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Aqueous Extract of *Psidium cattleianum* as Intracanal Medication: An *In Vitro* Study of Cell Viability

Christine Men Martins[1], Thayse Yumi Hosida[2], Ana Maria Sell[3], Eloi Dezan Junior[4] and Mirian Marubayashi Hidalgo[5]

1 Dentistry Department, Clinic Integrate Area, Municipal Foundation of Education and Culture FUNEC, Sao Paulo, Brazil
2 Restorative Dentistry Department, Pediatric Area, Aracatuba Dental School, Sao Paulo, Brazil
3 Basic Health Sciences Department, Immunology Area, Maringa State University, Parana, Brazil
4 Restorative Dentistry Department, Endodontic Area, Aracatuba Dental School, Sao Paulo, Brazil
5 Dentistry Department, Endodontic Area, Maringa State University, Parana, Brazil

Corresponding author:
Christine Men Martins

christinemen@hotmail.com

Dentistry Department, Clinic Integrate Area, Municipal Foundation of Education and Culture FUNEC, Santa Fe do Sul, Sao Paulo, Brazil.

Tel: +55 (44) 9 9927 8314

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Abstract

Psidium cattleianum extract has shown activity against oral microorganisms and anti-inflammatory activity, and also tissue biocompatibility. These characteristics aroused interest in its use as an alternative to traditional intracanal medication. The aim of this study was to evaluate the aqueous extract of *P. cattleianum* cytotoxicity by structural and functional cell viability, in order to use as intracanal medication. The hypothesis is that aqueous extract of *P. cattleianum* should be used as intracanal medications due to better cytotoxicity response than cell culture medium. Positive control used was RPMI cell culture medium and negative control was water. Cell viability was analyzed after 1 h, 3 h, 6 h, 10 h and 24 h of incubation by exclusion method with trypan blue and MTT assay, using human mononuclear cells (PBMC) and human Periodontal Ligament Cells (PDL) in culture. By trypan blue assay, both PBMC and PDL cells showed an average viability higher ($p>0.05$) than RPMI when the cells were maintained in aqueous extract. Distilled water showed lowest average viability ($p<0.05$). By MTT assay, PDL cells showed increasing viability over time when maintained in aqueous extract culture. *P. cattleianum* was able to maintain the structural and functional cell viability, shoeing a higher performance than positive control. Also, this extract neutralized the detrimental effect of distillated water. The results about biocompatibility assays are promising to indicate the use of aqueous extract of *P. cattleianum* as an alternative to traditional intracanal medications.

Keywords: *Psidium*; Cell survival; Materials testing

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Introduction

Endodontic treatments are closely associated with Orofacial pain of dental origin, which affect a large part of the population and they are the main representatives of emergency and emergency care, especially in public environments [1,2]. They can also be performed as a result of asymptomatic cases, but in any case, the infection may be present in the root canal system and or the apical region [3]. The suitable chemical-mechanical preparation as well as the use of intracanal medication eliminates infection, prevents recontamination [3-5], and may also prevent or reduce periapical inflammation through biocompatibility [6,7].

Calcium hydroxide is often used as intracanal medication [8,9]. However, new alternatives must be sought, especially considering the worldwide tendency toward the use of medicinal plants, due to advantages such as diversity, flexibility, accessibility, availability and wide acceptance. Furthermore, the World Health Organization reported that medicinal plants would be the biggest and best source of obtaining drugs for humanity [10].

Based on previous studies, *Psidium cattleianum* shows promise as intracanal medication during endodontic treatment [11-18]. The aqueous and ethanol extracts of *P. cattleianum* have shown remarkable antibacterial activity against oral microbiota,

anti-inflammatory and potential anticariogenic effects, and biocompatibility [11-15]. However, determining the use and clinical application of this extract requires testing for cytotoxicity [16], sensitization [17], anti-inflammatory and antimicrobial activities [12-15,18], and other issues.

Thus, this study evaluated the aqueous extract of Psidium cattleianum cytotoxicity by structural and functional cell viability, in order to use as intracanal medication. The hypothesis is that aqueous extract of *P. cattleianum* should be used as intracanal medications due to better cytotoxicity response than cell culture medium.

Material and Methods

The project was approved by the Ethics Committee in Research Involving Humans from the Maringa State University, with the protocol number CAAE: 0108.0.093.000-10.

Extract and controls

The aqueous extract was prepared accordingly to a described protocol [11]. Healthy leaves were selected, washed in tap and deionised water, dried at 37°C for 1 week, ground in a blender until a thin powder was achieved. Aqueous extract was obtained by decoction in deionised water (100 g/600 mL) for 5 min at 100°C and at 55°C for an additional 1 h. The solution was then filter sterilised with 0.22 µm mixed cellulose ester membranes (Millipore™; Billerica, MA, USA). The extract was stored in dark bottles at −20°C until further use.

Culture medium Roswell Park Memorial Institute 1640 (RPMI-Gibco®, Life Technologies, USA) was used as a positive control and distilled water served as negative control.

Human mononuclear cells (PBMC)

To obtain the PBMC, 15 ml of venous blood from four donors was obtained aseptically in heparinized tubes with 2 drops of Liquemine® (Roche, Switzerland). The cells were isolated by the method described by Boyum [19] modified. The tubes were centrifuged and the interface between plasma and red blood cells were collected and diluted in sterile Phosphate-Buffered Saline (PBS) and placed over a discontinuous gradient Histopaque® (Sigma Chemical Co., USA). After further centrifugation, the leukocytes were removed via Pasteur pipette and transferred to another tube. Then the washing process was carried out with PBS three times. The cell precipitated was resuspended in RPMI (Life Technologies, USA).

Human periodontal ligament cells maintained in culture (PDL)

The PDL cells were kindly provided by the laboratory of Applied Virology, Santa Catarina Federal University. The cells were kept in an incubator (5% CO_2/95% humidity, 37°C) in cell culture bottles (TTP Techno Plastic Products, Switzerland) containing Dulbecco's MEM (DMEM-Cultilab, Brazil) supplemented with 10% fetal bovine serum (FBS-Cultilab, Brazil) and 1% of a combination of penicillin 10,000 UI/mL, streptomycin 20 mg/ml and amphotericin B 2 mg/L (PSA-Cultilab, Brazil). The culture medium was changed every 48 or 72 h. After cell confluence the trypsinization was done using 0.25% trypsin solution and Ethylenediaminetetraacetic acid (EDTA-Sigma Chemical Co., USA) for several minutes to release the cells. After neutralization with RPMI (Life Technologies, USA), the cell precipitate was washed, dissolved in a fresh culture medium, and aliquoted in bottles. Each trypsinization yielded a new passage and throughout the experimental period when confluence, cells were replicated to maintain the culture's optimal viability. The experiments were performed when cells were between the eleventh and twelfth passages, as recommended by ATCC [20] and Oh et al. [21].

Trypan blue assay

The viability was determined by microscopic observation of the cells excluded by staining with trypan blue, a vital stain derived from toluidine. A final concentration of 1 x 10^6 cells/mL of the two types of cells was incubated in aqueous extract of *P. cattleianum* or controls during 24 h at 25°C. Samples were collected at 1, 3, 6, 10 and 24 h and analyzed in a Neubauer chamber with an equal volume staining. Cells were considered not viable when impregnated by staining or when degenerated. Three previously trained observers performed the readings under an optical microscope and expressed the results as percentages. They repeated the process four times.

MTT assay

The cells were adjusted to 1 x 10^4 cells/ml in RPMI (Life Technologies, USA) with 10% FBS (Cultilab, Brazil) and 1% PSA (Cultilab, Brazil), and seeded in 96-well plates (TPP Techno Plastic Products, Switzerland). After incubation (5% CO_2 and 95% humidity at 37°C incubator) for 24 h for cell adhesion, the culture medium was discarded, and the extract or control was added. After the incubation times of 1, 3, 6, 10 and 24 h, the experimental medium were removed and MTT solution was added (5 mg/ml, MTT-thiazole blue, Sigma Chemical Co., USA). The plates stayed in the incubator during 3 h. Then the MTT solution was removed and dimethylsulfoxide (DMSO-Merck, Brazil) was added to dissolve formazan crystals. Cell viability was determined by reading the absorbance of the wells under the wavelength of 550 nm (ASYS Expert plus Microplate Reader, Biochrom, UK). The test was also performed in triplicate with four repeats.

Statistical analysis

Data on the percentage of viable cells were obtained by means of exclusion with trypan blue and PDL to PBMC in culture were collected for the groups in a time-dependent manner. To analyze these results, we used linear mixed effects models with PROC NLMIXED of SAS version 9. The results of the viability of PDL cell culture by MTT method were subjected to ANOVA Factorial through SAS software 9.3.n. The level of statistical significance was set at 5% for all analyses.

Results

Average of cell viability

Table 1 represents the average viability of cells maintained in the aqueous extract and controls. By trypan blue methodology,

Table 1 Average of cell viability maintained in the aqueous extract and controls.

	Trypan Blue (%) PBMC	Trypan Blue (%) PDL	MTT (O.D.)[1] PDL
Extract of *Psidium cattleianum*	97.5[2]	83.3	2.07[3]
RPMI (positive control)	92	78.1	0.55
Distilled water (negative control)	21.5*	42.4*	0.03

[1]O.D.: Optical Density. [2]Readings performed by three previously trained observers. [3]Test was performed in triplicate with four repeats. *Statistically difference between the negative control and other media ($p<0.05$)

both PBMC and PDL cells maintained in aqueous extract showed greater viability than in the positive control RPMI, but there is no statistically significant difference ($p>0.05$). The negative control presented lowest viability ($p<0.05$). For the MTT, the average Optical Density (OD) was 3.76 times higher in the aqueous extract than the positive control RPMI and 69 times higher than negative control distilled water, but there is no statistically significant difference ($p>0.05$).

Cell viability in the aqueous extract of *Psidium cattleianum* and controls throughout the experimental period

The **Figure 1** represents the average cell viability in the aqueous extract and controls throughout the experimental period. Trypan blue assay to PBMC was shown in **Figure A1** and to PDL in **Figure A2**. Aqueous extract maintained structural viability, showing a homogeneous performance during the 24 h, with slightly higher values, but there is no statistically significant difference ($p>0.05$) than positive control RPMI. The negative control presented lowest viability ($p<0.05$). In B, the functional viability of PDL maintained in culture and analyzed by MTT is expressed in OD. It demonstrated that aqueous extract allowed higher performance than controls ($p>0.05$), increasing over time even without statistically significant difference. The diluent control was also the lowest since the first hour ($p<0.05$).

Discussion

This is the pioneer study of PBMC and PDL cytotoxicity cell viability maintained in the aqueous extract of *Psidium cattleianum* over 24 h. The aqueous extract was more effective in maintaining cell viability for both cell types, with a homogeneous and slightly higher performance than that of the positive control, and also permitted functional viability of PDL. Thus, regarding to this results, our hypothesis was accepted.

The PBMC was used due to the ease and speed of obtaining it. The PDL was used to approach the *in vitro* research with the reality, once they are the cells directly involved in the healing after endodontic treatment [22]. Another important factor in cell selection is because these types of cells permit the standardization of cell concentration, in order to detect possible changes.

The trypan blue assay is commonly used in assessing viability and allows the analysis of physical integrity of the cell membrane [19,23]. However, this methodology does not evaluate the real metabolic capacity of the cell. To overcome this gap, we carried out the MTT assay, quantifying the reduction of MTT salt. Through metabolism, MTT salt becomes formazan salt, which is purplish and soluble in DMSO. This makes it possible to quantify cellular metabolic activity via NADPH-dependent oxidoredutases. The reduction of MTT to formazan is directly proportional to mitochondrial activity and cell viability [20,21].

The cells were maintained viable with similar percentages if placed in aqueous extract of *P. cattleianum* or RPMI. RPMI is usually considered positive control because it is a mixture of salt fortified with amino acids, vitamins and other essential components for cell growth. This medium is a nutrient solution with pH and osmotic concentration ideal for cell culture [24]. The

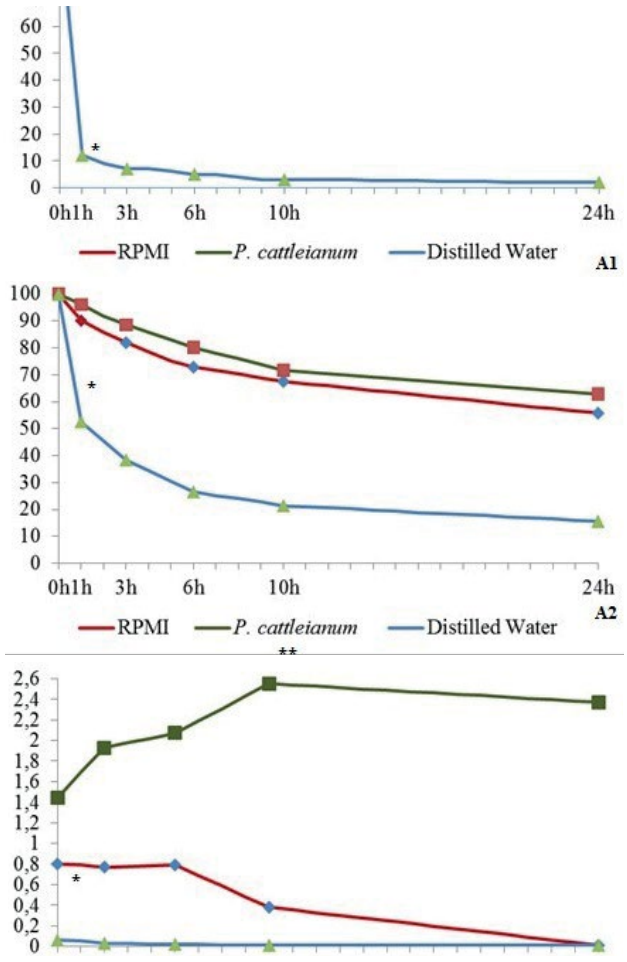

Figure 1 Average of cell viability in the aqueous extract and controls throughout the experimental period. **A:** Trypan blue assay, readings performed in triplicate by three previously trained observers with four repeats. **A1:** PBMC. **A2:** PDL culture cells. **B:** MTT assay, D.O. Reading. Test was performed in triplicate with four repeats. *Significant difference between negative control and other media ($p<0.05$). **Significant difference between aqueous extract and other media ($p<0.05$).

cells maintained in the aqueous extract showed higher viability then those maintained in the RPMI control. Although this is not a statistically significant difference, aqueous extract appears non-cytotoxic, biocompatible, and effective on viability and integrity maintenance.

The aqueous extract was obtained from *P. cattleianum* plant with distilled water as diluent. Compared to the aqueous extract, the control diluent caused a very high rate of cell death, by the trypan blue assay. Thus, the extract appears to have neutralized the detrimental effect that distillated water performed. The water is a hypotonic medium that can lead to rapid lysis of the cell membrane [25].

When analyzed throughout the experimental period by MTT, the aqueous extract was more efficient than the controls. Aqueous extract showed an increase in OD average, significant ($p<0.05$) after the tenth hour, which is an unusual finding. The medium usually keeps cell viability constant and its effectiveness to decrease over time. One possible explanation could be the natural antioxidants in the leaf extract of *P. cattleianum*, as discussed in previous studies [18]. Another possibility is the production and/or release of mediators or growth factors by the cells themselves after contact with aqueous extract. This increase in OD should be studied in the future, to verify whether it originates with a cell proliferation or increase the metabolic activity of the cells.

In addition to the aqueous extract's maintenance of the integrity of the membrane and the functional metabolic activity of the cells, it also appears to have favorable biological properties and antibacterial activity, and can influence the demineralization of hydroxyapatite by microorganisms [11-18]. Also, Brighenti et al. [11] showed it may reduce demineralization of the enamel, present acidogenic potential, the viability of microorganisms and the production of extracellular polysaccharide.

All of these properties can be compared with Calcium Hydroxide properties [2-9]. However, Calcium Hydroxide antimicrobial activity is mainly due to alkaline potential [4], while extract presents acidogenic potential [11], so the action path differs between the two substances. Thereby, the aqueous extract of *P. cattleianum* has potential to be an alternative or to complement intracanal medication. By this way, further studies are needed to analyze others aspects in order to its indication.

Conclusion

So, based on the methodology and results presented, it is concluded that the aqueous extract of *Psidium cattleianum* was able to maintain structural and functional cell viability, outperform the positive control and neutralize the deleterious effect of distilled water. The results about biocompatibility assays are promising to indicate the use of aqueous extract of *P. cattleianum* as an alternative to traditional intracanal medications.

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