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**BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY**

***A N I N T E R N A T I O N A L J O U R N A L***

# Viability of Human Gingival Fibroblast (FGH) Treated with Ethanolic "Aroeira" Extract (*Myracrodruon urundeuva* Allemão)

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## ABSTRACT

*The aim of this study was to evaluate the effect of ethanolic "aroeira" (Myracrodruon urundeuva) extract on the viability of human gingival fibroblast. For this, fibroblasts ($2x10^3$ cells/well) were plated in a 96-well* plate *and incubated for 24 h; the medium (Eagle's medium modified by Dulbecco - DMEM) supplemented with 10% fetal bovine serum was replaced by DMEM with different ethanolic extract concentration (0, 0.1, 1, 10, 100, and 1000µg / mL). The fibroblast viability was analyzed after 48,72, and 96 h by the neutral red capture test and violet crystal. The "aroeira" extract, at high concentrations (100 and 1000 µg/mL) caused decrease in both cellular viability tests ($p<0.05$). However, dilutions between 0.1 and 10 µg/mL did not affect the viability of the cells. It was concluded that "aroeira" extract was able to change the gingival fibroblast viability, and this effect was concentration dependent.*

**Keyword**: Medicinal Plant, fibroblast, *Myracrodruon urundeuva*, "aroeira", "aroeira-do-sertão".

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## INTRODUCTION

The phytotherapeutics are standardized vegetable preparations that consist of a complex mixture of plant-based substances that are adequately prepared and prescribed in compliance with applicable legislation (Bettega et al. 2011; Machado and Oliveira 2014, Fürst and Zündorf 2015). Phytotherapeutic compounds, in general, can be used in a variety of formula such as capsules, tablets, gels, ointments, aqueous solutions, and infusions, such as tea (Francisco 2010). The leaves, seeds, twigs, and stems of the "aroeira" (Myracrodruon urundeuva), a tree that is widely present throughout Brazil, have been studied because of their antifungal, anti-adherent, and antimicrobial activities (Machado and Oliveira 2014). Among "aroeira" extract properties, described in the literature with in vivo models, the following benefits can be pointed out: healing effects (Nunes Jr. et al. 2008); anti-inflammatory effects (Rodrigues et al. 2002; Souza et al. 2007), and antiulcerative effects (Souza et al. 2007).

Another extract property of "aroeira" (Machado and Oliveira 2014) is its antimicrobial effect. Menezes et al. (2010) evaluated the extract action of "araça" (Psidium cattleianum Sabine) and "aroeira" (Myracrodruon urundeuva Allemão) in experimental animals subjected to a cariogenic challenge and investigated the extracts' effect upon the cariogenic microbes introduced into these animals. The animal model and the experimental conditions were adequate to characterize the test extracts' effect upon the enamel microhardness and upon the cariogenic microbes, because the two tested extracts produced a substantial reduction in cariogenic microbes in the experimental animals and consumption of the same positively affected superficial enamel hardness.

Although, "aroeira" extract has shown some promising results, about 25% of the Anacardiaceae genus are known to be toxic and cause contact dermatitis such as for example, Anacardium, Astronium, Blepharocarya, Cotinus, Lithrea, Mangifera, Mauria, Myracrodruon, Schinopsis, Schinus, Semecarpus, Spondias, Swintonia, and Toxicodendron. The dermatitis caused by these plants is attributed mainly to the phenolic lipids (Pell 2004). Similarly, the Myracrodruon urundeuva species, i.e., the "aroeira-do-sertão", although presents desirable properties (antimicrobial, anti-inflammatory, etc.), its irritating and sensitizing power are high, and it causes allergies and dermatitis (Reis 2010). Another very important point is its dose, which represents an obstacle to guaranteeing the safety and efficacy. Even in the cases that confirm biological activity of the plants, the dosage band at which the desired effect is seen, can be restricted considerably so that the high dosage could induce the harmful effects (Reis 2010). Thus, the purpose of this work was to evaluate the effect of ethanolic extract of "aroeira" at different concentrations on the viability of human gingival fibroblasts.

## MATERIAL AND METHODS

Healthy and dry leaves were collected from *M. urundeuva* ("aroeira") from the Bauru municipality of SP and, after verifying the species, were prepared (Saldanha et al. 2013), identified, and stored in Rioclarense herbarium (HRCB) at the IB-UNESP-Rio Claro Botanic Department under the voucher species number HRCB59831. The leaves were dried to 40ºC for 48 h and powdered, which was mixed with EtOH/$H_2O$ (7:3, v/v) and macerated for a week. This method was repeated several times until the extraction process was complete. Then the solvent was filtered through filter paper and concentrated under minimal pressure at <40°C. The crude extract thus obtained was transferred to glass crucible and was completely dried (Saldanha et al. 2013).

### Experimental Groups

In these experiments, lineage human gingival fibroblasts (FGH), previously obtained from the primary culture (Damante et al. 2009) were used. The cells were grown in Eagle's medium, modified by Dulbecco (DMEM), supplemented with bovine fetal serum 10% and 1% antimycotic antibiotic solution, at 37°C in an incubator with a $CO_2$ level of 5% atmosphere. The experimental groups were divided based on different concentrations of the extract such as 0.1, 1, 10, 100, and 1000 µg/mL; a control group (without extract addition) was also used. The extract was diluted directly in DMEM, without solubilizing any agent. The cells (2 x

$10^3$ cells/well) were plated using 96-well plates (TPP®), with n=6 (six wells to each group). After incubation for 24 h, the culture medium was changed to a medium with different concentrations of extract; the control group was changed to a conventional culture medium without the extract.

The viability of the fibroblasts was analyzed at 48, 72, and 96 h after the addition of media with and without extract at different concentrations. After each experimental period, the culture medium containing the “aroeira” extract was removed; the cells were washed with a solution of saline phosphate, dried, and divided to analyze them. All the following assays were performed at least in triplicate.

## Cellular Viability Analyses

*Neutral Red Assay*

From a 0.4% neutral red stock solution, a neutral red 50 µg/mL solution was prepared in DMEM. The obtained solution was left at 37ºC overnight to precipitate the crystals and was then filtered with a Millipore filter (0.22 µm). The cells were treated with this solution (50 µg/mL) and the plates were incubated at 37ºC for 3h to allow the stain to be absorbed by the lysosomes in viable cells. The cells were then washed with phosphate buffered saline with calcium (PBS-$Ca^{+2}$) and the stain was extracted with a 50% ethanol solution and 1% acetic acid by placing 200 µL of this solution in each well (Volpato et al. 2011). Then, the absorbance was determined at 540 nm (FluoStar OPTIMA microplate fluorescence reader).

*Violet Crystal Assay*

The adhered cells were washed with phosphate buffered saline (PBS) and fixed in glacial ethanol and acetic acid (3:1, v/v) at ambient temperature for 10 min, left to dry by air and incubated at 37ºC in dark. The cells were then stained with violet crystal to 0.1% (w/v) at ambient temperature for 10 min. The excess stain was removed by decantation and the cells were washed with distilled water two times. The stain was extracted in 10% (v/v) acetic acid and optic density was available to 550 nm (FluoStar OPTIMA microplate fluorescence reader).

## STATISTIC ANALYSIS

Statistical analysis was done by ANOVA to a complementary criterion established by the Tukey test ($p<0.05$). All the statistical tests that were appropriate to the experiments performed, groups, and variables, were run using the Statistic 11.0.s program.

## RESULTS

**Neutral Red Assay**

By and large, the absorbance values achieved in all the groups varied in the periods during which they were measured. The higher values belonged to the groups with lower “aroeira” extract concentration and the control group. At 48 h, the 0.1, 1, and 100 µg/mL groups showed similar results but higher than the control; however, there was no significant difference with the control group ($p>0.05$). The 10 and 1000 µg/mL groups presented statistically different results from the control group ($p<0.05$) (Table 1). At 72 h, the highest value (0.133) corresponded to the 0.1 µg/mL group, while the lowest value (0.014) corresponded to the 1000 µg/mL group. These two groups were significantly different from the control group ($p<0.05$), while the others did not present a significant difference in the study period (Table 1). At 96 h, the groups presented significant absorbance values. The groups with smaller extract concentrations and the control group remained with values above 0.1; the higher concentration groups (10, 100, 1000 µg/mL) presented lower values than the control group ($p<0.05$) (Table 1).

**Table 1**- Absorbance Valuable (mean ± standard deviation) the neutral red tests in all the groups and periods.

| EXPERIMENTAL GROUPS | PERIOD (H) | | |
|---|---|---|---|
| | **48** | **72** | **96** |
| **Control** | 0.085±0.016 | 0.065±0.011 | 0.161±0.022 |
| **0.1µg/mL** | 0.122±0.026 | 0.133±0.038* | 0.165±0.031 |
| **1µg/mL** | 0.122±0.020 | 0.066±0.012 | 0.110±0.015 |
| **10µg/mL** | 0.136±0.013* | 0.048±0.096 | 0.054±0.016* |
| **100µg/mL** | 0.072±0.016 | 0.067±0.008 | 0.074±0.012* |
| **1000µg/mL** | 0.002±0.001* | 0.015±0.017* | 0.010±0.000* |

* Significant statistic difference ($p<0.05$) in related to the correspondent period control group

**Violet Crystal Assay**

In the 48 h period, the group majority presented absorbance band of 0.230–0.380, without a significant difference in the control group ($p>0.05$) (Table 2). The group with 1000 µg/mL concentration had less statistically significant absorbance (0.049) than the control group (0.292) ($p<0.05$). At 72 h, the majority of groups presented similar values to the control group (0.420), hence, no significant difference between the groups ($p>0.05$). Only the treated groups with extract concentrations of 100 (0.271) and 1000 µg/mL (0.124) presented smaller significant differences in relation to the control group (0.420) ($p<0.05$) (Table **2**). The 96 h period presented a similar profile to the 72 h, wherein the groups treated with smaller concentrations presented similar values to the control group ($p>0.05$). The groups corresponding to 100 µg/mL (0.219) and 1000 µg/mL (0.087) presented smaller values than the control group (0.623) ($p<0.05$) (Table 2).

**Table 2**-Absorbance Valuable (mean ± standard deviation) the Violet Crystal test in all groups and periods.

| EXPERIMENTAL GROUPS | PERIODS (H) | | |
|---|---|---|---|
| | **48** | **72** | **96** |
| **Control** | 0.292±0.496 | 0.420±0.493 | 0.623±0.053 |
| **0.1 µg/mL** | 0.349±0.350 | 0.417±0.019* | 0.617±0.059 |
| **1 µg/mL** | 0.382±0.020 | 0.301±0.045 | 0.532±0.060 |
| **10 µg/mL** | 0.236±0.033 | 0.386±0.043 | 0.409±0.035 |
| **100 µg/mL** | 0.196±0.226 | 0.271±0.010* | 0.219±0.229* |
| **1000 µg/mL** | 0.049±0.324* | 0.124±0.469* | 0.087±0.078* |

* Significant statistic difference ($p<0.05$) in related to the correspondent period control group

## DISCUSSION

Currently, an increasingly broad variety of phytoconstituents are being studied and understood in experimental models to achieve the understanding of their biologic activity *in vitro* and *in vivo* (Catão et al. 2006). An effort has been made by the Brazilian government (Medicinal Plants National Program and Phytotherapeutics in the Health Unique System-SUS; Interministerial Order 2.960/2008) for the development of phytotherapeutics products (Bettega et al. 2011) because the country possesses the greatest biodiversity in the world, with estimates hypothesizing that Brazil’s ecosystem comprises 22% of the world’s biologic species (Elisabetsky and Costa-Campos 1996). The few studies with “aroeira” extract that have been conducted thus far have presented evidences with promising benefits, such as the effects described (antimicrobial, anti-inflammatory, regenerator), which are of significant interest to a wide variety of health-related fields (physiology, dentistry, etc.) (Machado and Oliveira 2014).

In this present study, cell viability was determined by crystal violet assay, which is a test based on the ability of DNA from viable cells to capture the pigment (gentian violet), and neutral red assay (lysosomal function) (De Deus et al. 2009; Scelza et al. 2012). Both the tests (assays of some cellular functions) showed cell viability. These *in vitro* tests employing cell culture are advantageous due to their simplicity/quickness, low costs (Freshney 2005), and control of the some experimental conditions (pH, $CO_2$ concentration, and levels of some molecules) (Schweikl and Schmalz 1996; De Deus et al. 2009).

In general, there are contradictory results involving “aroeira” due to tissue biocompatibility (Nunes Jr. et al. 2008; Machado et al. 2012) and the induction of apoptosis in cellular lineages (Ferreira et al. 2011). However, comparision of these works with the present results showed some similarities between them. Different doses (extract concentration) might determine the effect in a positive, or negative manner. In general, low concentrations of “aroeira” extract do not promote cell death and are biocompatible in different experimental models (Nunes Jr. et al. 2008; Machado et al. 2012; Machado and Oliveira 2014), as confirmed by the present results. In the present study, the high values of cell viability at lower concentrations in 48 h could be an effect of some components of the extract, such as glucose molecules from tannins and flavonoid (Souza 2012; Machado et al. 2015). On the other hand, high-concentration extracts promoted cell death (Ferreira et al. 2011), or reduce viability, as demonstrated in this work. Polyphenol fractions, present in the plant extract (Souza 2012; Machado et al. 2015), are possible candidates to promote the cellular proliferation inhibition, and can induce cell death by apoptosis (Queires et al. 2006).

Many plants that belong to the Anacardiaceae family (which includes “aroeira”) have toxic potential due to the presence of phenolic derivatives such as tannin, the main chemical that constitutes “aroeira-do-sertão” (Monteiro et al. 2005). Polyphenols of the “aroeira” (*Schinus terebinthifolius Raddi*) showed ability to reduce the proliferation on cell lineage of the human prostate carcinoma *in vitro* (Queires et al. 2006). This study showed considerable modulation capability (proliferation, or inhibition) depending on the concentration of the extract (Queires et al. 2006). The results reported by Monteiro et al. (2005), Queires et al. (2006) and Souza (2012) matched with the prsent findings, suggesting a modulation of the cell viability dependent of the concentration of the extract, and of the possible components present in the extract. However, Pellegrina et al. (2005); Sakao et al. (2009); Ferreira et al. (2011) and Silva et al. (2011); Machado (2015) reported that cellular viability reduction at high “aroeira” extract concentrations could be associated with the presence of some components such as gallic acid and quercetin in high concentrations. Silva (2011) and Machado (2015) found these components in the “aroeira” extract. Machado (2015) findings seemed to be similar with the present findings as when the “aroeira” extract concentration was too high, it promoted a cellular viability reduction during the intervening treatment period.

## CONCLUSION

From the obtained results, it could be concluded that ethanolic “aroeira” extract at different

concentrations promoted a change in human gingival fibroblast viability. Higher concentrations decreased the cellular viability, while smaller dilutions did not change the viability considerably.

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