vanillic acid on vascular permeability (A) and TBARs evaluation (B). Each value represents mean ± SEM of results obtained from 6 mice. Statistical differences were determined by ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01 (compared with saline group).

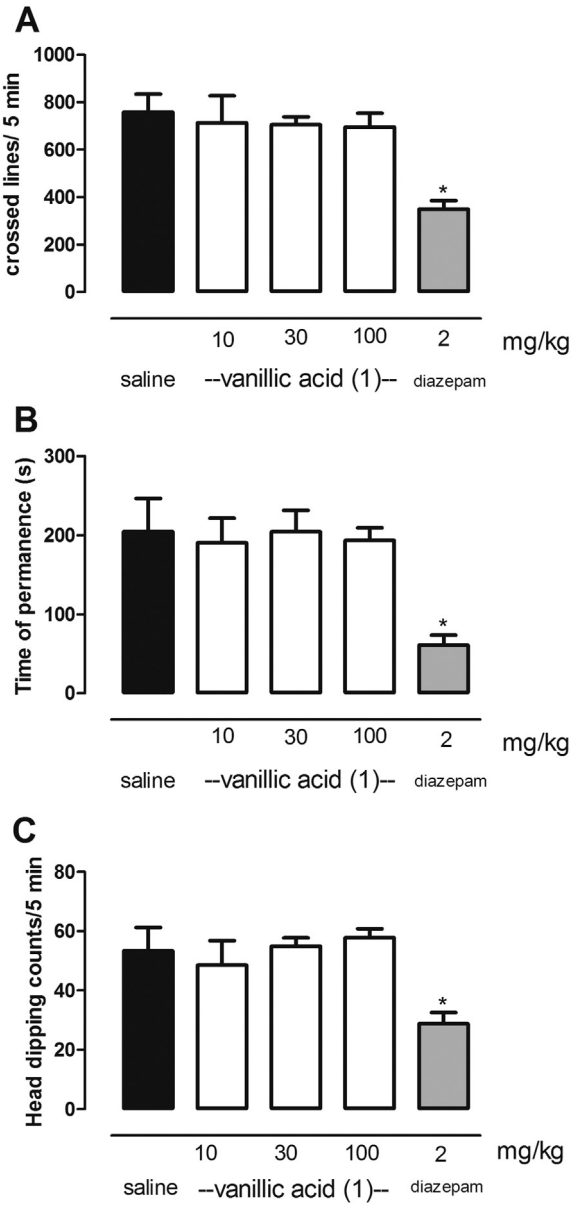

Fig. 4. Effect of vanillic acid in crossed lines in open field test (A), time of permanence in the Rota-rod test (B) and head dipping in Hole board test (C). Each value represents mean ± SEM of results obtained from 6 mice. Statistical differences were determined by ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01 (compared with saline group).

3.5. Pharmacokinetic profile

Chromatograms of phenol-free mice plasma demonstrated a good chromatographic selectivity with no endogenous plasma interferences at the retention time of vanillic acid (8 min) (Fig. 5A). A typical chromatogram from mice plasma dosed with vanillic acid at 10 mg/kg is shown in Fig. 5B. The calibration curve was linear ($y = 18491\ x + 17128$) with a correlation coefficient of 0.988. The linear range for the determination of vanillic acid was 0.1 to 25 μg/mL (Fig. 5C). Precision (percent relative standard deviation %RSD) was 1.17% to 5.46% while accuracy was 96% to 115%. Recovery of vanillic acid was obtained from the corresponding spiked plasma and mean recovery rate was 51.5% (n = 6). Taking into account these data, the performance of the HPLC was considered suitable for subsequent measurements. Plasma concentrations were fitted adequately using a non-compartment model to obtain the pharmacokinetic profile. Concentration–time curves after a single dose of 10 mg/kg revealed that the peak of vanillic acid concentration was reached approximately 5 min after administration (Fig. 5D) and the pharmacokinetic analysis of the plasma concentration–time curves showed that the $t_{1/2}$ was 0.123 h, the AUC_{0-45} was 1.35 μg·h/mL, the $AUC_{0-\infty}$ was 1.38 μg·h/mL, the mean residence time was 0.164 h, the systemic clearance was 1.01 mL/min and the volume of distribution was 11.2 mL. Finally, F value was 30% in mice, based on the AUC of i.p. and i.v. administration.

4. Discussion

This study demonstrated the antinociceptive action of vanillic acid in a chemical-induced pain model and the possible involvement of serotoninergic, adrenergic systems, ASIC channel and vanilloid type transient receptor potential vanilloid (TRPV). Taking into account that pain is the most common motivating factor to seek medical attention and, in spite of the progress that has taken place in the development of analgesic dugs, extensive studies in search for safer analgesic agents have been necessary. Since early studies have identified antinociceptive properties of vanillic acid (Leal et al., 2011; Morucci et al., 2012), the aim of this study was to investigate the mechanisms that might be involved in the observed effect.

Responses to noxious chemical stimuli were determined in the acetic acid-induced abdominal constriction assay, a classical, simple and sensitive model of acute pain for measuring antinociception induced by central or peripherally acting analgesics. In agreement with previous results, the vanillic acid reduced the visceral pain induced chemically (Morucci et al., 2012).

The induction of pain behaviors in animals relies upon a stimulus applied to a nociceptive neuron and the activation of a pain pathway. Several drugs induce analgesia or antinociception by interfering with the neuronal pathways involved in the receipt and transmission of the nociceptive information from the periphery to higher centers in the central nervous system. In this sense, the noradrenergic system would play a modulatory role in the expression of behavioral effects caused by an irritant agent (Korzeniewska-Rybicka and Plaźnik, 2001). Bezerra et al. (2008) have shown that α_2-adrenoceptor agonists significantly reduce the writhing response. Furthermore, these kinds of agonists are used in clinical practice for the treatment of acute pain events and prevention of postoperative pain (Eisenach et al., 1995). In this sense, the mechanism by which vanillic acid modulates pain transmission is likely to involve adrenergic pathways, since the action of the compound was reduced by prior treatment with yohimbine, a selective α_2-adrenoceptor antagonist.

The role played by serotoninergic receptors in the regulation of modulation of nociceptive processing has been demonstrated in many studies. Serotonin is a monoamine widely distributed both at the periphery and in the central nervous system and together with other proinflammatory mediators at the periphery, is one of the active compounds which contribute to inflammation-induced pain (Viguier et al., 2013). In agreement with the results obtained in the writhing test, ondansetron and pindolol exhibited a significant inhibitory effect on the activity of vanillic acid, suggesting that the $5HT_3$ and $5HT_1$ receptors would be involved in the antinociception caused by the studied compound. $5HT_2$ receptors were not involved, since ketanserin did not affect vanillic acid-induced antinociception. Therefore, it could be hypothesized that the serotoninergic and adrenergic systems are involved in the antinociceptive activity of vanillic acid in the pain induced by acetic acid.

The next step was to evaluate other possible mechanisms involved in the analgesic activity of the phenolic compound. Since acetic acid promotes local irritation mediated by the dissociation of protons stimulating TRP and ASIC channels located in primary afferent neurons, the involvement of these receptors in the antinociceptive mechanism of vanillic acid was evaluated.

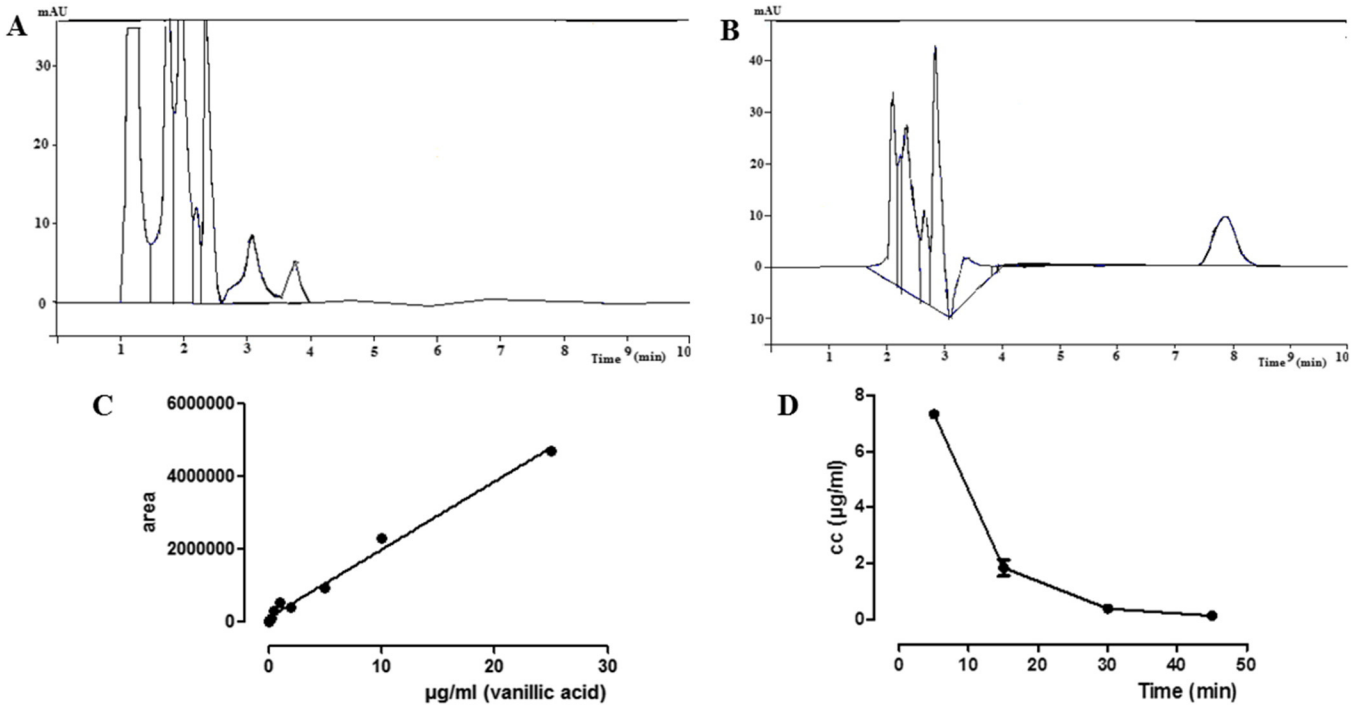

Fig. 5. Chromatogram of free drug in mice plasma (A), mice plasma obtained after the administration of vanillic acid 10 mg/kg (B), area vs concentration of vanillic acid curve (C) and concentration vs time curve (D).

ASIC channels play a critical role in the perception of a wide range of pH changes in conditions related to tissue acidosis. ASICs channels are voltage-insensitive, proton-gated channels which are activated by a decrease in the pH and are expressed throughout peripheral sensory and CNS neurons. These channels, particularly ASIC3 and ASIC1a, are highly sensitive to moderate pH changes and they are activated during tissue inflammation and participate in pain sensation (Chu et al., 2011). In this study, vanillic acid relieved acidosis evoked pain in the same fashion as amiloride, a classical nonselective ASIC inhibitor.

Since protons excite or sensitize nociceptors by interacting with different molecular targets, including members of the ASIC and the TRP channel families, the effect of vanillic acid on nociception induced by the activation of the TRPV1, TRPA1 and TRPM8 was also studied.

The TRPV1 receptor plays a prominent role in the acid-evoked sensitization of cutaneous and visceral nociception. This receptor features a population of non-myelinated neurons, whose activation promotes vascular leakage and vasodilatation, culminating in the production and release of proinflammatory and proalgesic factors. This neurogenic inflammation helps in initiating a cycle in which injury-evoked nociceptor activation initiates or exacerbates the inflammatory response (Damasceno et al., 2014; Julius, 2013). Capsaicin interacts directly with TRPV1 channel to serve as a positive allosteric modulator (Julius, 2013) and this study showed that vanillic acid produced inhibition against capsaicin-induced nociception, indicating that the phenol inhibits the nociceptive transmission initiated by TRPV1 activation. Therefore, not only did this phenol diminish pain but also it could facilitate healing by interrupting this neuroinflammatory cycle.

Since it is known that TRPA1 is co-expressed within a subset of TRPV1-expressing sensory neurons (Julius, 2013), and that they are some of the main transducers of the nociceptive response, the decrease of pain-related responses to cinnamaldehyde induced by vanillic acid, could suggest that these receptors could be involved in the antinociceptive activity mediated by the phenol. In the same sense, since menthol is known to be a positive allosteric modulator of TRPM8, the participation of this receptor in the antinociceptive activity of vanillic acid could not be ruled out.

TRP ion channel family activates sensory neurons to produce acute or persistent pain. The TRP channel signaling pathway has been investigated, the participation of phospholipase C, phosphatidyl-inositol-4,5-bisphosphate, inositol triphosphate, diacylglycerol and downstream polyunsaturated fatty acids has been described (Julius, 2013; Hardie, 2003). So, the effect of phenolic acid at this level cannot be discarded. More studies are necessary to confirm the intracellular pathway.

As nociceptors were chemically stimulated, not only were they able to transmit information to the CNS, but also they accomplished the antidromical release of neurotransmitter, which, in turn, have peripherical effects. Particularly, some nociceptors that are stimulated by acetic acid, release substances such as histamine, serotonin, cytokines, eicosanoids and neuropeptides (tachykinins and calcitonin gene-related peptide) in peritoneal fluid which elicit vasodilation, vascular leakage and other responses from nearby peripheral cell types, exacerbating the inflammatory process and leading to the perception of pain perception (Deraedt et al., 1980; Ikeda et al., 2001). In this context, the Evans blue dye has been widely used as a marker of arterial wall extravasation and it is an excellent indicator of increased vascular permeability to macromolecules, since it binds almost immediately to plasma albumin. The inhibition of the increase of abdominal capillary permeability induced by acetic acid demonstrated by vanillic acid could decrease the release of inflammatory mediators, thus modifying the threshold of inflammatory pain and contributing to explain the pharmacological profile of vanillic acid.

There are evidences indicating that the oxidative stress is critically involved in the development and maintenance of pain. The antioxidant activity of vanillic acid has been demonstrated in vitro (Tai et al., 2012); however, to study such properties in vivo is essential. The levels of plasma lipid oxidation products assessed by the TBARS assay were significantly modified when mice were treated with the highest dose of vanillic acid, showing that the phenolic compound possesses an antioxidant effect in vivo. Taking into account that the occurrence of oxidative stress has been described in several animal models of pain, and that this phenomenon has been demonstrated to be implicated in the induction and maintenance of NMDA receptor-mediated pain (Rossato et al.,

2011), it could be hypothesized that vanillic acid might accomplish its antinociceptive activity by protecting targets from reactive oxygen species.

A major concern in the evaluation of analgesic action of compounds is whether pharmacological treatment causes other behavioral alterations, such as motor incoordination and sedation, which might be misinterpreted as analgesic activity. A behavioral assessment was carried out to determine if the antinociceptive effects of vanillic acid were exerted by any disturbances on the central nervous system. The locomotor activity and neuromuscular coordination were not affected by vanillic acid. Besides in the Hole board test, a well-established method to assay potential anxiolytic and/or sedative effects caused by drugs, vanillic acid did not cause any change in the head-dipping response. Therefore, the inhibition of the nociceptive activity does not seem to result from a non-specific muscle-relaxant or sedative effect because vanillic acid did not show any alterations in the behavioral assessment.

Regarding the pharmacokinetics study, it was necessary to adapt the method of Don Farthing et al. (1999) who developed a useful methodology applicable to human fluids Modifications to the original protocol were introduced to apply such method to mice plasma. In this sense, the detection (0.05 μg/mL) and quantification (0.1 μg/mL) limits were improved significantly while maintaining a good precision. In addition, the measurement of drug exposure (AUC) and maximum time and plasma concentration of the efficacious dose in mice are in line with the study carried out by Luo et al. (2014). The vanillic acid circulating concentrations reached maximal blood levels in about 5 min and decreased rapidly after the peak. Nevertheless at lower plasma concentration of the phenolic acid, antinociception was observed. It has been described that this phenol was widely distributed in the kidney and to a lesser extent, in the liver, implying that the distribution of phenolic acid depends on the blood flow perfusion rate of the tissue, but also, trace amounts of vanillic acid were detected in the brain (Luo et al., 2014).

It has been reported that the influence of peripheral inflammatory disease reaches far beyond the affected region and that it could modulate the function of the central nervous system (Hopkins, 2007). Likewise other plant phenols, following i.p. administration the phenolic compound rapidly crosses the blood–brain barrier and enters the brain tissue (Guest and Grant, 2012); this phenomenon leads to a rapid decrease in the plasma concentration after a few minutes. While only the peripheral terminal of the nociceptor will respond to environmental stimuli, both the peripheral and central terminals can be targeted by analgesic drugs in order to influence the transmission of pain messages. Taking into account that 5-HT neurons within the rostroventral medulla (Viguier et al., 2013), TRP receptors within periaqueductal gray and rostral ventromedial medulla (Palazzo et al., 2012) and ASIC subunits within hippocampal neurons (Deval et al., 2010) are involved in the detection, transmission and regulation of pain, it could be possible that the low concentration of vanillic acid at the time that the antinociceptive effect was measured, could be due to a rapid redistribution of the compound into the CNS to exert its antinociceptive effect. In this sense, the antinociceptive activity observed by intracisternal injection of vanillic acid could be representing both spinal and/or supraspinal effect of the phenolic compound. But, the effect of vanillic acid at peripheral level could not be rejected since ipsilateral i.pl. administration of phenolic acid significantly inhibited capsaicin induced nociception in a dose-dependent manner without any effect of contralateral paw administration of vanillic acid.

Finally, bioavailability of vanillic acid (30%) could be enhanced in order to potentiate its benefits.

5. Conclusions

The present study indicates that vanillic acid can be effectively absorbed after systemic administration to modulate nociception, suggesting that vanillic acid might represent a potential therapeutic option for the treatment of pain-related diseases. The present observations will further contribute to a better understanding of the peripheral and central antinociceptive mechanisms of action induced by vanillic acid, which will give support for future investigations. The multiple targets involved in the mechanism of action offer a promising option as an effective treatment for visceral and inflammatory pain.

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