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Involvement of Cerebral Nervous System Areas and Cytokines on Antihyperalgesic and Anti-Inflammatory Activities of *Kielmeyera rugosa* Choisy (Calophyllaceae) in Rodents

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***Kielmeyera rugosa* is a medicinal plant known in Northeastern Brazil as 'pau-santo', and it is used in the treatment of several tropical diseases such as malaria, schistosomiasis, and leishmaniasis. We evaluated antihyperalgesic and anti-inflammatory activities of methanol stem extract of *K. rugosa* (MEKR) in mice. The mechanical hyperalgesia induced by carrageenan and tumor necrosis factor-alpha (TNF-α), prostaglandin E_2, and dopamine were assessed. We also investigated the anti-inflammatory effect of MEKR on carrageenan-induced pleurisy and paw edema. Ninety minutes after the treatment, the animals were submitted to an imunofluorescence for Fos protein. MEKR (100, 200, and 400 mg/kg; p.o.) inhibited the development of mechanical hypernociception and edema. MEKR significantly decreased TNF-α and interleukin 1β levels in pleural lavage and suppressed the recruitment of leukocytes. MEKR (1, 10, and 100 mg/mL) did not produce cytotoxicity, determined using the methyl-thiazolyl-tetrazolium assay *in vitro*. The locomotor activity was not affected. MEKR activated significantly the bulb olfactory, piriform cortex, and periaqueductal gray of the central nervous system. Our results provide first time evidence to propose that MEKR attenuates mechanical hyperalgesia and inflammation, in part, through an activation of central nervous system areas, mainly the periaqueductal gray and piriform cortex areas. Copyright © 2014 John Wiley & Sons, Ltd.**

Keywords: Kielmeyera rugosa; *Calophyllaceae*; hyperalgesia; pain; inflammation; Fos.

INTRODUCTION

Pain is the most common reason why individuals seek medical attention, yet the pain sensation is highly necessary to protect the organism from potentially tissue-damaging stimuli. Moreover, pain is one of the classic signs of the inflammatory process, whose treatment represents a major problem due to the use of available medications and their side effects (McCurdy and Scully, 2005; da Silva *et al*., 2012).

Inflammatory hyperalgesia, commonly associated with hypernociception in animals, is an increased response to a stimulus which is normally painful (Verri *et al*., 2006; Cunha *et al*., 2008a). For this, compounds derived from natural products have been utilized since the beginning of time for the treatment of inflammatory pain, as a challenge to reduce side effects of pain medications currently used (McCurdy and Scully, 2005; Guimarães *et al*., 2013).

Natural products have been important in the development of modern analgesics (Guimarães *et al*., 2014). Henceforth, the discovery of new analgesics may also be derived from recent work carried out with plant extracts and compounds (Balunas and Kinghorn, 2005; Quintans *et al*., 2013). The genus *Kielmeyera*, family Calophyllaceae, is present in the vast majority of the 47 species occurring exclusively in Brazil (Pinheiro *et al*., 2003; Sela *et al*., 2010). Some species, such as *Kielmeyera coriacea*, are popularly known in Brazil as 'pau-santo', used by the native population of Brazil in the treatment of several tropical diseases such as malaria, schistosomiasis, leishmaniasis, and fungal or bacterial infections (Audi *et al*., 2002).

Considering that this genus is explored in chemical studies of natural products due to its potential use in phytochemical and pharmacological products (Pinheiro *et al*., 2003), the species *Kielmeyera rugosa* Choisy has been the focus of biological evaluation. Xanthones, besides 4-alkyl and 4-phenylcoumarins, are important chemotaxonomic markers within the genus *Kielmeyera*, in a specific study on the composition parts of *K. rugosa* (Nogueira *et al*., 2009). Recently, it was demonstrated that the stem extract of *K. rugosa* possesses significant antitumoral activity (Ribeiro *et al*., 2012). It has also been observed in this genus that microinjections of xanthone from dichloromethane fraction of *K. coriacea* stems in the intra-median raphe nucleus reduce immobility time in the forced swimming test model; it also has an antidepressant effect on rats submitted to the test (Sela *et al*., 2010). In recent research, extracts from

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stems of *K. rugosa* showed positive result in a cytotoxic screening and antitumor activity on sarcoma 180 (Oliveira *et al.*, 2013).

In this context, the aim of this study was to investigate the effect of methanol stem extract of *K. rugosa* (MEKR) on inflammatory hyperalgesia induced by carrageenan (CG), tumor necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2), and dopamine (DA). In addition, the current study was designed to clarify the characteristics of pain-associated neuronal activities by immunofluorescence localization of the c-Fos protein on central nervous system areas.

MATERIALS AND METHODS

Plant material and preparation of extract. Stems of *K. rugosa* were collected in May 2010 from a 'restinga' (the vegetation mosaic found on Brazilian coastal sandy plains) near the Pomonga River [coordinates: 10°47′ 23.7″S 36°58′31.4″W] in the Municipality of Santo Amaro das Brotas, Sergipe State, Brazil. The species was identified by Dr. Volker Bittrich and Dr. Maria C. E. Amaral, plant taxonomists of the Institute of Biology at the State University of Campinas (UNICAMP). A voucher was registered under the code 206.

The methanolic extract of *K. rugosa* was obtained according to what has been described(Nogueira *et al.*, 2009). The stems (397.4 g) of *K. rugosa* were extracted at room temperature with methanol (1,5 L, twice). The solvent was removed under reduced pressure to give the correspondent crude extract (20.9 g).

LC-grade methanol (Tedia, Fairfield, OH, USA) and formic acid (JT Baker, Philipsburg, PA, USA) were used for high-performance liquid chromatography (HPLC) analysis. Deionized water was purified by a Milli-Q system (Millipore, São Paulo, SP, Brazil). All the solvents were filtered through nylon 0.45 μm membranes (MFS) and degassed by ultrasonic bath before use.

High-performance liquid chromatography–photodiode-array detection analysis. High-performance liquid chromatography analysis was performed using a Shimadzu-model (Kyoto, Japan) prominence liquid chromatograph equipped with a vacuum degasser (DGU-20A3), autosampler (SIL-10A), two high-pressure pumps (LC-6A), and a SPDM20Avp photodiode array detector (DAD) system coupled with a CBM 20A interface. Data collection was carried out using LC Solution software. Analysis was performed in an analytical Phenomenex Kinetex™ C18 column (250 × 4.6 mm i.d., 5 μm of particle diameter, Torrance, CA, USA) with a C18 guard column (4 × 3 mm, 4 μm, Phenomenex, Torrance, CA, USA) under the following conditions: flow rate at 0.8 mL/min, injection volume of 20 μL and a mobile phase consisting of 0.1% aqueous formic acid (v/v, A) and methanol (B). The gradient elution for MEKR sample was as follows: 10–30% (B) in 12 min, 30–100% (B) in 70 min, remaining at 100% (B) for 10 min. Photodiode array detector was set at 254 nm for acquiring chromatogram and ultraviolet spectra were recorded between 200 and 500 nm. Identification was based on comparisons of ultraviolet (UV) absorption.

A sample of MEKR was dissolved in methanol at a concentration of 2.25 mg/mL and then was submitted to filtration in a cellulose membrane (pore diameter of 0.45 μm) before HPLC injection.

Drugs and reagents. λ-Carrageenan, TNF-α, PGE_2, DA, Tween 80, fluoromount G, glycine and bovine serum albumin were purchased from Sigma (USA). Indomethacin and dipyrone were obtained from União Química (São Paulo, Brazil). Diazepam (DZP) was purchased from Cristália (Brazil). Rabbit anti-Fos and donkey anti-rabbit Alexa Fluor 594 was obtained from Santa Cruz Biotechnology (USA). The extract was freshly prepared with 0.9% saline and Tween 80 0.02% (vehicle) for pharmacological experiments. The other substances were solubilized with distilled water or saline.

Animals. Adult (3-month-old) male albino *Swiss* mice (28–32 g) were randomly housed in appropriate cages at 21 ± 2°C on a 12 h light/dark cycle (lights on from 06:00 a.m. to 6:00 p.m.), with free access to food (Purina®, Brazil) and water. All experiments were carried out between 09:00 a.m. and 14:00 p.m. in a quiet room. All hyperalgesic tests were carried out by the same visual blinded observer. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS 102/11) and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research (Zimmermann, 1983).

Hyperalgesia induced by carrageenan, tumor necrosis factor-alpha, prostaglandin E_2, and dopamine. Mouse paw hyperalgesia was performed as previously described (Cunha *et al.*, 2004; Vivancos *et al.*, 2004). The mice were divided into five groups ($n = 6$, per group), which were treated with vehicle (saline + Tween 80 0.02%, v/v, p.o.), MEKR (100, 200, or 400 mg/kg, p.o.), indomethacin (10 mg/kg, i.p.) or dipyrone (60 mg/kg, i.p.). Thirty minutes after treatment, 20 μL of CG (300 μg/paw), PGE_2 (100 ng/paw), DA (30 μg/paw), or TNF-α (100 pg/paw) were injected subcutaneously into the subplantar region of the hind paw. The degree of hyperalgesia was evaluated at 0.5, 1, 2, and 3 h after the injection of hyperalgesic agents.

Measurement of mechanical hyperalgesia. Mechanical hyperalgesia was tested in mice as reported by Cunha *et al.* (2004) (Cunha *et al.*, 2004). In a quiet room, the mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors for 15–30 min before starting the test. This method consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight®, Ribeirão Preto, São Paulo, Brazil) adapted with a polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the withdrawal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was automatically recorded. The intensity of stimulus was obtained by averaging four measurements

taken with minimal intervals of 3 min. The animals were tested before and after treatments. The results are presented as the Δ withdrawal threshold (g), calculated by the difference between the values obtained after the treatments and before the treatment.

Carrageenan-induced pleurisy. Pleurisy was induced by intrathoracic (i.t.) injection of CG (300 μg; 0.1 mL) diluted in sterile saline. Control animals received the same volume of vehicle. The animals were pretreated with MEKR (100, 200, and 400 mg/kg; p.o.) or vehicle (saline + Tween 0.02% v/v; p.o.) 30 min before injection of the inflammatory agent. Four hours after stimulation, the animals were sacrificed in a CO_2 chamber; the pleural cavities were opened and washed with 1 mL of PBS(1×) containing EDTA (10 mM). Total counts of leukocyte collected in the pleural lavage were performed on a Neubauer chamber under an optical microscope. The samples were diluted (40×) in Türk solution. The differential leukocyte analysis was performed under a light microscope with immersion oil objective in cytocentrifuged smears colored with May–Grunwald–Giemsa, where 100 cells per slide were counted. The amount of TNF-α and IL-1β produced in the pleural cavity were assessed 4 h after injection of CG. The recovered pleural lavage was centrifuged at 770 × g for 10 min. TNF-α and IL-1β were quantified on supernatant free of cells by enzyme immunoassay (ELISA) following the manufacturer's protocol (BD-Bioscience Pharmingen).

Measurement of paw edema. The effect of MEKR on edema formation caused by the intraplantar injection of CG was analyzed according to the method previously reported (Levy, 1969). A separate group of mice was divided into five groups ($n = 6$, per group) that were treated with vehicle (saline + Tween 80 0.02%; p.o.), MEKR (100, 200, and 400 mg/kg; p.o.), or indomethacin (10 mg/kg; i.p.). Right paw volume was measured by the dislocation of the water column of a plethysmometer (Insight®, Brazil) before (time zero) and at 1, 2, 3, 4, 5, and 6 h after subplantar injection of 50 μL of CG(1%). Paw edema was expressed (in milliliter) as the difference between the volume of the paw after and before CG injection. The area under the curve (AUC[0–240 min]; in milliliter per minute) was also calculated using the trapezoidal rule.

Methyl-thiazolyl-tetrazolium cell viability assay. The cytotoxic effect of MEKR on macrophages was determined using the methyl-thiazolyl-tetrazolium (MTT) assay method according to Mosmann (1983) (Mosmann, 1983). Murine peritoneal macrophages (2.5 × 105 cells) were treated with MEKR at concentrations ranging from 1 mg/mL to 1000 mg/mL and were later cultured in RPMI-1640 supplemented with 10% FBS for 24 h. Thereafter, the medium was replaced with fresh RPMI containing 5 mg/mL of MTT. After additional 4 h of incubation at 37°C, the supernatant was discharged, and dimethyl sulfoxide solution (150 μL/well) was added to each culture plate. After 15 min of incubation at room temperature, absorbance of solubilized MTT formazan product was spectrophotometrically measured at 540 nm. Five individual wells were assayed per treatment and percentage of viability was determined in relation to controls [(absorbance of treated cells/absorbance of untreated cells) × 100].

Evaluation of the motor activity. In order to investigate the motor activity of the animals treated with MEKR and the consequent impairment of the mechanical hyperalgesic assessment, the motor activity of the animals was evaluated in rota-rod apparatus, according to Dunham and Miya (1957) (Dunham and Miya, 1957) with some modifications. Initially, the mice able to remain on the Rota-rod apparatus (AVS®, Brazil) longer than 180 s (7 rpm) were selected 24 h before the test. Thirty minutes after the administration of either MEKR (100, 200, and 400 mg/kg, p.o.), vehicle (saline + Tween 80 0.02%; p.o.), or DZP (1.5 mg/kg, i.p.), each animal was tested on the Rota-rod apparatus and the time (s) remained on the bar for up to 180 s was recorded at 0.5, 1, and 2 after the administration.

Immunofluorescence. To evaluate the action of the test drug on the central nervous system, 90 min after the injection of MEKR (100, 200, or 400 mg/kg; p.o.) or vehicle (Saline + Tween 80 0.02%, p.o.), the animals ($n = 6$, per group) were perfused, and the brains were collected and cryoprotected for immunofluorescence processing to Fos protein.

Frozen serial transverse sections (20 μm) of all brain were collected on gelatinized glass slides. The tissue sections were stored at −80°C until use. The sections were washed with phosphate buffer (0.01 M) saline isotonic (PBS) 5× for 5 min and incubated with 0.1 M glycine in PBS for 10 min. Non-specific protein binding was blocked by incubation of the sections for 30 min in a solution containing 2% bovine serum albumin. After that, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for two hours with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000). The cover slip was mounted with Fluoromount G. As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS 5× for 5 min.

A striking attribute of Fos is that it is rapidly expressed in central neurons after noxious stimuli. As that is a very used way to visualize the pathways involved in the integration of noxious input. For this reason, we evaluated the action of the test drug on the central nervous system, 90 min after the injection of MEKR (100, 200, or 400 mg/kg; p.o.) or vehicle (Saline + Tween 80 0.02%; p.o.), after injection CG (300 μg/paw), 60 min after the treatment.

Acquisition and analyses of images. Pictures from Fos positive brain areas were acquired for each animal with an Axioskop 2 plus, Carl Zeiss, Germany. The brain regions were classified according to Paxinus and Watsu Atlas, 1997. Neurons were counted by the free software Image J (National Institute of Health) using a plug-in

(written by the authors) that uses the same level of label intensity to select and count the Fos positive cells.

Statistical analysis. Data were evaluated using GraphPad Prism Software Inc. (SanDiego, California, USA) version 5.0, through the analysis of variance followed by Tukey's test. The results are presented as mean ± SEM. In all cases, the differences were considered significant if $p < 0.05$.

RESULTS

High-performance liquid chromatography–photodiode-array detection analysis

High-performance liquid chromatography-DAD analysis of the methanol extract from stems of *K. rugosa* (MEKR) (Fig. 1) revealed the presence of peaks with phenolic compounds-like UV spectra. On the basis of elution order in C18 column and their UV absorption spectra, peaks 1–3 ($Rt = 11.0$ min, $Rt = 21.0$ min, and $Rt = 40.8$ min, respectively) were similar to hydroxybenzoic acids such as vanillic acid, protocatechuic acid, and syringic acid, respectively (Sun *et al.*, 2007). Peaks 4–8 ($Rt = 52.8$ min, $Rt = 68.1$ min, $Rt = 76.2$ min, $Rt = 79.1$ min, and $Rt = 81.0$ min, respectively) presented UV spectra similar to the one observed for alkyl and phenylcoumarins (Garazd *et al.*, 2003; Scio *et al.*, 2003). Peak 4 ($Rt = 52.8$ min) was identified by matching the retention time with that of an authentic sample of the disprorinol A, a 4-propylcoumarin previously isolated from *K. rugosa* (Nogueira *et al.*, 2009).

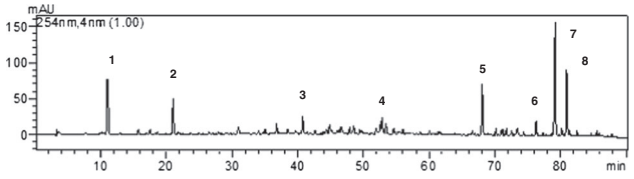

Figure 1. High-performance liquid chromatography-diode array detector chromatogram at 254 nm of methanolic extract from *Kielmeyera rugosa* stems.

Effect of methanol stem extract of *Kielmeyera rugosa* on carrageenan-induced mechanical hyperalgesia and mouse paw edema

Injection of CG in the subplantar region of the mouse paw induced a marked mechanical hyperalgesia characterized by an increased sensitivity as the intensity of stimulus was decreased, which remained throughout the 3 h. MEKR demonstrated an antihyperalgesic effect in this model, as mice treated with MEKR (100, 200, or 400 mg/kg; p.o.) 0.5 h before CG administration exhibited a significant reduction in mechanical hyperalgesia induced by CG at all evaluated times, when compared with animals of the control group that received only vehicle (Fig. 2A). These doses produced an effect similar to indomethacin (10 mg/kg). The group of animals that received saline in the subplantar region, instead of CG, did not present any alteration on the threshold of sensitivity to mechanical stimuli (data not shown).

Mouse paw edema induced by CG administration was also evaluated. As shown in Fig. 3, CG injection increased the mouse paw volume from 1 to 6 h after injection, and the treatment of mice with MEKR significantly decreased the edema. The doses of 100, 200, and 400 mg/kg of MEKR were able to maintain the reduction of the edema during the 6-h evaluation period, as did indomethacin. Animals that received only the vehicle of CG (sterile saline) did not present significant alteration in paw volume (data not shown).

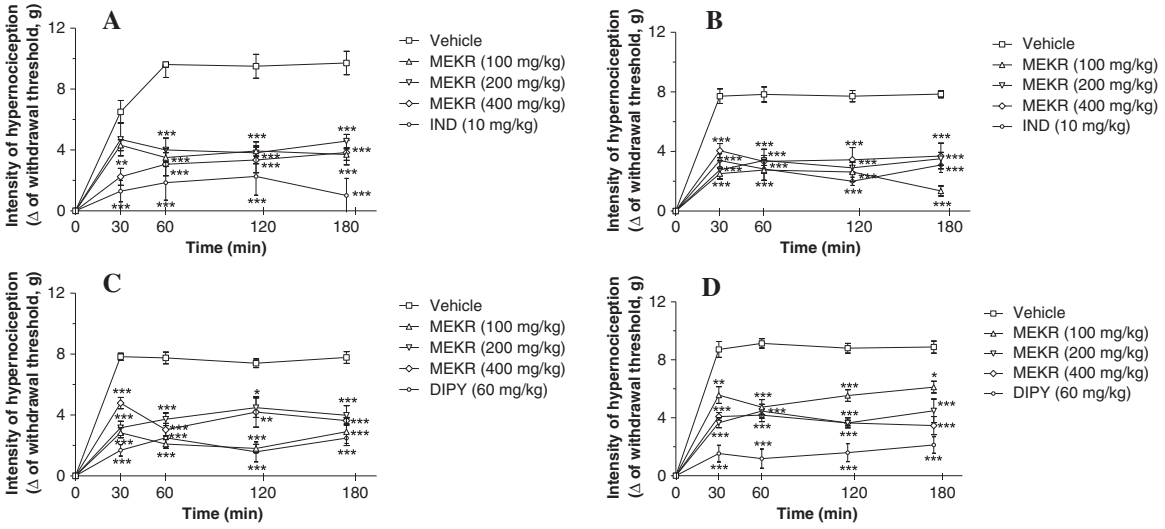

Figure 2. Effect of acute administration of vehicle, methanolic extract of stem of *Kielmeyera rugosa* (MEKR; 100, 200, or 400 mg/kg, p.o.), indomethacin (IND, 10 mg/kg, i.p.), or dipyrone (DIPY, 60 mg/kg, i.p.) on mechanical hyperalgesia induced by carrageenan (A), tumor necrosis factor-alpha (B), prostaglandin E_2 (C), or dopamine (D). Each point represents the mean ± SEM of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus control group (analysis of variance followed by Tukey's test).

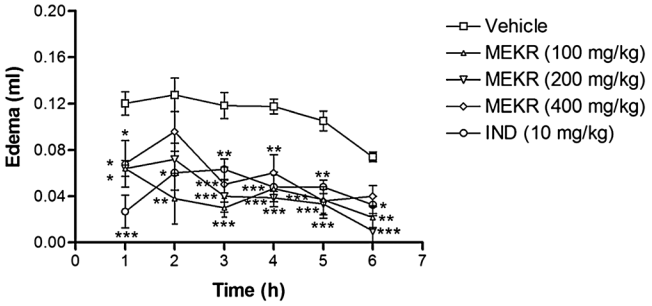

Figure 3. Anti-inflammatory effect of methanol stem extract of *Kielmeyera rugosa* treatment on carrageenan-induced paw inflammation. Methanolic extract of stem of *K. rugosa* (MEKR; 100, 200, or 400 mg/kg, p.o.), saline (control group, p.o.), or indomethacin (IND, 10 mg/kg, i.p.), was administered 6 h before carrageenan. Paw edema measured at 1, 2, 3, 4, 5, and 6 h after the carrageenan injection. Data are expressed as mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with control group (analysis of variance followed by Tukey's test).

Effect of methanol stem extract of *Kielmeyera rugosa* on tumor necrosis factor-alpha, dopamine, or prostaglandin E_2-induced mechanical hyperalgesia

The inhibitory effect of MEKR on the mechanical hyperalgesia induced by TNF-α is shown in Fig. 2B. MEKR (100, 200, and 400 mg/kg, p.o.) was able to reduce mechanical hyperalgesia induced by TNF-α, when compared with animals of the vehicle group, similarly to indomethacin.

The MEKR antihyperalgesic effects on PGE_2-induced and DA-induced hyperalgesia are shown in Fig. 2C and D, respectively. Acute treatment with MEKR (100, 200, and 400 mg/kg) can reduce the mechanical hyperalgesia induced by PGE_2 and DA when compared with vehicle group animals.

Effect of methanol stem extract of *Kielmeyera rugosa* on carrageenan-induced mouse pleurisy

All doses of MEKR were able to significantly suppress the recruitment of leukocytes to the mouse pleural cavity, as shown in Fig. 4A. Pretreatment with MEKR also significantly attenuated the number of neutrophils (Fig. 4B). MEKR (100, 200, and 400 mg/kg) also significantly reduced the TNF-α (Fig. 4C) and IL-1β (Fig. 4D) levels in the pleural exudates collected at 4 h after CG injection.

Lack of cytotoxicity effect of methanol stem extract of *Kielmeyera rugosa*

Increasing concentrations of MEKR (1, 10, 100, 500, and 1000 mg/mL) were unable to cause alteration of murine peritoneal macrophages viability of RPMI control 1–100 mg/mL), indicating that MEKR treatment did not significantly affect mitochondrial reduction of MTT to formazan resulting in an undetectable cytotoxic effect, in these concentrations (Figure 5).

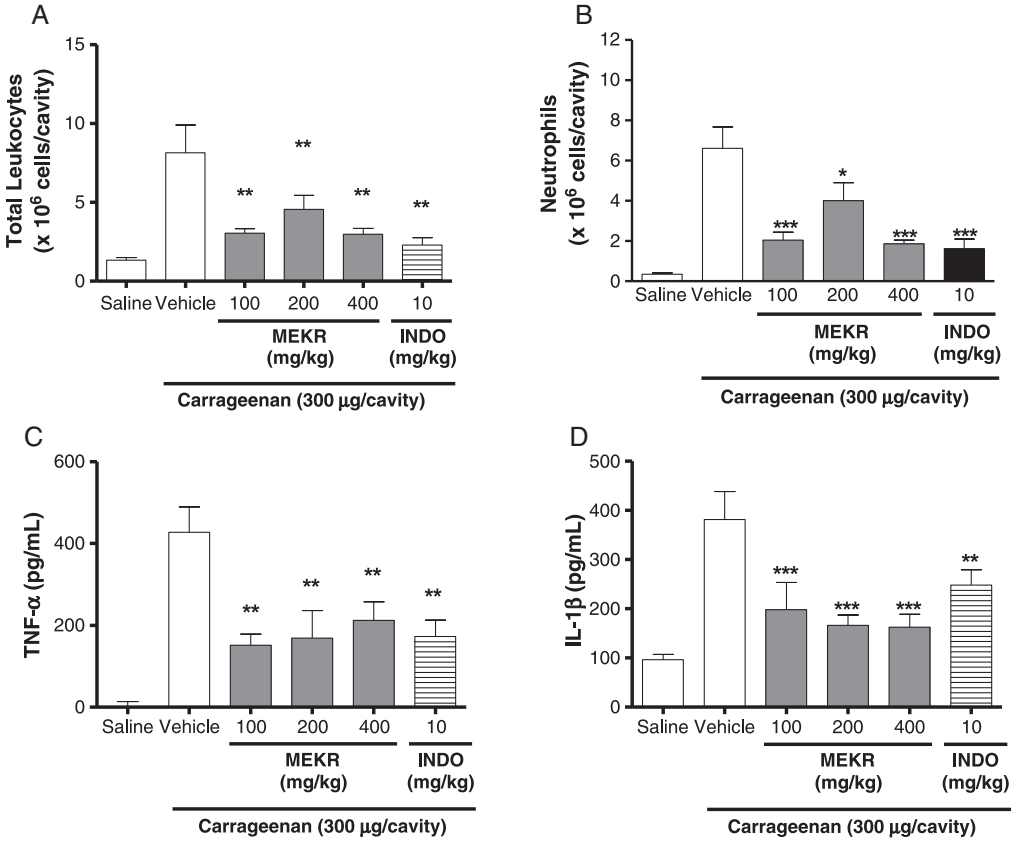

Figure 4. Effect of *Kielmeyera rugosa* (MEKR; 100, 200, and 400 mg/kg; p.o.) or indomethacin (IND, 10 mg/kg; i.p.) on the inflammation by carrageenan in mouse pleurisy. The analyses were performed 4 h after carrageenan injection (300 µg/cavity) to evaluate the recruitment of total leukocytes (A), neutrophils (B), and to assess tumor necrosis factor-alpha (TNF-α) (C) and interleukin-1β (IL-1β) levels (D). Data were expressed as mean ± SEM, for a minimum of six animals. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the control group (vehicle) (analysis of variance followed by Tukey's test).

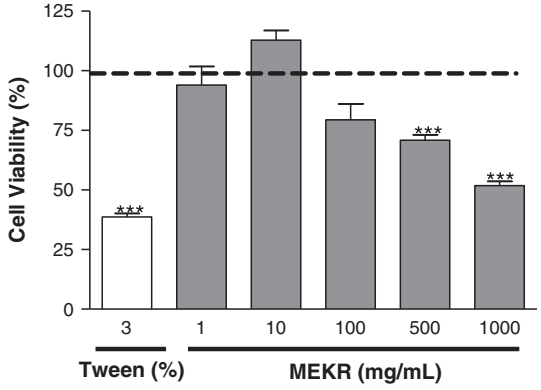

Figure 5. Effect of *Kielmeyera rugosa* (MEKR; 1, 10, 100, 500, and 1000 mg/mL) on the viability assay by the methyl-thiazolyl-tetrazolium (MTT) method for previously MTT-treated cells *in vitro*. Measurement of formazan absorbance in relation to the experimental design, showing mean values ± SEM at different times after the first MTT treatment; absorbance of solubilized MTT formazan product was spectrophotometrically measured at 540 nm. $***p < 0.001$ compared with the control group (analysis of variance followed by Tukey's test).

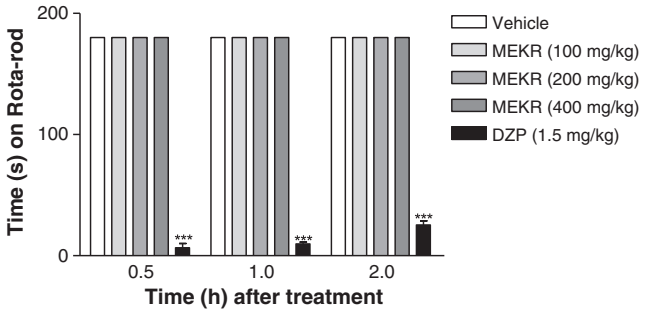

Figure 6. Time (s) on the rota-rod observed in mice after i.p. treatment with vehicle (control), *Kielmeyera rugosa* (MEKR; 100, 200, and 400 mg/kg; p.o.) or diazepam DZP (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s after drug treatment. Statistical differences versus vehicle-treated mice group were calculated using analysis of variance, followed by Tukey's test ($n = 6$, per group), $***p < 0.001$.

Lack of effect of methanol stem extract of *Kielmeyera rugosa* on motor activity

Fig. 6 shows the motor activity of mice treated with different doses of MEKR. In this test, MEKR, in all doses, was unable to cause a significant decrease of ambulation (number of crossings) at 0.5, 1, and 2 h after administration, unlike DZP.

Effect of methanol stem extract of *Kielmeyera rugosa* on immunofluorescence

In the olfactory bulb ($p < 0.01$), piriform cortex (100, 400 mg/kg, $p < 0.01$; 200 mg/kg, $p < 0.05$) and in the periaqueductal gray ($p < 0.01$) of the animals brains, the average number of neurons showing Fos protein was significantly increased by an oral injection of MEKR when compared with control (Figs 7 and 8).

DISCUSSION

Because the chemical profile of this genus is characterized by the occurrence of phenolic compounds such as xanthones, 4-phenyl, and 4-alkylcoumarins (Garcia Cortez *et al*., 1998; Cruz *et al*., 2001; Cruz *et al*., 2002; Scio *et al*., 2003; Nogueira *et al*., 2009) and on the basis of the results by HPLC-DAD analysis, we suggest that these compounds could be present in the methanolic extract from stems of *K. rugosa* (MEKR). Further investigations by liquid chromatography–mass spectrometry on the chemical composition of *K. rugosa* are ongoing.

The inflammatory hyperalgesia in mice is mediated by a cascade of cytokines (Cunha *et al*., 2005). CG induces a concomitant release of TNF-α, which stimulates the subsequent release of interleukin (IL)-6/IL-1β and keratinocyte-derived chemokine (KC/CXCL1), which ultimately induce the synthesis of prostaglandins and the release of sympathetic amines, respectively (Cunha *et al*., 1992; Cunha and Ferreira, 2003), causing the activation of fiber sensory nerve endings, types Aδ and C, increasing the local flow and vascular permeability by the release of neurokinin substance P and neurokinin A (Nakamura and Ferreira, 1987; Cunha *et al*., 2005). This can lead to the inflammatory process resulting in central and peripheral hyperalgesia (Guimarães *et al*., 2012).

The cytokine cascade begins with TNF-α, which stimulates two distinct pathways, as previously mentioned: IL-1β, which in turn activates cyclooxygenase to produce prostanoids, and KC/CXCL1 production, which stimulates the release of sympathetic amines (Verri *et al*., 2006). Prostanoids and sympathetic amines are ultimately responsible for nociceptor sensitization (Cunha *et al*., 2005). On the basis of these findings, we investigated the possible effect of MEKR on the hyperalgesia induced by PGE_2 and DA.

Studies have shown that the peripheral injection of PGE_2 and sympathomimetic amines, such as DA, triggers the activation of nociceptors and transmission impulse by primary nociceptive neurons (de Oliveira *et al*., 2012; Guimarães *et al*., 2012). That effect induces both allodynia and hyperalgesia in response to mechanical stimulation (Ferreira, 1972; Kuhn and Willis, 1973). This nociceptive effect seems to be related to the ability of PGE_2 to sensitize peripheral terminals of small diameter and high threshold, including primary afferent fibers sensitive to thermal, chemical, and mechanical stimuli (Kumazawa *et al*., 1993; Mizumura *et al*., 1993).

Prostaglandin E_2 acts on the EP_2 receptors, and DA acts on the metabotropic-type D_1 receptors. The nociceptive behavior and mechanical allodynia caused by i.pl. PGE_2 are mediated through activation of distinct EP receptors and PK-dependent mechanisms (Kassuya *et al*., 2007). Therefore, hyperalgesia elicited by PGE_2 and DA is independent on the production of other inflammatory mediators or recruitment of cells such as neutrophils (Cunha *et al*., 2008b). The fact that MEKR treatment also inhibited DA and PGE_2-induced hyperalgesia implies that either MEKR directly reduces nociceptor sensitization or MEKR can even induce an endogenous mediator through this action, or we cannot exclude the possibility that MEKR interacts even with other types of EP or DA receptors.

The injection of CG in mice produces a typical biphasic edema associated with the production of several inflammatory mediators, such as bradykinin, prostaglandins, nitric oxide, and cytokines (Henriques *et al*., 1987; Posadas *et al*., 2004). It is well accepted that cytokines

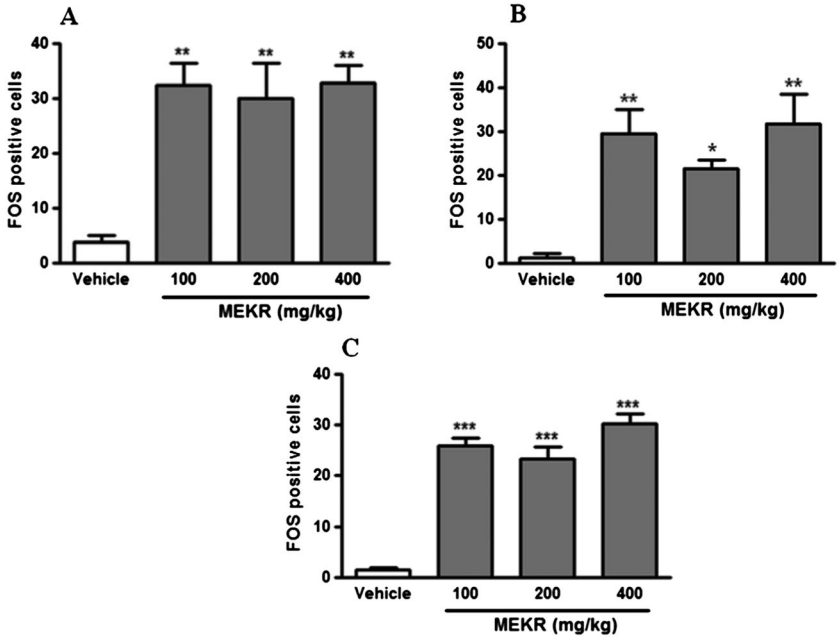

Figure 7. Neurons Fos positive in the bulb olfactory (A), piriform cortex (B), and periaqueductal gray (C). Vehicle (control) or *Kielmeyera rugosa* (MEKR; 100, 200, and 400 mg/kg, p.o.) were administered 1.5 h before the perfusion. Values represent in mean ± SEM ($n = 6$, per group). $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ versus control (one-way analysis of variance followed by Tukey's test).

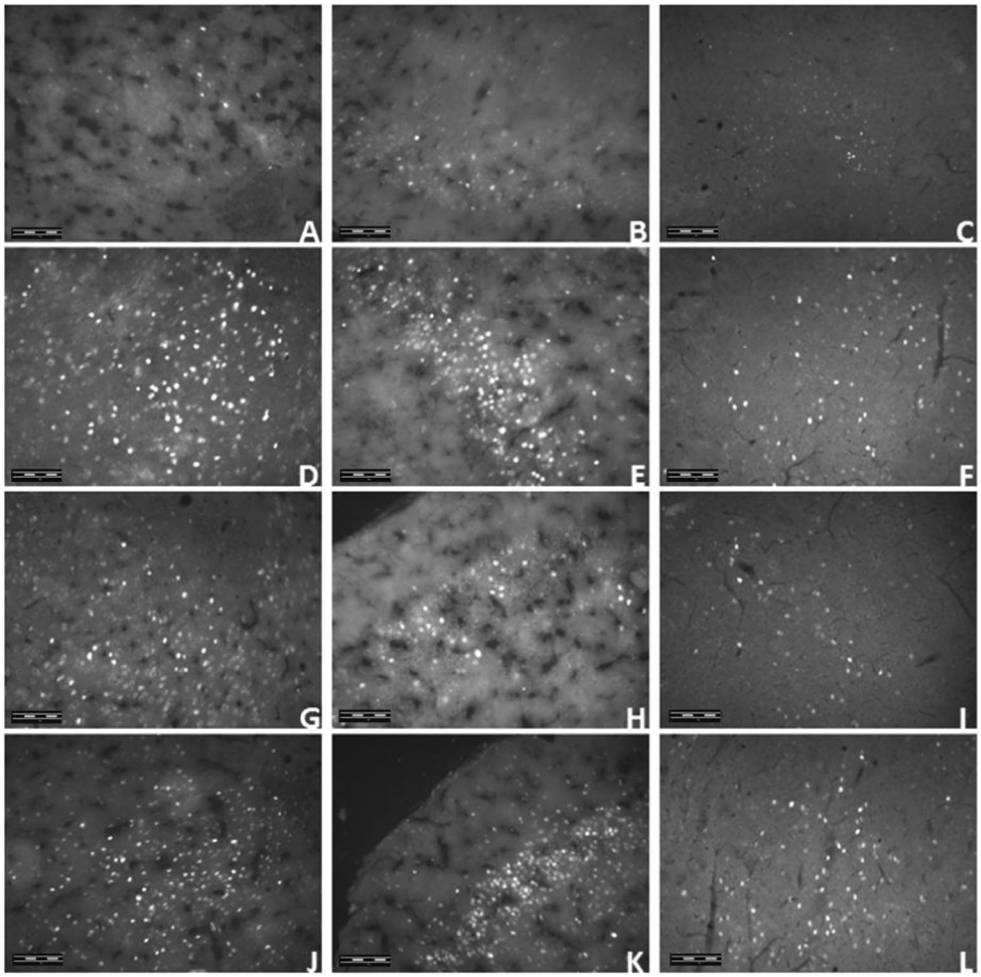

Figure 8. Immunofluorescence for Fos protein in the neurons of the olfactory bulb (A, D, G, J), piriform cortex (B, E, H, K), and periaqueductal gray (C, F, I, L), 1.5 h after the treatment with *Kielmeyera rugosa* (MEKR; 100, 200, and 400 mg/kg; p.o.) or vehicle (control), respectively in the figures of each central area, 20 μm.

constitute a link between cellular injuries or immunological recognition and the local or systemic signs of inflammation, for example, cell migration, edema, fever, and hyperalgesia (Ferreira *et al*., 1988; Faccioli *et al*., 1990; Dinarello, 2000). Different cell types, including macrophages, monocytes, and glial cells produce IL-1β, which in turn induces the production of other inflammatory mediators involved with cellular recruitment, fever, acute phase protein release, and increase of vascular permeability (Dinarello, 1998). We have shown that the doses of 100, 200, and 400 mg/kg of MEKR-induced antiedematogenic activity.

The mediators involved in the genesis of inflammatory pain also play an essential role in triggering other inflammatory events, including edema and leukocyte migration (Cunha *et al*., 2008a). Therefore, the production of cytokines, including TNF-α and IL-1β, in the site of inflammation is essential for the development of inflammatory hyperalgesia. For this reason, we performed a cell migration assay and measurement of IL-1β and TNF-α by CG-induced pleurisy. Inflammation induced by CG involves cell migration, exudation of plasma, and production of mediators such as nitric oxide, prostaglandin E_2, IL-1β, IL-6, and TNF-α (Ferreira *et al*., 1993; Cunha *et al*., 2005). These mediators are capable of recruiting leucocytes, such as neutrophils, in various experimental models. The results allowed us to detect a marked inhibitory effect of MEKR on neutrophil migration, besides a reduction of TNF-α and IL-1β level in the pleural exudate.

Evidences suggest the role of colorimetric assays using the MTT for assessment of cytotoxicity, and proliferation studies in cell biology (Berridge *et al*., 2005; van Meerloo *et al*., 2011). The concentrations used of MEKR (1–100 mg/mL) did not affect the MTT reduction in murine peritoneal macrophages, indicating a cell viability effect of this extract.

The fact that MEKR induces antihyperalgesic effect in the mechanical hyperalgesic models suggests that MEKR can block the neural transmission of pain, like other drugs do, and may induce analgesia. Moreover, it has been observed that many compounds derived from medicinal plants present a reduction of locomotor activity (Le Bars *et al*., 2001) by an inhibitory effect on the central nervous system (CNS) or by a non-specific muscle relaxation effect (Melo *et al*., 2011). Thus, these activities can reduce the motor coordination response, invalidating the nociceptive behavioral tests (de Sousa *et al*., 2006). However, relaxing or motor deficit effects were discarded, because MEKR administration, at the therapeutic doses, did not affect the motor performance of the mice, as tested in the rota-rod test.

The expression of immediate early genes, most notably c-Fos, has been used to map activation of neural circuits under a variety of experimental conditions. c-Fos is expressed in a variety of brain sites, like in the areas involved in the pain modulation (Barr, 2011), being, the Fos protein, a marker useful for the control of neuronal activities in central pathways of the sensory system, particularly in the nociceptive pathway (Williams *et al*., 1990). To demonstrate the influence of MEKR in the CNS areas, Fos protein labeled by immunofluorescence was performed, showing a significant activation of the olfactory bulb, piriform cortex and periaqueductal gray (PAG).

The piriform cortex (PC) is a three-layered structure in which the principal excitatory neurons are pyramidal cells. One attractive feature of the piriform cortical slice preparation is that functionally distinct inputs from the olfactory bulb via the lateral olfactory tract. The information arriving through theses distal synapses provides the vast majority of olfactory signals to the cortex that is presumably used for sensory tasks such as odor discrimination and recognition (Suzuki and Bekkers, 2006; Bathellier *et al*., 2009). The PC, beyond of the olfactory function due to its connections with olfactory bulb, presents an influence on the aggressive and mating behavior, once this area receives information from the amygdala and hippocampus and projects their axons to amygdala and hypothalamus. These areas make connection with the brain stem, including the raphe and parabrachial nuclei as well as the PAG, influencing the ascending and descending nociceptive circuits.

The PAG, the most important area of descending pain pathway, is interconnected with the hypothalamus and limbic forebrain structures and also receives direct inputs of spinomesencephalic. The PAG projects to the rostral ventromedial medulla, which in turn sends its output to dorsal horn, inhibiting the I-laminae, an important dorsal horn area involved in the nociception (Heinricher *et al*., 2009). Pain modulatory drugs such as opioids, serotoninergic, and cannabinoids exert central effects in the PAG, and several lines of evidence indicate a central role for prostaglandins in this brain region, what indicates that PAG is an important area involved in the control of inflammatory pain (Breder *et al*., 1995; Leith *et al*., 2007; Phillips and Clauw, 2011; Kraft, 2012; Siqueira-Lima *et al*., 2014). Thus, the attenuation of the mechanical hyperalgesia observed in the present study may be derived from the activation of descending pain pathway and consequent inhibition of spinal cord I-laminae. That can be suggested by the significant activation of the PAG observed in the immunofluorescence protocol.

In summary, the activation of the olfactory bulb and piriform cortex indicates that MEKR influences on the animal behavior and the activation of the PAG suggests the involvement of the central nervous system, more specifically of the pain descending inhibitory pathway, in the action of MEKR on the inflammatory pain observed in the hyperalgesia and inflammation protocols used in this study.

The exact mechanism through which MEKR exerts its antihyperalgesic profile remains to be elucidated. Previously, *Kielmeyera* species showed mainly xanthones and 4-alkyl and 4-phenyl coumarins, which are regarded as the characteristic constituents of plants belonging to this genus. Taechowisan *et al*. (2005) demonstrated the physiological roles of 5,7-dimethyloxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin for the development of biologically active substances (Taechowisan *et al*., 2005). Nevertheless, a previous chemical study of extracts of fruits, leaves, and stems of *K. rugosa* were identified on the basis of their spectral data, chemical compounds found in *K. rugosa* resemble those that were previously isolated from the other species of *Kielmeyera*, such as xanthones besides 4-alkyl and 4-phenylcoumarins (Nogueira *et al*., 2009).

Many coumarin derivatives have special ability to scavenge reactive oxygen species–free radicals and to influence processes involving free-radical injury they have also been found to possess vasorelaxant (Hoult and Paya, 1996) and anti-inflammatory activity

(Fylaktakidou *et al*., 2004; Nicolaides *et al*., 2004). Considering that Taechowisan *et al*. (2006) observed that 5,7-dimethyloxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin significantly reduced the formation of TNF-α, it is conceivable to suggest that these compounds are responsible for the pharmacological activity presented in this study (Taechowisan *et al*., 2006).

The present study demonstrates, for the first time, that systemic administration of MEKR, at doses that did not induce any motor performance alteration, produced consistent antihyperalgesic and anti-inflammatory effects in different models of hyperalgesia and inflammation, probably by interfering of CNS, through stimulation of areas that modulate pain perception or modulation, such as PAG. These effects seem to be associated with the power of MEKR to inhibit the cytokine cascade generated by CG and/or decrease the production of inflammatory mediators. However, further studies can clarify the exact mechanisms underlying the effects of MEKR.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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