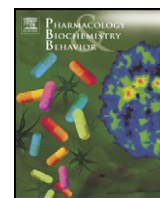


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## Peripheral antinociceptive action of mangiferin in mouse models of experimental pain: Role of endogenous opioids, $K_{ATP}$ -channels and adenosine

Synara C. Lopes<sup>a,b</sup>, Ana Virginia L. da Silva<sup>a,b</sup>, Bruno Rodrigues Arruda<sup>a,b</sup>, Talita C. Morais<sup>a,b</sup>, Jeison Barros Rios<sup>c</sup>, Maria Teresa S. Trevisan<sup>c</sup>, Vietla S. Rao<sup>a,b</sup>, Flávia A. Santos<sup>a,b,\*</sup>

<sup>a</sup> Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, 60430-270 Fortaleza, Ceará, Brazil

<sup>b</sup> National Institute of Science and Technology, Institute of Biomedicine of Brazilian Semi-arid, Faculty of Medicine, Federal University of Ceará, 60430-270 Fortaleza, Ceará, Brazil

<sup>c</sup> Department of Organic and Inorganic Chemistry, Federal University of Ceará, 60451-970 Fortaleza, Ceará, Brazil

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### ABSTRACT

This study aimed to assess the possible systemic antinociceptive activity of mangiferin and to clarify the underlying mechanism, using the acute models of chemical (acetic acid, formalin, and capsaicin) and thermal (hot-plate and tail-flick) nociception in mice. Mangiferin at oral doses of 10 to 100 mg/kg evidenced significant antinociception against chemogenic pain in the test models of acetic acid-induced visceral pain and in formalin- and capsaicin-induced neuro-inflammatory pain, in a naloxone-sensitive manner, suggesting the participation of endogenous opiates in its mechanism. In capsaicin test, the antinociceptive effect of mangiferin (30 mg/kg) was not modified by respective competitive and non-competitive transient receptor potential vanilloid 1 (TRPV1) antagonists, capsazepine and ruthenium red, or by pretreatment with L-NAME, a non-selective nitric oxide synthase inhibitor, or by ODQ, an inhibitor of soluble guanylyl cyclase. However, mangiferin effect was significantly reversed by glibenclamide, a blocker of  $K_{ATP}$  channels and in animals pretreated with 8-phenyltheophylline, an adenosine receptor antagonist. Mangiferin failed to modify the thermal nociception in hot-plate and tail-flick test models, suggesting that its analgesic effect is only peripheral but not central. The orally administered mangiferin (10–100 mg/kg) was well tolerated and did not impair the ambulation or the motor coordination of mice in respective open-field and rota-rod tests, indicating that the observed antinociception was unrelated to sedation or motor abnormality. The findings of this study suggest that mangiferin has a peripheral antinociceptive action through mechanisms that involve endogenous opioids,  $K_{ATP}$ -channels and adenosine receptors.

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### 1. Introduction

*Mangifera indica* L. (mango, Anacardiaceae) is an important medicinal and fruiting tree that grows abundantly in the tropical and subtropical regions. The medicinal usage of its leaf and stem bark extracts have been reported in several traditional systems of medicine (Scartezzini and Speroni, 2000; Ojewole, 2005). The main polyphenol present in extracts is mangiferin with C-glucosyl linkage and polyhydroxy component that determine its strong antioxidant and anti-inflammatory properties (Garrido et al., 2001). Mangiferin is also present in some other medicinal herbs, influencing their therapeutic and preventive properties. A number of biological activities of

mangiferin have been suggested, including antioxidant, antiviral, antitumor, anti-allergic, antidiabetic, hepato- and cardioprotective, anti-inflammatory and immunomodulatory effects (Sato et al., 1992; Guha et al., 1996; Garcia et al., 2003; Pardo-Andreu et al., 2006; Das et al., 2012; Vyas et al., 2012). Furthermore, mangiferin (10–40 mg/kg) has been shown to significantly improve the scopolamine-associated loss of learning ability in Elevated plus Maze, Water Maze, and Passive Shock Avoidance behavioral models through inhibitions of whole brain acetyl cholinesterase and lipid peroxidation, and by restoration of reduced glutathione (Jung et al., 2009; Biradar et al., 2012). We previously showed that mangiferin affords gastroprotection against ethanol or indomethacin-induced gastric damage (Carvalho et al., 2007), exerts prokinetic action on gastrointestinal transit (Cavalcante Morais et al., 2012), and neuroprotection in ketamine-induced schizophrenia model (Rao et al., 2012). Earlier works suggest that pain is one of the most important symptoms of inflammatory disease (Vane and Botting, 1990;

\* Corresponding author at: Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Rua Cel Nunes de Melo 1127, Rodolfo Teófilo, 60430-270 Fortaleza, Ceará, Brazil. Tel.: +55 85 3366 8341; fax: +55 85 3366 8333.

E-mail address: [flavia@ufc.br](mailto:flavia@ufc.br) (F.A. Santos).

Weisburger, 2002), and oxidative stress modifies experimental nociception (Viggiano et al., 2005; Hacimuftuoglu et al., 2006; Garrido-Suárez et al., 2010; Ma et al., 2009). In this regard, a recent study has shown that mangiferin can modulate or inhibit several key oxidant and inflammatory pathways such as Nrf2–NF $\kappa$ B signaling (Das et al., 2012), suggesting its ability to suppress pain. Acute nociceptive, inflammatory, and neuropathic pain all depend to some degree on the peripheral activation of primary sensory afferent neurons, which express a variety of inhibitory neuroreceptors such as opioid, alpha-adrenergic, cholinergic, adenosine and cannabinoid receptors, and agonists for these receptors also represent viable targets for drug development (Sawynok, 2003). Potential utility of mangiferin for treating and preventing neuropathic pain has been proposed based on preclinical studies that show the preventive effects of mangiferin on tumor necrosis factor  $\alpha$ -induced  $\kappa$ B degradation and the binding of nuclear factor  $\kappa$ B to 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-Suárez et al., 2010).

Current therapy for inflammatory pain includes the peripheral application of opioid receptor agonists. Activation of opioid receptors modulates voltage-gated ion channels, and can also influence ligand-gated ion channels like the transient receptor potential vanilloid type 1 (TRPV1) (Endres-Becker et al., 2007). Dar et al. (2005) reported for first time, the antinociceptive effect of mangiferin and its derivatives in mice, using the acetic acid-inducing writhing and hot-plate tests. Only mangiferin but not the derivatives manifested significant naloxone-sensitive antinociception after a subcutaneous dose of 42.2 mg/kg mangiferin, indicating an endogenous opioid-related mechanism. However, the antinociceptive efficacy of mangiferin on formalin- and capsaicin-induced neuropathic and inflammatory pain is unknown, wherein primary sensory afferent neurons can be activated by a range of inflammatory mediators such as prostanoids, bradykinin, ATP, histamine, and serotonin, and inhibiting their actions represents a strategy for the development of analgesics (Priestley and Hunter, 2006; Ortega-Álvarez et al., 2012).

To have greater insights into the mechanism (s) and the sites of action, this study examined the pain modulator effect of mangiferin, using several mouse models of chemical and thermal nociception.

## 2. Materials and methods

### 2.1. Plant material and isolation of mangiferin

Mangiferin (Fig. 1) used in this study was extracted and isolated from the bark of *M. indica* L. (Anacardiaceae) as per procedures reported earlier (Barreto et al., 2008). A voucher specimen (# 32628) of the plant material authenticated by Dr. Francisco Edson de Paula has been deposited at the Herbário Prisco Bezerra of the Federal University of Ceará. The isolated MGF was approximately of 95% purity having the molecular weight (MW) 422.5 and melting point (m.p.) 271 °C.

### 2.2. Animals

Male Swiss mice (20–25 g) obtained from the Central Animal House of Federal University of Ceará, Brazil were used. Experimental groups consisted of 8 animals per group. They were housed in

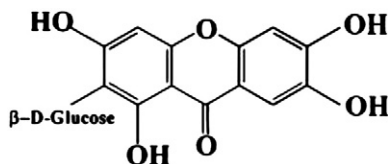


Fig. 1. Chemical structure of mangiferin.

environmentally controlled conditions ( $24 \pm 2$  °C, 12 h light/12 h dark cycle), with free access to standard diet (Purina Chow) and tap water. Each animal was used only once for experimentation. Mice were deprived of food for 15 h before the experimentation. The experimental protocols were approved by the Animal Care and Use Committee of the Federal University of Ceará in accordance with the ethical guidelines of the International Association for the Study of Pain (Proc. No. 24/2012). All efforts were made to minimize animal suffering and reduce the number of animals used.

### 2.3. Drugs and chemicals

Acetic acid, formaldehyde, capsaicin, capsazepine, ruthenium red, L-arginine, N<sup>ε</sup>-nitro-L-arginine methyl ester (L-NAME), 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ), diazoxide, glibenclamide, and 8-phenyltheophylline were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The other drugs were from Morphine hydrochloride (Dimorf®; Cristalia, SP, Brazil), naloxone chloridrate (Narcan®, Cristalia, SP, Brazil), diazepam (Valium®, Roche, SP, Brazil). All other chemicals used were of analytical grade. Capsaicin was dissolved in a vehicle comprising of ethanol, Tween 80 and normal saline (1:1:8). Mangiferin was dissolved in 2% Tween 80 in saline. All other drugs were dissolved in normal saline. The vehicles used alone had no effects per se on the nociceptive responses in mice.

### 2.4. Nociceptive tests

The analgesic activity of mangiferin was evaluated on the chemical nociception in the animal models of acetic acid-induced writhing, capsaicin and formalin-induced hind paw licking, and on the thermal nociception using the tail-flick and hot-plate tests. Conscious (un-anesthetized) mice were used in all the nocifensive tests. The doses selection for mangiferin and positive controls were based on literature findings. Control groups were treated with a similar volume of the vehicle that had been used to dilute this compound.

#### 2.4.1. Acetic acid-induced writhing

The acetic acid-induced nociception was performed as described previously by Koster et al. (1959). Groups of mice ( $n = 8$ ) were treated with vehicle (2% Tween 80, 10 mL/kg, p.o.) or mangiferin (10, 30 and 100 mg/kg, p.o.) 60 min before or morphine (5 mg/kg, s.c.) 30 min before the administration of acetic acid (0.6%, 10 mL/kg, i.p.). The number of abdominal constrictions (writhing) was counted for each animal, starting 10 min after acetic acid injection over a period of 20 min. In order to evaluate the participation of the opioid system in the antinociceptive property of mangiferin, different groups of mice were pretreated with naloxone (2 mg/kg, i.p.) 15 min before the administration of mangiferin (30 mg/kg, p.o.) or morphine (5 mg/kg, s.c.).

#### 2.4.2. Formalin-induced paw licking

The formalin-induced nociception was performed as described previously by Hunskaar and Hole (1987). Groups of mice ( $n = 8$ ) were treated with vehicle (10 mL/kg, p.o.) or mangiferin (10, 30 and 100 mg/kg, p.o.) 60 min before or morphine (5 mg/kg, s.c.) 30 min before the administration of 20  $\mu$ l of 1% formalin (in 0.9% saline) into the plantar surface of the right hind paw. The duration of paw licking (s) as an index of painful response was determined at 0–5 min (early phase, neurogenic) and 20–25 min (late phase, inflammatory) after formalin injection. In order to verify the possible mechanism of mangiferin antinociception (30 mg/kg) animal groups were pretreated with naloxone (2 mg/kg, s.c.), 15 min before the mangiferin or morphine.

#### 2.4.3. Capsaicin test

The capsaicin-induced nociception was performed as described previously by Santos and Calixto (1997). Groups of mice ( $n = 8$ ) were treated with vehicle (2% Tween 80, 10 mL/kg, p.o.) or mangiferin (10,

30 and 100 mg/kg, p.o.) 60 min before or morphine (5 mg/kg, s.c.) 30 min before the intraplantar administration of capsaicin (1.6  $\mu$ g in 20  $\mu$ l). The time the animals spent licking the injected paw in seconds (s) was registered for a period of 5 min.

To elucidate the possible mechanism in the antinociceptive effect of mangiferin in capsaicin test, the effects of morphine (5 mg/kg, s.c.), diazoxide (2 mg/kg, i.p.), L-arginine (600 mg/kg, i.p.), or the appropriate antagonist, naloxone (2 mg/kg, i.p., a non-selective  $\mu$ -opioid receptor antagonist), capsazepine (5 mg/kg, i.p., a competitive TRPV1 channel antagonist), ruthenium red (3 mg/kg, s.c., a non-competitive TRPV1 antagonist), 8-phenyltheophylline (8 mg/kg, i.p., the adenosinergic antagonist), glibenclamide (2 mg/kg, i.p., a blocker of  $K_{ATP}$ -channels), L-NAME (20 mg/kg, i.p., a non-selective NOS inhibitor) and ODQ (1 mg/kg, i.p., a blocker of soluble cGMP) or their combinations were analyzed. Sixty minutes after mangiferin (30 mg/kg) or 30 min following the agonist/antagonist drug administrations, capsaicin test was carried out as described earlier. When drugs were combined with mangiferin, the respective agent was administered 15 min before mangiferin. Vehicle-treated controls that received capsaicin were also included. The dose selections for capsaicin (1.6  $\mu$ g), the agonist and antagonist drugs were based on our pilot experimentation and from literature citations.

#### 2.4.4. Hot-plate and tail-flick tests

The hot-plate test (Eddy and Leimbach, 1953) and tail-flick test (Janssen et al., 1963) were used with slight modifications to evaluate the antinociceptive effect of mangiferin. The time taken to lick either hind paw or to jump up (reaction time) when placed on a hot-plate (Ugo Basile, model-DS 37, Italy) maintained at  $51 \pm 0.5$  °C was recorded. In the tail flick test, the time taken to flick the tail (the reaction time) when the tail was immersed (3–4 cm from its tip) in a water bath at 55 °C was noted. Mice showing a pretreatment reaction time greater than 15 s in the hot plate test and 5 s in the tail flick test were not used in the experiment. A cut off time of 45 s and 10 s were used to avoid tissue damage in the hot plate test and tail flick tests, respectively. Animal groups (n = 8) were treated with the vehicle (10 ml/kg, p.o.), mangiferin (10, 30 and 100 mg/kg, p.o.) or morphine (5 mg/kg, s.c.) and the reaction time was measured before and after 30, 60, 90, and 120 min of drug administrations.

#### 2.5. Open-field test

The open-field test (Capaz et al., 1981) was used to evaluate the effect of mangiferin on locomotor activity of mice. The apparatus consisted of an acrylic box (transparent walls and black floor) measuring  $30 \times 30 \times 15$  cm<sup>2</sup> and divided in nine squares of equal area. The number of squares crossed with all paws was counted in a 4 min session. Animal groups (n = 8) were treated with the vehicle (10 ml/kg, p.o.) or mangiferin (10, 30 and 100 mg/kg, p.o.) 60 min before or diazepam (1 mg/kg, i.p.) 30 min before the test.

#### 2.6. Rota-rod test

The rota-rod test was carried out according to the method described earlier (Rosland et al., 1990). The apparatus consisted of a horizontal bar with a diameter of 5 cm, subdivided into four compartments (Ugo Basile, model 7650, Italy). The mice were placed on the bar rotating at a speed of 4 rpm and mice that were able to remain on the rod longer than 120 s were selected 24 h before the test. Animal groups (n = 8) were treated with the vehicle (10 ml/kg, p.o.) or mangiferin (10, 30 and 100 mg/kg, p.o.) 60 min before or diazepam (1 mg/kg, i.p.) 30 min before the test. Each animal was tested on the rota-rod for the number of falls, during a 2 min period.

#### 2.7. Statistical analysis

The results are presented as the mean  $\pm$  S.E.M. for 8 animals per group. Statistical comparisons of the data were performed by one-way analysis of variance (ANOVA), followed by Newman Keul's test for multiple comparisons. Differences were considered statistically significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Writhing test

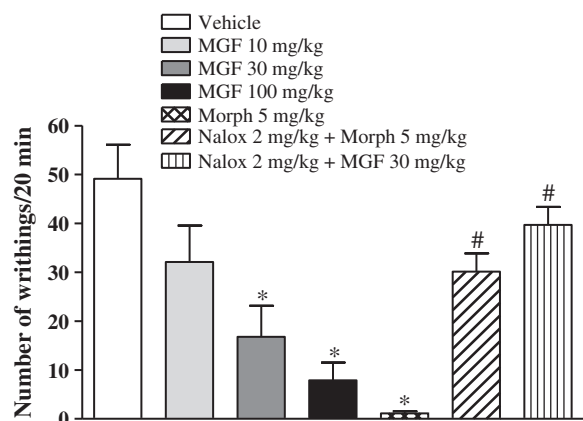
In acetic acid-induced writhing test, mangiferin suppressed the mean number of writhes, when compared to vehicle-treated control group (Fig. 2). These were in the order of  $49.13 \pm 6.99$ ,  $32.13 \pm 7.46$ ,  $16.78 \pm 6.36$ , and  $7.87 \pm 3.63$ , respectively, for the controls and mangiferin at the tested doses of 10, 30 and 100 mg/kg. The positive control group treated with morphine (5 mg/kg, s.c.) also manifested significantly diminished number of writhes ( $1.12 \pm 0.44$ ). Naloxone (2 mg/kg, i.p.) significantly inhibited the antinociceptive effect of morphine and mangiferin (30 mg/kg).

#### 3.2. Formalin test

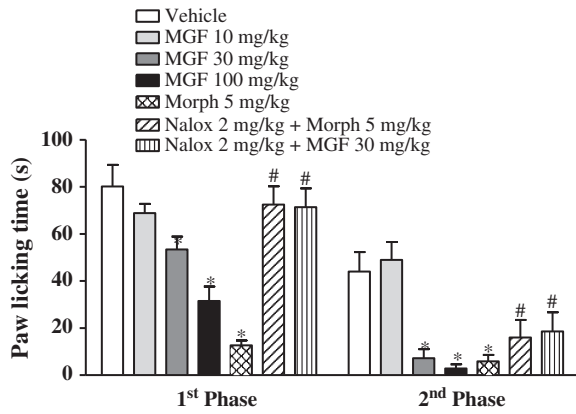
In formalin test, vehicle treated animals showed the mean licking times (s) of  $80.14 \pm 9.19$  in the first phase and  $44.00 \pm 8.31$  in the second phase (Fig. 3). Pretreatment with mangiferin 30 and 100 mg/kg caused significant diminutions of both first phase (neurogenic) ( $31.50 \pm 6.16$  s and  $53.40 \pm 5.49$  s, respectively) and second phase (inflammatory) ( $2.83 \pm 1.76$  s and  $7.17 \pm 3.86$  s, respectively) pain responses (Fig. 3). Morphine (5 mg/kg), the reference standard also significantly suppressed the formalin-response at both phases (first phase,  $12.67 \pm 2.11$  s and second phase,  $5.86 \pm 2.73$  s). Naloxone (2 mg/kg, i.p.) significantly blocked the antinociceptive effect of morphine and mangiferin (30 mg/kg) at both phases of the formalin test (Fig. 3).

#### 3.3. Capsaicin test

Fig. 4 shows the suppressive effect of mangiferin on paw-licking response to subplantar injection of capsaicin (1.6  $\mu$ g). The vehicle treated mice showed the paw-licking response of  $96.00 \pm 11.06$  s. Mangiferin significantly reduced the paw-licking response at doses

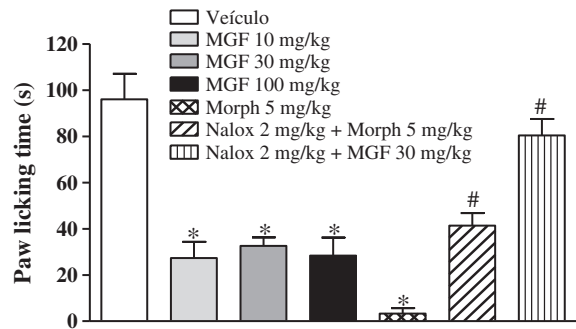


**Fig. 2.** Effects of mangiferin and morphine on acetic acid-induced writhing test in mice. The animals were pretreated with mangiferin (MGF, 10, 30 or 100 mg/kg, p.o.) or vehicle 60 min or with morphine (Morph, 5 mg/kg, s.c.) 30 min before the acetic acid (0.6%, 10 ml/kg, i.p.). Naloxone (Nalox, 2 mg/kg, i.p.) was administered 15 min before morphine or mangiferin (30 mg/kg). The number of abdominal constrictions (writhings) was counted over a period of 20 min. All data are mean  $\pm$  S.E.M. (n = 8). \* $p < 0.05$  compared with vehicle group. # $p < 0.05$  compared with morphine or mangiferin at dose of 30 mg/kg. ANOVA followed by Newman Keul's test.

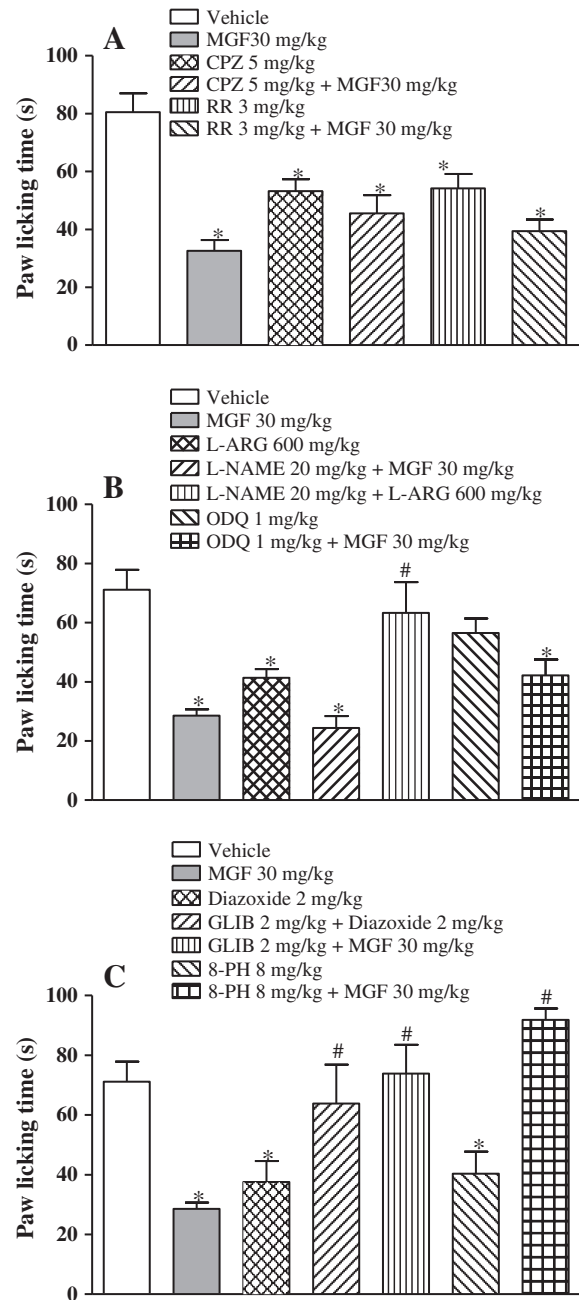


**Fig. 3.** Effects of mangiferin and morphine on formalin test in mice. The animals were pretreated with mangiferin (MGF, 10, 30 or 100 mg/kg, p.o.) or vehicle 60 min or with morphine (Morph, 5 mg/kg, s.c.) 30 min before the administration of 1% formalin (20  $\mu$ l in 0.9% saline) into the plantar surface of the right hind paw. Naloxone (Nalox, 2 mg/kg, i.p.) was administered 15 min before morphine or mangiferin (30 mg/kg). The duration of paw licking (s) was determined at 0–5 min (first phase) and 20–25 min (second phase) after formalin injection. All data are mean  $\pm$  S.E.M. (n = 8). \*p < 0.05 compared with vehicle group. #p < 0.05 compared with morphine or mangiferin at dose of 30 mg/kg. ANOVA followed by Newman Keul's test.

10 mg/kg ( $27.29 \pm 7.00$  s), 30 mg/kg ( $32.60 \pm 3.69$  s) and 100 mg/kg ( $28.40 \pm 7.78$  s) when compared to vehicle-treated control. Morphine (5 mg/kg) greatly reduced the paw-licking response ( $3.25 \pm 2.41$  s) and appeared much more potent than to mangiferin. Naloxone (2 mg/kg, i.p.) significantly inhibited the antinociceptive effects of both morphine and mangiferin (30 mg/kg) (Fig. 4). The capsaicin-induced nociception was also significantly blocked by capsazepine and ruthenium red, the respective competitive and non-competitive TRPV1 antagonists (Fig. 5A). However, their combinations with mangiferin failed to modify mangiferin antinociception. Similar to mangiferin (30 mg/kg), L-arginine (600 mg/kg) alone caused significant inhibition of capsaicin-induced nociception (Fig. 5B). However, in mice pretreated with L-NAME, a non-selective NOS inhibitor, the antinociceptive effect of L-arginine but not of mangiferin was significantly reversed. Further, a soluble cGMP inhibitor ODQ (1 mg/kg) failed to block the antinociceptive effect of mangiferin. Like mangiferin, diazoxide (2 mg/kg), an opener of  $K_{ATP}$ -channels significantly inhibited the nociception induced by capsaicin, which was however, reversed in animals pretreated with glibenclamide (2 mg/kg), a blocker of  $K_{ATP}$  channels (Fig. 5C). Mangiferin antinociception was significantly blocked by glibenclamide as well as by 8-phenyltheophylline (8 mg/kg), an adenosine receptor antagonist.



**Fig. 4.** Effects of mangiferin and morphine on capsaicin test in mice. The animals were pretreated with mangiferin (MGF, 10, 30 or 100 mg/kg, p.o.) or vehicle 60 min or with morphine (Morph, 5 mg/kg, s.c.) 30 min before the administration of capsaicin (1.6  $\mu$ g, 20  $\mu$ l) into the right hind paw. Naloxone (Nalox, 2 mg/kg, i.p.) was administered 15 min before morphine or mangiferin (30 mg/kg). The time the animals spent licking the injected paw was registered for a period of 5 min. All data are mean  $\pm$  S.E.M. (n = 8). \*p < 0.05 compared with vehicle group. #p < 0.05 compared with morphine or mangiferin at dose of 30 mg/kg (ANOVA and Newman Keul's test).



**Fig. 5.** Mechanistic studies on mangiferin antinociception in capsaicin test with mice: A. Effects of pretreatment with TRPV1 selective antagonist capsazepine or non-selective antagonist ruthenium red (RR) and their combinations with mangiferin; B. Pretreatment effects of NOS inhibitor L-NAME or soluble cGMP inhibitor ODQ and their combinations with mangiferin; C. Pretreatment effects of  $K_{ATP}$  channel blocker glibenclamide or adenosinergic antagonist 8-Phenyltheophylline (8-PH) and their combinations with mangiferin. The animals were pretreated with vehicle or mangiferin (MGF 30 mg/kg, p.o.) 60 min before or with glibenclamide (GLB, 5 mg/kg, i.p.), capsazepine (CPZ, 5 mg/kg, i.p.), ruthenium red (RR, 3 mg/kg, s.c.), L-arginine (L-ARG, 600 mg/kg, i.p.), L-NAME (20 mg/kg, i.p.), ODQ (1 mg/kg, i.p.), diazoxide (2 mg/kg, i.p.), and 8-phenyltheophylline (8-PH, 8 mg/kg, i.p.), 30 min before the administration of capsaicin (1.6  $\mu$ g, 20  $\mu$ l) into the right hindpaw. When drugs were combined, the respective agent was administered 15 min before mangiferin. The time in seconds (s) the animals spent licking the injected paw was registered for a period of 5 min. All data are mean  $\pm$  S.E.M. (n = 8). \*p < 0.05 compared with vehicle group. #p < 0.05 compared with morphine or mangiferin at the dose of 30 mg/kg (ANOVA and Newman Keul's test).

#### 3.4. Hot-plate and tail-flick tests

In the hot-plate and tail-flick test models of thermal nociception, while mangiferin (10, 30 and 100 mg/kg, p.o.) pretreatment manifested

**Table 1**  
Effects of mangiferin and morphine on hot-plate test in mice.

Groups	Reaction time (s) after the drug administration				
	0 min	30 min	60 min	90 min	120 min
Vehicle	17.31 ± 1.53	18.61 ± 2.88	18.60 ± 1.85	18.15 ± 1.20	20.63 ± 1.98
MGF 10 mg/kg	17.29 ± 1.66	18.01 ± 1.98	23.35 ± 2.45	23.84 ± 2.71	25.21 ± 2.44
MGF 30 mg/kg	16.05 ± 1.59	20.34 ± 2.44	21.75 ± 1.53	23.51 ± 2.96	27.48 ± 4.06
MGF 100 mg/kg	15.59 ± 1.48	22.89 ± 2.45	23.43 ± 2.37	25.26 ± 1.31	18.43 ± 1.67
Morphine 5 mg/kg	17.41 ± 6.35	44.99 ± 0.01*	38.76 ± 2.76*	35.08 ± 3.83*	26.14 ± 3.23

Mice were pretreated with vehicle, mangiferin (MGF, 10, 30 or 100 mg/kg, p.o.) 60 min or morphine (5 mg/kg, s.c.) 30 min before the test. Data are expressed as mean ± S.E.M. of reaction time in seconds (s) of 8 animals.

\*  $p < 0.05$  different from vehicle group (ANOVA followed by Newman Keul's test).

**Table 2**  
Effects of mangiferin and morphine on tail-flick test in mice.

Groups	Reaction time (s) after the drug administration				
	0 min	30 min	60 min	90 min	120 min
Vehicle	2.76 ± 0.32	2.79 ± 0.38	2.56 ± 0.49	2.34 ± 0.21	2.36 ± 0.33
MGF 10 mg/kg	2.97 ± 0.30	2.90 ± 0.13	2.89 ± 0.27	3.22 ± 0.30	2.75 ± 0.11
MGF 30 mg/kg	2.77 ± 0.36	2.20 ± 0.14	2.92 ± 0.27	3.24 ± 0.18	3.59 ± 0.27
MGF 100 mg/kg	2.50 ± 0.33	3.27 ± 0.50	3.47 ± 0.09	3.70 ± 0.10	3.58 ± 0.08
Morphine 5 mg/kg	3.15 ± 0.47	5.23 ± 0.49*	6.49 ± 0.94*	7.13 ± 0.74*	5.63 ± 0.75*

Mice were pretreated with vehicle, mangiferin (MGF, 10, 30 or 100 mg/kg, p.o.) 60 min or morphine (5 mg/kg, s.c.) 30 min before the test. Data are expressed as mean ± S.E.M. of reaction time in seconds (s) of 8 animals.

\*  $p < 0.05$  different from vehicle group (one way ANOVA followed by Newman Keul's test).

no significant analgesia when compared with control group, the opioid agonist, morphine (5 mg/kg, s.c.) demonstrated significant antinociception at the time points of 30, 60, 90 and 120 min (Tables 1 and 2).

### 3.5. Open-field and rota-rod tests

Mangiferin administered at the oral dose of 10, 30 and 100 mg/kg failed to produce any significant effect on locomotion frequency or on motor coordination on rota-rod in mice, indicating that the observed antinociception was unrelated to sedation or motor abnormality (Table 3). In contrast, diazepam (1 mg/kg, i.p.) significantly lowered the locomotion frequency (evidenced by reduced number of crossings) and motor coordination (evidenced by increased number of falls).

## 4. Discussion

Mangiferin was evaluated for analgesic activity in mice using experimental models of chemogenic and thermal nociception. The data from this investigation confirmed the antinociceptive effect of mangiferin against chemically induced nociception but not the thermal pain, in a naloxone reversible manner suggesting a peripheral opioid mechanism. Tail flick-induced thermal nociception has been

considered to involve spinal mechanisms while Eddy's hot-plate-induced thermal nociception involves the supraspinal mechanisms (Yaksh and Rudy, 1976). In both these experimental models, mangiferin failed to demonstrate antinociceptive activity at any one of the test doses, indicating that it had no spinal or supraspinal level of action. However, this observation differs from the studies of Dar et al. (2005) that reported a naloxone reversible antinociception against thermal pain in hot-plate test, following a single dose (42.2 mg/kg) subcutaneous/intraperitoneal injection. This discrepancy may be due to differences in experimental conditions and the route of mangiferin delivery. It is likely that enteral or parenteral administration may influence the plasma concentration kinetics differently and thus the test drug bioactivity. However, there is no available literature data on the absorption kinetics of mangiferin. Since the antinociceptive activity of mangiferin occurs without activation of opioid receptors in the central nervous system (CNS), centrally mediated side effects may be absent with peripheral opioid activity. Acting as a peripheral opioid agonist, mangiferin may possibly activate peripheral opioid receptors on sensory nerve fibers and suppress capsaicin algia by decreasing the excitability of sensory nerves and/or inhibiting release of pro-inflammatory neuropeptides. Thus an important finding from our study is the demonstration of peripheral opioidergic system involvement in the antinociceptive effect of mangiferin, which is possibly manifested through its anti-inflammatory and antioxidant effects in experimental models of chemogenic pain.

From this study, it could be assumed that the antinociceptive action of mangiferin was purely mediated through blockade of peripheral pain pathways without the any involvement of central action. In this regard, a recent pharmacokinetic study (Zajac et al., 2013) using the qualitative methods of thin-layered-chromatography and UV/VIS spectrophotometry failed to trace mangiferin in the brain fractions, which makes it unlikely that the compound traverse the blood-brain barrier after being systemically administered (300 mg/kg, i.p.) and concluded that it is improbable that mangiferin could act via direct interaction with central neural components, but rather has peripheral, target specific functions which could be secondarily reflected in brain metabolism. Because of this, the reported neuropharmacological effects of mangiferin following systemic administration and the claims or suggestions made on its

**Table 3**  
Effects of mangiferin and diazepam on open-field test and rota-rod test in mice.

Groups	Open-field test	Rota-rod test
	Number of crossing	Number of falls
Vehicle	45.25 ± 5.40	0.37 ± 0.26
MGF 10 mg/kg	44.25 ± 4.81	0.50 ± 0.27
MGF 30 mg/kg	38.38 ± 3.62	0.50 ± 0.27
MGF 100 mg/kg	43.25 ± 4.57	0.37 ± 0.26
Diazepam 1 mg/kg	23.17 ± 5.36*	2.67 ± 0.33*

Mice were pretreated with vehicle, mangiferin (MGF, 10, 30 or 100 mg/kg, p.o.) 60 min or diazepam (1 mg/kg, i.p.) 30 min before the test. Data are expressed as mean ± S.E.M. of number of crossing (open-field test) or number of falls (rota-rod test) of 8 animals.

\*  $p < 0.05$  different from vehicle group (one way ANOVA followed by Newman Keul's test).

usefulness against Alzheimer, Parkinson, and Schizophrenia diseases, and for the improvement of cognitive function (Biradar et al., 2012; Rao et al., 2012; Jung et al., 2009) need further analysis and explanation.

Pain is one of the most important symptoms of inflammatory disease because it directly affects people's daily lives and is the primary reason why patients seek medical expertise (Vane and Botting, 1990; Weisburger, 2002). Studies also suggest that oxidative stress modifies experimental nociception (Viggiano et al., 2005; Garrido-Suárez et al., 2010; Ma et al., 2009) and antioxidants ameliorate nociception (Rokyta et al., 2003; Hacimuftuoglu et al., 2006). Known antioxidants like PBN (phenyl-N-tert-butyl-nitron), TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy) and NAC (N-acetyl-L-cysteine) not only are effective in suppressing neuropathic and inflammatory pain (Viggiano et al., 2005; Hacimuftuoglu et al., 2006) but may even permit a decrease in the doses of analgesics and prevent the negative impact of reactive oxygen species on nociception (Rokyta et al., 2003). Mangiferin's xanthonoid structure with C-glucosyl linkage and polyhydroxy component is believed to be crucial for its free radical-scavenging ability leading to a potent antioxidant effect. The antinociceptive potential of mangiferin observed in present investigation is consistent with earlier reports on plant-derived substances like citronellal, a monoterpene (Brito et al., 2012), quercetin, a bioflavonoid (Valério et al., 2009), and the polyphenolic resveratrol (Granados-Soto et al., 2002) that largely possess the antioxidant property. Therefore, studying the pain modulation effects of antioxidants is considered an emerging area of interest.

In the present experiments, nociceptive responding, indicated by licking the affected hindlimb, was quantified for 30 min after formalin injection or for 5 min following intraplantar capsaicin or the number of abdominal constrictions (writhes) for 20 min, following intraperitoneal acetic acid. Mangiferin was effective in attenuating acute neurogenic phase, and tonic inflammatory phase of the formalin response and similarly the neuropathic and inflammatory pain induced by capsaicin as well the writhing response induced by acetic acid. The capsaicin test is a particularly relevant model since it is a selective TRPV1 agonist capable of inducing an acute nociception and neurogenic inflammation in experimental animals and pain in humans through activation of capsaicin-sensitive peripheral afferent fibers or sensory neurons (Saadé et al., 2002) that play an important role in nociceptive pain processing, thus involving the peripheral nervous system (Gold and Gebhart, 2010). Mangiferin, given orally, elicited a dose-unrelated antinociceptive effect on the capsaicin-induced neurogenic paw-licking response, just as observed in the second phase (inflammatory) of formalin test. Mangiferin was also effective in inhibiting the nociception induced by intraperitoneal acetic acid, confirming previously reported results (Dar et al., 2005). The writhing test is frequently used to evaluate visceral pain because acetic acid directly activates visceral and somatic nociceptors innervating the peritoneum and induces the inflammation in visceral organs, which is mediated by inflammatory mediators such as prostaglandins, bradykinin, substance P and cytokines (Le Bars et al., 2001). Reports suggest that pro-oxidant species may be important mediators of tissue injury-induced algia in rodents, and antioxidants are effective analgesics in neuropathic and inflammatory pain models (Hacimuftuoglu et al., 2006). Besides acting as an antioxidant, mangiferin is known to inhibit several key inflammatory pathways such as Nf2–NfκB signaling (Das et al., 2012). Thus the antioxidant and anti-inflammatory properties also may, in part, account for the antinociceptive activity of mangiferin.

It is currently accepted that an endogenous opioid analgesic system is present at peripheral level (Smith, 2008; Alves et al., 2012), and most of opioid antinociceptive effects are mediated via activation of opioid receptors (Stein and Lang, 2009) and opioid receptors have been identified on peripheral terminals of afferent nerves, which can be the sites of the intrinsic modulation of nociception (Vadivelu et al., 2011). Attempts to mimic or augment such peripheral analgesia may potentially

lead to analgesic effects in the absence of the central adverse effects caused by opioids. In capsaicin test, the antinociceptive effect of mangiferin was not modified by respective selective and non-selective TRPV1 antagonists capsaizepine or ruthenium red, but was reversed by naloxone, a non-selective  $\mu$ -opioid receptor antagonist, suggesting an opioid mechanism. Mangiferin may indirectly inhibit/attenuate the TRPV1 receptor in the skin sensory afferents to induce antinociception through stimulation of endogenous peripheral opioid system and involving the pathways that include adenosinergic, nitric oxide, and the opening of ATP-sensitive potassium channels. Therefore, so as to have greater insights into the molecular mechanism by which mangiferin promotes peripheral antinociception, the present study examined first the possible participation of NO/cGMP/K<sup>+</sup> + ATP pathway that appears to involve the peripheral antinociception produced by opioids (Ferreira et al., 2005; Rodrigues and Duarte, 2000). In capsaicin test, our results show that pretreatments with a K<sub>ATP</sub> blocker, glibenclamide but not the nonspecific NOS inhibitor, L-NAME, or the soluble guanylyl cyclase inhibitor, ODQ could effectively reverse the antinociceptive effect of mangiferin, suggesting that mangiferin's antinociception results from the modulation of K<sub>ATP</sub> currents.

The activation of adenosine receptors in peripheral afferents is also important in modulating pain and studies report on the release of endogenous adenosine in the presence of morphine (Sawynok, 1998; Peart and Gross, 2005). Adenosine has recently been proposed to be a significant anti-inflammatory autacoids released peripherally under conditions of inflammation (Cronstein, 1994). The antinociceptive actions of adenosine and adenosine analogs in animal models have been reported that could be reversed by caffeine or 8-phenyltheophylline (Homayounfar et al., 2005). This mechanism also seems to participate in mangiferin's antinociception, because pre-treatment with the adenosine antagonist, 8-phenyltheophylline effectively reversed its effect.

Postoperative pain management is an essential part of surgical management because inadequate treatment of pain is associated with postoperative complications and poor outcomes. Paracetamol, nonsteroidal anti-inflammatory drugs (NSAIDs) like celecoxib and, if necessary, opioids are the most commonly used drugs for perioperative analgesia. Opioid use is avoided mainly because of the fear of side effects such as respiratory depression, nausea, vomiting, urinary retention, and pruritis (Scott and Perry, 2000). It was observed that analgesia induced by celecoxib in a model of inflammatory pain was, in contrast with that induced by paracetamol could be reversed by opioid receptor antagonist naloxone (França et al., 2006; Pickering et al., 2011), suggesting that its antinociceptive effect is mediated by endogenous opioids, presumably indirectly, involving release from opioidergic nerves. Based on the present findings, mangiferin deserves a role in the management of post-operative pain.

Motor deficits may create confounds in studies in which antinociception is measured. To clarify if the analgesic effect is not a result of motor deficits, we assessed the effects of mangiferin on open-field and rota-rod tests that are classical models for screening central nervous system actions providing information on motor coordination and myorelaxant activity. Mangiferin (10–100 mg/kg, p.o.), neither impaired locomotor activity in open-field test nor presented myorelaxant activity as demonstrated in the rota-rod test that measures grip strength, suggesting that the mangiferin antinociception observed in this investigation is not exerted through peripheral neuromuscular blockade or induction of sedation.

Studies also have shown that mangiferin is free from cytotoxic or genotoxic effects (Rodeiro et al., 2012). Acute toxicity in mice showed that mangiferin was safe at an oral dose up to 25 g/kg (Niu et al., 2012). These findings demonstrate that mangiferin has the antinociceptive potential to be developed as a therapeutic agent. Based on our results obtained in capsaicin model of nociception, it was suggested that mangiferin's antinociception possibly involves the participation of endogenous opioids, K<sub>ATP</sub>-channels, and adenosine receptors. Nevertheless, studies are warranted, using nociceptive

models other than capsaicin so as to better understand its antinociceptive mechanism and exploit the antinociceptive property of this compound for clinical situations.

## 5. Conclusions

This study demonstrated the antinociceptive activity of a glucosylxanthone, mangiferin in experimental models of visceral pain induced by intraperitoneal acetic acid, and in the inflammatory and neurogenic pain evoked by subplantar formalin or intraplantar capsaicin in mice. In tests of thermal pain (hot-plate and tail-flick), mangiferin failed to manifest significant antinociception, indicating that its action is only peripheral and not central. The results also indicated that mangiferin's antinociception possibly involves the participation of endogenous opioids,  $K_{ATP}$ -channels, and adenosine receptors.

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## Conflict of interest statement

The authors declare that they have no conflicts of interest.

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