



The Dynamics of Subcutaneous Tissue Response to Microorganisms Associated with the Extract of Araçá (*Psidium cattleianum*): An Edemogenic and Microscopic Analysis

A Dinâmica da Resposta do Tecido Subcutâneo para Associação de Microorganismos com o Extrato de Araçá (*Psidium cattleianum*): Uma Análise Microscópica e Edemogênica

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ABSTRACT

Objective: To evaluate *in vivo* tissue reaction to the extract of araçá (*Psidium cattleianum*) associated with inactivated microorganisms. **Material and Methods:** A 0.1 mL suspension was used containing *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Peptostreptococcus micros*, and *Porphyromonas endodontalis*, which were inactivated by heat and mixed into a 1.0 mL saline (control group), an aqueous solution, or a hydroalcoholic extract of araçá. Eighteen male rats (*Rattus norvegicus*) under general anesthesia received 0.2 mL of 1% intravenous Evans blue. Thirty minutes later, 0.1 mL of one of the associations was injected into the animals' dorsal region. The animals were euthanized after 3 and 6 hours, and the materials obtained were placed in formamide for 72 hours then analyzed in a spectrophotometer ($\lambda=630 \text{ nm}$). For the morphological analysis, 30 rats received polyethylene tubes implants with the extracts or the saline with the associations in the dorsal region and euthanized after 7 and 30 days to be analyzed according to an inflammation cell score. **Results:** No significant difference ($p>0.05$) was observed in the edema among groups. The optical microscopy results showed a repair in the 30-day-period, which was higher when compared to the 7-day-period ($p<0.0001$). Nevertheless, in the 7-day-period, the hydroalcoholic extract presented a significant response compared to the aqueous extract ($p=0.05$) and a trend for better results than the control group. **Conclusion:** The aqueous and hydroalcoholic araçá extracts associated with inactivated microorganisms

RESUMO

Objetivo: Avaliar *in vivo* a reação tecidual do extrato de araçá (*Psidium cattleianum*) associado com microorganismos inativados. **Material e Métodos:** Uma suspensão de 0.1mL foi usada contendo *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, *Peptostreptococcus micros* e *Porphyromonas endodontalis* dos quais foram inativos por aquecimento e misturados a 1,0 mL de soro fisiológico (grupo controle), uma solução aquosa ou hidroalcoólica de araçá. Dezoito ratos machos (*Rattus norvegicus*) sob anestesia geral receberam 0,2mL de Azul de Evans a 1% intravenoso. Após trinta minutos, 0,1mL de um dos extratos (associado com microorganismos inativos) foi injetado nos animais na região dorsal. Os animais foram eutanasiados após 3 e 6 horas, e os materiais obtidos colocados em formamida por 72 horas para análise em espectrofotômetro ($\lambda=630 \text{ nm}$). Para análise morfológica, 30 ratos receberam implante subcutâneo de tubo de polietileno com as associações na região dorsal, eutanasiados após 7 e 30 dias para serem analisados de acordo com um escore de células inflamatórias. **Resultados:** Não houve diferença significativa ($p>0,05$) no edema entre os grupos. Os resultados obtidos em microscópio óptico apontaram reparo em 30 dias superior ao de 7 dias ($p<0,0001$). No período de 7 dias a solução hidroalcoólica apresentou resposta superior a solução aquosa ($p=0,05$) e uma tendência de melhor resultado que o controle. **Conclusão:** A solução aquosa e hidroalcoólica de extrato de araçá associadas a microorganismos inativados apresentaram respostas biológicas semelhantes

showed similar responses to control, indicating no interference on the toxic effects of the bacterial components in tissue repair.

KEYWORDS

Anaerobic bacteria; Edema; Inflammation; Plant extracts; *Psidium*.

ao controle, indicando que não há interferência sobre os efeitos tóxicos advindos dos componentes bacterianos, no sentido de favorecer o reparo.

PALAVRAS-CHAVE

Bactéria anaeróbica; Edema; Extrato de Plantas; Inflamação; *Psidium*.

INTRODUCTION

Bacteria are the primary cause of the development of necrotic pulps, periapical lesions, and post-treatment disease following root-canal treatment. Therefore, the eradication of microorganisms and their by-products from the root-canal system is primordial for the success of the endodontic treatment. Although mechanical instrumentation is one of the most important factor for controlling root-canal infection, it cannot achieve total elimination of bacteria when used alone [1]. The complexity of root canals, which is attributable to lateral canals and isthmus, means that almost half of root-canal walls are left unprepared with only instrumentation. Therefore, the use of antimicrobial irrigation solutions has been advised as an adjunct to mechanical instrumentation. Microorganisms with different characteristics (structural, metabolic, and pathogenic) reaching the periapical region stimulate the inflammatory and immunologic responses [2]. Also, the persistence of bacteria, endotoxins (lipopolysaccharides), products of bacterial metabolism, pulp necrosis (including dead bacteria), induce an inflammatory and immune response, with activation of the complement system and arachidonic acid metabolism, leading to processes that may induce or culminate in the development of a periapical lesion [3]

An alternative for decrease bacterial load is the use of plant extracts used in popular medicine. As presented in previous in vitro

studies, these extracts have antibacterial potential [4-6].

Medicinal plants are an alternative or even support protocols to traditional treatment [6]. Over the last few years, there has been a significant increase in scientific progress surrounding the pharmacological and chemical studies of medicinal plants aimed at obtaining new compounds with therapeutic properties [7,8].

Recent studies using Brazilian cerrado plant extracts provide evidence of antimicrobial activities, showing promising results for buccal microbial control [6,9-11].

Araçá (*Psidium cattleianum*) is a plant commonly found in the American tropics that has been studied and presented promising results. Araçá, which belongs to the Myrtaceae family, is also known as “araçá-do-campo” (field guava) or “araçá-comum” (common guava) [12,13].

According to a previous study, the *Psidium cattleianum* leaf extract has demonstrated antimicrobial activity and inhibits the growth of microorganisms such as *Streptococcus mutans*, *Porphyromonas gingivalis*, and *E. faecalis* among others, even killing *S. mutans* when applied at high concentrations [4,5]

Another microbiological research 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 in 24 h [14]. According to Medina et al. [8], the

abundance of phenolic compounds in *Psidium cattleianum* extracts was positively correlated with their antioxidant, antimicrobial, and antiproliferative effects.

Despite the limited number of studies, it has proven antimicrobial action [11,15] and possible anti-inflammatory potential, besides the already proved biocompatibility through in vivo study [16,17].

Therefore, the aim of this study was to evaluate in vivo, the tissue reaction to the extract of araçá (*Psidium cattleianum*) associated with inactivated microorganisms.

MATERIAL & METHODS

Animals

Forty-eight Wistar (*Rattus norvegicus*) male rats, 60 days old and 250 g - 300 g, were obtained from Araçatuba School of Dentistry Vivarium – UNESP for this research.

The animals were housed in temperature-controlled rooms and received water and food ad libitum through the pre-experimental period. The care of the animals was performed according to the Araçatuba School of Dentistry Ethical Committee (Process #2008 – 000165), which approved the project before the beginning of the experiments.

Extracts

The plants were collected from a permanent reserve area in Carolina (MA-Brazil) county rural property during the rainy season (December to February). The leaves used in the extracts were collected from healthy plants and then dried at ambient temperature until the condition of dry and breakable [18] allowing its grinding until powder.

Araçá (*Psidium cattleianum*) hydroalcoholic and aqueous leaf extract solution were prepared. For the hydroalcoholic solution was used 20 g of leaves and 250 mL of the 80% ethanol [11].

Microorganisms Association

All microorganisms (Table 1) were provided by the Microbiology Laboratory in Araçatuba School of Dentistry – UNESP.

The microbial containing all the bacteria was re-suspended in saline 1 mL (control), araçá hydroalcoholic or aqueous extract solution (1 mL). The experimental groups were:

1) Aqueous: bacteria pool (5x10⁶ cel/mL to each reference strain) + 1 mL aqueous araçá extract.

2) Hydroalcoholic: bacteria pool (5x10⁶ cel/mL to each reference strain) + 1 mL de hydroalcoholic araçá extract.

3) Saline: bacteria pool (5x10⁶ cel/mL to each reference strain) + 1 mL saline.

Table 1 - Bacterial species used in the suspension:

Bacterial Species	Strain Provenance	Morphotype	Physiology
<i>Porphyromonas gingivalis</i>	ATCC 33277	Gram-Negative	Anaerobic
<i>Peptostreptococcus micros</i>	ATCC 33270	Gram-Positive	Anaerobic
<i>Prevotella intermedia</i>	ATCC 25611	Gram-Negative	Anaerobic
<i>Fusobacterium nucleatum</i>	ATCC 25586	Gram-Negative	Anaerobic
<i>Porphyromonas endodontalis</i>	ATCC 35406	Gram-Negative	Anaerobic
<i>Enterococcus faecalis</i>	ATCC 29212	Gram-Positive	Facultative

Edemogenic Test – Immediate Reaction

Eighteen animals were used and separated to each experimental period of 3 and 6 hours of the three associations. The animals were submitted to general anesthesia with xylazine (Rompun 4 Bayer) at a ratio of 25 mg/Kg and ketamine (Francotar – Virbac), at a ratio of 50 mg/kg mixed in the same syringe, intramuscularly administered. Then, the animal received intravenous in the penile vein, a 0.2

mL of 1% Evans blue (Evans Blue Difco Lab) [17,19,20]. After 30 minutes, a solution (0.1 mL of aqueous or hydroalcoholic) was injected in the dorsal area in each animal, using an insulin syringe with a 0.70×25 mm hypodermic needle (22G \times 1 1/4") and the median line as reference [17]. The animals were injected with one araçá extract solution (aqueous or hydroalcoholic) added with the microorganism solution, or with saline added with the microorganism solution as a control group. After 3 and 6 hours, the animals were killed with an anesthetic overdose. Subsequently, the animal's dorsal area was treated with a manual trichotomy, until the edema area was visualized, characterized by a blue color zone. The tissue was standardized and removed by an iron mold, with 23 mm diameter. The standard tissue parts were cut and kept in vials containing 4 mL formamide (Vetec Química- RJ – Brazil), and then storage at 45° C for 72 hours. The solution was filtered with gauze and analyzed in spectrophotometer (Cary 50 Bio, Varian), using a wavelength of 630 nm [17,19,20].

Polyethylene Tubes

Sixty polyethylene tubes (Abbott Lab of Brazil, Sao Paulo, SP, Brazil) with 1.0 - mm internal diameter, 1.6 - mm external diameter and 3.0 - mm length were filled with the tested materials [17,21].

Subcutaneous Implants

Five animals for each group were used in this stage (aqueous, hydroalcoholic, and saline) in two experimental periods of 7 and 30 days [22-24], involving a total of 30 animals.

The animals were subjected to general anesthesia with xylazine (25 mg/kg) and ketamine (50 mg/kg) mixed in the same syringe, intramuscularly administered. After dorsal trichotomy and disinfection of the area with 5% iodine solution (Riodente, Rioquímica, São José do Rio Preto, Brasil), a 2 cm longitudinal incision was made with a No. 15 scalpel blade following

the median line with subcutaneous tissue and divulsion was performed [17,21].

The polyethylene tubes were filled with one of the associations (aqueous solution, hydroalcoholic, or saline added to inactive microorganisms). Then were implanted in the right and left side of the animals' subcutaneous tissue. The incision was sutured with 4.0 silk (Ethicon, Johnson & Johnson) and each animal received two implants containing the same solution, totalizing 10 implants per group.

After 7 and 30 days the animals were euthanized with an anesthetic overdose and the tubes were removed with the surrounding tissue and fixed in 10% formalin at pH 7.0 for 48 hours and then washed in water for 12 hours. The pieces were dehydrated, clarified, and included in paraffin, followed by longitudinally cut with 6 μ m thickness, to be stained with hematoxylin and eosin for microscopic analysis (Leica, Germany).

The results obtained for tissue response from extracts was compared to those of the control group. A descriptive analysis was performed for the three experimental groups. Tissue reactions at the open end of the tubes were scored according to previous studies [17,21] as follows: 0, few inflammatory cells or no reaction; 1, less than 25 cells and mild reaction; 2, between 25 and 125 inflammatory cells and moderate reaction; and 3, 125 or more inflammatory cells and severe reaction (40 \times magnification). Fibrous capsules were considered thin when $< 150 \mu$ m and thick when $> 150 \mu$ m.

Statistical Analysis

The edemogenic test results, relative to edema quantitative, were available using 2 factors ANOVA (time and solution) using the software GMC 2002 [25]. The results of the microscopic analysis in the form of scores, were submitted to the Mann-Whitney and Kruskal-Wallis test. The significance level was 5%.

RESULTS

Edemogenic Analysis

The results for edema quantification (Table 2) showed no statistically significant differences between the experimental groups ($P > 0.05$). Among groups, the aqueous extract and saline presented less edema than the hydroalcoholic extract at 3 hours. At 6 hours, all groups showed similar values (Table 2).

Table 2 - Optical density values

Time (Hours)	Groups		
	Aqueous	Hydroalcoholic	Saline
3	0.353±0.044	0.732±0.188	0.388±0.086
6	0.629±0.281	0.581±0.368	0.538±0.097

The edema values (mean ± standard deviation) on both time periods and the experimental groups under spectrophotometer ($\lambda=630$ nm).

Microscopy Analysis

Aqueous Araçá + Bacteria - Day 7: Median score 3 with a thick fibrous capsule; Inflammatory cells in large amount, with macrophage predominance, numerous lymphocytes, few leucocytes and other mononuclear cells, and chronic inflammation characteristics were observed. Some plasmocyte cells were noted. A few fibroblasts were seen around rare collagen fibers that presented complex arrangement (Figure 1).

Aqueous Araçá + Bacteria - Day 30: Median score 1 with a thin fibrous capsule; The microscopic image revealed a decrease in the inflammatory compared to the 7-day period. The cell inflammatory agglomerate was substituted by a thin and parallel collagen fiber capsule, willing to the implant area. Some fibrocytes were identified, as were some few

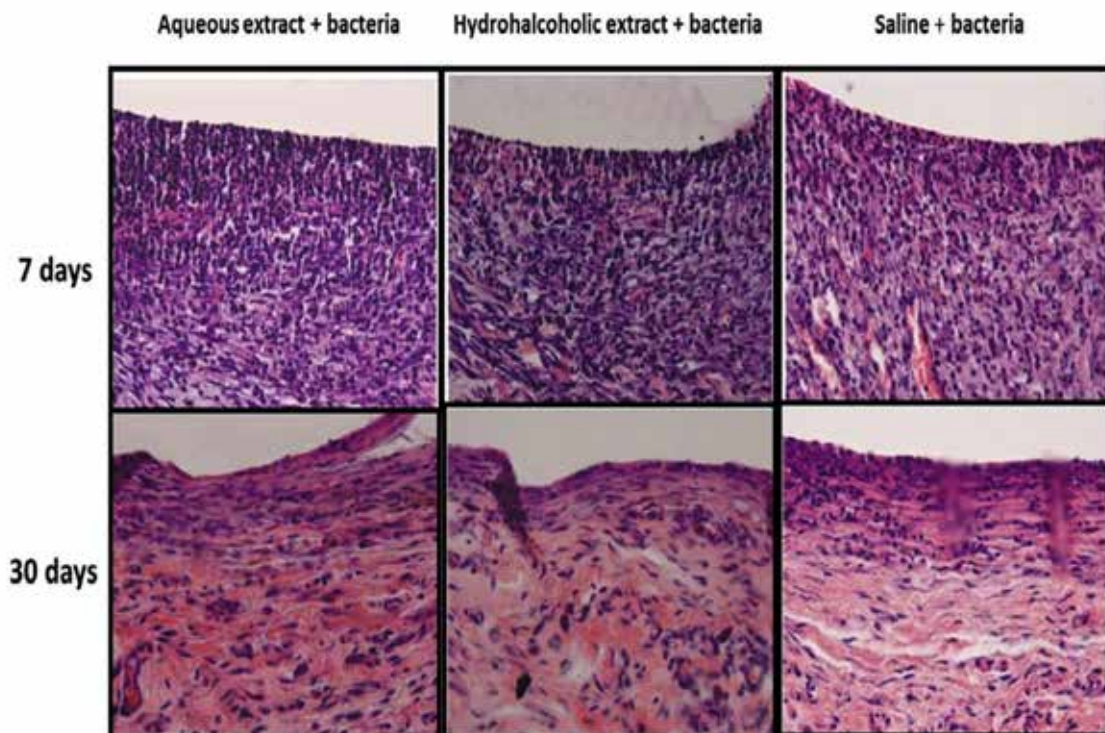


Figure 1 - At 7 days: Presence of inflammatory cells, with macrophage predominance, numerous lymphocytes, few leucocytes and mononuclear cells. At 30 days: There is a reduction of inflammatory infiltrate after 30 days. In general, for all groups the cell inflammatory agglomerate was substitute for a thin collagen fiber capsule, surrounding the implant area. A few fibrocytes were identified and some fibroblasts around the collagen fiber. Overall, the macrophages layer was low, the lymphocyte quantity and other mononuclear cells were reduced. Fewer and less dense blood vessels in the conjunctive tissue interior was observed. (H.E staining, 40X)

fibroblasts around collagen fiber. In general, the macrophages layer was low profile, and the lymphocyte quantity and other mononuclear cells were significantly reduced. Blood vessels were fewer and less dense in the conjunctive tissue interior (Figure 1).

Hydroalcoholic Araçá + Bacteria - Day 7: Median score 2, with mostly specimens presenting a thick fibrous capsule, but the presence of a thin capsule was also noted; Similar to the aqueous araçá group in the same period, there was rich macrophage quantity, lymphocytes, and few leukocytes and other mononuclear inflammatory cells. Some plasmocytes were observed. There was little fibroblasts predominance, and rare collagen fibers presented organized way. There were numerous blood capillaries diffused over the entire area (Figure 1).

Hydroalcoholic Araçá + Bacteria - Day 30: Median score 1 with a thin fibrous capsule; The collagen fibers were more organized and denser, at more advanced mature degree compared to the 7-day period. The fibroblasts present were thinner and had a linear nucleus (indicating a collagen metabolism decrease); in general, collagen fibers displayed parallel structure (Figure 1).

Saline + Bacteria - Day 7: Median score 3 with a thick fibrous capsule; Conjunctive tissue inflammation was observed near the implant. Fibroblast rare was present around few collagen fibers that performed complex and disorganized disposal. Dense blood-vessel networks were observed for all areas (Figure 1).

Saline + Bacteria - Day 30: Median score 2 with a thin fibrous capsule; This group presented major tissue organization relative to 7-day period. A macrophage agglomerate surface was in contact with the implant area; some lymphocytes were present and other few inflammatory cells was observed. The lower vessel caliber around all area was observed (Figure 1).

DISCUSSION

The araçá leaves used for extracts were the same as those used in previous studies that found evidence of antimicrobial action against buccal bacteria [11,15] and biocompatibility [16,17], thereby avoiding any interference of biologic activity.

Brighenti et al. [5] conducted a study which 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 Ruvière et al. [26] and confirmed by Valentim et al. [17] and our study.

The abundance of phenolic compounds such as kaempferol, quercetin, cyanidin and an ellagic acid (tannin) is directly related to the antimicrobial effect [8,27]. Microorganisms are sensitive to the phenolic toxicity caused by enzyme inhibition by the oxidized form of the phenolic compound [28].

In periapical diseases the presence of *P. gingivalis*, *P. endodontalis* and *P. intermedia* has been evidenced [29,30]. Those microorganisms were selected once they present lipopolysaccharide (LPS), produce histolytic enzymes and the antigens induce the inflammatory response [31]. The *F. nucleatum*, also presents a LPS, capable of inducing a cutaneous inflammatory reaction in rats, activating the complement system [32,33]. The choice of Gram-positive *P. micros* was due to its common prevalence in pulp necrosis and periapical lesion [29,34-36]. *E. faecalis* is found in 81.5% of endodontic problems and is considered a very resistant microorganism to irrigation and inter appointment medication [37,38].

Endotoxin from alive or dead bacteria acting on macrophages, neutrophils or fibroblasts, triggers the release of inflammatory

mediators such as TNF, interleukin-1, interleukin-6, interleukin-8, interferon-alpha and prostaglandins [39-43]. In rats, several studies have demonstrated that virulence factors, even in dead bacteria, are capable of promoting an inflammatory response in animals [45-48].

The subcutaneous polyethylene implantation started with Torneck [48], which became a commonly used primary compatibility test. In our research, the implantation of polyethylene tube was performed according to previous reports, which showed biocompatibility [17,21,24,49,50].

When comparing the results, the edema presented similarities in both time periods, independent of group. For the 6-hour period, a slight increase in its quantity, though not significant statistically, was observed. We concluded that the inactive-microorganism presence did not interfere with the initial inflammatory response standard.

However, because it is inactive microorganism subproducts and products, it is not known how much time elapsed before inflammatory response was elicited.

About the edemogenic tests results, though not statistically significant, it was found that saline presented better values and caused less edema, followed by aqueous and hydroalcoholic that presented higher initial edema. It was found that the inactive microorganism did not drastically change the proportion of edema relative to pure materials, which were similar to results obtained by Machado [50], but without inactive bacteria. The hydroalcoholic general result was high due the observed edema in the 3-hour period, which we believe was intensified due to the ethanol presence. This fact can likely be explained, probably, due the ethanol irritating effect in the initial period, and its metabolism in organism in the 6-hours period.

In the 7-day period, severe inflammatory response was observed, probably due to the inactive microorganism presence and its possible initial actions against the host, or even the initial surgical trauma, corroborating the results obtained by Machado [50].

In the 30-day period, there was an inflammatory process control in all groups, with very similar response. Thus, for the 3 groups in this period, inflammation level was reduced and compared to initial period, with evidence of repair process.

According to the results, the araçá extract deserves more attention for future research, once this plant shows vast antimicrobial and anti-inflammatory potential and has demonstrated biological compatibility. However, further researches are necessary to better analyze its behavior against active compounds which may become part of future drugs for use in dentistry.

CONCLUSION

Within the limitations of this study, extracts of araçá (*Psidium cattleianum*) have no effect on the bacterial components. However, the extracts do not interfere in the process of subcutaneous tissue repair, showing good biocompatibility.

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