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Article

Anti-inflammatory, analgesic, and immunostimulatory effects of *Luehea divaricata* Mart. & Zucc. (Malvaceae) bark

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Luehea divaricata (Malvaceae) is a plant widely used for treatment of various inflammatory and infectious conditions; however few reports discuss its biological properties. The aim of this study was to evaluate the anti-inflammatory and analgesic effects as well as the macrophage activity in mice treated with the hydroalcoholic crude extract of *L. divaricata* (CLD). Thin layer chromatography revealed presence of epicathequin, stigmasterol, lupeol and α,β-amyrin in the extract. To evaluate the anti-inflammatory and analgesic activities, animals were subjected to paw edema induced by carrageenan test, writhing, formalin and capsaicin tests. Immunomodulatory activity was evaluated by adhesion and phagocytic capacity, lysosomal volume, and reactive oxygen species (ROS) production by peritoneal macrophages, after daily treatment with CLD for 15 days. CLD promoted reduction in paw edema (36.8% and 50.2%; $p<0.05$ at doses of 100 and 300 mg/kg, respectively), inhibited writhing behavior at the higher dose (64.4%, $p<0.05$), reduced formalin reactivity (81.2% and 91.6% at doses of 100 and 300 mg/kg, respectively, $p<0.05$), and reduced capsaicin reactivity by 63.9% (300 mg/kg). CLD (200 $mg \cdot kg^{-1} \cdot day^{-1}$) increased phagocytosis capacity of macrophages (~3 fold, $p<0.05$), neutral red uptake (~50%, $p<0.001$), and ROS production (~90%, $p<0.001$). These data suggest that CLD possesses anti-inflammatory, analgesic and immunostimulatory properties.

Uniterms: *Luehea divaricata* Mart. & Zucc./pharmacognosy. *Luehea divaricata* Mart. & Zucc./ hydroalcoholic crude extract/properties. Anti-inflammatories. Analgesics. Reactive oxygen species.

Luehea divaricata (Malvaceae) é utilizada para o tratamento de várias condições patológicas, entretanto, há poucos relatos sobre sua bioatividade. O objetivo deste estudo foi avaliar o efeito anti-inflamatório e analgésico, bem como a atividade de macrófagos em camundongos tratados com extrato bruto hidroalcoólico (CLD) da planta. Cromatografia em camada delgada revelou a presença de epicatequina, estigmasterol, lupeol e α,β-amirina no material. Para avaliar a atividade anti-inflamatória e analgésica, animais foram submetidos a teste de edema de pata induzido por carragenana, teste de contorções, da formalina e da capsaicina. A atividade imunomodulatória foi avaliada pela capacidade de adesão e de fagocitose dos macrófagos, volume lisossômico e produção de espécies reativas de oxigênio (ROS), após tratamento diário com CLD por 15 dias. CLD promoveu redução do edema de pata (36,8% e 50,2%; 100 e 300 mg/kg, respectivamente; $p<0,05$), redução do número de contorções (64,4%; 300 mg/kg; $p<0,05$), redução da reatividade no teste da formalina (81,2% e 91,6%; 100 e 300 mg/kg, respectivamente; $p<0,05$), e no teste da capsaicina em 63,9% (300 mg/kg). CLD (200 $mg \cdot kg^{-1} \cdot day^{-1}$) aumentou capacidade

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de fagocitose dos macrófagos (~3 vezes, p<0,05), volume lisossômico (~50%, p<0,001) e produção de ROS (~90%, p<0,001). Estes dados sugerem que o CLD possui propriedades anti-inflamatórias, analgésicas e imunoestimulatórias.

Unitermos: *Luehea divaricata* Mart. & Zucc./farmacognosia. *Luehea divaricata* Mart. & Zucc./extrato bruto hidroalcoólico/propriedades. Antiinflamatórios. Analgésicos. Espécies reativas de oxigênio.

INTRODUCTION

Luehea divaricata Mart. & Zucc. (Malvaceae) is a plant found in the South Region of Brazil and popularly known as "açoita-cavalo" by the local inhabitants. Its barks have been empirically described by local herbalists in Santa Catarina - Brazil to possess antimicrobial and anti-inflammatory properties, and it has been used in the treatment of respiratory and gastrointestinal infections, arthritis, rheumatism, and leukorrhea, and as vermifuge (data not published).

Despite the consumption of bark infusions by the local population, there is little scientific information about its biological activities. Antifungal properties have been attributed to a dichloromethane extract of *L. divaricata* barks (25 µg/filter disk), which was able to inhibit polymer synthesis or the assembly of the cell wall of *Neurospora crassa* (Zacchino *et al.*, 1998). In 2003, Brazilian research characterized two triterpenes (a tormentic acid derivative and maslinic acid), which were obtained from a methanolic extract of *L. divaricata* leaves (Tanaka, Vidotti, Silva, 2003), but the biological properties of these compounds have not been assessed. The same research group identified a flavonoid, epicatechin, from a methanolic extract of the leaves of *L. divaricata*, which demonstrated antimicrobial activity against *Staphylococcus aureus* (250-500 µg/mL) (Tanaka *et al.*, 2005). Coelho de Souza *et al.* (2004) also verified the antimicrobial activity of a methanolic extract (50 mg/mL) of the aerial parts of *L. divaricata*. Although previous research suggests that *L. divaricata* possesses biological activity, no known studies have validated the effectiveness of *L. divaricata* in the control of inflammatory diseases and pain or investigated the immunomodulatory capacity of this plant.

Inflammation is a complex process involving a network of chemical signals that mediate the actions of the immune system. During inflammation, serum proteins and leukocytes migrate to areas of tissue injury. These inflammatory and activated cells release vasoactive mediators such as nitric oxide, bradykinin, and metabolites of arachidonic acid, all of which increase regional blood flow and promote microvascular permeability (Suffredini *et al.*, 1999). Several inflammatory mediators, mainly bradykinin and metabolites of arachidonic acid, are potent pain-producing agents that can excite and sensitize nociceptive primary afferent neurons (Petho, Reeh, 2012). Several plant extracts have been studied for their anti-inflammatory properties, by using *in vitro* and *in vivo* models (Ojewole, 2006; Siriwatanametanon *et al.*, 2010; Areej *et al.*, 2013), and existing data suggest that *L. divaricata* may also have similar characteristics.

Apart from anti-inflammatory qualities, natural compounds exhibit properties that may interfere with the modulation mechanisms of innate immunity, more specifically, the function of macrophages (Napolitano *et al.*, 2005; Lee *et al.*, 2007; Cruz *et al.*, 2007). Plant derivatives present an exciting opportunity for the discovery of new therapeutic agents and adjuvants that exhibit immunomodulatory properties, potentially representing suitable alternatives for the prevention and treatment of immune-related pathologies (Kalluf, 2008; Tiwari *et al.*, 2004). Plant extracts capable of stimulating the immune system might be useful in the treatment and prevention of infections. Immunostimulatory effects of plant-derived medicines following oral administration could be relevant in the search of new-generation vaccine adjuvants (Licciardi, Underwood, 2011). On the other hand, compounds that exert immunosuppression would be desirable in the treatment of autoimmune and chronic inflammatory diseases. Studies involving macrophages are very significant since they constitute the first line of defense of an organism and are responsible for activating many other processes involved in immune defense. In relation to macrophage functions, plant extracts have been shown to reduce nitric oxide production (Mehrotra *et al.*, 2002; Napolitano *et al.*, 2005), and reduce phagocytosis and expression of plasma membrane proteins (Lee *et al.*, 2007), as well as stimulate phagocytic capacity and production of nitric oxide (Cruz *et al.*, 2007).

To understand the biological potential of *L. divaricata*, the present study aimed to investigate the anti-inflammatory, analgesic, and immunomodulatory effects of the hydroalcoholic crude extract of *L. divaricata* (CLD) bark in mice. These data are an important starting point to characterize the potential biological benefits of

L. divaricata and define some of its practical therapeutic applications in drug development.

MATERIAL AND METHODS

Animal care

Adult female Swiss mice weighing 25-35 g were supplied by the West University of Santa Catarina. The animals were maintained in a controlled environment at 22 °C ± 2 °C with a 12 h light/dark cycle, with free access to water and food. All animals were acclimatized to the test laboratory 24 h prior to experiments. All procedures were approved by the Institutional Ethics Committee of West University of Santa Catarina (documents numbers: 011/2004, 135/2005 and 001/2010) and are in accordance with NIH Animal Care Guidelines.

Drugs and reagents

Carrageenan type IV, capsaicin, zymosan, nitroblue tetrazolium (NBT), horseradish peroxidase, neutral red, phenol red, phorbol myristate acetate (PMA), acetic acid, formaldehyde and dimethylsulfoxide (DMSO) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material and preparation of crude extract

The barks of *L. divaricata* were collected in March 2000, in Santa Clara, Joaçaba, Santa Catarina, Brazil (geographic coordinates: 27.1758°S, 51.5662°W). The specimen was identified with the collection documenting number 382.136 at the Municipal Botanical Museum, Curitiba-PR, Brazil, and stored at the same local. The collected barks of *L. divaricata* was then dried under ventilation in an air-flow chamber and pulverized. The powder was submitted to extraction by maceration (25-30 °C), with ethanol 80° GL for 14 days. The extract was filtered and concentrated in a vacuum with a rotatory evaporator, until completely dry. The dried extract was then stored at -20 °C until use.

Phytochemical analysis

1 g of crude extract of *L. divaricata* was dissolved in 5 mL of ethyl acetate or chloroform. After 5 min, the solution was filtered and dried to obtain ethyl acetate (SAE) or chloroform ($SCHCl_3$) samples. The yield of SAE and $SCHCl_3$ was 2.96% and 6.29%, respectively. Both fractions were checked by thin layer chromatography (TLC) according Hostettmann, Marston and Hostettmann (1998). In order to perform TLC, different eluents and systems as mobile phase were used, and silica gel 60 F_{254} (Merck) as stationary phase. It was used standards from the bank´s laboratory, stigmasterol, lupeol, α,β-amyrin, vitexin, rutin, quercetin and epicatechin. Some of these compounds were isolated from the plant under study.

The chromatographic $SCHCl_3$ profile was carried out with two different solvent systems, hexane:acetone 7:3 and 8:2, and the standards used were α,β-amyrin, lupeol and stigmasterol. The SAE profile was performed with chloroform: methanol 8:2, using vitexin, rutin, epicatechin and quercetin as standards. In both cases, the spots were revealed using sulfuric anisaldehyde to 110 °C and ferric chloride.

Paw edema induced by carrageenan test

Experimental animals (n=6-10) were pre-treated with the crude extract of *L. divaricata* (CLD) at doses of 10-300 mg/kg, orally administered (via oral, v.o.), and control animals (n=10) received a vehicle (distilled water; 0.1 mL/10 g) in place of the CLD. After 1 h, the mice were anesthetized with halothane and were administered carrageenan (300 µg/paw, subcutaneous injection) into the right paw (Morris, 2003). The contralateral paw received the same volume of sterile phosphate-buffered saline (PBS) and served as a control. The paw volume was measured with a plethysmometer (Panlab, Barcelona, Spain) immediately after carrageenan or PBS administration, at specified time points (30, 60, 120, and 240 min after injection of phlogistic agent). The results are expressed as the mean ± standard error of mean (SEM) values of the difference in volume (in mililiters) between the carrageenan- and saline-treated paws.

Writhing test

Writhing is induced by an intraperitoneal (i.p.) injection of acetic acid (0.6%) as an irritant, following which writhing movements are observed and evaluated for 30 min (Koster, Anderson, Debeer, 1959). Experimental animals (n=6-10) were pre-treated with the CLD at doses of 10 – 300 mg/kg, orally administered (via oral, v.o.) 1 h prior to injection of acetic acid. Control animals (n=10) received a vehicle (distilled water; 0.1 mL/10 g) in place of the CLD. The results are expressed as the mean ± SEM of the number of times the animal writhed.

Paw formalin and capsaicin tests

Nociceptive behavior was induced by the injection

of formalin (Hunskaar, Hole, 1987) or capsaicin (Sakurada *et al.*, 1992) in the ventral surface of the right hind paw. Animals (n=6–10) were pre-treated as specified in the above-described writhing test. After 1 h of pretreatment with CLD or vehicle, the animals received a 20 µL injection of either 2.5% formalin solution (0.92% formaldehyde) or capsaicin (1.6 µg/paw) prepared in PBS. Following formalin or capsaicin injection, the mice were immediately placed in glass chambers, and the time spent licking, flicking, and biting the injected paw was measured with a stopwatch; this time (expressed in seconds) was considered an indication of nociception. This nociceptive behavior is recorded in two phases for the formalin test and only in the one phase for the capsaicin test. The first phase of nociceptive response normally peaks at 0-5 min, and the second phase, 15-30 min after the formalin injection. The results are expressed as the mean ± SEM of nociceptive behavior (in seconds).

Immunostimulatory activity evaluation and macrophage isolation

Animals were randomly separated into three groups, n=5 animals/group: Control Group, animals received only a vehicle (a solution consisting of 10% DMSO and 40% Tween 80 in saline); Treatment Group 100, received 100 mg·kg body weight^{-1}·day^{-1} of CLD; Group 200, received 200 mg·kg body weight^{-1}·day^{-1}. Body weight was measured immediately before administration. All animals were fed a regular chow diet (Nuvital®) and received water *ad libitum*, and the treatments with CLD or vehicle were performed by gavage daily for 15 days. After this period, the animals were sacrificed in a CO_2 chamber, and the macrophages were collected. This experiment was performed in triplicate such that the total number of animals evaluated per group was 15.

Following euthanasia, the resident macrophages were obtained after i.p. injection of 5 mL of sterile PBS, massaging and opening the peritoneum, and drawing back of the fluid with a Pasteur pipette (~5 mL). Macrophages were collected by centrifugation (290xg, 4 °C for 5 min), the pellet was washed, and then resuspended in PBS after counting in a Neubauer chamber by optical microscopy, using a Trypan blue solution (1%). Adhesion assay and measurement of phagocytic capacity, lysosomal volume, superoxide anion production, and hydrogen peroxide production were performed using macrophage aliquots obtained in this manner.

Adhesion assay

Aliquots (0.1 mL) of macrophage suspension (5×10^6 cells/mL) were added to the wells of a 96-well flat-bottomed tissue culture plate and left to adhere for 1h under controlled temperature (37 °C). After washing with PBS, 0.15 mL of 50% methanol was added to each well, and incubated for 10min at room temperature. The supernatant was discarded, and 0.1 mL of 0.2% Giemsa solution was added. After incubating at room temperature for 40min, washing with distilled water, and incubating with 0.2 mL of 50% methanol at room temperature (Rosen, Gordon, 1987), the absorbance was measured at 550 nm (Microplate Reader 680; Biorad®). The mean of adhesion absorbance values of each group (C, 100 and 200) was used to normalize data for phagocytic capacity, lysosomal volume and ROS production assays (normalized value=absorbance value of group x/mean of adhesion absorbance of group x). This normalization was done to adapt results to the actual number of adherent cells on the plate.

Phagocytic capacity

Aliquots (0.1mL) of macrophage suspension (2×10^6 cells/mL) were added to the wells of a 96-well flat-bottomed tissue culture plate and left to adhere for 1h under controlled temperature (37 °C). Then, 10 µL of neutral red-stained zymosan (1×10^8 particles/mL) were added to each well. After incubation for 30 min at 37 °C, the cells were then fixed with Baker´s formol-calcium (4% formaldehyde, 2% sodium chloride, and 1% calcium acetate), 0.1 mL/well, for 30 min. The cells were washed two times and centrifuged, and then, 0.1 mL of acidified alcohol (10% acetic acid, 40% ethanol in distilled water) was added to each well (Bonatto *et al.*, 2004). After 30 min at 37 °C, the absorbance was measured at 550 nm using the microplate reader.

Lysosomal volume

Lysosomal volume was measured using the method described by Pipe, Coles and Farley (1995). A macrophage suspension (0.1 mL, 1×10^6 cells/mL) was incubated for 1 h at 37 °C, and then, 0.02 mL of 2% neutral red solution in PBS was added, and the samples were incubated for 30 min at 37 °C. The cells were washed twice with PBS and centrifuged. Neutral red was solubilized with 0.1 mL of the extraction solution (1% acetic acid in 50% ethanol) (Bonatto *et al.*, 2004). After 30min of incubation at 37 °C, the absorbance was measured at 550 nm using the microplate reader.

Superoxide anion production

Superoxide production was estimated by the NBT

reduction assay (Madhavi, Das, 1994). Macrophages (0.1 mL) suspended in PBS (2×10^6 cells/mL) were incubated for 1 h at 37 °C in the presence of 0.01 mL of 10 μM phorbol myristate acetate (PMA). Subsequently, 0.1 mL of NBT solution (0.25%) was added and the samples were incubated at 37 °C for 30 min. After centrifugation, cells were fixed with 0.1mL of 50% methanol. To assess the amount of superoxide anion production by the cells, the formazan resulting from NBT reduction was solubilized with 0.12 mL of 2 M potassium hydroxide (KOH) and 0.14 mL of DMSO. After 30 min of incubation, the absorbance was measured at 550 nm using the microplate reader.

Hydrogen peroxide production

Hydrogen peroxide production by macrophages was measured as described by Pick and Mizel (1981). This assay is based on horseradish peroxidase-dependent conversion of phenol red into a colored compound by H_2O_2. Macrophages (0.1 mL, 2×10^6 cells/mL) were incubated at 37 °C for 1 h and 10 μM PMA (0.01 mL) was added to all the wells. Cells were then incubated in the presence of 0.1 mL of phenol red solution (5 mM glucose, 0.56 mM phenol red, and 8.5 U/mL horseradish peroxidase) in the dark for 1h at 37 °C. The reaction was stopped by adding 0.01 mL of 1 M NaOH, and the absorbance was measured at 655 nm using the microplate reader.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by one way ANOVA followed by Tukey's post-hoc test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Phytochemical analysis

Phytochemical analysis by TLC suggested the presence of epicatechin in SAE (Figure 1A), and stigmasterol, lupeol and α and β-amyrin in the $SCHCl_3$ (Figure 1B). The other compounds investigated were not found.

Anti-inflammatory and analgesic effects

Pretreatment of animals with CLD reduced carrageenan-induced edema formation by 36.8% and 50.2% at the 100 and 300 mg/kg, respectively (Table I, p<0.05). The higher dose of CLD reduced writhing by 64.4% (Table II, p<0.05). Moreover, CLD significantly reduced the second phase of formalin reactivity (inflammatory phase) by 81.2% and 91.6% at 100 and 300 mg/kg, respectively (Table II, p<0.05). Capsaicin reactivity was also reduced; the time spent on painful activities was reduced by 63.9% in animals that received the higher dose of CLD (Table II).

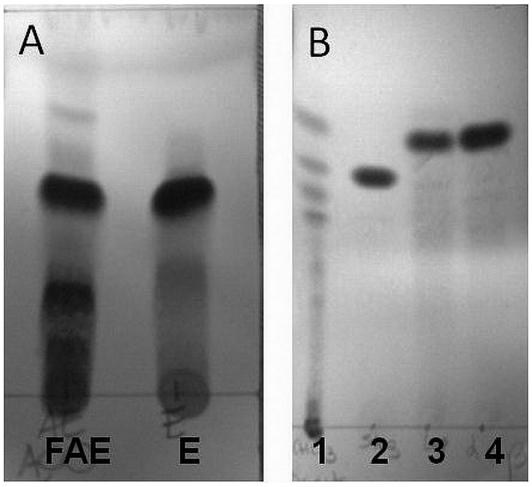

FIGURE 1 - Chromatographic profile of *L. divaricata*. Panel A: ethyl acetate fraction (SAE) and E, epicatechin. Panel B: chloroform fraction ($SCHCl_3$) (1), stigmasterol (2), lupeol (3) and α, β-amyrin (4).

Adhesion capacity

Animals that received 200 mg/kg of CLD (Group 200) significantly reduced macrophage adhesion compared to both the Control Group and Group 100 (~55%, p <0.05). Group 100 showed similar adhesion capacity to the Control Group (p >0.05) (Figure 2).

Phagocytic capacity

Macrophages from Group 200 showed ~3-fold increased phagocytic capacity than the Control Group (p <0.001) and Group 100 (p <0.001). There was no significant difference between the Control Group and Group 100 (p >0.05) (Figure 3).

Lysosomal volume

Neutral red uptake by the macrophages in Group 200 was ~50% greater than that observed to the Control Group (p <0.001), which was in contrast to the macrophages from Group 100, which showed a reduction of uptake capacity by ~20%. Group 200 presented ~90% higher uptake capacity than Group 100 (p <0.001, Figure 4).

TABLE I - Anti-inflammatory effect of crude extract of *L. divaricata* (CLD) on the paw edema induced by carrageenan test in mice

Groups	Dose mg/kg	Paw edema induced by carrageenan (300 µg/paw) measured with plethysmometer at different time points				Edema inhibition (%)
		30 min	60 min	120 min	240 min	
Control	distilled water	32.5 ± 6.1	62.5 ± 6.3	112.5 ± 13.1	120.0 ± 15.8	0.0
Crude Extract	10	33.3 ± 6.7	46.6 ± 4.2	71.6 ± 7.3	96.6 ± 6.7	27.3
	30	31.6 ± 8.7	43.3 ± 5.5	80.0 ± 10.6	106.6 ± 8.0	21.8
	100	18.0 ± 4.8	34.0 ± 8.1[a]	62.0 ± 10.6[a]	94.0 ± 7.5[a]	36.8
	300	4.0 ± 2.4[a]	24.0 ± 7.4[a]	60.0 ± 13.8[a]	64.0 ± 8.1[a]	50.2

Each group represents the mean for 6 to 10 animals. Significantly different from control group: [a] $p < 0.05$. Results are presented as mean ± EPM values of the difference in volume (mL) between the carrageenan- and saline-treated paws. The edema inhibition was determined by calculating the area under curve.

TABLE II - Analgesic effect of crude extract of *L. divaricata* (CLD) on the writhing test, paw formalin test and paw capsaicin test in mice

Models		Control	Dose of CLD (mg/kg)			
			10	30	100	300
Writhing test	number of writhings	35.4 ± 4.1	26.0 ± 3.4	26.6 ± 1.6	25.0 ± 4.1	12.6 ± 4.4[a]
Paw formalin test	1st fase (seconds)	57.0 ± 7.7	62.3 ± 7.3	41.3 ± 3.1	51.8 ± 14.5	50.6 ± 11.2
	2nd fase (seconds)	76.0 ± 16.4	51.3 ± 13.3	37.8 ± 14.1	14.3 ± 8.2[a]	6.4 ± 4.0[a]
Paw capsaicin test	Reactivity (seconds)	26.6 ± 3.0	30.3 ± 4.6	27.0 ± 2.2	9.6 ± 2.1[a]	n.d.

Each group represents the mean ± SEM for 6 to 10 animals. Significantly different from control group: [a] $p < 0.05$. n.d., not determined. The time in seconds indicates time spent licking, flicking, and biting the injected paw.

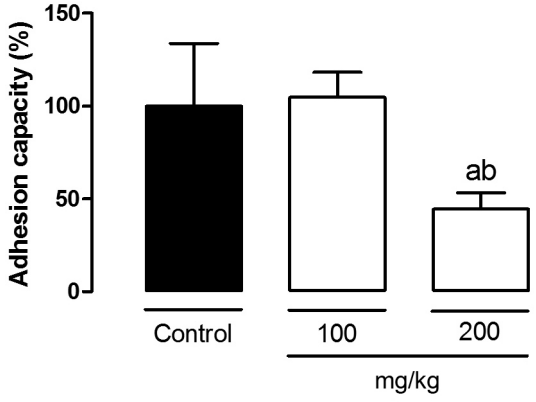

FIGURE 2 - Adhesion capacity of resident macrophages from mice fed regular chow (Control Group), treated with 100 mg/kg b.w. of CLD (Group 100), and 200 mg/kg b.w. (Group 200). [a] $p<0.05$ compared to Control. [b] $p<0.05$ compared to Group 100. Results are presented as % relative to Control.

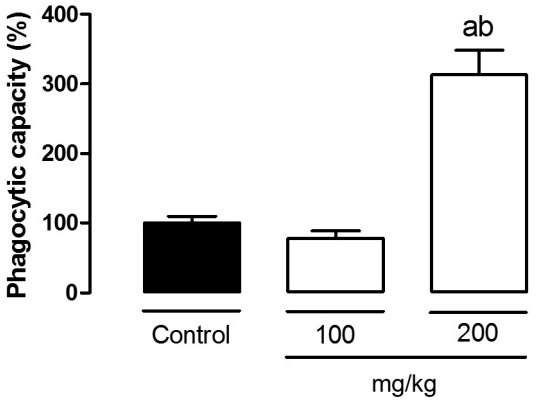

FIGURE 3 - Phagocytic capacity of resident macrophages from mice fed regular chow (Control Group), treated with 100 mg/kg b.w. of CLD (Group 100), and 200 mg/kg b.w. (Group 200). [a] $p<0.001$ compared to Control. [b] $p<0.001$ compared to Group 100. Results are presented as % relative to Control.

Reactive oxygen species (ROS) production

Superoxide anion (Figure 5) and hydrogen peroxide (Figure 6) production was ~90% greater in the macrophages from Group 200 than those from the Control Group ($p < 0.001$). The macrophages from Group 100 produced half the concentration of ROS compared to that of Group 200 ($p < 0.001$). No statistical difference was observed between the Control Group and Group 100.

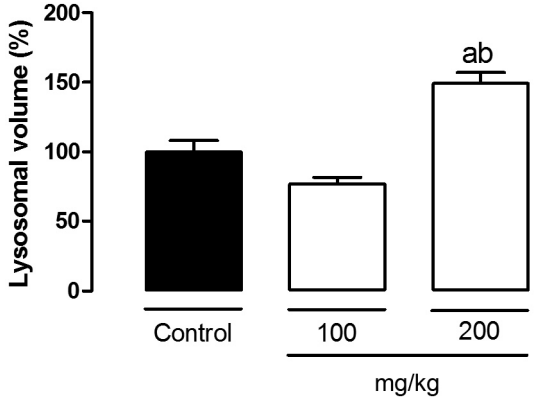

FIGURE 4 - Lysosomal volume of resident macrophages from mice fed regular chow (Control Group), treated with 100 mg/kg b.w. of CLD (Group 100), and 200 mg/kg b.w. (Group 200). [a] $p<0.001$ compared to Control. [b] $p<0.001$ compared to 100. Results are presented as % relative to control.

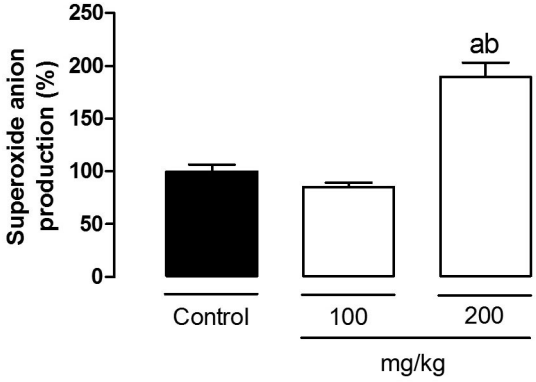

FIGURE 5 - Superoxide anion production of resident macrophages from mice fed regular chow (Control Group), treated with 100 mg/kg b.w. of CLD (Group 100), and 200mg/kg b.w. (Group 200). [a] $p<0.001$ compared to Control. [b] $p<0.001$ compared to 100. Results are presented as % relative to control.

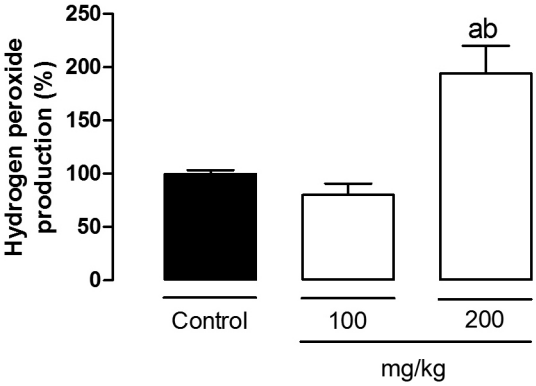

FIGURE 6 - Hydrogen peroxide production of resident macrophages from mice fed regular chow (Control Group), treated with 100 mg/kg b.w. of CLD (Group 100), and 200 mg/kg b.w. (Group 200). [a] $p<0.001$ compared to Control. [b] $p<0.001$ compared to 100. Results are presented as % relative to control.

DISCUSSION

Bioactive compounds found in medicinal plants may present anti-inflammatory and analgesic capacities, in addition to immunomodulation properties. Indeed, many compounds isolated from plant extracts have demonstrated anti-inflammatory properties as well as immunostimulatory activities (da Silva, Parente, 2001; Biella *et al.*, 2008; Halder *et al.*, 2009). The current study characterizes the biological activity of *L. divaricata* bark for the first time, specifically showing that CLD reduced inflammation in all assays performed and that it stimulated macrophage activity.

Thin layer chromatography (Figure 1) revealed the presence of epicatechin in the SAE, and stigmasterol, lupeol and α and β-amyrin in the $SCHCl_3$. Whereas some flavonoids and triterpenes exhibit immunomodulatory and anti-inflammatory activities (Saleem, 2009; Sabeva *et al.*, 2011; Ahmad *et al.*, 2013; Rosenblat, Volkova, Aviram, 2013), it is possible that they may be partly responsible for the activities found in the present study.

Carrageenan-induced paw edema is a well-characterized experimental model of inflammation, and it has been widely used to investigate the efficacy of natural compounds (Nardi *et al.*, 2003; Wang *et al.*, 2013). The acute inflammatory reaction elicited by carrageenan is characterized by sequential liberation of inflammatory mediators, promoting marked accumulation of exudate and intense migration of polymorphonuclear leukocytes in the affected paw (Morris, 2003). In the present study, we clearly show the anti-inflammatory properties of CLD, as demonstrated by a dose-dependent reduction in paw edema in mice (Table I). This effect was evident 30 min after the oral administration of CLD, and was maintained throughout the evaluation period.

The writhing test is an experimental model used to screen investigational drugs for analgesic activity. This is evaluated by the irritation caused by the i.p. injection of acetic acid, which causes the release of mediators such as bradykinin, prostaglandins, histamine, and 5-hydroxytryptamine (Whittle, 1964). Different of writhing test, in formalin test is possible to identify two phases of nociception. The first phase is characterized by direct activation of primary afferent fibers, followed by the second phase, which is characterized by the release of several inflammatory mediators in the immediate area of formalin injection (Hunskaar, Hole, 1987; Corrêa, Calixto, 1993; Choi, Lee, Suh, 2001). The CLD effectively reduced writhing behavior that suggests a level of pain in animals (Table II). This reduction in pain by CLD is further supported by the reduction in pain, as demonstrated in the

second phase of formalin test for CLD-treated animals (Table II). The lack of effect of CLD in the first phase of the formalin test supports our hypothesis that anti-inflammatory substances are responsible for the analgesic effect of CLD, rather than a direct excitation of the primary afferents.

Capsaicin is known to directly activate the vanilloid receptor (TRPV-1) in the primary afferent fibers, which transmits the nociceptive stimulus to the central nervous system, promoting the release of pro-inflammatory neuropeptides such as substance P, neurokinins, nitric oxide, and prostaglandins. These substances are responsible for producing the sensation of pain and inflammatory process (Holzer, 1991; Wu *et al.*, 1998; Palazzo, Rossi, Malone, 2008). The data on formalin test paired with the reduction in capsaicin-induced pain behavior suggests that CLD activity is due to the modulation of inflammatory mediators. Together with the reduction in edema in the carrageenan model, these data suggest that CLD contains anti-inflammatory substances, which act to reduce the sensation of pain by reducing inflammatory excitation of nociceptors in the primary afferent fibers.

Indigenous populations who use crude extract of *L. divaricata* to treat various conditions prepare infusions of the barks that are often administered orally. To simulate the effects of oral intake and evaluate the potential immunomodulatory activity of CLD, we orally administered CLD to animals for 15 days. According to Gertsch, Viveros-Paredes and Taylor (2011), the activity of the immune system can only unambiguously be evaluated *in vivo*, and the most ethnopharmacologically relevant way to test plant products is by oral administration. Here, we show that oral administration of a minimum of 200 mg/kg CLD produced an overall stimulatory effect on the peritoneal macrophages from mice.

The adhesion capacity of the macrophages from Group 200 was reduced by ~50% in comparison to those from both the Control Group and Group 100 (Figure 2). Modifications in the adhesion capacity of cells can be derived from changes in either the membrane fluidity (Calder *et al.*, 1990; Noudeh *et al.*, 2010) or in the distribution of adhesion molecules within the plasma membrane (KyungSeop *et al.*, 2000; Kim *et al.*, 2011). For example, there are reports showing that polyphenols and tannins contained in plant extracts are able to modify the properties of the plasma membrane, thereby affecting adhesion capacity (Cyboran, Oszmianski, Lkeszczynska, 2012; Olchowik *et al.*, 2012). Interestingly, the phagocytic capacity observed in Group 200 was 3-fold greater than that observed for Control Group ($p<0.001$) (Figure 3). This measurement was normalized by adhesion capacity, and although the adhesion capacity of the cells in Group 200 was 50% lower than that observed for the Control Group, the phagocytic capacity of adherent cells was still 3-fold greater. Together, these data indicate that the substances present in the barks of *L. divaricata* are able to stimulate a significant macrophage-mediated response, contributing to the body of evidence demonstrating that plant components influence immunomodulation by altering the phagocytic capacity of macrophages (Bin-Hafeez *et al.*, 2003; Lee *et al.*, 2007; Cruz *et al.*, 2007) and other phagocytic cells (Nudo, Catap, 2011). However, a dose of 100 mg/kg did not elicit an impact on the adhesion and phagocytic capacity of isolated macrophages, suggesting an insufficient concentration of bioactive molecules to provide immunomodulation. Notwithstanding the lack of response in Group 100, the results obtained in the current study, particularly in relation to phagocytosis modulation, suggest that CLD is able to strongly stimulate the innate immune system at sufficient concentrations.

Cellular digestion of agents contained in phagosomes is controlled by fusion to hydrolase-containing lysosomal vesicles. Neutral red uptake by macrophages in Group 200 was greater than that observed in Group 100 and the Control Group (Figure 4). This supports the dose-dependent response observed in phagocytic capacity (Figure 3), indicating that the cells were able to both phagocytose the cationic dye neutral red and process it. However, the lysosomal volume of Group 200 did not show a proportional increase in neutral red uptake with respect to phagocytic capacity. This mismatch between phagocytosis and dye uptake has also been observed by other research groups (Bonatto *et al.*, 2004). Swanson and Baer (1995) report that several events enable fusion between the lysosomal vacuolar membrane and phagosomes, which together form a phagolysosome. The rate of phagosome-lysosome fusion can vary dramatically depending on the nature of the encapsulated particles, suggesting that not all particles phagocytized stimulate a large change in lysosomal volume. This may partly explain the disparity between the results obtained for phagocytic capacity (Figure 3) and lysosomal volume (Figure 4) in Group 200.

Resting macrophages consume little oxygen, although activation by an appropriate stimulus leads to increased production of reactive oxygen species (ROS). ROS are produced by macrophages (superoxide anions and hydrogen peroxide) by a non-mitochondrial respiratory burst, which greatly increases consumption of oxygen and conversion to superoxide anions (Babior, 1984). The nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) enzyme that resides in the plasma membrane of phagocytes is responsible for this

conversion, and it is activated by phagocytosis as well as by compounds such as PMA via activation of kinase C proteins (Lunardi, Lima, 2006). Superoxide anions and hydrogen peroxide that are immediate products of this respiratory burst lead to the generation of hypochlorite and chloramines, which are potent microbicidal oxidized halogens, in addition to hydroxyl radicals, an oxidizing radical that can damage all types of macromolecules (Babior, 1984). Our results show an increase in the concentrations of superoxide anion and hydrogen peroxide produced by the activated macrophages from Group 200 in comparison to those from Control Group (Figure 5 and Figure 6). These data are consistent with the increased phagocytic capacity of the macrophages from Group 200 animals. In these experiments, the cells of all groups were treated with PMA, but a greater concentration of superoxide anions and hydrogen peroxides were observed in Group 200 compared to Group C, suggesting that the crude extract at 200 mg/kg was able to stimulate the production of these ROS by macrophages. Studying the molecular mechanisms by which the compounds from CLD stimulate ROS production in macrophages was not the aim of this study, but we found that the crude extracts from medicinal plants are able to interact directly on the NADPH oxidase enzyme (Mahomoodally *et al.*, 2012), suggesting that this action of the extract on this enzyme cannot be ruled out. Physiologically, the activation of phagocytic NADPH oxidase can be induced by microbial products such as bacterial lipopolysaccharide, by lipoproteins, or by cytokines such as interferon-γ, interleukin-1β, or interleukin-8 (Bonizzi *et al.*, 2000). In addition to exerting an important role as microbicidal agents, superoxides and related ROS contribute to signal transduction via various membrane receptors, and thus, to overall immunological function (Drogë, 2002).

Taken together, these results indicate that the CLD contains substances with both anti-inflammatory and analgesic effects, as well as immunomodulatory bioactive compounds capable of stimulating macrophage activity. These findings support the empirical use of this plant to combat different types of inflammatory diseases and infections. Further studies are necessary to elucidate the chemical compounds within CLD that contribute to these properties, and to evaluate the therapeutic effects exerted by these compounds on adaptive immune cells and their products.

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REFERENCES

AHMAD, A.; GUPTA, G.; AFZAL, M.; KAZMI, I.; ANWAR, F. Antiulcer and antioxidant activities of a new steroid from *Morus alba*. *Life Sci.*, v.92, p.202-210, 2013.

AREEJ, M.; ASSAF, A.M.; HADDADIN, R.N.; ALDOURI, N.A.; ALABBASSI, R.; MASHALLAH, S.; MOHAMMAD, M.; BUSTANJI, Y. Anti-cancer, anti-inflammatory and anti-microbial activities of plant extracts used against hematological tumors in traditional medicine of Jordan. *J. Ethnopharmacol.*, v.145, p.728-736, 2013.

BENTO, A.F.; MARCON, R.; CLAUDINO, R.F.; CALIXTO, J.B. Preventive and therapeutic oral administration of the pentacyclic triterpene α,β-amyrin ameliorates dextran sulphate sodium-induced colitis in mice: The relevance of cannabinoid system. *Mol. Immunol.*, v.54, p.482-492, 2013.

BABIOR, B.M. The respiratory burst of phagocytes. *J. Clin. Invest.*, v.73, p.599-601, 1984.

BIELLA, C.A.; SALVADOR, M.J.; DIAS, D.A.; DIAS-BARUFF, M.; PEREIRA-CROTT, L.S. Evaluation of immunomodulatory and anti-inflammatory effects and phytochemical screening of *Alternanthera tenella* Colla (Amaranthaceae) aqueous extract. *Mem. Inst. Oswaldo Cruz.,* v.103, p.569-577, 2008.

BIN-HAFEEZ, B.; HAQUE, R.; PARVEZ, S.; PANDEY, S.; SAYEED, I.; RAISUDDIN, S. Immunomodulatory effects of fenugreek (*Trigonella foenum* graecum L.) extract in mice. *Int. Immunopharmacol.*, v.3, p.257-265, 2003.

BONATTO, S.J.R.; FOLADOR, A.; AIKAWA, J.; YAMAZAKI, R.K.; PIZATTO, N.; OLIVEIRA, H.H.P.; VECCHI, R.; CALDER, P.C.; FERNANDES, L.C. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. *Cell. Immunol.*, v.231, p.56-62, 2004.

BONIZZI, G.; PIETTE, J.; MERVILLE, M.P.; BOURS, V. Cell type-specific role for reactive oxygen species in nuclear factor kB activation by interleukin-1. *Biochem. Pharmacol.*, v.59, p.7-11, 2000.

CALDER, P.C.; BOND, J.A.; HARVEY, D.J.; GORDON, S.; NEWSHOLME, E.A. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem. J.*, v.269, p.807-814, 1990.

CHOI, S.S.; LEE, J.K.; SUH, H.W. Antinociceptive profiles of aspirin and acetaminophen in formalin, substance P and glutamate pain models. *Brain Res.*, v.921, p.233-239, 2001.

COELHO DE SOUZA, G.; HAAS, A.P.S.; VON POSER, G.L.; SCHAPOVAL, E.E.S.; ELISABETSKY, E. Ethnopharmacological studies of antimicrobial remedies in the South of Brazil. *J. Ehtnopharmacol.*, v.90, p.135-143, 2004.

CORRÊA, C.R.; CALIXTO, J.B. Evidence for participation of B1 and B2 kinin receptors in formalin-induced nociceptive response in the mouse. *Br. J. Pharmacol.*, v.110, p.193-198, 1993.

CRUZ, G.V.B.; PEREIRA, P.V.S.; PATRÍCIO, F.J.; COSTA, G.C.; SOUSA, S.M.; FRAZÃO, J.B.; ARAGÃO-FILHO, W.C.; MACIEL, M.C.G.; SILVA, L.A.; AMARAL, F.M.M.; BARROQUEIRO, E.S.B.; GUERRA, R.N.M.; NASCIMENTO, F.R.F. Increase of cellular recruitment, phagocytosis ability and nitric oxide production induced by hydroalcoholic extract from *Chenopodium ambrosioides* leaves. *J. Etnopharmacol.*, v.111, p.148-154, 2007.

CYBORAN, S.; OSZMIANSKI, J.; KLESZCZYNSKA, H. Interaction between plant polyphenols and the erythrocytes membrane. *Cell. Mol. Biol. Lett.*, v.17, p.77-88, 2012.

DA SILVA, B.P.; PARENTE, J.P. An anti-inflammatory and immunomodulatory polysaccharide from *Orbignya phalerata*. *Fitoterapia.*, v.72, p.887-893, 2001.

DROGË, W. Free radicals in the physiological control of cell function. *Physiol. Rev.*, v.82, p.47-95, 2002.

GERTSCH, J.; VIVEROS-PAREDES, J.M.; TAYLOR, P. Plant immunostimulants - scientific paradigm or myth? *J. Ethnopharmacol.*, v.136, p.385-391, 2011.

HALDER, S.; BHARAL, N.; MEDIRATTA, P.K.; KAUR, I.; SHARMA, K.K. Anti-inflammatory, immunomodulatory and antinociceptive activity of *Terminalia arjuna* Roxb bark powder in mice and rats. *Indian J. Exp. Biol.*, v.47, p.577-583, 2009.

HOLZER, P. Capsaicin: cellular targets mechanisms of action and selectivity for thin sensory neurons. *Pharmacol. Rev.*, v.43, p.144-201, 1991.

HOSTETTMANN, K.; MARSTON, A.; HOSTETTMANN, M. *Preparative chromatography techniques*: applications in natural product isolation. 2.ed. Berlin: Springer, 1998. 244 p.

HUNSKAAR, S.; HOLE, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain*, v.30, p.103-114, 1987.

KALLUF, L.J.H. *Fitoterapia funcional*: dos princípios ativos à prescrição de fitoterápicos. São Paulo: VP Editora, 2008. 304 p.

KIM, Y.S.; JEON, J.H.; IM, J.; KANG, S.S.; CHOI, J.N.; JU, H.R.; YUN, C.H.; SON, C.G.; LEE, C.H.; HAN, S.H. Induction of intercellular adhesion molecule-1 by water-soluble components of *Hericium erinaceum* in human monocytes. *J. Ethnopharmacol.*, v.133, p.874-880, 2011.

KOSTER, R., ANDERSON, M., DEBEER, E.J. Acetic acid for analgesic screening. *Fed. Proc.*, v.18, p.418-420, 1959.

KYUNGSEOP, A.; JUNGHEE, K.; SEIRYANG, O.; SHIYONG, R.; HYEONGKYU, L. Inhibitory activity of stilbenes from medicinal plants on the expression of cell adhesion molecules on THP1 cells. *Planta Med.*, v.66, p.641-644, 2000.

LEE, Y.G.; BYEON, S.E.; KIM, J.Y.; LEE, J.Y.; RHEE, M.H.; HONG, S.; WU, J.C.; LEE, H.S.; KIM, M.J.; CHO, D.H.; CHO, J.Y. Immunomodulatory effect of *Hibiscus cannabinus* extract on macrophage functions. *J. Etnopharmacol.*, v.113, p.62-71, 2007.

LICCIARDI, P.V.; UNDERWOOD, J.R. Plant-derived medicines: A novel class of immunological adjuvants. *Int. Immunopharmacol.*, v.11, p.390-398, 2011.

LUNARDI, F.; LIMA, I.H.C.; ASSREUY, J. Comparative study of respiratory burst induced by phorbol ester and zymosan in human granulocytes. *Clin. Biochem.*, v.39, p.78-85, 2006.

MADHAVI N.; DAS, V.N. Effects of n-3 and n-6 fatty acids on the survival of vincristine sensitive and resistent human carcinoma cells, *in vitro*. *Cancer Lett.*, v.84, p.31-41, 1994.

MAHOMOODALLY, F.; MESAIK, A.; CHOUDHARY, M.I.; SUBRATTY, A.H.; GURIB-FAKIM, A. *In vitro* modulation of oxidative burst via release of reactive oxygen species from immune cells by extracts of selected tropical medicinal herbs and food plants. *Asian Pac. J. Trop. Med.*, v.5, p.440-447, 2012.

MEHROTRA, S.; MISHRA, K.P.; MAURYA, R.; SRIMAL, R.C.; SINGH, V. Immunomodulation by ethanolic extract of *Boerhaavia diffusa* roots. *Int. Immunopharmacol.*, v.2, p.987-996, 2002.

MORRIS, C.J. Carrageenan-induced paw edema in the rat and mouse. *Methods Mol. Biol.*, v.225, p.115-121, 2003.

NAPOLITANO, D.R.; MINEO J.R.; SOUZA M.A.; PAULA, J.E.; ESPINDOLA, L.S.; ESPINDOLA, F.S. Down-modulation of nitric oxide production in murine macrophages treated with crude extracts from Brazilian Cerrado. *J. Etnopharmacol.*, v.99, p.37-41, 2005.

NARDI, G.M.; FELIPPI, R.; DALBÓ, S.; SIQUEIRA-JUNIOR, J.M.; ARRUDA, D.C.; DELLE MONACHE, F.; TIMBOLA, A.K.; PIZZOLATTI, M.G.; CKLESS, K.; RIBEIRO-DO-VALLE, R.M. Anti-inflammatory and antioxidant effects of *Croton celtidifolius* bark. *Phytomedicine*, v.10, p.176-184, 2003.

NOUDEH, G.D., SHARIFIFAR, F.; KHATIB, M.; BEHRAVAN, E.; AFZADI, M.A. Study of aqueous extract of three medicinal plants on cell membrane-permeabilizing and their surface properties. *Afr. J. Biotechnol.*, v.9, p.110-116, 2010.

NUDO, L.P.; CATAP, E.S. Immunostimulatory effects of *Uncaria perrottetii* (A. Rich) Merr.(Rubiaceae) vinebark aqueous extract in Balb/C mice. *J. Etnopharmacol.*, v.133, p.613-620, 2011.

OJEWOLE, J.A.O. Antinociceptive, anti-inflammatory and antidiabetic properties of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract in mice and rats. *J. Etnopharmacol.*, v.103, p.126-134, 2006.

OLCHOWIK, E.; LOTKOWSKI, K.; MAVLYANOV, S.; ABDULLAJANOVA, N.; IONOV, M.; BRYSZEWSKA, M.; ZAMARAEVA, M. Stabilization of erythrocytes against oxidative and hypotonic stress by tannins isolated from sumac leaves (*Rhus typhina* L.) and grape seeds (*Vitis vinifera* L.). *Cell. Mol. Biol. Lett.*, v.17, p.333-348, 2012.

PALAZZO, E.; ROSSI, F.; MALONE, S. Role of TRPV1 receptors in descending modulation of pain. *Mol. Cell. Endocrinol.*, v.286, p.79-83, 2008.

PETHO, G.; REEH, P.W. Sensory and signaling mechanisms of bradykinin, eicosanoids, platelet-activating factor, and nitric oxide in peripheral nociceptors. *Physiol. Rev.*, v.92, p.1699-1775, 2012.

PICK, E.; MIZEL D. Rapid microassays for measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immunol. Methods*, v.46, p.211-226, 1981.

PIPE, R.K.; COLES, J.A.; FARLEY, S.R. Assays for measuring imune response in the mussel *Mytilus edulis*. *Tech. Fish Immunol.*, v.4, p.93-100, 1995.

ROSEN, H.; GORDON, S. Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells *in vitro* and inflammatory cell recruitment *in vivo*. *J. Exp. Med.*, v.166, p.1685-1701, 1987.

ROSENBLAT, M.; VOLKOVA, N.; AVIRAM, M. Pomegranate phytosterol (β-sitosterol) and polyphenolic antioxidant (punicalagin) addition to statin, significantly protect against macrophage foam cells formation. *Atherosclerosis*, v.226, p.110-117, 2013.

SABEVA, N.S.; McPHAUL, C.M.; LI, X.; CORY, T.J.; FEOLA, D.J.; GRAF, G.A. Phytosterols differentially influence ABC transporter expression, chlolesterol efflux and inflammatory cytokine secretion in macrophage foam cells. *J. Nutr. Biochem.*, v.22, p.777-783, 2011.

SAKURADA, T.; KATSUMATA, K.; TAN-NO, K.; SAKURADA, S.; KISARA, K. The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord. *Neuropharmacology*, v.3, p.1279-1285, 1992.

SIRIWATANAMETANON, N.; FIEBICH B.L.; EFFERTH, T.; PRIETO, J.M.; HEINRICH, M. Traditionally used Thai medicinal plants: *In vitro* anti-inflammatory, anticancer and antioxidant activities. *J. Ethnopharmacol.*, v.130, p.196-207, 2010.

SALEEM, M. Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene. *Cancer Lett.*, v.285, p.109-115, 2009.

SUFFREDINI, A.F.; FANTUZZI, G.; BADOLATO, R.; OPPNHEIM, J.J.; O'GRADY, N.P. New insights into the biology of the acute phaseresponse. *J. Clin. Immunol.*, v.19, p.203-214, 1999.

SWANSON, J.A.; BAER, S.C. Phagocytosis by zippers and triggers. *Trends Cell. Biol.*, v.5. p.89-93, 1995.

TANAKA, J.C.A.; SILVA, C.C.; DIAS FILHO, B.P.; NAKAMURA, C.V.; CARVALHO, J.E.; FOGLIO, M.A. Chemical constituents of *Luehea divaricata* Mart. (TILIACEAE). *Quím. Nova*, v.28, p.834-837, 2005.

TANAKA, J.C.A.; VIDOTTI, G.J.; SILVA, C.C. A New Tormentic Acid Derivative from *Luehea divaricata* Mart. (Tiliaceae). *J. Braz. Chem. Soc.*, v.14, p.475-478, 2003.

TIWARI, U.; RASTOGI, B.; SINGH, P.; SARAF, D.K.; VYAS, S.P. Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals. *J. Ethnopharmacol.*, v.92, p.113-119, 2004.

WANG, B.S.; HUANG, G.J.; LU, Y.H.; CHANG, L.W. Anti-inflammatory effects of an aqueous extract of Welsh onion green leaves in mice. *Food Chem.*, v.138, p.751-756, 2013.

WHITTLE, B.A. Release of a kinin by intraperitoneal injection of chemical agents in mice. *Int. J. Neuropharmacol.*, v.3, p.369-378, 1964.

WU, J.; LIN, Q.; MCADOO, D.J.; WILLIS, W.D. Nitric oxide contributes to central sensitization following intradermal injection of capsaicin. *NeuroReport*, v.9, p.589-592, 1998.

ZACCHINO, S.; SANTECCHIA, C.; LÓPEZ, S.; GATTUSO, S.; MUÑOZ, J.D.; CRUÃNES, A.; VIVOT, E.; CRUÃNES, M.C.; SALINAS, A.; RUIZ, R.E.; RUIZ, S. *In vitro* antifungal evaluation and studies on mode of action of eight selected species from the Argentina flora. *Phytomedicine*, v.5, p.389-395, 1998.

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