***Original Research Article***

# Anti-inflammatory effect of the hydroethanolic extract from *Dacryodeskukachkana*leaves in mice: Role ofmacrophage nitric oxide


## 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 inisolated macrophages stimulated with carrageenan, along with possible adverse effects in the central nervous system.

**Study design:**After characterization of phenolic constituents, HEDkLwas injected in the animals 60 min before the administration of inflammatory stimulis. 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 HEDkLwas 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 minutes before inflammatory stimulus. 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 (50mg/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 $NO_2^-/NO_3^-$(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 $NO_2^-/NO_3^-$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.*

## 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 phlogistic 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 $E_2$-$PGE_2$; tumor necrosis factor alpha-TNF-$\alpha$, nitric oxide-NO) [2]. In this line NO of macrophage source, activated viathe 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) [5,6,7]. Thus, it is necessary the search for alternative anti-inflammatory compounds presenting less adverse effects.

Plants belonging to the genus *Dacryodes*(Burseraceae), including *Dacryodeskukachkana*L.O. Williams (breu or breu-mescla), are widely found in the Amazonian Forest of Brazil and Peru[8] and popularly used to treatvarious 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. MATERIAL AND METHODS

### 2.1 Hydroethanolic extract preparation

The leaves of *Dacryodeskukachkana*were collected in Mâncio 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 µm; membrane filter: 0.22 µm - 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 µm; flow rate: 1.0 mL/min; injection volume: 20 µL; 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- 8 x – 0.0061; $r$ = 0.9997); catechin (y = 4.10-8 x + 0.006; $r$ = 0.997); ellagic acid (2.10-7 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 $ABTS^{+}$ after 10 minutes of incubation.

The results are expressed as percentage of inhibition (PI), calculated by PI (%) = [(AC -AS)/AC] × 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 DPPHor ABTS at final time [13].

### 2.4 Animals and treatment

Female *Swiss* mice (25 - 35 g), maintained at 22 ± 2 ºC, 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, and 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 $mm^3$ of peritoneal fluid [18]. Peritoneal fluid was also quantified for the levels of proteins by the Bradford method ($A_{595\ nm}$) [20] and $NO_2^-/NO_3^-$ by the Griess method ($A_{540\ nm}$) [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 dorse. At the 3rd day after induction, additional 3 mL of air was injected to maintain the air pouch integrity. At the 7th 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 $10^6$ macrophages (1 mL/well) and incubated for 24 hours (37°C in 5% $CO_2$) for adhesion to occur [23]. Non-adhered macrophages were removed by 3 successive washes with the medium cultureRPMI. 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 $NO_2^-/NO_3^-$ 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

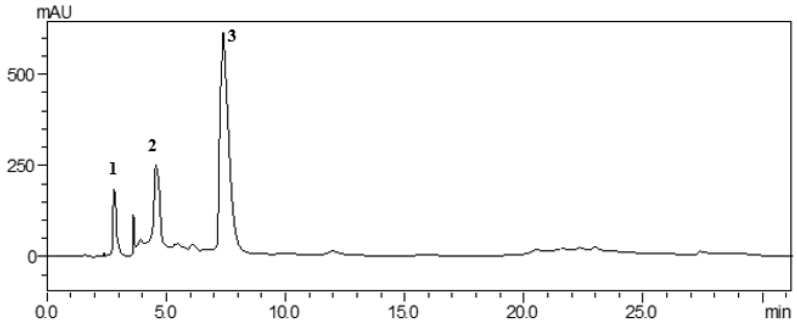
The characterization of EHDkL by HPLC-DAD revealed the presence of three peaks, characteristic of the phenolic compounds gallic acid (peak 1), epicatechin (peak 2) and ellagic acid (peak 3) (Figure 1).

HEDkL showed antioxidant activity ($IC_{50}$< 50 µg/mL), inhibiting both radicals ABTS ($IC_{50}$: 10.29 ± 0.32 µg/mL) and DPPH ($IC_{50}$: 8.46 ± 0.46 µg/mL) (Table 1).

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 ascyclooxygenase 2($COX_2$) and NO, and reducing the 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].




| Peak | $t_R$ | 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 |

**Figure 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 µm; flow rate: 1.0 mL/min; injection volume: 20 µL; 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.

**Table 1. $CI_{50}$ values for DPPH and $ABTS^+$ radicals**

| COMPOUND | $CI_{50}$ DPPH (µg/mL) | $CI_{50}$ $ABTS^+$ (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 |

$CI_{50}$ – Half maximal inhibitory concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); HEDkL – hidroethanolic extract of *D. kukachkana*leaves.

## 3.2 HEDkL inhibits the paw edema and MPO activity stimulated by carrageenan

HEDkL at 25, 50, 100 or 200 mg/kg (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 2D/F). HEDkL also inhibited the paw edema from 5 to 12h about 36% (166 ± 90 *vs.* carrageenan 262 ± 146 AUC), while the reference drug dexamethasone inhibited by 49% (132 ± 77 *vs*. carrageenan 262 ± 146 AUC) (Figure 2G).

Carrageenan increased the activity of MPO (1.1 ± 0.3 *vs*. saline: 0.7 ± 0.3 U/mg tissue) in the paw tissues at 5h that was inhibited by HEDkL by 48% (0.6 ± 0.3 mg *vs*. saline: 0.7 ± 0.3 mg) (Figure 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 involving TNF-α, interleukin 1 beta - IL-1β, 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.

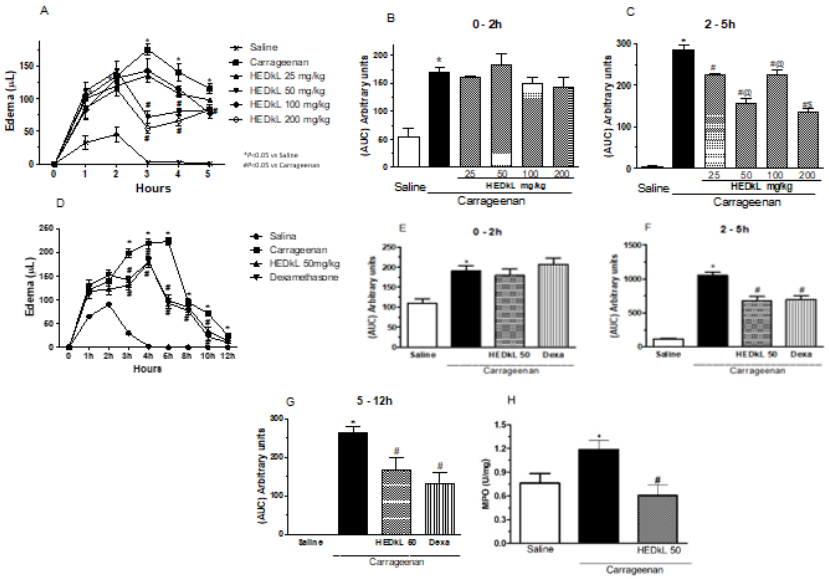



**Figure 2. HEDkL inhibits the paw edema induced by carrageenan.**
Mice received HEDkL (25 – 200 mg/kg; *per oral*) or dexamethasone (Dexa; 4 mg/kg; PO) 1 h before carrageenan (300 µg/SC) or saline (0.9% NaCl; IP). Paw edema was measured by hydroplethysmometry(0 - 5h and 0 - 24h) and expressed as the 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 ($A_{405nm}$). Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. $P<0.05$ *vs.* (*Saline, #Carrageenan, &HEDkL 25 mgkg, @HEDkL 50 mg/kg, $HEDkL 100 mg/kg); AUC: area under curve.

## 3.3HEDkL inhibits the increase of leukocyte migration, protein extravasation, hypernociception and $NO_2^-/NO_3^-$ induced by carrageenan

Intraperitoneal administration of carrageenan increased the n° of total leukocytes in 2-fold (4887 ± 1273 *vs*. saline: 2043 ± 512 cells/mm$^3$) and that of neutrophils in 19-fold (3655 ± 862 *vs.* saline: 190 ± 139 cells/mm$^3$), which migrated to the peritoneal fluid. HEDkL reduced the n° of total leukocytes by 45% (2664 ± 803 *vs*. carrageenan: 4887 ± 1273

cells/mm$^3$), primarily due to the decrease of the n° of neutrophils by 83% (629 ± 289 *vs*. carrageenan: 3655 ± 862 cells/mm$^3$) (Figure 3A).

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) (Figure 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) (Figure 3C) and that of $NO_2^-/NO_3^-$ by 23% (0.41 ± 0.08 $NO_2^-/NO_3^-$ nmol/mL) compared to carrageenan (0.53 ± 0.03 *vs*. saline: 0.40 ± 0.08 $NO_2^-/NO_3^-$ nmol/mL) at 4h (Figure 3D).

Carrageenan also evoked significant leukocyte migration to the peritoneal and the air pouch fluid, which was inhibited by HEDkL, being neutrophils the main targeted cells. These data are 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].

It is well known that inflammatory mediators from resident and infiltrated cells stimulate oxidative stress [39]. Since HEDkL reduced MPO and $NO_2^-/NO_3^-$ 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).

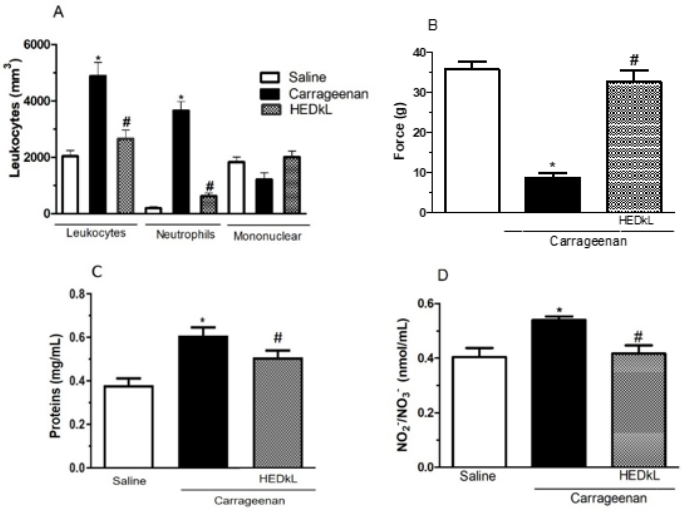



**Figure3. HEDkL inhibits peritoneal leukocyte migration, hypernociception, total protein and $NO_2^-/NO_3^-$stimulated by carrageenan**.

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/$mm^3$), (B) Total protein (mg/mL), (C) Hypernociception (g), (D) $NO_2^-/NO_3^-$(nmol/$mL^1$). Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. *P*<0.05 *vs.* (*Saline, #Carrageenan).

## 3.4HEDkL inhibits the increase of leukocyte migration to the subcutaneous air pouch andthe production of $NO_2^-/NO_3^-$ by macrophagesstimulated 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/$mm^3$), being reduced (76%) by HEDkL(600 ± 146 *vs*. carrageenan: 2517 ± 383 cells/$mm^3$) (Figure 4A). Carrageenan also increased the n° of neutrophils in 8-fold (2948 ± 404 *vs.* saline: 341 ± 171 cells/$mm^3$), that was reduced (88%) by HEDkL (374 ± 72 *vs*. carrageenan: 2948 ± 8404 cells/$mm^3$) (Figure 4B). Dexamethasone reduced both total leukocytes by 85% (368 ± 113 *vs.* carrageenan: 2517 ± 383 cells/$mm^3$) and neutrophils by 95% (162 ± 32 *vs*. carrageenan: 2948 ± 8404 cells/$mm^3$) (Figure 4A/B).

Isolated macrophages stimulated with carrageenan showed increased levels of $NO_2^-/NO_3^-$ 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$NO_2^-/NO_3^-$ levels(Figure 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 $NO_2^-/NO_3^-$ levels in the supernatant of cultured macrophages.

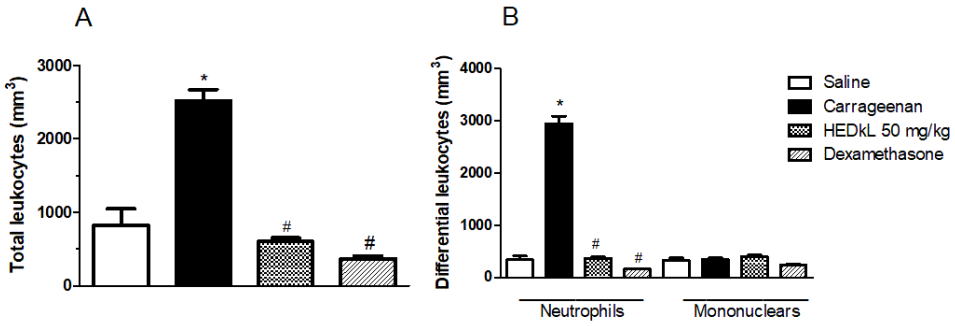



**Figure 4. HEDkL inhibits leukocyte migration stimulated by carrageenan to the subcutaneous air pouch**.
Mice received HEDkL (50 mg/kg; PO) 1 h before carrageenan (500 µg; SC) or saline (0.9% NaCl; IP). The pouch exudate was collected 5h after stimuli for quantification of (A) Total leukocytes, (B) Differential leukocytes. Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. *P*<0.05 *vs.* (*Saline, #Carrageenan).

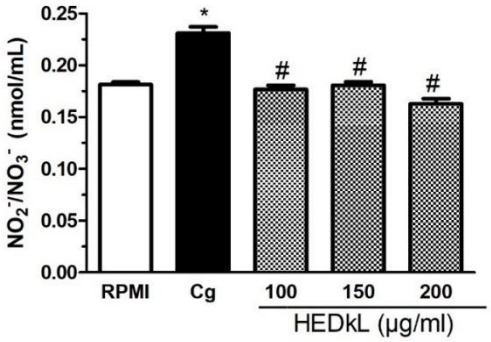



**Figure 5. HEDkL reduces $NO_2^-/NO_3^-$ in the supernatant of isolated macrophages stimulatedwith carrageenan.**
Macrophages were incubated (30 min, 37°C)with RPMI or HEDkL (100 - 200 µg mL) followed by additional incubation (60 min) with carrageenan (100 µg/mL) or RPMI. $NO_2^-/NO_3^-$ **(**nmol/mL) was measured by the Griess method.Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. *P*<0.05 *vs.* (*RPMI, #Carrageenan - Cg).

## 3.5HEDkL*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, rearingand grooming. Similarly, HEDkLdid 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 hips and permanence time). Besides, the depressor behavior was not altered in the tail suspension test, evaluated by the animal'simmobility 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.

**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 |
| **PLUS MAZE** | | |
| [1]NEOA | 5.6 ± 2.7 | 3.7 ± 3.8 |
| [2]TSOA (s) | 94 ± 59 | 81 ± 71 |
| [3]NECA | 7.2 ± 2.4 | 7.7 ± 3.8 |
| [4]TSCA (s) | 155 ± 37 | 175 ± 55 |
| **HOLE BOARD** | | |
| N° of head hips | 27 ± 10 | 26 ± 7 |
| Permanence time (s) | 17 ± 6 | 19 ± 10 |
| **TAIL SUSPENSION** | | |
| Immobility time (s) | 100 ± 23 | 94 ± 34 |

[1]n° of entries in the open arms; [2]time spent in open arms; [3]n° of entries in the closed arms; [4]time spent in closed arms.



## 4. CONCLUSION

The hydroethanolic extract from *Dacryodeskukachkana* 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

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

## REFERENCES


1. Larsen GL, HensonPM. Mediators of Inflammation. Annual Review of Immunology. 1983; 1: 335-359.
2. Nacife VP, Soeiro MD, Araújo-Jorge TC, Castro-Faria Neto HC, Meirelles MD. Ultrastructural, immunocytochemicaland flow cytometry study of mouse peritoneal cells stimulated with carrageenan. Cell Structure and Function. 2000; 25: 337–350.
3. Lind M, HayesA,CaprndaM, PetrovicD, RodrigoL,KruzliakP et al. Inducible nitric oxide synthase: Good or bad? Biomedicine & Pharmacotherapy. 2017; 93: 370–375.
4. Gray Z, Shi G, Wang X, HuY. Macrophage inducible oxide nitric synthase promotes the initiation of lung squamous cell carcinoma by maintaining circulation inflammation. Cell Death and Disease. 2018; 9: 642.
5. Perez A, Jansen-Chaparro S,SaigiI, Bernal-Lopez MR,Miñambres I, Gomez-Huelgas R. Glucocorticoid-induced hyperglycemia. Journal of Diabetes. 2014; 6: 9-20.
6. Judd LL, Schettler PJ, Brown ES, Wolkowitz OM, Sternberg EM, Bender BG, et al. Adverse consequences of glucocorticoid medication: psychological, cognitive, and behavioral effects. The American Journal of Psychiatry. 2014;171: 1045–1051.
7. Bindu S, Mazumder S, Bandyopadhyay U. Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. Biochemical Pharmacology. 2020; 180: 114147.
8. Daly DC. Notes on the Burseraceae in central Amazonia, including four new taxa. Studies in neotropical Burseraceae XXVI. Brittonia. 2018; 70: 427-444.
9. Tee LH, Yang B, Tey BT, Chan ES, Azlan A, Ismail A, Sun J, Lau CY, Palanisamy UD, Ramanan RN, et al. Valorization of *Dacryodes rostrata* fruit through the characterization of its oil. Food Chemistry. 2017; 235:257–264.
10. Swana L, Tsakem B, Tembu JV, Teponno RB, Folahan JT, Kalinski JC, Polyzois A, Kamatou G, Sandjo LP, Chamcheu JC, Siwe-Noundou X. The Genus *Dacryodes* Vahl.: Ethnobotany, Phytochemistry and Biological Activities. Pharmaceuticals. 2023;16:775.
11. Lopes RF, Silva APAB, Amorim, RMF, Araujo DF, da Silva FMA, Koolen HHF, et al. Hydroethanolic extract from barks of the Amazonian species *Dacryodeskukachkana*: potential usage in painful conditions. European Academic Research. 2021; 9: 5516.
12. Silva APAB, Amorim RMF, Lopes RF, Mota MRL, Da Silva FMA, Koolen HHF, et al. *Calycophyllumspruceanum* BENTH ameliorates acute inflammation in mice. JournalofEthnopharmacology. 2018;219: 103-109.
13. Frota LS, Alves DR, Marinho MM, da Silva LP, Almeida Neto FWDQ, Marinho ES, & de Morais SM. Antioxidant and anticholinesterase activities of amentoflavone isolated from Ourateafieldingiana (Gardner) Engl. through in vitro and chemical-quantum studies. Journal of Biomolecular Structure and Dynamics. 2023; 41: 1206-1216.
14. Becker MM, Nunes GS, Ribeiro DB, Silva FE, Catanante G, Marty JL. Determination of the antioxidant capacity of red fruits by miniaturized spectrophotometry assays. Journal of the Brazilian Chemical Society. 2019; 30: 1108-1114.

15.Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine. 1999; 26: 1231-1237.
16. LevyL. 1969. Carrageenan paw edema in the mouse. *Life Sciences*, 8: 601–606.
17. Bradley PP, Christensen RD, RothsteinG. Cellular and extracellular myeloperoxidase in pyogenic inflammation.Blood. 1982; 60: 618–622.
18.SouzaGEP, Ferreira SH.Blockade by antimacrophage serum of the migration of PMN neutrophils into the inflamed peritoneal cavity.AgentsandActions. 1985; 17: 97–103.
19. Cunha, T. M.; Verri JR. W. A.; Vivancos, G. G.; Moreira, I. F.; Reis, S.; Parada, C. A.; et al. 2004. An electronic pressure-meter nociception paw test for mice. *Brazilian Journal of Medical and Biological Research*, 37: 401-407.
20. BradfordMA. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry. 1976; 72: 248–254.
21. GreenLC, Wagner DA, Glogowski J, Skipper PL,Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and nitrate in biological fluids. Analytical Biochemistry. 1982;126: 131-138.
22. Edwards JC, Sedgwick AD, Willoughby DA. The formation of a structure with the features of synovial lining by subcutaneous injection of air: An *in vivo* tissue culture system.Journal of Pathology. 1981; 134: 147-156.
23. Alencar NM, Assreuy AM, Havt A, Benevides RG, de Moura TR, de Sousa RB, et al.*Vatairea macrocarpa* (Leguminosae) lectin activates cultured macrophages to release chemotactic mediators. Naunyn-Schmiedeberg's Archives of Pharmacology. 2007; 374: 275-282.
24. ArcherJ. Tests for emotionality in rats and mice: a review. Animal Behaviour. 1973; 21: 205-235.
25. Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology. 1987; 92: 180-185.
26. Echandia ER, BroitmanST, Foscolo MR. Effect of the chronic ingestion of chlorimipramine and desipramine on the hole board response to acute stresses in male rats. Pharmacology Biochemistry and Behavior. 1987; 26: 207-210.
27. Steru L,Chermat R, Thierry B, Simon P. Tail suspension test: A new method for screening antidepressants in mice.Psychopharmacology. 1985; 85: 367-370.
28. Saeed MK, Deng Y, Dai R, Li W, Yu Y, IqbalZ. Appraisal of antinociceptive and anti-inflammatory potential of extract and fractions from the leaves of *Torreya grandis* Fort Ex Lindl. Journal of Ethnopharmacology. 2010; 127: 414- 418.
29. Sofidiya MO, Imeh E, Ezeani C, Aigbe FR, Akindeleb AJ. Antinociceptive and anti-inflammatory activities of ethanolic extract of *Alafia barteri.*Revista Brasileira de Farmacognosia. 2014; 24: 348-354.
30. Falcão TR, De Araújo AA, Soares LAL, De Moraes Ramos RT, Bezerra ICF, Ferreira, M. R. A, et al.*Libidibia ferrea* fruit crude extract and fractions show anti-inflammatory, antioxidant, and antinociceptive effect in vivo and increase cell viability in vitro. Evidence-BasedComplementaryandAlternative Medicine. 2019; 2019: 2019.
31. Falcão TR, De Araújo AA, Soares LAL, De Moraes Ramos RT, Bezerra ICF, Ferreira, M. R. A, et al.Crude extract and fractions from *Eugenia uniflora* Linn leaves showed anti-inflammatory, antioxidant, and antibacterial activities. BMC Complementary and Alternative Medicine. 2018; 18: 1-12.
32. QuZ, Liu A, Li P, Liu C, Xiao W, Huang J, et al. Advances in physiological functions and mechanisms of (−)-epicatechin. Critical Reviews in Food Science and Nutrition. 2021; 61: 211-233.
33.Bai J, Zhang Y, Tang C, Hou Y, Ai X, Chen X, et al. Gallic acid: Pharmacological activities and molecular mechanisms involved in inflammation-related diseases. Biomedicine & Pharmacotherapy. 2021; 133: 110985.

34.Henriques MG, Silva PM, Martins MA, Flores CA, Cunha FDQ, Assreuy-Filho J et al. Mouse paw edema. A new model for inflammation. Brazilian Journal of Medical and Biological Research. 1987; *20*(2): 243-249.
35.Cunha TM, Verri Jr. WA, Silva JS, Poole S, Cunha FQ, Ferreira SH. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proceedings of the National Academy of Sciences. 2005; 102: 1755-1760.
36. Watanabe S, Alexander M,Misharin AV, Budinger GRS. The role of macrophages in the resolution of inflammation. The Journalof Clinical Investigation. 2019; 129: 2619-2628.
37. Alves NO, Costa CMC, Siqueira JTT, Teixeira MJ. Dor princípios e práticas. São Paulo: Artmed. 2009.
38. Cury Y, Picolo G, Gutierrez VP, Ferreira SH. Pain and analgesia: The dual effect of nitric oxide in the nociceptive system. Nitric Oxide. 2011; 25: 243–254.
39. Griffiths HR, Gao DE, Pararasa C. Redox regulation in metabolic programming and inflammation. Redox Biology. 2017; 12: 50-57.
40. Vanhamme L, Boudjeltia K, Van AP, Delporte C. The other myeloperoxidase: Emerging functions. Archives of Biochemistry and Biophysics. 2018; 649:1-14.
41. Cinelli MA, Do HT, Miley GP, Silverman RB. Inducible nitric oxide synthase: Regulation, structure, and inhibition. Medicinal Research Reviews. 2019; 40:158-189.
42. Varela ML, Mogildea M, Moreno I, Lopes A. Acute Inflammation and Metabolism. Inflammation. 2018; 41: 1115-1127.
43. Souza GE, Cunha FQ, Mello R, Ferreira SH. Neutrophil migration induced by inflammatory stimuli is reduced by macrophage depletion. Agents and Actions. 1988; 24: 377-380.
44. Damiani D, Kuperman H, Dichtchekenian V, Manna TD, Setian N. Corticoterapia e suasrepercussões: a relaçãocusto–benefício. Pediatria (São Paulo). 2001; 23: 71-82.
45.Ostensen M. Adverse effects of non-steroidal anti-inflammatory agents on the central nervous system. Tidsskrift for den Norske Laegeforening: Tidsskrift for PraktiskMedicin, nyRaekke, 1990; 110: 856.

**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 *Dacryodeskukachkana*leaves
HPLC - High performance liquidchromatography
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 $E_2$
Sisbio - Sistema de Autorização e Informação em Biodiversidade
TNF-α - Tumor necrosis factor alpha