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Pâmella Buenos Aires Domingues

**Estudo das propriedades antimicrobianas de *Caryocar villosum* (Aubl.)
Pers. sobre bactérias cariogênicas**

Governador Valadares

2021

Pâmella Buenos Aires Domingues

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Dissertação apresentada ao Programa de Pós Graduação Ciências Aplicadas à Saúde, da Universidade Federal de Juiz de Fora, Campus Governador Valadares, como requisito parcial à obtenção do título de Mestre em Ciências Aplicadas à Saúde, área de concentração Biociências.

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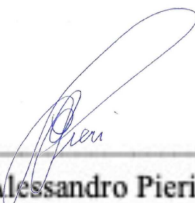
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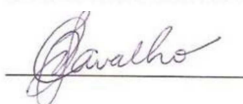
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RESUMO

A cárie dentária é um problema de saúde pública, que está associada ao aumento de *Streptococcus mutans* e *Lactobacillus* acidogênicos, envolvidos no início e progressão das lesões, respectivamente. Os antimicrobianos padrão-ouro no combate a essas bactérias vêm apresentando efeitos adversos que incentivam o estabelecimento de estudos para identificação de substâncias alternativas para a prevenção da doença. O objetivo deste trabalho foi avaliar o potencial antimicrobiano de extratos de *Caryocar villosum* contra bactérias responsáveis pela doença cárie, *Streptococcus mutans* e *Lactobacillus sakei*. Extratos de polpa, semente e casca de *C. villosum*, obtidos com diferentes solventes (etanol, hidroalcolico, hexano e metanol) em concentrações que variaram de 5 a 0,009 mg/mL, foram testados. Para determinar a Concentração Inibitória Mínima (CIM), foi adicionado à placa de 96 orifícios 50 µL das soluções dos extratos ou clorexidina em diferentes concentrações juntamente com 50 µL de cultura bacteriana. Como controle negativo foi utilizado Caldo Infusão Cérebro e Coração (BHI) estéril ao invés da solução dos extratos. Após incubação a 37° C por 48 horas, foi acrescentado resazurina para avaliar a viabilidade celular. A natureza da atividade antimicrobiana, foi avaliada através do tempo de morte dos micro-organismos desafiados a concentrações de ½ CIM e CIM dos extratos, incubadas a 37° C. Aliquotas de 10 µL foram plaqueadas, em intervalos de tempo crescentes até 24 horas, em placas de Petri com ágar BHI, incubadas a 37° C por 24 horas para contagem das unidades formadoras de colônias (UFC/mL). A liberação de proteínas intracelulares das bactérias foi avaliada para avaliação de possível ação dos extratos sobre a parede celular bacteriana, após incubação a 37°C por 5 horas. Para a avaliação da interferência dos extratos na capacidade de aderência à superfície pelas bactérias, concentrações entre 0,312 a 5 mg/mL foram utilizadas em placas de microtitulação com as culturas das duas bactérias, e incubadas a 37° C por 24 horas. Após esse período os poços foram lavados com água destilada, e adicionados metanol, cristal violeta e por último ácido acético glacial. A quantidade de micro-organismos aderidos à placa foi medida por absorbância a 540nm. Os extratos apresentaram atividade antimicrobiana em CIM variando entre 1,25 e 2,5 mg/mL, para as duas bactérias testadas. Todos os extratos testados contra *L. sakei* apresentaram ação bactericida, já contra *S. mutans* a atividade variou entre bacteriostática e bactericida. Mesmo resultados apontando capacidade bactericida para alguns extratos, no teste de liberação de proteínas, não houve indicativo de comprometimento da parede celular, sugerindo que o mecanismo de ação dos extratos sobre as bactérias seja em outro alvo celular. A capacidade

de reduzir a aderência bacteriana à superfície foi observada em todas as concentrações testadas, inclusive as subinibitórias, sendo um resultado importante, visto que a cárie é uma doença biofilme-dependente. Os resultados do presente estudo são relevantes para o uso dos diferentes extratos de *C. villosum* no combate e prevenção da cárie. Novos estudos devem ser conduzidos para identificação dos compostos ativos presentes nos extratos, além da avaliação da toxicidade e danos à mucosa oral para o uso seguro.

Palavras-chave: Frutos amazônicos. Extratos naturais. Cárie dental.

ABSTRACT

Dental caries is a public health problem that is associated with an increase in acidogenic *Streptococcus mutans* and *Lactobacillus*, involved in the onset and progression of lesions, respectively. The gold standard antimicrobials in combating these bacteria have shown adverse effects that encourage the establishment of studies to identify alternative substances for the prevention of the disease. The objective of this work was to evaluate the antimicrobial potential of *Caryocar villosum* extracts against bacteria responsible for caries disease, *Streptococcus mutans*, and *Lactobacillus sakei*. Pulp, seed and peel extracts of *C. villosum*, obtained with different solvents (ethanol, hydroalcohol, hexane and methanol) at concentrations ranging from 5 to 0.009 mg/mL, were tested. To determine the Minimum Inhibitory Concentration (MIC), 50 μ L of extract solutions or chlorhexidine at different concentrations was added to the 96-well plate along with 50 μ L of bacterial culture. As a negative control, sterile Brain and Heart Infusion Broth (BHI) was used instead of the extract solution. After incubation at 37° C for 48 hours, resazurin was added to assess cell viability. The nature of the antimicrobial activity was evaluated through the death time of the microorganisms challenged at concentrations of $\frac{1}{2}$ MIC and MIC of the extracts, incubated at 37° C. Ten μ L aliquots were plated, at increasing time intervals up to 24 hours, in Petri dishes with BHI agar, incubated at 37° C for 24 hours to count colony forming units (CFU/mL). The release of intracellular proteins from bacteria was evaluated to assess the possible action of the extracts on the bacterial cell wall, after incubation at 37°C for 5 hours. To assess the interference of extracts on the ability of bacteria to adhere to the surface, concentrations between 0.312 and 5 mg/mL were used in microtiter plates with cultures of the two bacteria and incubated at 37° C for 24 hours. After this period, the wells were washed with distilled water, and methanol, crystal violet and finally glacial acetic acid were added. The number of microorganisms adhered to the plate was measured by absorbance at 540nm. The extracts showed antimicrobial activity in MIC varying between 1.25 and 2.5 mg/mL, for the two tested bacteria. All extracts tested against *L. sakei* showed bactericidal action, whereas against *S. mutans* the activity varied between bacteriostatic and bactericidal. Even though results indicate a bactericidal capacity for some extracts, in the protein release test, there was no indication of cell wall impairment, suggesting that the mechanism of action of the extracts on bacteria is in another cell target. The ability to reduce bacterial adhesion to the surface was observed at all concentrations tested, including sub-inhibitory ones, being an important result, since caries is a biofilm-dependent disease. The results of the present

study are relevant for the use of different extracts of *C. villosum* in combating and preventing caries. New studies must be conducted to identify the active compounds present in the extracts, in addition to the evaluation of toxicity and damage to the oral mucosa for safe use.

Keyword: Amazonian fruits. Natural extracts. Dental caries.

LISTA DE ILUSTRAÇÕES

Quadro 1- Atividade biológica de espécies do gênero <i>Caryocar spp.</i>	12
Figura 1 – <i>Caryocar villosum</i>	13
Chart 1- Codes are used for naming the extracts obtained from different parts of the fruit of <i>Caryocar villosum</i> (piquiá) with different solvents by ultrasound.....	37
Figure 1- Time kill curve of <i>Streptococcus mutans</i> when treated with different concentrations, ranging from MIC to ½ MIC, of the <i>Caryocar villosum</i> extracts.....	37
Figure 2- Time kill curve of <i>Lactobacillus sakei</i> when treated with different concentrations, ranging from MIC to ½ MIC, of the <i>Caryocar villosum</i> extracts.....	38
Figure 3- Time kill curve of <i>Lactobacillus sakei</i> and <i>Streptococcus mutans</i> when treated with chlorhexidine at 0,12% and 2% concentration	38
Graph 1- Estimated coefficients of the protein release test on caries-causing microorganisms (<i>Streptococcus mutans</i> and <i>Lactobacillus sakei</i>) treated with different concentrations of extracts obtained from <i>Caryocar villosum</i> fruit parts, produced by different organic solvents and with chlorhexidine at 0,12% and 2% concentration, with significance, $p < 0,001$	39
Graph 2- Estimation of adhesion evaluation coefficients on caries-causing microorganisms (<i>Streptococcus mutans</i> and <i>Lactobacillus sakei</i>) treated with different concentrations of extracts obtained from <i>Caryocar villosum</i> fruit parts, produced by different organic solvents and with chlorhexidine at 0,12% and 2% concentration, with significance, $p < 0,001$	39

LISTA DE TABELAS

Table 1- The minimum inhibitory concentration of <i>Caryocar villosum</i> extracts obtained from different parts of the plant with four solvents against <i>Streptococcus mutans</i> and <i>Lactobacillus sakei</i> , in mg/mL.....	34
Table 2- Analysis of variance protein release in caries-causing microorganisms (<i>Streptococcus mutans</i> and <i>Lactobacillus sakei</i>) treated with different concentrations and extracts of <i>Caryocar villosum</i> fruit parts produced from different organic solvents.	34
Table 3- Estimates of the coefficients of the treatments for protein release.....	35
Table 4- Analysis of variance of the adhesion evaluation of caries-causing microorganisms (<i>Streptococcus mutans</i> and <i>Lactobacillus sakei</i>) treated with different concentrations and extracts of <i>Caryocar villosum</i> fruit parts produced from different organic solvents.....	35
Table 5- Estimates of the coefficients of the treatments in the adherence evaluation.....	36

LISTA DE ABREVIATURAS E SIGLAS

MUFA	Ácidos graxos monoinsaturados
CIM	Concentração Inibitória Mínima
MIC	Minimum Inhibitory Concentration
LPS	Lipopolissacarídeos
UFJF-GV	Universidade Federal de Juiz de Fora – Campus Governador Valadares
MRS	Mann Rogosa Sharpe
DMSO	Dimetilsufóxido
BHI	Brain Heart Infusion
CLSI	Clinical Laboratory Standards Institute
UFC	Unidades Formadoras de Colônia
BSA	Albumina de soro bovino
DO	Densidade óptica
PPE	Extrato da Polpa de Piquiá extraída com solvente Etanólico
PPHi	Extrato da Polpa de Piquiá extraída com solvente Hidroalcólico
PPHx	Extrato da Polpa de Piquiá extraída com solvente Hexano
PPM	Extrato da Polpa de Piquiá extraída com solvente Metanólico
SPE	Extrato da Semente de Piquiá extraída com solvente Etanólico
SPHi	Extrato da Semente de Piquiá extraída com solvente Hidroalcólico
SPHx	Extrato da Semente de Piquiá extraída com solvente Hexano
SPM	Extrato da Semente de Piquiá extraída com solvente Metanólico
CPHi	Extrato da Casca de Piquiá extraída com solvente Hidroalcólico
CPM	Extrato da Casca de Piquiá extraída com solvente Metanólico

CLX Clorexidina

SUMÁRIO

1	INTRODUÇÃO.....	11
2	OBJETIVOS.....	15
2.1	OBJETIVO GERAL.....	15
2.2	OBJETIVOS ESPECÍFICOS	15
3	ARTIGO CIENTÍFICO.....	16
4	CONCLUSÃO.....	40
	REFERÊNCIAS	41
	ANEXO A – Instruções aos autores preconizadas pelo periódico Archives of oral biology.....	44

1. INTRODUÇÃO

A cárie dentária, um problema de saúde pública que acomete entre 60 e 90% das crianças, além de grande parte dos adultos (ROCHA, 2017). A cárie se apresenta como uma doença crônica, multifatorial, sacarose dependente e associada ao aumento de *Streptococcus mutans* e *Lactobacillus* acidogênicos, que estão envolvidos no início e na progressão das lesões, respectivamente. Esses micro-organismos tem a capacidade de metabolizar carboidratos em ácidos, desencadeando a desmineralização do esmalte dental (PORTO et al., 2018).

Essa doença é biofilme-dependente, sendo o biofilme uma comunidade de micro-organismos aderidos à superfície, que geralmente são encapsulados e protegidos por uma matriz extracelular composta por vários biopolímeros, fazendo com que a eliminação não seja uma prática simples (JUN et al., 2018; PORTO et al., 2018). A principal medida efetiva para a prevenção da cárie é justamente a remoção mecânica do biofilme com uso de escovas e fio dental. Porém, a remoção mecânica do biofilme é dependente da destreza do operador e qualidade dos produtos utilizados. Para contornar essa limitação, métodos químicos são propostos para complementar a higienização bucal (JAIN e JAIN, 2016; JUN et al., 2018). Na Odontologia o digluconato de clorexidina é considerado a substância antimicrobiana padrão-ouro. Entretanto, esta substância apresenta efeitos adversos como escurecimento dental, hipersensibilidade, alteração do paladar, formação de cálculo supragengival e descamação da mucosa oral. Com isso, verifica-se a importância do estabelecimento de estudos que busquem novos tratamentos alternativos à clorexidina para o combate e prevenção às infecções bacterianas orais causadoras de enfermidades orais como a cárie (TAKENAKA, OHSUMI, NOIRI, 2019; JAIN e JAIN, 2016).

Como opção de superar a problemática da resistência, cientistas buscam nas plantas opções aos antimicrobianos convencionais, com intuito de diminuir seus efeitos adversos (NAZZARO et al., 2013). Os extratos obtidos de plantas já são incorporados na odontologia através de produtos de higiene oral, como exemplo, os cremes dentais e enxaguantes a base de própolis, carvão, cravo, *Azadirachta indica* (JANAKIRAM et al., 2020), *Melaleuca alternifolia* (PIEKARZ et al., 2017) e *Carica papaya* extrato (SALIASI et al., 2018).

Os compostos naturais utilizados na prevenção da cárie normalmente são metabólitos secundários necessários para sobrevivência da planta e que possuem atividades biológicas. Os metabólitos utilizados mais comuns são alcalóides, fenóis, flavonóides e ácidos orgânicos, que além de ser uma alternativa econômica, tem sua eficácia comprovada (CHEN et al., 2020).

As espécies do gênero *Caryocar*, são conhecidas popularmente como piquiá, pequi ou piqui de acordo com a espécie e local onde é encontrada. Dentre as espécies que possuem maior

estabelecimento de estudos científicos se encontram *Caryocar brasiliense*, *Caryocar villosum* e *Caryocar coriaceum* (NASCIMENTO-SILVA e NAVES, 2019). As árvores deste gênero são encontradas nas Américas Central e do Sul, e especialmente no território brasileiro são encontradas na região Centro Oeste, região ocidental do estado de Minas Gerais, no Nordeste e na região Amazônica (TORRES et al., 2018).

As frutas de *Caryocar* são fontes ricas em componentes nutricionais importantes como ácidos graxos monoinsaturados (MUFA), fibras, minerais, compostos bioativos (TORRES et al., 2018) e carotenóides (NASCIMENTO-SILVA e NAVES, 2019). As quantidades desses diferentes componentes, podem variar dependendo da espécie, de condições ambientais das regiões nas quais as árvores estão inseridas, parte da fruta analisada e o tipo de análise feita (TORRES et al., 2018). Além das características nutricionais importantes, os frutos também se apresentam como potenciais fontes de compostos bioativos com diversas atividades biológicas (Quadro 1).

Quadro 1- Atividade biológica de espécies do gênero *Caryocar spp.*

Atividade Biológica	Espécie	Parte da planta	Referências
Ação anti-inflamatória	<i>C. coriaceum</i>	Polpa	FIGUEIREDO et al., 2016
	<i>C. brasiliense</i>	Amêndoa	NASCIMENTO-SILVA e NAVES, 2019
	<i>C. brasiliense</i>	Polpa	TORRES et al., 2018
	<i>C. coriaceum</i>		
	<i>C. villosum</i>		
	<i>C. villosum</i>	Polpa e Casca	YAMAGUCHI et al., 2017
	<i>C. villosum</i>	Polpa	XAVIER et al., 2011
Ação anticancerígena	<i>C. brasiliense</i>	Polpa	NASCIMENTO-SILVA e NAVES, 2019
			TORRES et al., 2018
Ação hepatoprotetora	<i>C. brasiliense</i>	Amêndoa	NASCIMENTO-SILVA e NAVES, 2019
Ação lishmanicida	<i>C. coriaceum</i>	Casca	ALVES et al., 2017
		Folhas	TOMIOTTO-PELLISSIER et al., 2018
Antigenotóxico	<i>C. villosum</i>	Polpa	NASCIMENTO-SILVA e NAVES, 2019
Antifúngico	<i>C. brasiliense</i>	Amêndoa	PASSOS et al., 2002
	<i>C. coriaceum</i>	Casca	ALVES et al., 2017
	<i>C. villosum</i>	Polpa	GRENAND et al., 2003
Antibacteriano	<i>C. brasiliense</i>	Polpa	FERREIRA et al., 2011
	<i>C. coriaceum</i>	Casca	ALVES et al., 2017
		Polpa	COSTA et al., 2011
			SARAIVA et al., 2011
Nematicida	<i>C. coriaceum</i>	Polpa	ALVES et al., 2017

Efeito antioxidante	<i>C. brasiliense</i>	Polpa	NASCIMENTO-SILVA e NAVES, 2019
	<i>C. villosum</i>	Polpa	TORRES et al., 2018
	<i>C. brasiliense</i>	Polpa e amêndoa	
	<i>C. villosum</i>	Polpa e Semente	YAMAGUCHI et al., 2017
	<i>C. villosum</i>	Polpa	CHISTE et al., 2012
			CHISTE e MERCADANTE, 2012
			BARRETO et al., 2009
		Casca	ROXO et al., 2020
Hipolipidêmico	<i>C. coriaceum</i>	Polpa	NASCIMENTO-SILVA e NAVES, 2019
			FIGUEIREDO et al., 2016
	<i>C. brasiliense</i>		TORRES et al., 2018

Fonte: Elaborado pelo autor (2021).

Típicas da floresta amazônica brasileira, as árvores da espécie *C. villosum*, o “piquiá”, apresentam frutos ricos em carboidratos, aminoácidos, minerais e oligoelementos (CHISTE e MERCADANTE, 2012), baixo teor de água e alto teor de gordura (MAGID et al., 2006) (Figura1).

Figura 1 – *Caryocar villosum*.



Fonte: Arquivo pessoal dos autores (2021).

A composição nutricional e a presença de compostos bioativos em *C. villosum*, oferecem à fruta atividades biológicas importantes. Uma avaliação dos compostos bioativos presentes no “piquiá” realizada por Yamaguchi et al. (2017), verificou a presença de compostos fenólicos e flavonoides na casca e polpa da fruta. A presença desses compostos, é indicativa de atividade antioxidante e anti-inflamatória, as quais foram confirmadas, pois os extratos de polpa e casca mostraram alta capacidade de eliminar espécies reativas de oxigênio e óxido nítrico, que estão presentes no processo inflamatório, em baixas concentrações variando de 2 -50 µg/mL, com

baixo potencial tóxico.

A presença de ácidos graxos no óleo de *C. villosum* também foi associado à atividade anti-inflamatória por Xavier et al. (2011), tendo sua atividade comparada com a dexametasona, um corticosteroide que modula a resposta de linfócitos. Em outro estudo (ROXO et al., 2020), o extrato da casca de *C. villosum*, mostrou alta capacidade em eliminar radicais livres, com resultados compatíveis com o ácido ascórbico, um potente antioxidante. Os autores também sugeriram que essa ação esteja relacionada com a presença de ácidos graxos, principalmente gálico e elágico.

A atividade antioxidante e anti-inflamatória é bem relatada na literatura, conforme Quadro 1. Já quanto a atividade antimicrobiana, os estudos são restritos, sendo encontrada apenas atividade antifúngica (GRENAND et al., 2003). Outras espécies do gênero, apresentaram atividade importante contra bactérias Gram-positivas (COSTA et al., 2011; SARAIVA et al., 2011) e Gram-negativas (FERREIRA et al., 2011; SARAIVA et al., 2011).

De forma a buscar alternativas benéficas e acessíveis para o combate à problemática da cárie dental, alguns estudos têm apresentado potencial de algumas plantas para obtenção de compostos antimicrobianos no emprego de terapia e prevenção desta enfermidade. Assim, levando em consideração, a alta incidência da cárie e a atividade antimicrobiana apresentada por extratos de *Caryocar*, especialmente aqueles com ação sobre bactérias Gram-positivas, que é o caso daquelas com importância na etiologia da cárie, torna-se relevante o estudo do potencial antimicrobiano de extratos da espécie *Caryocar villosum*. Não há registros na literatura relatando atividade antibacteriana desta espécie em bactérias envolvidas na doença cárie, sendo esse um trabalho inédito na avaliação destes extratos sobre essas bactérias, na busca de alternativas à clorexidina para o combate da cárie dental.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial antimicrobiano de extratos de *Caryocar villosum* contra as bactérias envolvidas na doença cárie, *Streptococcus mutans* e *Lactobacillus sakei*.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar as concentrações inibitórias mínimas dos extratos de *Caryocar villosum*;
- Avaliar atividade bactericida e/ou bacteriostática dos extratos de *Caryocar villosum*;
- Avaliar a atividade de diferentes concentrações dos extratos de *Caryocar villosum* sobre a capacidade de aderência das estirpes de *Streptococcus mutans* e *Lactobacillus sakei*.
- Avaliar a possível atividade dos extratos de *Caryocar villosum* sobre a parede bacteriana de *Streptococcus mutans* e *Lactobacillus sakei*.

3. ARTIGO CIENTÍFICO

Artigo científico enviado para publicação no periódico **Archives of oral biology**, *Qualis* CAPES Interdisciplinar B1. A estruturação do artigo baseou-se nas instruções aos autores preconizadas pelo periódico (ANEXO A).

“Antimicrobial properties of *Caryocar villosum* (Aubl.) Pers. against bacteria involved in dental caries”

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ABSTRACT

Caries is a multifactorial oral disease associated with microorganisms. Chlorhexidine gluconate is the main antimicrobial used to control cariogenic bacteria, however it has side effects. The products based on natural compounds present broad biological activity, including those related to oral diseases. *Caryocar villosum*, as species with economic and nutritional importance in the Amazon region, has bioactive compounds with important biological activities. Belonging to this genus are found species that present antimicrobial activity against other Gram-positive bacteria, but *C. villosum* do not present any scientific evidence about its effect on bacteria involved in caries. The objective of this study was to evaluate the antimicrobial potential of *C. villosum* extracts against caries etiologic agents, *Streptococcus mutans* and *Lactobacillus sakei*. The Minimum Inhibitory Concentration (MIC) of the extracts was evaluated by microdilution. Complementary evaluations of time to death and protein

release were performed, besides the evaluation of the interference of the extracts in the ability of the bacteria to adhere to the surface. The extracts presented MIC between 1.25 and 2.5 mg/mL, some suggesting bacteriostatic potential, others bactericidal. Even with some extracts suggesting bactericidal capacity, in the protein release test, there was no indication of cell wall compromise. The ability to reduce bacterial adherence to the surface was observed in all concentrations tested, including the subinhibitory ones, being an important result, since caries is a biofilm-dependent disease. The results of the present study indicate *C. villosum* extracts as a potential alternative in preventing caries disease. Further studies are needed to evaluate the compounds present in the extracts, besides the evaluation of toxicity for safe use.

Keywords: Natural extracts, amazon fruits, biological activity, *Streptococcus mutans*, *Lactobacillus sakei*

1. Introduction

Caries is an oral disease with high incidence worldwide, affecting children and adults (Jeon et al., 2011; Ribeiro et al., 2019). It is the main pain-causing oral disease, which limits masticatory functions, and impacts people's aesthetics and quality of life (Bud et al., 2021). Caries is a chronic, multifactorial, sucrose dependent and infectious disease. The microorganisms involved in its progression metabolize carbohydrates into acids, affecting calcified tissues through enamel demineralization (Porto et al., 2018). Several microorganisms are associated with its development, including *Streptococcus mutans*, which plays a key role in the initiation of biofilm installation due to its acidogenic, aciduric and extracellular matrix-producing nature (Jeon et al., 2011; Ribeiro et al., 2019) and *Lactobacillus spp.*, involved in disease progression (Bud et al., 2021).

The prevention of caries is through the mechanical removal of bacterial biofilm, through brushing and flossing. However, this practice depends on the operator's dexterity, the amount of daily brushing and the quality of the products used, given these limitations, chemical agents are proposed as aids in cleaning (Jain & Jain, 2016; Jun et al., 2018). Chlorhexidine digluconate is an antimicrobial with high efficacy against oral infections with adsorption capacity in oral tissues and residual effect, which is considered the gold standard in dentistry (Hortense et al., 2010). However, its use is restricted and cannot be used daily for long periods, as it has adverse effects such as tooth darkening, hypersensitivity, taste alteration, formation of supragingival calculus and desquamation of the oral mucosa (Takenaka et al., 2019; Jain & Jain, 2016). Such effects search for therapeutic alternatives to control cariogenic bacteria and the onset of the disease essential.

There is an interest from researchers and industry in the development of new products based on natural products (Ribeiro et al., 2019). These products are important sources of bioactive compounds that offer broad biological activity, including oral diseases (Jeon et al., 2011), in addition to having fewer adverse effects when compared to conventional substances (Nazzaro et al., 2013). A promising but still poorly studied botanical genus is *Caryocar*, which is easily found in Central and South America, in Brazil it is present in the Midwest, Western region of Minas Gerais, Northeast and Amazon region (Torres et al., 2018). The species of the genus have important biological activities, such as improvement in cardiac activity, anticancer, hepatoprotective, anti-inflammatory, antioxidant, antigenotoxic and antimicrobial action (Nascimento-Silva & Naves, 2019; Torres et al., 2018).

Caryocar villosum, popularly known as “piquiá”, is native to the Amazon and is used in local cuisine, industry and culture (Yamaguchi & Souza, 2021). The fruits of the species are round, irregular, with an average of seven to nine centimeters in diameter. The fruit pulp is rich in fatty acids (Lorenzo et al., 2021), low in the water, sugars and organic acids (Magid et al., 2006). Both the pulp and the peel have considerable amounts of total phenolic compounds, flavonoids (Yamaguchi et al., 2017) and carotenoids (Chiste & Mercadante, 2012; Chiste et al., 2012). The presence of these bioactive compounds indicates their use as a nutritional supplement and promising herbal medicine. In the literature, there are reports of biological activities such as antioxidant (Chisté et al., 2012; Lorenzo et al., 2021), anti-inflammatory (Xavier et al., 2011) and antifungal (Grenand et al., 2004), however, there are still no records regarding the antimicrobial activity against bacteria associated with caries.

Studies in the literature have shown the correlation of bioactive natural products of the genus *Caryocar* and the antimicrobial capacity of fruits, as for example, the antimicrobial activity of *Caryocar coriaceum* pulp oil against Gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Costa et al. 2011). Thus, the composition rich in bioactive compounds and the records of promising activities for other species of the genus *Caryocar*, are fundamentals that make the establishment of the use of *C. villosum* in research in the search for effective and accessible alternatives to combat and prevent caries disease (Ferreira et al. 2011; Costa et al. 2011; Saraiva et al. 2011).

Given the above, this study aimed to evaluate the antimicrobial potential of *Caryocar villosum* extracts against bacteria involved in development of dental caries, for use in combating and preventing the public health problem that caries represents.

2. Materials and methods

2.1 Strains, culture media and storage conditions

The strains used of *Streptococcus mutans* (UA159) and *Lactobacillus sakei* (ATCC 15521) are part of the bacteriotheca of the Federal University of Juiz de Fora – Campus Governador Valadares (UFJF-GV). They are stored in microtubes containing Man Rogosa Sharpe broth (MRS) (HiMedia, Marg, Mumbai, India), added with 20% glycerol (v/v) at -20°C, for activation throughout the experimental process.

2.2 Preparation of *C. villosum* extract solutions

The extracts of *C. villosum* were extracted by ultrasound in partnership with the Federal University of Amazonas (ISB/UFAM), Coari – Amazonas, from different parts of the fruit, pulp, seed and peel, obtained with different solvents, hydroalcohol, ethanol, hexane and methanol. After processing the extraction of compounds by different solvents, they were evaporated to obtain the mass of different extracts of *C. villosum*. The extract solutions were obtained and solubilized with dimethylsulfoxide (DMSO) (Exodus scientific, Sumaré, São Paulo, Brazil) at the proportion of 10 mg/mL. A serial dilution was then established in Brain Heart Infusion Broth (BHI) (Titan Biotech LTD. Bhiwadi, Rajasthan, India), from 5 mg/mL to 0.009 mg/mL. The extracts were coded according to Chart 1.

2.3 Establishment of Minimum Inhibitory Concentrations (MIC)

The strains were subjected to the MIC test according to CLSI (2015) with modifications. The extracts were tested at different concentrations ranging from 5 to 0.009 mg/ml. For this, 50µL of culture in BHI broth (Titan Biotech LTD) of each strain at 1.5×10^5 CFU/mL were inserted into 96 orifices microtiter plates along with 50µL of the solutions of the extracts at different concentrations. As a positive control, 50µL of the culture of each strain plus 50µL of a known effective antimicrobial solution, in this case 2% chlorhexidine digluconate, was used. For the negative control, 50µL of culture from each strain plus 50µL of autoclaved BHI (Titan Biotech LTD) was used. In addition to the extracts, chlorhexidine was also tested at concentrations from 2% to 0.003% as an additional form of control. The plates were incubated at 37° C in a bacteriological oven for 48 hours.

After the 48 hours incubation time, 50µL of 0.01% resazurin solution (InLab Confiança, Diadema, São Paulo, Brazil) was added to each hole to verify the presence of cell viability. The orifices where there was a reduction of resazurin (blue color) into resorufin (pink color) indicated bacterial viability (Palomino et al., 2002). The MIC was defined as the lowest concentration of the solutions with the ability to prevent bacterial growth during 48h.

2.4 Time kill curve

The test was performed according to CLSI (2015) with modifications. Previously, a serial dilution was performed in base 10 of the inoculum in BHI (Titan Biotech LTD) to evaluate the possible concentration for colony counting.

Were used MIC and $\frac{1}{2}$ MIC of extracts and concentrations of 0.12% and 2% for chlorhexidine gluconate. Bacterial cultures standardized to the concentration of 5.5×10^5 CFU/mL, at the same volumes of the MIC assay, were incubated at 37°C in BHI broth (Titan Biotech LTD). At predetermined times (0, 4, 8, 10, 12 and 24h), a 10 μ L aliquot was obtained for plating on Petri plates with BHI agar (Titan Biotech LTD). Then, the Petri plates will be incubated at 37°C. The reading was performed 24 hours after each collection and plating time, for colony counting and obtaining the respective number of colony forming units (CFU/mL). The tests were performed in duplicate and the result considered, the calculation of the average of each sample.

2.5 Protein Release Test

Protein quantification was obtained from the suspension of bacterial cells treated with the extracts obtained from *Caryocar villosum*. The assay was performed according to Bradford (1976) with modifications. For the test, the concentration of MIC and 2MIC were used for the extracts and 1.5×10^5 UFC/mL for the strains. The samples were incubated at 37°C for 5 hours, then underwent agitation in a centrifuge (Nova Instruments, Piracicaba, São Paulo, Brazil) at 3000 RPM (revolutions per minute) for 30 minutes. A 5 μ L aliquot of the supernatant was obtained and 250 μ L of Bradford's reagent (Sigma Life Science, São Paulo, São Paulo, Brazil) was added to determine the release of intracellular protein content. Protein quantification was determined by spectrophotometry with λ of 594nm (Thermo Scientific, Waltham, Massachusetts, United States of America). BHI (Titan Biotech LTD), BHI added with each of the extracts, and BHI added with bacteria were used as controls. The standard curve was established with bovine serum albumin (BSA) (Sigma Life Science, São Paulo, São Paulo, Brazil) diluted in BHI broth (Titan Biotech LTD) at concentrations of 0.1 μ g/mL to 0.5 μ g/mL.

2.6 Assessment of Interference on Bacterial Adhesion

In this step, the concentrations between 0.312 to 5 mg/mL obtained for each extract were considered. Following protocol by Stepanovic et al., (2000) with modifications, 190 μ L of diluted culture of each strain was plated at 1.5×10^5 UFC/mL along with 60 μ L of each

antimicrobial in 96 orifices microtiter plates. For the positive control, autoclaved distilled water was added along with 190 μL of culture and for the negative control, autoclaved BHI (Titan Biotech LTD) without the addition of the strains. The plates were incubated at 37°C for 24h, after this period three washes with distilled water were done and dried at room temperature. Then 250 μl of methanol (Panreac Quimica SLU, Castellar dell Vallés, Barcelona, Spain) was added and left for 15 minutes. After removal of the methanol and natural drying, the holes were stained with crystal violet (Scientific Exodus, Sumaré, São Paulo, Brazil) for 10 minutes and washed with autoclaved distilled water, and again, dried at room temperature. Finally, 250 μl of glacial acetic acid (Sciavicco Comércio e Indústria LTDA, Sabará, Minas Gerais, Brazil) 33% per orifice was added.

The adhesion capacity was measured by absorbance in a spectrophotometer (Thermo Scientific) with λ of 540nm, comparing the optical density (OD) of the treatment with that of the controls. All procedures were performed in duplicate.

2.7 Statistical Analysis

The results obtained in the protein release test and adherence evaluation underwent a statistical analysis that initially consisted of a descriptive analysis of the data, by means of measures of central tendency, variability and shapes. The measures of shape, such as asymmetry, refer to the shape of the data distribution. The variables used for both tests were optical density data, plate factor, controls, concentrations, solvents, plant part, bacteria and treatments.

After the descriptive analysis, regression models were adjusted to explain and quantify the relationship between variables. To determine the standard curve of albumin, in the protein release test, the simple linear regression model was used, represented by $Y=X\beta+\epsilon$, where Y is the dependent variable, X the explanatory variables, β is the angular coefficient and ϵ represents all residual factors plus possible measurement errors (Reencher & Achaalje, 2008).

To analyze the results of the adherence and protein release tests, the mixed model and analysis of variance (ANOVA) were used, considering a significance level of 5%. In this model, besides the fixed effects, random effects are also evaluated, in this case the plate effect. The notation of the mixed model is represented by $Y=X\beta+Z\gamma+\epsilon$, where Z is the random effects and γ are the parameters associated with it (McCulloch & Searle, 2001).

The intercept is the value at which the fitted line crosses the y-axis ($y, x=0$). Due to the methodological limitations of the impossibility of randomizing the treatments in 96 orifices

microtiter plates, the estimates of some factor levels were omitted, because it generated a collinearity problem and confounding of effects. These values that were omitted are included in the value of the intercept so that it was possible to perform the estimation.

All analyses and results were obtained using the R programming language (R Core Team, 2021).

3. Results

3.1 Minimum Inhibitory Concentrations (MIC)

The antimicrobial evaluation of the extracts of *Caryocar villosum* fruit parts and their respective solvents against *S. mutans* showed the same MIC value, 1.25 mg/mL, as represented in Table 1. The MIC results of the extracts against *L. sakei* ranged from 1.25 mg/mL to 2.5 mg/mL, as represented in Table 1.

Chlorhexidine, the gold standard in dentistry, up to the time of the reading in all concentrations tested, from 2% to 0.003%, showed no viable bacterial cells.

3.2 Time kill curve

The death time curve test will suggest the nature of the action of the extracts according to the concentration tested. The extracts PPE, PPH_x, PPH_i, CPH_i, SPM and CPM, at MIC concentration suggest bactericidal action on *S. mutans* bacteria, according to Figure 1. Except for PPE, that the activity conferred complete inhibition starting at T₈, the others promoted inhibition of the viable bacterial load starting at T₀, with no growth of any colony until the last evaluated time. On the other hand, extracts PPM, SPH_x, and SPE, at MIC concentration, showed inhibition for a certain time, with growth returning after T₄, T₈, and T₁₂, respectively, suggesting a bacteriostatic nature of their activity. When evaluated at ½ MIC concentration, the SPE, SPH_x, PPM and SPM extracts showed a behavior very similar to the control, in other words, no antimicrobial action, whereas the PPH_x, CPH_i and CPM extract according to their behavior suggests a bacteriostatic nature, and the PPH_x among them showed the longest inhibition time.

The extracts obtained from *C. villosum* when tested against *L. sakei*, except for PPM, all showed suggestive activity as bactericidal at MIC concentration. The ½ MIC concentration of PPH_i, SPM and PPM behaved very similarly to the control, whereas PPE, SPE, PPH_x, SPH_x and CPH_i appeared to have bacteriostatic activity, with inhibition of bacterial activity for up to

8 hours, according to Figure 2.

Both 0.12% and 2% chlorhexidine inhibited the growth of *L. sakei* strains until the last time point evaluated (T24) (Figure 3). Against *S. mutans*, the 0.12% concentration inhibited the bacteria until reading T4, while the 2% concentration inhibited them until T8. This suggests that the standard antimicrobial used in dentistry is bactericidal against *L. sakei* and bacteriostatic against *S. mutans*.

3.3 Protein Release Test

The cell membrane integrity was determined by treating the bacterial cells with different concentrations of the plant extracts (control, MIC and 2 MIC) and measuring the amount of proteins released from the supernatant of the tested bacteria. When analyzing the optical density variable did not show normal distribution, and therefore the Box-Cox transformation was applied to the data to stabilize the variance and make the data more similar to the normal distribution (Box & Cox, 1964).

Two models were performed, in the first, represented in Table 2, the significance of the effects was evaluated. In this model, it was observed that only the controls and the bacteria showed a significant difference ($p < 0.05$).

In another model, the combinations of treatments whose effects were significant were evaluated (Table 3). Only 2% chlorhexidine was capable of damaging the cell wall of *S. mutans* and causing protein leakage up to the time of reading (Table 3, Graph 1). The other treatments, even though they presented a significant difference ($p < 0.05$), presented negative estimates, that is, did not increase the release of protein.

The standard protein used as bovine serum albumin (BSA) (Sigma Life Science), at concentrations ranging from 0 to 50 $\mu\text{g/mL}$. The recorded OD generated the following equation:

$$x = \frac{y - 0.416}{0.005}$$

Where 0.416 is the intercept and 0.005, the angular coefficient.

3.4 Assessment of Interference on Bacterial Adhesion

The optical density variable did not show normal distribution, and the Box-Cox transformation of the data was performed (Box & Cox, 1964). In the adherence evaluation assay, two models were also performed, and Table 4 shows the significance of the effects. When the adherence capacity of *S. mutans* and *L. sakei* was evaluated, there was no statistical difference between the two bacteria or between the different concentrations tested ($p < 0.05$)

(Table 4).

After analyzing the significance of the effects, each treatment was verified among those with significant effects (Table 5, Chart 2). Among the extracts evaluated, PPE, PPHx, SPE and SPHi showed significant difference ($p < 0.05$), with the pulp extracts (PPE and PPHx) reducing the adherence of bacteria to the surface (negative estimate), and the seed extracts (SPE and SPHi) inducing bacterial adherence (positive estimate). Chlorhexidine at both concentrations showed $p < 0.05$, with estimates higher than the positive control. Suggesting that this antimicrobial at the different concentrations induces the adherence of bacteria to the surface.

4. Discussion

The genus *Caryocar*, in addition to its nutritional importance, the plants of the genus are used sustainably, all their parts are used. In French Guiana, Colombia, Venezuela and the central-western and northern regions of Brazil they are used in cooking and the cosmetic industry and are part of the culture of these places (Magid et al., 2006).

The fruits of *C. villosum*, popularly called “piquiá”, are considered interesting sources of bioactive compounds. The fruits of this species, have phenolic compounds (Chiste et al., 2012), being the most abundant the gallic acid, rhamnoside ellagic acid and ellagic acid (Chiste & Mercadante, 2012). Twelve carotenoids are founding according to Chiste et al. (2012) and Chiste & Mercadante (2012). Boy et al., (2021) evaluated the efficiency of the extraction of phenolic compounds using two methods, agitation and ultrasound. The highest values were found for ultrasound extraction, the same method as in the present study. Phenolic compounds are involved with the ability to reduce oxidative stress and antioxidant activity (Hirata et al, 2004; Campos & Frasson, 2011), with anti-inflammatory, estrogenic, enzyme inhibition, antiallergic, vascular activity, cytotoxic antitumor activity (Cushine & Lamb, 2005), besides the antimicrobial activity well reported in the literature (Hintz et al., 2015; Ribeiro et al., 2019; Jeon et al., 2011; Cushine & Lamb, 2005). This activity is justified by the ability of these compounds to bind to soluble extracellular proteins, in addition to forming complexes with the bacteria cell wall (Ribeiro et al., 2019; Cowan, 1999). These authors even reported flavonoid activity against *S. mutans*.

The extracts tested against *S. mutans* showed the same Minimum Inhibitory Concentration (MIC) of 1.25 mg/mL for all treatments with extracts obtained from *C. villosum*. Against *L. sakei*, the MIC values varied between 1.25 and 2.5 mg/mL, as shown in Table 1. The extracts that presented MIC 1.25 mg/mL were very close to the limit considered by Ríos & Recio (2005), which is 1 mg/mL for extracts and 0.1 mg/mL for isolated compounds. The MIC test is a

preliminary analysis, requiring a further evaluation to assess other parameters, mechanisms and specificities.

Thus, to evaluate the nature of the activity of the extracts at different concentrations, the time kill curve test was performed. All extracts tested against *L. sakei* in MIC concentration suggest a bactericidal activity because until the last moment evaluated there was complete inhibition of bacteria, being that SPE and SPM inhibited the growth completely since the first evaluated time (T0), and PPHi took more time to start the process of bacterial inhibition, having the complete inhibition in T8. For the other extracts, there was complete inhibition from T4 until the last reading, taken 24 hours after plating.

For the extracts tested against *S. mutans*, only SPE, SPHx and PPM are suggested to possess bacteriostatic activity nature. Among these, PPM showed inhibition of bacterial growth only 4 hours (from T0 to T4), while the others, inhibition occurred during 8 hours. The SPHx inhibition happened from the first reading until T8, while the SPE interaction of the extract with the bacteria took a little longer, with complete inhibition happening from T4 to T12. The other extracts tested against this bacterium suggest that they are bactericidal because they inhibited the growth of the bacteria until the last reading.

For cases in which the concentrations of $\frac{1}{2}$ MIC and MIC behaved similarly in the time kill curve, as in the case of PPE and PPHi against *S. mutans* and CPM against *L. sakei*, one might think that in the previous evaluation of minimum inhibitory concentration made in microdilution at the time of the 48 hours reading the MIC would be 1.25 mg/mL for the mentioned extracts. However, in the methodology of the time to time kill curve, until the 24 hours reading the bacteria did not show growth, suggesting that the real MIC is 0.625 mg/mL, according to Holetz et al., (2002) with weak antimicrobial activity.

The bacterial cell wall when exposed to antibacterial agents can be compromised. Extravasation of cytoplasmic contents is an indication of damage to this structure (Wang et al., 2015). No studies evaluating the mechanism of action of *C. villosum* in bacterial cell walls were identified. However, studies showed that Gram-positive bacteria, such as *Staphylococcus aureus*, had the amount of protein release increased when compared to amoxicillin when treated with compounds from isolated *Gycosmis pentaphylla* (Murugan et al., 2020), when treated with oil of *Abelmoschus moschatus* seed, the result did not show higher protein release than the control (Arokiyaraj et al., 2015). This difference in activity can be explained by the difference in the composition of the analyzed products. In the present study, although there was evidence of extracts with bactericidal activity, there was no increase in protein release ($p < 0.05$) when the cariogenic bacteria were treated with the “piquiá” extracts. More studies need to be done to

evaluate if the extracts have an alternative action mechanism to the cell wall or if the protein leakage would happen in a longer time than the one evaluated in this study (5 hours). What can be noticed was a significant protein release ($p < 0.05$) for *S. mutans* when treated with 2% chlorhexidine. However, the same antimicrobial in a lower concentration of 0.12%, did not show protein release in any of the treated bacteria, corroborating with Cieplik et al. (2019) that presents the activity of this antimicrobial as concentration-dependent, in other words, in lower concentrations, it presents itself as bacteriostatic and in higher concentrations, bactericidal with extravasation of intracellular components.

Natural oils are expected to have better activity on Gram-positive bacteria due to their cell wall structure composed of up to 95% peptidoglycan, which enables the entry of lipophilic molecules. In contrast, Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane composed of a double layer of phospholipids bound internally by lipopolysaccharides (LPS). The outer membrane has many porins, which function as hydrophilic transmembrane channels, the set of these characteristics are responsible for the relative resistance of these bacteria to natural extracts (Costa et al., 2011; Orchard & Vuuren, 2017; Nazzaro et al., 2013).

Caries is disease dependent on biofilm formation. This physical-chemical process is formed by a mixed community of bacteria, *S. mutans* being the primary bacteria. The initial attachment and accumulation of microorganisms on the tooth surface is sucrose dependent. When the biofilm is not removed from the surface, they are exposed to carbohydrates from the diet that are metabolized by the bacteria into organic acids and synthesized into polysaccharides. The greater the number of extracellular polysaccharides, the greater the bacterial adhesion, biofilm cohesion, and the formation of a structure necessary for the establishment of this adhered biofilm. The biofilm matrix is an acidic environment, granting a competitive advantage to acidophilic bacteria such as *S. mutans* and *Lactobacillus spp.* (Jeon et al., 2011). Given the biofilm-dependent nature of caries disease, the need to search for agents capable of inhibiting or reducing the ability of bacteria to adhere to the tooth surface is a reality. Of the extracts obtained from *C. villosum* tested, the concentrations did not interfere in the capacity of adherence ($p < 0.05$), however, the part of the plant and the solvent used for extraction, was significant. The extracts obtained from the pulp, PPE and PPHx, that in the time kill curve suggest to be bactericidal in MIC concentration, showed ability to reduce the adherence of bacteria to the surface, including in sub-inhibitory concentrations. This behavior may be attributed to the compounds present, being necessary studies for the identification and

quantification of these compounds and their ability to inhibit bacteria adherence to the surface.

Despite its advantageous antimicrobial properties, chlorhexidine has adverse effects that contraindicate its daily use, such as staining of the teeth, alteration in the taste buds with consequent taste alteration, scaling of the oral mucosa, and formation of supragingival calculus (Zanatta & Rosing, 2007). In the adherence evaluation test, an increase in adherence of *S. mutans* and *L. sakei* to the surface was observed in the presence of chlorhexidine at the two concentrations evaluated. This result is consistent with the study by Zannata et al. (2010), which showed the paradoxical behavior of the antimicrobial, even with properties that reduce plaque formation, the increase in calculus formation is a reality when chlorhexidine is used as a mouthwash in concentrations from 0.12%, especially if the surfaces already have stains and plaque. This influence on the process of supragingival calculus formation is not fully understood, according to the authors. Biofilm is formed when bacteria are under stress as a defense mechanism. Environmental, nutritional and antimicrobial factors in sub-inhibitory concentrations can induce the formation of this biofilm (Costa et al., 2014). Therefore, one hypothesis is that chlorhexidine gluconate causes a stress on bacterial cells before inhibiting them.

The antibacterial activity of other species of the genus *Caryocar* is described in the literature, such as the pulp of *C. coriaceum*, with activity against *S. aureus* (Costa et al., 2011; Saraiva et al., 2011), Gram-positive bacteria, as well as cariogenic bacteria. According to Freese et al., (1973), this action is justified by the presence of high levels of fatty acids, compounds that are also present in *C. villosum* (Xavier et al., 2011; Roxo et al., 2020).

In the preliminary test of antimicrobial activity evaluation, MIC, *C. villosum* extracts presented inhibitory concentrations between 1.25 and 2.5 mg/mL and the results of the time kill curves showed that the extracts can moderate the growth of the main bacteria responsible for the development of caries. Moreover, the ability to reduce adherence to surfaces at concentrations starting at 0.312 mg/mL is important considering the pathophysiology of caries disease as dependent on the formation of bacterial biofilm. Considering the set of results found in this study, PPE was the extract considered the most promising with low MIC against *S. mutans* and *L. sakei*, suggesting that it has bactericidal activity in addition to reducing adherence at low concentrations. However, peel and seed extracts require special attention, as they are parts of the plant that would normally be discarded. The sustainable use of these by-products would add social value to the use of these extracts. The results of this study are relevant and innovative, however, further studies are needed to evaluate the active compounds

present in the extracts, in addition to the evaluation of toxicity and damage to the oral mucosa for the safe use and possible development of a product for use in dentistry.

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Tables

Table 1- The minimum inhibitory concentration of *Caryocar villosum* extracts obtained from different parts of the plant with four solvents against *Streptococcus mutans* and *Lactobacillus sakei*, in mg/mL.

	Ethanol	Hydroalcohol	Hexane	Methanol
<i>Streptococcus mutans</i>				
Pulp	1.25	1.25	1.25	1.25
Seed	1.25	1.25	1.25	1.25
Peel	*	1.25	*	1.25
<i>Lactobacillus sakei</i>				
Pulp	1.25	2.5	2.5	1.25
Seed	2.5	1.25	2.5	1.25
Peel	*	1.25	*	1.25

Source: Prepared by the author. * There was no extract with the solvent

Table 2- Analysis of variance protein release in caries-causing microorganisms (*Streptococcus mutans* and *Lactobacillus sakei*) treated with different concentrations and extracts of *Caryocar villosum* fruit parts produced from different organic solvents.

	Sum Sq	Mean Sq	NumDF	Pr(>F)
Control	0.113	0.028	4	≅ 0
Concentration	0.005	0.005	1	0.312
Solvent	0.020	0.007	3	0.231
Part of the plant	0.019	0.009	2	0.130
Bacteria	0.447	0.447	1	≅ 0

Table 3 - Estimates of the coefficients of the treatments for protein release.

	Estimate	Std. Error	Pr(> t)
Intercept	0.681	0.038	≅ 0
Chlorhexidine 0,12% (<i>L. sakei</i>)	-0.141	0.053	0.009
Chlorhexidine 0,12% (<i>S. mutans</i>)	0.029	0.053	0.584
Chlorhexidine 2% (<i>L. sakei</i>)	-0.049	0.053	0.365
Chlorhexidine 2% (<i>S. mutans</i>)	0.198	0.053	≅ 0
CPHi (<i>L. sakei</i>)	-0.108	0.046	0.021
CPHi (<i>S. mutans</i>)	-0.048	0.046	0.299
CPHx (<i>S. mutans</i>)	0.013	0.046	0.775
CPM (<i>L. sakei</i>)	-0.144	0.046	0.002
Positive (<i>L. sakei</i>)	-0.006	0.053	0.905
PPE (<i>L. sakei</i>)	-0.166	0.046	≅ 0
PPE (<i>S. mutans</i>)	-0.036	0.046	0.443
PPHi (<i>L. sakei</i>)	-0.129	0.046	0.017
PPHi (<i>S. mutans</i>)	-0.099	0.046	0.035
PPHx (<i>L. sakei</i>)	-0.119	0.053	0.028
PPHx (<i>S. mutans</i>)	-0.037	0.046	0.430
PPM (<i>L. sakei</i>)	-0.132	0.046	0.005
PPM (<i>S. mutans</i>)	-0.043	0.046	0.356
SPE (<i>L. sakei</i>)	-0.141	0.053	0.009
SPE (<i>S. mutans</i>)	-0.035	0.046	0.449
SPHi (<i>L. sakei</i>)	-0.157	0.046	≅ 0
SPHi (<i>S. mutans</i>)	-0.029	0.046	0.532
SPHx (<i>L. sakei</i>)	-0.173	0.053	0.001
SPHx (<i>S. mutans</i>)	0.008	0.046	0.833
SPM (<i>L. sakei</i>)	-0.145	0.046	0.002
SPM (<i>S. mutans</i>)	0.044	0.046	0.345

Table 4 - Analysis of variance of the adhesion evaluation of caries-causing microorganisms (*Streptococcus mutans* and *Lactobacillus sakei*) treated with different concentrations and extracts of *Caryocar villosum* fruit parts produced from different organic solvents.

	Sum Sq	Mean Sq	NumDF	Pr(>F)
Control	14.347	3.587	4	≅ 0
Concentration	0.365	0.365	1	0.101
Solvent	1.161	0.387	3	0.037
Part of the plant	3.249	1.625	2	0.018
Bacteria	0.631	0.316	2	0.106

Table 5- Estimates of the coefficients of the treatments in the adherence evaluation.

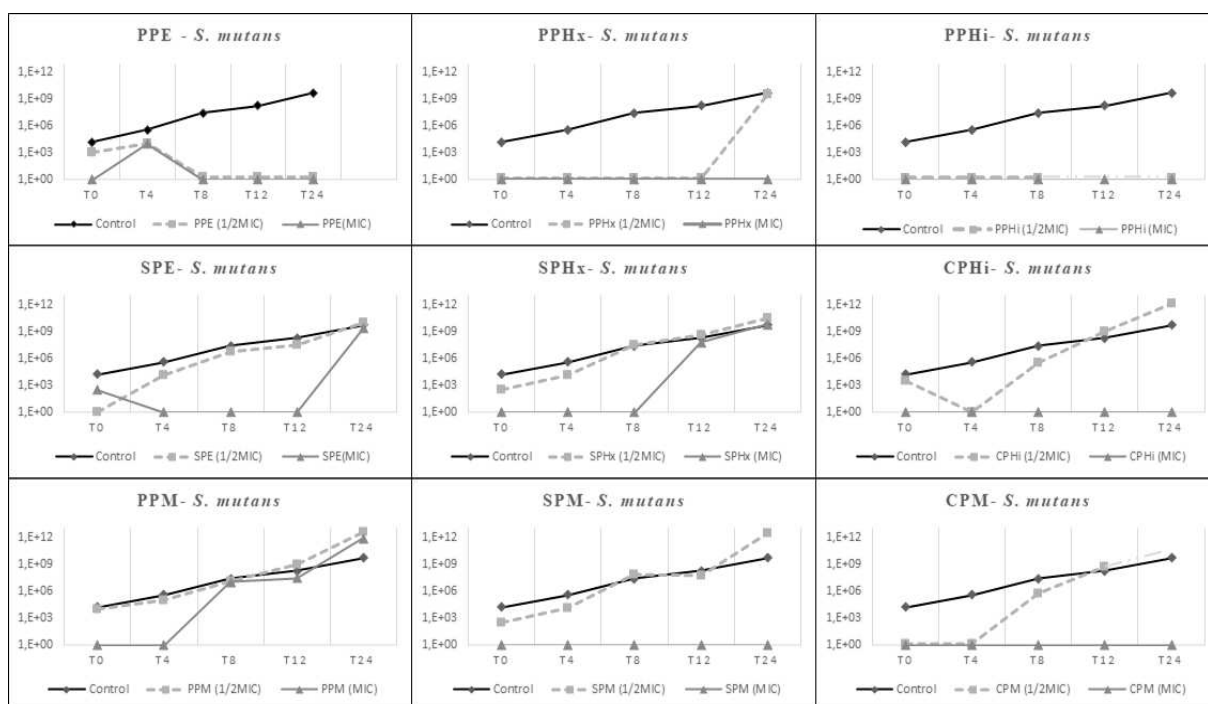
	Estimate	Std. Error	Pr(> t)
Intercept	0.975	0.094	≅ 0
Chlorhexidine 0,12%	1.639	0.199	≅ 0
Chlorhexidine 2%	1.687	0.199	≅ 0
CPHi	-0.092	0.113	0.417
CPM	-0.076	0.113	0.500
Positive	0.750	0.078	≅ 0
PPE	-0.334	0.108	0.002
PPHi	0.040	0.108	0.707
PPHx	-0.252	0.108	0.020
PPM	-0.150	0.108	0.168
SPE	0.418	0.108	≅ 0
SPHi	0.268	0.108	0.014
SPHx	0.092	0.108	0.398
SPM	0.335	0.108	0.002

Figures

Chart 1- Codes are used for naming the extracts obtained from different parts of the fruit of *Caryocar villosum* (piquiá) with different solvents by ultrasound.

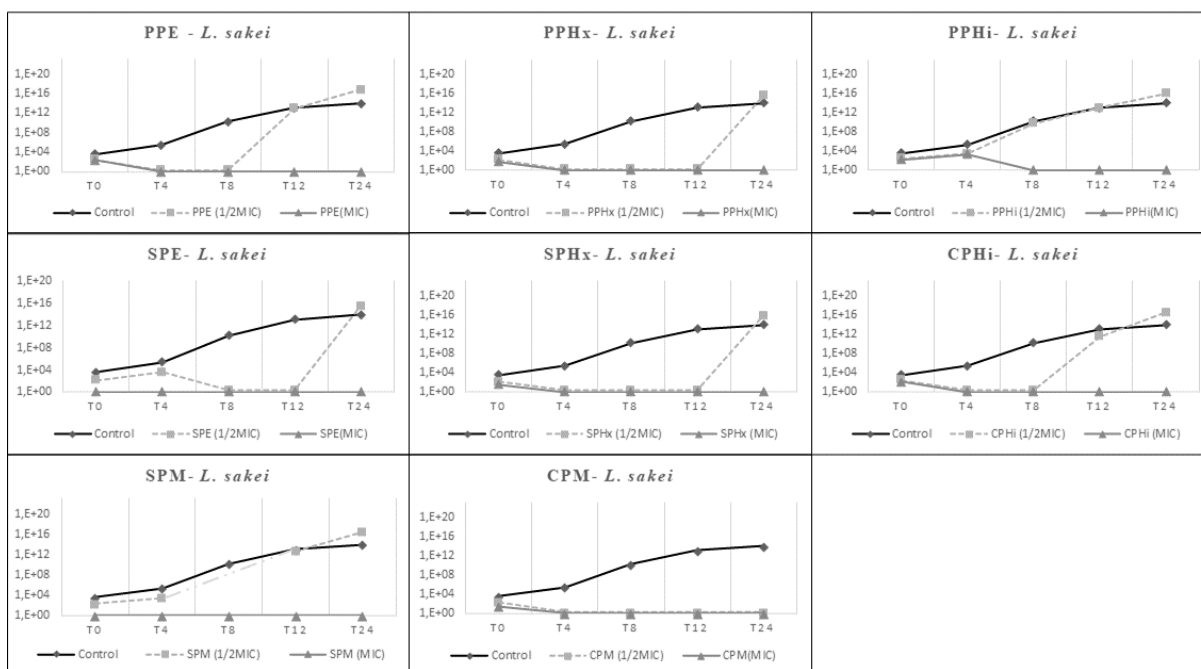
Plant part	Solvents	Code
Pulp	Ethanol	PPE
	Hydroalcohol	PPHi
	Hexane	PPHx
	Methanol	PPM
Seed	Ethanol	SPE
	Hydroalcohol	SPHi
	Hexane	SPHx
	Methanol	SPM
Peel	Hydroalcohol	CPHi
	Methanol	CPM

Figure 1- Time kill curve of *Streptococcus mutans* when treated with different concentrations, ranging from MIC to $\frac{1}{2}$ MIC, of the *Caryocar villosum* extracts.



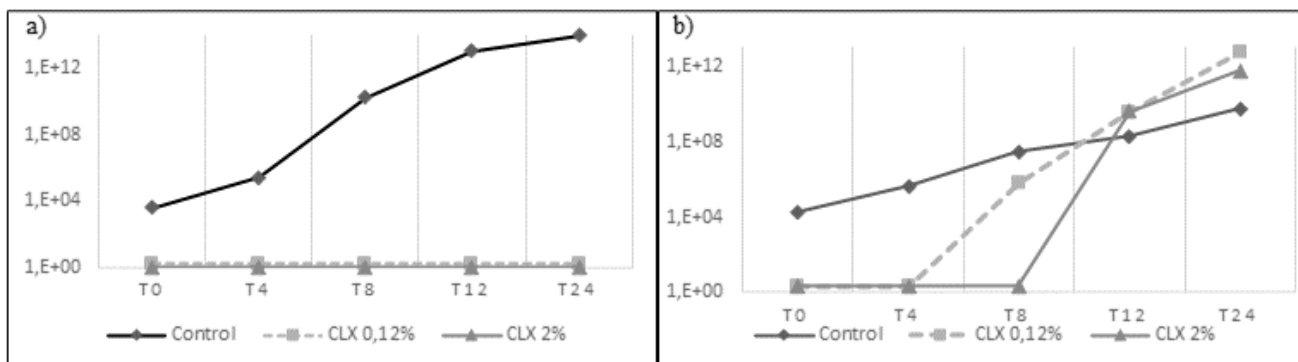
Source: Prepared by the author. *In the T12 reading of the PPHi extract and T24 of the CPM, it was a lost plot. A trend line was made at these times.

Figure 2- Time kill curve of *Lactobacillus sakei* when treated with different concentrations, ranging from MIC to $\frac{1}{2}$ MIC, of the *Caryocar villosum* extracts.



Source: Prepared by the author. *On the T18 reading of the SPM extract, it was a lost plot, and a trend line was performed at that point. There was not enough PPM extract to perform the test.

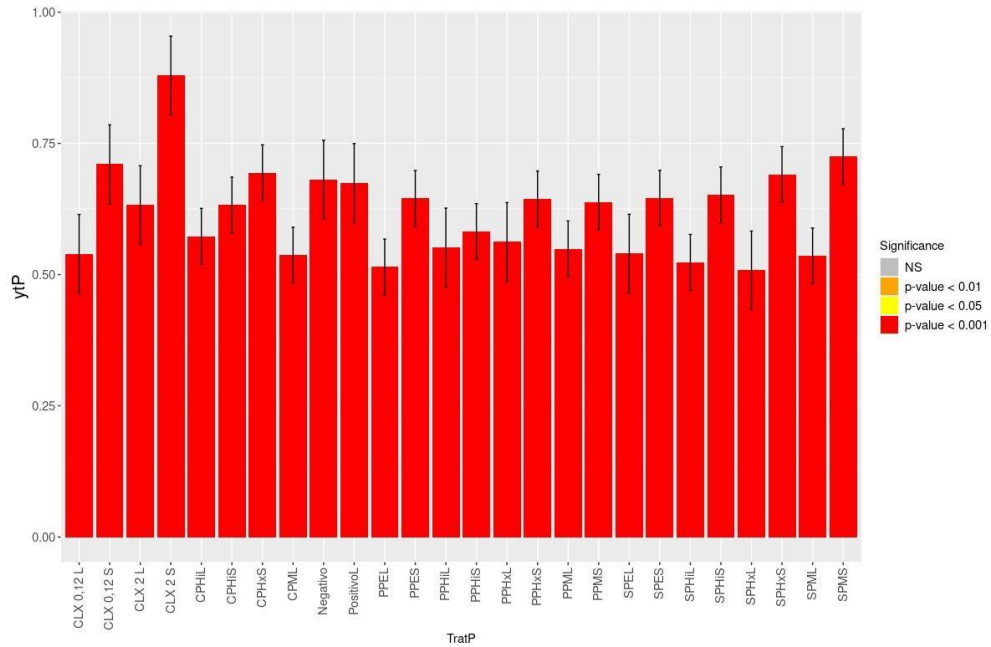
Figure 3- Time kill curve of *Lactobacillus sakei* and *Streptococcus mutans* when treated with chlorhexidine at 0.12% and 2% concentration.



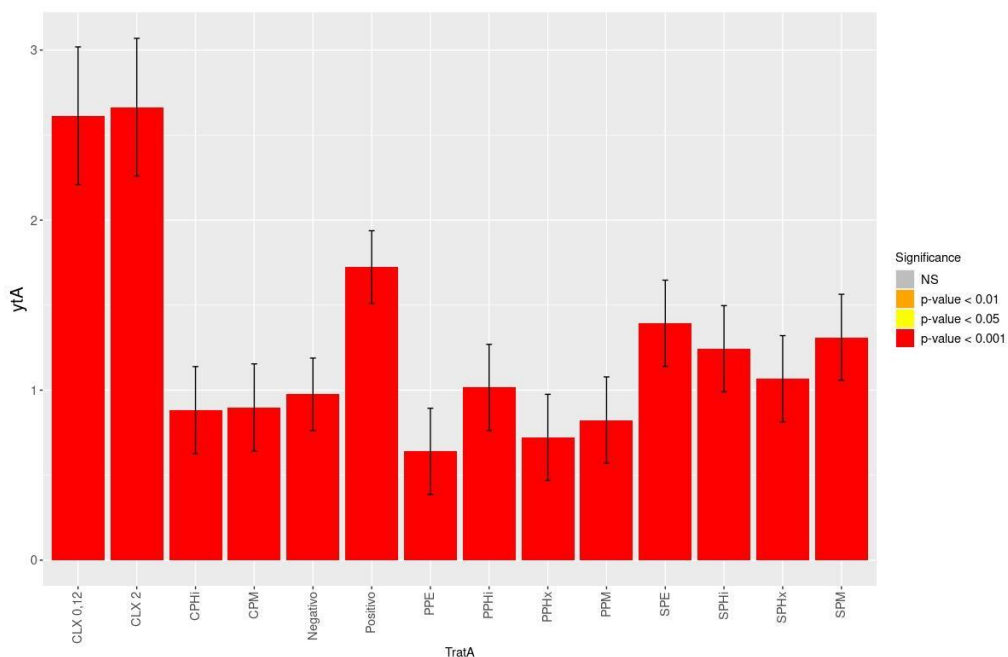
Source: Prepared by the author.

Figure 3- a) *L. sakei* time kill curve tested with 0.12% and 2% chlorhexidine. b) *S. mutans* time kill curve tested with 0.12% and 2% chlorhexidine.

Graph 1- Estimated coefficients of the protein release test on caries-causing microorganisms (*Streptococcus mutans* and *Lactobacillus sakei*) treated with different concentrations of extracts obtained from *Caryocar villosum* fruit parts, produced by different organic solvents and with chlorhexidine at 0.12% and 2% concentration, with significance, $p < 0.001$.



Graph 2- Estimation of adhesion evaluation coefficients on caries-causing microorganisms (*Streptococcus mutans* and *Lactobacillus sakei*) treated with different concentrations of extracts obtained from *Caryocar villosum* fruit parts, produced by different organic solvents and with chlorhexidine at 0.12% and 2% concentration, with significance, $p < 0.001$.



4. CONCLUSÃO

Os extratos de *C. villosum*, apresentaram potencial antimicrobiano em concentrações a partir de 1,25 mg/mL, tanto para *S. mutans* quanto para *L. sakei*. A atividade dos extratos contra *L. sakei*, se apresentou bactericida até o último momento avaliado inibindo o crescimento das bactérias. Já para *S. mutans*, a maioria dos extratos também apresentaram natureza bactericida, com exceção apenas do SPE, SPHx e PPM, os quais inibiram a multiplicação microbiana até um determinado período, após o qual houve retorno do crescimento bacteriano. Mesmo para aqueles extratos que sugerem ter ação bactericida, não houve extravasamento de proteínas intracelulares, sugerindo que ação deles seja alternativa à parede celular ou que a destruição da parede ocorra em um tempo maior ao avaliado nesse estudo. Outra vantagem identificada para os extratos, foi a capacidade de reduzir a aderência das bactérias em superfícies, inclusive em baixas concentrações, o que impediria a formação do biofilme, sem o qual não ocorreria a formação das cáries. A partir dos resultados desse trabalho, pode-se concluir que os extratos de *C. villosum* apresentam potencial antimicrobiano relevante sobre bactérias cariogênicas, e são promissores fontes de compostos para serem utilizados na prevenção da doença cárie. Considerando o conjunto de resultados encontrados neste estudo, o PPE foi o extrato considerado o mais promissor com baixa CIM contra *S. mutans* e *L. sakei*, sugerindo que possui atividade bactericida além de reduzir a aderência em baixas concentrações. No entanto, os extratos da casca e da semente requerem atenção especial, pois são partes da planta que normalmente seriam descartadas. O uso sustentável desses subprodutos agregaria valor social ao uso desses extratos. Sugere-se, que novos estudos sejam conduzidos, para identificação dos compostos ativos, e para avaliar a toxicidade dos mesmos, para o seu adequado e seguro emprego na prevenção da doença cárie.

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ANEXOS

ANEXO A – Instruções aos autores preconizadas pelo periódico Archives of oral biology

**ARCHIVES OF ORAL BIOLOGY**

A Multidisciplinary Journal of Oral & Craniofacial Sciences

AUTHOR INFORMATION PACK**TABLE OF CONTENTS**

• Description	p.1
• Audience	p.1
• Impact Factor	p.1
• Abstracting and Indexing	p.2
• Editorial Board	p.3
• Guide for Authors	p.4

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Archives of Oral Biology is an international journal which aims to publish papers of the highest scientific quality in the oral and craniofacial sciences including:
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