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ORIGINAL ARTICLE

Antiarthritic and Antinociceptive Potential of Ethanolic Extract from Leaves of *Doliocarpus dentatus* (Aubl.) Standl. in Mouse Model

Lidiane Schultz Branquinho, Maria Helena Verdan[1], Saulo Euclides Silva-Filho[2], Rodrigo Juliano Oliveira[3], Claudia Andrea Lima Cardoso[4], Arielle Cristina Arena[5], Candida Aparecida Leite Kassuya

Faculty of Health Sciences, Federal University of Grande Dourados, [1]Faculty of Exact Sciences and Technology, Postgraduate Program in Chemistry, Federal University of Grande Dourados, [4]Center of Studies in Natural Resources, State University of Mato Grosso do Sul, Dourados, [2]Faculty of Pharmaceutical Sciences, Federal University of Mato Grosso do Sul, [3]Faculty of Medicine (FAMED) "Dr. Helio Mandetta", Federal University of Mato Grosso do Sul,, Campo Grande, MS, [5]Department of Morphology, Institute of Biosciences of Botucatu, Paulista State University, Botucatu, SP, Brazil

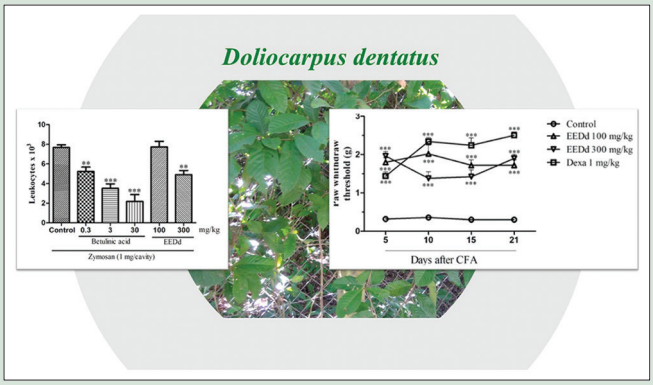
ABSTRACT

Objectives: The folk use of *Doliocarpus dentatus* for pain and inflammatory conditions led our group to evaluate the anti-inflammatory, antinociceptive, and antiarthritic effects of its ethanol extract from the leaves (EEDd) on mouse models. **Results:** Oral treatments with EEDd (100–300 mg/kg) significantly inhibited the formalin-induced nociceptive and cold sensitivity, prevented acetic acid-induced nociceptive behavior, and prevented articular inflammation (including knee edema, leukocyte infiltration, and mechanical hyperalgesia) induced by zymosan. In the peritonitis model, betulinic acid (BA, 0.3–30 mg/kg) and EEDd (300 mg/kg) significantly inhibited zymosan-induced leukocyte infiltration. In the complete Freund adjuvant (CFA) model, oral treatments with EEDd (100–300 mg/kg) for 21 days significantly inhibited mechanical hyperalgesia, cold response, and edema. In the MTT viability assay, EEDd (3–90 µg/mL) did not induce leukocytes cytotoxicity. cytotoxicity. Most models employed male and female *Swiss* mice or, for the CFA test, C57BL/6 mice. **Conclusion:** This study demonstrates that EEDd exhibited antinociceptive, antihyperalgesic, and antiarthritic potential in mice and BA contribute for the EEDd observed activities.

Key words: Arthritis, articular inflammation, betulinic acid, *Doliocarpus dentatus,* pain

SUMMARY

- We evaluated the antiarthritic and antinociceptive potential of the ethanolic extract from the leaves of *Doliocarpus dentatus* in mouse model, based on the folk use of the species. Three doses were used and betulinic acid, a previous identified compound on the extract, was also assessed. The experiments showed that EEDd inhibited nociception, mechanical hyperalgesia, edema, and cold sensitivity, prevented articular inflammation, and did not induce leukocytes infiltration and cytotoxicity in mice.



Abbreviations Used: BA: Betulinic acid; CFA: Complete Freund adjuvant; Dexa: Dexamethasone; DMSO: dimethylsulfoxide; EEDd: Extract in ethanol from the leaves of *Doliocarpus dentatus*; i.p.: Intraperitoneally; MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; NO: Nitric oxide; o.: Oral route; PBS/EDTA: Phosphate-buffered saline that contained ethylene-diaminetetraacetic; p NSAIDs: Non-steroidal anti-inflammatory drugs; RPMI: Roswell Park Memorial Institute 1640 cell culture medium; s.c.: Subcutaneous; SEM: Standard deviation.

Correspondence:
Dr. Maria Helena Verdan,
Federal University of Grande Dourados, Rodovia Dourados/Itahum, Km 12, Mailbox 364, Postal Code: 79804-970, Dourados,
Mato Grosso do Sul, Brazil.
E-mail: mhelenaverdan@gmail.com
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INTRODUCTION

Several rodent models which mimic human arthritis have been developed to study the inflammatory process, pain, and cardiovascular disease and these models are also relevant to the discovery of new drugs.[1,2] Medicinal plants are useful sources to develop new drugs to treat diseases.

Doliocarpus dentatus (Aubl.) Standl. (Dilleniaceae), popularly known as "cipó-vermelho" can be found in Mata Atlântica, Amazon forest and Cerrado biome of Brazil.[3,4] Leaves and roots of *D. dentatus* are used by the population to treat cystitis, pain, and swelling associated with inflammation, as well as diuretic and laxative.[3,5] Scientific studies demonstrated that the chloroform extract of the whole plant of *D. dentatus* has leishmanicidal activity,[6] the diethyl ether extract from lianas were cytotoxic,[4] ethanolic extract from the leaves of *D. dentatus* (EEDd) presented anti-inflammatory and antimycobacterial activities.[7] Ishikawa *et al*. identified betulinic acid (BA), betulin, kaempferol 3-*O*-β-L-rhamnopyranoside,

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sitosterol 3-*O*-β-D-glucopyranoside, quercetin, and kaempferol at EEDd.[7,8]

Thus, the objective of this study was to assess, in addition to the antiarthritic, antinociceptive, and anti-inflammatory potential, the cell viability of the EEDd.

MATERIALS AND METHODS

Vegetal material and extract preparation

Leaves from *D. dentatus* were collected at Federal University of Mato Grosso do Sul (Campo Grande) and were identified by Prof. Dr. Arnildo Pott. A specimen was deposited at UFMS herbarium (Number 49860) and the registration number in the National System for the Management of Genetic Heritage is A2CF88A. EEDd was prepared as described by Ishikawa *et al.*[7]

Chemical reagents

BA, CFA, zymosan and MTT were brought from Sigma-Aldrich (St Louis, MO, USA). The other reagents were acquired from good quality suppliers.

Animals

Male or female *Swiss* mice (30 g) or male C57BL/6 mice (25 g) were used and maintained in polypropylene boxes at the biotherium of the Health Sciences Faculty of UFGD, with controlled temperature (22 ± 2°C) and relative humidity (55 ± 10%). Animals feed and water were provided *ad libitum*. This study was approved by the Ethics Committee in Animal Experimentation of UFGD (39/2017).

Experimental design of the treatments

One hour before experimental procedures, mice were distributed in groups (n = 6/group) for all assays and received the samples in doses as follows: Groups 1 and 2 EEDd with 100 and 300 mg/kg, p.o., respectively; Group 3 vehicle (p.o., negative control). The positive control for Group 4 in experiments of formalin and acetic acid-induced abdominal contortions tests was morphine (5 mg/kg, intraperitoneally [i.p.]). For zymosan-induced articular inflammation and CFA models, dexamethasone (Dexa) (1 mg/kg, s.c.) was used as positive control. In the peritonitis model, BA was tested at doses of 0.3, 3, and 30 mg/kg. For the formalin test, the dose of 30 mg/kg of EEDd was also tested to observe if extract has dose response efficacy.

Formalin test

After 1 h of treatments, male *Swiss* mice received 20 μL of sterile saline with 2.5% of formalin into the right paw.[9] Subsequently, animals were placed individually in glass funnels and they were observed for paw licking from 0 to 5 min (Phase 1 – neurogenic) and 15–30 min (Phase 2 – inflammatory). In the sequence, the cold sensibility was determined by the acetone drop method.[10]

Acetic acid-induced abdominal contortions

One hour after the treatments, the amount of 0.8% of acetic acid (0.1 mL, i.p.) diluted in saline (0.9%) was injected in male *Swiss* mice. The number of abdominal contortions was observed in the sequence for 20 min.

Complete Freund adjuvant model

The animals received the treatments cited at the experimental design, daily, for 21 days. After first treatments, male C57BL/6 mice received an injection of 20 μL of CFA at the right paw.[11] The tests of mechanical hyperalgesia, paw edema measurement, and cold sensibility were performed at days 5, 10, 15, and 21 after CFA injection according to Kuraoka-Oliveira study.[12]

Zymosan-induced articular inflammation

The female *Swiss* mice of each treatment group received intra-articular injection of 20 μL of zymosan (200 μg/articulation) at the posterior right knee by the suprapatellar ligament.[13] Three and 4 h after zymosan injection, the paw lifting was measured using an electronic analgesimeter (InSight®). With the help of a digital micrometer (Mitotoyo®), the knee edema was determined by measuring the difference of the right and left knee diameter (μm) after 4 and 6 h of zymosan injection. After 6 h of zymosan injection, animals were euthanized with an overdose of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg, i.p.) and the knee articulation cavities were washed with 10 μL of phosphate-buffered saline that contained ethylene-diaminetetraacetic (PBS/EDTA).

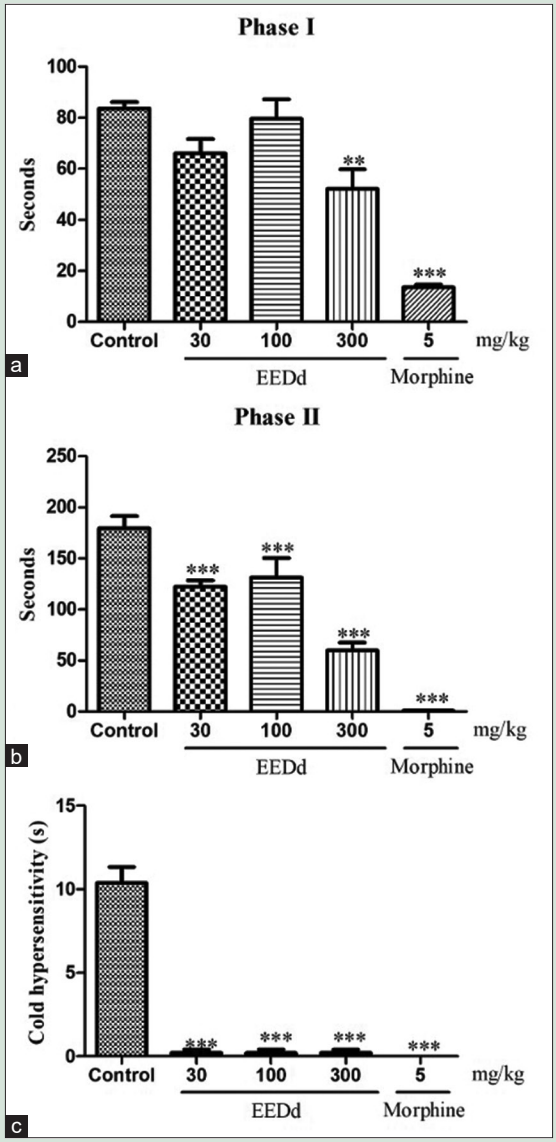

Figure 1: Effect of administration of EEDd (30, 100, and 300 mg/kg, p.o.) and morphine (5 mg/kg, i.p.) on Phase I (a), Phase II (b), and cold hypersensitivity (c) induced by formalin injection in Swiss mice. Each bar represents mean ± SEM of 6 animals. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (vehicle). One-way analyzed by variance followed by the Newman-Keuls test. i.p.: Intraperitoneally; EEDd: Ethanolic extract from the leaves of *Doliocarpus dentatus*

Zymosan-induced peritonitis

The male *Swiss* mice of each treatment group received an injection of 200 µL of zymosan (1 mg/cavity, i.p.).[14] Animals were euthanized with an overdose of inhaled isoflurane (1.5%) after 6 h and cells present at peritoneal cavity were collected by introducing 1 mL of PBS/EDTA. Leukocytes counts were performed with the help of a hematology analyzer (KX-21N Sysmex) and the nitric oxide (NO) concentration was determined according to Griess method.[15]

Cell viability analysis by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

Leukocytes were obtained from the male mice peritoneal cavities after 4 h of zymosan injection (1 mg/cavity, i.p.). At 96-well plates, cells (5×10^5 cells/well) were exposed to 100 µL of Roswell Park Memorial Institute (with 10% of fetal bovine serum + 100 U/mL penicillin + 100 µg/mL streptomycin). After 90 min, EEDd (3, 10, 30, 90 µg/mL) or vehicle and 10 µL of MTT (5 mg/mL) were added to each well and then incubated at 37°C and 5% of CO_2 for 2 h. Posteriorly, the supernatant was removed and 100 µL of dimethylsulfoxide were added to each well, and cells were again incubated at 25°C for 10 min. Absorbance was measured at 540 nm.[16] Viability was determined by the formula: viability (%) = (absorbance of treated cells − blank absorbance)/(control absorbance − blank absorbance) × 100. Data were presented as values of three independent experiments performed in triplicate.

Statistics

Results are expressed as mean ±SEM (standard deviation) employing the software GraphPad Prism (San Diego, CA, USA). The results were statistically analyzed by variance (ANOVA), followed by Newman-Keuls' or Tukey's or by two-way ANOVA followed by Bonferroni's tests. Differences were considered statistically different when $P < 0.05$.

RESULTS

Only the dose of 300 mg/kg of EEDd significantly reduced the nociceptive behavior at Phase I of the formalin-induced spontaneous nociception. All oral treatments with EEDd significantly decreased the nociception at Phase II with follow inhibitions: 32% (30 mg/kg), 27% (100 mg/kg), and 67% (300 mg/kg). The morphine decreased the nociception on both phases (84% and 99%, respectively). All doses tested of EEDd significantly reduced the cold hyperalgesia (maximum inhibition of 98%), while morphine blocked the induction by formalin [Figure 1].

EEDd demonstrated antinociceptive effect on the acetic acid-induced abdominal contortion assay, decreasing the number of contortions in 22% (100 mg/kg) and 29% (300 mg/kg), while morphine was capable to decrease the number of contortions in 98% [Figure 2].

In CFA model, the daily administration of both doses of EEDd showed a significant reduction in mechanical hyperalgesia at all analyzed days. Reductions induced by EEDd to cold sensibility were detected in all analyzed days (76% and 82% at day 5; 77% and 71% at day 10; 64% and 72% at day 15; and 75% and 50% at day 21 for doses of 100 and 300 mg/kg, respectively), as well with Dexa group (88%; 74%; 76%; and 79% at days 5, 10, 15, and 21, respectively). Edema reduction by Dexa was observed at all analyzed days, with maximum reduction of 83% at the 21st day after oral administration of CFA. The group treated with the dose of 100 mg/kg presented maximum inhibition of 59%, while the group of dose 300 mg/kg demonstrated 62% and 48% at days 15 and 21 of treatment, respectively [Figure 3].

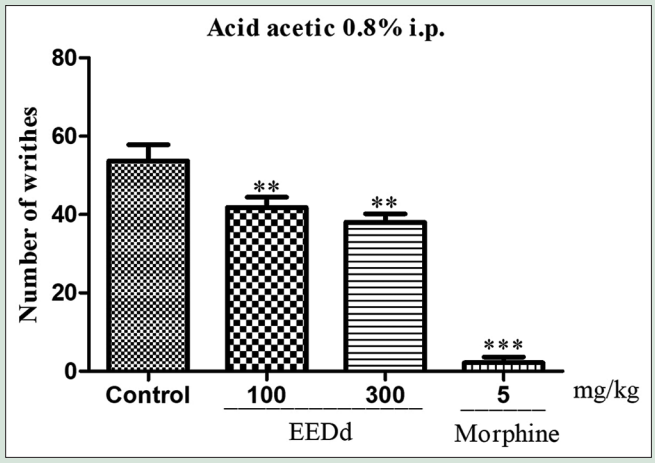

Figure 2: Effect of administration of EEDd (100 and 300 mg/kg, p.o.) and morphine (5 mg/kg, i.p.) on the acetic acid-induced abdominal writhing test in mice. Each bar represents mean ± SEM of 6 animals $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (vehicle). One-way analyzed by variance followed by the Newman-Keuls test. i.p.: Intraperitoneally; EEDd: Ethanolic extract from the leaves of *Doliocarpus dentatus*

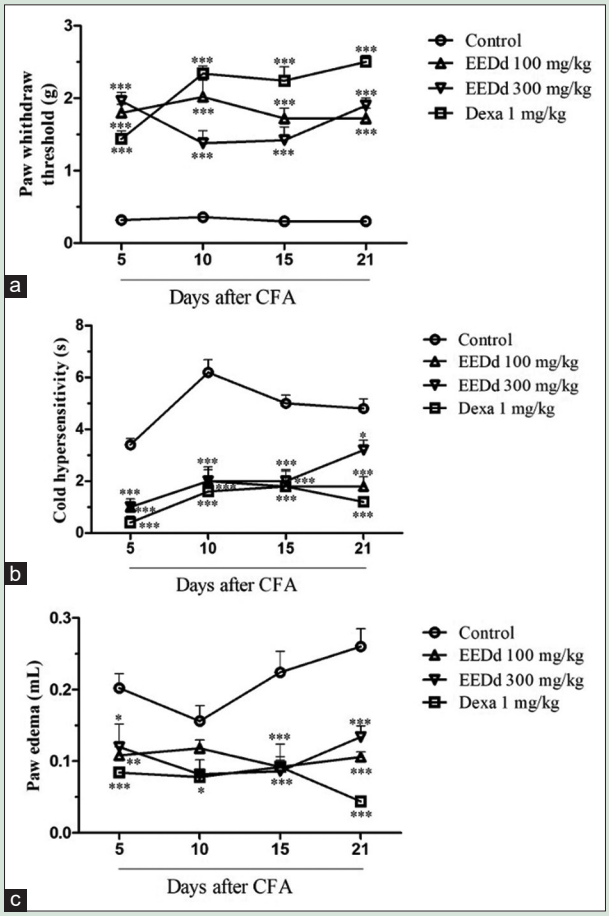

Figure 3: Effect of administration of EEDd (100 and 300 mg/kg, p.o.) and dexamethasone (1 mg/kg, s.c.) (Dexa) on the mechanical von Frey analgesimeter test (a) and cold hyperalgesia (acetone test) (b) and edema (c) after 5, 10, 15, and 21 days from complete Freund adjuvant paw injection in mice. Each bar represents mean ± SEM of 6 animals. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control (vehicle). Two-way analyzed by variance followed by the Bonferroni test. s.c.: Subcutaneous; EEDd: Ethanolic extract from the leaves of *Doliocarpus dentatus*

EEDd presented significant effects on the mechanical hyperalgesia at 4 h, but not 3 h, from zymosan-induced intra-articular inflammation. EEDd decreased the knee edema with inhibitions of 61% (100 mg/kg) and 47% (300 mg/kg) at 4 h and 71% (100 mg/kg) and 55% (300 mg/kg), at 6 h after injection. All treatments significantly reduced the leukocyte migration, showing an inhibition of 35% and 57% at doses 100 and 300 mg/kg, respectively [Figure 4]. Dexa presented significant inhibitions on all evaluations.

EEDd (300 mg/kg) decreased the number of leukocytes migration in 37%, but the treatment with dose of 100 mg/kg did not show significative difference on zymosan-induced peritonitis. The treatments with 0.3, 3, and 30 mg/kg of BA reduced the leukocytes infiltration in 32%, 54%, and 72%, respectively, compared to control group [Figure 5]. Despite that, treatment with EEDd did not produced significative effects at nitrite concentration (results are not shown). EEDd at concentrations of 3, 10, 30, and 90 μg/mL maintained leukocyte of 90.9%, 81.1%, 95.1%, and 75.5%, respectively, showing that EEDd did not provoked cytotoxicity.

DISCUSSION

Studies are relevant to obtain possible products that can combat pain and inflammatory processes. The concentration of BA found in EEDd is about 0.23%[7] and presents biological properties such as anti-inflammatory,[17,18] analgesic and antipyretic,[19] cytotoxic,[20] and antitumor activities.[21] BA could be the substance responsible for EEDd effects since it showed efficacy against inflammation and pain in zymosan-induced peritonitis. We did not test in other models since the literature showed that BA has

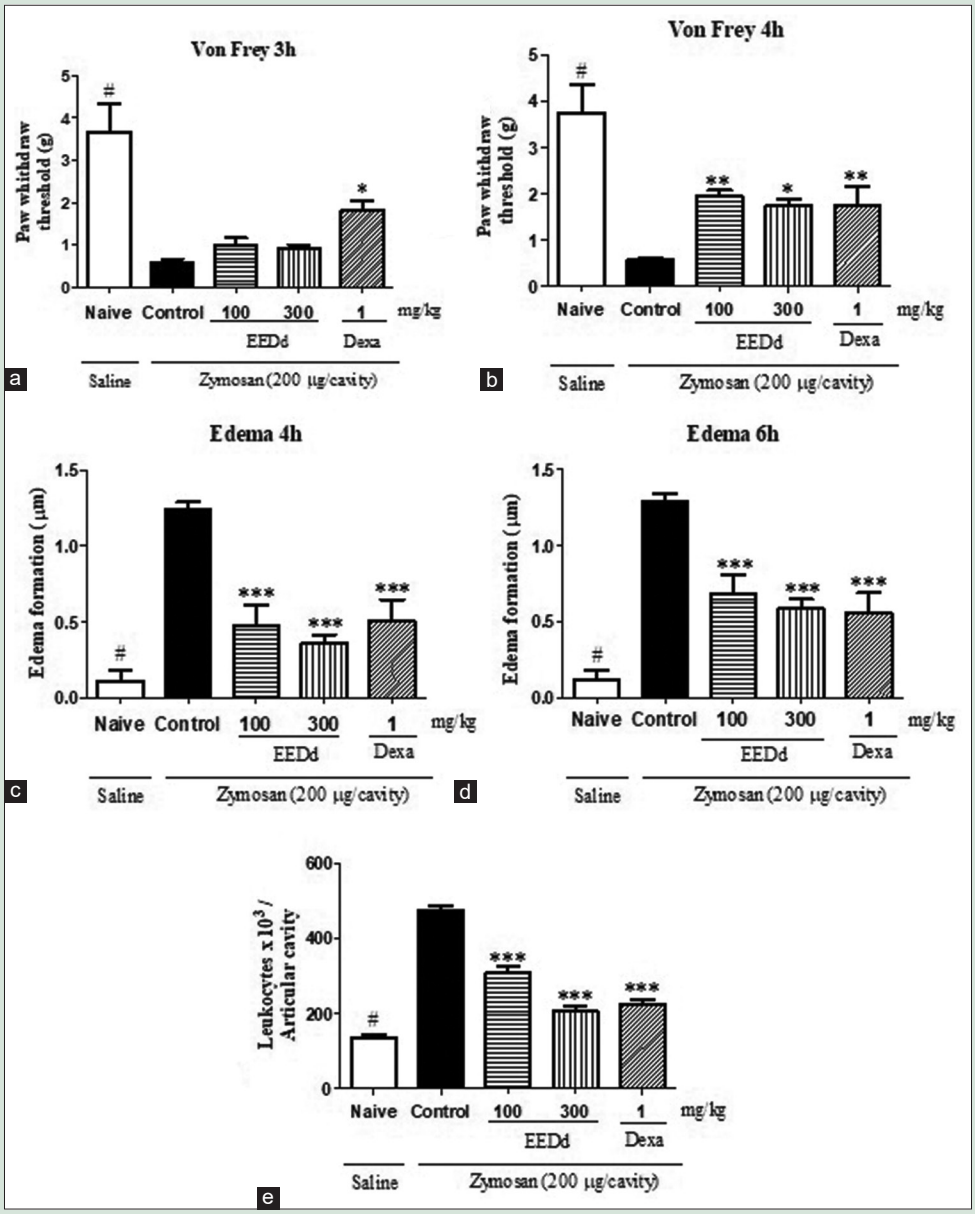

Figure 4: Effect of EEDd (100 or 300 mg/kg, p.o.), vehicle or Dexa (1 mg/kg, s.c.) on mechanical hyperalgesia, edema, and leukocyte recruitment in zymosan-induced articular inflammation test in mice. Analysis of mechanical hyperalgesia in 3 and 4 h after injection (a and b), edema in 4 and 6 h after injection (c and d), and leukocyte recruitment 6 h after injection (e). Each bar represents mean ± SEM of 6 animals. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to the control group, # indicates a statistically significant difference from the vehicle group. One-way analyzed by variance followed by the Newman–Keuls test. s.c.: Subcutaneous; EEDd: Ethanolic extract from the leaves of *Doliocarpus dentatus*

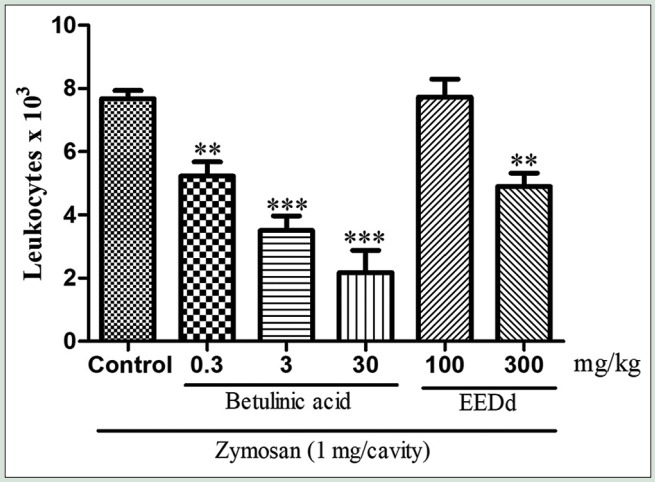

Figure 5: Effect of oral administration of betulinic acid (0.3, 3. 30 mg/kg) and EEDd (100 and 300 mg/kg) on leukocyte migration 6 h after zymosan injection (1 mg/cavity, i.p.) in *Swiss* mice. Each bar represents mean ± SEM of 6 animals. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (vehicle). One-way analyzed by variance followed by the Tukey's test. i.p.: intraperitoneally; EEDd: Ethanolic extract from the leaves of *Doliocarpus dentatus*

anti-inflammatory and analgesic effects.[22-24] The effective dose of EEDd to decrease the leukocyte infiltration at the zymosan-induced peritonitis was 300 mg/kg [Figure 5]. According to the concentration, the effective dose of BA should be 0.69 mg/kg, and the results showed that the doses from 0.3 to 30 mg/kg were effective in this model. In this way, we demonstrated that the analgesic activity of EEDd can be due to, in parts, the presence of BA in the extract.

The formalin assay is a model of nociception that has two phases, the first one is related to neurogenic pain since Type C and Aδ fibers are activated and the second phase related to inflammatory nociception, in which inflammatory mediators release and stimulate the nociceptors.[9] Both phases are antagonized by opioids, while the nonsteroidal anti-inflammatory drug compounds do not demonstrate great efficacy in Phase I.[25] The oral administration of EEDd, at all doses, substantially inhibited the inflammatory phase, in a dose-dependent manner and could interfere in peripheral and central pain mechanisms of formalin-induced nociception [Figure 1].

The administration of acetic acid at the peritoneal cavity promotes nociceptive pain and peripheral sensibilization.[26] EEDd substantially decreased the number of abdominal contortions [Figure 2]; confirming the studies performed by Oyebanji *et al*.[19] and Ali *et al*.[27] which correlated the antinociceptive effect of the acetic acid-induced abdominal contortions model in mice with BA and kaempferol. Both molecules are present in EEDd, and their effects may be responsible for the folk use of *D. dentatus* to treat pain and inflammatory problems.[7]

The intraplantar injection of CFA induce an inflammatory response of long duration.[12] The CFA-induced polyarthritis is characterized by causing similar effects such as the rheumatoid arthritis.[28] In our study, the animals were evaluated for 21 days to investigate the antiarthritic effects and the results demonstrated that the long-term treatment with EEDd exhibited mechanical and thermic antihyperalgesic properties at the mechanic and thermic hyperalgesia, as well as antiedematogenic effect. Ishikawa *et al*.[7] demonstrated that the treatment with doses of 100 or 300 mg/kg of EEDd inhibited the edema as well as the mechanical hyperalgesia induced by carrageenan.[29] In addition, BA and quercetin (both present in EEDd)[7] showed therapeutic action on rheumatoid arthritis, decreasing the amount of inflammatory cytokines and modulating the immune response at the CFA-induced arthritis.[17,30] The analgesic and anti-inflammatory effects of EEDd confirm the previous evidences of inflammatory stimulus by carrageenan, due to its antiarthritic potential.

Zymosan induces the release of pro-inflammatory cytokines, chemokines, NO,[31] products from arachidonic acid, complement system, endotelin-1, and neutrophils infiltration.[32] Our data showed that EEDd inhibited the mechanical hyperalgesia, edema, and the leukocytes infiltration at zymosan induced-inflamed knee [Figure 4]. Guazelli and coauthors demonstrated that quercetin decreased pain, edema, and leukocytes infiltration and the production and expression of inflammatory molecules at the zymosan-induced inflamed joint.[33] The *in vitro* treatment with EEDd did not compromised the cell viability in none of the tested concentrations, suggesting that the inhibitory effect at the leukocyte's migration was not due to toxic effects that cause cell death.

CONCLUSION

The data presented here demonstrated the anti-inflammatory, antinociceptive, and antiarthritic potential of EEDd in mice. The mechanism of EEDd is probably related to the reduction of leukocyte migration, inhibiting pro-inflammatory cytokines. These properties make a highly interesting perspective for the treatment of chronic inflammatory diseases.

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Conflicts of interest

There are no conflicts of interest.

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