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Anti-hyperalgesic effect of *Mangifera indica* L. extract on the sciatic chronic constriction injury model in rats

[*Efecto anti-hiperalgésico del extracto de Mangifera indica L. en el modelo de daño por constricción crónica del nervio ciático en ratas*]

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Abstract

Context: The aqueous extract of *Mangifera indica* L. stem bark (MSBE) shows antioxidant, anti-inflammatory and analgesic properties.

Aims: To test the MSBE in chronic constriction injury (CCI) of the sciatic nerve in rats, a classical model of neuropathic pain.

Methods: Given the possibility that some clinical effect of MSBE can appear only after its chronic administration, we designed a long term medication protocol with 500 mg/kg, p.o. or distilled water daily during 8 days from 9 days post-CCI. Pregabalin (10 mg/kg, p.o.) was used as reference drug. Sham CCI animals received vehicle. Behavioral tests were carried out before CCI, at 9 and 16 days after injury. A section of sciatic nerve, 5 mm distal to the ligature site was dissected for histopathological studies. A single oral similar dose or vehicle was administered to mononeuropathic rats, 14 days after surgery. Mechano-hyperalgesia and thermal hyperalgesia of the ipsilateral paw were determined using a modification of pin prick method and the unilateral hot plate, respectively before CCI, 0, 30, 60, 120 and 180 min post-administration.

Results: Repeated oral MSBE doses reduced nociceptive score, increased paw withdrawal latency and attenuated CCI-induced Wallerian degeneration-related changes involved in the hyperalgesic state of CCI rats. Likewise, MSBE shows significant mechanical and thermal anti-hyperalgesic effect from 1h after its single administration.

Conclusions: The study of MSBE should be focused in neuropathic pain models since this natural product could have a clinical relevance in the treatment of neuropathic pain syndromes.

Keywords: Hyperalgesia; mangiferin; neuropathic pain.

Resumen

Contexto: El extracto acuoso de la corteza del árbol del mango *Mangifera indica* L (ECAM) muestra propiedades antioxidantes, anti-inflamatorias y analgésicas.

Objetivos: Evaluar el ECAM en el modelo de daño por constricción crónica del nervio ciático (CCI) en ratas, un modelo clásico de dolor neuropático.

Métodos: Dada la posibilidad de que algunos efectos clínicos del ECAM puedan aparecer tras su administración crónica, se diseñó un protocolo de suministro a largo plazo con dosis orales de ECAM 500 mg/kg, p.o. o agua destilada durante 8 días desde el día 9 post-CCI. Se utilizó la pregabalina (10 mg/kg, p.o.) como fármaco de referencia. Los animales falsos operados recibieron vehículo. Las pruebas conductuales fueron conducidas antes del CCI, a los 9 y 16 días post-CCI. Una sección del nervio ciático 5 mm distal a la ligadura fue disecada para los estudios histopatológicos. Se estableció un protocolo de administración única a la misma dosis o vehículo 14 días tras la cirugía. Se evaluó la hiperalgesia mecánica y térmica de la pata ipsilateral utilizando una modificación del método de *pin prick* y el plato caliente unilateral, respectivamente antes del CCI, 0, 30, 60, 120 y 180 min post-administración.

Resultados: ECAM suministrado a dosis repetidas redujo las escalas nociceptivas, incrementó la latencia de retirada de la pata y atenuó la degeneración *Walleriana* implicada en la hiperalgesia de las ratas CCI. Igualmente mostró efecto anti-hiperalgésico desde la primera hora tras su administración.

Conclusiones: El estudio del ECAM debería ser enfocado hacia los modelos de dolor neuropático ya que el producto podría tener relevancia clínica en el tratamiento de estos síndromes.

Palabras Clave: Dolor neuropático; hiperalgesia; mangiferina.

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INTRODUCTION

Neuropathic pain is defined as a pain caused by a lesion or disease of the central or peripheral somatosensory nervous system that represents a debilitating disorder (Treede et al., 2008). The prevalence of chronic pain of predominantly neuropathic origin in the general population is 8% (Torrance et al., 2006). Primary neuropathic pain diseases include trigeminal neuralgia and genetic disorders such as erythromelalgia and extreme paroxysmal pain disorder. More frequent, secondary neuropathic pain may be further classified on the basis of the character of the insult of the nervous system (inflammatory, metabolic, vascular, autoimmune or mechanical) and its physiopathology (Woolf, 2004).

Cancer-induced neuropathic pain may result from compression of the nerve or direct infiltration by the growing tumor, secondarily from changes in the neuronal inflammatory milieu or as consequence of cancer-directed therapy (Bennett et al., 2012). The management of patients with chronic neuropathic pain is challenging since the medications and other treatment options target specific mechanisms of pain generation (Baron et al., 2010). Despite newer drugs and the increased use of rational poly-pharmacy that may improve therapeutic efficacy, the response to most treatments for neuropathic pain is modest (Finnerup et al., 2010).

Today it is recognized that the nervous system injuries results in maladaptative plasticity as a consequence of altered relationships between neurons and their innervation targets; changes in gene expression; and altered signaling between neurons, glia and other immune cells (Latremoliere and Woolf, 2009; De Leo et al., 2006). This plasticity alters function at multiple levels of the somatosensory system, from peripheral nerves to the cortex. Such changes include increased spontaneous activity in primary sensory neurons and altered transduction properties; enhanced transmission of nociceptive information at the level of the dorsal horn due to central sensitization, desinhibition and descending facilitation from the brainstem and synaptic plasticity at the level of cortex (Berger et al., 2011). Since new targets for therapeutic intervention are today available (Manning, 2006) it is necessary emerging strategies to solve this problem.

Neuroimmune activation and nitroxidative stress are the particular interest, since their implication in the spinal glutamatergic dysfunction that facilitates the N-methyl-D-aspartate (NMDA) receptors activation and excitotoxic apoptosis of inhibitory interneurons (Salvemini et al., 2011; De Leo et al., 2006).

Neurodegeneration as a reaction to nerve injury appears to be an underlying cause of neuropathic pain. Then, designing neuroprotective therapies as means either to prevent the onset, control the progression or even to reverse the nerve damage leading to these chronic pain syndromes, have been other novel alternative (Bordet and Pruss, 2009).

The standard aqueous stem bark extract of selected varieties of mango (*Mangifera indica* L.; MSBE), contains a definite mixture of components including poly-phenols, triterpenes, phytosterols, fatty acids and microelements (Núñez-Sellés et al., 2007). Previous experiments have shown that this extract has antioxidant (Garrido et al., 2008; Martínez et al., 2000), anti-inflammatory, analgesic (Garrido et al., 2004a; 2004b; 2001) and immunomodulatory (García et al., 2003; 2002) properties. MSBE prevents tumor necrosis factor alpha (TNFα)-induced IκB degradation and the binding of nuclear factor κB (NF-κB) to the DNA, which induces the transcription of genes implicated in the expression of some mediators and enzymes involved in inflammation, pain, oxidative stress and synaptic plasticity (Garrido et al., 2005; Leiro et al., 2004).

Highly significant are the brain-neuroprotective effects of MSBE on gerbils in ischemia-reperfusion and *in vitro* glutamate induced neuronal injury models (Lemus-Molina et al., 2009; Martínez et al., 2001). Mangiferin (MG), its major component (about 15-20% from the extract), decreases the glutamate-mediated Ca^{2+} influx through NMDA receptor and shows antioxidant activity related to its iron-chelating properties in addition to scavenging activity of free radicals (Pardo-Andreu et al., 2008; Gottlied et al., 2006). Also, it is able to limit microglial activation in terms of

attenuation of prostaglandins E_2 production, free radicals formation and reduction of cyclo-oxygenase-2 synthesis induced by lipopoly-saccharide (Bhatia et al., 2008). Based in these evidences, previously we hypothesized the potentiality of this extract to modulate some of the molecular targets implicated in peripheral and central neuropathic pain mechanisms, especially central sensitization (Garrido-Suárez et al., 2010). The aim of the present study was to test the MSBE on the sciatic chronic constriction injury model in rats, a classical model of neuropathic pain, in which main pathophysiological mecha-nism is neuroinflammation (Berger et al., 2011).

MATERIALS AND METHODS

Plant material, preparation and chemical characterization

Mangifera indica L. stem bark was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code 41722) were deposited at the Herba-rium of the Academy of Sciences, guarded by the Institute of Ecology and Systematics from the Ministry of Science, Technology and Environ-ment, Havana, Cuba and authenticated by MSc Ramona Prieto, curator, and MSc Isora Baró, Director of the Herbarium. Stem bark extract from *M. indica* was prepared by decoction in water for 1 h and then it was concentrated by evaporation and spray-dried in a Niro Atomizer Standard Spray Drying (Soeborg, Denmark) to obtain a fine homogeneous brown powder with a particle size of 30–60 mm (Acosta-Esquijarosa et al., 2009).

The chemical composition of this extract has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV-Vis spectrophotometry (Garcia Rivera et al., 2011; Núñez-Sellés et al., 2002). The batch used in this study was analyzed in the Quality Department of the Pharmaceutical Chemistry Center (Havana, Cuba) and was found to have the following content: moisture <10%, water-soluble substances >50%, total phenol (in anhydrous base) >30% and mangiferin >10%, according to the quality specifications established. The solid extract was dissolved in distilled water for pharmacological studies.

Experimental animals

Experimental procedures were carried out in accordance with European regulations on animal protection (Directive 86/609), the Declaration of Helsinki, and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulga-ted by the US National Institute of Health (NIH Publication № 85-23, revised 1996).

All experimental protocols were approved by the Institutional Animal Care and Ethical Com-mittee from the Center of Drugs Research and Development (CIDEM, La Habana, Cuba).

Male Sprague-Dawley rats weighing 200-250 g (8-10 weeks) were obtained from the Center for Experimental Animals Production (CENPALAB, La Habana, Cuba). They were kept in controlled conditions (22 ± 0.5°C, relative humidity 40-60%, a 7 a.m. to 7 p.m. alternate light-dark cycle, food and water *ad libitum*). The experiments took place during the light period and animals belonging to the various treatment groups (n=10 for each group) were tested in randomized order.

Neuropathic surgery (chronic constriction injury, CCI)

Animals were anesthetized with xylazine (13 mg/kg, i.m.) and ketamine (87 mg/kg, i.m.). A CCI was produced by ligating the common sciatic nerve on the left side (Bennett and Xie, 1988). Briefly, 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 its trifurcation, about 7 mm of nerve was freed of adhering tissue and three ligatures (using 4.0 chromic gut) were tied loosely around it at 1-1.5 mm intervals. The ligatures just barely constricted the diameter of the nerve when viewed by 40x magnification. This degree of constriction retarded, but did not arrest, the circulation through the superficial epineural vasculature and produced a small, brief twitch in the muscle around the exposure. The incisions were closed in layers. In sham-operated controls an identical operation were performed but without ligation of sciatic nerve. Mechano-hyperalgesia and thermal hyperalgesia were

measured before CCI surgery and every two days until 16 days post-CCI. These manifestations would fully develop 8-14 days after the nerve ligature.

Behavior tests

Hind paw mechano-hyperalgesia

Mechano-hyperalgesia of the ipsilateral hind paw was assessed using a modification of the pin prick method (Tal and Bennett, 1994). With the rats standing on the wire mesh floor and confined beneath an inverted plastic box (described above), the point of a blunted 23 gauge needle was applied to the skin of the heel (touching, but not penetrating). Normal rats respond with a very small and brief withdrawal. CCI rats respond most often with a withdrawal that is clearly exaggerated in amplitude and duration.

Behavioral responses to the pin prick were rated according to the following scale: 0 = no response; 1 = rapid paw flicking, stamping, or shaking (less than 1 s); 2 = repeated paw stamping, shaking, or paw lift less than 3 s; 3 = above behaviors or hindpaw licking for more than 3 s; 4 = above behaviors for more than 3 s and hindpaw licking for more than 3 seconds. An additional point was added if any vocalizations occurred.

Unilateral hot plate

Rats were gently restricted and after a few seconds necessary for the struggle to finish, the plantar side of the tested paw was placed on the hot plate surface (52 ± 0.2°C) and the other paw on a metallic surface at room temperature. The paw withdrawal latency (PWL) from the heated surface was recorder in either response to the thermal hyperalgesia. Only the clear unilateral withdrawal of the paw was taken into account, discarding the unspecific generalized struggle observed in some cases. Three measures at 2-min-intervals were taken before or after MSBE or vehicle treatment and their means were considered basal and experimental latencies, respectively In order to avoid tissue injury, the cut off was set at 20 seconds. Rats were not previously habituated to the test environment (Menéndez et al., 2002).

Medication protocols

Repeated oral MSBE administration

Given the possibility that some clinical effects of MSBE can appear only after its chronic administration we first designed a long term medication protocol to evaluate the effect of MSBE on mononeuropathic rats. The animals (n=10 per group) received oral MSBE (500 mg/kg), distilled water (10 mL/kg) or pregabalin (10 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) once daily during 8 days from 9 days post-CCI. The MSBE dose was selected according to previous report in formalin test (Garrido et al., 2005) and on pilot experiment in our laboratory utilized formalin test 5%. Since MSBE reducing tonic phase, which underlying central sensitization, also implicated in CCI pain mechanisms. Behavioral tests were carried out before CCI, at 9 and 16 days after injury.

Single oral MSBE medication

Single oral MSBE (500 mg/kg), distilled water (10 mL/kg) or pregabalin (10 mg/kg) were administrated (n=10 per group) to CCI rats only on day 14 post-CCI, when hyperalgesic state had fully developed after CCI surgery. Nociceptive score and PWL were measured before and at 30 min, 1, 2 and 3 h after oral administration.

Histopathological studies

Samples of 5 mm sciatic nerve located distal to the lesion were stored in the fixative solution (10% formalin) and cut into 4 µm thickness. Staining was carried out by using hematoxylin and eosin. Nerve sections were analyzed qualitatively under light microscope (40x) for axonal degeneration.

Statistical analysis

Data were analyzed using the statistical program Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group statistically significant differences were tested using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. The results are presented as mean ± SEM. $P<0.05$ was considered statically significant.

RESULTS

Effect of repeated oral dose of MSBE on mechanical and thermal hyperalgesia in chronic constriction injury model

Chronic constriction injury of sciatic nerve resulted in a significant development of mechanical hyperalgesia in ipsilateral left paw by the increase of nociceptive responses to pin prick (nociceptive score) when compared to sham group at 9 and 16 days (p<0.001) (Fig. 1A). Likewise after CCI surgery rats displayed a significant decrease in PWL with respect sham animals at the same time, indicating that thermal hyperalgesia was also established (p<0.001) (Fig. 1B). MSBE (500 mg/kg, p.o.) treatment during 8 days from 9 days post-CCI significantly attenuated CCI induced nociceptive responses to pin prick (p<0.001). In addition, the animals treated with this extract increased its PWL compared with water-treated group at 16 days (p<0.001) after injury. Treatment of pregabalin also produced similar effects.

Effect of MSBE histopathological changes in chronic constriction injury model

CCI induced abnormal histopathological changes observed by light microscopy in the longitudenal section of sciatic nerve undergoing Wallerian degeneration (WD). In Fig. 2A and B, the cellularity was increased in relation with the sham CCI as the result of Schwann cell proliferation and macrophage infiltration, fragmentation of the myelin sheath into myelin ovoids that are contained in the Schawan cell cytoplasm (digestion chambers), axonal degeneration that resulted in the loss of the orderly alignment of axons. The treatment with MSBE (500 mg/kg, p.o.) during 8 days from 9 days after injury attenuated CCI-induced WD-related changes (Fig. 2C). Pregabalin also attenuated these changes (data not shown).

Effect of single oral dose of MSBE on mechanical and thermal hyperalgesia in chronic constriction injury model

Nociceptive response to pin prick increased significantly by day 14 post-CCI (p<0.001). Also, PWL decreased significantly at this time point compared with sham CCI (p<0.001). Results indicated that both, mechanical and thermal hyperalgesia were developed in injured paw. After single oral MSBE administration on day 14 nociceptive score decreased (p<0.001) and PWL increased (p<0.001) compared with water-treated control group respectively from 60 min. These effects are maintained until the last evaluation at 3 h (Fig. 3A and B, respectively). The reference drug pregabalin also produced similar effects.

DISCUSSION

The most important feature of this study was to be the first approach to the evaluation of MSBE extract on a neuropathic pain model (Bennett and Xie, 1988). Chronic administration of this extract attenuated the mechano-hyperalgesia and thermal hyperalgesia on ipsilateral paw during the severity peaking at 16 days. In addition, acute oral administration of this extract also decreased neuropathic manifestations on 14 days post-CCI. These evidences suggest the potentiality of the MSBE to modulate some of the molecular targets implicated in neuropathic pain mechanisms through of its long term effects mediated mainly by transcriptional changes with its main implications on nitroxidative stress, inflammatory response, apoptosis and neuroplasticity (Latremoliere and Woolf, 2009; Garrido et al., 2005). Previously, we advised that MSBE could be used to treat neuropathic pain supported in preclinical data and some preliminary clinical reports with *Mangifera indica* extract formulations (Garrido-Suárez et al., 2010; 2011). Particularly, MG contained in this extract has an antinociceptive action mediated by endogenous opioids, K_{ATP} channels and adenosine (Lopes et al., 2013). At present is also recognized that this xanthone exerts peripheral antinociceptive actions via L-arginine-nitric oxide-cGMP pathway activation in formalin test (Izquierdo et al., 2013) and possibly a modulatory effect on noradrenergic system, also involved in pain transmission could be expected, since its reversible mono-amine oxidase inhibitory activity have been reported (Tomić et al., 2005).

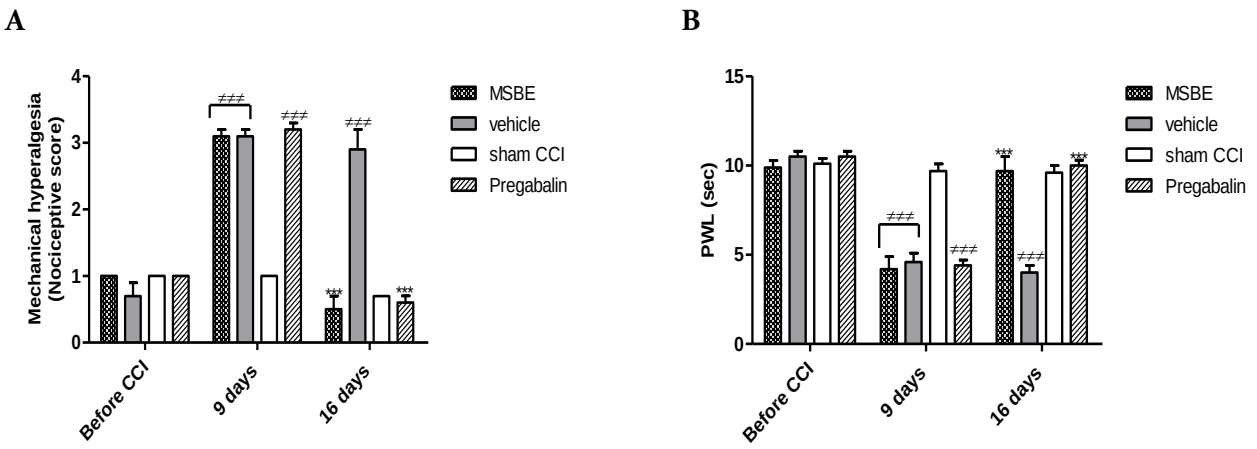

Figure 1A. Evaluation of the mechano-hyperalgesia in the ipsilateral hind paw of sham and chronic constriction injury (CCI) rats treated with *Mangifera indica* L extract (MSBE) (500 mg/kg, p.o.), pregabalin (10 mg/kg, p.o.) or vehicle during 8 days from 9 day post-CCI as determined by pin prick test. Nociceptive scores of sham rats did not change at 9 and 16 days. Nociceptive scores of CCI rats were significantly elevated with respect to sham animals. MSBE reduced nociceptive scores similar to sham animals compared to water-treated group. Pregabalin-treated group also shows similar results. **B.** Evaluation of the thermal hyperalgesia in the ipsilateral hind paw of sham and CCI rats with the same treatment. Paw withdrawal latency (PWL) of sham animals did not change at 9 and 16 days. CCI rats decreased significantly PWL with respect sham CCI. MSBE increased PWL similar to sham animals compared with water-treated group. Each column represents the responses of 10 animals per group as mean ± SEM. $^{***}p<0.001$ represents the statistical difference between treated groups and control (vehicle), while $^{###}p<0.001$ represents the statistical difference between CCI with respect sham CCI rats.

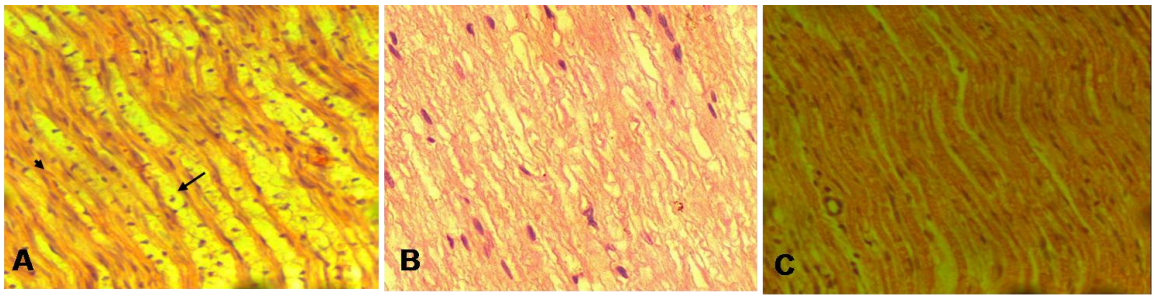

Figure 2. Effect of the treatment with repeated doses of *Mangifera indica* L. extract (MSBE, 500 mg/kg, p.o.) during 8 days on histopathological changes induced by CCI. **A**, **B** and **C** show the longitudinal-section of sciatic nerve 5 mm distal to the lesion of CCI rats treated with vehicle, sham CCI and CCI mononeuropathic rats treated with MSBE, respectively. In **B** note the low cellularity and the orderly alignment of axons and their associated with myelin sheaths in sham CCI. In figure **A** head arrow shows the cellularity increased relative with non-injured nerve (sham CCI) as result macrophage infiltration and Schwann cell proliferation. The black arrow indicates one of the several Schwann cell digestion chambers with myelin ovoid (the red roughly oval mass). **C** pre-treatment with MG shows decrease in the CCI-induced derangement of nerve fibers and increase in the number of cells and degradation of myelin sheath.

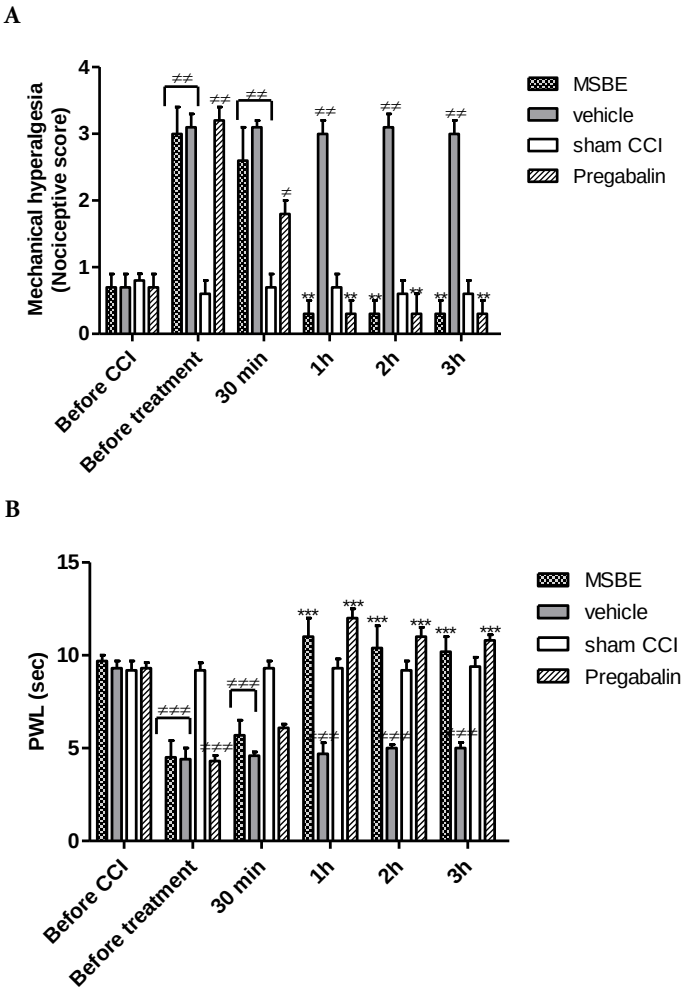

Figure 3A. Evaluation of the mechano-hyperalgesia in the ipsilateral hind paw of sham and chronic constriction injury (CCI) rats treated with single dose of *Mangifera indica* L. (MSBE 500 mg/kg, p.o.), pregabalin (10 mg/kg, p.o.) or vehicle on 14 days post-CCI as determined by pin prick test. Nociceptive scores of CCI rats increased compared with sham rats. Nociceptive scores of MSBE-treated group decreased significantly with respect to CCI rats treated with water from the first hour, this effect was maintained during 3 h. Pregabalin-treated group also showed similar results. **B.** Evaluation of the thermal hyperalgesia in the ipsilateral hind paw of sham and CCI rats with the same acute treatment. Paw withdrawal latency (PWL) of CCI rats decreased significantly compared with sham rats. PWL of MSBE-treated group increased significantly from the 1 h, the effect was also maintained until the end of experiment. Pregabalin-treated group also showed similar results. Each column represents the responses of 10 animals per group as mean ± SEM. $^{**}p<0.01$ and $^{***}p<0.001$ represents the statistical difference between treated group and control (vehicle), while $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ represents the statistical difference between CCI with respect sham CCI rats.

MSBE also attenuated CCI-induced WD-related changes. This process is tightly linked to the development of neuropathic pain following CCI (Ramer et al., 1997). WD involves an increase in free intracellular Ca^{2+} and the activation of calpains leading to a massive decrease of microtubular and neurofilament protein levels and to axonal fragmentation (Debový, 2011). MG inhibition of calpains activity could be implicated in its beneficial neuroprotective effect and subsequently decreased of neuropathic pain manifestations (Campos-Esparza et al., 2009). WD also can be viewed as the inflammatory response to axonal injury and is primarily attributable to the production of cytokines and chemokines from Schwann cells, which regulate macrophage responses and may facilitate myelin breakdown and clearance (Debový, 2011). Specifically TNFα has been implicated in both, thermal and mechanical hyperalgesia (Manning, 2006). This cytokine induces long term potentiation on spinal cord C fibres in nerve injury rats mediated by NF-κB that is localized to dorsal root ganglia neurons and Schwann cells following partial sciatic nerve injury (Lin et al., 2007, Manning, 2006). Then the primary inhibitory effect of MSBE on TNFα-induced activation of NF-κB could explicate its anti-hyperalgesic effect. Moreover, neuronal apoptosis in a small subpopulation of cell has been reported in the dorsal horn after CCI, which contributed to pain hypersensitivity through temporary loss of inhibitory neurons (Latremoliere and Woolf, 2009). Nitroxidative stress enhances glutamatergic signalling by nitrating and inactivating glutamate transporters and neuronal death appears to be the result of NMDA

receptors-induced excitotoxicity (Salvemini et al., 2011). Subsequently MSBE, that exhibits a potent antioxidant effect, shows potential in order to stabilize glial cell and to modulate NMDA receptors; may be an attractive product for studying in neuropathic pain models (Garrido-Suárez et al., 2010; Pardo-Andreu et al., 2008; Garrido et al., 2001).

Some preliminary results in clinical case reports and case series have been published (Garrido-Suárez et al., 2011), but more preclinical and clinical evidences are yet necessary to demonstrate its possible clinical relevance.

CONCLUSIONS

Chronic administration of MSBE decreased mechanical and thermal hyperalgesia in mono-neuropathic rats. This extract also attenuated CCI-induced Wallerian degeneration-related changes involved in this hyperalgesic state. In addition, acute oral administration of MSBE also decreased neuropathic manifestations on 14 days post-CCI. Then, the study of MSBE should be focused in neuropathic pain models since this natural product could have a clinical relevance in the treatment of neuropathic pain syndromes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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