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Regular Article

Analgesic Effect of *Ilex paraguariensis* Extract on Postoperative and Neuropathic Pain in Rats

Dong Wook Lim,[a,#] Jae Goo Kim,[a,#] Taewon Han,[a] Sung Keun Jung,[b,c] Eun Yeong Lim,[a,c] Daeseok Han,[a] and Yun Tai Kim*[,a,c]

[a] *Research Group of Innovative Special Food, Korea Food Research Institute; 62 Anyangpangyo-ro, Bundang-gu, Seongnam 463–746, Republic of Korea:* [b] *Research Group of Nutraceuticals for Metabolic Syndrome, Korea Food Research Institute; 62 Anyangpangyo-ro, Bundang-gu, Seongnam 463–746, Republic of Korea: and* [c] *Department of Food Biotechnology, Korea University of Science & Technology; 217 Gajeong-ro, Yuseong-gu, Daejeon 305–333, Republic of Korea.*

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***Ilex paraguariensis*, known as "Yerba Mate," is an herb used in a beverage that is widely consumed in southern Latin American countries. Furthermore, it has been traditionally used to treat depression, and as an analgesic to manage both nerve pain and headache. The pain-related experimental evidence regarding the analgesic effects of Mate is unclear. Therefore, this study was designed to investigate whether Mate extract exhibits analgesic effects in both the plantar incision and spared nerve injury (SNI) models in rats. We tested the mechanical withdrawal threshold (MWT) using von Frey filaments. We also tested pain-related behavior using ultrasonic vocalization (USV). Neuropeptide Y (NPY) and pain-related cytokines were also determined in the dorsal root ganglia in a rat model of SNI. Our results showed that oral administration of Mate extract significantly increased MWT values, and reduced the number of 22–27 kHz USVs 24 h after the plantar incision operation. Moreover, after 15 d of continuous treatment with Mate extract, the SNI-induced hypersensitivity, cytokine levels, and NPY expression were significantly reduced compared to the corresponding findings in the control group. These results suggest that the intake of Mate extract has potential as a treatment for both postoperative pain and neuropathic pain.**

Key words Yerba Mate; analgesic effect; mechanical hyperalgesia; neuropeptide Y; pain

Pain is a common and distressing symptom of many diseases. Its management remains a major clinical challenge, because the mechanisms that cause pain are poorly understood, and there is a lack of effective treatments.[1)] Prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) may cause adverse effects such as renal and liver failure, and gastrointestinal lesions.[2)] Moreover, the use of current analgesic drugs, opioids in particular, is limited by side effects such as tolerance and physical dependence.[3)] Therefore, it is necessary to identify new analgesic drugs that are both tolerable and effective.[4)] Alternative approaches, such as the use of natural products, have been tested for therapeutic application, including the management of pain.[5–7)]

Ilex paraguariensis Aquifoliaceae St. Hilaire, known as "Yerba Mate" or simply "Mate," is used to make an herbal tea beverage that is widely consumed in southern Latin American countries (southern Brazil, Argentina, Paraguay, and Uruguay) and is gaining rapid penetration into world markets, including the United States.[8)] Recently published evidence has shown some beneficial effects of Mate, including antioxidant,[9)] anti-inflammatory,[10)] antiobesity,[11)] antihyperlipidemic,[12)] and antidiabetic properties.[13)] To our knowledge, based on pain-related experimental evidence, little is known about the potential analgesic effects of Mate.

The present study was designed to investigate whether Mate extract exhibits antinociceptive effects by using a plantar incision model of postoperative pain,[14)] and a spared nerve injury (SNI) rat model of neuropathic pain.[15)] To evaluate pain-related behavior, we studied the mechanical withdrawal threshold (MWT), as measured using von Frey filaments, and pain-induced ultrasonic vocalizations (USVs), measured using ultrasonic microphones.[16)] In addition, levels of neuropeptide Y (NPY) and pain-related cytokines were determined in the dorsal root ganglia (DRG) in a SNI rat model.

MATERIALS AND METHODS

Preparation of the *Ilex paraguariensis* Sample Dried leaves of *Ilex paraguariensis* were purchased from Kapdang Co. (Seoul, Korea). The sample was identified by Dr. Yun Tai Kim, and a specimen (#NP-1208) was deposited with the research group of Innovative Special Food, Korea Food Research Institute. Dried leaves of *I. paraguariensis* (300 g) were extracted in a reflux apparatus by using 70% ethanol (3000 mL) at 80°C for 4 h. The process was repeated once; the extracts were combined and filtered through a membrane filter (0.45 μm; Millipore, Billerica, MA, U.S.A.). After removing the solvents *via* rotary evaporation, the remaining extracts were freeze-dried, yielding about 20.2% of the dried leaf weight (w/w). The crude extract powder (100 mg) was dissolved in methanol (5 mL) and filtered through a 0.45 μm filter (Sartorius Stedim, Goettingen, Germany). Then, 10 μL was injected into an HPLC system (Jasco, Hachioji, Tokyo, Japan) comprising a PU-980 pump, an AS-950-10 autosampler, and an MD-2010 Plus multi-wavelength detector. Chromatic separation was conducted at 30°C on Waters Symmetry C18 5-μm column, (4.6×250 mm) with gradient elution using a mobile phase composed of water, 0.1% (v/v) acetic acid, and

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: ytkim@kfri.re.kr

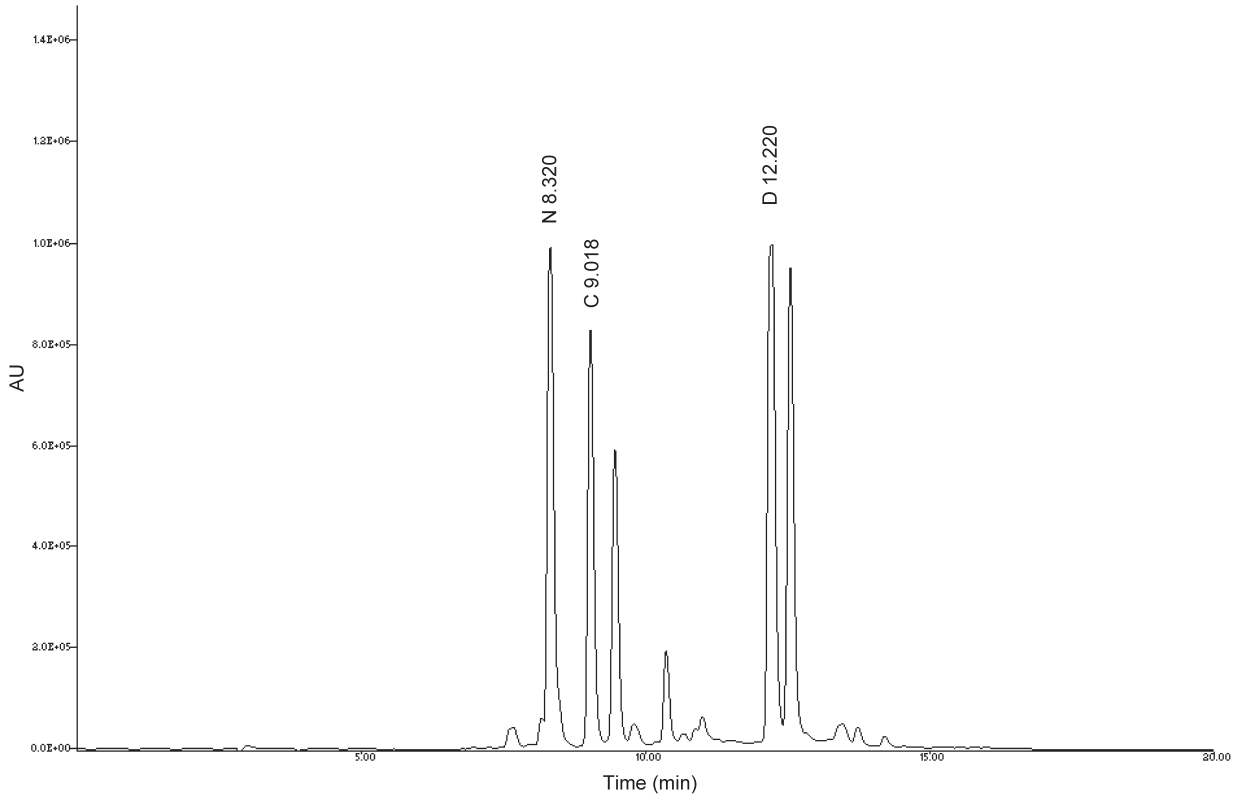

Fig. 1. HPLC Chromatogram of the Composition of *I. paraguariensis*
N: Neochlorogenic acid; C: Chlorogenic acid; D: 3,5-Dicaffeoylquinic acid.

acetonitrile. The analytes were eluted from the column using a linear gradient of acetonitrile, which increased from 0 to 40% (v/v) within 30 min, held at 40% for an additional 10 min, and then returned to the initial gradient. The run time was set at 40 min, the flow rate was set at 1.0 mL/min, and the samples were detected at 280 nm (Fig. 1). The quantitative analysis was replicated three times. The regression equation and correlation coefficient (R^2) of each standard curve were automatically determined using Excel (Microsoft, Redmond, WA, U.S.A.). The regression equations for 3,5-dicaffeoylquinic acid, chlorogenic acid, and neochlorogenic acid were y=35105.0573x, y=30439.1576x–51133.2739, and y=28909.6621x, respectively. R^2 ranged from 0.99986 to 0.99993, indicating that a high linear correlation was achieved for all standard curves. The concentrations of 3,5-dicaffeoylquinic acid, chlorogenic acid, and neochlorogenic acid were found to be 23.79±0.2681, 16.2±1.295, and 22.6±3.21 mg/g using the peak area in the chromatogram and the regression equation.

Animals and Treatments Male Sprague-Dawley (SD) rats (160–200 g) were purchased from Samtako (Gyeonggi-do, Korea). The animals were housed two rats per cage in an air-conditioned room at 23±1°C, 55–60% relative humidity, and a 12-h light/dark cycle (07:00 lights on, 19:00 lights off). The rats were given a regular laboratory rodent diet and water *ad libitum*. After acclimatization for one week, eight-week-old male SD rats were anesthetized with 2% isoflurane and the pain-related surgeries were performed. After the plantar incision operation, the rats were divided into four groups: (1) control+vehicle, (2) control+naproxen (30 mg/kg) (3) control+*I. paraguariensis* extract (100 mg/kg), and (4) control+*I. paraguariensis* extract (300 mg/kg). Naproxen was diluted in 0.9% (w/v) saline solution with 5% Tween 20. It was injected intraperitoneally (i.p., using an injection volume of 0.3 mL/100 g body weight) 1 h after the plantar incision operation. The *I. paraguariensis*-treated groups were orally administered the extract 1 h after the plantar incision operation. After the SNI operation, the rats were divided into five groups: (1) SNI-control+vehicle, (2) SNI-control+naproxen (30 mg/kg), (3) SNI-control+chlorogenic acid (30 mg/kg), (4) SNI-control+*I. paraguariensis* extract (100 mg/kg), and (5) SNI-control+*I. paraguariensis* extract (300 mg/kg). An oral dose of *I. paraguariensis* extract was given immediately after the surgery, and once a day for 15 consecutive days. All animal experiments were performed according to the guidelines of the Korea Food Research Institutional Animal Care and Use Committee (KFRI-M-12024).

Plantar Incision Model of Postoperative Pain Rats were anaesthetized with 2% isoflurane. A 1 cm longitudinal incision was made with a scalpel, through the skin and fascia of the plantar aspect of the paw, starting 0.5 cm from the proximal edge of the heel and extending toward the toes. The plantar muscle was elevated and incised longitudinally. Following hemostasis with gentle pressure, the skin was opposed with two single interrupted sutures using polyamide monofilaments. The animals were allowed to recover in their home cages.

USV Analysis Pain-induced USVs in rats were tested as previously described.[17] After the plantar incision was complete, the 22–27 kHz USVs that were emitted by adult rats were monitored and scored for 10 min by using Sonotrack ultrasonic microphones (Metris B.V., KA Hoofddorp, the Netherlands) placed at a distance of 25–30 cm from the heads of the animals. The emitted "calls" were counted using Sono-

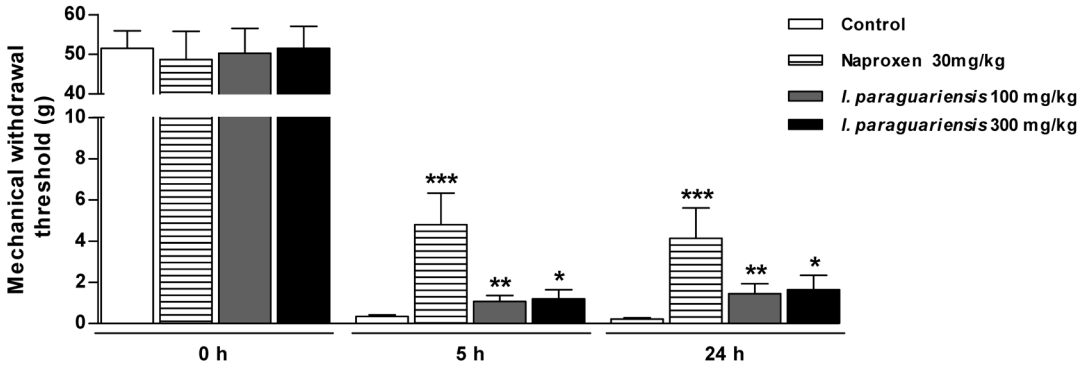

Fig. 2. Effect of *I. paraguariensis* Extract on Mechanical Hypersensitivity Induced by a Plantar Incision in Rats

Baseline assessment of animals before surgery (day 0) showed no significant variation between groups. Six or 24 h after surgery, rats treated with the *I. paraguariensis* extract had a significantly attenuated hypersensitivity in response to von Frey stimulation of the injured hind paw. Data are mean±S.E.M. (n=8 per group). ***$p<0.001$, **$p<0.01$, and *$p<0.05$, compared to the control group.

track 2.2.1 software (Metris B.V.).

SNI Model of Neuropathic Pain Surgery was performed as previously described,[18] with minor modifications. The SNI procedure comprised axotomy and ligation of the tibial and common peroneal nerves, leaving the sural nerve intact. The common peroneal and the tibial nerves were tight-ligated with 5.0 silk and sectioned distal to the ligation, removing 2±4 mm of the distal nerve stump. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The skin was opposed with two single interrupted sutures using polyamide monofilaments.

MWT Analysis Animals were placed on an elevated wire grid and the plantar surface of the paw was stimulated with a series of ascending force von Frey monofilaments (Stoelting, Wood Dale, IL, U.S.A.). The threshold was taken as the lowest force that evoked a brisk withdrawal response to one of three repetitive stimuli. To determine the time course of hyperalgesia, a baseline measurement was performed prior to surgery; thereafter, measurements were performed at 6 and 24 h after plantar incision surgery and at 3, 6, 9, 12, and 15 d after surgery for SNI.

Immunofluorescence SNI-injured rats were sacrificed following the MWT analysis, and their ipsilateral lumbar 4 (L4), L5, and L6 DRG were fixed through the ascending aorta with 0.9% saline (Sigma, St. Louis, MO, U.S.A.), followed by 500 mL of cold 0.1 M phosphate buffer (PB) (Sigma) containing 4% paraformaldehyde (PFA) (Sigma). The fixed DRG was cut into 20 μm sections on a cryostat (CM1850; Leica, Heidelberg, Germany). Immunofluorescence staining was performed on 20 μm sections using polyclonal antibodies specific for anti-neuropeptide Y (1 : 1000 dilution; AB 10980, AbCam, Cambridge, U.K.), followed by exposure to a Alexa Flour 594-labeled goat anti-rabbit antibody (1 : 500 dilution; Molecular Probes, Eugene, OR, U.S.A.). The images were observed and analyzed on a fluorescence microscope using Zeiss software (Axio Observer A1, Carl Zeiss, Jena, Germany).

Cytokine Analysis We used the multiplex enzyme-linked immunosorbent assay (ELISA) cytokine assay (Quansys Biosciences, Logan, UT, U.S.A., and BioLegend, San Diego, CA, U.S.A.) to measure the interleukin (IL)-6, IL-2, and interferon (IFN)-γ levels in the isolated L4, L5, and L6 DRG from SNI-injured rats. All ELISAs were conducted according to the manufacturer's protocols

Statistical Analysis The data was analyzed by one-way ANOVA, followed by Tukey's *post hoc* test for multigroup comparisons, using Prism 5 (GraphPad Software, Inc., San Diego, CA, U.S.A.). All of the data are presented as the mean±standard error of the mean (S.E.M.). Significance was set at $p<0.05$.

RESULTS

Effects of *I. paraguariensis* Extract on Mechanical Hyperalgesia Induced by Plantar Incision Incision of the plantar surface of the hind paw resulted in a significant reduction in the MWT, as measured using von Frey stimulation. The plantar incision induced marked mechanical hyperalgesia in the incised paw (MWT reduced from 51.50±4.43 g at baseline to 0.22±0.45 g 24 h after plantar incision). Treatment with naproxen (30 mg/kg) significantly attenuated the plantar incision-induced mechanical hyperalgesia in response to von Frey stimulation. Importantly, the oral administration of *I. paraguariensis* extract (100 and 300 mg/kg) significantly attenuated the mechanical hyperalgesia, as demonstrated by increased MWT values compared to control values, in response to von Frey stimulation of the injured hind paw (Fig. 2).

Effects of *I. paraguariensis* Extract on USVs Induced by Plantar Incision The analgesic activity of *I. paraguariensis* extract was also examined by measuring pain-induced USVs using ultrasonic microphones. Six and 24 h after plantar incision, the control group emitted 22–27 kHz USV calls, which are characteristic of pain-related behaviors. Compared to the control treatment, treatment with 30 mg/kg of naproxen significantly reduced the number of 22–27 kHz USV calls, demonstrating antinociceptive effects. Similarly, treatment with either 100 or 300 mg/kg *I. paraguariensis* extract significantly reduced the number of 22–27 kHz USV calls (Fig. 3).

Effects of *I. paraguariensis* Extract on Mechanical Hyperalgesia Induced by SNI Next, we evaluated the potential efficacy of systemic administration of *I. paraguariensis* extract using a SNI rat model of neuropathic pain. At baseline (day 0), no significant changes were observed between the group that was treated with *I. paraguariensis* extracts and the control group. Three days after operation, the animals began to exhibit hypersensitivity in response to von Frey stimulation. This hypersensitivity continued for the remainder of the study. In contrast, administration of *I. paraguariensis* extracts (100 or 300 mg/kg) significantly increased the MWT response

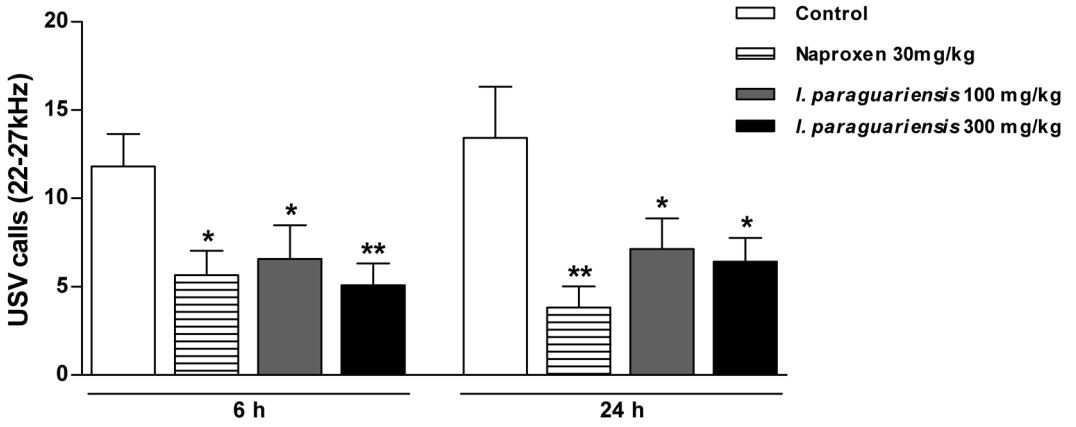

Fig. 3. Effect of *I. paraguariensis* Extract on USV Induced by Plantar Incision in Rats

The number of 22–27 kHz USV calls significantly reduced in the *I. paraguariensis* extract-treated group compared to the number in the control group. Data are mean±S.E.M. (n=8 per group). **p<0.01, and *p<0.05, compared to the control group.

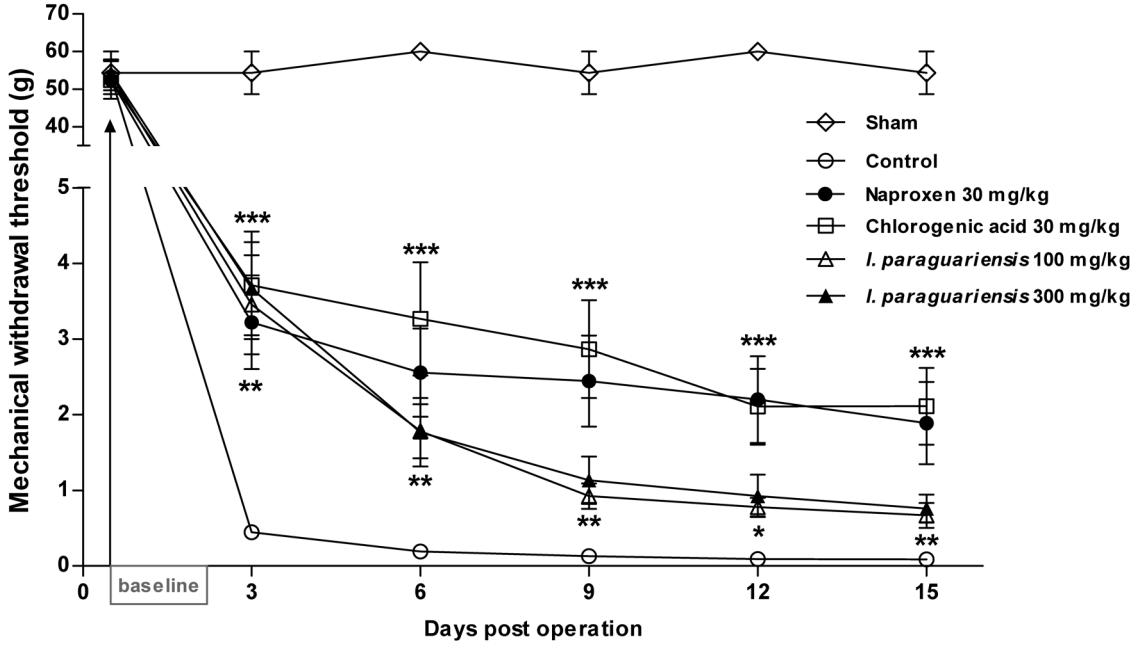

Fig. 4. Effect of *I. paraguariensis* Extract on the SNI Rat Model of Neuropathic Pain

Administration of *I. paraguariensis* extract (300 mg/kg, *p.o.*) significantly attenuated the SNI-induced hypersensitivity in response to von Frey stimulation of the hind paw from days 3 to 15 after treatment. Data are mean±S.E.M. (n=8 per group). ***p<0.001, **p<0.01, and *p<0.05, compared to the control group.

to von Frey stimulation of the hind paw, compared to the response in SNI-control rats from days 3 to 15 after surgery, indicating attenuated hyperalgesia (Fig. 4). In addition, chlorogenic acid (30 mg/kg), one of the primary phenolic acids in *I. paraguariensis* extract, significantly attenuated hyperalgesia in response to von Frey stimulation of the surgically injured hind paw.

Effects of *I. paraguariensis* Extract on the Expression of Cytokines and NPY Induced by SNI in the Rat DRG IL-6, IL-2, and IFN-γ levels were measured in the isolated L4, L5, and L6 DRG. The cytokine levels significantly increased in the SNI control group in comparison to the corresponding levels in the sham group. There was a significant reduction in the IL-2, IL-6, and IFN-γ levels in the SNI+*I. paraguariensis* extract treated-group compared to the corresponding levels in the SNI-control group (Fig. 5). Consistent with the hypersensitivity response shown in Fig. 4, we observed large up-regulation of NPY expression in the DRG of SNI-control rats (Fig. 6A). Similar results have been reported in the DRG and trigeminal ganglion in other models of peripheral neuropathic pain.[19] These changes are accompanied by increased NPY expression in the central terminals of these neurons, and in the dorsal column nuclei.[20] Treatment with the *I. paraguariensis* extract (either 100 or 300 mg/kg) significantly reduced the SNI-induced NPY expression (Fig. 6B).

DISCUSSION

The analgesic activity of *I. paraguariensis* extract was determined using a postoperative pain model in rats. Postoperative pain in humans has been mimicked in rats using the plantar incision model.[14] An incision through the skin, fascia, and plantar muscle causes primary hyperalgesia in response to mechanical stimuli at the site of the injury, and secondary hyperalgesia in the surrounding area.[21] Incision of the plantar surface of the hind paw resulted in a significant reduction in

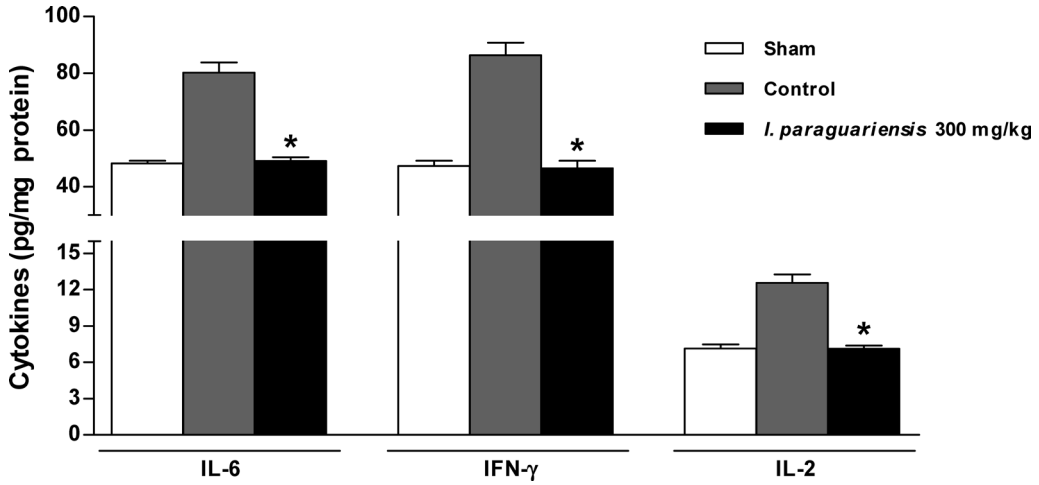

Fig. 5. Effect of *I. paraguariensis* Extract on Cytokine Expression Induced by SNI in the Rat DRG

Treatment with *I. paraguariensis* extract significantly reduced the SNI-induced increases in IL-2, IL-6, and IFN-γ levels. Data are mean±S.E.M. (n=8 per group). *p<0.05, compared to the SNI-control group.

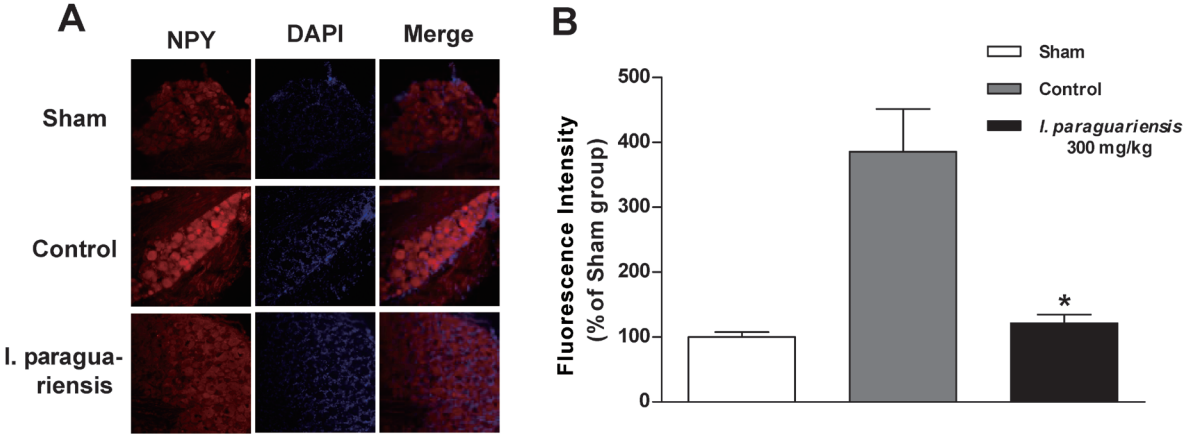

Fig. 6. Effect of *I. paraguariensis* Extract on NPY Expression Induced by SNI in the Rat DRG

(A) Representative images of NPY-positive cells in the rat DRG. (B) Treatment of SNI rats with *I. paraguariensis* significantly reduced the average number of NPY-positive cells in the DRG. The bars represent the mean±S.E.M. (n=4 per group). *p<0.05, compared to the SNI-control group.

MWT, as measured using von Frey stimulation. Analgesic compounds reversed the incision-induced decreases in MWT in response to mechanical stimulation.[22] Analgesics are majorly used in postoperative pain relief; therefore, we previously employed the plantar incision model to study the antihyperalgesic effects of conventional analgesics including naproxen and gabapentin.[17,23] The present study demonstrated that administration of *I. paraguariensis* extract significantly attenuated mechanical hyperalgesia in response to von Frey stimulation of the injured hind paw. This is evidenced by increased MWT values compared to control MWT values. The analgesic effects of the *I. paraguariensis* extract were also confirmed by studying the postoperative pain-induced USV using ultrasonic microphones. Adult rats produce two distinct types of USV calls that appear to reflect the caller's emotional state: a positive state (a high-pitched and short *ca.* 50 kHz USV), and a negative state (a low-pitched and longer *ca.* 27 kHz USV).[24] In particular, the number of 22–27 kHz USVs has been suggested as a measure of affective shifts in rats,[25] and has been used in a variety of unconditioned models, such as pain, anxiety, and stress-related models.[26,27] Because vocalization is an objective measure that can be quantified with easily, pain-induced USV has been examined using ultrasonic microphones, particularly in rats. Six or 24 h after plantar incision, the control group emitted 22–27 kHz USV calls, consistent with pain-related behavior.[28] A significant reduction in 22–27 kHz USV calls was observed after the administration of *I. paraguariensis* extract at a concentration of 300 mg/kg. These results indicate that the *I. paraguariensis* extract might have analgesic effects on plantar incision in the postoperative pain model in rats.

NPY is a highly conserved peptide from the pancreatic polypeptide family; it has marked and diverse biological effects.[29] In DRG neurons, NPY is normally undetectable or present in very low levels.[30] Following peripheral axotomy or other types of experimental nerve injury, dramatic up-regulation of NPY occurs primarily in medium and large DRG neurons.[31] Previous work by others has suggested NPY may sensitize primary sensory afferents. The Y1 and Y2 receptors are the receptors believed to be involved in the transmission and modulation of pain after SNI.[32] These anatomical findings indicate the importance of the NPY system in the development of pathological neuropathic pain.[33] Neuropathic pain is a form of chronic pain caused by nerve injury, disease states, or toxic insults. We evaluated the use of *I. paraguariensis* extract in a rat model of SNI to determine whether *I. paraguariensis* extract affected the expression of NPY. We used a model of

SNI-induced neuropathic pain, because it mimics the symptoms of chronic nerve compression in humans.[34] In our study, the SNI-control group exhibited hypersensitive behavioral responses and considerable up-regulation of NPY expression in the DRG. Treatment with *I. paraguariensis* extract reduced the SNI-induced NPY expression, and significantly attenuated hyperalgesia in response to von Frey stimulation of the hind paw. Together, these results suggest that *I. paraguariensis* extract should be considered as a possible analgesic for neuropathic pain.

Pro-inflammatory cytokines, such as IL-6 and TNF-α facilitate neuropathic pain,[35] while inhibition of pro-inflammatory cytokines or administration of anti-inflammatory cytokines, such as IL-10, reduce neuropathic pain in animal models.[36] Recently, Li *et al.* reported that administration of the corticosteroid anti-inflammatory drug triamcinolone acetonide (TA) significantly reduced mechanical hyperalgesia in response to von Frey stimulation of the hind paw, and was very effective at reducing SNI-induced increases in cytokine levels.[37] These reports suggest that various inflammatory processes are important in the development of neuropathic pain. In our results, the *I. paraguariensis* extract-treated group had significantly lower IL-6, IL-2, and IFN-γ levels than the corresponding levels in the SNI-control group. The potential analgesic effects of the *I. paraguariensis* extract are related to the bioactive compounds, particularly to the phenolic acids present. The phenolic acid composition of *I. paraguariensis* extract is similar to that of coffee, with chlorogenic acids being the most abundant compounds.[38] *In vitro* studies have provided evidence suggesting that the phenolic acids present in *I. paraguariensis* extract modulate the inflammatory response.[39] Our results demonstrated that chlorogenic acid, a major phenolic acid in *I. paraguariensis* extract, significantly attenuated hyperalgesia in SNI-induced neuropathic pain in the rats. The evidence presented in the above reports led to the hypothesis that *I. paraguariensis* extract might have potential analgesic effects through the regulation of the inflammatory processes during the development of neuropathic pain.

In conclusion, the administration of *I. paraguariensis* extract led to a reduction in the number of USVs after plantar incision in rats, and to a decrease in hypersensitivity in response to von Frey stimulation of the hind paw, as evidenced by an increased MWT in the SNI model of neuropathic pain in rats. Although more pharmacological and toxicological investigations are needed to elucidate the mechanism of action and conduct safety evaluations, our results suggest that Mate extract could be useful for the treatment of both postoperative and neuropathic pain.

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Conflict of Interest The authors declare no conflict of interest.

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