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Anti-inflammatory effect of the monoterpene myrtenol is dependent on the direct modulation of neutrophil migration and oxidative stress

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A B S T R A C T

Myrtenol is a bicyclic monoterpene with anti-inflammatory properties. However, the mechanisms involved are partially unknown. Here, we investigated the effect of myrtenol during experimental chronic arthritis and the possible modulating activity of oxidative stress and neutrophil migration. Complete Freund's Adjuvant (CFA)-sensitized rats were treated with vehicle (1 mL/kg, po), myrtenol (12.5, 25 or 50 mg/kg, po), indomethacin (10 mg/kg, po) or dexamethasone (0.4 mg/kg) followed by intra-articular injection of CFA (0.5 mg/mL, 50 μL per joint). Then, paw edema and articular incapacitation (paw elevation time) were evaluated for 14 days. On the last day, a blood concentration superoxide dismutase (SOD) and nitrite was determined. In another experimental setting, human neutrophils were incubated with vehicle (sterile saline, 1 mL) or myrtenol (10–100 ng/mL) and the *in vitro* chemotaxis to N-formylmethionine-leucyl-phenylalanine (fMLP) (10^{-7} M/well) was evaluated. In addition, antiinflammatory effect of myrtenol was investigated in carrageenan-induced peritonitis. We found that CFA induced a prominent paw swelling and incapacitation of the joint, which were significantly prevented by myrtenol ($P < 0.05$). In addition, blood accumulation nitrite was attenuated by myrtenol when compared with vehicle-treated CFA group ($P < 0.05$). Furthermore, plasma levels of SOD were significantly increased by myrtenol versus vehicle-treated CFA group ($P < 0.05$). Moreover, fMLP-triggered neutrophil chemotaxis and carrageenan-induced peritonitis were markedly prevented by myrtenol ($P < 0.05$). Therefore, myrtenol showed anti-inflammatory and antinociceptive effects on experimental chronic arthritis, which seems to be related to the direct modulation of neutrophil migration and antioxidant activity.

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1. Introduction

Rheumatoid arthritis (RA) is a systematic, chronic, immune-derived inflammatory disease, it damages the peripheral joint, ligaments, and tendons, which leads to joint deformity and destruction, as well as physical disability [33]. Currently, anti-inflammatory therapy is done with the nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic agents, and monoclonal antibodies [1]. However, these drugs are associated with gastrointestinal or cardiovascular side effects and either are expensive [5]. Thereby, it is necessary to find new drugs for RA treatment and other chronic diseases.

Essential oils-producing medicinal plants are important source of compounds with therapeutic potential for the management of several diseases, including central nervous system disorders, cancer, arthritis, and painful conditions [22]. Volatile essential oils are produced by more than 17,500 aromatic plant species, such as *Lamiaceae, Rutaceae, Myrtaceae, Zingiberaceae* and *Asteraceae* [54].

Essential oils components include two groups of distinct biosynthetic origin. The main group is composed of terpenes

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(monoterpenes, sesquiterpenes, phenylpropanoids) and terpenoids [48]. The other group of essential oils components includes low molecular weight aromatic and aliphatic constituents [48]. Monoterpenes, such as alpha- and beta-pinene, thymol, menthol, 1,8-cineole, and myrtenol, are described as the most significant constituents of essential oils (approximately 90% of all compounds) [52].

(−)-Myrtenol (**Fig. 1**) is a bicyclic alcohol monoterpene with pleasant odor and commonly used in the production of cosmetics. This monoterpene is found in essential oils of several medicinal plants, such as *Rhodiola rosea* L., *Paeonia lactiflora* Pall, *Cyperus rotundus.* L. and *Tanacetum vulgare* L., whose crude extract shows anti-inflammatory potential [41,45].

Myrtenol-containing plant extracts are used in folk medicine for treatment of anxiety symptoms, gastrointestinal pain, inflammation and infection [11,16,35]. The literature also reports acute anti-inflammatory and antinociceptive effects of myrtenol [57]. According to that study, myrtenol is suggested to modulate acute inflammation through the inhibition of cytokines release and neutrophil migration [57]. In addition, the anxiolytic effect and antiproliferative effects on human cancer cells are also reported [42,69]. However, myrtenol effect on chronic inflammatory conditions is unknown. In addition, whether the modulation of neutrophil migration by myrtenol occurs through direct or indirect mechanisms is a matter of debate.

Then, this study aimed to investigate the effects of myrtenol during chronic arthritis in animals models and the possible mechanisms involved.

2. Materials and methods

2.1. Animals

Male Wistar rats (180–230 g) were obtained from the Local Animal Facility of the Federal University of Piauí and the Federal University of Ceará, and divided into experimental groups (n = 6). The animals were kept in a temperature-controlled room (24 ± 1 °C) with free access to food and water, under 12 h/12 h dark-light cycles, and were deprived of food 12 h previous to the experiments. Following the experimental procedures, animals were euthanized by an overdose of sodium pentobarbital (100 mg/kg, i.p.) followed by cervical dislocation. The local Ethics Committee for Animal Experiments approved the whole protocol that strictly follows the NIH guidelines for care and use of laboratory animals (CEEA-PI No.008/12). We also used human neutrophils for *in vitro* chemotaxis study. Human neutrophil isolation from healthy volunteers was in accordance with the Human Ethics Committee of Federal University of Ceará (protocol 543.774).

2.2. Chemicals and drugs

The following drugs were used in this study: λ-Carrageenan (Sigma-Aldrich, Saint Louis, MO, USA), dexamethasone (Decadron®, São Paulo, SP, Brazil), Indomethacin (Sigma-Aldrich, Saint Louis, MO, USA), Tween 80 (Sigma-Aldrich, Saint Louis, MO, USA), N-formylmethionine-leucyl-phenylalanine (fMLP, Sigma-Aldrich, Saint Louis, MO, USA), Complete Freund's Adjuvant (Sigma-Aldrich, Saint Louis, MO, USA), myrtenol (2-Pinen-10-ol; Pin-2-ene-10-ol; 6,6-dimethylbicyclo (3.1.1) hept-2-ene-2-methanol) provided by Professor Dr. Damião Pergentino de Sousa, Department of Pharmaceutical Sciences at Federal University of Paraíba. The chemicals used and other solutions were all of analytical grade. Myrtenol was dissolved in a 3% tween 80-containg saline solution (NaCl 0.9%). Drug concentrations were adjusted for treatment to yield a volume of 10 mL/kg.

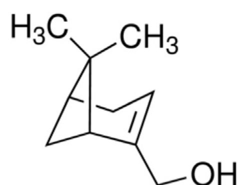

Fig. 1. Chemical structure of myrtenol.

2.3. Assessment of toxicity

Myrtenol doses used in the experimental protocols were selected based on LD50 orally already described in literature for mice being 2457 mg (in males), 632 mg (females) and 1432 mg (males and matched females) [10]. The doses of myrtenol used were 12.5, 25 and 50 mg/kg and the volume administered varied according to the weight of the animals in the proportion of 1 mL of solution per 100 g of the animal's weight (rat) or 0.1 mL of solution for each 10 g of animal weight (mouse).

2.4. Complete Freund's adjuvant (CFA)-induced chronic monoarthritis in rats

Chronic monoarthritis was induced according to the method proposed by Ref. [49] with modifications. The animals were given a subcutaneous injection of Complete Freund's Adjuvant, 50 μL, containing 0.5 mg/mL *Mycobacterium tuberculosis* in the base of the tail. After 21 days, the rats were divided into 6 groups (n = 6/group) and treated orally with vehicle (0.9% NaCl + 3% Tween 80, 10 mL/kg), myrtenol (Mir 12.5, 25 or 50 mg/kg), indomethacin (Ind, 10 mg/kg) or dexamethasone (Dex 0.4 mg/kg) followed by injection of CFA in the right tibio-tarsal articulation. The day of the second dose of CFA was considered the initial day of arthritis (D0).

2.5. Assessment of inflammatory edema in CFA-induced chronic monoarthritis

The rats were evaluated immediately before the intra-articular injection of CFA (basal measurement). Edema variation was determined hourly from 1 h to 5 h post-CFA injection (acute phase protocol) and also once a day (D1), (D3), (D5), and (D7) (subacute phase protocol) and (D10) and (D14) (chronic phase). The severity of arthritis was assessed with a plethysmometer (Insight™) by measuring the right hind paw volume (mL) subtracted by the volume of basal measurement. All measurements made by the same observer and in duplicate. Edema was arrested in milliliters by the difference from baseline values for the other hours of observation (Tx-T0).

2.6. Assessment of CFA-induced joint incapacitation

The rat knee joint incapacitation test is described in detail elsewhere [62,63]. In this test, a computer-assisted device measures the time that a specific hind paw fails to touch the surface of a rotating cylinder over a one-min period (paw elevation time), in seconds. Control (normal) paw elevation time is approximately 10–15 s. In our experiment, articular incapacitation was quantified as the increase in paw elevation time after intra-articular injection of CFA (0.5 mg/mL, 50 μL per joint) into the right tibio-tarsal joint, and the period during which the hind paw failed to touch the rotating cylinder was interpreted as being proportional to the pain felt by the animal. In this model, articular incapacitation is used as a measure of articular inflammatory hyperalgesia. Paw elevation

time was measured before (control time, T0) and 1, 2, 3, 4 and 5 h post-CFA injection and also once a day up to day 14 after CFA administration.

2.7. Concentration of superoxide dismutase (SOD) in rats

To determine if the superoxide dismutase activity was used, the method was described by Ref. [20]; in which 100-μL plasma sample is added to 1110 μL phosphate buffer (50 mM, pH 7.4), 75 μL L-methionine (20 mM), 40 μLTriton X-100 (1% v/v), 75 μL hydroxylamine hydrochloride (10 mM), and 100 μL EDTA (50 mM), followed by incubation in a water bath at 37 °C for 5 min. Then, 80 μL riboflavin (50 mM) was added and samples were exposed to light for 10 min. At the end, 100-μL sample was added to the 96-well plate plus 100-μL Griess reagent, and after 10 min at room temperature, the optical density was measured by spectrophotometry in ELISA Reader (BioTek EL800) using a 550-nm filter [26]. Enzyme activity unit is defined as the superoxide dismutase (SOD) amount able to inhibit the nitrite formation by 50%. The calculation is done using the following formula: SOD = v0/v1, where v0 is the control absorbance and v1 is the test absorbance. In this method, 36 ng SOD inhibits 50% nitrite formation.

2.8. Plasma nitrite concentration in CFA-induced monoarthritis

Briefly, blood of CFA-induced monoarthritis animals (200 μL) were collected from the tail vein and were diluted in distilled water (1:4 v/v) for subsequent deproteinization by addition of 1/20 volume of a zinc sulfate solution at a concentration 300 g/L to achieve a final concentration of 15 g/L. Then, the samples were centrifuged at 1000 g for 15 min at room temperature [43,46]. For the determination of nitrite concentration, 100 μL of the supernatant was incubated for 10 min at room temperature with 100 μL of Griess reagent. The optical density (absorbance) was measured in a plate reader (BioTek EL800, 550 nm filter). The calibration curve consisted of a standard NaNO2 (sodium nitrite) at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0 μMol diluted in distilled water [26].

2.9. Myrtenol evaluation on spontaneous motor activity in mice

Rats were observed for spontaneous locomotion in an open-field apparatus. This test was performed 45 min after oral administration of vehicle (0.9% saline, 10 mL/kg), myrtenol (25 mg/kg) or diazepam (4 mg/kg). The animals were individually brought into the open field for a 1-min adaptation time followed by a period of 4 min recording of the number of fields explored as described by Ref. [17].

2.10. Isolation of human neutrophils

Heparinized human blood from healthy volunteers was collected by venipuncture into a 15 mL Falcon tubes containing a Percoll gradiente of four layers (72, 63, 54, and 45%, 2 mL each). After centrifugation at 650x g for 30 min at 25° C, the lower layer containing neutrophils was collected and washed with Hank's balanced salt solution (HBSS) by centrifugation at 450x g for 8 min at 25 C. The pellet, red cell − free was resuspended in RPMI 1640 containing 0.1% BSA. Total counts were performed with a Neubauer chamber, and differential cell counts were performed on cytospin slides. Preparation of neutrophils that were 98% pure were considered ideal for use in the assay [29], (Souto et al., 2011).

2.11. Migration assay in Boyden chamber

The chemotaxis assay was performed in 48-well microchamber (Boyden Chamber, Neuro Probe Inc., Gaithersburg, MD, USA) as previously described Souto et al., 2011. Human neutrophils were incubated with sterile saline or myrtenol (10, 30, 100 ng/mL, diluted in sterile saline) for 30 min. Then, fMLP (chemotactic agent, 10^{-7} M/well) or RPMI medium (negative migration control) were added to the wells of the bottom chamber, which was later covered by a polycarbonate membrane (Neuro Probe, 5 mM pore). In the upper wells 5×10^{4} neutrophils were added and the chamber was incubated for 1 h at 37 °C and 5% CO_2. After incubation, the membrane was washed in PBS, fixed in 80% methanol, and stained using a Romanowsky staining system (Dade Behring Inc., Newark, DE). The number of cells counted in five random fields per well was registered using light microscopy (1000× magnification) in triplicate. Results are expressed as number of neutrophils per field.

2.12. Real time measurement of leukocyte rolling and adhesion by intravital microscopy

Intravital microscopy was used to establish the extent of leukocyte rolling and adhesion to the mesenteric microcirculation, as previously described [25]. Mice were treated with vehicle (0.9% NaCl + 3% Tween 80, po), myrtenol (Myr, 25 mg/kg, po) or dexamethasone (Dex, 1 mg/kg, po) followed by intraperitoneal injection of 1% carrageenan (Cg, 500 μL/cavity). A saline-treated control group (10 mL/kg, p.o.) was also included. Four hours after the injection of the carrageenan, the mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p., União Química, Brazil). Following laparotomy, the mesenteric tissue was exteriorized and placed in a temperature controlled (37 °C) transilluminated platform and observed by intravital microscopy. The images were recorded at ×200 magnification. The preparation was kept moist and warm by irrigation with warmed (37 °C) Ringer Locke's solution, pH 7.2–7.4, containing 1% gelatin during the whole procedure. Third-order venules, defined according to their branch-order location within the microvascular network, were selected for the study. These vessels corresponded to post-capillary venules, with a 12–18 μm diameter. Rolling leukocytes were defined as cells that moved along an 100 μm length segment of a venular bloodstream at a sufficient slow pace to be individually visible. The number of rolling leukocytes per 100 μm venule was determined at 10-min intervals. Leukocytes were considered to be adherent to the venular endothelium when stationary for a period over than 30 s. The number of adherent cells per 100 μm^2 venule was recorded. Cells were counted in the recorded images, using five different fields for each animal to avoid sampling variability. Data were then averaged for each animal. (Baez, 1969; Rhodin, 1986; Strong et al., 1991).

2.13. Statistical analysis

Data were submitted to Two-way ANOVA followed by Boferroni's test using the GraphPad Prism® Software version 5.0. Values were expressed as Mean ± Standard Error of Mean. The level of significance was set at $P < 0.05$.

3. Results

3.1. Myrtenol prevents edema and joint incapacitation in CFA-associated chronic monoarthritis

Fig. 2 shows that vehicle-treated CFA group presented a prominent time-dependent paw edema (2.30 ± 0.08 mL as detected at day 14, Fig. 2A) and progressive joint incapacitation (41.4 ± 3.88 s at

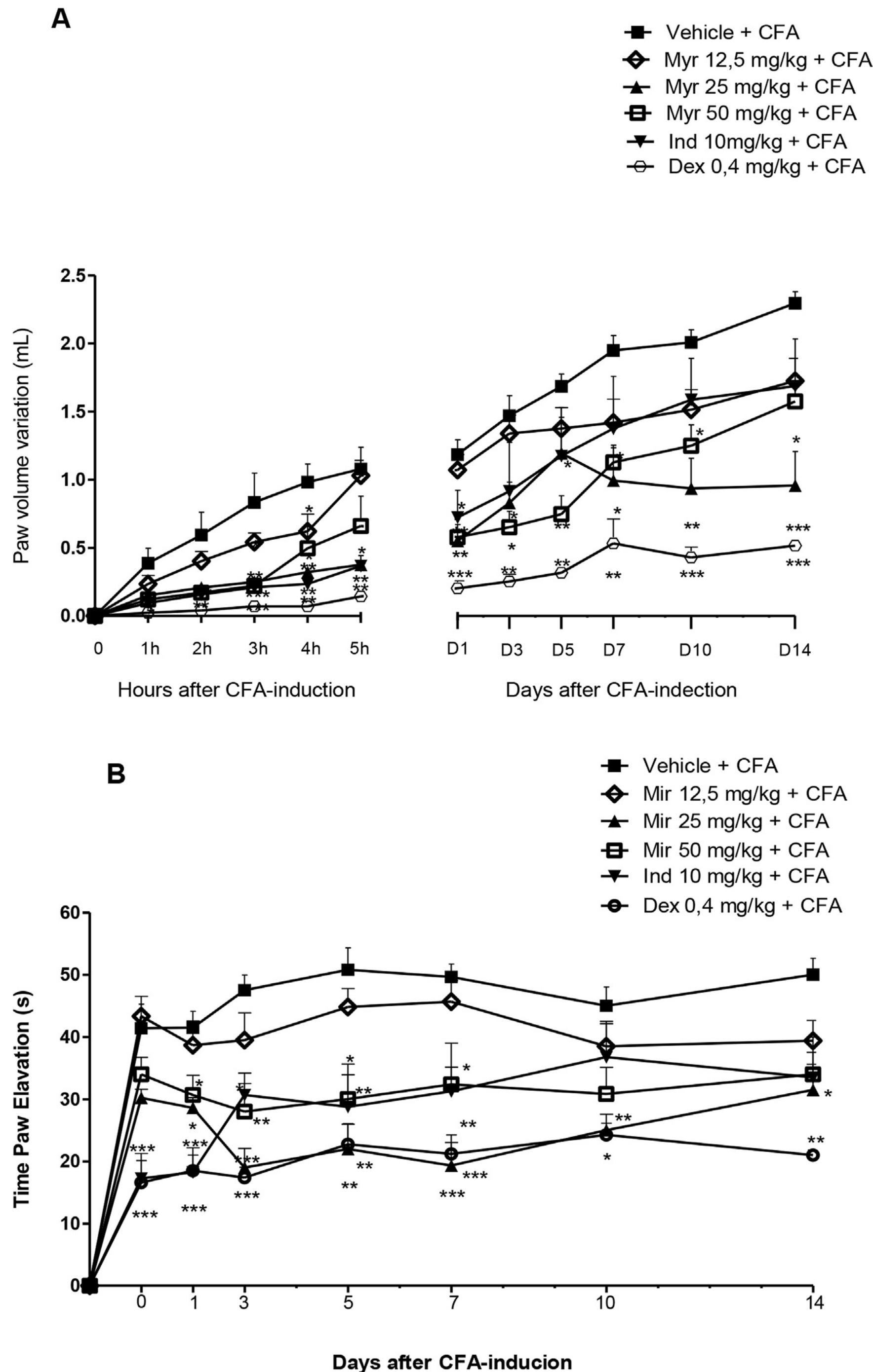

Fig. 2. Myrtenol prevents CFA-induced arthritis. Complete Freund's Adjuvant (CFA)-sensitized rats were treated with vehicle (0.9% NaCl + 3% Tween 80, 10 mL/kg, po), myrtenol (Mir 12.5, 25 or 50 mg/kg, po), indomethacin (Ind, 10 mg/kg, po) or dexamethasone (Dex 0.4 mg/kg) followed by intra-articular injection of CFA (0.5 mg/mL, 50 μL per joint). Paw edema (Panel A) and articular incapacitation (Panel B) were evaluated for 14 days. Values are expressed as mean ± SEM * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs vehicle-treated CFA control group (one-way ANOVA and Student Newman Keuls test).

day 1 and 50.0 ± 2.64 s at day 14, Fig. 2B). In addition, myrtenol-treated animals (at doses of 25 and 50 mg/kg) showed a significant reduction of CFA-elicited edema (0.25 ± 0.03 and 0.22 ± 0.02 mm, respectively) and incapacitation (19.0 ± 3.09 s and 28.0 ± 6.20 s, respectively) as detected in the third hour versus vehicle-injected CFA group ($P < 0.05$, Fig. 2A and B). Such protective effect persisted until the last observed time (day 14, Fig. 2A and B). Similarly, the positive controls indomethacin and dexamethasone also reduced edema and nociception observed over the time when compared with vehicle-injected CFA animals ($P < 0.001$). However, myrtenol at the lowest dose (12.5 mg/kg) failed to protect animals from either edema or joint incapacitation (Fig. 2A and B).

3.2. Myrtenol shows decreased levels of nitrite and SOD

As observed in Fig. 3A, groups treated with myrtenol (12.5, 25 and 50 mg/kg) showed a significant decrease ($P < 0.05$) in nitrite (1.20 ± 0.03, 1.26 ± 0.05, 1.40 ± 0.02, respectively) when compared

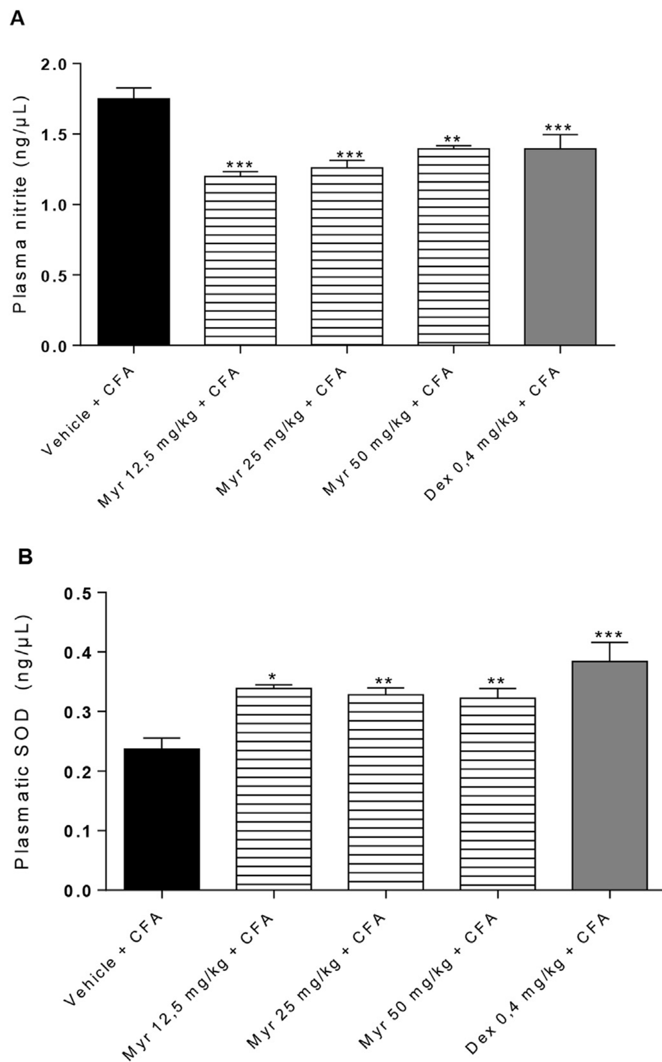

Fig. 3. Antioxidant effect of Myrtenol. Complete Freund's Adjuvant (CFA)-sensitized rats were treated with vehicle (0.9% NaCl + 3% Tween 80, po), myrtenol (Mir 12.5, 25 or 50 mg/kg, po), indomethacin (Ind 10 mg/kg, po) or dexamethasone (Dex 0.4 mg/kg) followed by intra-articular injection of CFA (0.5 mg/mL, 50 μL/joint). On day 14 post CFA injection, blood samples were collected for measurement nitrite levels (Panel A) and superoxide dismutase (SOD) (Panel B). Values are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs vehicle-treated CFA control group (one-way ANOVA and Student Newman Keuls test).

to vehicle-treated CFA control group (1.75 ± 0.07). The group treated with dexamethasone (0.4 mg/kg) also showed significant reduction in blood concentration of nitrite versus CFA group. Plasma levels of SOD were determined as antioxidant parameter. In regard to SOD levels (Fig. 3B), all groups treated with myrtenol (12.5, 25 and 50 mg/kg) showed a significant increase ($P < 0.05$) in SOD production (0.33 ± 0.01, 0.32 ± 0.01 and 0.32 ± 0.01, respectively) in comparison to vehicle-injected CFA control group (0.23 ± 0.01). Additionally, dexamethasone (0.4 mg/kg) treatment also increased the levels of SOD (0.38 ± 0.03) when compared to CFA group (Fig. 3B).

3.3. Myrtenol does not impair rat spontaneous locomotor activity

As observed in Table 1, administration of myrtenol (25 mg/kg) did not affect rat locomotion (37.13 ± 6.32 fields explored) in comparison to vehicle-treated animals (36.88 ± 6.10). However, diazepam (4 mg/kg) significantly reduced the number of invaded fields (2.75 ± 1.30) versus vehicle-treated control group (Table 1).

Table 1
Myrtenol does not impair animal exploratory activity.

Treatment	Dose (mg/kg, p.o.)	Numbers of fields explored
Vehicle	–	36.9 ± 6.1
Myrtenol	25	37.13 ± 6.32
Diazepam	4	2.75 ± 1.3***

Mice were given either vehicle (0.9% NaCl + 3% Tween 80, po), Myrtenol (Mir 25 mg/kg, po), or diazepam (4 mg/kg, po) and submitted to the open field test. Values are expressed as mean ± SEM $^{***}p < 0.001$ vs vehicle control (one-way ANOVA and Student Newman Keuls test).

3.4. Myrtenol inhibits neutrophil migration

Neutrophil migration was observed in *in vitro* (Fig. 4A) and *in vivo* (Fig. 4B and C) conditions. As observed in Fig. 4A, the chemotaxis of neutrophils that were incubated with myrtenol (30 and 100 ng/mL) was pronounced inhibited (3.83 ± 0.60 and 4.20 ± 1.46, respectively) when compared to neutrophils that were incubated with vehicle only (14.00 ± 0.57) (Fig. 4A). RPMI, the vehicle of fMLP, was used as a reference negative control. Additionally, real-time measurement of leukocyte rolling Fig. 4B and adhesion (Fig. 4C) in mesenteric microcirculation of rats showed that intraperitoneal injection of carrageenan resulted in a significant increase in the number of rolling and adherent leukocytes (13.96 ± 3.37 and 1.60 ± 0.59, respectively) when compared to the saline-treated normal control group (rolling cells: 1.93 ± 0.86, and adherent cells: 0.02 ± 0.02, $P < 0.05$). Furthermore, treatment of animals with either myrtenol (25 mg/kg) produced a significant reduction in the number of rolling and adherent leukocytes (7.44 ± 1.89 and 0.20 ± 0.05, respectively) versus vehicle group that were administered carrageenan ($P < 0.05$). Similarly to myrtenol group, the animals treated with dexamethasone (1 mg/kg) showed a marked decrease number of rolling and adhered leukocytes (3.18 ± 1.21 and 0.25 ± 0.13, respectively) versus carrageenan group (Fig. 4A and B).

4. Discussion

The migration of neutrophils to the inflammatory focus is a multifactorial event that involves many steps, such as rolling and adhesion of neutrophils to endothelial cells, followed by a group-induced chemotaxis of inflammatory mediators known as chemotactic agents Souto et al., 2011. The process of leukocyte chemotaxis depends on the release of chemotactic factors at the injury site, such as a peptide fMLP produced by bacteria that causes inflammation or substances released by resident cells, for example, complement fragments and lipid mediators chemokines as leukotriene B4 (LTB4), able to stimulate neutrophils and monocytes in human and other animal species (Sugawara et al., 1995; Katanaev, 2001). The data obtained from the *in vitro* chemotaxis assay induced by fMLP in Boyden chamber revealed that incubation of human neutrophils with myrtenol (30 and 100 ng/mL) significantly inhibited chemotaxis of these cells. The cell migration mediated by chemotactic factors released into the lesion site is essential for the initiation and regulation of inflammatory/immune response (Fine et al., 2001). Their recruitment to sites of inflammation depends on a gradient of chemotactic factors produced locally (Bruno et al., 2007). Therefore, the results obtained in *vitro* assay, suggests that anti-inflammatory activity of myrtenol involve direct inhibition of human neutrophil chemotaxis induced by fMLP (Saltatici et al., 2006).

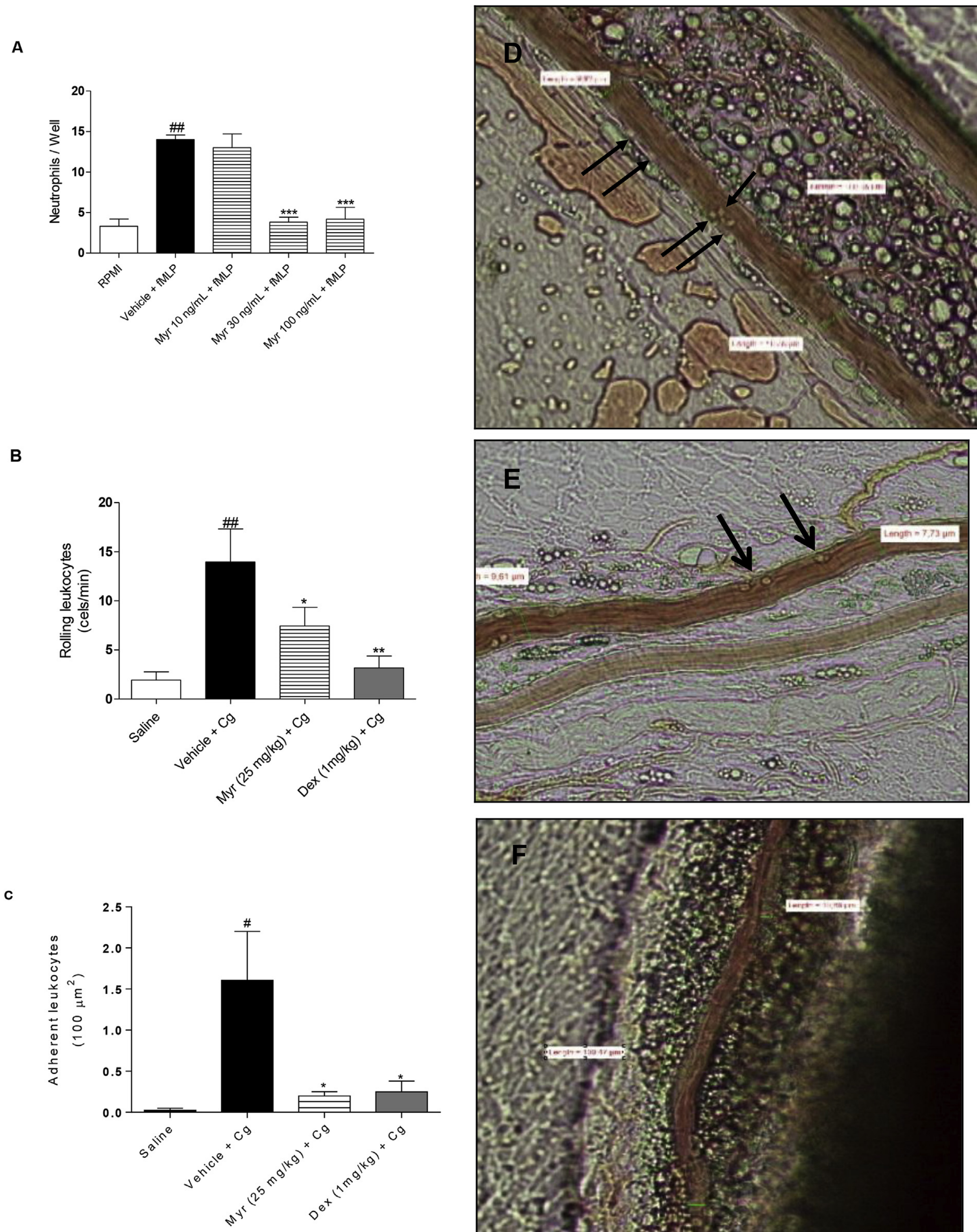

Fig. 4. Myrtenol controls *in vitro* and *in vivo* neutrophil migration. Human neutrophils were incubated with vehicle (sterile saline, 1 mL) or myrtenol (Myr, 10, 30 and 100 ng/mL) for 30 min. Then, *in vitro* fMLP-induced (10^{-7} M/well) neutrophil chemotaxis was evaluated in a Boyden chamber (panel A). RPMI medium was adopted as negative chemotactic control. In another experimental setting, the rats were treated with vehicle (0.9% NaCl + 3% Tween 80, po), myrtenol (Myr, 25 mg/kg, po) or dexamethasone (Dex, 1 mg/kg, po) followed by intraperitoneal injection of 1% carrageenan (Cg, 500 μL/cavity) for real-time measurement of leukocyte rolling (Panel B) and adhesion (Panel C) in mesenteric microcirculation. Saline-injected rats were adopted as a normal control. Video photomicrographs displaying leukocyte-endothelium interaction is shown in Panel D, E and F. Panel D: carrageenan group, showing leukocyte rolling significantly increased on the vascular endothelium Panel E: myrtenol (25 mg/kg, p.o.) group, showing significantly reduction of leukocyte rolling in the mesenteric microvasculature., show leukocyte rolling basal.:. Panel F: dexamethasone group. Values are expressed as mean ± SEM. $^{\#\#}P < 0.001$ vs RPMI or saline; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs vehicle-treated Cg group; $^{***}P < 0.001$ vs vehicle-treated fMLP group (one-way ANOVA and Newman Test Keuls).

Motivated by the results obtained in the in *vitro* chemotaxis model (Boyden chamber), we investigated the anti-inflammatory potential of myrtenol on leukocyte bearing and adhesion model, and in real time, in mice mesenteric microcirculation by intravital microscopy technique in carrageenan-induced peritonitis model. In inflammatory response, the neutrophil migration is one of the important events that occur in microvascular level, been those the first cells to be recruited to an inflammatory site (Carlos and Harlan, 1994; Phillipson and Kubes, 2011; Kolaczkowska and Kubes, 2013). Our results demonstrate that the myrtenol decreased rolling and especially neutrophil adhesion, as revealed by intravital microscopy. The results corroborate with the literature, which shows that the myrtenol was able to reduce the inflammatory edema, total and differential leukocyte number as well as myeloperoxidase activity (MPO) and the levels of pro-inflammatory cytokines TNF-α, IL -1β and IL-6 in the peritoneal exudate of mice with carrageenan-induced peritonitis (Smith et al., 2014). The carrageenan-induced acute inflammation is characterized by infiltration of phagocytes and overproduction of free radicals and the release of inflammatory mediators such as IL-1β, IL-6 and TNF-α (Huang et al., 2011), which promote expression of adhesion molecules, leukocyte migration, increased vascular permeability and transendothelial migration (Hallegua and Weisman, 2002). The recruitment of neutrophil requires rolling and adhesion of cells to the luminal surface of vascular endothelium. The rolling is mediated by selectins and firm adhesion of cells to the endothelium is mediated by the integrins and cellular adhesion molecules that, in turn, lead to changes in the morphology of the neutrophils resulting in the transmigration through the endothelial cell barrier and extravasation tissues [67]. It is possible, therefore, suggest that myrtenol may show anti-inflammatory properties by competitive inhibition of the selectin or by changing the functional state of the integrins [2,6,14].

Recent studies with essential oil of *Rosmarinus officinalis* L., which main constituent is the β-myrcene monoterpene with anti-inflammatory activity previously described, also showed significant inhibition in adhesion and rolling of leukocytes in mice microcirculation [40]. The β-myrcene and myrtenol are unsaturated monoterpenes [4]. Thus, substances which inhibit leukocyte migration can provide means to regulate or modulate the inflammatory and immune response and reduce tissue damage [18,28].

Neutrophils have a key also role in the development of chronic inflammatory and autoimmune diseases, by tissue infiltration and causing diseases such as rheumatoid arthritis [15]. It is well known that during chronification of inflammation occurs the replacement of polymorphonuclear cells found mostly in acute inflammation for giant mononuclear cells such as fibroblasts, monocytes, lymphocytes, macrophages and plasma cells [30,60]. Based on the results, we evaluate the effects of myrtenol in chronic inflammation in arthritis model CFA induced in rats. Among various experimental models of arthritis animals, arthritis induction by complete Freund's adjuvant (CFA) is a method that mimics the human pathophysiological state, including chronic edema in multiple joints due to inflammatory cell accumulation, erosion of cartilage joint and bone destruction, and is therefore widely used to investigate the activity of several anti-inflammatory and antiarthritic agents [44,58]. Expressed data showed a reduction of tibial-tarsal joints edema of animals treated with myrtenol (25 and 50 mg/kg) compared to the control group. This reduction was significant to the 14 days of observation. FCA-induced arthritis has a primary phase and a secondary phase of chronic arthritis. The primary is the inflammatory phase, which occurs generation of prostaglandins and in the second phase there is the generation antibodies [64]. Ref. [49] standardized an experimental model of CFA induced arthritis reproducing signs and symptoms similar to human rheumatoid disease, in other words, a chronic, symmetrical polyarthritis with joint destruction, deformity and disability [61]. Therefore, another test was used to evaluate the degree of disability joint in animals with CFA induced arthritis. The myrtenol (25 mg/kg) reduced the paw elevation time of the animals with arthritis. This finding provides additional evidence that one of the possible mechanisms of action of anti-inflammatory activity of myrtenol is the attenuation of neutrophil recruitment to the focus of inflammation, or inhibiting production of prostaglandins in the generation of antibodies. Moreover, literature data demonstrate that leukocytes migrate into the joint of patients with rheumatoid arthritis, causing damage to the synovial tissue, bone and cartilage through the secretion of reactive oxygen species, proteases, secretion cytokines and prostaglandins [68]. Similar results have been observed with natural products such as Taraxasterol, a pentacyclictriterpene, was one of the main active constituents in genus *Taraxacum* and extract of *Swertia chirayita* leaves [34,65].

As reported in the literature, drugs with sedative activity can interfere in locomotor activity [66]. However, the dose of myrtenol used in the assay did not change the exploratory activity of the animals, indicating that a possible sedative action of myrtenol is not involved in the joint disabling mechanism.

Because of the well-known importance of neutrophils in chronic inflammatory process, it can be suggested that one of the possible explanations of anti-inflammatory activity of this monoterpene is the attenuation of neutrophil recruitment to the focus of inflammation. Furthermore, the migration of neutrophils results in increased generation of reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals [13].

Free radicals reactive oxygen species (ROS) and nitrogen (ERNS) also play a key role in the worsening of chronic inflammatory processes, acting either directly or indirectly as damaging agents inducing transcription of pro-inflammatory factors and pro-apoptotic in all organic systems [9,27].

Reactive oxygen species induce many tissue problems by damaging biomembranes, attacking their proteins and inducing destructive oxidation of membrane lipids, a process known as lipid peroxidation [53]. Reactive nitrogen species, such as NO were also involved in the development of joint destruction in rheumatoid arthritis [21]. High levels of ROS and ERNS been reported in patients with rheumatic disease [46]. Thus, oxidative damage of the vital organs is considered as secondary complications of arthritis [59]. From this information, the next step was to evaluate the antioxidant activity of myrtenol in animals with CFA induced arthritis in order to expand the possible myrtenol antioxidant action of this monoterpene, by quantifying levels of SOD *in vitro*.

SOD is considered one of the most important endogenous enzymatic antioxidant defenses of the organism for fighting the production of free radicals, the increasing of their activity suggests an antioxidant effect related to the modulation of enzymatic activity [3]. The experiments demonstrated the inhibitory effect of myrtenol (12.5, 25 and 50 mg/kg) on the production of nitrite *in vivo*, and significantly increased SOD activity. Geraniol, a monoterpene alcohol, important constituent of essential oil of ginger and lemon, also showed antioxidant activity similar to myrtenol in nitric oxide production, superoxide dismutase activity and ROS measurement [32]. These results, therefore, suggest that myrtenol has a possible antioxidant effect by reducing the level of lipid peroxidation and nitrite content, since it was able to reduce the formation of reactive species derived from oxygen and nitrogen. Furthermore, myrtenol increased activity of superoxide dismutase, suggesting that its antioxidant role may be due to the upregulation of these enzymes.

The presence of NO was indirectly determined by nitrite dosage and SOD activity in the blood of animals treated with myrtenol. NO is an important signaling molecule involved in many physiological

and pathological processes, including the signaling of pain and inflammation, playing an important role in several types of inflammatory processes and, moreover, in inflammation, nitric oxide (NO) acts as a mediator pro-inflammatory in the pathogenesis of several diseases, and is importance to search for new safe and effective antioxidants drugs [31,39].

In summary, these results suggest that chronic and acute anti-inflammatory effect of myrtenol might be due in part to reduced leukocyte influx to the site of inflammation and reduction of oxidative stress, especially the reduction of NO and lipid peroxides and increased levels of endogenous enzymatic antioxidants, such as SOD.

In this context, the results obtained with the myrtenol extend evidence already found for the anti-inflammatory action of this monoterpene. However, future studies will be needed to use the myrtenol as an effective anti-inflammatory agent and its safety profile in clinical use.

Conflicts of interests

The authors declare no conflicts of interest.

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