# *Serjania marginata* induced inhibition of mycobaterial growth, reduced cytokine and inflammatory parameters

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# Abstract

**Objectives:** This study investigated the antimycobacterial, anti-inflammatory and antihyperalgesic effects of hydroalcoholic extract from leaves of *S. marginata* (EESM) in *in vitro* and *in vivo* models.

**Methods and Results:** EESM (0.98–1000 µg/ml) was evaluated in *in vitro* against *Mycobacterium tuberculosis, M. bovis, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis.* The EESM oral administration (p.o.) (30, 100 and 300 mg/kg) and dexamethasone subcutaneous injection (s.c.) (1 mg/kg) were tested against the carrageenan-induced inflammatory paw edema and pleurisy in *Swiss* mice. The EESM (30 and 100 mg/kg, p.o.) and dexamethasone (1 mg/kg, s.c.) were tested against the CFA-induced paw inflammation and *M. bovis* (bacillus Calmette-Guerin - BCG)-induced pleurisy in C57bL6 mice. The minimum inhibitory concentration (MIC) of EESM in the presence of *M. tuberculosis* was 62.4 µg/ml. The values of MIC of EESM in the presence *S. epidermidis, K. pneumoniae* were 1000 µg/mL while EESM did not interfere against *P. aeruginosa* growth. EESM significantly inhibited paw edema/mechanical hyperalgesia in carrageenan induced paw inflammation and leukocytes migration/proteins exudation in carrageenan-induced pleurisy model. In the BCG-induced pleurisy model, the daily treatment for 7 days, with EESM inhibited the levels of IL-1β in blood and in pleural exudate. The EESM did not alter the mycobacterial growth in the cell culture from pleural lavage, spleen and liver samples collected from BCG-treated animals. The EESM significantly inhibited the persistent edema and mechanical hyperalgesia induced by CFA.

**Conclusion:** This study confirms the EESM anti-inflammatory property and showed that EESM has high potency in inducing inhibition of mycobaterial growth and low potency or no effects in relation to other microorganisms.


# Introduction

The immune and inflammatory process respond promptly to the presence of infectious pathogens. If the infectious pathogens persist, the body develops an adaptative immune and inflammatory response [1,2]. For the discovery of potential anti-inflammatory/anti-infective agents, the infectious pathogens or PAMPs (Pathogen Associated Molecular Patterns) could be used in experimental models. The inflammatory response to live infectious agents and PAMPs depends on several components, such as leukocytes, receptor pattern recognition receptors (PRR), the complement system, pro-inflammatory mediators, cytokines, among others [2–5]. Pharmacological control of an inflammatory response to an aggressive pathogen is carried out using antibiotics. Depending on the development of multi-resistant strategies of pathogen and host response, endogenous and pharmacological control is often not fully effective.

In relation to the prevalence of tuberculosis, a disease caused by *Mycobacterium tuberculosis* has increased due to the development of resistant strains, the use of anti-tumor necrosis fator (TNF)s and the co-infection by the human immunodeficiency virus [1,6,7]. In addition, the currently recommended vaccine, *M. bovis* - bacillus Calmette-Guerin (BCG), attenuates the development of tuberculosis in severe form in

children, however it does not prevent primary infection or reactivation of latent tuberculosis infection in adults and new vaccines have been developed [6,7].

The alternative strategies are needed to eliminate the pathogens and also harmful inflammation [4]. The plant candidate as alternative source is *Serjania marginata* Casar. (Sapindaceae) found in Brazil, Paraguay, Bolívia and Argentina [8–10]. The traditional knowlegde calls this plant as "cipó-uva" or "cipó-timbó" [11,12] or blancuzco [10] and the leaves, prepared in the form of juice, are used to treat stomachache by the Guarani Indians [13].

Gastroprotective activity of *S. marginata* leaves aqueous [14] and hydroalcoholic extracts [15] were scientifically shown. [15] also showed that hydroalcoholic extract from leaves of *S. marginata* was antimicrobial, antidiarrheal, and (anti)mutagenic. [16] demonstrated that *S. marginata* leaves aqueous extract was not toxic after a single exposure, however if it was used after prolonged periods it affected some parameters. [15] showed that after 14 days of administration hydroalcoholic extract also affected some parameters such as induce gastric lesions.

The present work was motivated since the description of *in vitro* antimicrobial property of *S. marginata* was related by [15] and by *Serjania sp* are used against other infections and inflammatory conditions in folk medicine [13,17,18]. This study investigated the anti-inflammatory and anti-mycobacterial activity of hydroalcoholic extract from leaves of *S. marginata* (EESM) in several models contributing to the pharmacological knowledge of this plant.

# Material And Methods

### *Vegetal material and extract preparation*

Leaves from *S. marginata* were collected in February 2011 in an area of Cerrado in Dourados, Mato Grosso do Sul, Brazil (longitude of 55°19'24.9" W, and an altitude of 429 m). The dried (500 g) and pulverized leaves were extracted by percolation at room temperature with EtOH/$H_2O$ (7:3, v/v). The hydroalcoholic extract was filtered, concentrated under vacuum (± 40 °C), and lyophilized (yield: 33% w/w). The phytochemical analysis of EESM was performed by [12].

### *Chemical reagents*

Complete Freund's Adjuvant (CFA), dexamethasone (DEXA), isoniazid, and carrageenan were brought from Sigma-Aldrich (St Louis, MO, USA). Ketamine and xylasine were obtained from Syntec (Santana de Paraíba, SP, Brazil). BCG was obtained from Fundação Ataulpho de Paiva (Rio de Janeiro, Brazil). D*imethylsulfoxide (*DMSO) and other reagents were acquired from other suppliers.

### *In vitro antimycobacterial and antibacterial activity*

The *in vitro* antimycobacterial activity was performed according to [19]. The EESM was dissolved in 5% of DMSO in a solution of pure water and the minimum inhibitory concentration (MIC) values for EESM was assayed at range of 0.98 – 250 µg/ml and for isoniazid between 0.004 – 1 µg/ml in the presence of the *M. tuberculosis* strain H37Rv (ATCC27294).

The *in vitro* antibacterial activity methodology was performed according to [20] and [21]. The EESM was dissolved in 5% of tween 80 in a solution of pure water and the minimum inhibitory concentration (MIC) values for EESM was assayed at 8.125, 16.25, 32.5, 75, 125, 250, 500, 1000 µg/ml. Bacterial cultures (*Klebsiella pneumoniae* ATCC *700603*, *Pseudomonas aeruginosa* ATCC *27853*, and *S. epidermidis* ATCC *12228*) will be activated through subcultures on Mueller-Hinton agar for 24 h at 37 °C.

After activation and/or subculture, the bacterial inoculum will be standardized, which consisted of the preparation of a bacterial suspension in sterile saline, standardized on the McFarland 0.5 da scale (1 x $10^8$ CFU/ml) and diluted 1:10 also in saline (1 x $10^7$ CFU/ml). After dilution, 10 µL volumes will be transferred to the sterile microplate wells, containing a final volume of 100 µL of Mueller-Hinton broth plus the different final concentrations of EESM, resulting in a final inoculum of approximately 10 x $10^5$ CFU/ml and later taken to greenhouse at 37 ºC for 24 h. The MICs (minimum inhibitory concentrations) in microdilution will be determined by reading in a spectrophotometer at 610 nm in a microplate observing the lowest concentration in which the EESM will completely inhibit microbial growth [20,21].

### *Animals, control drugs' administration, EESM dilution and doses for in vivo experiments*

Female and male *Swiss* (28 – 32 g) and C57BL/6 mice (25 – 30 g) were provided by the central biotherium at the Federal University of Grande Dourados (UFGD). Animals were kept in polypropylene boxes in a sectorial Biotherium at Faculty of Health Sciences at UFGD with the following conditions: 22 °C, in the presence of a 12 h light/dark cycle, with food and water *ad libitum.* The EESM was dissolved in saline solution (0.9 %) and the dose was calculated according to mice weight. Vehicle solution is the saline solution since it was used to dissolve the EESM. The EESM oral administration (p.o.) doses used in the carrageenan-induced paw inflammation and pleurisy were 30, 100 and 300 mg/kg to verify if the EESM presents a dose-response profile. As the doses of 30 and 100 mg/kg of EESM were effective against inflammatory parameters, these doses were tested also against BCG-induced pleurisy and CFA-induced inflammatory reaction. To verify the inhibitory efficacy of the commercial drugs, the dose of 1 mg/kg (by subcutaneous route – s.c.) of dexamethasone was used as control group in *in vivo* models while the dose of 25 mg/kg (p.o.) of isoniazid was used in BCG model.

### *Paw edema, mechanical and cold hyperalgesia evaluation in carrageenan induced paw inflammation*

The *male Swiss mice* were distributed in five experimental groups (n = 6) and one hour before the carrageenan injection, they were treated (p.o.) with 30, 100 and 300 mg/kg of EESM, with the vehicle and dexamethasone (1 mg/kg, s.c.). Subsequently, the animals received a subcutaneous injection (100 µL) of a solution containing 300 µg of carrageenan [22] dissolved in sterile 0.9 % saline in the right hind paw. The

contralateral paw, which was used as a control, received 100 µL of saline. Paw edema was analyzed using a plethysmometer (Panlab, Spain) at 1, 2 and 4 h after injection. In addition, mechanical hyperalgesia was assessed using an electronic version of the von Frey test (Analgesimeter von frey, InSight) and cold sensitivity was analyzed with the acetone drop test [23] at 3 and 4 h after carrageenan injection.

### *Leukocyte migration and protein analysis in pleural exudate collected from carrageenan induced pleurisy*

Female *Swiss* mice were distributed in five experimental groups (n = 6) and one hour before the pleurisy, they were treated (p.o.) with 30, 100 and 300 mg/kg of EESM, with the vehicle and dexamethasone (1 mg/kg, s.c.). Subsequently, with the aid of an adapted needle, 100 µL of carrageenan (containing 100 µg) was injected into the right side of the animals' chest cavity to induce inflammation [24]. Four hours after the injection of carrageenan, the animals were euthanized (100 mg/kg of ketamine and 10 mg/kg of xylasine by intraperitoneal route (i.p.)) and, afterwards, the exudate was collected by washing the chest cavity with 1 mL of phosphate buffered saline (PBS). Having made the collection, the total number of leukocytes in the exudate was determined by the KX-21N Sysmex apparatus and the protein exudation was evaluated by the Bradford reaction, using the commercially available Bradford kit (Bioagency, São Paulo, Brazil).

### *Leukocyte migration,* Il-1β *dosage and CFU growth in BCG-induced pleurisy model*

Groups of six male C57BL/6 mice were treated orally with EESM (30 or 100 mg/kg), isoniazid (25 mg/kg), saline solution (0.9 %; control group) and saline solution (0.9 %; naive group). Pleurisy was induced 1 h after the previous treatment with an intrapleural injection with 0.1 mL of a suspension of BCG (4 X $10^5$ colony forming units (CFU) into the right pleural cavity. In the naïve group, instead of a BCG injection, the mice received a saline injection in the pleural space. The animals were treated orally with EESM, isoniazid, saline solution (0.9 %) once daily for 7 days [25]. After 7 days, the animals were euthanized (100 mg/kg of ketamine and 10 mg/kg of xylasine, i.p.) and the pleural cavity was washed with 1 ml of sterile phosphate buffered saline. The washed sample of 50 µL was diluted with Evans blue to determine the total number of leukocytes. The rest of the sample was centrifuged, and the supernatant was stored for the Il-1β measurements using ELISA. The blood was also collected from each animal for Il-1β measurements. The precipitate was suspended in 0.5 mL of sterile ultrapure water and 0.1 mL of Ogawa Kudu, and 0.1 mL of the suspension was plated onto 7H11 agar. The spleen and liver were macerated with 1 mL of sterile saline solution, and 0.1 mL of the suspension was plated on 7H11 agar. The cells were cultured for 60 days at 37°C in 5% $CO_2$ [26].

### *Paw edema, mechanical and cold hyperalgesia evaluation in CFA induced paw inflammation for 22 days*

Groups of six male C57BL/6 mice were treated with vehicle (control group), EESM (30 and 100 mg/kg, p.o.), and dexamethasone 1 mg/kg (s.c., the positive control group) every day, once a day for 22 days. After the first treatment, the animals received 30 µL of Freund's complete adjuvant (CFA) (oil suspension

containing inactive *M.tuberculosis*) via intraplantar injection in the right paw. Mechanical and cold sensitivity and paw edema were measured 6, 11, 16 and 22 days after the injection of CFA.

### *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 Newman-Keuls test was chosen as a post hoc (GraphPad Prism Software). The percentage of inhibition was calculated from the control group. Differences were considered to be significant when $p < 0.05$.

## Results

### *Effects of EESM on M. tuberculosis and other microorganisms in vitro*

The MIC value of EESM in the presence of the *M. tuberculosis* strain was 62.4 µg/ml, while the MIC value of isoniazid was 0.030 µg/ml. EESM was effective against *S. epidermidis*, *K. pneumoniae* in 1000 µg/ml and was not effective against *P. aeruginosa.*

### *Effects of EESM on paw edema induced by carrageenan*

The paw edema was significantly inhibited at doses of 100 and 300 mg/kg of EESM after 2 h of carrageenan injection with inhibition of 38 % and 44 %, respectively ($p < 0.05$) (Figures 1B). After 4 h, all doses showed significant differences from the control group with inhibition of 36 %, 33 % and 58 % in doses 30, 100 ($p < 0.05$) and 300 mg/kg ($p < 0.01$). The dexamethasone group showed significant inhibition at 1, 2 and 4 h.

Dexamethasone was different from all treated groups when the comparison among groups was perfomed (Figures 1A-C). The groups treated with doses of 100 and 300 mg/kg differed from the control and from the group treated with 30 mg/kg of EESM (Figure 1B) and in figure 1C the groups treated with doses of 30 and 100 mg/kg differed from the control and from group treated with 300 mg/kg of EESM.

In the carrageenan induced mechanical hyperalgesia, 100 % inhibition was observed in two evaluations at the dose of 300 mg/kg and the dexamethasone group ($p < 0.001$) (Figures 2A and 2B). The dose of 100 mg/kg also demonstrated prevention of hyperalgesia development, with 100 % ($p < 0.001$) and 96 % ($p < 0.01$) efficacy, in 3 and 4 h after carrageenan injection, respectively (Figures 2A and 2B). The dose of 30 mg/kg was not effective for the prevention of carrageenan paw hyperalgesia. The control and the group treated with 30 mg/kg of EESM differed significantly from the other treated groups (Figures 2A and 2B). The groups treated with doses of 100, 300 mg/kg and dexamethasone did not differ among themselves (Figure 2A) and in figure 2B the groups treated with doses of 300 mg/kg and dexamethasone differed from the other treated groups.

In the cold nociceptive response, no doses showed a significant difference from control group, however the dexamethasone group obtained an inhibition of 52 % and 49 % ($p < 0.05$) in 3 and 4 h, respectively

(Figures 2C and 2D).

### *Effects of EESM on pleurisy induced by carrageenan*

In pleurisy induced by carrageenan, EESM 100 and 300 mg/kg significantly reduced leukocyte migration ($p < 0.001$) by 59 and 67 %, respectively, compared to the control group (Figure 3A). In protein exudation evaluation, EESM demonstrated significant difference ($p < 0.001$), with 53, 42 and 60 % of inhibition, at doses 30, 100 and 300 mg/kg, respectively. The dexamethasone group also demonstrated a significant decrease in both evaluations ($p < 0.001$) (Figure 3).

The statistical comparison among groups showed that control and the group treated with 30 mg/kg of EESM differed from the other groups in Figure 3A while only control group differed from others groups in Figure 3B.

### *Effects of EESM on Leukocyte migration,* Il-1β *dosage and CFU growth in BCG-induced pleurisy model*

After 7 days of oral treatment, EESM (30 and 100 mg/kg) did not decrease the leukocyte migration into the pleural spaces after intrathoracic injection of BCG, however isoniazid reduced significantly the leukocyte invasion to the pleura (Figure 4A). In pleural exudates, the reduction of IL-1β levels were significant with inhibition of 72 % and 76 % with the treatment of 30 and 100 mg/kg EESM (p.o.) and 76 % isoniazid (25 mg/kg, p.o.) (Figure 4B). In serum, the reduction of IL-1β levels were significant with inhibition 50 % and 59 % with the treatment of 30 and 100 mg/kg EESM (p.o.) and 67 % isoniazid (25 mg/kg, p.o.) (Figure 4C). The statistical comparison among groups showed that only the control group differed from the other groups in Figures 4B and 4C.

During the cell culture of the pleural lavage, spleen and liver samples collected from BCG treated animals, the EESM (30 or 100 mg/kg) did not alter the mycobacteria growth (results not shown) and the UFC count did not differ from control group (results not shown) until 60 days. The negative control group samples presented mycobacterial growth in all cultivated plaques, with a mean of 54 CFU after 60 days of culture, while the isoniazid group samples did not show any growth after 60 days (results not shown).

### *Effects of EESM on CFA induced paw inflammation*

A single daily oral treatment with EESM for 6 days at doses of 30 and 100 mg/kg and dexamethasone (1 mg/kg, s.c.) reduced mechanical hyperalgesia in 100 % respectively (Figure 5A). After 11 and 16 days from CFA injection, no significant inhibitions in mechanical response were observed with 30 mg/kg of EESM, however a significant inhibition was detected with dexamethasone (Figure 5B and 5C). At 22 days after CFA, the inhibition observed with 30 mg/kg of EESM was 100 % (Figure 5D). The groups treated with EESM (30 and 100 mg/kg) did not differ statistically among themselves at days 6, 11 and 16, however they were different from the dexamethasone group on 11 and 16 days (Figures 5A-C). The group treated with 100 mg/kg differed from the group treated with 30 mg/kg at 22 days after CFA injection (Figure 5D).

The dose of 100 mg/kg, but not the dose of 30 mg/kg of EESM and dexamethasone groups showed a significant difference in paw edema on days 6 and 11 after the administration of CFA with the inhibition of 21 % and 47 %, respectively (Figures 6A and 6B). On days 16 and 22, the doses of 30, 100 mg/kg of EESM and dexamethasone groups were different from the control groups with inhibitions of 33 %, 50 % and 65 % on day 16 and 57 %, 67 % and 87 % on day 22, and 57 %, 67 % and 87 % on day 22, respectively (Figures 6C and 6D). The treatment with 100 mg/kg did not statistically differ from dexamethasone group on days 6, 11 and 16, however on day 22 these groups were statistically different (Figure 6). Both treatments with EESM were not statistically different to dexamethasone group on day 16, nevertheless on day 22 both treatments with EESM differed from dexamethasone group.

In cold hyperalgesia induced by CFA, the treatment with EESM at dose of 100 mg/kg was statistically different from the control group, with the inhibition 52 % showing similarity inhibition to dexamethasone group on day 6. The treatments with both doses of EESM did not differ from the control group on days 11, 16 and 22.

# Discussion

Species of the *Serjania* genus have been used in folk medicine for their anti-inflammatory potential in Brazil and in Mexico [27] while *S. marginata* leaves are squeezed for juice by the Bolivian people as an analgesic agent for stomach [13]. The present study was the first one to show the anti-inflammatory, analgesic and potential antibiotic properties of EESM in inflammatory/infection/hyperalgesic models. The data of the present work corroborate with the traditional folk use of *S. marginata* as an analgesic and expand the information regarding its use.

Some inflammatory models used in this present research involve carrageenan and CFA. The carrageenan was used in two acute inflammatory models to analyze the efficacy and potency of anti-inflammatory agents until 48 h [28]. When the carrageenan is administered into the mice paw the edema, mechanical and cold hyperalgesia can be measured [29] and when it is injected by intrapleural route the leukocyte and protein can be measured in pleural exudate [30]. In the present study the EESM showed to be effective against acute edema (2, and 4 h, Figure 1B and 1C), mechanical hyperalgesia (3 and 4 h, Figures 2A and 2B), however the cold hyperalgesia (Figures 2C and 2D) was not inhibited in carrageenan paw inflammatory model. In the carrageenan induced-pleurisy model, both leukocyte migration and protein exudation were inhibited by EESM (Figures 3A and 3B). In this model, the EESM induced a dose dependent inhibition (Figures 1B, 1C, 2A, 2B and 3A) in which the efficacy response to EESM differed significantly when its dose was increased. In Figure 2B the classical dose-response effect could be detected since each dose of EESM significantly differed in efficacy among themselves. As far as we know, only the research of [15] showed the myeloperoxidase activity inhibition by EESM in ulcer models. The present study showed the anti-inflammatory and mechanical anti-hyperalgesic (analgesic) response of EESM occurs a dose-dependent manner.

The major compounds such as flavonoids, proanthocyanidins and saponins have already been described in EESM [12]. Flavonoids and proanthocyanidins (tannins) are the chemical classes related to the antioxidant and anti-inflammatory activities of some plant species. Flavonoids, due to their chemical structure and their redox capacity, can interfere in important mechanisms that are related to inflammatory processes such as enzyme inhibition [31,32]. Proanthocyanidins have anti-inflammatory potential that could modulate the arachidonic acid and the nuclear factor NF-kB [33]. [12,15] showed that EESM gastroprotective property is associated with the presence of proanthocyanidins and flavonoids. Other substances present in EESM are pulsatilla D, hederacolchiside A1, salzmannianosid B and serjanioside D (saponins), epicatechin, cassiaoccidentalin A, tetrastigma B and serjanone A (flavonoids), proanthocyanidins A- 1 and A-2 and cinnamtannin B-1 [12]. The flavonoids, proanthocyanidins and saponins could be responsible for the EESM anti-inflammatory activity.

The EESM was also tested *in vitro* against gram-negative and gram-positive bacteria. The MIC of EESM in the presence of the *M. tuberculosis* was 62.4 µg/ml showing to be a promising antibacterial natural agent. [34] indicated that an ethanolic extract of *S. erecta,* another species of *Serjania genus,* demonstrated a MIC of 128.0 µg/ml and EESM showed to be twice more potent than this extract. The values of MIC of EESM in the presence *S. epidermidis* and *K. pneumoniae* was 1000 µg/ml indicating that EESM had low potency in inducing inhibition of other bacteria growth. [15] showed that the hydroethanolic extract from *S. marginata* had *in vit*ro antibacterial and antifungal activities of the HESM against *S. aureus* (MIC of 125 mg/mL), *E. coli* (MIC of 250 mg/mL), *S. setubal* (MIC of 250 mg/mL), and *C. albicans* (MIC of 250 mg/mL). EESM was not effective against *P. aeruginosa* growth. These set of results showed that EESM has high potency in inducing inhibition of mycobaterial growth and low potency or no effects in relation to other microorganisms.

Since the EESM activity seems to be more selective against *M. tuberculosis* than to other microorganisms the *in vivo* EESM potential against mycobacteria was performed. The EESM was tested in BCG-induced pleurisy since it is not possible to work with *M. tuberculosis in vivo* in our University. Another reason to work with BCG as a mycobacteria model, is that its immunization schedule is applied against tuberculosis at birth in various countries. The licensed vaccine with attenuated BCG is performed in health care and several new vaccines are in clinical phases [6]. BCG was inoculated in the interpleural space, inducing an infectious and inflammatory mycobacterial response confirmed by the results of the control groups compared to the naive group (Figure 4). The results of leukocyte migration into the pleural space, **IL-1β levels in serum and in pleural exudate in the negative control group significantly differed from those of the naïve group** after 7 days. In BCG pleurisy model, oral doses of 30 and 100 mg/kg of EESM did not interfere with leukocyte migration to the pleural exudate however it induced a significant inhibition on the interleukin-1 beta (IL-1**β)** levels in serum and pleural exudate. **The increase in IL-1β levels after 7 days in BCG model may be correlated with the mycobacterial growth, as showed previously by our group** [26]**.** [35] **indicated that mice deficient in interleukin (IL) showed reduction in bacterial load 35 days' post infection**. Tumor necrosis factor alpha (TNF-α) and IL-1β are produced by immune cells that are

involved in pulmonary tuberculosis [36,37]. These results showed that doses of EESM controlled the BCG-inflammation.

An aliquot of precipitate sample from pleural exudate and also spleen and liver macerate from all BCG-injected animals were platted in Middlebrook 7H9/Ogawa Kudu culture medium for analysis of BCG growth. The BCG culture was found positive for Ziehl-Neelsen stain confirming that this microorganism is a *Mycobacterium sp*. The isoniazid inhibited significantly all the aspects of *in vivo* inflammatory response (Figure 4) including mycobacterial growth *in vitro* and *in vivo*. The EESM was not able to inhibit the formation of CFUs in pleural exudate, liver, and spleen tissues from *in vivo* experiments. The absence of EESM antibacterial effects may be related to the route of administration, dose and pharmacokinetics/pharmacodynamics of the EESM. To verify if EESM inhibited the BCG *in vitro*, the attenuated BCG was reactived in Lowenstein Jensen medium (an inclined tube under standard growth conditions) [38] and subsequently EESM was added to evaluation of antimicrobial activity *in vitro*. Several concentrations until 4 mg/ml of EESM were tested against BCG *in vitro* therefore no inhibitions were detected demonstrating no EESM antibacterial and/or bacteriostatic effects.

The EESM was also tested against CFA (an oil suspension containing killed *M. tuberculosis*), an immunogenic/inflammatory stimulus since it is not possible to work with viable *M. tuberculosis* [39]. The CFA induced persistent edema, mechanical hyperalgesia and cold sensitivity in control animals for 22 days. We observed the inhibition of the formation of paw edema with similar results to the dexamethasone group for 22 days, with a significant difference in the dose of 100 mg/kg of EESM in all evaluations and on days 16 and 22 in the dose of 30 mg/kg, comparing to the group control (Figures 6A-D). In the assessment of mechanical hyperalgesia, the EESM showed a significant difference compared to the control group on day 22 (Figure 5D). These results demonstrated that chronic anti-inflammatory ability of EESM even also in the persistent CFA model of inflammation.

# Conclusion

The present study was the first one to show the anti-inflammatory, anti-hyperalgesic and potential antibiotic properties of EESM in inflammatory/infection/hyperalgesic in acute and persistent models. EESM has high potency in inducing inhibition of mycobaterial growth and low potency or no effects in relation to other microorganisms in *in vitro*. EESM chemical composition showed the presence of several classes of compounds such as flavonoids, proanthocyanidins and saponins could be responsible for the EESM anti-inflammatory activity.

# Declarations


### Financial Support and Sponsorship

Nil.


### Conflict of interest

The author(s) confirm that this article content has no conflicts of interest.

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### Ethics approval

The register code for research A9CDAAE was obtained from National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN). A specimen was deposited at the Herbarium of the Federal University of the Mato Grosso do Sul, Brazil under the number 41054. Approval of the animal protocols were granted by the UFGD ethics committee (33/2016) and of University center of Grande Dourados (UNIGRAN) ethics committee (No. 050/2020).

### Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; Maicon Matos Leitão and Silvia Cristina Heredia Vieira conducted the anti-inflammatory and analgesic evaluation; Luis Fernando Benitez Macorini, Thiago Leite Fraga and Joyce Alencar Santos Radai conducted the antimicrobial and antimycobacterial evaluation; Claudia Andrea Lima Cardoso and Silvia Cristina Heredia Vieira were involved in the preparation of extract; Candida Aparecida Leite Kassuya, Arielle Cristina Arena and Maicon Matos Leitão performed data analyses and wrote the manuscript. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

## Abbreviations

Complete Freund's Adjuvant (CFA), Dimethylsulfoxide (DMSO), Interleukin-1β (IL-1β), Mycobacterium bovis - Bacillus Calmette-Guerin (BCG), Receptor pattern recognition receptors (PRR), Tumor necrosis fator (TNF).

## Summary

This study investigated the antimycobacterial, anti-inflammatory and antihyperalgesic effects of *S. marginata* (EESM) in in vitro and in vivo models. The minimum inhibitory concentration in the presence of *M. tuberculosis* was 62.4 µg/ml, *S. epidermidis* and *K. pneumoniae* were 1000 µg/mL while did not interfere against *P. aeruginosa* growth. EESM significantly inhibited paw edema, mechanical

hyperalgesia, leukocytes migration and proteins extravasation. Inhibited the levels of IL-1β in blood and in pleural exudate, did not altering the mycobacterial growth in the cell culture from pleural lavage, spleen and liver. This study confirms the EESM anti-inflammatory property and showed that EESM has high potency in inducing inhibition of mycobaterial growth.

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# Figures

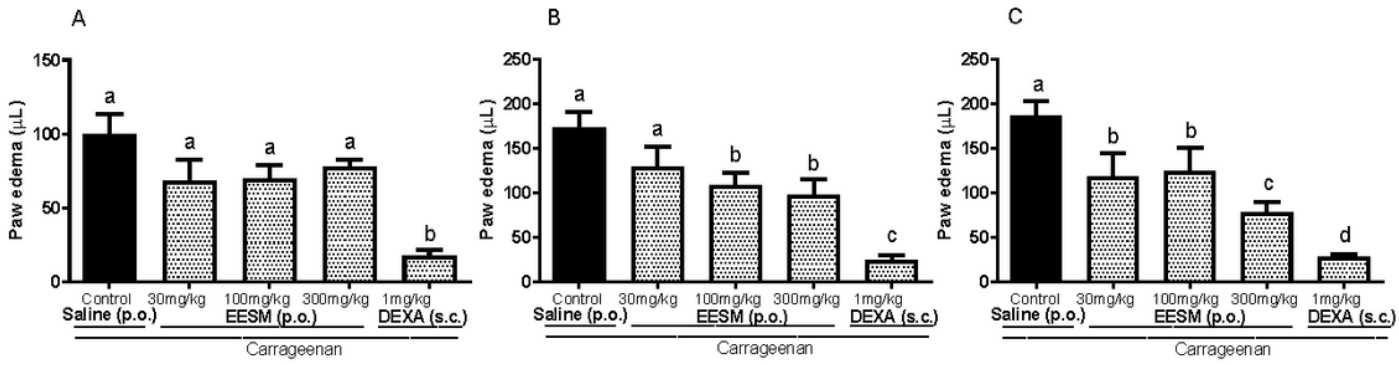



**Figure 1**

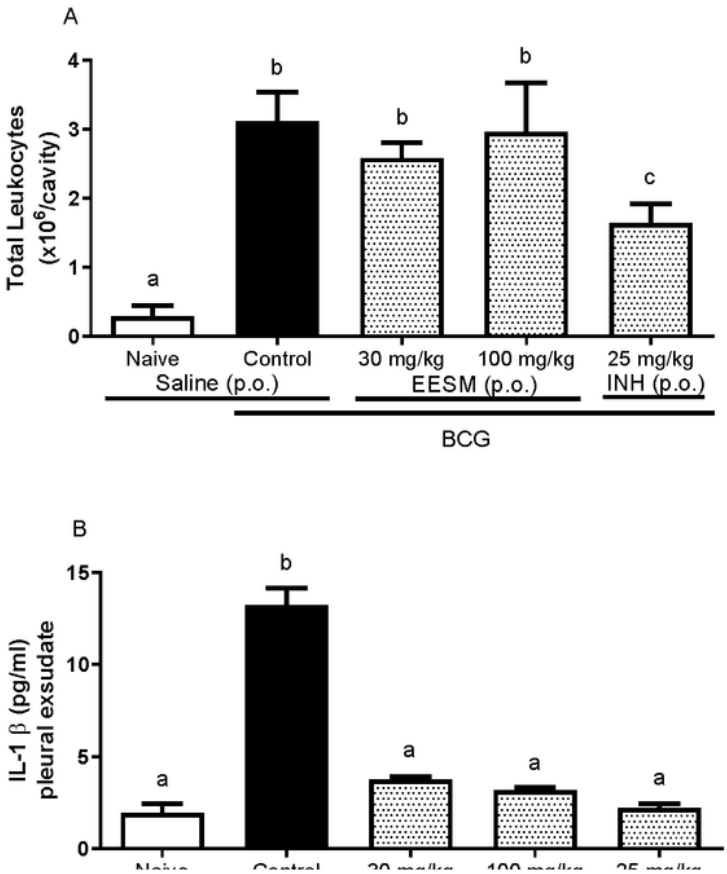
Effect of EESM oral treatments at 1 (A), 2 (B) and 4 (C) hour after carrageenan-induced edema. The control (saline 0.9 %, p.o.), EESM (30, 100, or 300 mg/kg, p.o.), and DEXA (dexamethasone 1 mg/kg, i.p.) groups were treated 1 h before the carrageenan injection. Each bar represents the mean ± SEM. The letters a, b, c, and d indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test

**Figure 2**

Effect of EESM oral treatment at 3 and 4 h after carrageenan-induced mechanical sensitivity (Figures 2A and 2B) and cold hypersensitivity (Figures 2C and 2D). The control (saline 0.9 %, p.o.), EESM (30, 100, or 300 mg/kg, p.o.), and DEXA (dexamethasone 1 mg/kg, s.c.) groups were treated before 1 h of carrageenan injection in the paw. Each bar represents the mean ± SEM. The letters a, b and c indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test

## Figure 3

Effect of EESM oral administration on (A) leukocyte migration x $10^6$ cells/cavity, (B) proteins (mg/ml) induced by intrapleural injection of carrageenan in mice. The control group received saline solution (0.9 %), and the EESM groups received 30, 100, or 300 mg/kg of EESM. Each bar represents the mean ± SEM. The letters a and b indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test




## Figure 4

Effects of EESM oral treatment on leukocyte (A) in the pleurisy, Il-1 levels pleural exudate (B), and in blood (C) induced by BCG. The animals received EESM (30, or 100, p.o.), vehicle (control) or isoniazid (ISO, 25 mg/kg, p.o.) for 7 days and an intrathoracic injection of BCG was administered since the first day. The naive group received an intrapleural injection of sterile saline instead of BCG and was also treated with saline solution. Each bar represents the mean ± SEM of 6 animals. The letters a, b, and c

indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test

**Figure 5**

Effects of oral administration of EESM on mechanical hyperalgesia induced by CFA. The animals received EESM (30, or 100, p.o., daily, once a day), vehicle (control) or dexamethasone (DEXA, 1 mg/kg, s.c., daily, once a day) for 6 (A), 11(B), 16 (C), and 22 (D) days. The CFA injection was performed on the first day. Each bar represents the mean ± SEM of 6 animals. The letters a, b, and c indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test

**Figure 6**

Effects of oral administration of EESM on edema induced by CFA. The animals received EESM (30, or 100, p.o., daily, once a day), vehicle (control) or dexamethasone (DEXA, 1 mg/kg, s.c., daily, once a day) for 6 (A), 11(B), 16 (C), and 22 (D) days. The CFA injection was performed on the first day. Each bar represents the mean ± SEM of 6 animals. The letters a, b, and c indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test

**Figure 7**

Effects of oral administration of EESM on cold hyperalgesia induced by CFA. The animals received EESM (30, or 100, p.o., daily, once a day), vehicle (control) or dexamethasone (DEXA, 1 mg/kg, s.c., daily, once a day) for 6 (A), 11(B), 16 (C), and 22 (D) days. The CFA injection was performed on the first day. Each bar represents the mean ± SEM of 6 animals. The letters a and b indicate significant differences among groups according to One-way ANOVA followed by the Newman-Keuls test

# Supplementary Files

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