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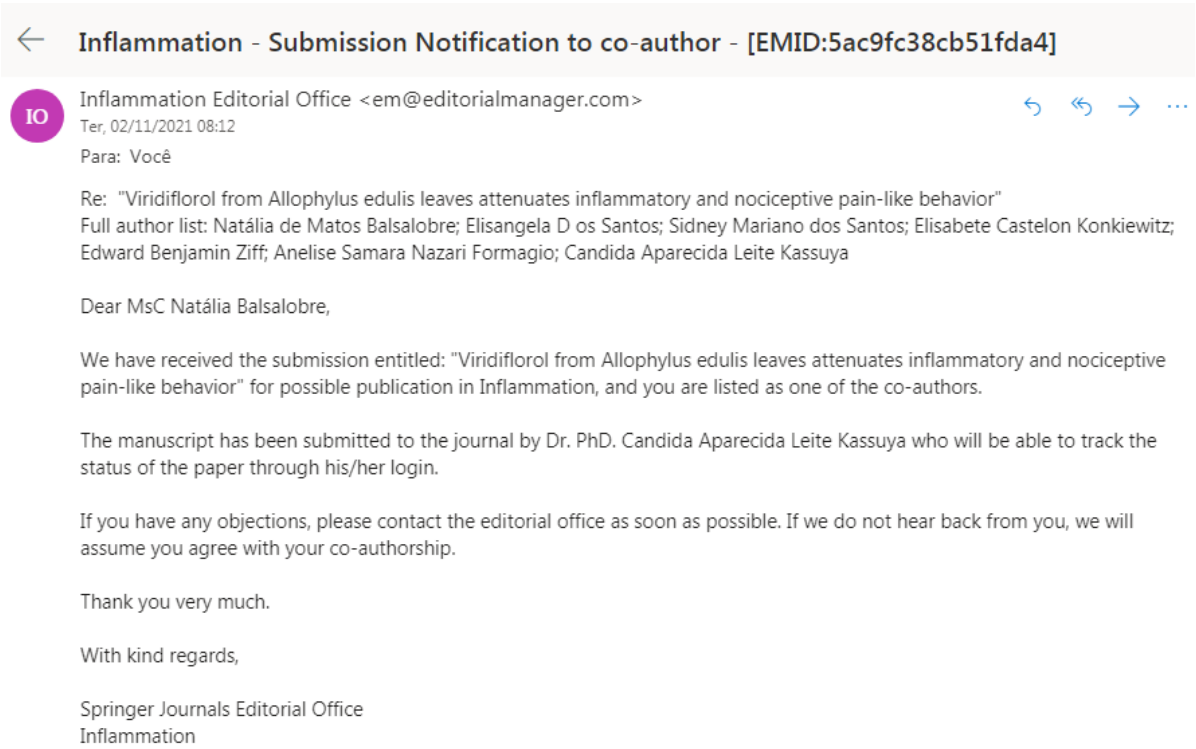

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Re: "Viridiflorol from Allophylus edulis leaves attenuates inflammatory and nociceptive pain-like behavior"
Full author list: Natália de Matos Balsalobre; Elisangela D os Santos; Sidney Mariano dos Santos; Elisabete Castelon Konkiewitz; Edward Benjamin Ziff; Anelise Samara Nazari Formagio; Candida Aparecida Leite Kassuya

Dear MsC Natália Balsalobre,

We have received the submission entitled: "Viridiflorol from Allophylus edulis leaves attenuates inflammatory and nociceptive pain-like behavior" for possible publication in Inflammation, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. PhD. Candida Aparecida Leite Kassuya who will be able to track the status of the paper through his/her login.

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Thank you very much.

With kind regards,

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Inflammation

Viridiflorol from *Allophylus edulis* leaves attenuates inflammatory and nociceptive pain-like behavior

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ABSTRACT

Viridiflorol was identified and isolated from the essential oil of *Allophylus edulis* leaves (OEAE). Viridiflorol has not been scientifically studied in relation to anti-inflammatory and anti-nociceptive pain-like properties. The aim of the present study was to investigate the analgesic (anti-hyperalgesic and anti-nociceptive) properties of OEAE and viridiflorol using *in vivo* models. The oral administration (p.o.) of OEAE (30, 100 and 300 mg/kg), viridiflorol (30, 100 and 200 mg/kg), morphine (1 mg/kg, subcutaneous route (s.c.) and the local administration (i.pl.) of OEAE (100 µg/paw) and viridiflorol (100 µg/paw) were tested using the formalin model in *Swiss* mice. OEAE (100 mg/kg, p.o.), viridiflorol (200 mg/kg, p.o.), and dexamethasone (1 mg/kg, s.c.) were tested by the zymosan-articular inflammation and in open-field (to verify effects on locomotor performance) models. Viridiflorol (0.3, 20 e 200

µg/paw) was also tested in the carrageenan induced-mechanical hyperalgesia model. Viridiflorol (200 µg/paw) was also tested in the TNF, and dopamine induced-mechanical hyperalgesia and edema models. The administration of OEAE (100 and 300 mg/kg, p.o.), viridiflorol (200 mg/kg, p.o.), morphine (1 mg/kg, s.c.) and local administration of OEAE (100 µg/paw) and viridiflorol (100 µg/paw) significantly inhibited edema, and the first and second phases of formalin induced-nociception. OEAE, viridiflorol and dexamethasone significantly reduced mechanical hyperalgesia, edema, total leukocytes, polymorphonuclear, nitric oxide and protein exudation in the zymosan-induced articular inflammation model. Oral treatments with OEAE and viridiflorol (200 mg/kg) did not cause *motor impairment* in the open field test since they did not reduce locomotor activity. The local administration of viridiflorol (200 µg/paw, i.pl.) significantly inhibited carrageenan-, TNF, and DOPA induced-mechanical hyperalgesia and edema.

This study confirms the anti-nociceptive and anti-hyperalgesic activities of OEAE and viridiflorol. Viridiflorol could be partially responsible for the anti-hyperalgesic properties and its mechanism of action could involve inhibition of the TNF and DOPA pathways.

Keywords: Sapindaceae; terpenes; pain; formalin; mice; zymosan.

1. INTRODUCTION

Natural products have historically been a source of medicines for the prevention and treatment of disease (1). The use of the plants with medicinal properties has increased and medicinal plants have been widely investigated for their biological activities (2). Secondary plant metabolites are affected by genetic, environmental, and agronomic factors (3,4). Essential oils and their components have been used therapeutically for centuries and have

importance for several commercial and clinical applications (*e.g.* from the food industry to aromatherapy) (4-6).

Biological effects of essential oils in pain and inflammation models have been attributed to terpenes (7). Natural oils and substances isolated from these oils have been increasingly used in the search for bioactive natural products that can be used, whether associated or not with drugs, to treat diseases (8). The leaves of *Allophylus edulis* (A. St.-Hil., Cambess. & A. Juss.) (Sapindaceae), known as "chal-chal" or "cocu" in Brazil are used in folk medicine as an anti-inflammatory agent (9-11). Studies conducted by our research group showed that the essential oil obtained from the leaves of *A. edulis* (EOAE) and its major constituent, viridiflorol (30.88%), exhibited anti-inflammatory, *in vitro* antimycobacterial and antioxidant activities (12). These models were used to determine the dose response curves, time response curves, and anti-inflammatory profiles of these substances. Santos et al. (2021) (13) showed that essential oils obtained from *A. edulis* leaves collected at different months of the year and in two cities had differences in chemical composition, however all had anti-inflammatory activity.

In the present work we evaluated the potential analgesic and anti-hyperalgesic effects of oral treatment with EOAE and viridiflorol in models of pain, including inflammatory and spontaneous pain models. In addition, viridiflorol was tested locally (in mice) against the carrageenan, tumor necrosis factor (TNF) and dopamine (DOPA) induced-hyperalgesia.

2. MATERIAL AND METHODS

2.1. Plant material, EOAE extraction and isolation of viridiflorol

Leaves of *A. edulis* were collected in Dourados, MS, Brazil, in March 2015 (22 ° 11'43.7 "S, 54 ° 56'08.5" W and 430m) and deposited in the herbarium (number 342) of the Federal University of Grande Dourados (UFGD). The authorization to access and study

samples from the Brazilian genetic heritage was obtained from the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen -A51F665).

EOAE extraction, chemical composition, isolation and identification of viridiflorol were performed according to Trevizan et al. 2016 (12). Fresh leaves (200 g) of *A. edulis* were subjected to hydrodistillation in a Clevenger apparatus for approximately 4 h. After drying and filtration, OEAE with a yield of 6.5% v/p, was obtained and stored at - 4 ° C. The OEAE was fractionated by silica gel column chromatography containing the viridiflorol that eluted with n-hexane-chloroform 7:3. Viridiflorol was identified by the comparison with literature spectroscopic data (1H and 13C NMR) (14-15).

2.2. Animals

Male (n=96) and female (n=36) *Swiss* mice were used for the experiments. The animals weighed between 28-30 g and were provided by the Central Animal Facility of the Federal University of Grande Dourados (UFGD). The animals were kept in polypropylene boxes, in the maintenance vivarium of UFGD, with a controlled temperature of 22 ± 2 ° C, and with free access to water and food (Nuvilab CR-1). The experiments were performed after approval by the Ethics Committee on Animal Use of UFGD under protocol number 03/2020.

2.3. Chemical agents

All reagents used for this work have a high analytical grade.

2.4. Oral and topical effects of OEAE and viridiflorol in the formalin-induced paw nociception and edema test

To evaluate the anti-nociceptive effect of OEAE and viridiflorol the formalin-induced nociception test was conducted, using 30 male *Swiss* mice, which were divided into six groups (n=5). Paw licking reactions were considered as an indication of a nociception response (16).

The mice received treatment or vehicle, 1 hour before the injection of 20 μL of 2.5% formalin solution into the plantar surface of the right hind paw. The groups for this test were: the positive control group, which received subcutaneous injection (s.c.) of morphine 1 mg/kg; the negative control group, which received 0.9% saline solution orally (p.o.); groups treated with 30 mg/kg or 100 mg/kg or 300 mg/kg of OEAE; and a group that received oral treatment with viridiflorol at doses of 30, 100, and 200 mg/kg. Immediately after the formalin injection, pain responses (paw licking) were recorded in phase I (0-5 min) and phase II (15-30 min) oof the test. Paw edema was measured using a plethysmometer.

To evaluate the local anti-nociceptive effect of OEAE and viridiflorol the formalin-induced nociception test was conducted using 20 male *Swiss* mice, which were divided into four groups (n=5). The groups were: viridiflorol 100 μg/paw treated group; OEAE 100 μg/paw treated group; positive control group treated with Morphine 1 mg/kg (s.c.); negative control group, which received 0.9% saline solution. Fifteen minutes after the treatment or placebo, all animals received an intraplantar injection of 20 μL of 1% formalin into the right hind paw (17).

After the induction of nociception, the animals were individually placed in glass funnels and the time of the animals licking reactions were measured in phase I (0-5 min) and phase II (15-30 min). Paw edema was measured using a plethysmometer at the end of the test.

2.5. Oral effects of OEAE and viridiflorol on Zymosan-induced joint inflammation test

To evaluate the anti-inflammatory and anti-hyperalgesic effects of OEAE and viridiflorol, the Zymosan-induced joint inflammation test was conducted using 24 female Swiss mice, divided into six groups. One hour before receiving an intra-articular injection of Zymosan, the animals were treated orally with: OEAE at a dose of 100 mg /kg (p.o.), or viridiflorol at a dose of 200 mg/kg (p.o.), dexamethasone (1 mg/kg, s.c.) (DEXA) or 0.9%

saline solution. Fifty minutes later the open field test was performed to verify the locomotor activity. After 1 hour following the respective treatments, for induction the of intra-articular inflammation, a 20 µL aliquot of zymosan (200 µg/article) was injected into the posterior right knee joint (18). Mechanical hyperalgesia was assessed at the 3rd and 4th hour after zymosan injection by measuring the paw withdrawal reflex with an electronic analgesimeter device (InSight®, Ribeirão Preto, Brazil) and the Von Frey method. Paw edema was measured using a digital micrometer (Digimess 110-284), evaluating the difference in diameters of the contralateral knees (in µm), at the 4th and 6th hour after zymosan injection.

Six hours after zymosan injection, the animals were euthanized, with a lethal injection of ketamine (300mg/kg) and xylazine (30mg/kg), and cervical dislocation and the knee joint cavities were washed with a solution of PBS/EDTA. For the determination of leukocyte migration into the intra-articular fluid, a total leukocyte count and a differential leukocyte count were performed on the collected wash fluid. The total number of leukocytes was counted in a Neubauer chamber using 20 µL of joint wash solution diluted in Turk solution (1:20 dilution). A 20 µL aliquot of this preparation (Turk's fluid + joint lavage) was placed in the Neubauer chamber, and the total number of cells was counted in the four quadrants of the chamber using an optical microscope (10x magnification). The counts were expressed as the total number of leukocytes $\times 10^6$/ml.

For the differential leukocyte counting, aliquots of the intra-articular lavage were centrifuged at 1,500 rpm for 10 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 200 µl of a PBS/EDTA solution. Slides for differential counts were prepared using an aliquot of the intra-articular lavage (50 µl) subjected to centrifugation at 1,500 rpm for 10 min. The slides were then mounted, fixed, and stained with eosin and hematoxylin. Subsequently, differential cell counts were performed on slides under a microscope. The number of leukocytes present in the intra-articular lavage was obtained by

calculating the percentage of leukocytes (differential count) and the total number of cells present in the joint lavage fluid. The results were expressed as the number of leukocytes × 10^6/ml. Nitrite evaluation was performed by Griess' method while the total dosage of protein was done with the Bradford kit (19).

2.6. Mechanical hyperalgesia and carrageenan-induced paw edema (Cg)

To evaluate the anti-hyperalgesic and anti-edematogenic effects of viridiflorol, the Carrageenan (Cg)-induced mechanical hyperalgesia test was conducted using 30 male *Swiss* mice, which were divided into six groups (n=5). The groups for this test were: the control group, which received saline solution 0.9% intraplantarly; the treated groups that received intraplantarly: viridiflorol 0.3 µg/paw, viridiflorol 20 µg/paw and viridiflorol 200 µg/paw.

Fifteen minutes after administration of the treatments, the mice received an intraplantar injection of 50 µl of carrageenan (300 µg/paw) in the right hind paw. Mechanical hyperalgesia was evaluated by the Von Frey method 3 and 4 hours after carrageenan injection and paw edema was evaluated with the digital plethysmometer apparatus 3 and 4 hours after carrageenan injection (20). The hyperalgesia in response to cold was measured with the acetone test in which 100 µl of acetone was instilled under the right hind paw (21).

2.7. Mechanical hyperalgesia and paw edema induced by tumor necrosis factor (TNF) and dopamine (DOPA)

To evaluate the possible mechanism of action of viridiflorol, the mechanical hyperalgesia test induced by TNF and DOPA, was conducted using 30 male *Swiss* mice, which were divided into six groups (n=5). The groups were: treated with viridiflorol 200 µg/paw, and the negative control group, which received saline solution, and the basal group, which received no treatment.

After 30 minutes of treatment, the animals received a subcutaneous injection in the right hind plantar region with DOPA (30 μg/paw) or TNF (100 μg/paw); the basal group did not receive this injection. Mechanical hyperalgesia was assessed at the 3rd and 4th hour after DOPA or TNF injection with Von Frey method. Paw edema was measured using a plethysmometer, at the 3rd and 4th hour after DOPA or TNF injection (22). The animals were subjected to mechanical stimuli before and after treatments.

2.8. Statistical Analysis

The data are presented as the mean ± standard error (SEM). The determination of significant differences among groups was made via one-way analysis of variance (ANOVA) and the comparisons among groups were performed by Tukey's multiple comparisons test. The percentage of inhibition was calculated in relation to the control group. Differences were considered to be significant when $P < 0.05$.

3. RESULTS

3.1. Effects of oral or local treatment with OEAE and viridiflorol in the formalin-induced paw nociception and edema model

Oral treatment with OEAE at doses of 100 and 300 mg/kg significantly inhibited nociception in both phases and also inhibited edema. When compared to the control group, the dose of 100 mg/kg reduced 58% of nociception reaction in the first phase, 58% of nociception in the second phase of formalin and 54% of edema (Figure 1). Oral treatment with OEAE at a dose of 300 mg/kg inhibited nociception by 70% in the first phase, 59% in the inflammatory phase (Fig. 1b) inhibiting 69% of edema (Fig. 1c). The treatment with viridiflorol at a dose of 200 mg/kg (p.o.) inhibited nociception by 70% in the first phase (Fig.

1b), 41% in the inflammatory phase (Fig. 1b) inhibiting 66% of edema (Fig. 1c). The treatment with dose of 30 mg/kg of viridiflorol did not interfere in nociception or in edema induced by formalin. The treatment with dose of 100 mg/kg of viridiflorol interfere inhibiting significantly in 54% edema induced by formalin. Morphine significantly reduced 83% of nociception in the first phase (Fig. 1a), 95% in the second phase (Fig. 1b), inhibiting 81% of edema (Fig. 1c).

Statistical comparison among groups showed that the control and the group treated with 30 mg/kg of EOAE and 30 and 100 mg/kg viridiflorol did not differ among themselves, however they differed from the other groups in Figure 1a, 1b and 1c. EOAE showed a dose-dependent effect since the groups treated with 100 and 300 mg/kg of EOAE did not differ among themselves, and they differed from the group treated with 30 mg/kg. Viridiflorol showed a dose-dependent effect since the groups treated with 100 and 200 mg/kg of viridiflorol did not differ among themselves, and they differed from the group treated with 30 mg/kg.

The intraplantar injection of OEAE at a dose of 100 µg/paw was effective in the reduction of nociception inhibiting 65% in the first phase of the formalin-induced nociception test (Fig. 2a) and in the second phase inhibiting 47% of nociception when compared to the control group (Fig. 2b). The intraplantar injection of viridiflorol at a dose of 100 µg/paw inhibited nociceptive behavior in 83% in the first phase of the test, although the second phase was not inhibited significantly by the compound. Morphine inhibited 78% in the first phase and 76% in the second phase of the test (Fig. 2a). The inhibitions of edema were 57% for OEAE, 36% for viridiflorol and 61% for Morphine (Fig. 2c). Statistical comparison among groups showed that the groups treated with EOAE, viridiflorol and morphine differed from the control group in Figure 2a. In Figure 2b, the viridiflorol and control groups differed from

the other groups. And in Fig. 2c, the groups EOAE and morphine differed from the other groups.

3.2. Anti-hyperalgesic and anti-inflammatory activity of oral treatment with OEAE and viridiflorol in the model of joint inflammation induced by zymosan

In the model of joint inflammation induced by zymosan, statistical analysis indicated that OEAE at a dose of 100 mg/kg and viridiflorol at a dose of 200 mg/kg were effective in mitigating Zymosan-induced mechanical hyperalgesia (Fig. 3a and 3b). Four hours after Zymosan injection, oral treatment with OEAE at 100 mg/kg inhibited mechanical hyperalgesia (Fig. 3a) and edema (Fig. 3c). Treatment with viridiflorol at a dose of 200 mg/kg was able to inhibit mechanical hyperalgesia 59% (Fig. 3a), and edema 78% (Fig. 3c). The group treated with DEXA, as expected, reduced mechanical hyperalgesia about 88% (Fig. 3a) and edema 71% (Fig. 3c).

Six hours after Zymosan injection, oral treatment with OEAE at 100 mg/kg inhibited 66% mechanical hyperalgesia (Fig. 3b) and 60% edema (Fig. 3d). Treatment with viridiflorol at a dose of 200 mg/kg was able to inhibit mechanical hyperalgesia 63% (Fig. 3b), and edema 79% (Fig. 3d). The group treated with DEXA, as expected, a reduction of mechanical hyperalgesia of about 66% (Fig. 3b) and edema 82% (Fig. 3d).

In relation to the mechanical hyperalgesia and knee edema, the groups treated with EOAE, dexamethasone and viridiflorol differed from the control groups when the statistical comparison among groups was performed (Fig. 3a, 3b, 3c and 3d).

Six hours after zymosan injection, oral treatment with OEAE at 100 mg/kg inhibited total (Fig. 3e) and polymorphonuclear leukocytes (Fig. 3f) with inhibitions of 69% and of 63%, respectively. Treatment with viridiflorol at a dose of 200 mg/kg was able to inhibit total

(Fig. 3e) and polymorphonuclear leukocytes (Fig. 3f) and the reductions were 67% and 64%, respectively (Fig. 3e and 3f).

Nitric oxide production was inhibited by OEAE treatment (81%), viridiflorol treatment (84%) DEXA treatment (86%) when compared to the control group (Fig. 3g). The total protein extravasation was inhibited by OEAE treatment (60%), viridiflorol treatment (62%) and DEXA treatment (74 %) when compared to the control group (Fig. 3h).

The administration of EOAE and viridiflorol produced no significant differences in the open field test when compared to vehicle-treated animals (results not shown).

3.3. Anti-hyperalgesic and anti-inflammatory activities of viridiflorol in local treatment in the carrageenan (Cg) model

Intraplantar treatment with viridiflorol at a dose of 200 µg/paw inhibited mechanical hyperalgesia in the Von Frey test 3 and 4 hours after Cg injection, inhibiting the pain response by 71.6% and 58%, respectively (Fig. 4a and 4b).

Viridiflorol treatment at a dose of 200 µg/paw significantly inhibited cold hyperalgesia 3 and 4 hours after Cg injection, inhibiting 88.8% and 83% cold hyperalgesia, respectively (Fig. 4c and 4d).

Intraplantar treatment with viridiflorol at doses of 0.3, 20 and 200 µg/paw significantly inhibited edema formation 3 hour after Cg injection inhibiting 42%, 58% and 70% edema (Fig. 4e). Four hours after Cg injection viridiflorol at a dose of 200 µg/paw inhibited edema by 81% (Fig. 4f) compared to the control group.

3.4. Mechanical hyperalgesia induced by carrageenan (CG), TNF, dopamine (DOPA)

Statistical analysis showed that local treatment with viridiflorol at a dose of 200 µg/paw was able to inhibit pain induced by TNF and DOPA (Fig. 5). Three hours after the application of TNF (100 pg/paw), viridiflorol was able to inhibit 71% of the pain (Fig. 5a). Three hours after the application of DOPA (30 µg/paw), statistical analysis showed that Viridiflorol was able to inhibit 72% of the pain (Fig. 5c).

Four hours after TNF injection, Viridiflorol was able to inhibit pain 72% (Fig. 5B). Four hours after DOPA injection, Viridiflorol was able to inhibit hyperalgesia 53%. These data indicate that the possible mechanism of action of viridiflorol is related to the inflammatory TNF pathway.

Statistical analysis showed that local treatment with viridiflorol at a dose of 200 µg/paw was able to inhibit paw edema after TNF and DOPA application, attenuating edema compared to the basal group (Fig. 6). Viridiflorol treatment three hours after TNF application was able to inhibit edema by 31% (Fig. 6a) and 4 hours after was able to inhibit edema by 65% (Fig. 6b). Viridiflorol treatment three hours after DOPA application inhibited edema by 83% (Fig. 6c) and at 4 hour inhibited edema by 89% (Fig. 6d).

4. DISCUSSION

The present study describes the anti-hyperalgesic and anti-nociceptive effects of OEAE and viridiflorol obtained from *A. edulis* leaves in several models. OEAE and viridiflorol administered orally, or by a local route, showed an anti-nociceptive effect in a dose-dependent manner in the formalin test and they also presented efficacy against hyperalgesia/ inflammation in the zymosan-articular inflammation and carrageenan models in mice. Viridiflorol and other compounds were isolated from the essential oil of *A. edulis* leaves (OEAE) and viridiflorol presented a yield of 30 % (12). Viriflorol together with other

compounds are responsible for the OEAE anti-nociceptive responses since the treatment with viridiflorol alone did not reproduce the inhibition induced by OEAE in equivalent doses of vidiriflorol present in the oil. The viridiflorol mechanism of action could involve TNF and DOPA pathways since this compound inhibited the hyperalgesia/oedema induced by these mediators.

A. edulis is known as “chal” or “cocu” in Brazil and its leaves have been used as an infusion for inflammatory disorders (9) and our group showed the anti-inflammatory effects of the essential oil obtained from this plant (12). The dose of 100 mg/kg of the essential oil obtained from *A. edulis* was shown to be anti-oedematogenic and the same effect was achieved with the dose of 30 mg/kg of viridiflorol in oedema and pleurisy induced by carrageenan (12). This led us to conclude that viridiflorol is responsible for the anti-inflammatory action of the essential oil obtained from *A. edulis* (12). Other species from the same genus are used in folk medicine to treat pain. A. cobbe (Sapindaceae) leaves are used to treat pain in hand or leg (23-24). In the present study, the dose of 30 mg/kg of viridiflorol, by the oral route, did not present anti-nociceptive effects while the oral dose of 100 mg/kg of OEAE inhibited the formalin induced nociception and edema (Fig. 1). These results showed that viridiflorol alone is not responsible for the OEAE anti-nociceptive responses.

Nociception induced by formalin in experimental mice is a relevant model to measure the anti-nociceptive effects of new products and the possible effects on pain mediators *in vivo* (25-26). *Noxious stimuli like formalin could lead to a direct activation of afferent primary neuron (nociceptor), called the neurogenic phase (Phase I) and the inflammatory reaction (activating the release and production of several inflammatory/nociceptive mediators) characterizes the second phase of nociception induced by formalin (16-27).* The results of this model are reported in Figures 1 and 2, and showed that OEAE and viridiflorol induced anti-nociceptive and anti-edematogenic properties inhibiting the first and second phases of

nociception and oedema. The inhibition of the edema and nociception of the second phase of formalin treatment by OEAE and viridiflorol were partially expected since both products are anti-inflammatory agents (12). The best inhibition value was achieved by OEAE with the dose of 100 mg/kg (p.o.) and 200 mg/kg of viridiflorol (p.o.), which showed inhibitions of 58% and 70%, respectively. In relation to nociception, in both phases and in edema, the comparison among groups showed that the control and the group treated with 30 mg/kg of EOAE differed from the 100 and 300 mg/kg (Figure 1a – c).

For the viridiflorol results in nociception and edema, the groups treated with 100 and 200 mg/kg differed from the group treated with 30 mg/kg (Fig. 1a – c). Local administration of OEAE and viridiflorol, both with a 100 µg/paw dose, significantly prevented licking behavior in phase 1 and phase 2 and on edema in mice (Fig. 2a, 2b, and 2c) suggesting that viridiflorol and other compounds directly reduced the sensitization of the nociceptive nerve fibers. Oral treatments with OEAE and viridiflorol did not cause *motor impairment* in the open field test since they did not reduce locomotor activity. The present study is the first to show that the anti-nociceptive properties of EOAE and viridiflorol are in a dose dependent manner without interfering in the locomotor performance.

The EOAE (100 mg/kg) and viridiflorol (200 mg/kg) *inhibited the mechanical hyperalgesia induced by zymosan. Zymosan is an inflammatory and immunomodulatory stimulator and did not directly evoke spontaneous pain (28). The spontaneous nociception induced by formalin is different from hyperalgesia evoked by mechanical stimulus in zymosan model. In the zymosan mechanical hyperalgesia model, a mechanical stimulus is needed and* EOAE and viridiflorol *inhibited this stimulus showing that these products antagonized mediators that induce central/peripheral sensitization. Peripheral sensitization is an increased sensitivity to an afferent nerve stimulus and in the zymosan model, peripheral sensitization could be mediated by prostaglandin and cytokines (29-30).* OEAE and

viridiflorol significantly reduced other inflammatory parameters such as knee edema, total leukocytes, polymorphonuclear cells, nitric oxide and protein exudation in zymosan-induced articular inflammation. *The present study showed the anti-hyperalgesic and anti-inflammatory properties induced by* EOAE and viridiflorol *involve the mediators produced by zymosan.*

TNF, dopaminergic, prostanoid and other systems are involved in the sensitization of nociceptors induced by inflammation in mice (31). *Considering that* viridiflorol *is most likely the principal candidate for causing the analgesic effects of OEAE,* it *was tested directly in the mouse paw. The dose of 200 µg/paw affected the ability of carrageenan to induce mechanical hyperalgesia and in the present work, when animals were treated by local administration, this compound also reduced the intensity of nociceptive/inflammatory parameters. The local application of viridiflorol reduced edema formation (at 3 and 4 h after stimulus), reducing the response to mechanical and cold hyperalgesia. The local injection of 200 µg/paw of* viridiflorol *changed the threshold of mechanical and cold sensitivity and decreased the edema induced by Cg. These results suggested that the direct injection of the compound could reduce the sensitization of the nociceptive nerve fibers and modulation of the inflammatory mediators. The local administration of viridiflorol inhibited the hyperalgesic effects of TNF and DOPA and significantly prevented the decrease in the threshold of sensitivity (Fig. 5), suggesting the involvement of the inflammatory pathway of TNF and DOPA in the mechanism of action however not discounting other pathways.*

OEAE, viridiflorol and dexamethasone significantly reduced mechanical hyperalgesia, edema, total leukocytes, polymorphonuclear cells, nitric oxide and protein exudation in zymosan-induced articular inflammation. The local administration of viridiflorol (200 µg/paw, i.pl.) significantly inhibited carrageenan, TNF, and DOPA induced-mechanical hyperalgesia.

This study confirms the analgesic and/or anti-hyperalgesic activity of OEAE and viridiflorol. Viridiflorol could be responsible for the anti-hyperalgesic properties of OEAE and its mechanism of action could involve the inhibition of the TNF and DOPA pathways.

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Authors' contributions

Natália de Matos Balsalobre, Elisangela Dos Santos, Elisabete Castelon Konkiewitz and Candida Aparecida Leite Kassuya performed the *in vivo* inflammatory and nociceptive pain-like behavior assays. Sidney Mariano dos Santos and Anelise Samara Nazari Formagio prepared the EOAE and isolated Viridiflorol. Natália de Matos Balsalobre, Elisangela Dos Santos, Elisabete Castelon Konkiewitz, Candida Aparecida Leite Kassuya and Edward Benjamin Ziff ensured statistical analysis, wrote, and corrected the manuscript.

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Ethics declarations

Ethics approval

Animals obeyed by the norms established by the ethics committee in animal experimentation of the Federal University of Grande Dourados and approved by the ethics committee in animal experimentation (03/2020) of the Federal University of Grande Dourados. All animal experiments were conducted in accordance with the "National Council for the Control of Animal Experimentation (CONCEA)."

Competing Interests

The authors declare that they have no conflict of interest.

For consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Availability of data and materials

All data generated or analyzed during this study are included in the present manuscript.

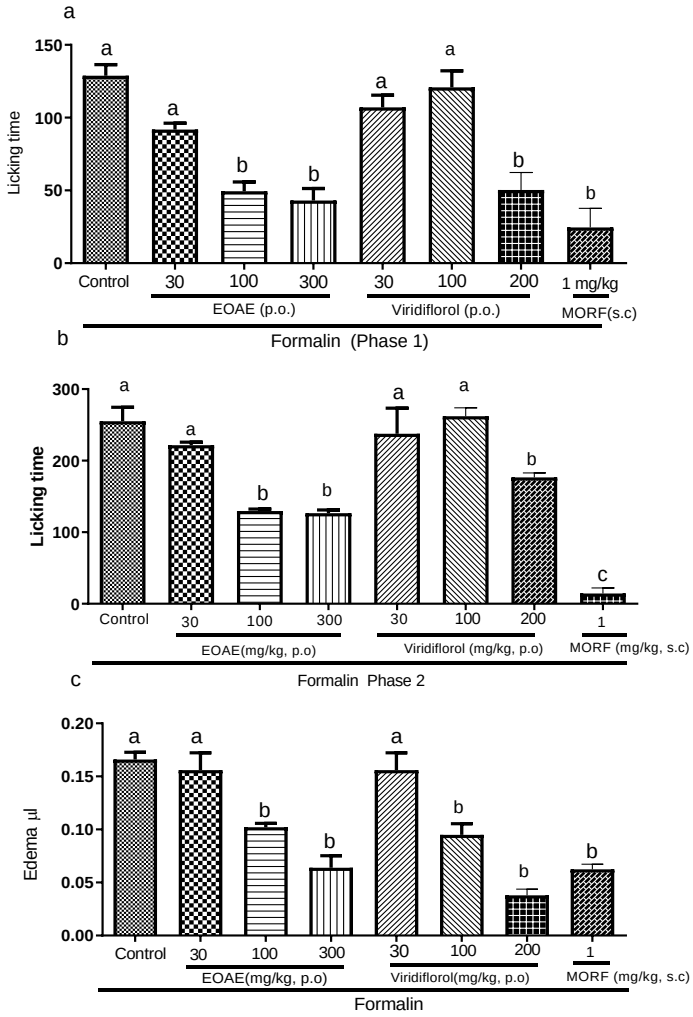


Fig. 1 Effects of oral administration of *A. edulis* essential oil (OEAE) and viridiflorol on nociception by formalin on licking behavior in phase 1 (a) and phase 2 (b) and on edema (c) in mice. The animals received OEAE (30, 100, and 300 mg/kg) or viridiflorol (30, 100, and 200 mg/kg), morphine (MORF- 1 mg/kg/s.c.), or vehicle, and after 1h, intraplantar injection of formalin 2.5% (20 µg/paw) was performed and spontaneous licking behavior and edema were measured. Each bar represents the mean ± SEM of 5 animals. The letters "a" and "b" indicate significant differences among groups according to Tukey´s multiple comparisons test

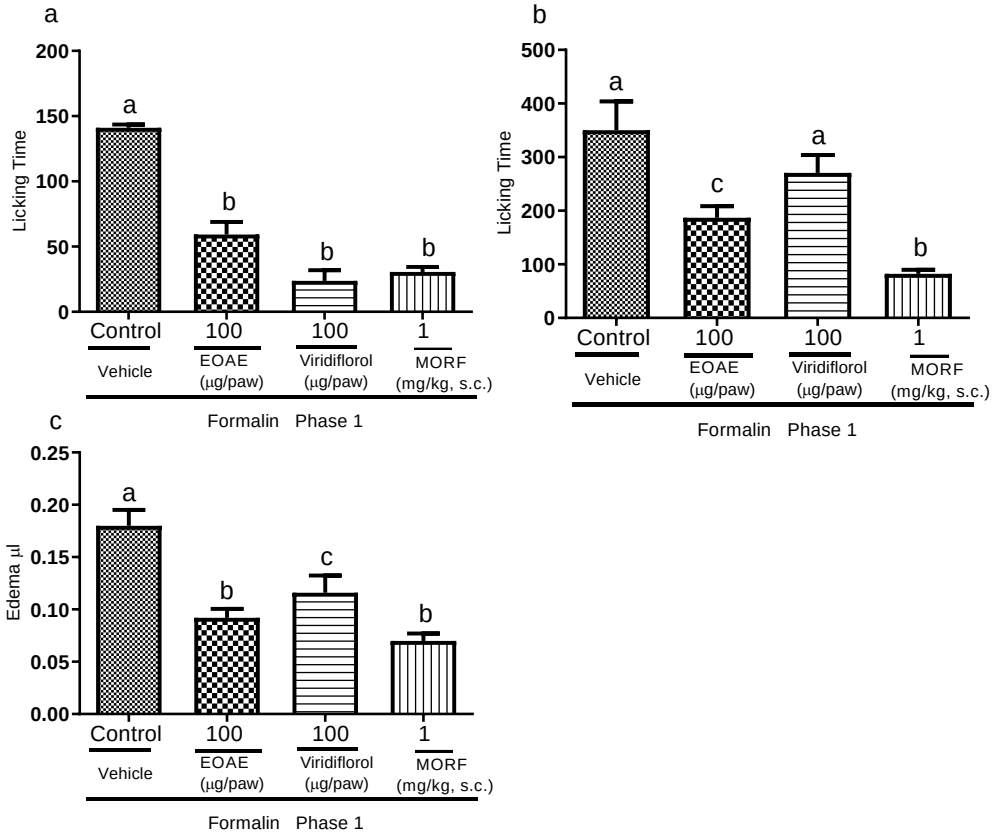
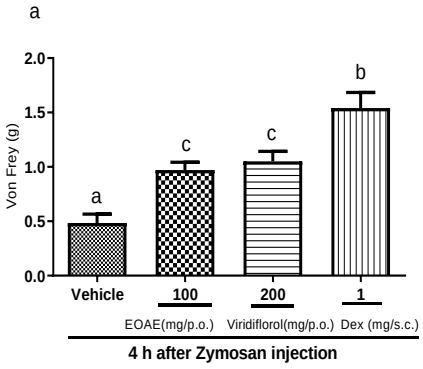
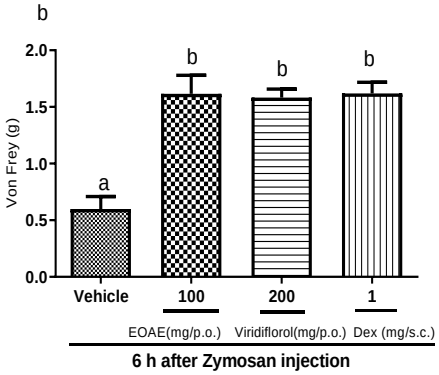
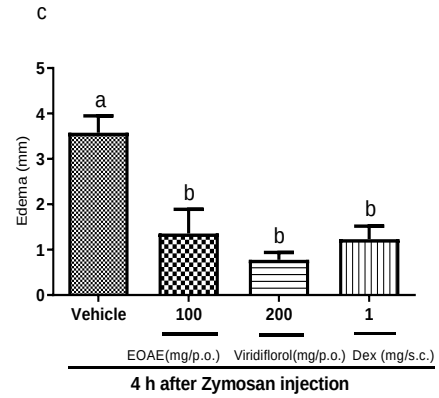


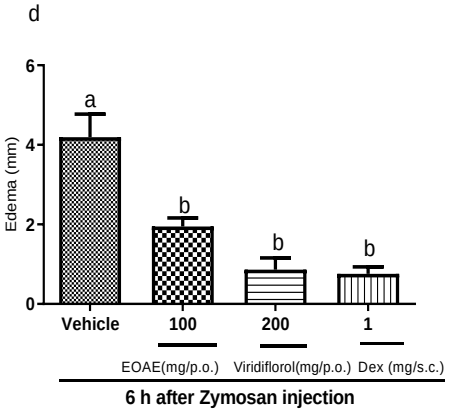
Fig. 2

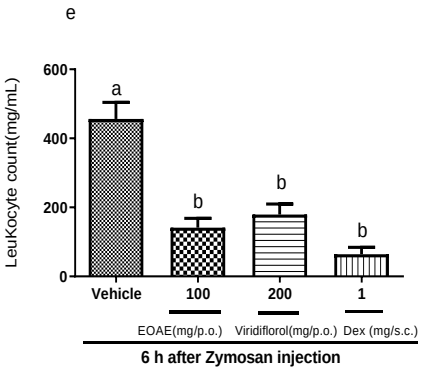
Effects of local administration of *A. edulis* essential oil (OEAE) and viridiflorol on nociception by formalin on licking behavior in phase 1 (a) and phase 2 (b) and on edema (c) in mice. The animals received, intraplantarly, OEAE (100 µg) or viridiflorol (100 µg), or vehicle. After 15 min, intraplantar injection of formalin 2.5% was performed and spontaneous licking behavior and edema were measured. Each bar represents the mean ± SEM of 5 animals. The letters “a”, “b”, and “c” indicate significant differences among groups according to Tukey´s multiple comparisons test

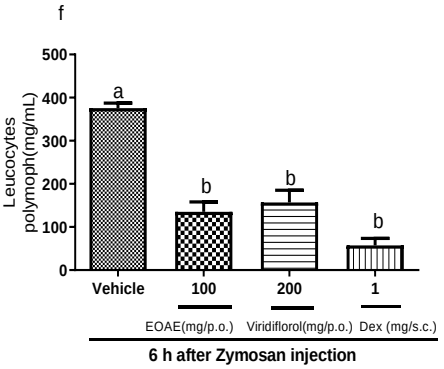

a
Von Frey (g)
2.0
1.5
1.0
0.5
0.0
a
c
c
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
4 h after Zymosan injection

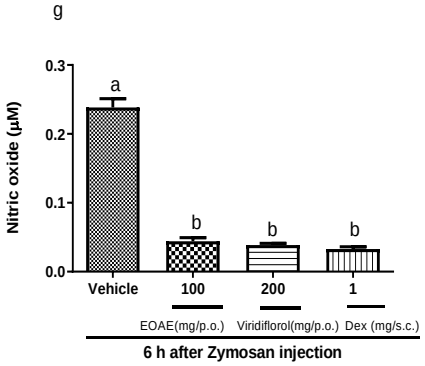

b
Von Frey (g)
2.0
1.5
1.0
0.5
0.0
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
6 h after Zymosan injection


c
Edema (mm)
5
4
3
2
1
0
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
4 h after Zymosan injection


d
Edema (mm)
6
4
2
0
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
6 h after Zymosan injection


e
LeuKocyte count(mg/mL)
600
400
200
0
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
6 h after Zymosan injection


f
Leucocytes polymoph(mg/mL)
500
400
300
200
100
0
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
6 h after Zymosan injection


g
Nitric oxide (µM)
0.3
0.2
0.1
0.0
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
6 h after Zymosan injection

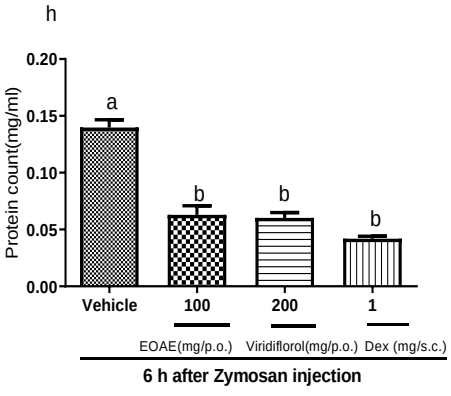

h
Protein count(mg/ml)
0.20
0.15
0.10
0.05
0.00
a
b
b
b
Vehicle
100
200
1
EOAE(mg/p.o.)
Viridiflorol(mg/p.o.)
Dex (mg/s.c.)
6 h after Zymosan injection

Fig. 3 Effect of OEAE or viridiflorol on mechanical hyperalgesia, edema and leukocyte recruitment in the zymosan-induced joint inflammation test in mice. Animals received OEAE (100 mg/kg, p.o.) or viridiflorol (200 mg/kg, p.o.) and control group (vehicle, p.o.) or DEXA (Dexamethasone 1 mg/kg, s.c.). After 1 h, 200 μg of zymosan was injected into the left knee of each animal. Figures 3a and 3b represent mechanical hyperalgesia 4 and 6 hours after injection, respectively. Figures 3c and 3d represent the analysis of knee edema 4 and 6 hours after injection, respectively. Figure E represents the analysis of leukocyte recruitment while Figure 3f represents the analysis of leukocyte recruitment 6 hours after injection. Figure 3g represents nitric oxide analysis and Figure H represents protein analysis 6 hours after injection. Each bar represents the mean ± SEM of 5 animals. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. The letters “a”, “b”, and “c” indicate significant differences among groups according to Tukey´s multiple comparisons test

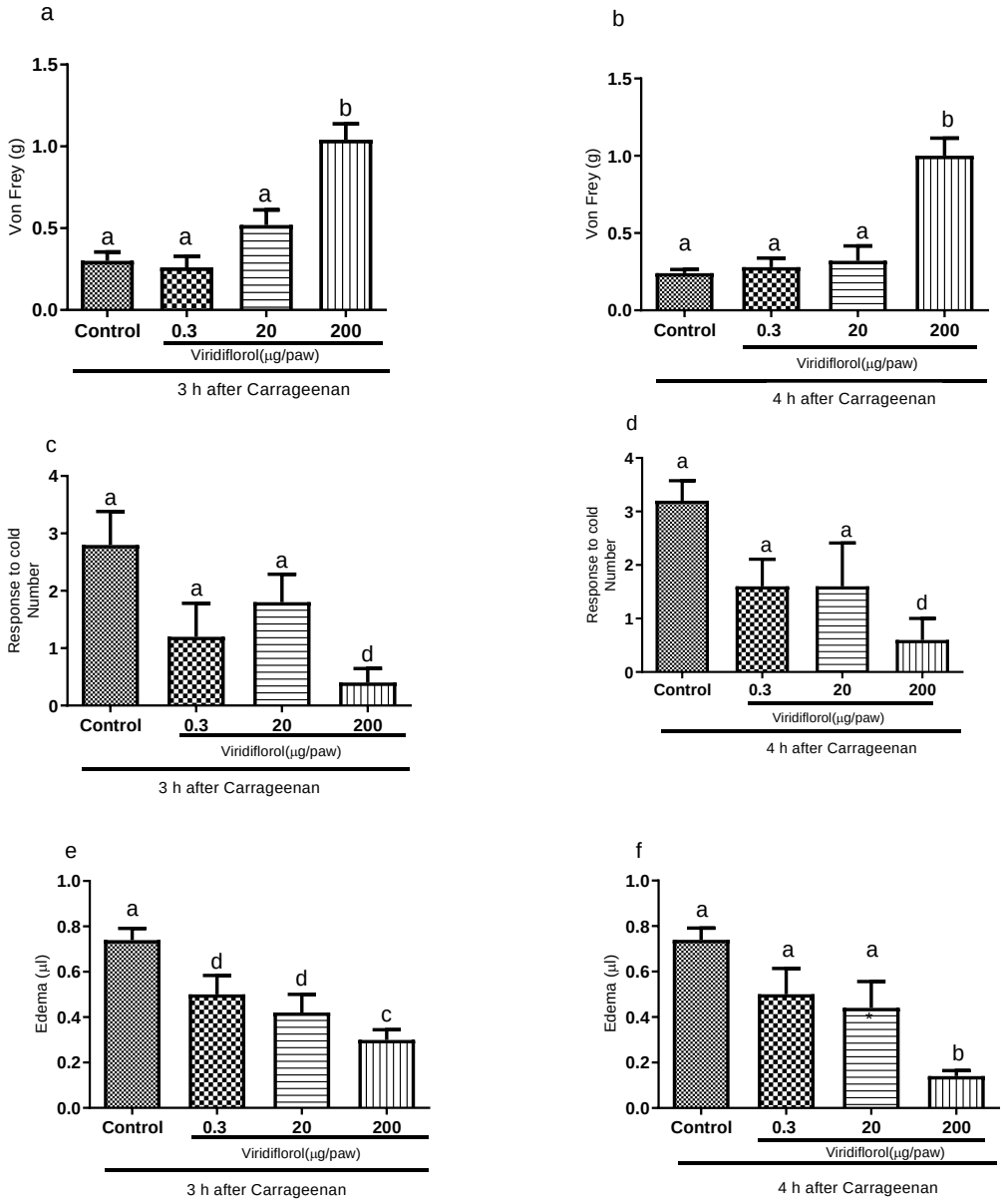


Fig. 4 Effect of intraplantar administration of viridiflorol on mechanical hyperalgesia (4a and 4b), cold hyperalgesia (4c and 4d) and carrageenan (Cg)-induced paw edema (4e and 4f) in mice. The animals received viridiflorol (0.3, 20 or 200 μg/paw) or vehicle (control), and after 1 h, intraplantar injection of Cg (300 μg / paw) was performed. The graphs represented the evaluation of paw edema after 3 (4e) and 4 (4f) hours after Cg injection. Each bar represents the mean ± SEM of 5 animals. Differences among groups were analyzed by one-way analysis

of variance (ANOVA) followed by Newman-Keuls test. The letters “a”, “b”, “c”, and “d” indicate significant differences among groups according to Tukey´s multiple comparisons test

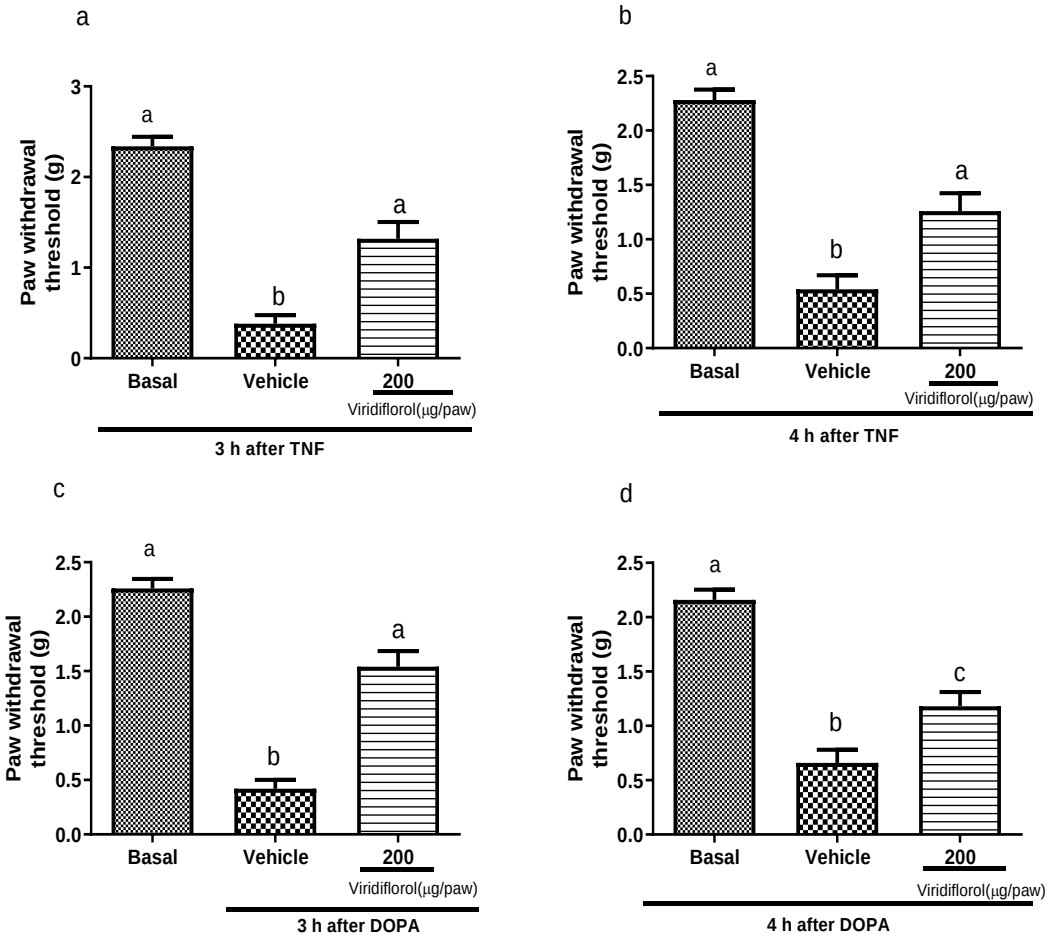


Fig. 5 Effect of intraplantar administration of viridiflorol on mechanical hyperalgesia induced by TNF (5a-b) and DOPA (5c-d). Animals received intraplantar injection (200 µg/paw) of the viridiflorol or vehicle and after 15 min (local treatment), DOPA (30 µg/paw) or TNF (100 pg/paw) were injected into the paw. The basal values were determined before the experiments. Mechanical hyperalgesia was measured 3 and 4 hours after TNF or DOPA injection. Bars express the mean ± SEM of five animals. The letters “a”, “b”, “c” indicate significant differences among groups according to Tukey´s multiple comparisons test

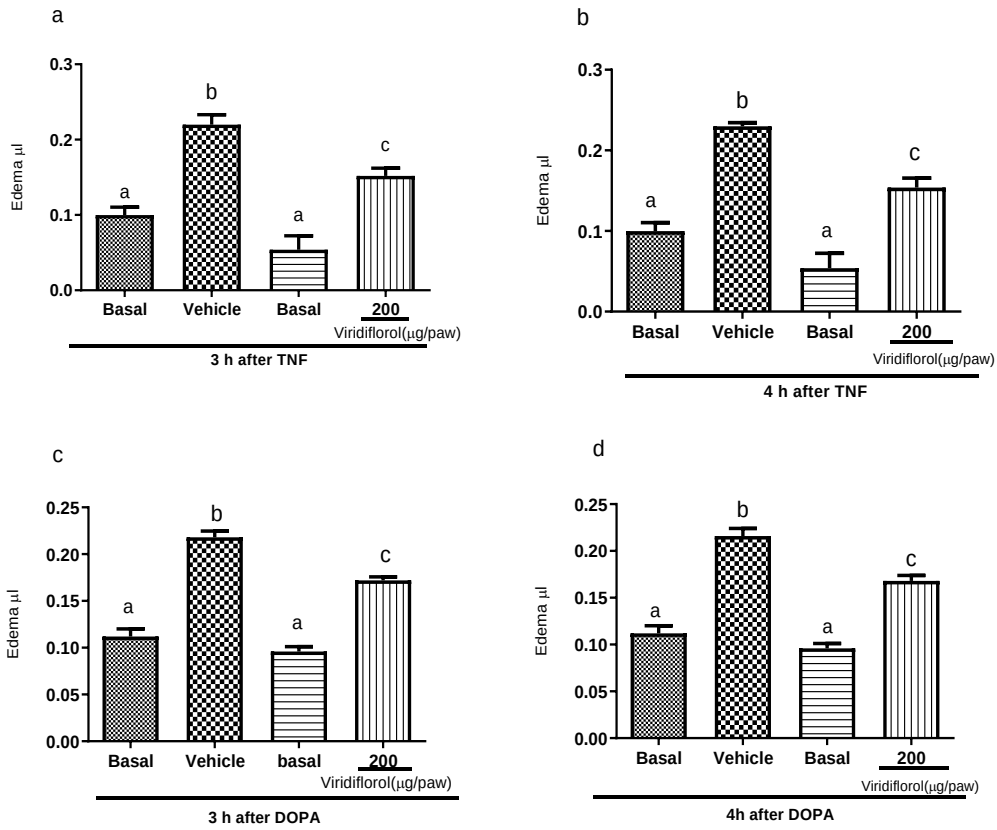


Fig. 6 Effect of intraplantar administration of viridiflorol substance on TNF (6a-b) and DOPA (6c-d) induced paw edema. Animals received intraplantar injection (200 µg/paw) of the substance viridiflorol or vehicle and after 15 min (local treatment), DOPA (30 µg/paw) or TNF (100 pg/paw) were injected into the paw. Paw edema was measured 3 and 4 hours after TNF or DOPA injection. The letters "a", "b" and "c" indicate significant differences among groups according to Tukey´s multiple comparisons test

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ANEXOS

Aprovação no Comitê de Ética do uso de Animais (CEUA):

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA

Dourados-MS, 12 de Novembro de 2020.

CERTIFICADO

Certificamos que a proposta intitulada ***"Estudo do Potencial Farmacológico e Toxicológico de Allophylus edulis"***, registrada sob o protocolo de nº 03/2020, sob a responsabilidade de *Candida Aparecida Leite Kassuya e Natália de Matos Balsalobre* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal Da Grande Dourados, em reunião de 30/04/2020.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*01/07/2020 a 01/02/2021*
Espécie/linhagem/raça	*Rattus norvegicus e Mus musculus - Swiss*
Nº de animais	291 / 63 Wistar e 228 Swiss
Peso/idade	42 dias
Sexo	Machos
Origem	Biotério Central UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA

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