Anti-inflammatory and antioxidative effects of the methanolic extract of the aerial parts of *Mitracarpus frigidus* in established animal models

Rodrigo Luiz Fabria, Roberta Alvim Garciaa, Jônatas Rodrigues Florêncioa, Nicolas de Castro Campos Pintoa, Luiz Gustavo de Oliveirab, Jair Adriano Kopke Aguiarb, Antônia Ribeiroa and Elita Scioa

aBioactive Natural Products Laboratory and bGlycoconjugate Analysis Laboratory, Department of Biochemistry, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora, Brazil

Keywords
catalase; cyclooxygenase; inflammation; *Mitracarpus frigidus*; myeloperoxidase

Correspondence
Elita Scio, Bioactive Natural Products Laboratory, Department of Biochemistry, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, CEP 36036 900, Brazil.
E-mail: elita.scio@ufjf.edu.br

Received May 2, 2013
Accepted November 7, 2013
doi: 10.1111/jphp.12189

Abstract

Objectives This study reports the in vivo anti-inflammatory and antioxidative effects of the methanolic extract of the aerial parts of *Mitracarpus frigidus* (MFM) and its chemical fingerprint.

Methods The acute anti-inflammatory activity was performed using the carrageenan-induced paw oedema and peritonitis, ear oedema induced by croton oil and ethyl phenylpropiolate methods. Total COX, COX-1 and COX-2 expression was also evaluated. Chronic activity was determined by cotton pellet granuloma model. The antioxidative activity was assessed using liver tissue malondialdehyde, catalase and myeloperoxidase activities.

Key findings *M. frigidus* showed an intense acute anti-inflammatory action (100 and 300 mg/kg) in a nondose-dependent manner with selective inhibition of COX-2 expression. This activity may be also related to the strong antioxidative effect observed. By the other side, the chronic anti-inflammatory activity of MFM was not expressive. Kaempferol, kaempferol-O-rutenoside, rutin, ursolic acid and psychorubrin were identified in MFM.

Conclusions The anti-inflammatory activity of MFM was probably due to inhibition of COX expression in a selective manner for COX-2. Other mechanisms, such as inhibition of inflammatory mediators and of the oxidative stress were possibly involved in the effects observed. To the best of our knowledge, it is the first time those activities are reported for *M. frigidus*.

Introduction

Inflammation is a reaction of the tissue blood vessels against an aggressor agent, characterized by the access of liquids and cells to interstice.[1] The inflammatory reaction symptoms include blush, heat, tumour, pain and loss of function.[2] The use of anti-inflammatory agents is helpful as a therapeutic option, however, unfortunately, the drugs currently available to treat inflammation are associated with several side effects and low efficacy, specially for chronic diseases.[3]

Medicinal plants are widely used in the traditional medicine systems of numerous countries to treat different inflammatory conditions and, in particular, skin inflammation processes. However, for many of these plants, the real efficacy and the relevant active principles are unknown. Consequently, experimental studies are needed to verify the pharmacological properties and to identify the active principles of these plant species.[3]

Some species of the genus *Mitracarpus* (Rubiaceae) have many ethnopharmacological uses. For example, *Mitracarpus scaber* Zucc. ex Schult. & Schult. F. is used in traditional medicine in West Africa for headaches, toothache, amenorrhea, dyspepsia, hepatic and venereal diseases and leprosy. The juice of the plant is applied topically for the traditional treatment of diseases of the skin.[4]
However, there are no reports on the traditional uses of Mitracarpus frigidus (Willd. ex Roem. & Schult.) K. Schum., an annual shrub commonly found in South America including Brazil. Previous studies showed that the methanolic extract obtained from this plant (MFM) revealed the presence of flavonoids, tannins, alkaloids, terpenes and quinones, and presented antimicrobial, leishmanicidal, cytotoxic and laxative activities. Recently, the pyranonaphthoquinone psychorubrin was first isolated from this extract. Moreover, MFM revealed no toxicity signs in rat models.

Based on these considerations, the present study was undertaken to evaluate the anti-inflammatory and anti-oxidative effects of the methanolic extract obtained from the aerial parts of M. frigidus in well-established animal models, and to identify some of its major compounds.

To the best of our knowledge, it is the first time those activities are reported for M. frigidus.

Materials and Methods

Plant material and extraction

Mitracarpus frigidus aerial parts were collected in Juiz de Fora, Minas Gerais, Brazil in May 2011. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of the Federal University of Juiz de Fora. Oven dried and powdered aerial parts of the plant (1000 g) were extracted by maceration with methanol (5 × 2000 ml) for 5 days at room temperature, and the MFM was obtained by evaporation (yield 10% w/w).

High-pressure liquid chromatography analysis

High-pressure liquid chromatography (HPLC) analysis was performed using an Agilent Technologies 1200 Series, with a photodiode array detector and an automatic injector. The column employed was a Zorbax SB-18; 250 × 4.6 mm, 5 μm particle size. Solvents that constituted the mobile phase were A (water pH adjusted to 4.0 with H3PO4) and B (acetonitrile). The elution conditions applied were: 0–20 min, 5–80% B and 20–30 min, 80–95% B. The mobile phase was returned to the original composition over the course of 30 min, and an additional 5 min were allowed for the column to re-equilibrate before injection of the next sample. The sample volume was 20 μl at a concentration of 1 mg/ml, the flow rate of 1 ml/min, and the temperature was maintained at 25°C during the analysis. Detection was performed simultaneously at 210, 230, 254 and 280 nm. Four pure standards kaempferol, kaempferol-3-O-rutinoside, rutin and ursolic acid, previously identified in Mitracarpus genus, were used in this experiment as markers, and psychorubrin isolated from this species was also added. For all experiments, MFM and the standards were dissolved in methanol.

Quantitative analysis

Determination of the content of the compounds (kaempferol, rutin, psychorubrin and ursolic acid) in MFM was performed by the external standard method. Stock solutions of 5, 25, 50, 100, 200 and 500 μg/ml were used. Each determination was carried out in triplicate.

Animals

Female Swiss albino mice weighing 20–25 g, or male Wistar albino rats with 200–250 g, were used for the in vivo assays. Animals were kept under standard laboratory conditions of temperature (25°C ± 2°C) and light/dark cycles (12/12 h). They were provided with standard pellets and tap water ad libitum. During the experiments, animals were maintained according to the recommended laboratory ethical guidelines. The study was approved by the Brazilian College of Animal Experimentation (Protocol n°022/2012).

Carrageenan-induced rat paw oedema

Rats were divided into five groups of six animals each as follows: Group A: negative control (0.6% sodium lauryl sulfate); Group B: dexamethasone – 1 mg/kg; Group C: indomethacin – 10 mg/kg; Group D: MFM – 100 mg/kg; Group E: MFM – 300 mg/kg. One hour after the oral treatment, acute paw oedema was induced by injecting 0.1 ml of 1% carrageenan in 0.9% saline. Paw volume was measured using plethysmometer by the mercury displacement method between 0 and 3 h. The inhibition percentage (%) of paw oedema in treated groups was then calculated using the formula: Inhibition (%) = (1 – Vt/Vc) × 100, where Vt = is the oedema volume in the drug-treated group; Vc = is the oedema volume in the control group.

At the third hour, animals were euthanized. Liver tissues were taken, rinsed in ice-cold normal saline and immediately placed in buffer pH 7.4 (140 mM KCl + 20 mM phosphate) of the same volume and finally homogenized at 4°C. Then, the homogenate was centrifuged at 11 270 g for 5 min. The supernatant was obtained by the external standard method. Stock solutions of 5, 25, 50, 100, 200 and 500 μg/ml were used. Each determination was carried out in triplicate.

Malondialdehyde determination

MDA was evaluated by the thiobarbituric acid reacting substance (TBARS) method. Briefly, MDA reacted with thiobarbituric acid at a high temperature (100°C) and
formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm. MDA levels were expressed as nmol/mg protein.

**Catalase activity**

Decomposition of H$_2$O$_2$ in the presence of catalase was measured at 240 nm in accordance with a standard procedure.[12] Catalase activity was defined as the amount of enzyme required to decompose 1 nmol of H$_2$O$_2$ per minute at 37°C. Results were expressed as U/ml in tissue.

**Cyclooxygenase activity (COX) assay**

Liver tissue levels of total COX and its two isoforms (COX-1 and COX-2) were determined using a commercially available COX activity assay kit according to the manufacturer’s instructions (Cayman no. 760151). The absorbance at 590 nm was measured on a microplate reader. The concentration of total COX was expressed as U/ml in tissue. COX-1 and COX-2 were expressed in % of total COX expression.

**Carrageenan-induced mice peritonitis**

Inflammation was induced by the modified method of Griswold et al.[13] Mice were divided into five groups of six animals each (Groups A–E), as described previously. The oral treatments were performed 1 h prior to the induction of peritonitis. After this time, carrageenan (0.25 ml, 0.75% w/v in saline) was injected intraperitonially. Four hours later, the animals were euthanized by cervical dislocation, and 2 ml of heparinized phosphate buffered saline was injected into the peritoneal cavity. Posteriorly, a gentle massage was made and peritoneal exudates were removed. The total leukocyte count was performed in a Neubauer chamber, and the differential cell determination was established. The percentage of leukocyte inhibition was calculated using the following formula: % of leukocyte inhibition = (1 – T/C) × 100, where T represents the treated groups’ leukocyte count, and C represents the control group leukocyte count. Inhibition of neutrophil migration was calculated by the following equation: Inhibition of neutrophil migration = (1 – NT/NC) × 100, where NT = neutrophil counts of treated groups, and NC = neutrophil counts of the control group.

**Ear oedema induced by croton oil**

The anti-inflammatory activity of MFM was evaluated by the ability of extract to prevent local inflammation induced by croton oil to the rat ear.[14] MFM was tested both orally and topically. For oral treatment, rats were divided into five groups of six animals each, as follows: (Groups A–E), as described previously. One hour after oral treatment, 50 μl of a fresh solution of croton oil (20% in acetone v/v) was applied topically to the right ear and the same volume of acetone to the left ear of each animal. In the other experiment, the animals treated topically were divided into five groups of six animals each, as follows: Group F: negative control (acetone); Group G: dexamethasone – 0.1 mg/50 μl; Group H: indomethacin – 0.1 mg/50 μl; Group I: MFM – 0.5 mg/50 μl; Group J: MFM – 1.0 mg/50 μl, diluted in acetone. Those animals were treated topically immediately after the application of croton oil. Four hours after treatment, all animals were sacrificed by cervical dislocation and identical discs of 6 mm, with the aid of a punch, were obtained from both the treated and the untreated ear. The discs were weighed and the weight difference of the right and the left ear discs was used as an index of the oedema level. A smaller weight difference indicates a more significant inflammation inhibition potential. The results were presented as mean ± S.E.M of weight differences within each group.

**Myeloperoxidase assay**

Myeloperoxidase activity was measured according to the modified method of Bradley et al.[15] To prepare the tissue homogenates, ear oedema tissues were ground with liquid nitrogen in a mortar. The discs were then treated with 1.0 ml of 10 mmol/l phosphate buffer (pH 6.0). The mixtures were homogenized and centrifuged using a refrigerated centrifuge at 1500 g for 10 min at 4°C and the supernatants used for the determination of enzyme activity. Myeloperoxidase activity was determined by adding 100 μl of the supernatant to 190 μl of 10 mmol/l phosphate buffer (pH 6.0) and 100 μl of 1.5 mmol/l o-dianisidine hydrochloride containing 0.0005% (w/v) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a UV-vis spectrophotometer. Myeloperoxidase levels in tissues were expressed as μmol/min/μg tissue.
hour after treatment, all animals were euthanized by cervical dislocation and identical discs of 6 mm, with the aid of a punch, were obtained from both the treated and the untreated ear. The discs were weighed, and the weight difference of the right and the left ear discs was used as an index of the oedema level. The smaller weight difference indicates a more significant inflammation inhibition potential. The results were presented as mean ± S.E.M of weight differences within each group.

**Cotton pellet granuloma**

The granulomas were developed in accordance to the method described by Naik et al.[17] Rats were divided into five groups of six animals each (Groups A–E), as described previously. After one hour of oral administration, sterile cotton pellets weighing 10 mg were subcutaneously implanted in both axillae of rats under anaesthesia. The treatments were daily given for 10 days. On the 11th day, the rats were euthanized, and the cotton pellets with the surrounding granulomas were resected out, and their wet and dry weights were recorded.

**Plasma malondialdehyde estimation**

After 11 days of treatment of the cotton pellet-granuloma experiment, 2–4 ml of blood was collected from retro orbital plexuses from each animal using capillary tube in a vial containing EDTA. Plasma was separated by centrifugation at 3000 g for 10 min. The sample was stored at −20°C and used to estimate MDA levels. The reduced levels of MDA were taken as an indicator of antilipoperoxidative activity, which can be considered as the index of the reduced oxidative stress. MDA levels were expressed as nmol/mg protein.

**Statistical analysis**

Statistical differences between the treatments and the control were evaluated by the analysis of variance (ANOVA) test followed by the Bonferroni test. A difference in the mean values of $P < 0.05$ was considered to be statistically significant.

**Results and Discussion**

**High-pressure liquid chromatography assay**

The HPLC chromatogram profile for MFM is shown in Figure 1. Five compounds were identified as kaempferol-O-rutenoside, rutin, kaempferol, psychorubrin and ursolic acid. The first compound was detected by its ultraviolet spectrum[18] and the others by external standard.

For better global positioning of any herbal drug, it has become important to generate chemoprofiling data on the basis of maximum available number of markers for the extract.[19] For this, the chemoprofiling data of MFM based on four markers (rutin, kaempferol, ursolic acid and psychorubrin) was performed. Ursolic acid was found to be the most abundant compound with 275.3 ± 0.8 mg/g in MFM, while contents of 56.8 ± 1.2, 21.5 ± 0.5 and 4.2 ± 0.2 mg/g were found for rutin, kaempferol and psychorubrin, respectively.

**Figure 1** High-pressure liquid chromatography chromatogram of *Mitracarpus frigidus* methanolic extract (MFM). The analysis was performed using a linear gradient of a binary solvent system A (water pH adjusted to 4.0 with H$_3$PO$_4$) : B (acetonitrile). The elution conditions applied were: 0–20 min, 5–80% B and 20–30 min, 80–95% B. It was run at a flow rate of 1 ml/min over 30 min, with an injection volume ("loop") of 20 μl and UV detection was at 230 nm.
Carrageenan-induced paw oedema

The acute anti-inflammatory effects of MFM on carrageenan-induced oedema in the hind paws of rats are shown in Table 1. Carrageenan-induced paw oedema remained even 3 h after infection into the subplantar region of rat paw. MFM showed a significant reduction in the paw oedema volume in relation to the negative control, and the maximum inhibition percentage was observed 1 h and 30 min after the oedema formation for 100 and 300 mg/kg, respectively. Treatments with MFM showed similar activity compared with dexamethasone and indomethacin used as positive controls (Table 1).

Carrageenan-induced paw oedema is a well-established in vivo model of inflammation and has been commonly used to evaluate the anti-oedematous effect of natural products. Oedema formation, in this classical experiment, is a biphasic response. In the early phase, 0–2 h after carrageenan injection, there is a release of histamine, serotonin and bradykinin on vascular permeability. The inflammatory oedema reaches the maximum level at the third hour due to the potentiating effect of bradykinin on mediator release and prostaglandins, producing oedema after mobilization of the leukocytes. In this study, MFM and positive controls showed anti-inflammatory effects in carrageenan-induced rat paw oedema in both phases, suggesting that the significantly higher anti-inflammatory activity may be due to the inhibition of inflammatory mediators, such as histamine and serotonin released during the first phase, and prostaglandins and bradykinin released during the second phase of inflammation.

Some studies have indicated that inflammatory effect induced by carrageenan is correlated with free radicals. Carrageenan-induced inflammatory response has been associated with neutrophil infiltration and with the production of neutrophil-derived free radicals, for instance superoxide, hydroxyl radicals and hydrogen peroxide, as well as due to the release of other neutrophils-derived mediators. A previous study had established that malondialdehyde (MDA) production was due to the free radical attack in the plasma membrane. Hence, carrageenan-induced inflammation results in an accumulation of MDA, and these data are used to evaluate the free radical generation in liver tissues after inflammatory states.

Therefore, in order to explore the effects of the antioxidant defences on the acute inflammation process in liver tissues, the MDA and the antioxidant enzyme catalase levels were evaluated. In this step, it was observed that the MDA level was significantly decreased in the animals treated with MFM (Figure 2a), indicating a reduction of oxidative stress as a result of antilipoperoxidative activity.

Catalase is a highly reactive enzyme, reacting with hydrogen peroxide to form water and molecular oxygen, and can form methanol, ethanol, formic acid or phenols by donating hydrogen. During normal body conditions, a balance between free radicals and the endogenous antioxidants exists but in a traumatic state, like inflammation, this event diminishes and the reactive oxygen metabolites dramatically increase. The increased generation of oxygen free radicals in the extracellular space is verified in the inflammatory state, in which the relatively low concentrations of catalase increase the susceptibility of extracellular components to oxygen radical injury, and may stimulate chemotaxis for other inflammatory cells. In the present study, it was observed that MFM decreased catalase activity, which was probably due to the inhibition of inflammation observed for MFM (Figure 2b).

Since its identification in 1971, there have been many studies on the actions of cyclooxygenase (COX or prostaglandin H2 synthase) and its inhibition, which has been associated with antilipid, antiplatelet, antipyretic, antinociceptive and anti-inflammatory effects. There are two isozymes of COX, COX-1 and COX-2. COX-1 is constitutively produced by the body and is involved in protecting the gastrointestinal mucosal lining, as well as maintaining kidney and platelet function, and COX-2 is involved with pain, inflammation, fever and possibly tumour growth.

Table 2 shows the effect of MFM on COX expression. MFM (100 mg/kg) significantly reduced total COX expression, similar to indomethacin, which is a COX inhibitor. By the other side, as MFM (300 mg/kg) showed strong anti-

Table 1 Effects of the methanolic extract from the aerial parts of M. frigidus (MFM) on carrageenan-induced rat paw oedema

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>0 h</th>
<th>1/2 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>–</td>
<td>0.24 ± 0.08</td>
<td>0.32 ± 0.05</td>
<td>0.38 ± 0.10</td>
<td>0.60 ± 0.10</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1</td>
<td>0.02 ± 0.01</td>
<td>0.19 ± 0.05*</td>
<td>0.16 ± 0.03*</td>
<td>0.23 ± 0.03*</td>
<td>0.29 ± 0.02*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>0.06 ± 0.03</td>
<td>0.11 ± 0.02*</td>
<td>0.10 ± 0.04*</td>
<td>0.20 ± 0.04*</td>
<td>0.25 ± 0.05*</td>
</tr>
<tr>
<td>MFM</td>
<td>100</td>
<td>0.11 ± 0.04</td>
<td>0.17 ± 0.04*</td>
<td>0.12 ± 0.02*</td>
<td>0.21 ± 0.03*</td>
<td>0.25 ± 0.03*</td>
</tr>
<tr>
<td>MFM</td>
<td>300</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.01*</td>
<td>0.10 ± 0.04*</td>
<td>0.13 ± 0.03*</td>
<td>0.29 ± 0.05*</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M. (n = 6). *Statistically different from the negative control (vehicle). †Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, P < 0.05).
inflammatory activity on the animal models and also high total COX expression, its mechanism of action was probably not only due to COX inhibition. Its behaviour was similar to dexamethasone, a steroid anti-inflammatory, which mechanism of action involves inhibition of several inflammatory mediators synthesis. The distinct results presented for the different doses of MFM, may be related to their different chemical constituents concentrations. However, MFM 100 and 300 mg/kg showed relative percentage inhibition of COX-2 (54% ± 1.0 and 55% ± 1.2, respectively), which suggested that MFM acted selectively on inhibition of COX-2 expression. Those results were important, since most anti-inflammatory drugs available for therapy, such as indomethacin, act in a nonselective manner, resulting in adverse reactions, mainly gastric disturbance, due to COX-1 inhibition.

**Carrageenan-induced peritonitis**

Mouse carrageenan peritonitis was also used to evaluate anti-inflammatory activity. MFM also inhibited peritoneal leukocyte migration (Table 3).

Leukocyte aggregation at the site of inflammation is a fundamental event in the inflammatory process. Intrapерitoneal injection of carrageenan leads to inflammation of the peritoneum as a result of macrophages in the carrageenan insulated tissue. This is followed by an influx of neutrophils and mononuclear leukocytes, with increased levels of plasma/peritoneal fluid chemoattractants, and with the sequential appearance of exudate proinflammatory cytokines. In this investigation, MFM (100 and 300 mg/kg) inhibited the carrageenan-induced leukocyte migration in the peritonitis model in mice, and significantly reduced the migration of neutrophils, indicating their anti-inflammatory action by inhibiting chemotaxis (Table 3).

**Ear oedema induced by croton oil**

Croton oil-induced dermatitis in the rat ear was used as a model of acute inflammation. The inflammatory reaction induced by the phorbol esters of croton oil is an acute response characterized by oedema, neutrophil infiltration, prostaglandin production and increases in vascular permeability. The action of croton oil was believed to be involved...
in, or to be dependent on, arachidonic acid release and metabolism by both the cyclooxygenase and lipoxygenase enzyme pathways. In this study, the anti-inflammatory effect of MFM was evaluated for oral and topical treatments. As shown in Figure 3a and 3c, when used orally and topically, respectively, the anti-inflammatory effect of MFM was similar to that of the dexamethasone and indomethacin used as positive controls. Those results together suggest that MFM presented similar pharmacological properties to those of lipoxygenase and cyclooxygenase inhibitors.

In the present study, the changes of myeloperoxidase activity in ear tissues, an index of neutrophil infiltration into inflammation tissues, were also determined (Figure 3b and 3d). As can be observed, the oral and topical administration of MFM and positive controls, indomethacin and dexamethasone, significantly decreased the level of myeloperoxidase activity in comparison with negative control (vehicle). Myeloperoxidase is an enzyme found primarily in azurophilic granules of neutrophils, which is used as a marker for tissue neutrophil content, and its inhibition implies the presence of anti-inflammatory activity. Tissue myeloperoxidase activity is a sensitive and specific marker for acute inflammation and reflects polymorphonuclear cell infiltration of the parenchyma.

### Table 3  Effect of methanolic extract from *M. frigidus* on leukocyte migration and neutrophil migration in peritoneal exudation in carrageenan-induced mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Leukocytes (10^5/ml)</th>
<th>Leukocyte inhibition (%)</th>
<th>Neutrophils (10^5/ml)</th>
<th>Neutrophil inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>–</td>
<td>8.5 ± 0.6</td>
<td>–</td>
<td>5.9 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1</td>
<td>2.1 ± 0.4*</td>
<td>75.3</td>
<td>1.3 ± 0.3*</td>
<td>77.4</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>2.3 ± 0.3*</td>
<td>73.5</td>
<td>1.4 ± 0.3*</td>
<td>75.8</td>
</tr>
<tr>
<td>MFM</td>
<td>100</td>
<td>1.8 ± 0.4*</td>
<td>78.8</td>
<td>1.2 ± 0.3*</td>
<td>78.9</td>
</tr>
<tr>
<td>MFM</td>
<td>300</td>
<td>1.4 ± 0.4*</td>
<td>83.5</td>
<td>0.9 ± 0.3*</td>
<td>83.4</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M. (n = 6). *Statistically different from the negative control (vehicle). **Statistically different from the positive controls (dexamethasone and indomethacin, respectively) (ANOVA followed by Bonferroni, *P* < 0.05).
Ear oedema induced by ethyl phenylpropiolate

EPP causes instant irritation of the rat ear, which leads to fluid accumulation and oedema characteristic of the acute inflammatory response. Suppression of this response is a likely indication of anti-inflammatory effect. EPP causes a release of many inflammatory mediators such as kinin, serotonin (5-HT) and prostaglandins (PGs). MFM (100 and 300 mg/kg) and positive controls (dexamethasone and indomethacin) exerted an inhibitory effect, both for oral and topical treatments, on the ear oedema formation induced by EPP in comparison with negative control (vehicle) (Figure 4a and 4b).

Cotton pellets-induced granuloma

The effects of MFM on the proliferative phase of inflammation are summarized in Figure 5. It was seen that MFM was responsible for an anti-inflammatory effect, which would be calculated depending on the moist and dry weight of cotton pellets. According to these results, the antiproliferative effects of MFM (100 and 300 mg/kg) and dexamethasone were calculated as 19, 25 and 50% inhibition, respectively (Figure 5a). After they were dried, the antiproliferative effects were calculated on the basis of dry weight pellets. The inhibition of inflammation by MFM (100 and 300 mg/kg) and dexamethasone were established as 20, 28 and 48%, respectively (Figure 5b).

The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of chronic inflammation. The moist weight of the cotton pellet correlates with the transudate. The dry weight of the pellet correlates with the amount of the granulomatous tissue. This effect may be due to the cellular migration to injured sites and accumulation of collagen and mucopolysaccharide. Also, an imbalance between free radical-generating and radical-scavenging systems results in oxidative stress, and is also documented in chronic inflammation. MFM presented chronic anti-inflammatory effects, but although it was not so evident, in an oxidative stress model, MFM (100 and 300 mg/kg) demonstrated significant reduction in MDA level in plasma, indicating reduction of oxidative stress by antilipoperoxidative activity (Figure 5c).

It is important to point out that the animals treated with indomethacin died after 4 days of treatment. Haematuria and melena were observed, suggesting gastric bleeding, probably due to COX-1 inhibition. The rats treated with MFM (100 and 300 mg/kg) did not show any gastric disturbance, which reinforces the possibility of a mechanism of action for MFM which does not involve only COX inhibition. The animals treated with dexamethasone had a significant weight loss during the days of treatment, unlike the vehicle and MFM treated groups (Figure 5d), probably due to protein catabolism induced by corticosteroids, resulting in the reduction of muscle mass. On the other side, the animals treated with MFM experienced normal weight gain.

Some of the compounds identified in MFM, such as kaempferol, rutin and ursolic acid, are reported to have anti-inflammatory and antioxidant activities. Thus, those compounds may be, at least in part, involved with the results reported.

Conclusion

The anti-inflammatory activity of MFM was probably due to inhibition of COX expression in a selective manner for COX-2. It is important, since most anti-inflammatory drugs available for therapy act in a nonselective manner, resulting in adverse reactions, mainly gastric disturbance, due to COX-1 inhibition. Other mechanisms, such as inhibition of inflammatory mediators and of the oxidative stress were possibly involved in the effects observed. To the best of our knowledge, it is the first time those activities are reported for *M. frigidus*. Studies focusing on the isolation and
structure elucidation of the anti-inflammatory and antioxidative constituents from *M. frigidus* are in progress.

**Declarations**

**Conflict of interest**

The authors declare that they have no conflicts of interest to disclose.

**Funding**

This work was supported by the grant from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG – CEX APQ 2874-5.02/07, CEXAPQ 01137-09, CBB PPM- 00149-10 and CBB APQ-02740-09.

**Acknowledgements**

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and the Universidade Federal de Juiz de Fora (UFJF)/Brazil for financial support, and also to Dr. Tatiana Konno from the Nucleus of Ecology and Socio-Environmental Development of Macaé/Federal University of Rio de Janeiro for the botanical identification of the species, and to Delfino Campos for technical assistance.

**References**

7. Fabri RL et al. *In vivo* laxative and toxicological evaluation and *in vitro* antitumor effects of *Mitracarpus*...


