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Calcium hydroxide associated with a new vehicle: *Psidium cattleianum* leaf extracts. Tissue response evaluation

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Abstract: The aim of this study was to evaluate edemogenic activity and subcutaneous inflammatory reaction induced by *Psidium cattleianum* leaf extracts associated with $Ca(OH)_2$. Thirty male Wistar rats, split equally into three groups [aqueous extract + $Ca(OH)_2$; ethanolic extract + $Ca(OH)_2$; and propylene glycol + $Ca(OH)_2$], were assessed every 3 h or 6 h (five animals in each period). Under general anesthesia, 0.2 mL of 1% Evans blue per 100 g of body weight was injected into the penile vein and each combination to be evaluated was subcutaneously injected into the dorsal region 30 min thereafter. Edemogenic activity was analyzed by spectrophotometry (λ=630 nm). For inflammatory reaction analysis, 50 rats received four polyethylene tubes (three experimental groups) and an empty tube (control group). The assessments were made at 7, 15, 30, 60, and 90 days, followed by hematoxylin-eosin staining and by the assignment of scores for evaluation of tissue response intensity. Ethanolic extract + $Ca(OH)_2$ yielded the largest edemogenic activity at 3 h. Intergroup differences at 6 h were not significant. The histological analysis showed progressive repair over time ($p<0.05$) and aqueous and ethanolic extracts produced similar responses to those of the control and $Ca(OH)_2$ + propylene glycol groups. *Psidium cattleianum* leaf extracts used as $Ca(OH)_2$ vehicles evoked similar tissue response when compared to $Ca(OH)_2$ associated with propylene glycol.

Keywords: Inflammation; Edema; Plants, Medicinal; Plant Extracts

Introduction

Microorganisms and their products are present in pulp and periapical diseases[1,2,3,4] and may be found in dental pulp necrosis, in persistent or refractory infections, and throughout the root canal system.[5,6] Even after cleaning and shaping the root canal with proper sodium hypochlorite irrigation, microorganisms may survive, especially in the apical third or in isthmus, due to the anatomic complexity of the root canal system.[7,8]

Many drugs have been tested over the years to reduce bacterial load, such as calcium hydroxide [$Ca(OH)_2$], one of the most widely used endodontic intracanal medications.[9,10] $Ca(OH)_2$ acts by direct contact, but its action against microorganisms that penetrate deep into dentinal tubules may be impaired.[11]

Contact time is another factor to be considered. Sjögren et al.[12] recommended using an interappointment medication to enhance the antibacterial effects of instrumentation, reducing bacterial count, and they proved that more than 7 days are required for its high efficacy. However, some microorganisms such as *Enterococcus faecalis* and *Candida albicans* are more resistant to $Ca(OH)_2$.[6,13,14]

Different microorganisms exhibit different levels of resistance to $Ca(OH)_2$ activity, and new vehicles or products that improve the effects of $Ca(OH)_2$ are being explored.

Psidium cattleianum belongs to the *Myrtaceae* family and is commonly found in the American tropics and locally known as "araçá-do-campo" (field guava) or "araçá comum" (common guava).[15,16] *Psidium* spp. are used to treat various diseases (e.g., scurvy,[17] cough, and lung diseases[18]) and as anti-inflammatory and hemostatic agents.[19,20] In addition, plants belonging to this species decrease the metastasis of cancer cells.[21]

Psidium cattleianum leaf extract has demonstrated antimicrobial activity and inhibits the growth of microorganisms such as *Streptococcus mutans, Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Actinobacillus actinomycetemcomitans*, and *E. faecalis*,[22] killing *S. mutans* when applied at high concentrations or reducing lactic acid production when used at low concentrations.[23]

A microbiological research study revealed the potential of *Psidium cattleianum* ethanolic extract when used as a vehicle for $Ca(OH)_2$, which boosted antimicrobial activity against *E. faecalis* and achieved total inhibition within 24 h.[24] According to Medina *et al.* (2011),[25] the abundance of phenolic compounds in *Psidium cattleianum* extracts was positively correlated with their antioxidant, antimicrobial, and antiproliferative effects.

Since the combination of *Psidium cattleianum* leaf extracts used as vehicle for $Ca(OH)_2$ has already proven effective in eliminating *E. faecalis*, tissue response evaluation is necessary as a preliminary compatibility test, and this may aid in endodontic therapy in the future.

The aim of this study was to evaluate the tissue response of rats to aqueous and ethanolic leaf extracts of *Psidium cattleianum* as a vehicle for $Ca(OH)_2$ through edemogenic and microscopic analyses.

Methodology

Animals

Eighty male Wistar albino rats (3 to 4 months old) weighing between 200 and 250 g were assessed: 30 animals for the analysis of edemogenic activity (10 for each one of the three extracts, and five animals for two assessment periods: at 3 h and 6 h) and 50 for subcutaneous implantation (10 for each experimental time period – 7, 15, 30, 60, and 90 days). The animals were kept in temperature-controlled rooms and received water and food *ad libitum*. This study was approved by the local Ethics Committee on the Use of Animals (CEUA, process no. 07457/2010).

Preparation of extracts

Psidium cattleianum leaves were obtained from the Araçatuba School of Dentistry, Universidade Estadual Paulista - UNESP, Araçatuba, SP, Brazil. The specimen (HLF2006/7) was deposited at the Pharmacology and Research Herbarium, Laboratory of Phytotherapy, São José do Rio Preto, Brazil. The leaves free from pests or diseases were washed and dried at 40 °C until they were brittle. The leaves were immediately ground to a powder.

The preparation of ethanolic and aqueous extracts followed Machado et al.[26] For ethanolic extract preparation, 20 g of powdered leaves was mixed with 250 mL of 80% ethanol. The mixture was then manually stirred inside an amber bottle for 3 min, 5 times a day, for 12 days. Subsequently, the mixture was filtered. The product was sterilized by filtration using a 0.22-µm cellulose ester membrane (Millipore™, Billerica, USA). For aqueous extract preparation, 20 g of powdered leaves was added to 250 mL of distilled water and boiled at 100° C for 5 min; the mixture was maintained at 55° C for 1 h and at room temperature for 72 h being stirred every 24 h. The solution was then filtered and sterilized according to the method described above.

After extract preparation, the following combinations were mixed at a ratio of 1 g of $Ca(OH)_2$ to 1 mL of the vehicle:

a. aqueous extract + $Ca(OH)_2$,
b. ethanolic extract + $Ca(OH)_2$,
c. propylene glycol + $Ca(OH)_2$.

Edemogenic activity analysis

Thirty animals were intramuscularly anesthetized with xylazine (10 mg/kg) and ketamine (25 mg/kg) and received an intravenous injection of 1% Evans blue solution – 0.2 mL per 100 g of body weight (Evans Blue; Difco Lab. Detroit, Michigan, USA) – into the penile vein. After 30 min, the animals were divided into three groups (n = 10), and each animal was injected with 0.1 mL of each combination (A, B, or C) using an insulin syringe with a 0.70 × 25 mm hypodermic needle (22G×1 1/4") in the dorsal region, next to the tail, using the midline as reference.[26] Edema formation was quantified at 3 h and 6 h.

After 3 h and 6 h, five animals from each group were killed with an anesthetic overdose. The back of each animal was shaved, and an edematous area was evidenced and removed with a safety margin. The specimens were standardized to 23 mm in diameter, perforated, and placed into individual bottles containing 4 mL of formamide (Vetec Química, RJ, Brazil). After 72 h at 45° C, gauze filtration was performed and absorbance of the specimens was measured using a spectrophotometer at 630 nm.[26]

Subcutaneous implants

Fifty rats (10 for each experimental period – 7, 15, 30, 60, and 90 days), received four subcutaneous implants corresponding to each experimental group (A-C) or an empty tube (control D).

Two hundred polyethylene tubes (Abbott Labs of Brazil, São Paulo, SP, Brazil), with an outer diameter of 1.6 mm and length of 7.0 mm, were filled with the test materials using an insulin syringe and a hypodermic needle (22G × 1 1/4"). The animals were anesthetized as previously described and the dorsal region was shaved before antisepsis with 5% iodine solution. A 1-cm incision was performed in the shaved area with a 15c blade. Each animal received four tubes followed by a 4-0 silk suture.[26]

The animals were euthanized with anesthetic overdose within 7, 15, 30, 60, and 90 days after implantation. The tubes and the surrounding tissues were removed and fixed in 10% formalin at pH 7.0. The specimens were processed and embedded in glycol methacrylate. Serial 3-µm sections were obtained for hematoxylin-eosin staining. Tissue reaction was evaluated as follows: score 0 – no inflammatory cell; score 1 – fewer than 25 cells; score 2 – between 25 and 125 cells; and score 3 – 125 or more cells. A fibrous capsule was considered to be thin when its thickness was less than 150 µm and thick when it was greater than 150 µm.[26,27,28]

The results obtained for edemogenic activity were analyzed by two-way analysis of variance (ANOVA) and Tukey's test, whereas the results of the histological examination were analyzed by the Kruskal-Wallis test. The significance level was set as $p < 0.05$ for all statistical analyses.

Results

Edemogenic activity analysis

Ethanolic extract + $Ca(OH)_2$ revealed greater edema formation at 3 h compared to the other groups ($p < 0.05$). There was no statistical difference between the groups ($p > 0.05$) at 6 h; however, the group treated with ethanolic extract + $Ca(OH)_2$ had a lower incidence of edema, whereas all the other groups showed a higher incidence, $p > 0.05$ (Figure 1).

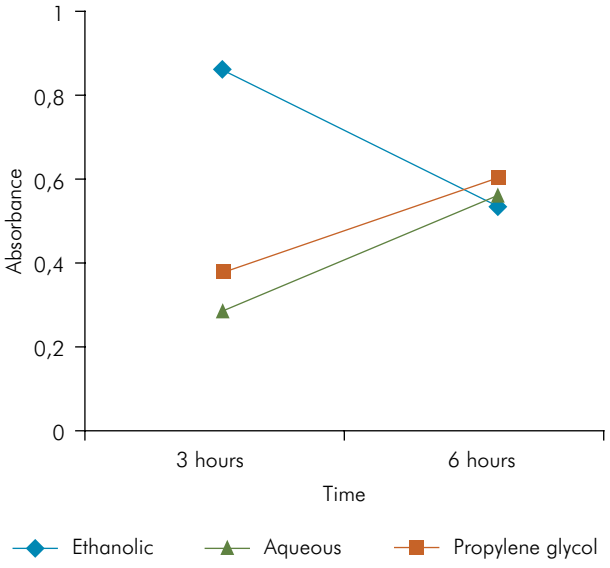

Figure 1. Graphical representation of mean value of edema in both periods studied.

Histological analysis

Aqueous extract + $Ca(OH)_2$ (Group A)

A moderate number of inflammatory cells were observed on days 7 and 15, indicating presence of lymphocytes, neutrophils, eosinophils, macrophages, and a thick fibrous capsule (Figure 2 A,E). The intensity of the inflammatory infiltrate decreased and thinning of the fibrous capsule was observed after 30, 60, and 90 days; the number of inflammatory cells observed on day 90 was significantly different from those observed on day 7, $p < 0.05$, (Figures 2 I,M,Q and Figure 3).

Ethanolic extract + $Ca(OH)_2$ (Group B)

A moderate number of inflammatory cells were observed on days 7 and 15, indicating

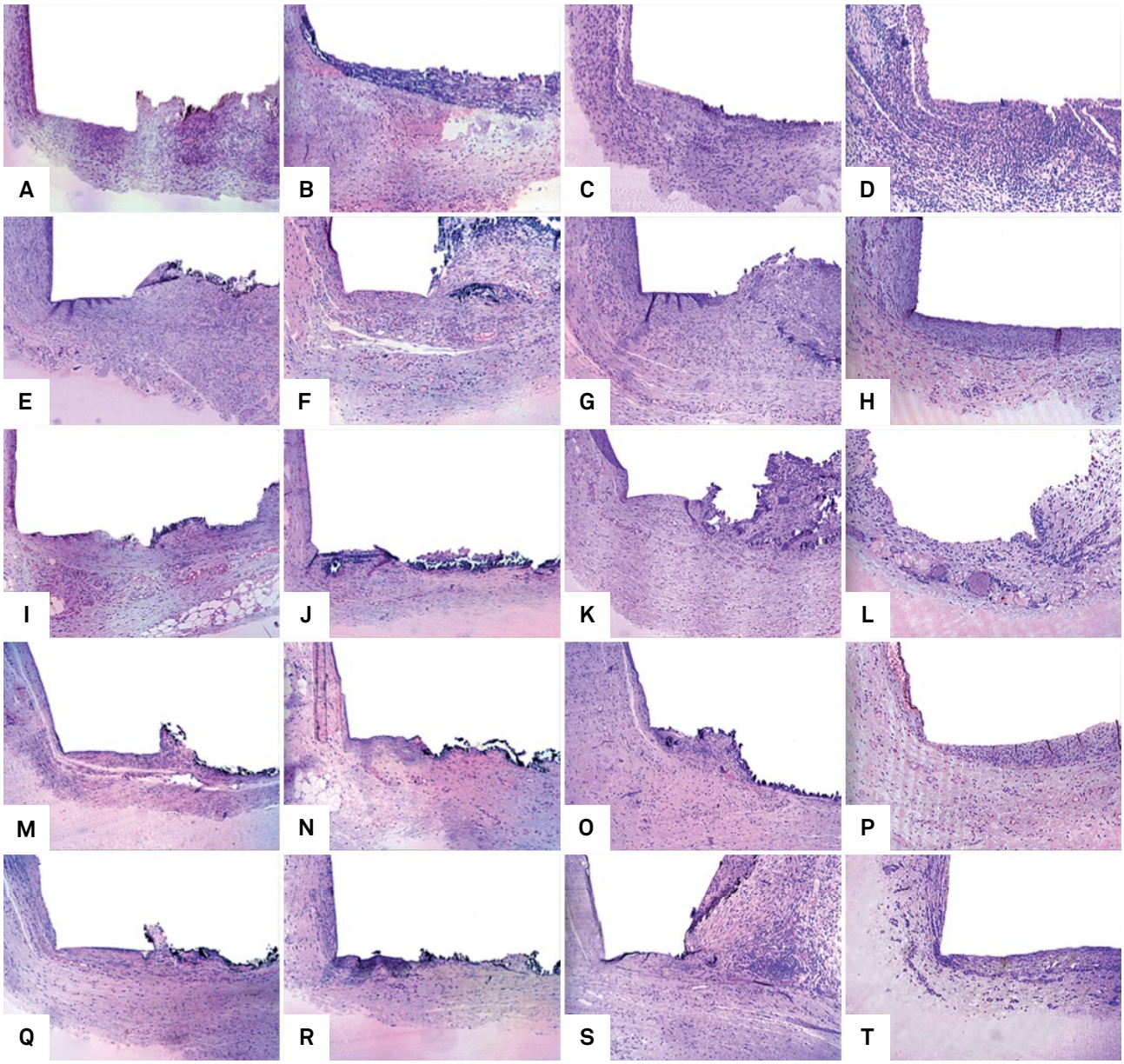

Figure 2. Histological analysis of experimental groups on postoperative days 7, 15, 30, 60 and 90 (HE, 100x).

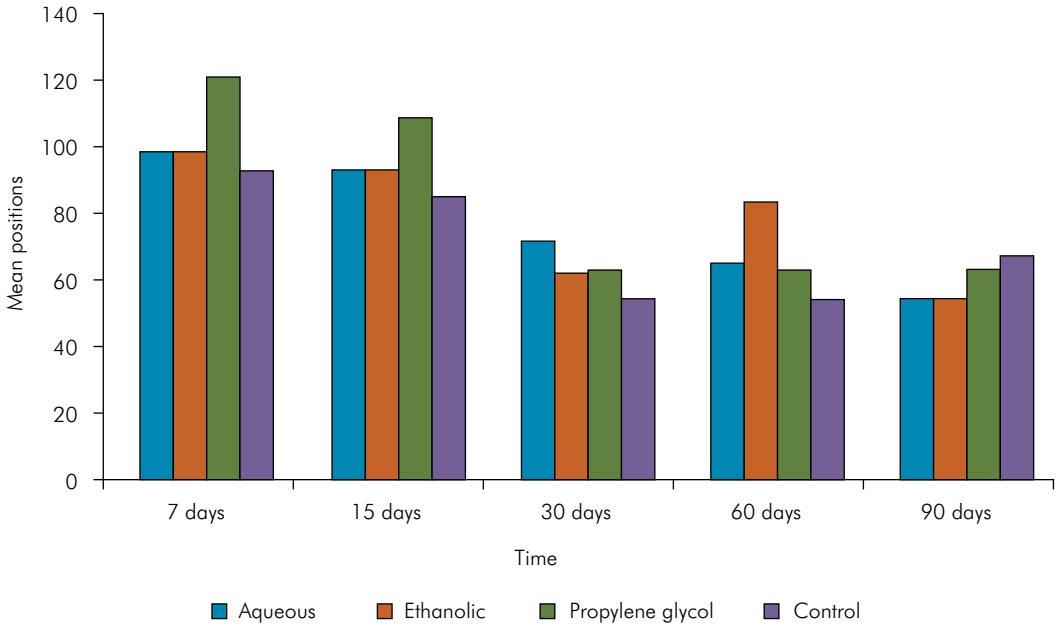

Figure 3. Graphical representation of the position occupied by the mean scores of the groups in the four periods studied.

presence of lymphocytes, neutrophils, eosinophils, macrophages, and a thick fibrous capsule (Figure 2 B,F). The intensity of the inflammatory infiltrate decreased ($p<0.05$) and thinning of the fibrous capsule was also observed after 30, 60, and 90 days (Figure 2 J,N,R and Figure 3).

Propylene glycol + $Ca(OH)_2$ (Group C)

A moderate number of inflammatory cells were observed on days 7 and 15, indicating presence of lymphocytes, neutrophils, macrophages, eosinophils, and a thick fibrous capsule (Figure 2 C,G). After 30 days, the number of inflammatory cells and capsule thickness decreased ($p > 0.05$, Figure 2 K,O,S and Figure 3).

Control - empty tube (Group D)

Similarly to what was observed in the other groups, the control group showed a moderate number of inflammatory cells on days 7 and 15, indicating presence of lymphocytes, neutrophils, macrophages, and a thick fibrous capsule. (Fig.1 D,H). The number of inflammatory cells decreased, and thinning of the fibrous capsule was observed after days 30, 60, and 90. (Figure 2 L,P,T and Figure 3).

Comparison between groups as a function of time

When comparing time periods, repair improved over time, showing similar results between the initial periods – at 7 and 15 days ($p > 0.05$), and also in the final ones – at 30, 60 and 90 days (Figure 3).

No difference was observed between the different combinations in the experimental periods ($p > 0.05$, Figure 3).

Discussion

Besides the beneficial antimicrobial properties of an intracanal medication, attention should be paid to its biological response. Based on this assumption, Holland et al.[29] evaluated the association of intracanal medications and observed severe inflammatory response when using camphor alone. The same result was obtained by Soekanto et al.[30] from the culture of rat dental pulp cells, indicating camphor cytotoxicity. However, the biocompatibility of camphorated paramonochlorophenol has been reported for its association with $Ca(OH)_2$, probably due to the lower penetration of camphor into tissues, once $Ca(OH)_2$ has a denaturing effect on connective tissue, reducing its cytotoxicity[31,32].

In an attempt to sidestep these limitations, association of $Ca(OH)_2$ with other antibacterial substances has been suggested. With this purpose, Midena et al.[33] evaluated subcutaneous tissue response in rats and the antimicrobial activity of intracanal $Ca(OH)_2$ with different substances and reported high inflammatory response with the addition of chlorhexidine. Notwithstanding, the antibacterial effect did not improve.

The choice of *Psidium cattleianum* leaf extract in this study is due to its previously described antimicrobial properties.[22,24] The study by Brighenti et al.[23] demonstrated its antibacterial potential at high concentrations, killing *S. mutans* grown on biofilms. At low concentrations, the extract inhibited acid production by *S. mutans* and reduced the expression of proteins involved in general metabolism, glycolysis, and lactic acid production. Its biocompatibility was introduced by Ruviére et al.[34]

The antibacterial activity of *P. cattleianum* is due to the presence of phenolic compounds, which contain flavonoids (kaempferol, quercetin, and cyanidin) and an ellagic acid, known as tannin.[35] According to Medina et al.,[25] the abundance of phenolic compounds is directly related to the antimicrobial effect. This phenolic toxicity to microorganisms is caused by enzyme inhibition by the oxidized form of the phenolic compound.[36]

The edemogenic activity analysis was performed using a vital dye (Evans Blue), which binds to albumin plasma protein. The presence of this dye at edematous sites allows quantification of the edema.[37]

The combination of ethanolic extract and $Ca(OH)_2$ showed significantly larger edema formation at the initial 3 h, probably because of the presence of ethanol. This group showed a decrease in edema formation after 6 h, whereas the group treated with the combination of aqueous extract and propylene glycol with $Ca(OH)_2$ showed larger edema formation (Figure 2). These findings were consistent with those previously reported using the ethanolic extracts of *Psidium cattleianum*[34] and of *Myracrodruon urundeuva*[26] administered separately.

The histological analysis demonstrated that the inflammatory reaction in the control group (empty tube) was similar to that which had been previously reported, indicating a minor tissue response caused by the polyethylene tube.[28,38,39] The highest scores were observed on days 7 and 15, in the initial period, and were associated with the surgical trauma. The control and experimental groups showed similar levels of inflammation in early periods, and our findings were similar to those reported by Torneck.[40] Because of a decrease in the influence of surgical procedure on day 30, a significant improvement was observed in inflammation in all groups, a characteristic that was maintained on days 60 and 90.

This study shows that the aqueous and ethanolic leaf extracts of *Psidium cattleianum* used as a vehicle for $Ca(OH)_2$ exhibited similar tissue response when compared to the propylene glycol and control groups.

Conclusion

Aqueous and ethanolic leaf extracts of *Psidium cattleianum* used as a vehicle for $Ca(OH)_2$ show favorable tissue response, similarly to that obtained from the combination of $Ca(OH)_2$ and propylene glycol, indicating that these extracts have promising applications in the field of dentistry.

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The authors deny any conflict of interest.

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