



# Anti-inflammatory Effect of the Hydroethanolic Extract from *Dacryodes kukachkana* Leaves in Mice: Role of Macrophage Nitric Oxide

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## Authors' contributions

This work was carried out in collaboration among all authors. Author ABSP performed the experimental protocols, analyzed the data, and wrote the first draft of the manuscript. Authors BFH and CVGQ performed part of the experimental protocols and analyzed the data. Authors RFL and RMC prepared the hydroethanolic extract for the study. Author DFA contributed to write the manuscript. Authors LSF, SICGB and SMM collaborated with the chemical analyses and *In vitro* antioxidant activity. Author Ana AMSA designed, managed, and supervised the study and manuscript. All authors read and approved the final manuscript.

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## ABSTRACT

**Aims:** This study aimed to characterize and evaluate the effect of the hydroethanolic extract from *D. kukachkana* leaves (HEDkL) in mice models of acute inflammation and in isolated macrophages stimulated with carrageenan, along with possible adverse effects in the central nervous system.

**Study Design:** After characterization of phenolic constituents, HEDkL was injected in the animals 60 min before the administration of inflammatory stimulus. The anti-inflammatory effect of HEDkL was evaluated in the models of paw edema, peritonitis and air pouch and in peritoneal macrophages stimulated with carrageenan *in vitro*. The *per se* effect of HEDkL was evaluated for behavioral alterations.

**Place and Duration of Study:** Higher Institute of Biomedical Sciences and Animal Health Research Center, State University of Ceara; Physiopharmacology Laboratory, Federal University of Acre; between March 2021 and March 2023.

**Methodology:** The characterization of phenolic compounds was performed by HPLC-DAD.

Swiss mice (25 - 35 g) received HEDkL (25 - 200 mg/kg; *per oral*) 60 min before carrageenan. Macrophages were incubated with HEDkL (100 - 200 µg/mL) before being stimulated. The anti-inflammatory effect was assessed for edema, hypernociception, vascular permeability, leukocyte infiltrate, oxidative stress and inflammatory. Behavioral alterations (exploratory, anxiolytic, depressant activities) were evaluated in the animals treated with HEDkL (50 mg/kg).

**Results:** The HPLC-DAD revealed the presence of ellagic acid, epicatechin and gallic acid. In the paw edema model, HEDkL (50 mg/kg) inhibited the edema late phase (2 - 5h) by 45% and the activity of the enzyme myeloperoxidase by 48%. In the peritonitis model, HEDkL (50 mg/kg) reduced leukocyte migration (45%), hypernociception (73%), total proteins (20%) and NO<sub>2</sub>/NO<sub>3</sub><sup>-</sup> (23%). In the air pouch model, HEDkL (50 mg/kg) reduced the migration of total leukocytes (76%) and neutrophils (88%). *In vitro* HEDkL (200 µg/mL) reduced by 31% the NO<sub>2</sub>/NO<sub>3</sub><sup>-</sup> in the supernatant of macrophages. HEDkL did not alter the animals exploratory, anxiolytic, or depressant behaviors.

**Conclusion:** HEDkL containing flavonoids and phenols presents anti-inflammatory effect *in vivo* and *in vitro* involving nitric oxide inhibition, being devoid of behavioral alterations.

**Keywords:** Breu-mescla; HPLC-DAD; inflammation; antioxidant; isolated macrophages; NO.

## ABBREVIATIONS

ABTS : 2,2'-Azino-Bis (3-ethylbenzothiazoline-6-sulfonic acid

AUC : Area Under Curve

DPPH : 2,2-Diphenyl-1-Picrylhydrazyl

HEDkL : Hydroethanolic Extract of *Dacryodes kukachkana* Leaves

HPLC : High Performance Liquid Chromatography

ICMBio : Instituto Chico Mendes de Conservação da Biodiversidade

iNOS : Inducible Isoform of the Nitric Oxide Synthase

MPO : Myeloperoxidase

NO : Nitric Oxide

NOS : Nitric Oxide Synthase

PGE : Prostaglandin E<sub>2</sub>

Sisbio : Sistema de Autorização e Informação em Biodiversidade

TNF-α : Tumor Necrosis Factor Alpha

## 1. INTRODUCTION

Acute inflammation is a defense response of the body against infections, injuries, traumas or immune reactions, involving inflammatory and vascular cells and mediators, to attain homeostasis and tissue repair. However, when

this response is exacerbated induces pain, fever, edema, tissue damage and function loss [1].

Carrageenan is a proinflammatory agent used to elucidate the anti-inflammatory mechanisms of new substances in rodents. The inflammatory effect induced by carrageenan involves activation

of resident cells, such as macrophages, and release of leukocyte chemotactic factors (prostaglandin E<sub>2</sub>-PGE<sub>2</sub>; tumor necrosis factor alpha-TNF- $\alpha$ , nitric oxide-NO) [2]. In this line, NO of macrophage source, activated via the inducible isoform of the nitric oxide synthase (iNOS), causes vasodilation, increased permeability, modulation of inflammatory cytokines and oxidative stress [3,4].

The anti-inflammatory drugs currently used in clinical practice, although effective, may cause adverse effects, such as gastrointestinal ulcers, renal and cardiac dysfunction, hyperglycemia and alterations in the central nervous system (psychological, cognitive, behavioral) [5-7]. Thus, it is necessary the search for alternative anti-inflammatory compounds presenting less adverse effects.

Plants belonging to the genus *Dacryodes* (Burseraceae), including *Dacryodes kukachkana* L.O. Williams (breu or breu-mescla), are widely found in the Amazonian Forest of Brazil and Peru [8] and popularly used to treat various inflammatory states [9], probably due to the antioxidants, including polyphenols, present in several *Dacryodes* species [10].

Experimental *in vivo* studies demonstrated for the hydroethanolic extract of *D. kukachkana* barks the antinociceptive effect via inhibition of inflammatory mediators [11]. In addition, non-published data obtained by our research group demonstrated the wound healing effect of the hydroethanolic extract prepared from its barks via reduction of inflammatory parameters (exudate, leukocyte infiltrate, oxidative stress).

Considering the lack of experimental studies with *D. kukachkana* leaves, the aim of this study was to characterize and evaluate the effect of its hydroethanolic extract in mice models of acute inflammation induced by carrageenan, the participation of NO from macrophage source and possible behavioral alterations.

## 2. MATERIALS AND METHODS

### 2.1 Hydroethanolic Extract Preparation

The leaves of *Dacryodes kukachkana* were collected in Mãnco Lima - Acre, Brazil (18 UTM 723548 7439155643 471) under authorization of Sisbio/ICMBio (n° 45912-1) and a voucher specimen (n° 20410) was deposited at the

herbarium of the Federal University of Acre. Leaves of *D. kukachkana* were dried (35°C) for 4 days, grounded into fine particles, subjected to percolation (72h, r.t.) in 70% ethanol, filtered and concentrated in rotary evaporator (45°C) [12]. The extract was frozen, lyophilized and named hydroethanolic extract of *D. kukachkana* leaves (HEDkL).

### 2.2 High Performance Liquid Chromatography (HPLC-DAD) of HEDkL

HEDkL (20 mg/mL) was dissolved in methanol to identify phenolic compounds. All samples and solutions were filtered (nylon membrane: 0.45  $\mu$ m; membrane filter: 0.22  $\mu$ m - Millipore) before use.

The chromatography was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AD alternative pumps connected to a DGU 20A5 degasser with CBM 20A integrator, SPD-M20A diode array detector, and LC 1.22 SP1 software and analyzed by C18 reverse-phase column (4.6 x 250 mm; 5  $\mu$ m; flow rate: 1.0 mL/min; injection volume: 20  $\mu$ L; wavelength: 350 nm). Acetonitrile and Milli-Q water were the mobile phases C and D, adjusted to pH 2.8 with phosphoric acid. The solvent gradient used was: 0-15 min - isocratic elution with C: D (20:80 v/v); 17-25 min - linear variation up to C: D (40:60 v/v); and 25-40 min - isocratic elution with C: D (20:80 v/v).

Gallic acid (0.001–0.5 mg/mL), epicatechin (0.000064–1.0 mg/mL) and ellagic acid (0.0001–1.0 mg/mL) (Sigma Chemical Co. - St. Louis, MO, USA) were used as analytical standards. The chromatographic peaks of HEDkL, analyzed in triplicate, were compared to the retention time and UV spectrum (200 to 400 nm) of the standards. The calibration curves were as the following: gallic acid ( $y = 3.10 \cdot 10^{-8} x - 0.0061$ ;  $r = 0.9997$ ); catechin ( $y = 4.10 \cdot 10^{-8} x + 0.006$ ;  $r = 0.997$ ); ellagic acid ( $2.10 \cdot 10^{-7} x + 0.003$ ;  $r = 0.999$ ) [13].

### 2.3 *In vitro* Antioxidant Activity of HEDkL

The antioxidant activity was evaluated by the methods of DPPH (2,2-diphenyl-1-picrylhydrazyl) [14] and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) [15]. The activity was measured in 96-well

flat-bottom plates by Elisa BioTek reader (ELX 800).

Serial dilutions of HEDkL at 2 µg/mL and control samples were prepared in methanol to obtain the following concentrations: 100, 50, 25, 12.5, 6.25 and 3.12 µg/mL, before addition of DPPH or ABTS. Methanol was used as the negative control and quercetin and ascorbic acid as antioxidant standards. Absorbances were measured at 515 nm for DPPH until 60 minutes of incubation, and at 630 nm for ABTS<sup>+</sup> after 10 minutes of incubation.

The results are expressed as percentage of inhibition (PI), calculated by  $PI (\%) = [(AC - AS)/AC] \times 100$ , where AC is absorbance of the DPPH or ABTS control solution at initial time and AS is the absorbance of the sample solution containing DPPH or ABTS at final time [13].

## 2.4 Animals and Treatment

Female Swiss mice (25 - 35 g), maintained at 22 ± 2 °C, light/dark cycle of 12:12h with free access to water and food, were used in the *in vivo* tests.

HEDkL (25 - 200 mg/kg) or dexamethasone (4 mg/kg) was administered *per oral* to the animals. After 60 minutes, 0.9% NaCl (saline) or the phlogistic agent carrageenan was administered by subcutaneous (SC) or intraperitoneal (IP) route. The anti-inflammatory effect was assessed in the acute models of paw edema, peritonitis and subcutaneous air pouch, and *in vitro* at 100, 150, 200 µg/mL on isolated macrophages stimulated by carrageenan (100 µg/mL).

The *per se* effect of HEDkL (50 mg/kg; *per oral*) after 60 minutes was evaluated for behavioral parameters (exploratory, locomotor, anxiety and depressor) in the following tests: open field, plus maze, hole board and tail suspension.

## 2.5 Paw-Edema Model

Paw edema [16] was induced by λ- carrageenan (300 µg; SC intraplantar) and measured by plethysmometry before (zero time) and up to 12 hours thereafter. Edema was calculated as the difference in paw volume displacement (µL) or area under curve-AUC (arbitrary units). The animals were euthanized, and the paw pads collected to determine the myeloperoxidase-MPO activity ( $A_{405nm}$ ) [17].

## 2.6 Peritonitis Model

Peritonitis was induced by carrageenan (500 µg; IP) [18] and evaluated 4 hours later for the following inflammatory parameters: abdominal hypernociception, leukocyte migration, total protein extravasation, and oxidative stress markers in the peritoneal fluid.

The evaluation of abdominal hypernociception was performed according to Cunha and collaborators (2004) [19] with modifications. For this, the animals were placed in acrylic boxes with mesh floor, stimulated in the peritoneum (average of 4 values) with a rigid tip connected to an electronic analgesimeter and the response (peritoneum withdrawal) expressed in g.

After euthanasia, the peritoneal cavity was washed (5 UI heparine + saline) and the peritoneal fluid collected for quantification of total leukocytes performed in Neubauer chamber (20 µL peritoneal fluid + 80 µL Turk reagent), and differential leukocytes (neutrophil, mononuclear) performed by smears on slides stained with hematoxylin-eosin (H&E). The results were expressed as the n° of cells per mm<sup>3</sup> of peritoneal fluid [18]. Peritoneal fluid was also quantified for the levels of total proteins by the Bradford method ( $A_{595nm}$ ) [20] and NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> by the Griess method ( $A_{540nm}$ ) [21].

## 2.7 Subcutaneous air Pouch Model

The air pouch was formed with an injection of 6 mL of sterile air (SC intrascapular) into the animal dorso. At the 3<sup>rd</sup> day after induction, additional 3 mL of air was injected to maintain the air pouch integrity. At the 7<sup>th</sup> day, mice were treated with HEDkL, being 60 minutes later stimulated with carrageenan (1 mL, SC) [22]. Animals were euthanized 5 hours after the induction and the pouch exudate collected for evaluation of the leukocyte infiltrate (total leukocytes, neutrophils, mononuclears) [18].

## 2.8 Macrophage Isolation

Naive macrophages were collected from peritoneal fluid of animals previously injected (IP) with 3 mL of heparinized PBS. Macrophage monolayers were prepared by addition of 10<sup>6</sup> macrophages (1 mL/well) and incubated for 24 hours (37°C in 5% CO<sub>2</sub>) for adhesion to occur [23]. Non-adhered macrophages were removed by 3 successive washes with the medium culture RPMI. Plate-adhered macrophages were incubated with HEDkL (100, 150, 200 µg/ml) or RPMI for 30 minutes at 37° C, followed by

additional incubation with carrageenan (100 µg/ml) or RPMI for 60 minutes. The supernatants were collected, centrifuged for 5 minutes and quantified for NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels [21].

## 2.9 Behavioral Tests

To evaluate the *per se* effect of HEDkL in the central nervous system, the animals received HEDkL (50 mg/kg) or saline *per oral* 60 minutes before observation of behavioral parameters (exploratory, locomotor, anxiety and depressor) in the tests: open field [24], plus maze [25], hole board [26] and tail suspension [27].

## 2.10 Statistical Analysis

Data were expressed as Mean ± SEM and analyzed by analysis of variance (ANOVA) followed by Bonferroni tests. The data of behavioral tests was expressed by Student t-test. Values of p<0.05 were considered significant.

## 3. RESULTS AND DISCUSSION

### 3.1 HEDkL Characterization by HPLC-DAD and *in vitro* Antioxidant Activity

The characterization of EHDkL by HPLC-DAD revealed the presence of three peaks, characteristic of the phenolic compounds gallic acid (peak 1), epicatechin (peak 2) and ellagic acid (peak 3) (Fig. 1).

HEDkL showed antioxidant activity (IC<sub>50</sub> < 50 µg/mL), inhibiting both radicals ABTS (IC<sub>50</sub>: 10.29 ± 0.32 µg/mL) and DPPH (IC<sub>50</sub>: 8.46 ± 0.46 µg/mL) (Table 1).

**Table 1. CI<sub>50</sub> values for DPPH and ABTS<sup>+</sup> radicals**

Compound	CI <sub>50</sub> DPPH (µg/mL)	CI <sub>50</sub> ABTS <sup>+</sup> (µg/mL)
Quercetin (standart)	1.61 ± 0.04	0.95 ± 0.06
Ascorbic acid (standart)	1.94 ± 0.27	11.90 ± 0.26
HEDkL	8.46 ± 0.46	10.29 ± 0.32

CI<sub>50</sub> – Half maximal inhibitory concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); HEDkL – hydroethanolic extract of *D. kukachkana* leaves

The inhibitory activity of HEDkL could be ascribed for the synergistic activities of its constituents (flavonoids and phenols), since they are known to present anti-inflammatory and antinociceptive activities [28,29].

Experimental studies demonstrated that ellagic acid, the major constituent found in HEDkL, has anti-inflammatory, antioxidant and antinociceptive activities, via inhibition of MPO in the mice model of peritonitis induced by carrageenan [30,31]. Epicatechin, the second major constituent of HEDkL, is also described for the anti-inflammatory and antioxidant activities via inhibition of inflammatory factors and reactive oxygen species [32]. Gallic acid has anti-inflammatory activity, inhibiting inflammatory mediators such as cyclooxygenase 2 (COX<sub>2</sub>) and NO, and reducing inflammation-related diseases via anti-oxidative stress [33].

Furthermore, the hydroethanolic extract of *D. kukachkana* barks, obtained by the same extraction protocol, also containing polyphenols (mainly ellagic acid), was shown to present antinociceptive activity in mice, but at a dose 10-fold higher that used in our study; and inhibited the inflammatory phase (15 - 30min) of the formalin test, suggesting the involvement of peripheral components and late phase inflammatory mediators [11].

### 3.2 HEDkL Inhibits the Paw Edema and MPO Activity Stimulated by Carrageenan

HEDkL at 25, 50, 100 or 200 mg/kg (Fig. 2A - C) inhibited the late phase (2 - 5h) of the carrageenan-induced paw edema (285 ± 29 vs. saline: 3.75 ± 10 AUC) by 21% (223 ± 14 AUC), 45% (157 ± 26 AUC), 21% (224 ± 30 AUC) and 53% (134 ± 22 AUC), respectively (Fig. 2C). The HEDkL inhibitory effect on the edema time-course at 50 mg/kg did not differ from that attained at the dose of 200 mg/kg (Fig. 2C), being the dose of 50 mg/kg chosen to perform the next protocols.

HEDkL (50 mg/kg) inhibited the paw edema from 2h to 5h (Fig. 2D). The inhibitory effect of HEDkL was about 56% (98 ± 12 vs. carrageenan 222 ± 8.6 AUC) similar to dexamethasone by 59% (92 ± 10 vs. carrageenan 222 ± 8.6 AUC) in the same hours (Fig. 2D/F). HEDkL also inhibited the paw edema

from 5 to 12h about 36% ( $166 \pm 90$  vs. carrageenan  $262 \pm 146$  AUC), while the reference drug dexamethasone inhibited by 49% ( $132 \pm 77$  vs. carrageenan  $262 \pm 146$  AUC) (Fig. 2G).

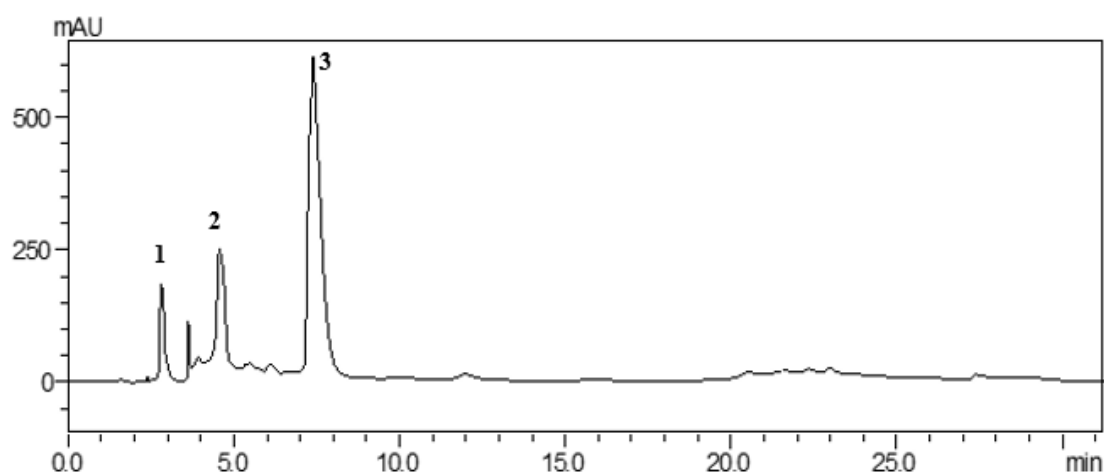
Carrageenan increased the activity of MPO ( $1.1 \pm 0.3$  vs. saline:  $0.7 \pm 0.3$  U/mg tissue) in the paw tissues at 5h that was inhibited by HEDkL by 48% ( $0.6 \pm 0.3$  mg vs. saline:  $0.7 \pm 0.3$  mg) (Fig. 2H).

The literature reports that the paw edema induced by carrageenan in mice is biphasic and characterized by protein leakage and release of vasoactive mediators (histamine, serotonin, bradykinin, substance P) in the initial phase (0 - 2h), and leukocyte migration by mechanisms involving TNF- $\alpha$ , interleukin 1 beta - IL-1 $\beta$ , prostaglandins and NO in the late phase (2 - 4h) [34,35], being these mediators released from resident cells such as macrophages [36]. In our study, HEDkL dose-dependently inhibited the cellular phase

of the carrageenan-induced paw edema, an effect that was sustained up to 12 hours, similar to the anti-inflammatory steroid dexamethasone. This inhibition corroborates the decreased activity of MPO, a marker of neutrophil infiltration.

### 3.3 HEDkL Inhibits the Increase of Leukocyte Migration, Protein Extravasation, Hypernociception and NO<sub>2</sub>/NO<sub>3</sub><sup>-</sup> Induced by Carrageenan

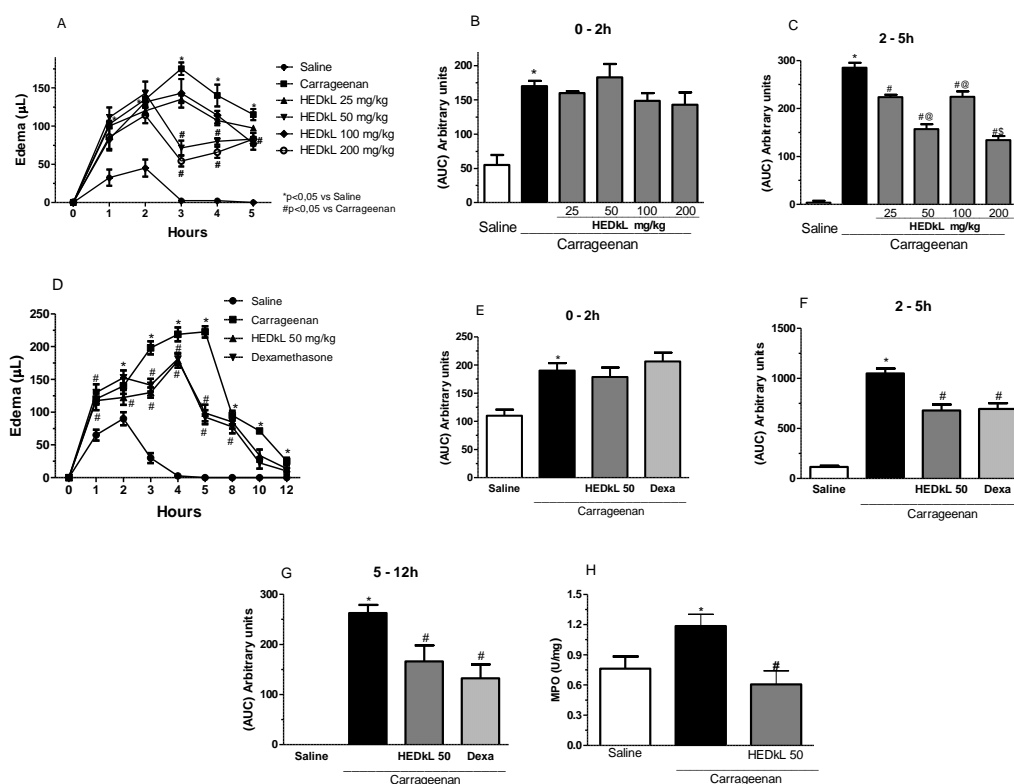
Intraperitoneal administration of carrageenan increased the n° of total leukocytes in 2-fold ( $4887 \pm 1273$  vs. saline:  $2043 \pm 512$  cells/mm<sup>3</sup>) and that of neutrophils in 19-fold ( $3655 \pm 862$  vs. saline:  $190 \pm 139$  cells/mm<sup>3</sup>), which migrated to the peritoneal fluid. HEDkL reduced the n° of total leukocytes by 45% ( $2664 \pm 803$  vs. carrageenan:  $4887 \pm 1273$  cells/mm<sup>3</sup>), primarily due to the decrease of the n° of neutrophils by 83% ( $629 \pm 289$  vs. carrageenan:  $3655 \pm 862$  cells/mm<sup>3</sup>) (Fig. 3A).



Peak	t <sub>R</sub>	Component	Area	Maximum wavelength
1	3.61 min	Gallic Acid	2.43 ± 0.72	214 nm; 273 nm
2	4.56 min	Epicatechin	3.66 ± 0.63	218 nm; 277 nm; 355 nm
3	7.38 min	Ellagic Acid	7.12 ± 0.70	255 nm; 366 nm

**Fig. 1. HEDkL high performance liquid chromatography**

The chromatography was performed in the HPLC system (HPLC-DAD) and analyzed by C18 reverse-phase column (4.6 x 250 mm; 5  $\mu$ m; flow rate: 1.0 mL/min; injection volume: 20  $\mu$ L; wavelength: 350 nm). Gallic acid, epicatechin and ellagic acid were used as standards. The chromatographic peaks of HEDkL were compared to the retention time and UV spectrum (200 to 400 nm) of the standards



**Fig. 2. HEDkL inhibits the paw edema induced by carrageenan**

Mice received HEDkL (25 – 200 mg/kg; per oral) or dexamethasone (Dexa; 4 mg/kg; PO) 1 h before carrageenan (300 µg/SC) or saline (0.9% NaCl; IP). Paw edema was measured by hydroplethysmometry (0 - 5h and 0 - 24h) and expressed as the variation in paw volume displacement (mL) or area under curve – AUC (arbitrary units). (A) Edema time-course: 0 - 5h, (B) AUC (0 - 2h), (C) AUC (2 - 5h), (D) Edema time-course: 0 - 24h, (E) AUC (0 - 2h), (F) AUC (2 - 5h), (G) AUC (5 - 12h), (H) AUC (12 - 24h), (I) Myeloperoxidase-MPO activity (A<sub>405nm</sub>). Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. P<0.05 vs. (\*Saline, #Carrageenan, &HEDkL 25 mg/kg, @HEDkL 50 mg/kg, \$HEDkL 100 mg/kg); AUC: area under curve

Moreover, carrageenan reduced the abdomen nociceptive threshold in response to the mechanical stimulation 3h later (866 ± 2.96 vs 35.50 ± 5.33 saline), inducing hypernociception, which was reversed by HEDkL (32.4 ± 8.6 g) (Fig. 3B).

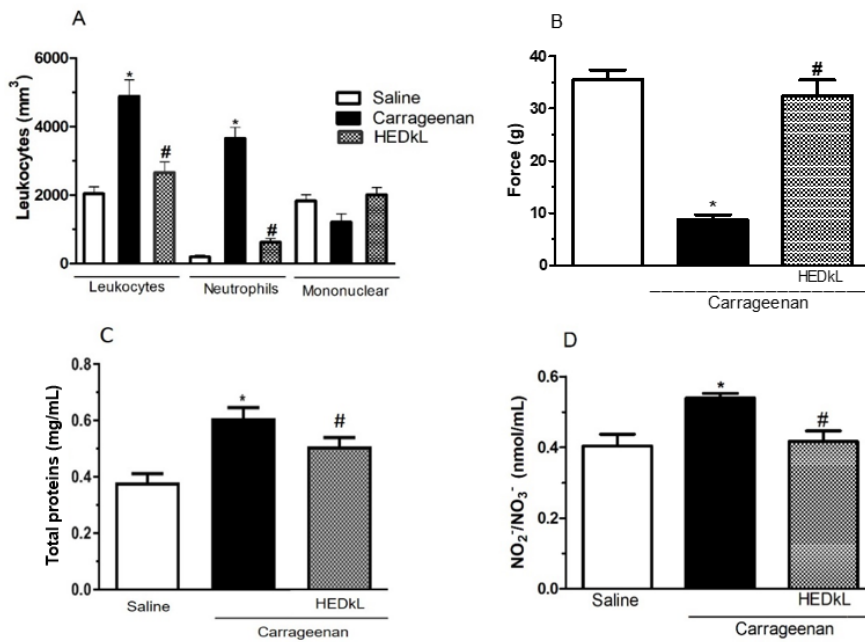
HEDkL also reduced the levels of total proteins in the peritoneal fluid by 20% (0.51 ± 0.08 mg/mL) compared to carrageenan (0.63 ± 0.03 vs. saline: 0.39 ± 0.08 mg/mL) (Fig. 3C) and that of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> by 23% (0.41 ± 0.08 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> nmol/mL) compared to carrageenan (0.53 ± 0.03 vs. saline: 0.40 ± 0.08 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> nmol/mL) at 4h (Fig. 3D).

The HEDkL inhibitory effect on the migration of leukocytes, mainly neutrophils, induced by carrageenan to the peritoneal and air pouch fluids is in accordance with the inhibition of the

edema late phase (cellular phase) and MPO activity stimulated by carrageenan.

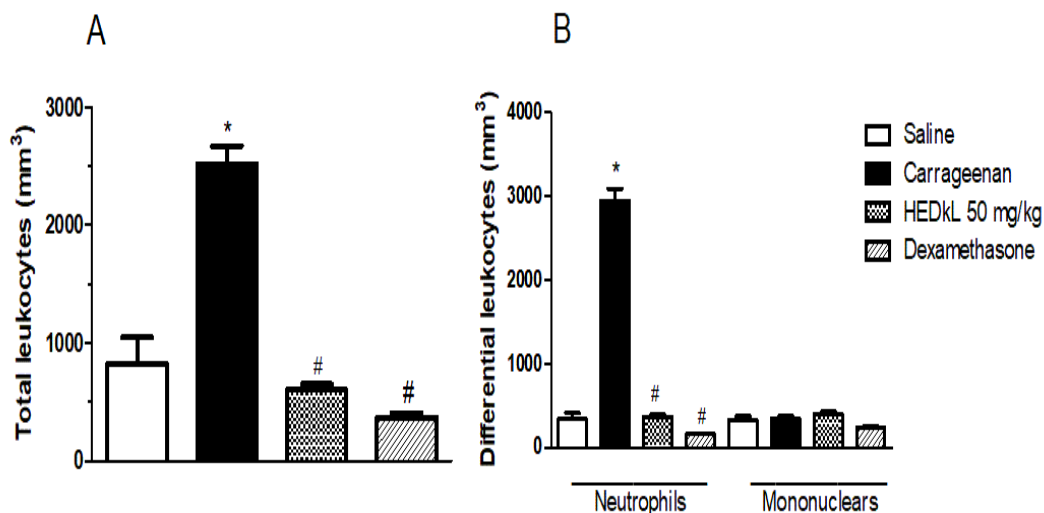
In addition, the inhibitory effect of HEDkL on hypernociception is correlated with the effect on paw edema and neutrophil migration and could be resulted from the increased release of inflammatory mediators that sensitize nociceptors, such as PGE, NO and pro-inflammatory cytokines [37]. Moreover, the HEDkL inhibitory effect on the carrageenan initial inflammatory phase (osmotic phase) can be correlated with the reduced protein levels that had been increased by carrageenan in the peritoneal fluid. The antinociceptive effect had already been demonstrated for the hydroethanolic extract of *D. kukachkana* barks in mice models [11]. It is possible to be hypothesized the involvement of NO in the HEDkL anti-inflammatory response, as the late-stage inflammatory mediators present in the

carrageenan model stimulate leukocyte migration and peripheral nociception by mechanisms that include the participation of the nitric oxide synthase (NOS) [38].



**Fig. 3. HEDkL inhibits peritoneal leukocyte migration, hypernociception, total protein and NO<sub>2</sub>/NO<sub>3</sub><sup>-</sup> stimulated by carrageenan**

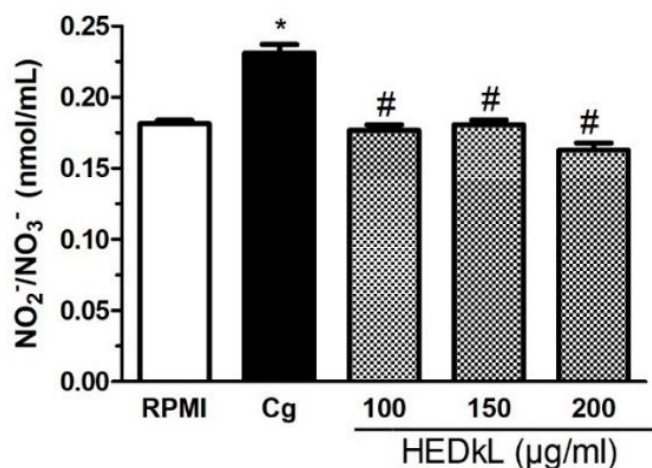
Mice received HEDkL (50 mg/kg; PO) 1 h before carrageenan (500 µg; IP). Control animals received saline (0.9% NaCl; IP). After 4 h, peritoneal fluid was collected for evaluation of (A) Total and differential leukocytes (cells/mm<sup>3</sup>), (B) Total protein (mg/mL), (C) Hypernociception (g), (D) NO<sub>2</sub>/NO<sub>3</sub><sup>-</sup> (nmol/mL). Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. P < 0.05 vs. (\*Saline, #Carrageenan)



**Fig. 4. HEDkL inhibits leukocyte migration stimulated by carrageenan to the subcutaneous air pouch**

Mice received HEDkL (50 mg/kg; PO) 1 h before carrageenan (500 µg; SC) or saline (0.9% NaCl; IP). The pouch exudate was collected 5h after stimuli for quantification of (A) Total leukocytes, (B) Differential leukocytes. Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. P < 0.05 vs. (\*Saline, #Carrageenan)





**Fig. 5. HEDkL reduces NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> in the supernatant of isolated macrophages stimulated with carrageenan**

Macrophages were incubated (30 min, 37°C) with RPMI or HEDkL (100 - 200 µg/mL) followed by additional incubation (60 min) with carrageenan (100 µg/mL) or RPMI. NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> (nmol/mL) was measured by the Griess method. Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. P < 0.05 vs. (\*RPMI, #Carrageenan - Cg)

**Table 2. HEDkL per se does not alter exploratory, locomotor, anxiety or depressor behaviors in mice**

Open field	Saline	EHDkL
N° of crossing	46 ± 6	39 ± 9
N° of rearing	5 ± 3	5 ± 4
N° of grooming	0.7 ± 0.7	1.2 ± 1.05
<b>Plus maze</b>		
<sup>1</sup> NEOA	5.6 ± 2.7	3.7 ± 3.8
<sup>2</sup> TSOA (s)	94 ± 59	81 ± 71
<sup>3</sup> NECA	7.2 ± 2.4	7.7 ± 3.8
<sup>4</sup> TSCA (s)	155 ± 37	175 ± 55
<b>Hole board</b>		
N° of head dips	27 ± 10	26 ± 7
Permanence time (s)	17 ± 6	19 ± 10
<b>Tail suspension</b>		
Immobility time (s)	100 ± 23	94 ± 34

<sup>1</sup>n° of entries in the open arms; <sup>2</sup>time spent in open arms; <sup>3</sup>n° of entries in the closed arms; <sup>4</sup>time spent in closed arms

It is well known that inflammatory mediators from resident and infiltrated cells stimulate oxidative stress [39]. Since HEDkL reduced MPO and NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> in the paw tissue, peritoneal fluid and in the supernatant from isolated macrophages, it seems to play important role as antioxidant. MPO is a peroxidase present in the neutrophils azurophilic granules possessing phagocytosis role [40]. NO interacts with superoxide anions, resulting in peroxynitrite, which mediates cytotoxic reactions, vasodilation, peripheral, central nociception, increased vascular permeability and leukocyte chemotaxis [41]. Besides, the antioxidant activity of HEDkL in the

inflammation models could reinforces the effect demonstrated by the *in vitro* protocols (DPPH, ABTS).

### 3.4 HEDkL Inhibits the Increase of Leukocyte Migration to the Subcutaneous Air Pouch and the Production of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> by Macrophages Stimulated with Carrageenan

Administration of carrageenan in the subcutaneous air pouch increased the n° of total leukocytes in 3-fold (2517 ± 383 vs. saline: 818 ±

646 cells/mm<sup>3</sup>), being reduced (76%) by HEDkL (600 ± 146 vs. carrageenan: 2517 ± 383 cells/mm<sup>3</sup>) (Fig. 4A). Carrageenan also increased the n° of neutrophils in 8-fold (2948 ± 404 vs. saline: 341 ± 171 cells/mm<sup>3</sup>), that was reduced (88%) by HEDkL (374 ± 72 vs. carrageenan: 2948 ± 8404 cells/mm<sup>3</sup>) (Fig. 4B). Dexamethasone reduced both total leukocytes by 85% (368 ± 113 vs. carrageenan: 2517 ± 383 cells/mm<sup>3</sup>) and neutrophils by 95% (162 ± 32 vs. carrageenan: 2948 ± 8404 cells/mm<sup>3</sup>) (Fig. 4A/B).

Isolated macrophages stimulated with carrageenan showed increased levels of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> by 27% (0.23 ± 0.01 vs. saline: 0.18 ± 0.007 nmol/mL) in the supernatant. The incubation with HEDkL at 100 µg/ml (0.17 ± 0.01 nmol/mL), 150 µg/mL (0.18 ± 0.009 nmol/mL) and 200 µg/mL (0.16 ± 0.01 nmol/mL) reduced NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels (Fig. 5).

The air pouch is formed by a cell layer almost exclusively composed by resident macrophages [22]. In this model, HEDkL reversed leukocyte migration induced by carrageenan. This effect is correlated with inhibition of NO and neutrophil migration demonstrated in peritonitis model. Neutrophils are the first immune cells to migrate into tissues and release chemoattractant substances, promoting migration of monocytes, which are differentiated into macrophages [42]. In the acute phase of carrageenan-induced inflammation, resident macrophages release the mediators NO, cytokines and neutrophil-specific chemokines, increasing neutrophil migration [43]. Accordingly, the anti-inflammatory effect of HEDkL was reproduced *in vitro* via reduction of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels in the supernatant of cultured macrophages.

### 3.5 HEDkL Per Se does not Change Mice Behavior Parameters in the Animals Central Nervous System

HEDkL did not alter the exploratory behavior in the open field test evaluated by the n° of crossing, rearing and grooming. Similarly, HEDkL did not alter the anxiety behavior either in the elevated plus maze (n° of entries in open and closed arms, and the time spent in open and closed arms), or in the hole board (n° of head dips and permanence time). Besides, the depressor behavior was not altered in the tail suspension test, evaluated by the animal's immobility time (Table 2).

The most used anti-inflammatory drugs in the clinical practice may cause adverse effects on the central nervous system such as anxiety, depression and sleep disorders, among others [44,45]. However, the *per oral* treatment in mice with HEDkL did not alter the behavioral parameters (exploratory, locomotor, anxiety or depressor), suggesting its potential use as an anti-inflammatory drug with low or absent adverse effects in the central nervous system.

## 4. CONCLUSION

The hydroethanolic extract from *Dacryodes kukachkana* leaves, composed by ellagic acid, epicatechin and gallic acid, presents anti-inflammatory, antioxidant and antinociceptive effects on acute inflammation models induced by carrageenan, involving NO and macrophages, being devoid of adverse effects in the central nervous system.

## ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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