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Anti-inflammatory and antinociceptive effects of kaempferide from the Brazilian green propolis

Efeitos anti-inflamatórios e antinociceptivos do kaempferide da própolis verde brasileira

Efectos antiinflamatorios y antinociceptivos del kaempferide del propóleo verde brasileño

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Abstract

The Brazilian green propolis is produced by *Apis mellifera* from *Baccharis dracunculifolia*. Kaempferide, which was isolated from green propolis and *B. dracunculifolia* is an O-methylated flavonol bearing gastroprotective, anti-allergic and anti-hypertensive activities. Thus, the aim of this study was to evaluate the anti-inflammatory and antinociceptive potentials of kaempferide, as well as its possible toxicological effect on kidney and liver. For that, nociception was assessed by thermal (hargreaves apparatus) and mechanical (Dynamic

Plantar Aesthesiometer) sensitivities; anti-inflammatory potential was assessed by carrageenan induced edema; and the toxicological profile was assessed by blood biochemical assessment. Kaempferide significantly reduced mechanical nociception and displayed anti-inflammatory effect, which was evidenced by the reduction of the volume of the paw edema, as well as by the reduction of infiltrates, cellularity and area of the injured tissue. On the other hand, it was inactive in the thermal nociception protocol either with or without an inflammatory process, as well as it was inactive in the mechanical nociception protocol after an inflammatory process. In addition, central nervous system depression, liver and kidney toxicities were not observed.

Keywords: Ehermal and mechanical nociception; Paw edema; Toxicity.

Resumo

A própolis verde brasileira é produzida por *Apis mellifera* a partir de *Baccharis dracunculifolia*. Kaempferide foi isolado da própolis verde a partir de *B. dracunculifolia*, é um flavonoide O-metilado com atividades gastroprotetora, antialérgica e anti-hipertensiva. Assim, o objetivo deste estudo foi avaliar os potenciais anti-inflamatórios e antinociceptivos do Kaempferide, bem como seu possível efeito toxicológico no rim e no fígado. Para tanto, a nocicepção foi avaliada pelas sensibilidades térmica (aparelho *hargreaves*) e mecânica (*Dynamic Plantar Aesthesiometer*); o potencial anti-inflamatório foi avaliado pelo edema induzido por carragenina; e o perfil toxicológico foi avaliado pela avaliação bioquímica do sangue. Kaempferide reduziu significativamente a nocicepção mecânica e apresentou efeito anti-inflamatório, o que foi evidenciado pela redução do volume do edema da pata, bem como pela redução dos infiltrados, celularidade e área do tecido lesado. Por outro lado, foi inativo no protocolo de nocicepção térmica com ou sem processo inflamatório, bem como foi inativo no protocolo de nocicepção mecânica após um processo inflamatório. Além disso, não foram observadas depressão do sistema nervoso central e toxicidades hepáticas e renais.

Palavras-chave: Nocicepção térmica e mecânica; Edema de pata; Toxicidade.

Resumen

El propóleo verde brasileño es producido por *Apis mellifera* de *Baccharis dracunculifolia*. Kaempferide, que se aisló del propóleo verde y *B. dracunculifolia*, es un flavonol O-metilado con actividades gastroprotectoras, antialérgicas y antihipertensivas. Así, el objetivo de este estudio fue evaluar los potenciales antiinflamatorios y antinociceptivos de la kaempferide, así como su posible efecto toxicológico en riñón e hígado. Para ello, la nocicepción se evaluó

mediante sensibilidades térmicas (aparato de Hargreaves) y mecánicas (*Dynamic Plantar Aesthesiometer*); el potencial antiinflamatorio se evaluó mediante el edema inducido por carragenina; y el perfil toxicológico se evaluó mediante evaluación bioquímica sanguínea. Kaempferide redujo significativamente la nocicepción mecánica y mostró un efecto antiinflamatorio, que se evidenció por la reducción del volumen del edema de la pata, así como por la reducción de infiltrados, celularidad y área del tejido lesionado. Por otro lado, estuvo inactivo en el protocolo de nocicepción térmica con o sin proceso inflamatorio, así como inactivo en el protocolo de nocicepción mecánica tras un proceso inflamatorio. Además, no se observaron depresión del sistema nervioso central, toxicidad hepática y renal.

Palabras clave: Nocicepción térmica y mecánica; Edema de la pata; Toxicidad.

1. Introduction

Secondary biologically active metabolites obtained from plants are used for the development of drugs due to the singularity of the molecules and their biological activities (Cragg and Newman, 2013). It is visible the amount of bioactive compounds found in national and international literature from natural products, with wide application within the pharmaceutical sciences with a special focus on analgesic and anti-inflammatory activities (Azab et al., 2016). In Brazil, the magnitude of biodiversity is not precisely known due to its remarkable complexity, since it is estimated that there are more than two million distinct species of plants, animals and microorganisms. Brazil is the country with the greatest plant genetic diversity in the world, with more than 55,000 species cataloged out of an estimated total between 350,000 and 550,000 (Fabricant and Farnsworth, 2001).

Propolis was widely used in ancient Egyptian folk medicine, including the Greeks, Romans and Incas, with its antiseptic, healing and as a material for embalming dead in antiquity (Toreti et al., 2013). Brazilian green propolis is composed of high amounts of phenolic compounds, such as artepelin C, baccarin, kaempferide, isosakuranetina, diidrokaempferide, drupanine, *p*-coumaric acid, caffeic acid, aromadendrine and caffeoylquinic acid derivatives, among other compounds (Huang et al., 2014).

Brazilian green propolis display anti-inflammatory, anti-diabetic and anti-Alzheimer's activities (Shahinozzaman et al., 2018), as well as antimicrobial (Zancanela et al., 2019), chemopreventive (Doi et al., 2017), antioxidant (Zaccaria et al., 2017) and antigenotoxic (Roberto et al., 2016) activities. Kaempferide (4′-Methoxy-3,5,7-trihydroxyflavone) is a major compound of Brazilian green propolis that display gastoprotective (Costa et al., 2018)

and anticancer (Matsuda et al., 2009) actives. Thus, in view of the potential of green propolis, the present study aimed to evaluate the anti-inflammatory, analgesic and toxicological effects of kaempferide.

2. Material and Methods

2.1 General experimental procedures and reagents

The NMR spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The samples were dissolved in DMSO, and the spectra were calibrated with the solvent signals at 2.50 (^{1}H) and 39.5 (^{13}C) respectively. Classic and Vacuum liquid chromatography, respectively CC and VLC (glass columns of 450 X 25 mm and 50-100 mm i.d.) were used for chromatographic fractionation of green propolis extract by using silica gel 60H (Merck, art. 7736). Thin Layer Chromatography (TLC) was undertaken by using plates of 1 mm thickness PF254 and art. 9385 from Merck. Sephadex LH-20 and λ-carrageenan were acquired from Sigma-Aldrich, St. Louis, MO, USA. Commercial hexanes, methanol and ethyl acetate (EtOAc) were purified by distillation in our facilities.

2.2 Brazilian green propolis raw material

Brazilian green propolis was produced by *Apis mellifera* and collected from their hives. It was provided by Apis Flora Commercial Ltda, Ribeirão Preto, SP, Brazil. The raw Brazilian green propolis (300 g) was freezed and crushed in a blender. Propolis powder was extract by dynamic maceration using ethanol: hexanes (Hex) solution (7:3), followed by a percolation process. The filtered extract was concentrated under vacuum. The presence of a precipitate was observed, which was separated from the aqueous solution furnishing two extracts: the precipitated (FrPr) and the aqueous (FrAq) extracts.

2.3 Isolation of kaempferide from green propolis extract

The extract (FrPr) was fractionated over silica gel 60H (Merck, art. 7736) using VLC with increasing amounts of ethyl acetate in hexanes from 0 to 50% as eluent, and collecting fractions of 2 L each. This procedure furnished seven fractions that were analyzed using TLC and hexanes: ethyl acetate 7:3 as mobile phase. The fifth fraction was submitted to separation

on Sephadex LH20 chromatography using methanol furnishing pure kaempferide (200.0 mg). The isolated compound was identified after ^{1}H and ^{13}C NMR analyses in comparison with literature data (Nath *et al*. 2015). NMR data also allowed us to conclude that the purity of the isolated compound was above 99%.

2.4 Animals and treatments

Male Rattus norvegicus rats of the Wistar strain weighing approximately 150 g came from the vivarium of the Ribeirão Preto Campus of the University of São Paulo, Brazil. The animals were acclimated for one week in an experimental room, at 23 ± 2 ºC, with humidity of 50 ± 10%, with 12 h of light-dark cycle, and with ad libitum access to food and water. The used protocols were approved by the ethics committee on the use of animals of the University of Franca (process: 2655301116).

The animals were randomly divided into groups of five, and at the end of the tests the animals were euthanized with sodium thiopental (0.84 g/kg). The treatment groups were negative control (vehicle), positive control (indomethacin 10 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and kaempferide (5, 10 and 20 mg/kg).

2.5 Locomotor performance and toxicity

The animals of the negative control and kaempferide 20 mg/kg groups were placed in a plastic box measuring 45 x 45 x 20 cm, with a bottom divided into nine equal areas (15 x 15 cm). The number of crosses by the four legs was determined in 6-minute sessions and taken as a behavioral index. The animals were subsequently observed in the first four hours and signs of general toxicity, such as effects on locomotion, behavior (agitation, lethargy and aggression), breathing, salivation, tearing, cyanosis of the extremities, piloerection and complications were noted (Furtado et al., 2015; Walsh and Cummins, 1976).

2.6 Thermical paw withdraw (Hargreaves Apparatus) test

Paw withdraw by radiant thermal stimulation was measured with three measurements, with at least 5 min between tests. A beam of light (Hargreaves, Ugo Basile, Italy) was placed on the hind paw plantar surface of the animals through the glass plate. To avoid possible damage to the tissue, a cut time of 20 s was imposed. Withdraw latencies were measured at

hour zero and 60 min after administration of treatments to assess analgesic potential. The inflammatory process was induced by the injection of 100 µL of λ-carrageenan (100 µg/paw) in the subplantar area of the right hind paw of each animal. After 60 min, the analgesic effect on inflammation was evaluated. The data were calculated by subtracting the baseline values (hour 0). The used protocol was adapted from Tsiklauri et al. (2017).

2.7 Mechanical paw withdraw (Dynamic Plantar Aesthesiometer (DPA) test

Paws withdraw by mechanical stimulation (Dynamic Plantar Aesthesiometer, Ugo Basile, Italy) was measured for three times, with at least 5 min between tests The maximum force was fixed at 50 g and the ramp speed was 20 s. Measurement was performed at hour zero and 60 min after administration of treatments to assess analgesic potential. The inflammatory process was induced with λ-carrageenan (100 µg/paw) and after 60 min the analgesic effect on inflammation was evaluated. The data were calculated by subtracting the baseline values (hour 0). The method was adapted from Di Cesare Mannelli et al. (2013).

2.8 Carrageenan-induced rat paw edema

After 60 min of treatment, edema was induced by injecting 100 µL of λ-carrageenan (100 µg/paw) into the subplantar area of right posterior paw (Winter et al., 1962) and the volume of the paw was measured using a Plesthymometer (Ugo Basile, Italy) after 1, 2, 3 and 4 h after administration of carrageenan. The percentage of inhibition of edema was calculated for each animal group in comparison with the control group.

2.9 Histopathological evaluation

After euthanizing de animals, the right and left pelvic plantar surfaces were removed and conditioned in a flask containing 10% buffered formalin (pH 7.4) for histotechnical processing. The material fragments were embedded in paraffin, cut into a microtome (3 µm thick) and stained with hematoxylin and eosin (HE). The analyses were carried out by two pathologists independently, and the disagreements were resolved by consensus. The degree of the inflammatory process of the lesions was assessed using an optical microscope with 100x magnification, with a count reading in four fields. Subsequently, it was used a score to classify the infiltrate intensity and edema of the acute inflammation process as absent (0),

discrete (1), moderate (2) and intense (3). Additionally, a video analysis was undertaken using the ImageJ image analyzer to determine the area, perimeter and amount of cellularity present from the basement membrane of the epithelial tissue.

2.10 Hepatotoxicity and nephrotoxicity

From the animals used in the paw edema test, intracardiac blood was collected after anesthesia. The blood was transferred to a microtube (2 mL), centrifuged (3500 rpm) for separation of the serum, which was used for measurement of hepatic ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase), as well as renal urea and creatinine biomarkers in automated Mindray BS200 equipment.

2.11 Statistical analysis

The data were evaluated by analysis of variance (One-way ANOVA), for entirely random experiments, with the calculation of the F statistic and its respective "p-value". The means were compared using the Tukey method, with the calculation of the minimum significant difference for $p<0.05$. For nonparametric tests (scores), the Kruskal Wallis test with Dunns posttest was used with the calculation of the minimum significant difference for $p<0.05$.

3. Results and discussion

The inflammatory process involves the activation of different mechanisms, and non-steroidal drugs are commonly used for its treatment. However, these drugs can display serious undesirable effects (Taghi et al., 2013). Thus, the search for new active ingredients with analgesic and anti-inflammatory activities becomes important.

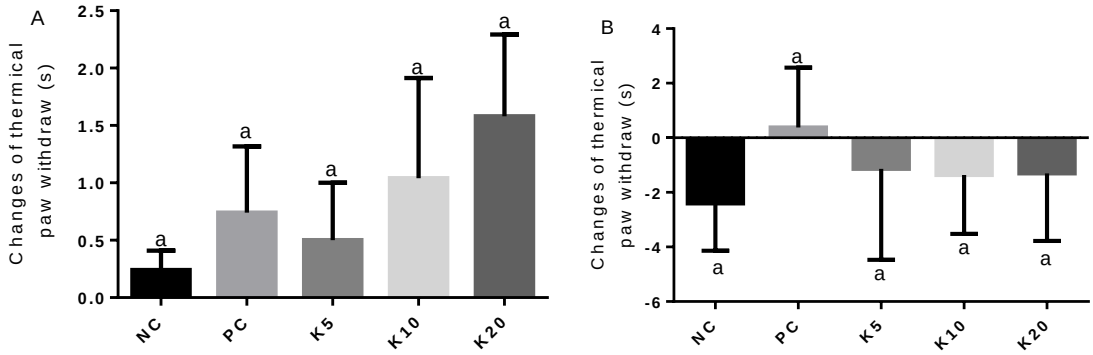
The evaluation of locomotor capacity is a test to determine a possible depressant effect on the central nervous system, in which it could leave the animal lethargic or unresponsive when stimulated (Walsh and Cummins, 1976). The use of this test in analgesic trials is extremely important to understand whether the effect found is really the blockade of pain or depression of the central nervous system (Kuniishi et al., 2017). The test has already been validated in several tests for anxiolytic and antidepressant compounds to understand the effect

on neurotransmitters and systems, such as gabaminergic, monoaminergic and serotonergic, among others (Monteiro et al., 2020).

In the evaluation of locomotor capacity, similar crossing values were observed, with 74.0 ± 2.0 cm of the kaempferide and 73.2 ± 1.0 cm of the negative control with p = 0.9647, indicating the absence of depressive activity in the central nervous system in the tested dosage. These data corroborate the findings by Ueda (2017) who evaluated flavonoids from Brazilian green propolis demonstrating the absence of toxicity induction in N2a cells (neuronal lineage) and protective activity against mutant copper-zinc superoxide dismutase-mediated toxicity.

The hargreaves test is effective for using analgesic tests for thermal stimulation without damage the animals tested (Cheah et al., 2017). The obtained data demonstrate the absence of analgesic activity in the tested dosages (Figure 1). In a similar trial, Paulino et al. (2006) observed the absence of the analgesic effect on hot plates, which, despite being different tests, have similar effect and activation of mechanisms in the neural region related to thermal pain, corroborating with the data found in this study. Dos Santos et al. (2010) carried out trials with *B. dracunculifolia*, the main botanical source of the Brazilian green propolis, demonstrating an antinociceptive effect by formalin and von Frey assays. However, in this study, there were no changes in the response to thermal sensitivity on inflammation after induction with λ- carrageenan. Thus, the different responses can be attributed to the different inducers of the inflammatory process and to the different pain receptors involved in each trial.

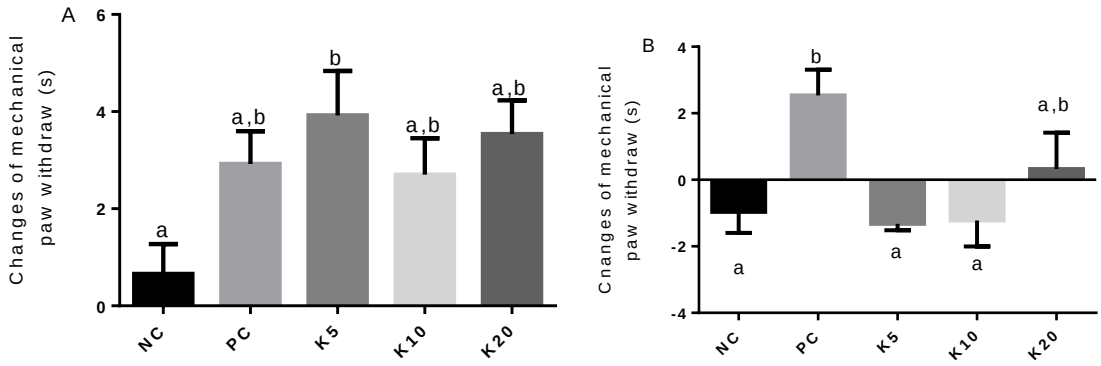
Figure 1 - Effect of kaempferide on thermal analgesia 1 hour (A) and 2 hours (B) after treatments.



NC - negative control Tween80 5% in distilled water; PC - indomethacin 10 mg/kg; K5, K10 and K20 - kaempferide 5, 10 and 20 mg/kg. Each bar represents mean and standard deviation; different letters represent statistical difference (p> 0.05).

The DPA test was selected because it resembles the von Frey test. However, it exerts pressure through monofilaments automatically, providing more accurate response data from mechanical nociceptors. Kaempferide significantly decreased mechanical sensitivity in comparison with negative control (Figure 2 A). These results were previously corroborated by demonstrating that the extract of *B. dracunculifolia* has antinociceptive activity in mechanical hypernociception (dos Santos et al., 2010). On the other hand, kaempferide was unable to modulate the analgesic response to mechanical sensitivity after induction of an inflammatory process (Figure 2 B). Thus, these data indicate that the activity on nociception induced by carrageenan of the extract of *B. dracunculifolia* (dos Santos et al., 2010) is not related to kaempferide.

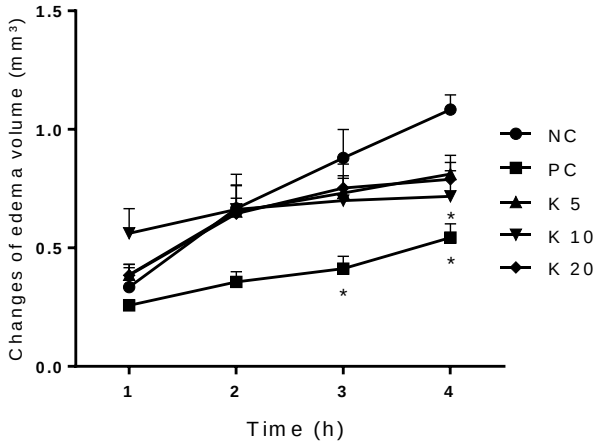
Figure 2 - Effect of kaempferide on mechanical analgesia 1 hour (A) and 2 hours (B) after treatments.



NC - negative control Tween80 5% in distilled water; PC - indomethacin 10 mg/kg; K5, K10 and K20 - kaempferide 5, 10 and 20 mg/kg. Each bar represents mean and standard deviation; different letters represent statistical difference (p> 0.05).

In the carrageenan-induced paw edema test (Figure 3), it is observed that indomethacin significantly reduced the volume of the edema, as expected (Furtado et al., 2015; dos Santos et al., 2010). The dose of 10 mg/kg was significantly different from the negative control in the fourth hour of evaluation, showing the anti-inflammatory potential of kaempferide. On the other hand, the other dosages neither differed significantly in comparison with the negative control nor among the three tested dosages. Thus, these data indicate that kaempferide is correlated with the anti-inflammatory potential of *B. dracunculifolia* (dos Santos et al., 2010), as well as with the anti-inflammatory activity of Brazilian green propolis (Paulino et al., 2006).

Figure 3 – Effect of kaempferide and controls on carrageenan induced edema.

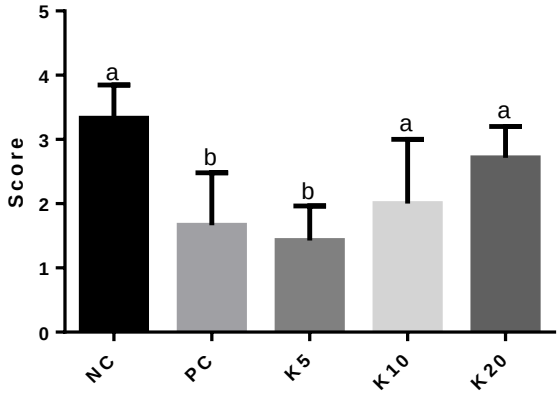


NC - negative control Tween80 5% in distilled water; PC - indomethacin 10 mg/kg; K5, K10 and K20 - kaempferide 5, 10 and 20 mg/kg. Each bar represents mean and standard deviation; different letters represent statistical difference (p> 0.05).

The carrageenan-induced paw edema assay correlates with different mediators of the inflammatory process, depending on the induction time. Thus, the effect of kaempferide may be correlated with inhibition on modulatory pathways of the arachidonic acid cascade, such as prostaglandins, cyclooxygenase products, and nitric oxide (NO). On the other hand, it is not correlated to mediators such as histamine, serotonin, and bradykinin on vascular permeability (Furtado et al., 2015). The modulation on inflammatory pathways of green propolis extract is reported in the literature, and the extract rich in artepillin C and kaempferide modulates the expression of different mediators, such as NO and several cytokines (Szliszka et al., 2013).

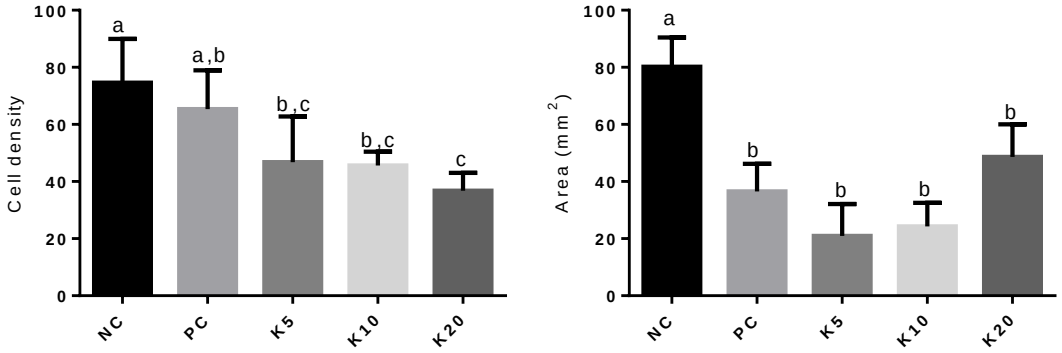
In the histopathological evaluation (Figures 4 and 5), it is visible that the modulation of chemokines occurred due to the decrease of cellular migration to the tissue, which was observed in both reference control and kaempferide groups.

Figure 4 - Effect of kaempferide in cell migration by histopathological analysis of the paw in HE staining.



NC - negative control Tween80 5% in distilled water; PC - indomethacin 10 mg/kg; K5, K10 and K20 - kaempferide 5, 10 and 20 mg/kg. Each bar represents mean and standard deviation; different letters represent statistical difference (p> 0.05).

Figure 5 - Effect of kaempferide in the cell density (A) and extension of the tissue area covered by inflammatory cells (B) in rat paw.



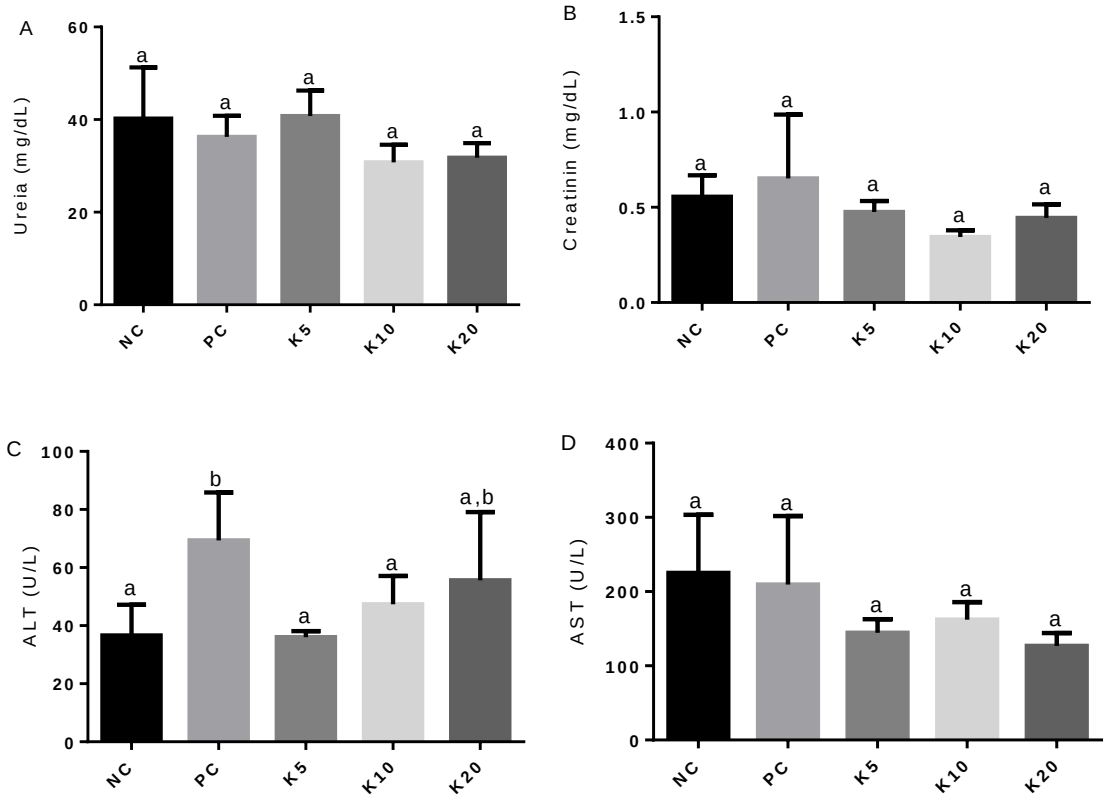
NC - negative control Tween80 5% in distilled water; PC - indomethacin 10 mg/kg; K5, K10 and K20 - kaempferide 5, 10 and 20 mg/kg. Each bar represents mean and standard deviation; different letters represent statistical difference (p> 0.05).

In the video analysis, in the cellular infiltrate, it is observed that the different doses of kaempferide statistically reduced the cell density in the injured tissue. The area of the inflamed process from the basal layer of the epithelial region decreased in all treated animals, in comparison with the negative control. The decrease of cell density in inflammatory infiltrate is probably linked to the myeloperoxidase enzyme (MPO), which is one of the

pathways responsible for the recruitment and alteration of homeostasis (Dai et al., 2019; Paulino et al., 2006).

The use of natural products and their isolated compounds has been used by the pharmaceutical industries over the years, requiring trials to prove the effectiveness of the pharmaceutical prototype and its clinical safety when evaluating systems, mainly in liver and kidneys (Thomford et al., 2018). There was no statistically significant difference among the treatment groups, demonstrating the absence of liver and renal toxicity (Figure 6). These data are corroborated by the literature, which shows that green propolis has no toxic effect on liver and kidney (Batista et al., 2012).

Figure 6 - Effect of kaempferide on renal toxicity assessed by urea (A) and creatinine (B) liver toxicity by ALT (C) and AST (D) tests.



NC - negative control Tween80® 5% in distilled water; PC - indomethacin 10 mg/kg; K5, K10 and K20 - kaempferide 5, 10 and 20 mg/kg. Each bar represents mean and standard deviation; different letters represent statistical difference ($p > 0.05$).

4. Final Considerations

Overall, it is observed that kaempferide, isolated from green propolis, display

analgesic activity on mechanical receptors. However, it has no analgesic activity on thermical receptors. Kaempferide displayed anti-inflammatory activity, which was evidenced by the reduction in the volume of paw edema, as well as by the reduction of infiltrate, area and cell density in the injured tissue. These effects may be related to the modulatory pathways of the arachidonic acid cascade (Paulino et al., 2006; Tan-No et al., 2006) and prostacyclins, due to the decrease in area (Coutinho et al., 2009), decrease in the number of cellularity and permeability (Dai et al., 2019). In addition, kaempferide did not cause central nervous system depression, as well as toxicity to liver and kidney, by the analyzed chemical markers.

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