DANIELLY DAVI CORREIA LIMA

Avaliação *in vitro* das atividades antibacteriana e citotóxica do extrato bruto e frações de *Euclea natalensis* frente aos agentes de infecções bucais

In vitro evaluation of the antibacterial and cytotoxic activities of the crude extract and fractions of Euclea natalensis against agents of oral infections

Dissertação apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do Título de Mestre em Odontologia na Área de Clínica Odontológica Integrada.

Uberlândia, 2021

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Uberlândia, 2021

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“Sonho que se sonha só é só um sonho, mas sonho que se sonha junto é realidade”

Raul Seixas

SUMÁRIO

RESUMO

RESUMO

O objetivo desse estudo foi avaliar as atividades antibacteriana e citotóxica do extrato bruto e frações obtidos das raízes de *Euclea natalensis* frente as bactérias causadoras da doença periodontal e cárie, bem como, identificar os compostos isolados. A concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) foram determinadas usando o ensaio de diluição em microplaca. A avaliação citotóxica do extrato e frações foram realizadas com células de fibroblastos humanos, através do ensaio do colorimétrico XTT e a elucidação dos compostos presentes na fração mais promissora foi determinada pela cromatografia líquida acoplada a espectrometria de massa (CLAE), por meio de uma análise qualitativa. Os resultados da CIM variaram de 25 a >400 μg / mL para o extrato e 1,56 a >400 μg / mL para as frações. As concentrações testadas para a avaliação citotóxica foram de 19,5 a 2.500 μg / mL, com IC_{50} entre 625 e 1250 μg / mL, e a presença de terpenoídes pentacíclicos e naftoquinonas foram identificados. O extrato e as frações apresentaram boa atividade antibacteriana frente bactérias periodontopatogênicas e cariogênica, podendo ser justificadas pelos compostos presentes e também apresentaram baixa citotoxicidade para células humanas. Esses dados são relevantes e podem ser um incentivo para novas pesquisas com essa espécie vegetal, o que pode contribuir para a descoberta de novos medicamentos fitoterápicos, a fim de ajudar na redução desses agravos bucais.

PALAVRAS-CHAVE: Cárie Dentária, Plantas Medicinais; Doença Periodontal; Naftoquinonas, Terpenoídes, *Euclea Natalensis*

ABSTRACT

ABSTRACT

The aim of this study was to evaluate the antibacterial and cytotoxic activities of the crude extract and fractions obtained from the roots of *Euclea natalensis A.D.C* against bacteria causing periodontal disease and caries, as well as to identify the isolated compounds**.** The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined using microplate dilution assay. The cytotoxic evaluation of the extract and fractions was performed with human fibroblast cells (GM07492A, lung fibroblasts) through the XTT colorimetric assay and the elucidation of the compounds present in the most promising fraction was determined by liquid chromatography coupled mass spectrometry (HPLC-ESI-MS/MS) by means of a qualitative analysis. The MIC results ranged from 25 to >400 μg / mL for the extract and 1.56 to >400 μg / mL for the fractions. The concentrations tested for cytotoxic evaluation were 19.5 to 2500 μg / mL, with IC_{50} between 625 and 1250 μg / mL.The presence of pentacyclic terpenoids and naphthoquinones were identified.The extract and fractions showed good antibacterial activity against periodontal pathogenic and cariogenic bacteria, which can be explained by the present compounds and showed low cytotoxicity to human cells. These data are relevant and encourage further research with this plant species, which can contribute to the discovery of new herbal medicines in order to help reduce these oral health problems.

KEYWORDS: Dental Caries, Periodontal Diseases, Medical Plants, Naphthoquinones, Terpenes, *Euclea Natalensis*

INTRODUÇÃO E REFERENCIAL TEÓRICO

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

As espécies vegetais são usadas para diversos fins, dentre eles, o fim medicinal é bastante utilizado sendo os primeiros relatos de seu uso em meados de 3.000 a.C (Mahomoodally, 2013; Perpétuo *et.al*, 2019). A medicina tradicional, portanto, é a forma mais antiga de tratar e curar doenças, foi desenvolvida por diferentes povos em todo mundo e sua prática consiste na aplicação dos conhecimentos populares dentro do contexto local a qual é inserido. As informações são repassadas de geração em geração devido aos bons resultados em cuidados relacionados à saúde (Tabuti, Lye & Dhillion, 2003; Abdullahi,2011; Tinitana *et. al*, 2016).

Os países em desenvolvimento tendem a sofrer mais com as desigualdades sociais o que torna evidente os problemas de saúde pública, pois o acesso fica restrito a uma pequena parcela da população (Oliveira & Artmann, 2009; Woolcock, 2018). Muitos dos problemas enfrentados são corriqueiros e recorrentes como a falta de acesso a vacinas e ao tratamento de infecções que resulta em um quadro persistente de várias doenças como febre amarela e tuberculose (Saliou , 2007; Fortes & Ribeiro, 2014). Outros como a cólera, hepatite e febre tifoide são devido à falta de água tratada, saneamento e condições básicas de higiene, com isso, a busca por tratamentos com plantas nativas de caráter medicinal é tido como principal e único recurso disponível e acessível, sendo essencial para sobrevivência de determinadas comunidades (Khan *et. al*, 2012; Wanderley *et. al*,2015; Oguntibeju, 2019).

No continente africano, devido a dificuldade de acesso a tratamento e recurso para adquirir medicamentos farmacêuticos, as plantas medicinais são utilizadas para tratar diversas doenças no sistema circulatório ao sistema neurológico e também como problemas relacionados a câncer, dores no geral, problemas nutricionais e até mesmo para problemas bucais (Conde *et. al*,2014; Martínez, Gómez & Sook Oh, 2017; Hlafa, Sibanda & Hompashe, 2019). A dor de origem bucal é algo prevalente na África, principalmente em regiões mais pobres devido à falta de clínicas odontológicas nessas áreas e a condição socioeconômica da população, tal sintomatologia influencia fortemente na qualidade dos indivíduos e mesmo que a causa dela não leve a morte diretamente por atingirem grande número de pessoas, a dor irá influenciar na saúde geral dos indivíduos acometidos (Ayo-Yusuf & Naidoo,2016; Abid *et. al*, 2016).

Os medicamentos fitoterápicos são utilizados para tratar diversas desordens bucais, pois muitos possuem efeitos anti-inflamatórios, antibacterianos, ajudando no controle da inflamação gengival e prevenção de cáries (Kumar *et. al*,2013). A doença periodontal e cárie são doenças de alta prevalência que necessitam da presença do biofilme dentário para inicializarem e prejudicar os dentes e tecidos adjacentes, esse biofilme é composto por diversos microrganismos que associado a fatores intrínsecos do indivíduo pode facilitar ou dificultar o desenvolvimento de tais doenças (Durant *et. al*, 2019).

Em Moçambique estimasse que das 5500 espécies vegetais encontradas cerca de 15% são utilizadas para fins medicinais, dentre eles, muitas são eficazes para dores e infecções bucais (Barbosa *et.al*, 2020), uma dessas plantas é a *Euclea Natalensis* A.D.C que é um arbusto de pequeno porte encontrada desde savanas a florestas, sendo as suas raízes comumente utilizadas para tratar doenças toráxicas, lesões cutâneas provocadas pela hanseníase e problemas bucais (Osthuizen & Lall, 2020). As raízes são secas, descascadas e esfregadas nos dentes e gengivas (Stander & Van Wyk,1991). Estudos atuais têm avaliado seu uso para lesões erosivas e abrasivas e também para sensibilidade dentinária (Sales- Peres *et. al*,2012; Sales- Peres *et. al*, 2016).

As raízes de *E. natalensis* são ricas substâncias químicas da classe das naftoquinonas que podem ajudar a explicar e justificar seu uso em diversas comunidades africanas, pois esse composto tem demostrado ação antifúngica, antibacteriana, anticancerígena, outras substâncias químicas da classe dos terpenoídes também já foram descritas (Weigenand *et. al*, 2004; More *et. al*,2008). Na literatura, ação antibacteriana contra ao *Mycobacterium Tuberculosis* e *Mycobacterium Bovis* é bem discutida (Lall & Meyer, 2001; Weigenand *et. al*, 2004; Lall *et. al*,2005; Van der Kooy, Meyer & Lall, 2006), entretanto, são escassos estudos correlacionando seu efeito antibacteriano frente as bactérias relacionadas a doença periodontal e a cárie (More *et. al*,2008). Diante da falta de estudos que justifiquem precisamente o uso de suas raízes para tratar e ajudar a prevenir a doença periodontal e a cárie, o objetivo desse trabalho foi avaliar o efeito antibacteriano, efeito citotóxico do extrato bruto e frações de *E.natalensis* em células humanas e caracterizar sua composição química.

CAPÍTULO I

2. CAPÍTULO 1

ARTIGO 1

In vitro evaluation of the antibacterial and cytotoxic activities of the crude extract and fractions of *Euclea natalensis* against agents of oral infections.

***Artigo a ser enviado para o periódico "ARCHIVES ORAL BIOLOGY"**

In vitro **evaluation of the antibacterial and cytotoxic activities of the crude extract and fractions of** ***Euclea natalensis*** **against agents of oral infections.**

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***In vitro* evaluation of the antibacterial and cytotoxic activities of the crude extract and fractions of *Euclea natalensis* against agents of oral infections**

ABSTRACT

Objective: The aim of this study was to evaluate the antibacterial and cytotoxic activities of the crude extract and fractions obtained from the roots of *Euclea natalensis A.D.C* against bacteria causing periodontal disease and caries, as well as to identify the isolated compounds**. Design:** The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined using microplate dilution assay. The cytotoxic evaluation of the extract and fractions was performed with human fibroblast cells (GM07492A, lung fibroblasts) through the XTT colorimetric assay and the elucidation of the compounds present in the most promising fraction was determined by liquid chromatography coupled mass spectrometry (HPLC-ESI-MS/MS) by means of a qualitative analysis. **Results:** The MIC results ranged from 25 to >400 µg / mL for the extract and 1.56 to >400 µg / mL for the fractions. The concentrations tested for cytotoxic evaluation were 19.5 to 2500 µg / mL, with IC_{50} between 625 and 1250 µg / mL.The presence of pentacyclic terpenoids and naphthoquinones were identified. **Conclusions:** The extract and fractions showed good antibacterial activity against periodontal pathogenic and cariogenic bacteria, which can be explained by the present compounds and showed low cytotoxicity to human cells. These data are relevant and encourage further research with this plant species, which can contribute to the discovery of new herbal medicines in order to help reduce these oral health problems.

KEYWORDS: Antibacterial Activity, Cytotoxic Activity, Medical Plants, Oral Microorganisms, *Euclea Natalensis*

INTRODUCTION

Periodontal disease and caries are highly prevalent oral health problems, being the main causes of tooth loss, strongly interfering in the quality of life and self-esteem of affected patients and contributing to the development of chronic diseases (PETERSEN & OGAWA et al., 2012; CHAPPLE et.al, 2017). These diseases are biofilm dependent, multifactorial and several bacteria participate in the process of installation and progression of these diseases, such as: *Actinomyces naeslundii, Porphyromonas gingivalis, Streptococcus sanguinis, Streptococcus oralis* , *Streptococcus intermedius , Streptococcus gordonii , Fusobacterium nucleatum, Tannerella forsythia* (HONG et al. ., 2015) as causal factors for periodontitis and *Streptococcus mutans, Streptococcus sobrinus Lactobacillus salivarius , Lactobacillus paracasei* and *Bifidobacterium dentium,* related to the process of initiation and formation of caries (SELWITZ et al., 2007; HENNE et al., 2015; SANZ et al., 2017). Some of these microorganisms are also related to other non-odontogenic infections, such as pulmonary actinomycosis (HUANG et al., 2021), bacterial endocarditis (NOMURA et al., 2013; HOLLAND et al., 2016) and cancer-related events such as progression and growth of breast tumors (PARHI et.al, 2020) and increased predisposition to head and neck cancer (MALINOWSKI et al., 2019; BRONZATO et al., 2020).

The prevention of periodontal disease and caries essentially consists in the daily removal of biofilm from the tooth through mechanical and chemical methods, the first being pointed out as the most effective and consisting in the association of floss with a toothbrush (FIGUERO et al, 2017). The chemical method is complementary to the mechanical method and has an antiseptic action conferred by its active principles (chlorhexidine gluconate, triclosan and cetylpyridinium), which promote an effective

reduction of bacterial plaque. On the other hand, plaque control through the chemical method can cause an imbalance of the host's microbiota if used continuously and for a long time, causing mouth sores, decreased salivary flow, tooth staining and taste changes (GUNSOLLEY, 2010; BEDOUX et al., 2012; RILEY & LAMOT, 2013; JAMES et al., 2017; DA COSTA et al., 2017). In order to avoid the side effects caused by chemical agents and assist in the prevention and treatment of periodontal disease and caries, the use of medicinal plants in oral health has been studied (KHALID et al., 2017; TANIDEH et al., 2019).

In Africa in particular, several plants have been used as sources of products with nutritional and therapeutic values, being an alternative for the cure and prevention of numerous diseases, contributing to the reduction of mortality, morbidity, disability and the control of oral infections (CONDE et al., 2014), although the mechanisms of action, active principles and cytotoxicity characteristics of most plant species are not yet fully understood (AGBOR & NAIDOO, 2019).

One of these plants is *Euclea natalensis A.D.C* popularly known as “Mulala”, from the Ebenaceae family, which is very common in tropical and subtropical regions of Africa (LALL et al; 2016). Its roots stem and leaves are used for various medicinal purposes (STANDER & VAN, 1991; MCGRAW et al., 1997; SALESPEZES et al., 2016; MAROYI, 2017). Its use in the treatment of oral diseases is preferably through the roots, which are peeled, chewed and rubbed on teeth and gums, helping to prevent periodontal disease, caries and dentinal hypersensitivity (STANDER & VAN WYK, 1991; MORE et. al, 2008; SALES-PERES et al., 2016; AGBOR & NAIDOO, 2019).

Previous studies isolated some chemical components of *E. natalensis* and found naphthoquinones and terpenoids, such as betulin, lupeol, diospirin, neodiospirin, 7

- methyljuglone (VAN DER KOOY, MEYER & LALL, 2006) and were correlated with their antibacterial properties (LALL & MEYER,2000), antifungal, (LALL et al.,2006), antimalarial (NGARIVHUME et al,2015) anticarciogenic (KISHORE et al, 2014), among others (OTIENO et al.,2008; CHAUKE et al.,2015). However, studies evaluating the use of its roots specifically for periodontal disease and caries pathogens (STANDER & VAN WYK, 1991; MORE et. al, 2008) as well as its cytotoxicity in human cells are still scarce in the literature (LALL et al., 2005; LALL, MEYER & TAYLOR, 2005).

Therefore, we evaluated the antibacterial effect of the crude extract and fractions of *E. natalensis* against the main aggressors of periodontal disease and caries, as well as analyzing its cytotoxic effect on human normal cells and characterizing the chemical composition. The hypothesis of this work is that the extracts obtained from this plant will be effective in causing the death or inhibiting the growth of most oral bacteria used without being cytotoxic.

MATERIAL AND METHOD

Collection and Authentication

The roots of *E. natalensis* were collected by the researcher Tássio Edno Atanásio Pitorro in Southern Mozambique, Inhambane Province, Zavala District (Latitude: 24°31'19"S/ Longitude:34°58'36"E) and transported to Brazil under an authorization issued by the Ministry of Agriculture and Food Security of Mozambique, through the phytosanitary certificate No. 131PIF/2020. A sample was identified by biologist Rodrigo Rodrigues Franco and deposited at the Herbarium of the Federal University of Uberlândia (HUFU) in Uberlândia, Minas Gerais, Brazil under voucher number HUFU79,555. The plant roots were dried, crushed and frozen at -20°C until the extraction and fractionation processes were carried out.

Process for obtaining the Extract and Fractions

For the extraction process, 500g of dry plant material was used, applying the adapted method of static maceration, that is, without agitation (FRANCO et al., 2020). The material remained immersed in 2.5 L of ethanol (1:5 m/v ratio) for six days. After this period, the solution with extractives was filtered and the solvent removed in a rotaevaporator under reduced pressure at 35º C. Then, the residue of plant material was macerated twice more, using the solvent recovered in the rotaevaporation. For fractionation, 10 g of ethanolic extract solubilized in 50 mL of methanol:water (9:1) solution were used. Then, with the aid of a bromine funnel, the fractionation of the extract was carried out using increasing polarity solvents: hexane (HEX), dichloromethane (DCM), ethyl acetate (AcOEt), n-butanol (ButOH) and water (H_20). For this process, three extractions were performed with 50 mL of each solvent. The fractions solvents were totally removed by rotaevaporation with reduced pressure at 35ºC, lyophilized and stored at -20ºC.

Antibacterial Activity

Bacteria Used in Tests

The bacteria used in this study were obtained from the American Type Culture Collection (ATCC) and are related to periodontal disease: *F. nucleatum* (ATCC 25586), *Actinomyces naeslundii* (ATCC 19039), *Actinomyces viscosus* (ATCC 43146), *Porphyromonas endodontalis* (ATCC 35406) *P. gingivalis* (ATCC 33277) and *Prevotella intermediate* (ATCC 15033). Related to caries disease: *Streptococcus salivarius* (ATCC 25975), *S. mutans* (ATCC 25175), *S. mitis* (ATCC 49456), *S. sanguinis* (ATCC 10556),

S. oralis (ATCC 55229), *Streptococcus sobrinus* (ATCC 33478) *Enterococcus faecalis* (ATCC 4082) and *L.paracasei* (ATCC 11578). The bacteria are kept in the Laboratory of Antimicrobial Assays (LEA) of the Federal University of Uberlândia, under freezing at -20°C in an 80% glycerol solution.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum inhibitory concentration (MIC) is defined as the lowest sample concentration that can inhibit bacterial growth. The experiment was performed in 96-well microplates and repeated three times.

1.0 mg of crude extract and fractions solubilized in 32 µL of dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) and diluted with 593 µL in brain heart infusion broth (BHI, Difco, Detroit, MI, United States) were used for the aerobic bacteria. Brucella broth (Difco) supplemented with hemin (5mg/mL, Sigma, St. Louis, MO, USA) and menadione (1mg/mL, Sigma) was used to dilute the extract and fractions for anaerobic bacteria. The tested concentrations ranged from 0.195 to 400 µg / mL and the inocula were adjusted to a cell concentration of 5 × 105 CFU / mL for aerobic bacteria (CLSI, 2012) and 1 × 106 CFU/mL for anaerobic bacteria (CLSI, 2007). 5% DMSO (v/v) was used as a negative control, and chlorhexidine and metronidazole (Sigma) were used as positive controls for aerobic and anaerobic bacteria, respectively. An inoculum was included to monitor bacterial growth. The 96-well microplates for aerobic bacteria were incubated at 37 °C for 24hrs, whereas the microplates containing the anaerobic microorganisms were incubated at 36 °C for 72 h in an anaerobic chamber containing 5–10% H2, 10% CO2 and 80-85% N2 (Don Whitley Scientific, Bradford, UK). After incubation, 30 µL of an aqueous solution of resazurin (Sigma) 0.02% was

added to each well. Resazurin is an oxido-reducing microbial growth to be observed, the blue and red colors represent the absence and presence of microbial growth (SARKER et al., 2007).

The Minimum Bactericidal Concentration (MBC) was defined as the lowest concentration of the sample in which no bacterial growth occurred. MBC was tested for all bacteria investigated in order to assess whether the crude extract and fractions have bactericidal effects or bacteriostatic action. To determine MBC, a 10 μL aliquot of the inoculum was removed from each well before resazurin (Sigma) was added and plated on blood agar supplemented with 5% defibrinated horse blood for aerobic strains. For anaerobic bacteria, Schaedler agar (Difco) was used, supplemented with hemin, menadione and 5% defibrinated horse blood.

Cytotoxicity Assessment

Cell Lineage and Cultivation Conditions

A normal human fibroblast strain (GM07492A) was used. The cells were stored in liquid nitrogen (-195°C), in aliquots of 1 x 106 cells/mL in a freezing solution composed of 50% of culture medium (HAM F10 + DMEM, in the proportion 1:1, Sigma-Aldrich), 40% fetal bovine serum (Nutricell, Campinas, São Paulo, Brazil) and 10% dimethylsulfoxide (Sigma-Aldrich).

To carry out the experiments, the cells were thawed and placed in culture in a 1:1 HAM F10 + DMEM culture medium (Sigma-Aldrich, St Louis, MO, USA). The culture medium was supplemented with 10% fetal bovine serum, 1.2 g/ml sodium bicarbonate (Sigma-Aldrich), 0.1 g/ml streptomycin (Sigma-Aldrich) and 0.06 g/ml penicillin (Sigma-Aldrich). Then, they were cultivated until the 4th passage in monolayer

with 10 mL of culture medium using 25 cm2 disposable flasks (Corning, Corning, New York, USA) at 36.5°C in a CO2 oven (Sanyo, Osaka, Osaka, Japan). Every two or three days, the cells were subcultured using PBS (Phosphate Buffered Saline) to wash them and trypsin (10x Solution, Sigma-Aldrich, St Louis, MO, USA) at ratio of 1 trypsin: 10 PBS, to detach cells from the inner surface of the culture flask. After cell detachment 1.5 ml of complete culture medium (supplemented with 10% fetal bovine serum) was added to the flask for trypsin-versene (ATV) inactivation and homogenized. Then, 200 µL of cells were cultured in new flasks containing 10 mL of complete culture medium, and incubated at 36.5°C.

Cytotoxic Activity

The assessment of cytotoxicity was performed using the in vitro toxicology colorimetric assay - Kit XTT (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's guidelines. To carry out the experiments, 1x104 cells were seeded in microplates containing 96 wells, each well of which received a maximum of 100 µL of culture medium (HAM-F10 + DMEM, 1:1) supplemented with 10% fetal bovine serum containing different concentrations of the samples, which ranged from 19.53 to 2500 µg/mL. Wells for negative (untreated), solvent (DMSO 1%) and positive control (doxorubicin hydrochloride, Bergamo, São Paulo, Brazil) were included. After incubation with the treatments at 36.5°C for 24 hours, the culture medium was removed and the cells were washed with 100 µL of PBS to remove the treatments and exposed to 100 µL of HAM-F10 culture medium without red de phenol (Sigma-Aldrich). Then, 25 µL of XTT {sodium 3'-[1-(phenylaminocarbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} was added to each well. at 36.5ºC for 17 hours. The absorbance of

the samples was determined by means of a multi-plate reader (ELISA – Asys – UVM 340/ MikroWin 2000) at a wavelength of 450 nm and a reference length of 620 nm. For data analysis were performed ANOVA variance test followed by Tukey test with 5% significance level ($p<0.05$) by GraphPad Prism program in order to calculate the sample concentration that inhibits 50% of cell growth (IC_{50}) (SILVA et. al, 2019).

Selectivity Index

The selectivity index (SI) is used to verify whether the extract and tested fractions have more affinity for bacteria than for human cells, being calculated for the extract and fractions in which their concentrations reduced cell viability by 50% through IC_{50} / MIC ratio. Results greater than 10 (SI >10) are considered promising because they are more active against microorganisms than against human normal cells (PAVAN et al., 2010; SILVA et.al, 2019).

Analysis by Liquid Chromatography and Mass Spectrometry

Analysis by liquid chromatography and mass spectrometry was performed on the fraction that presented the best results in the antibacterial and cytotoxic assay. The compounds present in this fraction were identified by high performance liquid chromatography coupled to an electrospray ionization mass spectrometer (ESI, "Electrospray Ionization") MeOH-H2O (4:1) (HPLC-ESI-MS/MS, Agilent Q -TOF, model 6520), a solvent system was used and the samples were infused in the ESI source at a flow rate of 200 μL/h. Nitrogen gas was used as a drying gas at a rate of 8 L/min and also as a nebulizer gas at 58 psi. The nebulizer temperature was set at 200 °C and a potential of 4.5 kV was applied to the capillary tube. The impact energy on the electron

was fixed at 20, 25 and 30 eV. The HPLC parameters were: Agilent Zorbax model 50×2.1mm column, 1.8 µm particles and 110 Å pore diameter, mobile phase: water acidified with formic acid (0.1 % v/v) (A) and methanol (B). Gradient solvent system (B) was: 2% (0 min); 98% (0-15 min); 100% (15-17 min); 2% (17-18 min); 2% (18-22 min), with a flow of 0.35 ml/min and UV detection of 280 and 360 nm. Data were acquired in negative and positive modes, with adjustment for a range of 20-1000 *m/z*. In mass spectrometry, the data were evaluated using the Agilent Mass Hunter B.07.00 software, the molecular ions and their fragments were compared with the results of other studies in the literature (FRANCO et al., 2020).

RESULTS

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The values obtained for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the crude extract of *E. natalensis* against anaerobic bacteria causing periodontal infections ranged from 25 to >400 µg/mL and from 200 to >400 µg/ mL, respectively **(Table 1).** Regarding caries bacteria, MIC ranged from 100 to >400 µg/mL and MBC from 200 to >400 µg/mL **(Table 2).**

The MIC related to periodontal disease bacteria that showed the best results were: *P. gingivalis* (25 µg/mL), *P. endodontalis* (25 µg/mL), *P. intermedia* (100 µg/mL). For MBC, the effect was bacteriostatic for the extract in all bacterial strains studied **(Table 1).** For caries bacteria only *S. mitis* showed a more promising result with a MIC of 100 µg/mL. The effect of MBC was bactericidal for *S. salivarius, E. faecalis, S. mutans, S.oralis, S. sobrinus* and *L. paracasei* and bacteriostatic for *S. mitis* and *S. sanguinis* **(Table 2).**

The values obtained from MIC and MBC for *E. natalensis* fractions against anaerobic bacteria causing periodontal infections ranged from 1.56 to >400 μg/mL and 3.12 to >400 μg/mL, respectively **(Table 1).** Regarding caries bacteria, MIC ranged from 25 to >400 μg/mL and MBC 25 to >400 μg/mL **(Table 2).**

Among the hexane and dichloromethane fractions were the ones that showed the best results for MIC both for periodontal and cariogenic bacteria. The promising results of MIC for HEX fraction against bacterial strains of periodontal disease were*: A. naeslundii* (6.25 μg/mL), *P. gingivalis* (12.5 μg/mL), *P. endodontalis* (12.5 μg/mL) and *P. intermedia* (25 μg/mL) exhibiting bacteriostatic effect for all of these **(Table 1).** And for caries causing bacteria were: *S. mitis* (25 μg/mL), *S. mutans* (50 μg/mL) exhibiting bacteriostatic effect, and *S. sanguinis* (25 μg/mL) and *S. sobrinus* (100 μg/mL) with bactericidal effect **(Table 2).**

For the DCM fraction, the best results found in the MIC were: *P. intermedia* (1.56 μg/mL), *P. endodontalis* (6.25 μg/mL), *P. gingivalis* (12.5 μg/mL) with bacteriostatic effect **(Table 1)** and for *S. sanguinis* a MIC of 25 μg/mL*, S. mutans* 100 μg/mL with a bacteriostatic effect and *S. mitis* with a MIC of 100 μg/mL with a bactericidal effect **(Table 2).**

Cytotoxic Evaluation

In the evaluation of cytotoxicity with human fibroblast cells, the concentrations used ranged from 19.5 μg/mL to 2500 μg/mL as described in **Table 3**. The value of the concentration needed to reduce cell activity by half was between 625 μg/mL and 1250 μg /mL. The crude extract had an IC_{50} of 841.6 ± 52.8 μg/mL and the fractions

had their IC_{50} between 681.8 to >2500 µg/mL. The HEX fraction had an IC_{50} of 681.8 ± 54.5 µg/mL and DCM 1030.3 ± 56.3 µg /mL **(Table 3).**

The crude extract showed satisfactory cell viability, but HEX was the fraction that presented the lowest IC_{50} value among all those evaluated, being, therefore, the least cytotoxic fraction followed by the DCM fraction.

Selectivity Index (SI)

The SI was calculated for the crude extract and the HEX and DCM fractions based on the result of the cytotoxic activity. Among the bacteria studied, the crude extract presented an SI of 33.7 for *P. gingivalis* and *P. endodontalis*. The HEX fraction showed results higher than 10 for the following bacteria: *A. naeslundii* (109.1), *P.gingivalis* (54.5), *P.endodontalis* (54.5) *P.intermedia* (27.3), *S. mutans* (13.6), *S. mitis* (27.3) and *S. sanguinis* (27.3). The DCM fraction also exhibited selectivity with SI of 82.4 for *P.gingivalis*, 164.8 for *P.endodontalis*, 660.4 for *P.intermedia*, 10.3 for *S. mutans* and *S. mitis* and 41.2 for *S. sanguinis* **(Table 4).**

Analysis by Liquid Chromatography coupled with Mass Spectrometry

In order to understand the promising results of *E. natelensis* demonstrated in the studied methodologies, an analysis of liquid chromatography coupled to mass spectrometry was performed on the DCM fraction. The bioactive compounds found were: betulin, lupeol, euclanone, euclein, methylnaphthazarin, natalenone, ramentaceone, rossoliside, shinanolone and their molecular masses are described in **Table 5**.

DISCUSSION

The use of medicinal plants and their derivatives in oral health has been heavily studied, in order to prevent and help solve problems with products that have low toxicity without causing secondary damage to mucous membranes and teeth (FREIRES et al., 2016; VELOSO et al., 2020). The hypothesis of this study was accepted, as it was shown that the roots of *E. natalensis* had good antibacterial activity with low cytotoxicity to human cells.

According to Rios & Recio (2005) antibacterial assays with plant extracts are considered promising when they present results below 100 μg/mL, this parameter was used to discuss the results found in this work, thus, it is possible to state that the crude extract of the roots of *E. natalensis* showed a promising antibacterial activity against *P. endodontalis, P. intermedia*, *P. gingivalis* and *S. mitis* strains, as they present a MIC equal to or less than 100 μg/mL.

The antimicrobial activity of the crude extract obtained from *E. natalensis* leaves was evaluated by More et al. (2008) against oral pathogens and presented a MIC of 25,000 μg/mL for *A.naeslundi* (ATCC 19039) and 630 μg/mL for *P. gingivalis* (ATCC 33277), *P. intermedia* (ATCC 25611) and *S. mutans* (ATCC 25175). The discrepancy in the values found for the same strains evaluated in our study can be explained by the difference in the part of the plant chosen to obtain the crude extract.

Regarding the fractions, each one exhibited an antibacterial behavior, however, only the HEX and DCM fractions were those that presented a MIC < 100 μg/mL for at least three different strains related to periodontal disease and caries evaluated in this study. The lowest MIC value found among the fractions was 1.56 μg/mL in the DCM

for *P. intermedia*, which is the first study to evaluate this effect against these bacteria and fractions.

Lall & Meyer (2000) evaluated the effect of the aqueous fraction obtained from *E.natalensis* roots against several microorganisms related to respiratory infections, where the Gram-negative bacteria tested did not show antibacterial activity and the Gram-positive ones had a MIC varying from 500 μg/mL to 600 μg/mL, in the current study correlating the results for the same fraction but for other pathogens with the lowest MIC value found for the Gram-negative bacteria *P. intermedia* with a value of 50 μg/mL and for Gram- positive *S. mitis* with 200 μg/mL.

Regarding MBC, neither the crude extract nor the fractions showed a bactericidal effect at a concentration lower than 100 μg/mL for periodontal disease pathogens, this effect was verified only in cariogenic pathogens and for HEX fractions for *S. sanguinis* with MBC of 25 μg/mL and *S. sobrinus* at 100 μg/mL and for AcOEt for *S. mitis* at a concentration of 50 μg/mL, no studies were found that have evaluated MBC precisely for *E. natelensis*, however, Mbanga (2013) evaluated the extract of other species of the Ebenaceae family obtained from trunks and roots against *S.mutans* among them only *E.undulata* exhibited this bactericidal effect at a concentration of 2424.00 μg/mL for the methanolic fraction.

The terpenoids and naphthoquinones found in HPLC-ESI-MS/MS carried out in the DCM fraction are chemical compounds with several pharmacological activities, among them the antibacterial activity is highlighted against several Gram-negative and Gram-positive bacteria (SIDDIQUE & SALEEM, 2011; AMIRI et al., 2019; AHMADI et al., 2020). Weigenand et al. (2004) also found the compounds: lupeol, betulin and shinanolone from the crude ethanol extract of *E. natalensis* roots, the last compound

described being the one with the best antibacterial activity against *Mycobacterium tuberculosis*. Lall et al. (2005) using a similar methodology found lupeol, betulin, diospirin and ramentaceone (7-methyljuglone) which was the compound with the most promising antibacterial activity for *M. tuberculosis* among those evaluated, the substances described are in agreement with those found in the present work. The crude chloroform extract of this same plant was obtained and analyzed by Van Der Kooy, Meyer & Lall, (2006) who found diospirin, isodiospirin, neodyspirin, mamegaquinone, shinanolone corroborating the compounds found for the same extract by McGaw et al. (2008).

For the cytotoxicity assay, the crude extract and the HEX and DCM fractions were the ones that showed statistical difference with IC_{50} values between 625 μg/mL to 1250 μg/mL, confirming its low cytotoxic activity, since, in this work, the highest concentration used in the antibacterial assay it was 400 μg/mL and most bacteria were inhibited at a lower concentration. Lall et al. (2005) evaluated the IC_{50} of the crude extract obtained from *E. natalensis* roots in Vero-type cells and obtained 64.87 μg/mL, in another study More et al. (2008) evaluated the IC_{50} for the same cell line with the crude extract obtained from *E. natalensis* leaves and obtained an IC_{50} of 285.1 μg/mL, the divergences between the results found in this work for the crude extract can be justified by the origin of the extract obtained is not only from roots, by the solvent used in the process of obtaining the extract and by the cell line analyzed.

The results obtained by SI demonstrate the effective potential of the extract and fractions on pathogens in relation to cytotoxicity, that is, the higher the index, the greater the affinity for the pathogen and the lower the toxicity to human cells, ensuring its safe use in the development of possible drugs (PAVAN et. al, 2010). In our study, the crude extract presented an SI of 33.7 for *P.gingivalis* and *P.endodontalis* against human

fibroblast cells, the HEX and DCM fractions had an SI > 10 for most anaerobic and aerobic bacteria. Lall et al. (2005) evaluated the SI against the Vero cell lineage and mouse macrophages against *Mycobacterium tubercolosis* and obtained results above 10 only for the substance 7-methyljuglone (IS=30.2), the good selectivity of this compound may help to clarify the good results found in the present study for the fraction of DCM.

Although the study of the effect of *E. natalensis* specifically for periodontal disease are few described in the literature and for caries no study has been found, the application of its crude extract and some of the fractions for microorganisms related to these diseases showed promising results, which may legitimize the use of its roots for inflammation of teeth and oral tissues in some regions of the African continent (TSHIKALANGE et al., 2016), and other studies with other methodologies to validate and confirm the possibility of these compounds being used in the development of herbal medicines applied to dentistry are fundamental.

CONCLUSION

Within the limitations of this study, it is possible to conclude that the extract and fractions obtained from *E. natalensis* roots present promising results for most bacteria evaluated. The concentrations tested in MIC/ MBC are well below that necessary to maintain 50% of cell viability in human fibroblasts, in addition, the extract and fractions of HEX and DCM are more selective for pathogens than for human cells. The promising results may be correlated with the presence of terpenoids and naphthoquinones, these results legitimize the use of *Euclea natalensis* roots for oral hygiene purposes in some regions of the African continent.

TABLE 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract and fractions obtained from *Euclea natalensis* against bacteria related periodontal disease

ANAEROBIC BACTERIA	CRUDE EXTRACT (µg/mL)		FRACTIONS (µg/mL)										CONTROL (µg/mL)	
			HEX		DCM		AcOEt		ButOH		H_2O		Metronidazole	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
F. nucleatum (ATCC 25586)	-	-	400	400	400	400	400	400	-	-	-	-	NT	NT
A. naeslundii (ATCC 19039)	400	-	6.25	12.5	400	400	-	-	-	-	400	-	NT	NT
A. viscosus (ATCC43146)	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT
P. gingivalis (ATCC 33277)	25	-	12.5	100	12.5	400	12.5	-	-	-	-	-	NT	NT
P. endodontalis (ATCC 35406)	25	-	12.5	400	6.25	-	50	-	50	-	-	-	NT	NT
P. intermedia (ATCC 15033)	100	200	25	50	1.56	3.12	-	-	-	-	50	100	NT	NT
*Bacteroides Fragilis**	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	1.48	1.48
*Bacteroides thetaiotaomicron**	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	2.95	2.95

NT: Not tested; * Control strains; - : >400 was considered inactive; HEX, Hexane; DCM, Dichloromethane; AcOEt, Ethyl Acetate; ButOH, Butanol; H_20, Water.

TABLE 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract and fractions obtained from *Euclea natalensis* against bacteria related to dental caries.

CARIES BACTERIA	CRUDE EXTRACT (µg/mL)		FRACTIONS (µg/mL)										CONTROL (µg/mL)	
			HEX		DCM		AcOEt		ButOH		H_20		Chlorhexidine	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. salivarius (ATCC 25975)	400	-	200	200	200	200	200	200	-	-	-	-	0.92	0.92
S. mutans (ATCC 25175)	400	400	50	100	100	200	200	400	-	-	-	-	0.92	0.92
S. mitis (ATCC 49456)	100	200	25	200	100	100	50	50	200	200	200	400	3.69	3.69
S. sanguinis (ATCC 10556)	200	400	25	25	25	50	-	-	-	-	-	-	1.84	1.84
S. oralis (ATCC 55229)	-	-	-	-	400	-	400	400	-	-	-	-	7.38	7.38
S. sobrinus (ATCC33478)	400	400	100	100	200	-	200	400	400	-	400	400	1.84	1.84
E. faecalis (ATCC 4082)	-	-	-	-	-	-	-	-	-	-	-	-	7.38	7.38
L. paracasei (ATCC 11578)	400	400	400	400	400	-	200	400	400	-	400	-	0.92	0.92

- : >400 was considered inactive; HEX, Hexane; DCM, Dichloromethane; AcOEt, Ethyl Acetate; ButH20OH, Butanol; H_20, Water.

TABLE 3: IC_{50} values obtained against strain GM07492A after 24 hours of treatment with different concentrations (19.53 – 2500 µg/mL) of *E. natalensis* and its fractions.

Sample	Cell lineage
	GM07492A
Crude extract	841.6 ± 52.8*
Hexane	681.8 ± 54.5*
Dichloromethane	1030.3 ± 56.3*
Ethyl Acetate	> 2500
Butanol	> 2500
Water	> 2500
DXR	20.0 ± 0.8

DXR – doxorubicin hydrochloride; dimethylsulfoxide (1%); GM07492A - Human Fibroblast. * Indicates statistical difference between groups ($p < 0.05$).

TABLE 4: Determination of the Selectivity Index (SI) of the extract and fractions of *E. natalensis* for caries pathogens and periodontal disease.

BACTÉRIA ANAEROBIES	SELECTIVITY INDEX			**BACTÉRIA CÁRIES**	SELECTIVITY INDEX		
	CRUDE EXTRACT	**HEX**	**DCM**		**CRUDE EXTRACT**	**HEX**	**DCM**
F. nucleatum (ATCC 25586)	-	1.7	2.6	*S. salivarius* (ATCC 25975)	2.1	3.4	5.2
A. naeslundii (ATCC 19039)	2.1	109.1	2.6	*S. mutans* (ATCC 25175)	2.1	13.6	10.3
A. viscosus (ATCC43146)	-	-	-	*S. mitis* (ATCC 49456)	8.4	27.3	10.3
P. gingivalis (ATCC 33277)	33.7	54.5	82.4	*S. sanguinis* (ATCC 10556)	4.2	27.3	41.2
P. endodontalis (ATCC 35406)	33.7	54.5	164.8	*S. oralis* (ATCC 55229)	-	-	2.6
P. intermedia (ATCC 15033)	8.4	27.3	660.4	*S. sobrinus* (ATCC33478)	2.1	6.82	5.2
				E. faecalis (ATCC 4082)	-	-	-
				L. paracasei (ATCC 11578)	2.1	1.7	2.6

- : not calculated; HEX, hexane; DCM, dichloromethane.

TABLE 5: Composition of the dichloromethane fraction of the crude extract of *E. natalensis* roots by HPLC-ESI-MS/MS (positive mode at 5,10,15,20,25 and 30 eV)

Suggested Compounds	Retention Time (min)	Formula	Calculated Mass $[M-H]^-$	*m/z* of $[M+H]^+$	Erro (ppm)	*m/z* for fragments of [M-H]-	References
Pentacyclic terpenoids							
Betulin	5.900	$C_{30}H_{51}O_2^+$	443.1329	443.1322	1.57	412, 288, 248, 207	Jumpantong et al., 2007; Adnan Ibrahin &Yaacob, 2020; Njanpa et al.,2021
Lupeol	14.978	$C_{30}H_{51}O^+$	427.3794	427.3786	1.87	409, 357, 203, 139	Pereira, 2016; Njanpa et al.,2021
Naphthoquinones							
Euclanone	13.006	$C_{22}H_{15}O_7^+$	391.2862	391.2856	1.53	361, 335, 308, 150	Ferreira, 1977; Ibrahim et al.,2020
Euclein	11.612	$C_{22}H_{15}O_6^+$	375.2529	375.2515	3.73	347, 329, 178, 97	Weigenand et al,2004.
Methynaphthazarine	5.871	$C_{11}H_9O_4^+$	205.0845	205.0839	2.92	187, 175, 160, 89	Ferreira, 1977.
Natalenona	8.999	$C_{22}H_{17}O_6^+$	377.1021	377.1033	3.18	359, 319, 239, 188	King et al, 1976; Ferreira, 1977.
Ramentaceone	8.048	$C_{11}H_9O_3^+$	189.0554	189.0550	2.11	174, 161, 97, 84	Mebe; Cordell; Pezzuto, 1998; Kawiak et al., 2012; Kawiak & Lojkowska,2016;
Rossoliside	12.152	$C_{17}H_{20}O_8^+$	353.2685	353.2675	2.83	335, 320, 313, 180	Budzianowski, 1995; Budzianowski 1996
Shinanolone	0.490	$C_{11}H_{13}O_3^+$	193.0857	193.0848	4.66	175, 160, 147, 98	Aung et al, 2002, Lall, Weiganand & Meyer, 2006.

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CONCLUSÃO

3. CONCLUSÃO

As seguintes conclusões podem ser obtidas:

1. O uso de plantas medicinais para tratamento de diversas doenças é uma das práticas mais antigas que irá seguir as crenças de cada região e grupo étnico, sendo transmitidas de geração em geração.
2. Países em desenvolvimento devido a problemas socias e econômicos possuem acesso limitado a médicos e medicamentos farmacêuticos sendo os medicamentos fitoterápicos muitas vezes o único recurso terapêutico disponível.
3. *Euclea Natalensis* apresentou boa atividade antibacteriana frente as bactérias periodontopatogênicas e cariogênicas, com baixa citotoxicidade para células humanas.
4. A presença de naftoquinonas e terpenoídes pentaciclícos podem justificar seu bom comportamento antibacteriano e citotóxico.

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* De acordo com a Norma da FOUFU, baseado nas Normas de Vancouver. Abreviaturas dos periódicos com conformidade com Medline (Pubmed).

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