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A Potential Natural Alternative to Chlorhexidine Mouthwashes: An in Vivo Study in Rats

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Abstract

Background: In the Brazilian northeast region, the leaves of a plant scientifically known as *Aristolochia cymbifera* (*A. cymbifera*) are therapeutically used to treat different conditions, including fevers, ulcers and stomach disorders.

Objective: To explore its effects in the oral cavity, this study aimed to evaluate the presence of morphological, cellular, and tissue modifications in the oral mucosa of rats treated with different *A. cymbifera* solutions.

Materials and Methods: The rats were divided into 6 groups: Group 1 was treated with 0.3 ml of vehicle (DMSO and water); Group 2 treated with 0.3 ml of A. cymbifera-ethanolic extract 4mg/ml; Group 3 treated with 0.3 ml of A. cymbifera-hexane extract 4mg/ml; Group 4 treated with 0.3 ml of A. cymbifera-ethanolic extract 10mg/ml; Group 5 treated with 0.3 ml of A. cymbifera-hexane extract 10mg/ml; and Group 6 treated with 0.3 ml of chlorhexidine gluconate 0.12%. All groups were treated twice daily by applying the solutions into the oral cavity, over a total period of 24 days. To analyze cytotoxicity on cells, the micronucleus test was performed, and an additional histopathologic analysis was conducted in order to evaluate tissue changes such as inflammatory infiltration, desquamation, and degeneration of cells.

Results: Results showed that the cytotoxicity of the *A cymbifera* -based solutions was similar to those of chlorhexidine, and all groups showed morphological characteristics of normal mucosa after treatments.

Conclusion: It is concluded that no cytotoxicity was present after treatment with extracts of *Aristolochia cymbifera*, showing its potential for the development of future clinical trials.

Keywords: Alternative medicine, Cytotoxicity, Plant extract, *Aristolochia cymbifera,* Oral medicine.

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Introduction

The daily use of toothbrushes, toothpaste and dental floss is essential to control plaque, avoiding the occurrence of dental caries and periodontal disease [1]. Nevertheless, in periods of higher cariogenic risk, when quantitative and qualitative changes of oral microbiota occur [2], the use of mouthwashes is highly recommended. The anti-plaque action of these mouthwashes consists of the reduction of bacterial adhesion to the teeth surfaces, inhibition of growth and proliferation of microorganisms and plaque's intercellular matrix formation [3]. Furthermore, mouthwashes have also shown to modify bacterial biochemistry, leading to a less pathogenic biofilm overall [4].

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For everyday plaque control in chronic situations of cariogenic challenge, such as in patients subjected to orthodontic appliances, regular mouthwash with fluoride and triclosan are commonly indicated [5]. However, the incidence of white spot lesions around brackets is still extremely high, highlighting the need for new therapies to prevent this outcome [6]. For short periods of increased cariogenic challenge, such as during treatment of periodontal disease and pericoronitis, the gold standard is the use of chlorhexidine rinses for 14 days. Chlorhexidine mouthwashes, although extremely effective, have significant side effects such as alteration of taste perception and teeth staining that limit their long term use [7]. Because of the side effects of these mouthwashes commonly used in dentistry, there is a need for safe, effective and affordable alternatives [8].

In the pharmaceutical industry, rinses derived from essential oils and plant extracts have been explored due to their ability to inhibit growth of fungi and bacteria and the effectiveness in reducing plaque and changing the composition of biofilm [9]. For everyday use and for long periods, these natural rinses might be preferable in comparison to chlorhexidine in controlling plaque and mild to moderate gingivitis because they may present less side-effects. Among the essential oils, an in vitro study performed by our group with the alcoholic extract of *Aristolochia cymbifera* (*A. cymbifera*) showed high effectiveness, reducing the microbial population by about 94.2% after one-hour in vitro tests with the reproduction of biofilms of human saliva [10].

Aiming to translate this research into future clinical application of the *A. cymbifera* extract, the objective of this study was to assess the cytotoxicity of this substance in the oral mucosa of rats, as the experimental animal model.

Materials and Methods

Experimental design

All procedures have been conducted according to the guidelines described by the ethics committee of the Federal University of Rio de Janeiro (CEUA-UFRJ).

The sample consisted of 30 Wistar rats, approximately 8 weeks old and weighing approximately 250g. The animals were kept under controlled temperature (22º ± 2ºC), humidity (50 ± 10%) and cycle of 12 hours light/ dark. Food and water were available *ad libitum*. The rats were divided into 6 groups: Group 1 (negative control) was treated with 0.3 ml of vehicle (DMSO and water); Group 2 treated with 0.3ml of *A. cymbifera* - ethanolic extract 4mg/ml; Group 3 treated with 0.3 ml of *A. cymbifera* -hexane extract 4mg/ml; Group 4 treated with 0.3 ml of *A. cymbifera* - ethanolic extract 10mg/ml; Group 5 treated with 0.3 ml of *A. cymbifera* -hexane extract 10mg/ml; and Group 6 treated with 0.3 ml of chlorhexidine gluconate 0.12% (positive control). All groups were treated twice daily with the solutions applied into the oral cavity by syringes. The experiment was carried out for 24 days. Collection of cells occurred three times, with 8 days interval between them: T1 – after 8 days, T2 – after 16 days, and T3 after 24 days.

Cells were collected from the right and left buccal mucosa and hard palate by scraping

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these areas using sterile spatulas. The collection was done in two different ways: a) a smear directly on the slide, wetted with PBS; b) cells collected from each animal were placed in an Eppendorf with 0.5 ml of PBS. For fixation of the cells to the Eppendorf, 0.5 ml of methanol: acetic acid (3:1 v / v) was added. Thereafter, the resulting solution of each collection was distributed into two slides (0.5 mL in each slide). The slides were dried for 24 hours at room temperature before being stained. At the end of the experimental period all animals were sacrificed, and tissue samples were collected from the right and left buccal mucosa, palate, and tongue for histopathology.

Cytology and Imaging

We performed an analysis of micronucleus formation since its appearance has been shown to be a reliable marker for cytogenetic damage [11, 12].

The Feulgen-Fast Green method was used for staining. Initially the slides were dipped in 1N HCl solution for 15 minutes for acid hydrolysis of nitrogenous bases purine and pyrimidine, allowing subsequent staining of the nuclear material. The slides were then washed with distilled water, where they remained for 15 minutes. Subsequently they were immersed in Schiff reagent (Merck, Darmstadt, Hessen, Germany) for 90 minutes, which is a specific dye for the nuclear chromosomal material exposed by acid hydrolysis. After removal of the Schiff reagent, the slides were dipped in 0.5% Fast Green for cytoplasmic staining (Merck, Darmstadt, Hessen, Germany) for 60 seconds and washed with distilled water again.

The slides were then subjected to four batteries of absolute alcohol in a row for 2 minutes each, and after dehydration in alcohol, the slides were subjected to three batteries of xylol for 5 minutes each. Final preparation of the slides with Entellan was then performed (Merck, Darmstadt, Hessen, Germany), covering them with coverslips to avoid bubble formation. The slides dried for 24 hours at room temperature.

Finally, the cells were evaluated with the Nikon Eclipse E600 microscope (Nikon Instruments, Melville, New York, USA) at a 400x magnification, to determine the frequency of micronucleated cells indicating mutagenicity. **Figure 1** illustrates possible nuclear changes that indicate genotoxicity and cytotoxicity. Data were recorded in tables and analyses of variance with *post hoc* Tukey (intergroup) and the Student *t* test (intragroup) were performed.

Histopathology

The tissue samples collected from right and left buccal mucosa, palate, and tongue were processed, embedded with paraffin, cut into 5µm sections and adhered to glass slides. Finally, the slides were stained by hematoxylin and eosin and analyzed by light microscopy. Histopathology analysis evaluated the presence of tissue changes such as inflammatory infiltration, desquamation, degeneration of cells and other injuries. Photomicrographs were taken using a microscope coupled to a Leica DM500 digital camera of 5 mega pixels ICC50.

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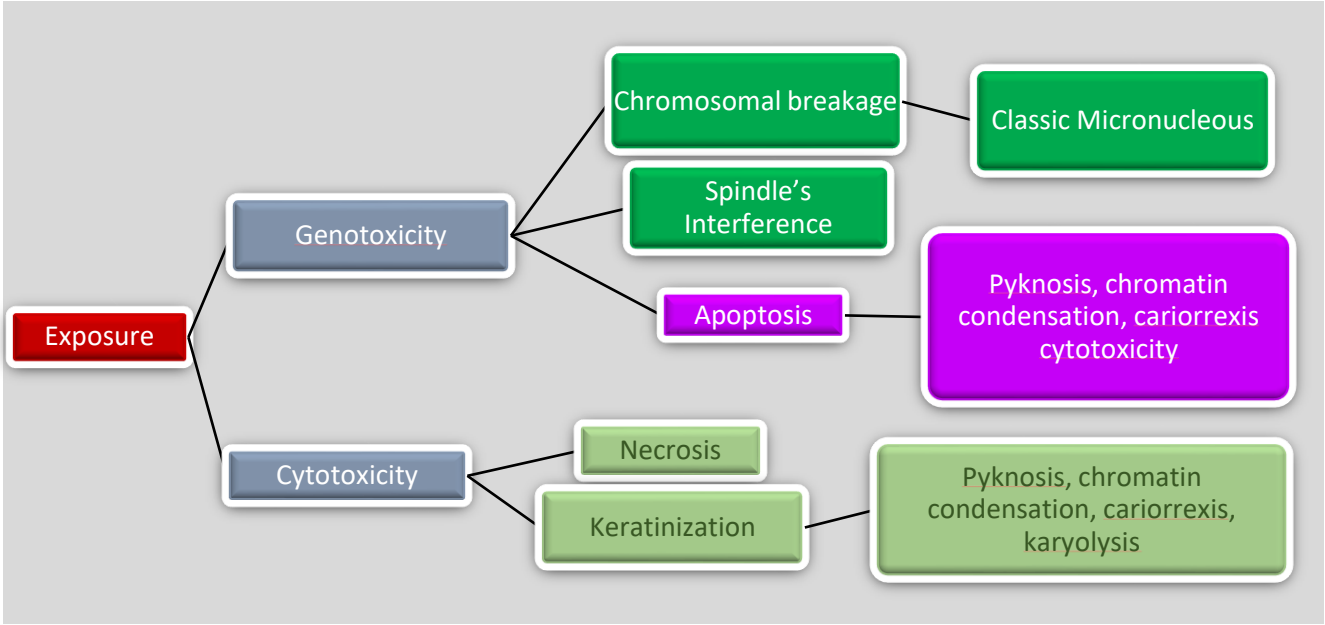

Figure 1. *Schematic of potential nuclear changes.*

Results

In the cytologic analysis we were able to observe the presence of different nuclear changes, such as cells without nucleus and with or without granules, illustrated in **Figure 2.**

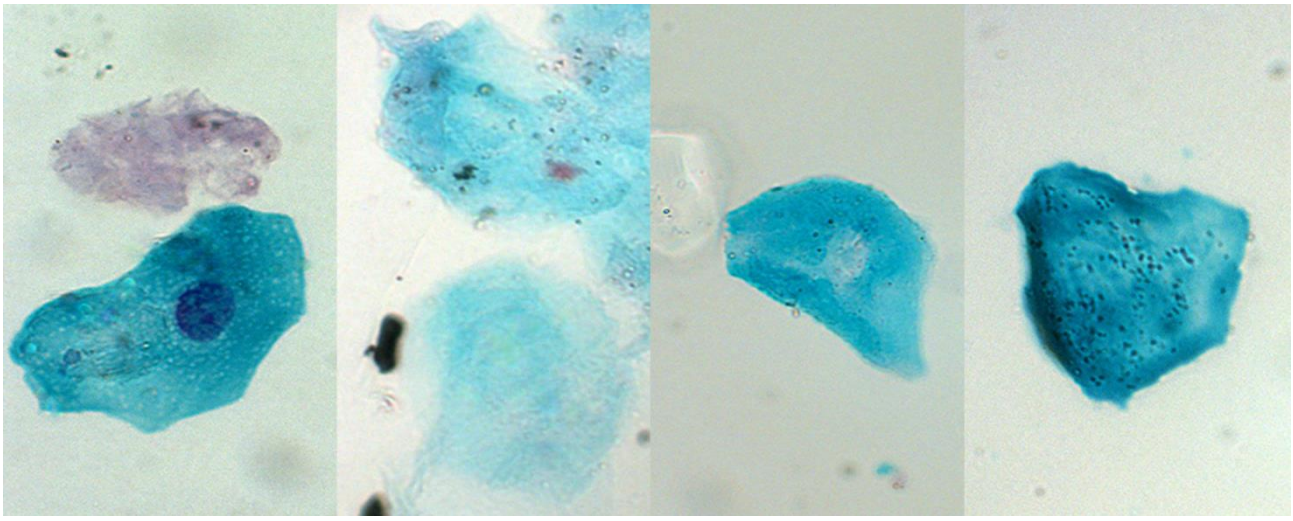

Figure 2. *Nuclear changes (100x): A) Control cell, B) pyknosis, C) cell without nucleus and without granules, D) karyolysis, E) cell without nucleus and with granules.*

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The *A. cymbifera* -hexane extract 10mg/ml (group 5) showed a significant increase in the count of cells without nucleus with time. However, no other treatment groups showed the same trend **(Table 1**). On the other hand, when the count of cells without nucleus and without granules at different intervals was analyzed, group 2 treated with 4mg/ml of the *A. cymbifera* -ethanolic extract showed a significant increase count from day 8 to day 16 and a significant decrease from day 16 to day 24 **(Table 2).** The cytotoxicity capacity of the *A. cymbifera* -based solutions was similar to those of chlorhexidine and no significant differences intergroups were observed **(Tables 1 and 2)**.

Table 1. *Count of cells without nucleus at intervals of 8, 16, and 24 days.*

GROUP	8 days (T8)		16 days (T16)		24 days (T24)	
	Average (SD)	**intergroup comparison***	**Average (SD)**	**intergroup comparison***	**Average (SD)**	**intergroup comparison***
Group 1	59.50 (62.93)[a]	A	31.50 (30.40)[a]	A	54.50 (19.09)[a]	A
Group 2	60.50 (33.23)[a]	A	59.50 (21.92)[a]	A	40.50 (9.19)[a]	A
Group 3	69.50 (24.74)[a]	A	42.50 (0.70)[a]	A	51.00 (19.79)[a]	A
Group 4	38.00 (18.38)[a]	A	32.00 (5.65)[a]	A	41.00 (16.97)[a]	A
Group 5	36.00 (11.31)[a]	A	45.50 (10.60)[b]	A	62.00 (14.14)[b]	A
Group 6	66.50 (27.57)[a]	A	80.50 (57.27)[a]	A	44.50 (7.77)[a]	A

a, b, c, - Different lowercase letters mean that there is statistically significant difference ($p < 0.05$) between different time in the same group (same row). *Different uppercase letters mean that there is a statistically significant difference ($p < 0.05$) between groups at each time (same column). SD – Standard deviation.

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Table 2. *Count of cells without nucleus and without granules at intervals of 8, 16, and 24 days*

GROUP	8 days (T8)		16 days (T16)		24 days (T24)	
	Average (SD)	**intergroup comparison***	**Average (SD)**	**intergroup comparison***	**Average (SD)**	**intergroup comparison***
Group 1	16.50 (3.53)[a]	A	15.50 (0.70)[a]	A	23.50 (6.36)[a]	A
Group 2	9.50 (6.36)[a]	A	29.00 (7.07)[b]	A	21.00 (16.97)[ab]	A
Group 3	12.50 (3.53)[a]	A	18.50 (16.26)[a]	A	24.50 (20.50)[a]	A
Group 4	11.00 (1.41)[a]	A	13.00 (1.41)[a]	A	10.00 (7.07)[a]	A
Group 5	7.00 (1.41)[a]	A	13.50 (4.94)[a]	A	23.00 (2.82)[a]	A
Group 6	12.50 (12.02)[a]	A	24.00 (7.07)[a]	A	10.50 (9.19)[a]	A

a, b, c, - Different lowercase letters mean that there is statistically significant difference ($p < 0.05$) between different time in the same group (same row). *Different uppercase letters mean that there is a statistically significant difference ($p < 0.05$) between groups at each time (same column). SD – Standard deviation.

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Upon examination of the tissues previously submitted to treatments, all groups showed morphological characteristics of normal mucosa in the histopathologic analysis. **Figure 3** shows the most representative photomicrographs of each group.

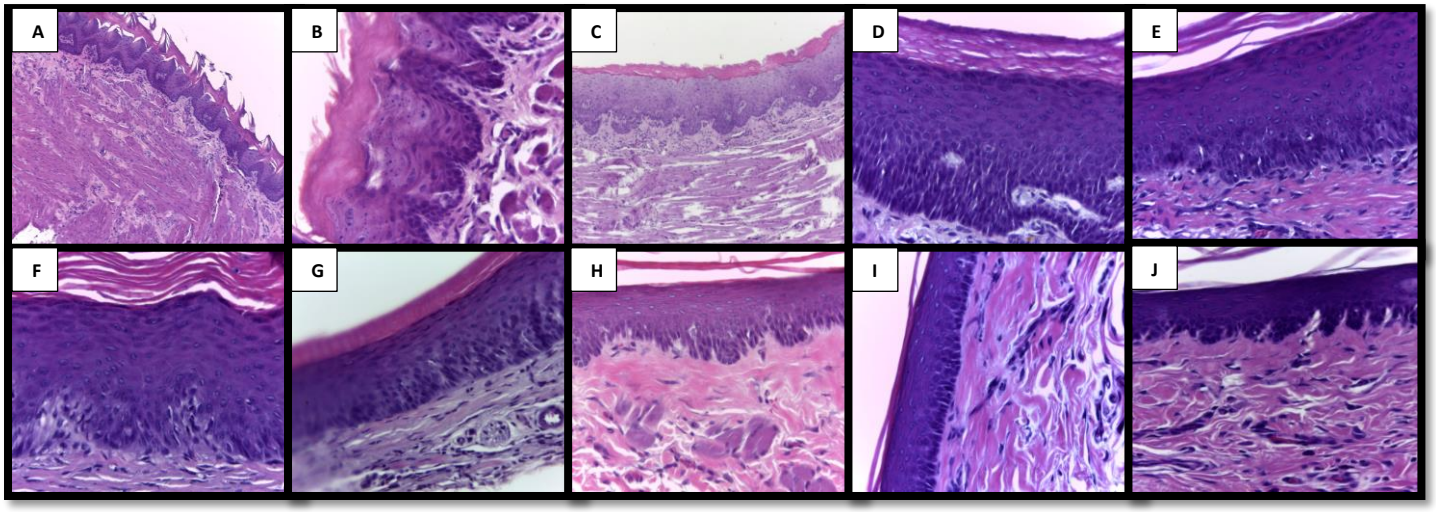

Figure 3. *Selection of tissue structures in different treatment groups: A) Tongue (group 4 – 4x), B) Tongue (group 3 – 40x); C) Tongue (group 1 – 4x), D) Palate (group 4 – 40x), E) Palate (group 5 – 40x), F) Palate (group 2 – 40x); G) Palate (group 3 -10x), H) Buccal mucosa (group 4 – 10x), I) Buccal mucosa (group 3 – 10x), J) Buccal mucosa (group 5 -10x).*

Discussion

In the northeast of Brazil, leaves of *A. cymbifera* are used to treat liver and stomach disorders, snake bites, fever and ulcer [13]. The root of *A. cymbifera* is believed to have therapeutic properties [13]. This study aimed to evaluate the presence of morphological, cellular, and tissue changes in the oral mucosa of 30 Wistar rats after topical application of different *A. cymbifera* extract solutions.

Our results showed that cytologically, most of the extract concentrations tested did not show significant nuclear changes that would indicate cytotoxicity. Histologically, all tissue samples were also considered normal. Both the efficacy and the absence of cytotoxicity of this plant extract suggests the possibility of a natural alternative for chlorhexidine mouthwashes. A disadvantage of chlorhexidine is the alteration of taste perception that it causes after a few days of use. Chlorhexidine is amphiphilic, which means it's both lipophilic and hydrophilic, a characteristic suggested to be the main cause of the taste alteration as side effect [7]. On the other hand, *A. cymbifera* has a strong bitter taste that we have tried to overcome with different dilutions. Even though natural and herbal substances

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popularly used for nutritional purposes, medications and even mouthwashes are taken considerably well by users, additional experiments are warranted in order to provide the extract with a more pleasant taste before commercialization. Additional tests to determine whether *A. cymbifera* would cause any teeth staining would also be desirable as the next step.

Using the kill-kinetics assay, the *A. cymbifera* alcoholic extract has previously demonstrated favorable results in microorganism's growth inhibition after 1 hour of in vitro exposure [10]. In conclusion, although the extract of *A. cymbifera* showed no cytotoxicity, no abnormal tissue characteristics, there is a need for clinical trials to confirm the usability and efficacy of this substance in humans before it is applied clinically or commercialized.

Disclosure of interest

The authors declare that they have no competing interests.

Authors' contributions

These authors contributed equally to this work.

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