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Antimicrobial Effect of *Arrabidaea chica* Polyphenolic Extract Used as Dentin Pre-treatment against Cariogenic Microbiota

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Authors' contributions

This work was carried out in collaboration among all authors. Authors LTV and ECB performed data acquisition and wrote de paper. Author Rosanna Tarkany Basting performed data acquisition and revised the paper. Author IMOS developed the Arrabidaea chica extraction. Author FMGF revised the paper and responsible for interpreting the data. Author FLBA performed data acquisition and revised the paper carefully for important intellectual content. Author CPT responsible for interpreting the data. Author MAF responsible for the conception and design of the study, interpretation of the data and writing the paper. Author Roberta Tarkany Basting responsible for the conception and design of the study, interpretation of the data and writing the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study tested the antimicrobial effect of *Arrabidaea chica* (Ac) polyphenolic extract in different concentrations incorporated into an aqueous solution or into a primer of an adhesive system used for cavities pre-treatment against *Streptococcus mutans* (Sm) and *Lactobacillus casei* (Lc).

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Study Design: *In vitro* experimental study design to evaluate the minimum inhibitory concentration (MIC) of different solutions containing Ac against Sm and Lc.
Methodology: Aqueous solution and primer (from a self-etching adhesive system - Clearfil SE Bond/Kuraray) were prepared containing Ac extract in six different concentrations: 0.0% (null); 0.25%; 0.5%; 1.0%; 1.5%; 2.0%; 2.5%. To determine the MIC, tubes with the inoculates of Sm and Lc were added with the solutions described above. Serial dilutions were inoculated in plates with culture medium (n=3). The colony forming unit count (CFU/mL) was carried out after 48 hours. Negative controls (aqueous or primer solutions without Ac incorporation) were used in both tests.
Results: Regression model showed that all Ac concentrations inhibited Sm growth ($p>0.05$) in aqueous solution. Negligible or no Sm growth was observed in the presence of primer in Ac concentrations above 0.25% ($p<0.05$). Negligible or no Lc growth was found in aqueous solution for all Ac concentrations, and in the presence of primer in Ac concentrations above 1% ($p>0.05$). All concentrations of Ac tested here maintained the acidic characteristics of the solutions.
Conclusion: *Arrabidaea chica* extract into an aqueous solution shows antimicrobial effect against Sm and Lc in all concentrations tested. When incorporated into the primer, the antimicrobial activity is observed in concentrations above 1%.

Keywords: Arrabidaea chica; antimicrobial effect; dentin; adhesive system.

1. INTRODUCTION

Streptococcus mutans and *Lactobacillus casei* are microorganisms associated with the development of dental caries [1-3]. *Streptococcus mutans* is a species found in biofilm formed on dental surfaces and on restoration edges, and increase the chances of formation of dental caries [3-4]. *Lactobacillus casei* are not implicated in the formation of caries, but contribute to the progression of the lesions under specific circumstances - a high and frequent intake of saccharose [3,5].

When dental caries leads to the loss of enamel and dental substrates, a dental restoration - mainly in composite resin - needs to be performed. Adhesive systems are designed to provide resistant and efficient bond between composite resin and dental substrate through the establishment of a hybrid layer [4,6]. However, it may lose stability over time, a process that may be mediated by metalloproteinases and cysteine cathepsins, which are capable of degrading protein components of the extracellular matrix [6]. Added to that is hydrolytic degradation, highlighting the importance of an adequate sealing of the adhesive interface [7-8]. The degradation of the adhesive system may lead to failures on the interface due to polymer contraction [8], and to formation of marginal gaps, which may result in the infiltration of bacteria between the margins of the cavity and the restoration material, leading to secondary caries [9].

Secondary caries is one of the main causes of restoration replacement. Therefore, the addition of antimicrobial agents into the adhesive systems may prevent recurrent caries, hindering bacterial colonization and growth, as well as the production of acids that cause demineralization of dental substrates [10]. Furthermore, given that the strategy for removal of carious tissue is based on maintenance of demineralized dentin, the cavity contains residual bacteria; in this sense, adhesive systems with antimicrobial agents can reduce the microbiota in the cavity [11].

Self-etching adhesives have low pH [7,12,13]. Although this property is related to an antimicrobial effect, it is gradually neutralized during the demineralization of smear layer [14]. Thus, application of an acidic adhesive system per se is not enough to promote an antimicrobial effect, not to mention its questionable action on acidogenic bacteria such as *Streptococcus mutans* and *Lactobacilos casei* [13,15].

The inhibition of growth of cariogenic bacteria by antimicrobial agents might be accomplished using plant extracts, which seem to show greater biocompatibility and lower side effects [16]. *Arrabidaea chica* (Humb. & Bonpl) Verlot (Ac) is commonly found in the Amazon Forest, known as pariri (in the state of Para), crajiru (in the state of Amazonas), puca-panga, coapiranga, chica or cipó-cruz [17]. This plant has been listed as on of those used for therapeutic use, and related to diseases such as microbial infections, gastrointestinal disorders, and inflammation [16]. Ac is rich in anthocyanin [18], a phenolic

compound widely spread in nature and responsible by the reddish coloration in some flowers, fruits, leaves and roots [19]. The main anthocyanin found in this species is the dye known as carajurina [19]. Ac extract is rich in polyphenols [20], and shows antioxidant [21], anti-inflammatory and antitumor activities [22,23], improves synthesis and organization of collagen fibrils, being considered an inhibitor of metalloproteinase (MMP) -2 and 9 on tendinous tissue healing [24].

Arrabidaea chica extract has been studied as dentin pre-treatment and when added to acid or primer of a conventional three-step adhesive system [25]. It is worth noticing that incorporation of the extract into primer reduces bond strength to dentin, but has no effect when it is incorporated into acid or when used in an aqueous solution, despite the lack of influence in the degree of conversion of conventional adhesive system [25]. However, its use as an antimicrobial agent against cariogenic microorganisms is still unknown, particularly when applied as a dental cavity pre-treatment or when incorporated into a two-step self-etching adhesive system.

This study tested the antimicrobial effect of the polyphenolic extract of *Arrabidaea chica* in different concentrations into an aqueous solution used as a dental cavity pre-treatment or or into the primer of an adhesive-system against *Streptococcus mutans* (Sm) and *Lactobacillus casei* (Lc).

2. MATERIALS AND METHODS

2.1 *Arrabidaea chica* Extract Identification

Arrabidaea chica Verlot voucher was deposited at Herbarium of University of Campinas under number 1348 and the study registered at the Nacional Patrimony Heritage number SISGEN: 010150/2012-9. The Ac extract was obtained following the method described in Servat-Medina et al. [26] and was used in all experiments. Ac leaves were collected at the Center for Research in Chemistry, Biology and Agricultural Sciences of the University of Campinas (CPQBA - UNICAMP) located in the city of Paulínia, São Paulo, Brazil (22º45`00`` S and 47º10`21`` W). The dried leaves were delivered to the Herbarium of the University of Campinas. One kilogram of dried and minced leaves were extracted three times, using 5 L of acidified (citric acid at 0.3%) hydroethanolic solution 70% for intervals of 1.5 hours, at room temperature, and using mechanical stirring. This procedure was repeated a number of times. The extract was filtered and the organic solvent was removed under a vacuum. The raw extract was then dried (Mini Spray Dryer B-290, ansa B-295, BÜCHI Labortechnik AG, Flawil, Switzerland).

2.2 Aqueous and Primer Solutions Preparation

Aqueous solutions and primer solutions were prepared in different concentrations of Ac: 0.0 (no Ac); 0.25%; 0.5%; 1.0%; 1.5%; 2.0% and 2.5%. The experiment used a two-step self-etching adhesive system primer (Clearfil SE Bond/ Kuraray; Primer: MDP, HEMA, hydrophilic dimethacrylate, camphorquinone, N.N-diethyl-P-toluidine, water; batch number 9N0168/9V0269). The extract was weighed on an analytic scale (OHAUS Corp, Adventure, Parsippany-Troy Hills, New Jersey, USA), added to Eppendorf tubes with distilled water or the self-etching adhesive system primer, and then stirred (Solution Stirrer, AP56, Phoenix Luferco, Araraquara, SP, Brazil) for 2 minutes. The tubes were covered with electrical tape to avoid photodegradation of the polyphenol extract. Solution pH (with water or primer) was measured by means of a pH meter (mPA 210, MS Tecnopon Equipamentos Especiais Ltda., Piracicaba, SP, Brazil) immediately after the constitution of the solutions.

2.3 Minimum Inhibitory Concentration (MIC) Tests of Solutions Containing Ac against Sm and Lc

Standard strains of Sm and Lc were obtained from the American Type Culture Collection (ATCC) with certificate of origin via Fundação André Tosello (*Streptococcus mutans* - ATCC 25175 and *Lactobacillus casei* - ATCC 393). Stationary phase cultures were prepared from lyophilized ones following the instructions given in the certificate. The primary culture was preserved to keep its morphological, physiological and genetic characteristics and its complete viability during storage.

For the preparation of the secondary culture, a tube of the frozen primary culture was separated and reactivated. Thawing was carried in an ice bath. Immediately after that, the content of the tube was transferred to another tube containing

10 mL of Brain Heart Infusion broth (BHI) with 20% glycerol. The tubes were kept in a CO_2 incubator (TE 399, Tecnal, Equipamentos para Laboratório, Piracicaba – SP – Brazil) for 24 h (Sm) and 48 h (Lc) at 36ºC ± 1°C.

Following growth, the microorganisms were inoculated in Petri dishes containing agar BHI and incubated in the conditions described above. The bacteria were frequently checked for morphology and gram coloration, as well as contamination.

Isolated colonies were removed from the Petri dish and suspended in 5 mL of sterilized NaCl (sodium chloride) solution at 0.89% until reaching turbidity of approximately 0.135 at 660 nm, which is equivalent to a semi-confluent growth on agar surface of 1-2 x 108 UFC/mL.

100 µL of the bacterial suspension was inoculated in 100 ml of BHI to obtain a concentration around 1-2 x 105 UFC/mL. The mixture was homogenized using a mechanical stirrer. Immediately after homogenization, 4960 µL of medium and 40 µL of the Ac solutions (aqueous or with primer) were added to each tube, totalizing 5mL. The tubes were then stirred and incubated at 37°C with partial CO_2 pressure of 10% for 24 hours.

Following incubation, tubes were agitated and turbidity was measured on a spectrophotometer at 660 nm to verify the impossibility of obtaining a minimal inhibitory concentration. Once verified the impossibility, the tests proceeded to microbial counting for Sm and Lc.

After that, 100 mL from each test tube were added to eppendorf tubes with 900 mL of sterile saline, which constituted a 1:10 dilution of the solution (dilution 1). The tube was homogenized in a shaker. Two sequential dilutions were obtained following this procedure, resulting in dilutions 2 and 3.

A total of 25 mL from each dilution were inoculated in sterile disposable Petri dishes containing BHI medium for Sm and MRS for Lc. After the absorption of the inoculate by the culture medium, the Petri dishes were incubated at 37°C and partial CO_2 pressure of 10% for 24/48 hours. The dishes were then removed from the incubator and the CFU/mL count was determined with a manual colony counter (CP 608 – Phoenix Luferco, Araraquara - São Paulo, Brazil).

Controls consisted of culture medium inoculated with the bacteria (Sm or Lc), and culture medium inoculated with the bacteria plus 40 µL of ultrapure water or primer (without Ac).

2.4 Statistical Analysis

The average pH of the aqueous and primer solutions for the different concentrations of Ac extract were analyzed in a descriptive way. Regression models were built between the Ac extract concentrations. All analyses were conducted in SAS (SAS Institute Inc., Release 9.2, 2010, Cary, NC, USA), considering 5% of significance.

3. RESULTS AND DISCUSSION

Table 1 shows pH values found for the aqueous and primer solutions with different concentrations of Ac extract. Ac incorporation into both solutions kept their acidic characteristics in different concentrations.

No Sm growth was observed in the presence of aqueous solution with Ac extract in all concentrations. Similarly, negligible or no Sm growth was found in the presence of primer solution with Ac concentrations above 0.25% (Table 2).

Table 1. pH values found for the aqueous and primer solutions in different concentrations of Ac extract

Concentration of Ac extract	pH	
	Aqueous solution	Primer solution
0.0	6.83	2.36
0.25	5.40	2.24
0.5	5.32	2.26
1.0	5.24	2.26
1.5	5.24	2.27
2.0	5.29	2.29
2.5	5.14	2.3

Table 2. Microbial count of Sm expressed as CFU/mL (standard deviation) for aqueous and primer solutions in different concentrations of Ac extract

Concentration of Ac extract	Solution	
	Aqueous	**Primer**
Strain (in the absence of solution)	**2.60 x 107 (0.47 x 107)**	**0.40 x 107(0.00)**
0.00	17.73 x 107 (16.78 x 107) a	38.67 x 103 (9.43 x 103) a
0.25	0.00 (0.00) b	34.00 x 103 (2.83 x 103) a
0.50	0.00 (0.00) b	0.00 (0.00) b
1.00	0.00 (0.00) b	0.00 (0.00) b
1.50	0.00 (0.00) b	0.00 (0.00) b
2.00	0.00 (0.00) b	0.00 (0.00) b
2.50	0.00 (0.00) b	0.00 (0.00) b

Averages followed by different lowercase letters are statistically different ($p \leq 0.05$)

In this study, Ac incorporation into aqueous and primer solutions kept their acidic characteristics in different concentrations (Table 2). The pH of the aqueous solution decreased with the increase of the extract concentration. On the other hand, the primer solution kept its pH value regardless of the extract concentration, possibly due to the primer's elevated acidity in comparison with the water. Thus, Ac incorporation into the aqueous solution may lead to more significant alterations of its physical chemical characteristics depending on the extract concentration. On the other hand, lower pH values lead to a reduction in microbial growth and biofilm formation [7,11,13], which is desirable when treatment is applied to surfaces contaminated with cariogenic microorganisms.

Aqueous Ac solutions showed negligible or no Lc growth in all concentrations of the extract. Primer solutions showed negligible or no Lc growth in concentrations above 1% of extract (Table 3).

Here, no Sm growth was observed in the presence of aqueous solution with Ac extract in all concentrations. Similarly, negligible or no Sm growth was found in the presence of primer solution with Ac concentrations above 0.25% (Table 3). The same pattern was observed for Lc cultures: negligible or no growth found in the presence of aqueous solution for all concentrations of Ac extract, and in the presence of primer, non-negligible microbial counts were found only for concentrations of 1% or less of Ac extract (Table 3).

It is known that self-etching adhesive systems can present antimicrobial activity due to low pH and incorporation of monomers with this property [11,13]. The incorporation of Ac into primer of the adhesive system used here showed antimicrobial properties without changing its pH. Due to Lc's acidophilic characteristics, higher concentrations of Ac extracts were needed to inhibit its growth [3]. However, considering the concentration of 2.5%, as used in previous study, one may define this concentration to be effective against Sm and Lc both in aqueous or primer solution.

Table 3. Microbial count of Lc expressed in CFU/mL (standard deviation) for aqueous and primer solutions in different concentrations of the Ac extract

Concentration of Ac extract	Solution	
	Aqueous	**Primer**
Strain (in the absence of solution)	**399.60 x 107 (0.00)**	**399.60 x 104 (0.00)**
0.00	52.67 x 107 (14.14 x 107) a	4.87 x 104 (0.28 x 104) a
0.25	0.33 x 107 (0.28 x 107) b	9.13 x 104 (0.85 x 104) a
0.50	0.00 (0.00) b	8.07 x 104 (4.62 x 104) a
1.00	0.00 (0.00) b	0.40 x 104 (0.00) b
1.50	0.00 (0.00) b	0.27 x 104 (0.38 x 104) b
2.00	0.00 (0.00) b	0.00 (0.00) b
2.50	0.00 (0.00) b	0.00 (0.00) b

Averages followed by different lowercase letters are statistically different ($p \leq 0.05$)

4. CONCLUSION

All concentrations of Ac tested here maintained the acidic characteristics of the solutions.

There was no Sm growth in aqueous solutions containing Ac extract in all concentrations. Sm growth was negligible or absent in primer solutions containing Ac extract in concentrations above 0.25%.

Lc growth was negligible or absent in all concentrations of Ac extract in aqueous solutions and above 1% in primer solution.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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