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The biocompatibility of *Achyrocline satureioides* plant extract over human gingival fibroblasts

A biocompatibilidade do extrato da planta *Achyrocline satureioides* sobre fibroblastos gengivais humanos

La biocompatibilidad del extracto de la planta *Achyrocline satureioides* en fibroblastos gingivales humanos

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Abstract

Objective: *Achyrocline satureioides* "*A.satureioides*" is a subshrub, widely distributed in South America because of its medicinal properties. Although it is widely used in folk medicine, there is still no approval for its therapeutic use, and its biocompatibility is little explored. This study aimed to evaluate its cytotoxicity and genotoxicity over human gingival fibroblasts (FMM-1). **Methodology:** Ten different concentration of the glycolic extract of *A.satureioides* were tested for 5min and 24h of contact with the cells to evaluate its cytotoxicity using the MTT colorimetric assay and to evaluate its genotoxicity using the micronucleus assay. Data were analyzed with one-way ANOVA and Tukey test with a significance level (α=0.05). **Results:** All tested concentrations of the extract presented cell viability more than 70% and has no significant difference of the control group after 5min and 24h. However, for 5min the 100 mg/mL was cytotoxic and for 24h the 1.56 mg/mL stimulated cell proliferation. For genotoxicity analysis, only the concentration 6.25 mg/mL showed results similar to the control of cell culture in the micronucleus count after 5min and 24h. **Conclusions:** The glycolic extract of *A.satureioides* doesn't have cytotoxic and genotoxic effects in concentrations up to 6.25 mg/mL, but in high concentrations it is considered genotoxic.

Keywords: Fibroblasts; Plant extracts; Phytotherapy.

Resumo

Objetivo: *Achyrocline satureioides* "*A.satureioides*" é um subarbusto amplamente distribuído na América do Sul por suas propriedades medicinais. Embora seja amplamente utilizado na medicina popular, ainda não há aprovação para seu uso terapêutico e a sua biocompatibilidade é pouco explorada. Este estudo teve como objetivo avaliar sua citotoxicidade e genotoxicidade sobre fibroblastos gengivais humanos (FMM-1). **Metodologia**: Dez diferentes concentrações do extrato glicólico de *A. satureioides* foram testadas por 5min e 24h de contato com as células para avaliar sua citotoxicidade pelo ensaio colorimétrico MTT e para avaliar sua genotoxicidade pelo ensaio do micronúcleo. Os dados foram analisados com ANOVA one-way e teste de Tukey com nível de significância (α = 0,05). **Resultados**: Todas as concentrações testadas do extrato apresentaram viabilidade celular superior a 70% e não houve diferença significativa do grupo controle após 5min e 24h. Porém, por 5min a 100 mg / mL foi citotóxica e por 24h o 1,56 mg / mL estimulou a proliferação celular. Para a análise de genotoxicidade, apenas a concentração 6,25 mg / mL apresentou resultados semelhantes ao controle de cultura de células na contagem de micronúcleos após 5min e 24h. **Conclusão:** O extrato glicólico de *A. satureioides* não apresenta efeitos citotóxicos e genotóxicos em concentrações de até 6,25 mg / mL mas em altas concentrações é considerado genotóxico.
Palavras-chave: Fibroblastos; Extratos vegetais; Fitoterapia.

Resumen

Objetivo: *Achyrocline satureioides* "*A.satureioides*" es un subarbusto ampliamente distribuido en América del Sur por sus propiedades medicinales. Aunque es muy utilizado en la medicina popular, todavía no hay aprobación para su uso terapéutico y su biocompatibilidad está poco explorada. Este estudio tuvo como objetivo evaluar su citotoxicidad y genotoxicidad en fibroblastos gingivales humanos (FMM-1). **Metodología**: Se probaron diez concentraciones diferentes del extracto glicólico de A. satureioides durante 5min y 24h de contacto con las células para evaluar su citotoxicidad mediante el ensayo colorimétrico MTT y su genotoxicidad mediante el ensayo de micronúcleos. Los datos fueron analizados con ANOVA de una vía y prueba de Tukey con nivel de significancia (α = 0.05). **Resultados**: Todas las concentraciones probadas del extracto mostraron una viabilidad celular superior al 70% y no hubo diferencias significativas en el grupo de control después de 5 min y 24 h. Sin embargo, durante 5 minutos a 100 mg / ml fue citotóxico y durante 24 horas, 1,56 mg / ml estimuló la proliferación celular. Para el análisis de genotoxicidad, solo la concentración 6.25 mg / mL presentó resultados similares al control de cultivo celular en el recuento de micronúcleos a los 5min y 24h. **Conclusión**: El extracto glicólico de A. satureioides no tiene efectos citotóxicos y genotóxicos en concentraciones de hasta 6.25 mg / mL pero en concentraciones altas se considera genotóxico.
Palabras clave: Fibroblastos; Extractos vegetales; Fitoterapia.

1. Introduction

Phytotherapy was used in dentistry and more specifically in endodontics since the last century (Groppo et al., 2008) due to its antimicrobial, anti-inflammatory and antifungal actions and because it is considered biocompatible (Guandalini Cunha et al., 2020; De La Chapa, Singha, Lee, & Gonzales, 2018; Marcia C Valera et al., 2016; Viegas et al., 2020) in addition to other techniques like photodynamic therapy and passive ultrasonic irrigation (Abu Hasna, Ferrari, & Talge Carvalho, 2019; Abu Hasna, Khoury, et al., 2020; Abu Hasna, Pereira Da Silva, et al., 2020). This use was encouraged by the World Health Organization (WHO); however, the agency establishes specific safety requirements including traditional use certificate and toxicity analyzes of the extracts (Organization, 2019).

Achyrocline satureioides "*A. satureiroides*" is a plant belonging to the Asteraceae family, popularly named "macela" or "marcela", it is a subshrub, widely distributed in South America and very common in Brazil (Ferraro et al., 2008; Lorenzo et al., 2000). Different extracts and purified fractions (polysaccharides and flavonoids) from *A. satureiroides* inflorescences, leaves and stems have been studied and their therapeutic use is related to their antibacterial, antiviral, analgesic, Immunomodulatory, hepatoprotective, muscle relaxant, anti-herpetic and antioxidant effects (Calvo, Cariddi, Grosso, Demo, & Maldonado, 2006; Desmarchelier, Coussio, & Ciccia, 1998; Hnatyszyn et al., 2004; Joray, del Rollán, Ruiz, Palacios, & Carpinella, 2011; Kadarian et al., 2002; Santos, Ripoll, Nardi, & Bassani, 1999; Zanon, Ceriatti, Rovera, Sabini, & Ramos, 1999). Recently, it was suggested as alternative approach for management of viral respiratory infections, including the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV2) (Siqueira, Simões, & Bassani, 2020).

Even *A. satureioides* extracts are widely used in folk medicine (Polydoro et al., 2004). The literature reported divergent results about its biocompatibility by few in vitro and in vivo studies (Calvo et al., 2006; Polydoro et al., 2004; Rivera, Gervaz, Sere, & Dajas, 2004; M C Sabini et al., 2013). To the best of our knowledge there is not studies evaluated the biocompatibility of *A. satureioides* extracts over the oral cells.

Therefore, this study aimed to evaluate the cytotoxic and genotoxic effects of *A. satureoides* extracts over human gingival fibroblasts (FMM-1) in periods of 5 min and 24 h of contact by MTT and micronucleus assays respectively, to assist in the future development of dental materials like intracanal medications, endodontic irrigants, mouthwashes and toothpastes.

2. Methodology

2.1 Extract and cells lineage

The glycolic extract of *A. satureiroides* was used at a concentration of 200 mg / mL (20%) diluted in propyleneglycol (Mapric - São Paulo, SP).

Human gingival fibroblasts (FMM-1) (Faculty of Dentistry, University of São Paulo, São Paulo, Brazil) were cultured routinely in Dulbecco's modified Eagle's medium "DMEM" (DMEM - LGC Biotecnologia, Cotia, Brazil) and supplemented with 10% fetal bovine serum (SFB - Gibco, USA) at 37 ºC in an atmosphere of 5% CO_2. The Trypan blue exclusion test (0.5%, Sigma-Aldrich, St. Louis, MO, USA) was performed to quantify viable cells.

2.2 Cytotoxicity analysis by MTT assay

In 96-well plates, 200 µL of DMEM + 10% SFB medium containing 2 x 10^4 viable cells were added. These plates were incubated at 37 °C with 5% CO2 for 24 h. Then, the cells were exposed to 200 µL / well of 10 different concentrations of *A. satureioides* extract (0.195, 0.390, 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg / mL), n = 12 for each group, and incubated for a period of 5 min and 24 at 37ºC with 5% CO2. Culture medium cells were used as a positive control group without any extract.

The plates were washed with phosphate buffered saline (PBS - Cultilab, Campinas, SP, Brazil) and the MTT solution (100 µL/well) was added to the 96-well plate. The plates were incubated at 37 °C with 5% CO2 for 1 h. Later, the MTT solution was discarded and 100 µL / well of dimethyl sulfoxide (DMSO - Sigma) was added and the plated incubated again fot 10 min. Lastly, the plates were shaken for 10 min and read by a multi-well spectrophotometer (Bio-Tek, Winooski, Vermont, USA) at 570 nm. The obtained optical densities (OD) were converted into a percentage of cell viability using the following formula: % Viability = (OD of treated group x 100) / average OD of the control group (Memari Trava et al., 2020).

2.3 Genotoxicity analysis by micronucleus assay

In 24-well plates, FMM-1 were grown in DMEM (LGC Biotechnology) medium supplemented with 10% fetal bovine serum (SFB) (Invitrogen) where 2 x 10^4 cells / well were plated and incubated at 37 °C with 5% CO2 for 24 h. Then, the cells were exposed to *A. satureioides* extracts diluted in DMEM + 10% SFB and to ethyl methane sulfanate (EMS); 5 mM as a positive control group for 24 h.

Later, the cells were fixed with 4% formaldehyde. Then, 500 µL of PBS and 1 drop of Fluoroshield solution with DAPI (Sigma-Aldrich) were added to the wells, which were photographed with a digital camera (Sony F828 digital, CyberShot, 8.0 megapixels) coupled with an inverted light microscope. At least 10 photos per well were taken and the number of micronuclei was determined to be 2,000 cells / well. DNA structures contained in the cytoplasm separated from the main nucleus were identified as micronuclei, with an area less than 1/3 of the main nucleus area. Cells in mitosis and that exhibited

nuclear fragmentation by apoptosis were not considered in the count (Oliveira et al., 2017; Sousa et al., 2020).

2.4 Statistical analysis

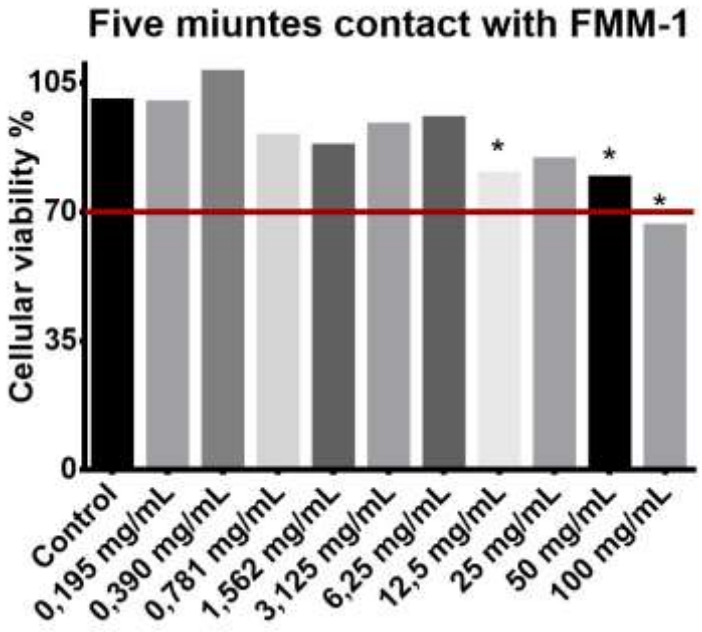
The data were statistically analyzed using the one-way ANOVA and Tukey tests, with a significance level of 5% by GraphPad Prism 6 (La Jolla, CA, USA).

3. Results

3.1 MTT assay

In the 5 min contact period, all the tested concentration of the extract presented cell viability more than 70% and has no significant difference of the control group. Conversely, the 100 mg/ mL of the extract presented 65.9% of cell viability and had significant difference of the control group ($p < 0.05$) (Figure. 1).

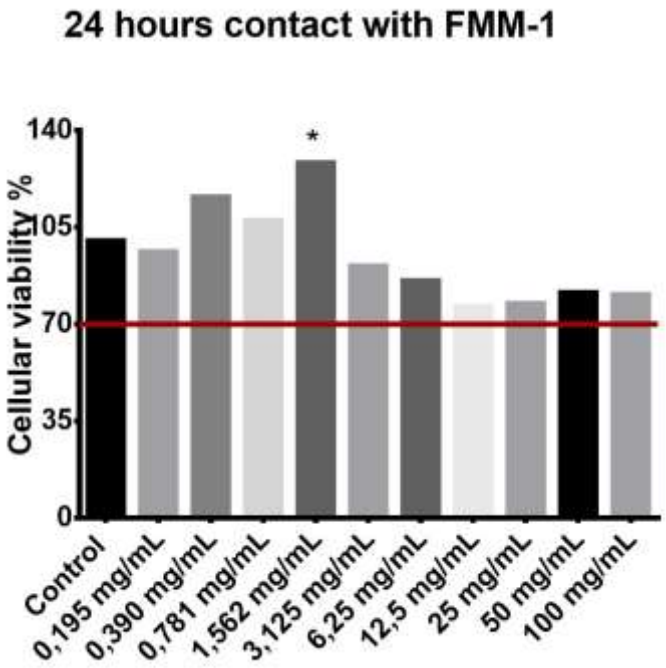
Figure 1 - Percentage (%) of the viability of FMM-1 in contact with different concentrations of A. satureioides extract for 5 min.



Source: Authors.

In the 24 h contact period, all the tested concentrations showed cell viability greater than 70% and has no significant difference of the control group except the concentration of 1.56 mg / mL that significantly stimulated cell proliferation compared to the control group ($p < 0.05$) (Figure. 2).

Figure 2 - Percentage (%) of the viability of FMM-1 in contact with different concentrations of A. satureioides extract for 24 hours.

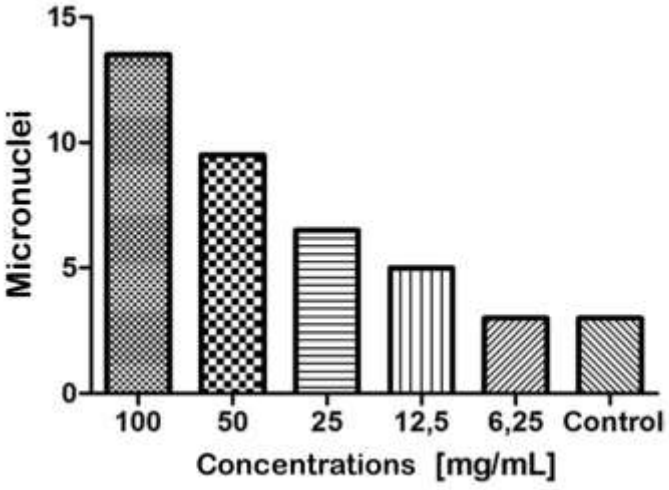


Source: Authors.

3.2 Genotoxicity assay

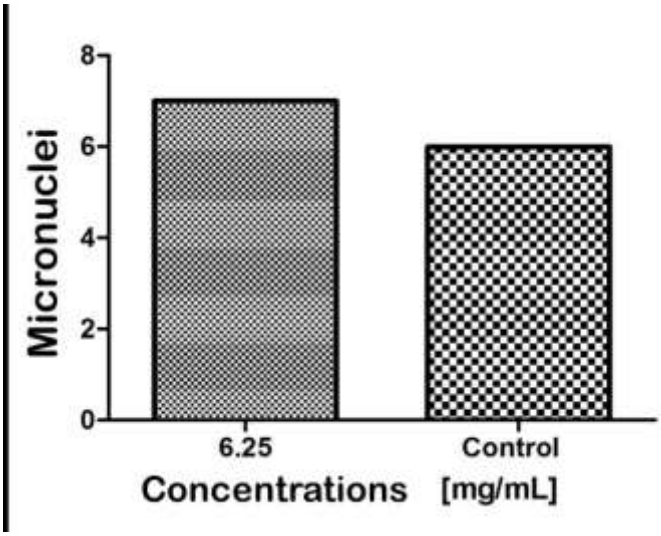
In the 5 minutes contact period, the micronucleus count increased according to the extract concentration, with the highest concentration analyzed (100 mg / mL) averaging 13.5 micronuclei. Only the concentration of 6.25 mg / mL of the extract showed similar results to the control (Figure. 3). As well, in the 24 h contact period, the concentration 6.25 mg / mL also showed results similar to the control of cell culture in the micronucleus count (Figure. 4).

Figure 3 - Counting of micronuclei after contact of FMM-1 with different concentrations of *A. satureioides* extract for 5 min.



Source: Authors.

Figure 4 - Counting of micronuclei after contact of FMM-1 with the concentration of 6.25 mg / mL of *A. satureioides* extract for 24 hours.



Source: Authors.

4. Discussion

Diverse plant extracts were used in dentistry principally as endodontic irrigant or intracanal medication (Horiba, Maekawa, Ito, Matsumoto, & Nakamura, 1991; Maekawa et al., 2015; Qunqiang & Farnham, 1989; Marcia C Valera et al., 2016) or even associated with other intracanal medication (Marcia Carneiro Valera et al., 2015) and this may be related to the fact of the increased resistance of micro-organisms against antibiotics overtime (Jungermann et al., 2011).

The aim of this study was to introduce the *A. satureioides* extract to be used like others (Ghonmode et al., 2013) as an effective substance over the endodontic and oral pathogens. For this, the cytotoxicity of the extract was evaluated over the human gingival fibroblasts (FMM-1). In this study, it was founded that the different concentrations of the extract were biocompatible and had no significant difference of the control group (without extract) when applied for 5 minutes or 24 hours, except of the 100 mg / mL concentration when applied for 5 minutes. Even more, the concentration of 1.56 mg / mL that significantly stimulated cell proliferation compared to the control group when applied for 24 hours.

To the best of our knowledge, there is no studies in the literature evaluated the effect of *A. satureioides* extract over oral cells. The study of Guss et al. (2017) evaluated this effect over mice fibroblasts and macrophage cell lines; however, it did not define the fibroblast cell line exactly, in this study, it was concluded that the extract show no toxicity to fibroblasts agreeing with the results of the present study (Guss et al., 2017). Even more, the extract has no toxic effect over the human peripheral blood mononuclear cells (M C Sabini et al., 2013). It was also verified that only high concentrations of cold extract of *A. satureioides* induced cytotoxicity, corroborating the current study, when the concentration of 100 mg / mL of glycolic extract of *A. satureioides* promoted viability of FMM-1 below 70% (65.9%), demonstrating that higher concentrations of the extract are cytotoxic (María Carola Sabini et al., 2012). Low toxicity was also identified by Salgueiro et al. (2016), the authors used extracts obtained by water infusion and tested its cytotoxic over human lymphocytes and it presented low toxicity (Salgueiro et al., 2016).

In this study as well, the genotoxicity of the extract was evaluated, and it was founded that, the major the extract concentration, the major the genotoxicity, being its dose-dependent genotoxic potential. Only the concentration of 6.25 mg / mL of the extract showed similar results to the control group in both 5 min and 24 h contact period.

In the literature, similar results were obtained, Sabini et al. (2013) found that the cold aqueous *A. satureioides* extract did not show genotoxicity in vitro against Vero cells in the concentration of 10 to 50 μg / mL, but in high concentrations it was genotoxic by the micronucleus assay (M C Sabini et al., 2013) and in a previous study, it was concluded the absence of genotoxicity of cold aqueous extract of *A. satureioides* by Allium test. These findings show the existence of a limit between safe and toxic concentrations that need to be further studied in order to guarantee safety for clinical use.

Beside the extract concentration, another point to be emphasized is the extract temperature, it seems that the hot aqueous extract of *A. satureioides* is more cytotoxic and genotoxic over cells than cold one and this may be related to high concentrations of flavonoids including luteolin, quercetin, and 3-O-methylquercetin (Cariddi et al., 2015). In this study, the extract was used at room temperature.

Then, the results obtained in the present study suggest the use of the *A. satureioides* at low concentration (below 6,25 mg / mL) as it presents lower cytotoxicity and genotoxicity and preferably to use the cold or room-temperature extract when used as a component of dental products like mouthwashes, toothpastes, or as endodontic irrigant and intracanal medication bringing benefits to the population.

5. Conclusion

It may be concluded that:

- The glycolic extract of *A. satureioides* (≤ 50 mg / mL) was not cytotoxic to gingival fibroblasts, in the periods of 5 min and 24 h of contact.
- The concentration of 6.25 mg / mL of the glycolic extract of *A. satureioides* did not induce an increase in micronucleus production in the two periods tested, with no genotoxicity.
- The concentration of 6.25 mg / mL of the glycolic extract of *A. satureioides* is indicated to be used as component of mouthwashes, toothpastes, or as endodontic irrigant and intracanal medication bringing benefits to the population.

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