

Gabriela Lopes Fernandes

**Extrato da casca de *Punica granatum*
potencializa o efeito anti cárie de um
enxaguatório bucal contendo trimetafosfato de
sódio e flúor**

ARAÇATUBA - SP

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sódio e flúor**

*Tese apresentada à Faculdade de
Odontologia de Araçatuba da
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Dedicatória

Dedico este trabalho

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“Deus é como o vento, que tudo toca.”

**O menino que
descobriu o vento**

Resumo

FERNANDES, G.L. **Extrato da casca de *Punica granatum* potencializa o efeito anti carie de um enxaguatório bucal contendo trimetafosfato de sódio e flúor**. 2020 80f. Tese (Doutorado em Ciência Odontológica, área de Biomateriais) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

A cárie dentária está entre as principais e mais comuns doenças bucais. É causada por ácidos produzidos pelo biofilme microbiano que levam à desmineralização do esmalte. A prevenção e controle dessa doença crônica consistem na desorganização periódica do biofilme e na promoção da remineralização dentária. Para resolver esse problema, associamos o extrato de casca de *Punica granatum* (romã) (PPE) ao trimetafosfato de sódio (TMP) e fluoreto (F) em formulações para uso como enxaguatório bucal, e avaliamos sua eficácia na redução do processo de desmineralização do esmalte dental, bem como seu potencial anti biofilme contra importantes patógenos orais presentes na cárie dentária (*Streptococcus mutans* ATCC 25175 e *Candida albicans* ATCC 10231). Blocos de esmalte bovino (4 mm × 4 mm) selecionados por dureza superficial inicial (SHi) foram alocados aleatoriamente de acordo com grupos de tratamentos de formulação (n = 12 / grupo): ETF1 (3,0% PPE + 0,2% TMP + 100ppmF), TF1 (0,2% TMP + 100ppmF), ETF2 (3,0% PPE + 0,3% TMP + 225ppmF), TF2 (0,3% TMP + 225ppmF), F1 (100 ppmF), F2 (225 ppmF) e P (formulação sem E / T / F - placebo). Os blocos foram tratados 2x / dia com cada formulação e submetidos a cinco ciclos de pH (soluções desmineralizantes / remineralizantes) a 37° C. A seguir, determinaram-se a dureza superficial final (SHf), a dureza integrada da subsuperfície de perda (Δ KHN) padronizada e as concentrações de fluoreto de esmalte (F) de cálcio (Ca) e fósforo (P). A porcentagem de perda de dureza superficial (% SH) foi calculada ($\% \text{ SH} = [(\text{SHf} - \text{SHi}) / \text{SHi}] \times 100$), e as formulações que promoveram menores porcentagens de desmineralização do esmalte (% SH) e suas contrapartes foram selecionadas para os ensaios anti biofilme, bem como a formulação contendo apenas PPE (E). Para isso, ETF2 e TF2 (% SH = -34,5% e -53,1%, respectivamente), e a formulação E foram usadas para tratar por 1 ou 10 minutos biofilmes duplos de *C. albicans* e *S. mutans* crescidos por 24 horas em discos de hidroxiapatita (HA). A desmineralização da superfície do esmalte foi menor nas amostras tratadas com a formulação ETF2, resultando em uma diminuição de 46% na % SH em comparação com a F2. Novamente, a capacidade de reduzir o corpo da lesão (Δ KHN) foi maior (~ 26%) com ETF2 em relação a F2, e F2 proporcionou a maior concentração de F, Ca e P na superfície do

esmalte. Entre as formulações de enxaguatório bucal ETF2, TF2 e E, as maiores taxas de redução de células viáveis foram exibidas tratando o biofilme com ETF2 por 10 minutos, independentemente do microrganismo testado. Em conclusão, a adição de PPE (3%) em enxaguatórios bucais contendo TMP (0,3) e F (225ppm) promoveu uma diminuição considerável no mineral sem perda de esmalte dental, além de reduzir consideravelmente o biofilme cariogênico formado por *S. mutans* e *C albicans*. Assim, cria uma perspectiva promissora para o desenvolvimento de um produto comercial dental sem álcool com os benefícios de milênios reconhecidos à saúde do *Punica granatum*.

Palavras-chave: *Punica granatum*; polifosfatos; desmineralização, esmalte dental, fluoreto, antimicrobiano.

Abstract

FERNANDES, G.L. ***Punica granatum* peel extract enhances the anti caries effect of mouthwash containing sodium trimetaphosphate and fluoride.** 2020 80f. Tese (Doutorado em Ciência Odontológica, área de Biomateriais) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2019.

Dental caries is among the main and most common oral diseases. It is caused by acids produced by microbial biofilm that lead to enamel demineralization. The prevention and control of this chronic disease consist of periodic disorganization of the biofilm and the promotion of dental remineralization. To address this problem, we associate *Punica granatum* (pomegranate) peel extract (PPE) with sodium trimetaphosphate (TMP), and fluoride (F) in formulations for being used as mouthwash, and evaluate its efficacy on reducing dental enamel demineralization process as well as its antibiofilm potential against important oral pathogens present in dental caries (*Streptococcus mutans* ATCC 25175 and *Candida albicans* ATCC 10231). Bovine enamel blocks (4 mm × 4 mm) selected by initial surface hardness (SHi) were randomly allocated according to groups of formulation treatments (n= 12/group): ETF1 (3.0%PPE+0.2%TMP+100ppmF), TF1 (0.2%TMP+100ppmF), ETF2 (3.0%PPE+0.3%TMP+225ppmF), TF2 (0.3%TMP+225ppmF), F1 (100 ppmF), F2 (225 ppmF), and P (formulation without E/T/F - placebo). The blocks were treated 2×/day with each formulation and submitted to five pH cycles (demineralizing/remineralizing solutions) at 37°C. Next, final surface hardness (SHf), integrated loss subsurface hardness (Δ KHN), and enamel fluoride (F) calcium (Ca) and phosphorus (P) concentrations were determined. The percentage of surface hardness loss (%SH) was calculated ($\%SH = [(SHf - SHi)/SHi] \times 100$), and the formulations which promoted lower percentages of enamel demineralization (%SH) and its counterparts were selected to the antibiofilm assays, as well as formulation containing only PPE (E). For that, ETF2 and TF2 (%SH= -34.5% and -53.1% respectively), and formulation E were used to treat for 1 or 10 minutes dual biofilms of *C. albicans* and *S. mutans* grown for 24 hours on hydroxyapatite discs (HA). Demineralization of the enamel surface was lower in samples treated with formulation ETF2, resulting in a 46% decrease in %SH in comparison with F2. Again, the capacity to reduce the lesion body (Δ KHN) was higher (~ 26%) with ETF2 in relation to F2, and F2 provided the highest concentration of F, Ca and P in enamel surface. Amongst the mouthwash formulations ETF2, TF2 and E, the highest rates of viable cells reduction were exhibited by treating biofilm with ETF2 for 10 minutes regardless of the microorganism tested. In

conclusion, the addition of PPE (3%) in mouthwashes containing TMP (0.3) and F (225ppm) promoted a considerably decrease in the mineral loss of dental enamel besides considerable reducing cariogenic biofilm formed by *S. mutans* and *C. albicans*. It thus creates a promising prospect for the development of an alcohol free dental commercial product with the millennial recognized health benefits of *Punica granatum*.

Keywords: *Punica granatum*; Polyphosphates; Desmineralization; Dental Enamel; Antimicrobial

Introdução Geral

Dentre as principais e mais comuns infecções orais estão a cárie dentária, a

gingivite e a periodontite, e uma característica comum nessas patologias é a presença de microrganismos que estão diretamente relacionados com o desenvolvimento ou persistência dessas infecções. O controle ou a remoção desses microrganismos através da escovação dental, utilização de fio dental e enxaguatórios bucais, auxiliam no controle e prevenção dessas doenças (Batista, Lins et al. 2014)

Muitas revisões têm abordado a utilização de enxaguatórios bucais como uma alternativa benéfica para o controle de biofilmes microbianos relacionados à carie (Bhadbhade, Acharya et al. 2011) devido a facilidade de utilização, acesso a microrganismos não alcançadas por processos mecânicos e baixo custo. Além disso, os enxaguatórios bucais podem ser uma medida tópica para aplicação de flúor devido ao contato com os dentes, realizando um efeito protetor (Favretto, Danelon et al. 2013). Dentre os agentes químicos utilizados está a clorexidina que é considerada como padrão ouro. Contudo, sua prescrição deve ser limitada a curtos períodos de tempo por poderem provocar pigmentação dos dentes e língua, alterações no paladar, descamação epitelial, sensações de queimação e sabor amargo (Bhadbhade, Acharya et al. 2011); (Dabholkar, Shah et al. 2016). Além da clorexidina, os óleos essenciais são muito utilizados, porém o alto teor alcoólico e o sabor desagradável muitas vezes não são bem tolerados pelos pacientes (Bhadbhade, Acharya et al. 2011); (Dabholkar, Shah et al. 2016).

O aumento na resistência dos microrganismos aos tratamentos convencionais e efeitos colaterais causados por esses produtos associados a uma maior abertura da medicina tradicional a novas alternativas de tratamento, vem fazendo com que a pesquisa e o desenvolvimento de produtos utilizando compostos naturais cresça consideravelmente (Bhadbhade, Acharya et al. 2011); (Akca, Akca et al. 2016).

A romã tem sido utilizada no Brasil no tratamento de infecções de garganta, rouquidão, febre, anti-séptico e antiviral em processos inflamatórios da mucosa oral

(Matos, Garland et al. 2002). A sua casca contém aproximadamente 20% de taninos ativos, incluindo punicalagina, punicalina e corilagina (Menezes, Cordeiro et al. 2006). Além disso, seu efeito benéfico em processos inflamatórios como a gengivite pode estar relacionado com a presença de flavonoides que possuem uma alta atividade antioxidante e estimulam enzimas endógenas com essa mesma função (Bhadbhade, Acharya et al. 2011). Estudos mostram que a romã apresenta atividade antimicrobiana contra uma variedade de microrganismos presentes no biofilme dental, incluindo o *Streptococcus mutans* (Bhadbhade, Acharya et al. 2011). Acredita-se que a ação da romã relaciona-se com a redução da capacidade de adesão desses microrganismos na superfície do dente, e da produção de enzimas responsáveis pelo metabolismo da sacarose, dificultando assim o desenvolvimento da cárie (Bhadbhade, Acharya et al. 2011). Além disso, bochechos com extrato da romã reduziram os níveis salivares de outras enzimas como, por exemplo, a aspartato-aminotransferase, que está relacionada com lesões celulares e é encontrada em altos níveis em pacientes com doença periodontal (Bhadbhade, Acharya et al. 2011).

Devido a sua facilidade de uso, a literatura sugere que além de agentes antimicrobianos, os enxaguatórios bucais contenham outros componentes para auxiliar no controle do desenvolvimento de patologias bucais. Nesse contexto a associação de flúor e fosfatos inorgânicos como o trimetafosfato de sódio (TMP) atuaria na redução do processo de desmineralização presente na cárie dentária. Já é demonstrado que a adição de TMP a uma solução fluoretada otimiza a capacidade do flúor na redução da desmineralização do esmalte (Takeshita, Castro et al. 2009); (Moretto, Magalhaes et al. 2010); (Manarelli, Vieira et al. 2011); (Takeshita, Exterkate et al. 2011); (Favretto, Danelon et al. 2013), e isso ocorreria provavelmente devido a capacidade de se ligarem e permanecerem ligados à superfície do esmalte modificando a permeabilidade da

mesma aos íons cálcio, flúor e aos ácidos presentes nos desafios cariogênicos (McGaughey and Stowell 1977); (van Dijk, Borggreven et al. 1980); (Takeshita, Exterkate et al. 2011). Sendo assim, pensando-se nas propriedades da romã e desse fosfato juntamente com o fluoreto de sódio, o desenvolvimento de um enxaguatório bucal contendo esses compostos poderia contribuir consideravelmente na prevenção e controle da doença cárie.

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Lista de Abreviaturas e Símbolos

LISTA DE ABREVIATURAS E SÍMBOLOS

UNESP	Universidade Estadual Paulista
PPE	<i>Punica granatum</i> peel extract
TMP	Sodium trimetaphosphate
F	Fluoride
NaF	Sodium fluoride
ANVISA	Agência Nacional de Vigilância Sanitária
CETESB	Companhia Ambiental do Estado de São Paulo
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
FDA	Food and Drug Administration
GL	Gay Lussac
pH	Potencial Hidrogeniônico
SHi	Initial surface hardness
SHf	Final surface hardness
KHN	Integrated loss subsurface hardness
Ca	Calcio
P	Fosforo
ppm	Partes por milhão
DE	Desmineralizador
RE	Remineralizador
HCl	Ácido clorídrico
NaOH	Hidroxido de sódio
SD	Standard Deviation
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CFU(s)	Colony forming unit (s)
CO₂	Carbon dioxide
h	Hora/Hour
min	Minuto/Minute
mL	Mililitro/Milliliter
nm	Nanômetro/Nanometer
°C	Graus Celsius/Degrees Celsius
SAB	Sabouraud dextrose
UFC(s)	Unidades Formadoras de Colônias
Mm³	Milímetro cúbico
µg	Micrograma

Lista de Abreviaturas e Símbolos

μL	Microlitro
μm	Micrômetro
μM	Micromolar
Rpm	Rotações por minute
NaCl	Cloreto de sódio
CaCl₂	Cloreto de cálcio
KCl	Cloreto de potássio
HA	Hidroxiapatita
CO₂	Dióxido de carbono

Sumário

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***Punica granatum* peel extract enhances the anti caries effect of mouthwash containing sodium trimetaphosphate and fluoride**

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*De acordo com as instruções aos autores do periódico *Phytotherapy Research*

Abstract

Dental caries is among the main and most common oral diseases. It is caused by acids produced by microbial biofilm that lead to enamel demineralization. The prevention and control of this chronic disease consist of periodic disorganization of the biofilm and the promotion of dental remineralization. To address this problem, we associate *Punica granatum* (pomegranate) peel extract (PPE) with sodium trimemphosphate (TMP), and fluoride (F) in formulations for being used as mouthwash, and evaluate its efficacy on reducing dental enamel demineralization process as well as its antibiofilm potential against important oral pathogens present in dental caries (*Streptococcus mutans* ATCC 25175 and *Candida albicans* ATCC 10231). Bovine enamel blocks (4 mm × 4 mm) selected by initial surface hardness (SH_i) were randomly allocated according to groups of formulation treatments (n= 12/group): ETF1 (3.0%PPE+0.2%TMP+100ppmF), TF1 (0.2%TMP+100ppmF), ETF2 (3.0%PPE+0.3%TMP+225ppmF), TF2 (0.3%TMP+225ppmF), F1 (100 ppmF), F2 (225 ppmF), and P (formulation without E/T/F - placebo). The blocks were treated 2×/day with each formulation and submitted to five pH cycles (demineralizing/remineralizing solutions) at 37°C. Next, final surface hardness (SH_f), integrated loss subsurface hardness (ΔKHN), and enamel fluoride (F) calcium (Ca) and phosphorus (P) concentrations were determined. The percentage of surface hardness loss (%SH) was calculated ($\%SH = [(SH_f - SH_i)/SH_i] \times 100$), and the formulations which promoted lower percentages of enamel demineralization (%SH) and its counterparts were selected to the antibiofilm assays, as well as formulation containing only PPE (E). For that, ETF2 and TF2 (%SH= -34.5% and -53.1% respectively), and formulation E were used to treat for 1 or 10 minutes dual biofilms of *C. albicans* and *S. mutans* grown for 24 hours on hydroxyapatite discs (HA). Demineralization of the enamel surface was lower in samples treated with formulation ETF2, resulting in a 46% decrease in %SH in comparison with F2. Again, the capacity to reduce the lesion body (ΔKHN) was higher (~ 26%) with ETF2 in relation to F2, and F2 provided the highest concentration of F, Ca and P in enamel surface. Amongst the mouthwash formulations ETF2, TF2 and E, the highest rates of viable cells reduction were exhibited by treating biofilm with ETF2 for 10 minutes regardless of the microorganism tested. In conclusion, the addition of PPE (3%) in mouthwashes containing TMP (0.3) and F (225ppm) promoted a considerably decrease in the mineral loss of dental enamel

besides considerable reducing cariogenic biofilm formed by *S. mutans* and *C. albicans*. It thus creates a promising prospect for the development of an alcohol free dental commercial product with the millennial recognized health benefits of *Punica granatum*.

Keywords: *Punica granatum*; Polyphosphates; Desmineralization; Dental Enamel; Antimicrobial

1 INTRODUCTION

Dental caries is the most common polymicrobial oral disease in the world (Philip and Walsh 2019) and although knowledge about caries increases, researchers and dentists still struggle for better alternatives for the prevention and treatment of this disease (Cheng, Li et al. 2015). The mouth has a polymicrobial flora composed of bacteria and yeast that play an important role in the fermentation of sugar in acids that lead to enamel demineralization. The main microorganisms involved in this process are *Streptococcus sp.*, *Staphylococcus sp.*, *Lactobacillus sp.* and *Candida sp* (Takahashi and Nyvad 2011, Emerenciano, Botazzo Delbem et al. 2018, Hemani and Gheenias 2018 , Arifa, Ephraim et al. 2019).

The prime mode of preventing the development and progression of dental caries are: dental brushing, flossing, and the use of mouthwashes (Megalaria, Thirumurugan et al. 2018, Pinni, Sakar Avula et al. 2018, Dentistry 2018). Mouthwashes have advantages as easy application, antimicrobial and anti-inflammatory action in less accessible regions for brush and floss, as well as for being a topical source of fluoride (Favretto, Danelon et al. 2013, Megalaria, Thirumurugan et al. 2018). Studies showed the effectiveness of sodium fluoride (NaF) solution at 0.02% (Chedid and Cury 2004, Delbem, Tiano et al. 2006, Favretto, Danelon et al. 2013), and to further improve its effectiveness this solution should be acidified (Delbem, Tiano et al. 2006) or supplemented with phosphates (Favretto, Danelon et al. 2013). It has been proved that the addition of sodium trimetaphosphate (TMP) can optimize the ability of fluoride (F) to reduce enamel demineralization, and, this action would be related to the ability of this phosphate to bind the dental surface, changing its permeability to calcium and fluoride

ions (Takeshita, Castro et al. 2009, Moretto, Magalhaes et al. 2010, Manarelli, Vieira et al. 2011, Takeshita, Exterkate et al. 2011, Favretto, Danelon et al. 2013).

Listerine[®] is a commercial mouthwash option, however studies show that the presence of alcohol in these solutions can be irritating to the gums and cheeks (El-Sharkawy, Mostafa et al. 2019, making the use uncomfortable for the patient, in addition, there are few studies in the literature on the effectiveness of Listerine[®] without alcohol (Guandalini Cunha, Duque et al. 2020).

In this sense, nature has enormous plant source with medicinal properties, including *Punica granatum*. Considered "a pharmacy unto itself," this fruit features important properties such as antimicrobials, anti-inflammatories, astringents, and antioxidant action, and with no significant toxic effect. (Ahuja, Dodwad et al. 2011, Kukreja and Dodwad 2012). *Punica granatum* has been related as being beneficial in reducing recurrent aphthous stomatitis pain and reducing the time of complete healing (Ghalayani, Zolfaghary et al. 2013), as a preventive and therapeutic aid to periodontal disease (Hajimahmoodi, Shams-Ardakani et al. 2011, Prasad and Kunnaiah 2014) and as presenting antifungal activity against *Candida* spp. (Endo, Cortez et al. 2010). Plants might use multiple strategies to deal with microorganisms that they are evolved over time (Abreu, Coqueiro et al. 2017). For this reason, the secondary their metabolites represent a large library of compounds that could potentiate the effects of known antibiotics, and can be important sources for new drugs or compounds suitable for further modification (Lu et al., 2017). The main chemical compounds present in *Punica garantum* are: eicosanoic, linolenic conjugated, linolenic alpha, oleic, palmitic, punicic, stearic, citric and malic acids, phenolic compounds such as gallic, coumaric acid, catechin, phloridzin and quercetin, protocatechuic, chlorogenic, cafeic, and ferulic

acid(Ahmadiankia 2019). According to Al Obaidi et al, 2017, ellagic acid, ellagitannins (including punicalagin), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones are the compounds with greater therapeutic activity (Al-obaidi, Muhsin et al. 2017).

Regardless of the antimicrobial and antiinflammatory effects of *Punica granatum* have been well known in the literature, its action on the demineralization dental process when associated with TMP and F need to be investigated. Furthermore, considering the health risks lead by prolonged use of chemicals in the conventional mouthwashes, the benefices of the bioactive compounds present in plants in preventing oral and dental diseases, as well as the remarkable increase in interest of the population in herbal medicine, the present study determined the anti caries effect of non-alcoholic mouthwashes produced with pomegranate peel extract, TMP and low concentrations of fluoride.

2 MATERIAL AND METHODS

2.1 Plant material and extraction procedure

Punica granatum (pomegranate) peel (dehydrated, crushed and sterilized) was obtained from a single allotment of the Company Santos Flora (Santosflora Com. De Ervas, Mairiporã, SP - Brazil). The product has certificates from regulatory agencies ANVISA, CETESB, IBAMA and FDA, in addition to pharmacopoeal and microbiological analyzes. According to the characteristics of pomegranate peel, the maceration process was used for the extraction process. Ethanol 70% GL (70° GL) was used in a 1:3 ratio for 24 hours. This mixture was vacuum filtered, separating the crude extract and draff. In this draff, ethanol 70° GL was added again, and the maceration

process was accomplished again. This process was repeated five times until no soluble solids are present in the extract (“dry matter”), indicating possible drug exhaustion by the extractor process. These 5 fractions were mixed and concentrated in a vacuum rotoevaporator until the soft extract was obtained. Subsequently, the soft extract was diluted in propyleneglycol, and characterized as the solids content (oven drying) where it was standardized in % solids (30.1%).

2.2 Preparing the mouthwash formulations

The formulations were standardized according to their active principle in: 3% PPE (E), 0.2 or 0.3% sodium trimetaphosphate (TMP), 100 or 225 ppm of fluoride (F), besides these, were also used stabilizers, microbiological preservatives, chelators, sweeteners, humectant and water and all formulations had their pH adjusted to 7.0 (Table 1).

Formulations ETF1(3% PPE + 0.2% TMP + 100 ppm F), TF1 (0.2% TMP + 100 ppm F), ETF2 (3% PPE + 0.3% TMP + 225 ppm F), TF2 (0.3% TMP + 225 ppm F), F1 (100 ppm F), F2 (225 ppm F) and P (placebo) were subjected to a pH cycling test and the solutions with best performing were tested in mixed biofilm models of *C. albicans* and *S. mutans*. in addition to these formulations, the formulation containing only 3% pomegranate peel extract (E) and Listerine[®] (alcohol-free) were also tested on these microorganisms (Figure 1).

2.3 Total phenolic quantification by colorimetric assay

To establish the total phenolics present in formulations containing *Punica granatum* peel extract (PPE), an analytical curve of gallic acid was carried out

(Waterman and Mole 1994, Fernandes, Berretta et al. 2018). All formulations and a standard solution of gallic acid were prepared in a 50 mL volumetric flask using water as a solvent. The formulations remained in an ultrasonic bath for 30 minutes. A 0.5 mL aliquot was transferred to another 50 mL flask where 2.5 mL of Folin-Denis reagent (Qhemis-High Purity, Hexis, São Paulo, Brazil) and 5.0 mL of 29% sodium carbonate (Cinética, São Paulo, Brazil) were added. The solutions were protected from light and the readings were performed after 30 min in a UV-Vis spectrophotometer at 760 nm (de Oliveira, de Castro et al. 2013).

2.4 Experimental design - pH cycling

Enamel blocks (4×4 mm, $n = 84$) of bovine incisors were stored in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. The enamel surfaces of the blocks were sequentially polished and selected by an initial surface hardness (SHi) test of the total of blocks and the trust interval and then randomized (320.0 to 380.0 KHN) into 7 groups ($n = 12$ per group) (Figure 1): formulation containing 3.0% PPE + 0.2% TMP + 100ppm F (ETF1), 0.2%TMP+100 ppm F (TF1) 3.0%PPE+0.3%TMP+225 ppm F (ETF2), 0.3%TMP+225 ppm F (TF2), 100 ppm F (F1), 225 ppm F (F2), and formulation without F/TMP/PPE (P), (Table 1). The enamel blocks were submitted to a pH cycling for five days and treated with each formulation twice a day (1 minute), totaling seven days. After this, the blocks were submitted to a final surface hardness (SHf), percentage of surface hardness loss (%SH), subsurface hardness integrated loss (Δ KHN), fluoride (F), calcium (Ca) and phosphorus (P) in the enamel.

2.4.1 Determination of fluoride in solutions

The F concentration in the solution was determined using a specific electrode for the F ion (9609 BN; Orion Research Inc., Beverly, MA, USA) attached to an ion analyzer (Orion 720 Aplus; Orion Research Inc., Beverly, MA, USA) and calibrated with standards containing 0.25 to 4.00 ppm F. Firstly, 1.0 mL of each product was dissolved in deionized water and transferred to a volumetric flask. The volume was then adjusted to 100 mL using deionized water. For each product, 3 dilutions were made. Subsequently, 2 samples of 1 mL were buffered with total ionic strength adjustment buffer (TISAB II) (Delbem, Sasaki et al. 2003). The pH levels of the solutions were determined with a pH electrode (2A09E, Analyser, São Paulo, Brazil) that was calibrated with standard pH levels of 7.0 and 4.0 (Danelon, Takeshita et al. 2014)

2.4.2 Experimental solutions

The blocks from each group were subjected to five pH cycles at 37°C during a procedure that lasted seven days (Vieira, Delbem et al. 2005). The blocks were immersed in a demineralizing solution (DE: 2.0 mmol/L calcium and phosphate in 75 mmol/L acetate buffer, pH 4.7; 0.04 µg F/mL; 2.2 mL/mm²). After 6 hours, the blocks were transferred to a remineralizing solution (RE: 1.5 mmol/L calcium, 0.9 mmol/L phosphate, and 150 mmol/L KCl in 0.1 mol/L cacodylic buffer, pH 7.0; 0.05 mg F/mL; 1.1 mL/mm²) for 18 hours. Deionized water rinses were performed between each step. The treatment regimen consisted of 60 seconds soak in 1 mL/block of solutions under agitation on a rotatory shaker, before the solution changes from DE (demineralizing) to RE (remineralizing) and from RE to DE (twice a day). Deionized water rinses were done between every step. In the last two days the blocks were kept in remineralizing solution.

2.4.3 Hardness measurements

The hardness of the enamel surface was determined using a microhardness tester (Shimadzu HMV-2000e) and a Knoop diamond under a 25 g-load for 10 seconds. Five indentations spaced 100 μm apart were made at the center of the initial surface hardness determination (SHi). After the pH cycling, final surface hardness (SHf) was calculated by producing five other indentations (100 μm from the baseline indentations). The percentage of surface hardness loss (%SH) was calculated using the following formula: $\%SH = [(SHf - SHi)/SHi] \times 100$. For the analysis of longitudinal hardness, a section was made in the center of each block and one of the halves included in acrylic and polished resin. Micromet 5114 hardness tester microdurometer (Buehler, Lake Bluff, USA) and Buehler Omni Met software (Buehler, Lake Bluff, USA) were used, loading 5 g for 10 s. A sequence of 14 impressions at distances of 5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 and 330 μm from the outer surface of the enamel was performed in the central area of the blocks (Spiguel, Tovo et al. 2009). The integrated hardness area (KHN $\times \mu\text{m}$) from the lesion to the hard enamel will be calculated using the trapezoidal rule (Graph Pad Prism, version 3.02) and subtracted from the hard enamel hardness integrated area to obtain the integrated loss of subsurface hardness (Δ KHN).

2.4.4 Analysis of F, Ca e P concentration in the enamel

Blocks (n=12/per group, 2 mm \times 2 mm) were obtained from the halves of the original 4 mm \times 4 mm specimens not embedded, and fixed with adhesive glue on a mandrel for straight. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit

silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of $50.0 \pm 0.03 \mu\text{m}$ each enamel block was removed. The vials, after the addition of 0.5 mL HCl 1.0 mol/L, were kept under constant stirring for 1 hour (Weatherell, Robinson et al. 1985) modified by (Alves, Pessan et al. 2007). For F analysis, specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, MA, USA) was used. The electrodes were calibrated with standards containing from 0.25 to 4.00 $\mu\text{g F/mL}$ (100 ppm F, Orion 940907), under the same conditions as the samples. The readings were conducted using 0.25 mL of the biopsy solution, buffered with the same volume of TISAB II modified with 1.0 mol/L NaOH (Akabane, Delbem et al. 2018). The results were expressed in $\mu\text{g/mm}^3$. Calcium (Ca) analysis was performed using the Arsenazo III colorimetric method (Vogel, Chow et al. 1983). The absorbance readings were recorded at 650 nm by using a plate reader (PowerWave 340, Biotek, Winooski, VT, USA). Phosphorus (P) was measured according to (Fiske and Subbarow 1925), and the absorbance readings were recorded at 660 nm. The results were expressed as $\mu\text{g/mm}^3$.

2.5 Antibiofilm activity

2.5.1 Artificial saliva

Artificial saliva used in this experiment was based on the protocol of Lamfon et al, (2013) and is composed by: 1L of deionized water was as follows: 2g yeast extract (Sigma-Aldrich), 5g bacteriological peptone (Sigma-Aldrich), 2g glucose (Sigma-Aldrich), 1g mucin from porcine stomach (Sigma-Aldrich), 0.35g NaCl (Sigma-Aldrich),

0.2g CaCl₂ (Sigma-Aldrich) and 0.2g KCl (Sigma-Aldrich). The final pH was adjusted to 6.8 using NaOH (Sigma-Aldrich) (Lamfon, Porter et al. 2003).

2.5.2 Microorganism strains and growth conditions

Two reference strains of American Type Culture Collection (ATCC) were used in this study: *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175. The strains were grown likewise as described by Arias et al, (2016). *Candida albicans* cell quantities were adjusted using a Neubauer chamber and resuspended in saliva at 1×10^7 cells ml⁻¹ and *Streptococcus mutans* was adjusted by spectroscopy (Optical density_{640 nm}=1.6). at 1×10^8 cells (Arias, Delbem et al. 2016).

2.5.3 Preparation of hydroxyapatite discs (HA)

The HA discs were made and characterized according to Arias et al, (2016), 0.650 g of HA powder (Sigma-Aldrich) was added to a stainless steel die (SKAY, São José do Rio Preto, Brazil) and taken to a hydraulic press where it was subjected to a load of 25 t, with this process, discs were 13 mm diameter and 3 mm of thickness were obtained (Arias, Delbem et al. 2016). Then, the disks were sterilized using ethylene oxide (Oximed – Tecnologia em esterelização – São José do Rio Preto, SP – Brazil).

2.5.4 Biofilm formation

According to Pandit et al, (2018), the discs were positioned vertically on supports made with orthodontic wires in 24-well plates (Pandit, Jung et al. 2018). Before contacting a mixed biofilm of *Candida albicans* and *Streptococcus mutans*, the discs were conditioned in 2 mL of artificial saliva for 1 hour incubated in 37°C at 5% CO₂. Then, the saliva was removed and 2 ml of mouthwashes ETF2, TF2, E and Listerine® (positive control) were added and kept in contact with the discs for 1 minute.

The discs were washed in saline solution, and subsequently 2 ml of the dual-inoculum (*C. albicans* and *S. mutans*) was added to the HA discs which were previously conditioned with each mouthwash. After, the 24-well plates were incubated (37°C; 5% CO₂) for 24 hours and treated for 1 minute and 10 minutes with mouthwashes.

2.5.5 Number of cultivable cells (CFU)

The number of cultivable cells present in the mixed biofilm formed on the HA discs was quantified by the number of colony forming units (CFU). HA discs were added into falcon tubes containing 3 mL of saline solution, then tubes were taken to an ultrasonic bath (Elmasonic p 30 H, Elma Schmidbauer GmbH, Baden-Württemberg, Germany) for 10 minutes and next, vortexed for 30 seconds and serial decimal dilutions (saline solution) were plated on CHROMagar Candida (Difco) and BHI agar (BHI; Difco) supplemented with amphotericin B at 7 µg ml⁻¹, respectively, for counting of colonies of *Candida albicans* and *Streptococcus mutans*. After 24 and 48 hours of incubation, the number of viable colonies was quantified and expressed in log¹⁰ and standardized per unit area (Log¹⁰ CFU cm⁻²) of HA discs.

2.5.6 Biofilm pH assessment

The biofilms treated with the mouthwash ETF2; TF2; E and Listerine[®], were transferred to new sterile falcon tubes and centrifuged for 5 minutes at 8000 rpm (Combi – 514, Hanil Scientific Inc., Aayukro – Rep da Korea), then the supernatant was filtered with 0.22 µm membrane filter (Corning Inc., Corning, USA) and subsequently the biofilm pH was measured (pH electrode - 2A09E, Analyser, São Paulo, Brazil).

2.6 Statistical analysis

All assays were performed in triplicate on at least three independent days. The SigmaPlot 12.0 program (Systat Software Inc., San Jose, USA) was employed for the statistical analysis with a confidence level of 95%. The tests were subjected to the normality test (Shapiro-Wilk) and after the appropriate parametric tests for each group were conducted.

3 RESULTS

3.1 Total phenolics in mouthwash formulations

In 1915 Otto Folin and W. Denis (Folin and Denis 1915) were the first to describe a colorimetric method for the determination of phenols and its derivatives in a liquid. It determines free and conjugated phenols, and it is based on the formation of colored complex with metals by the reduction of the phosphotungstic-phosphomolybdic acid reagent in a slightly alkaline medium. The wavelength absorbance of that colored complex formed is measured and related to the concentration of the phenolic mixtures in a plant extract. Table 2 illustrates the mean of total phenols expressed as gallic acid (mg/mg), found in each formulation containing 3% PPE (ETF1, ETF2 and E formulations), using the Folin-Denis colorimetric test. The total phenolics content (mg/g) in each formulation containing PPE was very similar (an average of 11.54 mg/g), and corresponded to 10% of the pomegranate glycolic extract produced by the extraction method described in section 2.1.

3.2 PPE enhances the TMP and F potential in reducing the demineralization of the enamel surface

The pH cycling model involves the exposure of substrates (enamel or dentin) to combinations of demineralization and remineralization experiments, which are designed for the dynamics of loss and gain of minerals involved in the dental caries process. Due to these fundamental advantages, pH cycling models collaborate to improve the understanding of the caries process and the possible mechanisms by which fluoride, phosphate and PPE exert on the anti-caries effect.

The average pH of all mouthwash solutions was adjusted to 7.0. The mean SH of all blocks was 364.6 (SD 9.8) KHN ($p = 0.533$). No significant differences were observed among the groups after random allocation ($p = 0.474$). The use of solution F2 (225F) resulted in a 13% decrease in %SH in comparison with solution F1 (100F) (Table 3). The demineralization of the enamel surface was lower in samples treated with solutions ETF2, resulting in a 46% decrease in %SH in comparison with solution F2 ($p < 0.001$). In addition, the capacity to reduce the lesion body (Δ KHN) was higher (~26%) with the solution ETF2 ($p > 0.001$) when compared to solution F2 ($p < 0.001$) followed by F2 = solution ETF1= solution TF2 > solution ETF1 > solution F1 > solution P ($p < 0.001$) (Table 3).

Similar and high amounts of F were observed for solution F2, ETF2 and TF2 ($p > 0.001$). With solution F2, enamel Ca concentration was increased by ~ 30% when compared to solution F1 ($p < 0.001$). No significant difference was observed among the solution F1, ETF1, ETF2 and TF2 regarding enamel Ca concentrations except for the Placebo (P), which showed a lower concentration ($p < 0.001$). Phosphorus concentrations in enamel were higher for the solution F2 when compared with the solution F1 = solution ETF1 = solution TF1 = solution ETF2 = solution TF2 ($p >$

0.001), and solution P the lowest concentration when compared to the other solutions ($p < 0.001$) (Table 3).

3.3- Mouthwash formulations inhibited biofilm cells proliferation and interfered in the biofilms pH

Pomegranate peel extract associated with TMP and F in mouthwash formulation reduces viable cells of *Candida albicans* and *Streptococcus mutans* biofilm.

In vitro biofilm assays were conducted using reference strains of important oral pathogens involved with dental caries to verify if PPE could act in association with TMP and F inhibiting *C. albicans* and *S. mutans* proliferation cells in biofilms grown for 24 hours on HA discs by exposition of mouthwash formulations. To further determine whether increasing exposure time of the biofilm to mouthwash formulations improved their effectiveness, we treated 24h-biofilm for 1 and 10 minutes. Also, in order to elucidate the effect of mouthwashes in the biofilms environmental, the pH of 24-hours biofilms treated for 1 and for 10 minutes was measured.

As presented in Figure 2 and 3, all mouthwashes significantly reduced the biofilm cells for both *C. albicans* and *S. mutans* respectively. Similarly as the positive control (Listerine®), formulations ETF2, TF2 and E produced an expressive significant reduction of *C. albicans* biofilm cells in comparison with the negative control group ($p < .001$). Although there was no significant difference between 1 and 10 minutes of treatment with ETF2, TF2 and E, the percentages of *C. albicans* biofilm reduction were considerably increased with 10 minutes exposure (Table 4). The results also demonstrated that the *S. mutans* biofilm cells exposed for 10 minutes with formulations ETF2 and TF2 were significantly more susceptible than for 1 minute exposure ($p <$

.001), while for formulation E and positive control, time of exposition had no significant influence on the reduction of *S. mutans* cells ($p > .05$). Among the mouthwash formulations ETF2, TF2 and E, the highest rates of viable cells reduction were exhibited by formulation ETF2 at 10 minutes of treatment regardless of the microorganism tested (Table 4).

As shown in Table 5, the highest pH were found in biofilms which received no treatment (negative control) and those treated with Listerine® (positive control), followed by biofilms treated with E, ETF2 and TF2 mouthwash formulations.

4 DISCUSSION

Known as the most common microbial dental tissue disease, the dental caries, is caused by the lack of dynamic balance between the processes of remineralization and demineralization of the enamel surface. Rich in ions, like calcium and phosphate, saliva has an important role in this process determining that the pH value where demineralization occurs is 5.5, below this reference value, the acids produced by oral biofilms can act in the enamel structure which can lead to an excessive loss of mineral and consequent cavitation (Jyotika, Anil et al. 2019)

The main finding of this study is that association of PPE (3%) with TMP (0.3%) and F (225 ppm) (ETF2) resulted in a 46% decrease in % SH compared to the formulation containing only 225 ppm F (F2) and a 35% reduction in % SH when comparing the TF2 and ETF2, namely, the presence of pomegranate peel extract in association with TMP and F encourages a reduction of mineral loss on the enamel surface.

The action of TMP and fluoride in the anti-caries process is already well established (Favretto, Danelon et al. 2013, Takeshita, Danelon et al. 2015, Danelon, Pessan et al. 2017, Manarelli, Delbem et al. 2017, Akabane, Delbem et al. 2018, Emerenciano, Botazzo Delbem et al. 2018). Studies show that TMP can be adsorbed on the enamel surface, reducing demineralization since this process can hinder acid diffusion and alter the affinity between enamel and saliva proteins (Nordbo and Rolla 1972, Takeshita, Castro et al. 2009, Danelon, Takeshita et al. 2014, Cavazana, Hosida et al. 2019)

However the action of the pomegranate isolated and in association with these active ingredients had not yet been investigated. Although the literature doesn't show studies evaluating the anti-caries pomegranate action, this effect is proven by several other extracts such as: cacao bean, liquorice, brazilian green propolis, tea leaves, nutmeg, and etc, and these studies show that this activity is associated with polyphenols present in extracts. (Onishi, Umemura et al. 2008, Zhang, Xue et al. 2009). The literature clearly shows how pomegranate is rich in polyphenols (as punicalin, punicalagin, gallic and ellagic acid amongst other)(Ismail, Sestili et al. 2012), and also calcium, magnesium, phosphorus, potassium and sodium, mostly found in the peel of this fruit (Esawya, Ragaba et al. 2019). Zhang et al, (2009) evaluated the action of *Galla chinensis* extract on the bovine enamel matrix subjected to acidic challenges, and found that the monomeric and polymeric polyphenols interacted with this organic enamel matrix (through covalent, ionic, hydrogen bonding or hydrophobic processes) (Pierpoint 1969, Loomis 1974, Han, Jaurequi et al. 2003, Zhang, Xue et al. 2009) leading to a metamorphism of this matrix that precipitated and decreased loss of ions in the enamel structure. Another possible action is the binding of the compounds present in the extract

to the crystals surface of enamel thus preventing its demineralization, in addition to facilitating the deposition of more ions on the surface (through ion carrier) (Tian, Li et al. 2009, Kim and Jin 2018). There are also studies showing that gallic acid (present in pomegranate) can function as a calcium ion transporter, favoring the remineralization process (Cheng, Li et al. 2008).

Although the beneficial effects of *Punica granatum* in process of demineralization and remineralization of tooth enamel have still not been evaluated in literature, there are plenty studies that prove its anti-inflammatory, antioxidant and antimicrobial activity (Alkathiri, El-Khadragy et al. 2017, Vucic, Grabez et al. 2019, Baradaran Rahimi, Ghadiri et al. 2020) that are compatible with our results.

The antimicrobial effect of the mixed biofilm formulations of *C. albicans* and *S. mutans* formed for 24 hours and treated for 1 and 10 minutes showed that, although there is no statistical difference between formulations ETF2 TF2 and E, they all significantly reduced the number of viable cells. And formulation ETF2 was superior to the others, except for *S. mutans* with 1 minute of treatment. The mechanism of action of the compounds present in the formulations and the specific characteristics of the microorganisms tested (bacteria and fungus), may have directly influenced these results.

S. mutans is a facultative anaerobic gram-positive bacteria present in dental biofilm and is one of the numerous etiological factors of dental caries (Farkash, Feldman et al. 2019). The virulence factors of these bacteria are related to acidogenic (production of lactic acid mainly), aciduric properties, in addition, these bacteria use sucrose from the diet to synthesize large amounts of extracellular polysaccharides (EPS) mostly glucans synthesized by glucosyltransferases (Brighenti, Luppens et al. 2008,

Gulube and Patel 2016, Cavazana, Hosida et al. 2019, Farkash, Feldman et al. 2019, Philip, Leishman et al. 2019).

C. albicans, on the other hand, is an opportunistic pathogenic fungus (Kim and Sudbery 2011, Farkash, Feldman et al. 2019) and its virulence is related to the transition from its yeast form to hyphae (Finkel and Mitchell 2011). This fungus is known to be a colonizer of caries lesions (Philip, Leishman et al. 2019) and also has the ability to produce acids and EPS (Farkash, Feldman et al. 2019).

The symbiotic relationship between *S. mutans* and *C. albicans* enables the formation of an improved biofilm *in vitro* and *in vivo*.(Farkash, Feldman et al. 2019, Philip, Leishman et al. 2019).

The phenolic compounds present in the extracts are responsible for the antimicrobial activity (Brighenti, Luppens et al. 2008). In general, the antimicrobial activity of *Punica granatum* peel extracts might be due to the presence substantial quantities of phytochemicals like flavonoids (such as Quercetin, Rutin, Naringenin, Luteolin, Pelargonidin, Prodelphinidin, Kaempferol, and Flavan) (Shafiqhi, Amjad et al. 2012), and tannins hydrolyzable (including methyl gallate, pedunculagin, punicalin, , punicalagin, gallic and ellagic acid). These phytochemicals, can act on the cell wall and cell membrane of microorganisms since they can precipitate proteins, in addition to inhibiting enzymes such as glycosyltransferase, making hard the adhesion of microorganisms. (Ismail, Sestili et al. 2012, Aravindraj, M. Preethi et al. 2017).

Scalbert in 1991 showed that tannins have antimicrobial activity against fungi and bacteria (Scalbert 1991), Koo et al. (2006) observed that cranberry juice inhibited the activity of the enzyme glycosyltransferase in biofilms of *S. mutans* (Koo, Nino de Guzman et al. 2006). Brighenti et al. (2007) observed through proteomic studies that the

P. cattleianum extract inhibited proteins responsible for RNA synthesis, and in *S. mutans* was observed the reduction of seven important proteins in carbohydrate metabolism and lactic acid production, this mechanism is directly linked to the development of dental caries (Brighenti, Luppens et al. 2008). Farkash et al. (2019) observed that the combination of polyphenols from Tibetan medicine and green tea has the ability to inhibit the growth of *S. mutans* and *C. albicans* by reducing the number of cells and the production of EPS (Farkash, Feldman et al. 2019).

Vasconcelos et al. (2006) who tested a gel formulated with *Punica granatum* in *Candida albicans* and *Streptococcus mutans* (isolated and combined) and observed a significant action against *Candida albicans*, but not as higher as the commercial antifungal Miconazole.(Vasconcelos, Sampaio et al. 2006). Endo et al. (2010) also demonstrated a strong activity of *Punica granatum* crude extract against *C. albicans*, showing through transmission electron microscopy morphological changes on the cells such as irregular budding patterns and pseudohyphae, thickening of the cell wall, changes in the space between the cell wall and the plasma membrane, as well as a reduction in the cytoplasmic content (Endo, Cortez et al. 2010). Gulube and Patel (2016) showed that *P. granatum* extract can affect the production of acid and EPS in a biofilm of *S. mutans* and these results did not harm the balance of the oral microbiota (Gulube and Patel 2016) .

In addition, the association of TMP and F with the extract may have contributed to the antimicrobial action. Cavazzana et al, (2019) showed that TMP and F reduced the number of viable *S. mutans* cells, and TMP (0.25% without F) decreased the total biomass and extracellular matrix components of a *C. albicans* and *S.mutans* biofilm (Cavazana, Hosida et al. 2019).

Another interesting data found in our study is that the pH of biofilms after treatment for 1 and 10 minutes were acidic, mainly for biofilms treated with formulations ETF2 and TF2, and even with these values we can observe reductions between 1 and 2 logs for the biofilms of *C. albicans* and *S. mutans* when treated with the developed formulations. These pH values were also found by other authors when the biofilms were treated with extracts and F. Pandit et al. (2018) where a biofilm of *S. mutans* formed for 22 hours and treated for one minute with 100 and 300 ppm of F (Pandit, Jung et al. 2018). Brighenti et al. (2008) evaluated the evaluated the pH below 5.0 for a biofilm of *S. mutans* 30 minutes after the start of measurements (Brighenti, Luppens et al. 2008).

In addition, to the scarcity of studies proving the effectiveness of Listerine[®] without alcohol, Guandalini et al. (2020) showed that Listerine[®] without alcohol was diluted four times to avoid being considered toxic in HaCaT epithelial cells (Guandalini Cunha, Duque et al. 2020). Park et al. (2014) also showed that Listerine[®] without alcohol reduced the cell viability of stem cells of the buccal adipose. Unlike Listerine[®] (Park, Lee et al. 2014), the extract of *P. granatum* used for the development of the formulations did not show a cytotoxic effect on L929 fibroblast cells (unpublished data).

In conclusion, the addition of PPE (3%) in mouthwashes containing TMP (0.3) and F (225ppm) promoted a considerably decrease in the mineral loss of dental enamel besides substancial reducing cariogenic biofilm formed by *S. mutans* and *C. albicans*. It thus creates a promising prospect for the development of an alcohol free dental commercial product with the millennial recognized health benefits of *Punica granatum*.

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DECLARATION OF CONFLICTS OF INTEREST

We declare no conflicts of interest.

5 REFERENCES

- Abreu, A. C., A. Coqueiro, A. R. Sultan, N. Lemmens, H. K. Kim, R. Verpoorte, W. J. B. van Wamel, M. Simoes and Y. H. Choi (2017). "Looking to nature for a new concept in antimicrobial treatments: isoflavonoids from *Cytisus striatus* as antibiotic adjuvants against MRSA." Sci Rep **7**(1): 3777.
- Ahmadiankia, N. (2019). "Molecular targets of pomegranate (*Punica granatum*) in preventing cancer metastasis." Iran J Basic Med Sci **22**(9): 977-988.
- Ahuja, S., V. Dodwad, B. J. Kukreja, P. Mehra and P. Kukreja (2011). "A comparative evaluation of efficacy of *Punica granatum* and chlorhexidine on plaque and gingivitis." Journal of the international Clinical Dental Research Organization **3**(1): 29-32.
- Akabane, S., A. C. Delbem, J. Pessan, L. Garcia, N. Emerenciano, D. F. Goncalves and M. Danelon (2018). "In situ effect of the combination of fluoridated toothpaste and fluoridated gel containing sodium trimetaphosphate on enamel demineralization." J Dent **68**: 59-65.
- Akca, A. E., G. Akca, F. T. Topcu, E. Macit, L. Pikdoken and I. S. Ozgen (2016). "The Comparative Evaluation of the Antimicrobial Effect of Propolis with Chlorhexidine against Oral Pathogens: An In Vitro Study." Biomed Res Int **2016**: 3627463.
- Al-obaidi, D. M., S. A. Muhsin and A. A. Ibrahim (2017). "In Vivo Antimicrobial Inhibition Of *Punica Granatum* Extracts As Mouthwash." Russian Open Medical Journal **6**(4): 1-5.
- Alkathiri, B., M. F. El-Khadragy, D. M. Metwally, E. M. Al-Olayan, M. A. Bakhrebah and A. E. Abdel Moneim (2017). "Pomegranate (*Punica granatum*) Juice Shows Antioxidant Activity against Cutaneous Leishmaniasis-Induced Oxidative Stress in Female BALB/c Mice." Int J Environ Res Public Health **14**(12).
- Alves, K. M., J. P. Pessan, F. L. Brighenti, K. S. Franco, F. A. Oliveira, M. A. Buzalaf, K. T. Sasaki and A. C. Delbem (2007). "In vitro evaluation of the effectiveness of acidic fluoride dentifrices." Caries Res **41**(4): 263-267.
- Aravindraj, S., M. M. Preethi and B. Sivapathasundharam (2017). "Antimicrobial Effects of *Punica granatum* Extracts on *Staphylococcus aureus*, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Enterococcus faecalis* and *Candida albicans*." International Journal of Current Microbiology and Applied Sciences **6**(9): 2762-2774
- Arias, L. S., A. C. Delbem, R. A. Fernandes, D. B. Barbosa and D. R. Monteiro (2016). "Activity of tyrosol against single and mixed-species oral biofilms." J Appl Microbiol **120**(5): 1240-1249.
- Arifa, M. K., R. Ephraim and T. Rajamani (2019). "Recent Advances in Dental Hard Tissue Remineralization: A Review of Literature." Int J Clin Pediatr Dent **12**(2): 139-144.
- Baradaran Rahimi, V., M. Ghadiri, M. Ramezani and V. R. Askari (2020). "Anti-inflammatory and anti-cancer activities of pomegranate and its constituent, ellagic acid: Evidence from cellular, animal, and clinical studies." Phytother Res.
- Batista, A. L., R. D. Lins, R. de Souza Coelho, D. do Nascimento Barbosa, N. Moura Belem and F. J. Alves Celestino (2014). "Clinical efficacy analysis of the mouth rinsing with pomegranate and chamomile plant extracts in the gingival bleeding reduction." Complement Ther Clin Pract **20**(1): 93-98.
- Bhadbhade, S. J., A. B. Acharya, S. V. Rodrigues and S. L. Thakur (2011). "The antiplaque efficacy of pomegranate mouthrinse." Quintessence Int **42**(1): 29-36.
- Brighenti, F. L., S. B. Luppens, A. C. Delbem, D. M. Deng, M. A. Hoogenkamp, E. Gaetti-Jardim, Jr., H. L. Dekker, W. Crielaard and J. M. ten Cate (2008). "Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production." Caries Res **42**(2): 148-154.
- Cavazana, T. P., T. Y. Hosida, J. P. Pessan, C. Sampaio, D. R. Monteiro and A. C. B. Delbem (2019). "Activity of sodium trimetaphosphate, associated or not with fluoride, on dual-species biofilms." Biofouling: 1-9.

Chedid, S. J. and J. A. Cury (2004). "Effect of 0.02% NaF solution on enamel demineralization and fluoride uptake by deciduous teeth in vitro." Braz Oral Res **18**(1): 18-22.

Cheng, L., J. Li, Y. Hao and X. Zhou (2008). "Effect of compounds of *Galla chinensis* and their combined effects with fluoride on remineralization of initial enamel lesion in vitro." J Dent **36**(5): 369-373.

Cheng, L., J. Li, L. He and X. Zhou (2015). "Natural products and caries prevention." Caries Res **49 Suppl 1**: 38-45.

Dabholkar, C. S., M. Shah, R. Kathariya, M. Bajaj and Y. Doshi (2016). "Comparative Evaluation of Antimicrobial Activity of Pomegranate-Containing Mouthwash Against Oral-Biofilm Forming Organisms: An Invitro Microbial Study." J Clin Diagn Res **10**(3): ZC65-69.

Danelon, M., J. P. Pessan, F. N. Souza-Neto, E. R. de Camargo and A. C. Delbem (2017). "Effect of fluoride toothpaste with nano-sized trimetaphosphate on enamel demineralization: An in vitro study." Arch Oral Biol **78**: 82-87.

Danelon, M., E. M. Takeshita, L. C. Peixoto, K. T. Sasaki and A. C. B. Delbem (2014). "Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization." Clin Oral Investig **18**(4): 1119-1127.

de Oliveira, J. R., V. C. de Castro, P. das Gracias Figueiredo Vilela, S. E. Camargo, C. A. Carvalho, A. O. Jorge and L. D. de Oliveira (2013). "Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry." BMC Complement Altern Med **13**: 208.

Delbem, A. C., K. T. Sasaki, A. M. Castro, L. M. Pinto and M. Bergamaschi (2003). "Assesment of the fluoride concentration and pH in different mouthrinses on the brazilian market." J Appl Oral Sci **11**(4): 319-323.

Delbem, A. C., G. C. Tiano, K. M. Alves and R. F. Cunha (2006). "Anticariogenic potencial of acidulate solutions with low fluoride concentration." J Appl Oral Sci **14**(4): 233-237.

Dentistry, A. A. o. P. (2018). "Policy on Use of Fluoride." Oral Health Policies **40**(6): 49-50.

Emerenciano, N. G., A. C. Botazzo Delbem, J. P. Pessan, G. P. Nunes, F. N. Souza Neto, E. R. de Camargo and M. Danelon (2018). "In situ effect of fluoride toothpaste supplemented with nano-sized sodium trimetaphosphate on enamel demineralization prevention and biofilm composition." Arch Oral Biol **96**: 223-229.

Endo, E. H., D. A. Cortez, T. Ueda-Nakamura, C. V. Nakamura and B. P. Dias Filho (2010). "Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*." Res Microbiol **161**(7): 534-540.

Esawya, M. A., T. I. M. Ragaba, A. S. G. I Shalabya, M. Bashab and M. Emam (2019). "Evaluated bioactive component extracted from *Punica granatum* peel and its Ag NPs forms as mouthwash against dental plaque." Biocatalysis and Agricultural Biotechnology **18**: 1-9.

Farkash, Y., M. Feldman, I. Ginsburg, D. Steinberg and M. Shalish (2019). "Polyphenols Inhibit *Candida albicans* and *Streptococcus mutans* Biofilm Formation." Dent J (Basel) **7**(2).

Favretto, C. O., M. Danelon, F. C. Castilho, A. E. Vieira and A. C. Delbem (2013). "In vitro evaluation of the effect of mouth rinse with trimetaphosphate on enamel demineralization." Caries Res **47**(5): 532-538.

Fernandes, R. A., A. A. Berretta, E. C. Torres, A. F. M. Buszinski, G. L. Fernandes, C. C. Mendes-Gouvea, F. N. de Souza-Neto, L. F. Gorup, E. R. de Camargo and D. B. Barbosa (2018). "Antimicrobial Potential and Cytotoxicity of Silver Nanoparticles Phytosynthesized by Pomegranate Peel Extract." Antibiotics (Basel) **7**(3).

Finkel, J. S. and A. P. Mitchell (2011). "Genetic control of *Candida albicans* biofilm development." Nat Rev Microbiol **9**(2): 109-118.

Fiske, C. H. and Y. Subbarow (1925). "The colorimetric determination of phosphorus. ." J Biol Chem **66**: 375-400.

Folin, O. and W. Denis (1915). "A colorimetric method for the determination of phenols (and phenol derivatives) in urine." Biol. Chem **22**: 305-308.

Ghalayani, P., B. Zolfaghary, A. R. Farhad, A. Tavangar and B. Soleymani (2013). "The efficacy of *Punica granatum* extract in the management of recurrent aphthous stomatitis." J Res Pharm Pract **2**(2): 88-92.

Guandalini Cunha, B., C. Duque, K. Sampaio Caiaffa, L. Massunari, I. Arague Catanoze, D. M. Dos Santos, S. H. P. de Oliveira and A. M. Guiotti (2020). "Cytotoxicity and antimicrobial effects of citronella oil (*Cymbopogon nardus*) and commercial mouthwashes on *S. aureus* and *C. albicans* biofilms in prosthetic materials." Arch Oral Biol **109**: 104577.

Gulube, Z. and M. Patel (2016). "Effect of *Punica granatum* on the virulence factors of cariogenic bacteria *Streptococcus mutans*." Microb Pathog **98**: 45-49.

Hajimahmoodi, M., M. Shams-Ardakani, P. Saniee, F. Siavoshi, M. Mehrabani, H. Hosseinzadeh, P. Foroumadi, M. Safavi, M. Khanavi, T. Akbarzadeh, A. Shafiee and A. Foroumadi (2011). "In vitro antibacterial activity of some Iranian medicinal plant extracts against *Helicobacter pylori*." Nat Prod Res **25**(11): 1059-1066.

Han, B., J. Jaurequi, B. W. Tang and M. E. Nimni (2003). "Proanthocyanidin: a natural crosslinking reagent for stabilizing collagen matrices." J Biomed Mater Res A **65**(1): 118-124.

Hemani, K. and S. Gheenass (2018). "Evaluation of antimicrobial property of extract of *Punica granatum* (L.) on oral pathogens." International Journal of Pharma and Bio Sciences **8**(3): 35-40.

Ismail, T., P. Sestili and S. Akhtar (2012). "Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects." J Ethnopharmacol **143**(2): 397-405.

Jyotika, S., G. Anil, S. Ankit and K. Shikha (2019). "Agents to Maintain Tooth Integrity: An Equilibrium between Remineralization and Demineralization-A Review." International Journal of Dental and Medical Specialty **6**(1): 9-14.

Kim, E. J. and B. H. Jin (2018). "*Galla chinensis* extracts and calcium induce remineralization and antibacterial effects of enamel in a *Streptococcus mutans* biofilm model." Journal of Korean Academy of Oral Health **42**(3): 90-96.

Kim, J. and P. Sudbery (2011). "*Candida albicans*, a major human fungal pathogen." J Microbiol **49**(2): 171-177.

Koo, H., P. Nino de Guzman, B. D. Schobel, A. V. Vacca Smith and W. H. Bowen (2006). "Influence of cranberry juice on glucan-mediated processes involved in *Streptococcus mutans* biofilm development." Caries Res **40**(1): 20-27.

Kukreja, B. J. and V. Dodwad (2012). "Herbal Mouthwhases - A gift of nature." International Journal of Pharma and Bio Sciences **3**(2): 46-52.

Lamfon, H., S. R. Porter, M. McCullough and J. Pratten (2003). "Formation of *Candida albicans* biofilms on non-shedding oral surfaces." Eur J Oral Sci **111**(6): 465-471.

Loomis, W. D. (1974). "Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles." Methods Enzymol **31**: 528-544.

Manarelli, M. M., A. C. B. Delbem, L. C. Baez-Quintero, F. R. N. de Moraes, R. F. Cunha and J. P. Pessan (2017). "Fluoride varnishes containing sodium trimetaphosphate reduce enamel demineralization in vitro." Acta Odontol Scand **75**(5): 376-378.

Manarelli, M. M., A. E. Vieira, A. A. Matheus, K. T. Sasaki and A. C. Delbem (2011). "Effect of mouth rinses with fluoride and trimetaphosphate on enamel erosion: an in vitro study." Caries Res **45**(6): 506-509.

Matos, A., J. L. Garland and W. F. Fett (2002). "Composition and physiological profiling of sprout-associated microbial communities." J Food Prot **65**(12): 1903-1908.

McGaughey, C. and E. C. Stowell (1977). "Effects of polyphosphates on the solubility and mineralization of HA: relevance to a rationale for anticaries activity." J Dent Res **56**(6): 579-587.

Megalaa, N., K. Thirumurugan, G. Kayalvizhi, R. Sajeev, E. B. Kayalvizhi, V. Ramesh and A. Vargeese (2018). "A comparative evaluation of the anticaries efficacy of herbal extracts (Tulsi

and Black myrobalans) and sodium fluoride as mouthrinses in children: A randomized controlled trial." *Indian J Dent Res* **29**(6): 760-767.

Menezes, S. M., L. N. Cordeiro and G. S. Viana (2006). "Punica granatum (pomegranate) extract is active against dental plaque." *J Herb Pharmacother* **6**(2): 79-92.

Moretto, M. J., A. C. Magalhaes, K. T. Sasaki, A. C. Delbem and C. C. Martinhon (2010). "Effect of different fluoride concentrations of experimental dentifrices on enamel erosion and abrasion." *Caries Res* **44**(2): 135-140.

Nordbo, H. and G. Rolla (1972). "Desorption of salivary proteins from hydroxyapatite by phytic acid and glycerophosphate and the plaque-inhibiting effect of the two compounds in vivo." *J Dent Res* **51**(3): 800-811.

Onishi, T., S. Umemura, M. Yanagawa, M. Matsumura, Y. Sasaki, T. Ogasawara and T. Ooshima (2008). "Remineralization effects of gum arabic on caries-like enamel lesions." *Arch Oral Biol* **53**(3): 257-260.

Pandit, S., J. E. Jung, H. M. Choi and J. G. Jeon (2018). "Effect of brief periodic fluoride treatments on the virulence and composition of a cariogenic biofilm." *Biofouling* **34**(1): 53-61.

Park, J. B., G. Lee, B. G. Yun, C. H. Kim and Y. Ko (2014). "Comparative effects of chlorhexidine and essential oils containing mouth rinse on stem cells cultured on a titanium surface." *Mol Med Rep* **9**(4): 1249-1253.

Philip, N., S. J. Leishman, H. Bandara and L. J. Walsh (2019). "Polyphenol-Rich Cranberry Extracts Modulate Virulence of Streptococcus mutans-Candida albicans Biofilms Implicated in the Pathogenesis of Early Childhood Caries." *Pediatr Dent* **41**(1): 56-62.

Philip, N. and L. J. Walsh (2019). "Cranberry Polyphenols: Natural Weapons against Dental Caries." *Dent J (Basel)* **7**(1).

Pierpoint, W. S. (1969). "o-Quinones formed in plant extracts. Their reactions with amino acids and peptides." *Biochem J* **112**(5): 609-616.

Pinni, J., J. S. Sakar Avula, S. Mukthineni, S. Bandi and T. Gokul (2018). "Antimicrobial Activity of Pomegranate (*Punica Granatum*) Pericarp Extract against *Streptococcus Mutans*- A Source For Natural Mouth Rinse: An *In-vitro* and *In-vivo* Study." *Biomedical & Pharmacology Journal* **11**(4): 2025-2030.

Prasad, D. and R. Kunnaiah (2014). "Punica granatum: A review on its potential role in treating periodontal disease." *J Indian Soc Periodontol* **18**(4): 428-432.

Scalbert, A. (1991). "Antimicrobial properties of tannins." *Phytochemistry* **30**(12): 3875-3883.

Shafiqhi, M., L. Amjad and M. Madani (2012). "In vitro antifungal activity of methanolic extract of various parts of *Punica granatum* L." *International Journal of Scientific & Engineering Research* **3**(12): 1-4.

Spiguel, M. H., M. F. Tovo, P. F. Kramer, K. S. Franco, K. M. Alves and A. C. Delbem (2009). "Evaluation of laser fluorescence in the monitoring of the initial stage of the de-/remineralization process: an in vitro and in situ study." *Caries Res* **43**(4): 302-307.

Takahashi, N. and B. Nyvad (2011). "The role of bacteria in the caries process: ecological perspectives." *J Dent Res* **90**(3): 294-303.

Takeshita, E. M., L. P. Castro, K. T. Sasaki and A. C. Delbem (2009). "In vitro evaluation of dentifrice with low fluoride content supplemented with trimetaphosphate." *Caries Res* **43**(1): 50-56.

Takeshita, E. M., M. Danelon, L. P. Castro, K. T. Sasaki and A. C. Delbem (2015). "Effectiveness of a Toothpaste with Low Fluoride Content Combined with Trimetaphosphate on Dental Biofilm and Enamel Demineralization in situ." *Caries Res* **49**(4): 394-400.

Takeshita, E. M., R. A. Exterkate, A. C. Delbem and J. M. ten Cate (2011). "Evaluation of different fluoride concentrations supplemented with trimetaphosphate on enamel de- and remineralization in vitro." *Caries Res* **45**(5): 494-497.

-
- Tian, F., B. Li, B. Ji, J. Yang, G. Zhang, Y. Chen and Y. Luo (2009). "Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*:The polarity affects the bioactivities." Food Chemistry **113**(1): 173-179.
- van Dijk, J. W., J. M. Borggreven and F. C. Driessens (1980). "The effect of some phosphates and a phosphonate on the electrochemical properties of bovine enamel." Arch Oral Biol **25**(8-9): 591-595.
- Vasconcelos, L. C., F. C. Sampaio, M. C. Sampaio, S. Pereira Mdo, J. S. Higino and M. H. Peixoto (2006). "Minimum inhibitory concentration of adherence of *Punica granatum* Linn (pomegranate) gel against *S. mutans*, *S. mitis* and *C. albicans*." Braz Dent J **17**(3): 223-227.
- Vieira, A. E., A. C. Delbem, K. T. Sasaki, E. Rodrigues, J. A. Cury and R. F. Cunha (2005). "Fluoride dose response in pH-cycling models using bovine enamel." Caries Res **39**(6): 514-520.
- Vogel, G. L., L. C. Chow and W. E. Brown (1983). "A microanalytical procedure for the determination of calcium, phosphate and fluoride in enamel biopsy samples." Caries Res **17**(1): 23-31.
- Vucic, V., M. Grabez, A. Trchounian and A. Arsic (2019). "Composition and Potential Health Benefits of Pomegranate: A Review." Curr Pharm Des **25**(16): 1817-1827.
- Waterman, P. G. and S. Mole (1994). " Analysis of Phenolic Plant Metabolites." Blackwell Scientific: Oxford, UK; Boston, MA, USA.
- Weatherell, J. A., C. Robinson, M. Strong and H. Nakagaki (1985). "Micro-sampling by abrasion." Caries Res **19**(2): 97-102.
- Zhang, L., J. Xue, J. Li, L. Zou, Y. Hao, X. Zhou and W. Li (2009). "Effects of *Galla chinensis* on inhibition of demineralization of regular bovine enamel or enamel disposed of organic matrix." Arch Oral Biol **54**(9): 817-822.

FIGURES E TABLES

Constituent	Mouthwash Formulation							
	ETF1	TF2	ETF2	TF2	E	F1	F2	P
Pomegranate Peel Extract (PPE)	10,40	-	10,40	-	10,40	-	-	-
Stabilizers	0,50	0,50	0,50	0,50	0,50	0,50	0,50	0,50
Microbiological Conservator	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
Chelating	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01
Sodium Fluoride (F)	0,02	0,02	0,05	0,05	-	0,02	0,05	-
Sodium Trimetaphosphate (TMP)	0,20	0,20	0,30	0,30	-	-	-	-
Sweetener I	7,50	7,50	7,50	7,50	7,50	7,50	7,50	7,50
Humectant	10,00	10,00	10,00	10,00	10,00	10,00	10,00	10,00
Purified water q.s.	100	100	100	100	100	100	100	100

TABLE 1 Groups of mouthwash formulations designed according its constituents (g)

Samples	Total phenols expressed as gallic acid (mean±SD)
Pomegranate Glycolic Extract	114,98 ± 3,55

TABLE 2 Concentration of phenolic compounds (mg/g) in samples.

Formulation ETF1	11,48 ± 0,22
Formulation TF1	0,54 ± 0,07
Formulation ETF2	11,59 ± 0,55
Formulation TF2	0,53 ± 0,07
Formulation E	11,56 ± 0,01
Formulation P	0,49 ± 0,06

TABLE 3 Mean (SD) of variables analyzed according to mouthwash formulations treatments.

Formulation	%SH (KHN)	ΔKHN (KHN × μm)	F (μg/mm ³)	Ca (μg/mm ³)	P (μg/mm ³)
P	-87.4 ^a (3.2)	6734.9 ^a (1217.9)	0.5 ^a (0.2)	156.6 ^a (33.1)	155.3 ^a (33.6)
F1	-73.4 ^b (4.5)	5046,4 ^b (778.5)	0.6 ^b (0.4)	207.5 ^b (63.7)	211.1 ^b (75.4)
F2	-64.1 ^c (5.0)	3810.1 ^c (842.2)	1.2 ^c (0.4)	269.4 ^c (51.2)	281.1 ^c (94.0)
ETF1	-51.7 ^d (7.1)	3710.7 ^c (973.0)	0.7 ^b (0.3)	218.1 ^b (65.6)	224.5 ^b (72.6)
TF1	-60.5 ^e (8.1)	4510.2 ^d (621.9)	0.7 ^b (0.2)	212.3 ^b (85.3)	227.0 ^b (99.1)
ETF2	-34.5 ^f (4.4)	2814.2 ^e (975.3)	1.0 ^c (0.3)	253.2 ^d (71.9)	192.4 ^b (73.0)
TF2	-53.1 ^d (5.0)	3878.1 ^c (853.8)	0.9 ^c (0.9)	188.2 ^b (28.4)	221.7 ^b (79.1)

Different superscript letters indicate significant differences among the treatments for each variable separately. (One-way ANOVA, followed by Student-Newman-Keuls test; p<0.001).

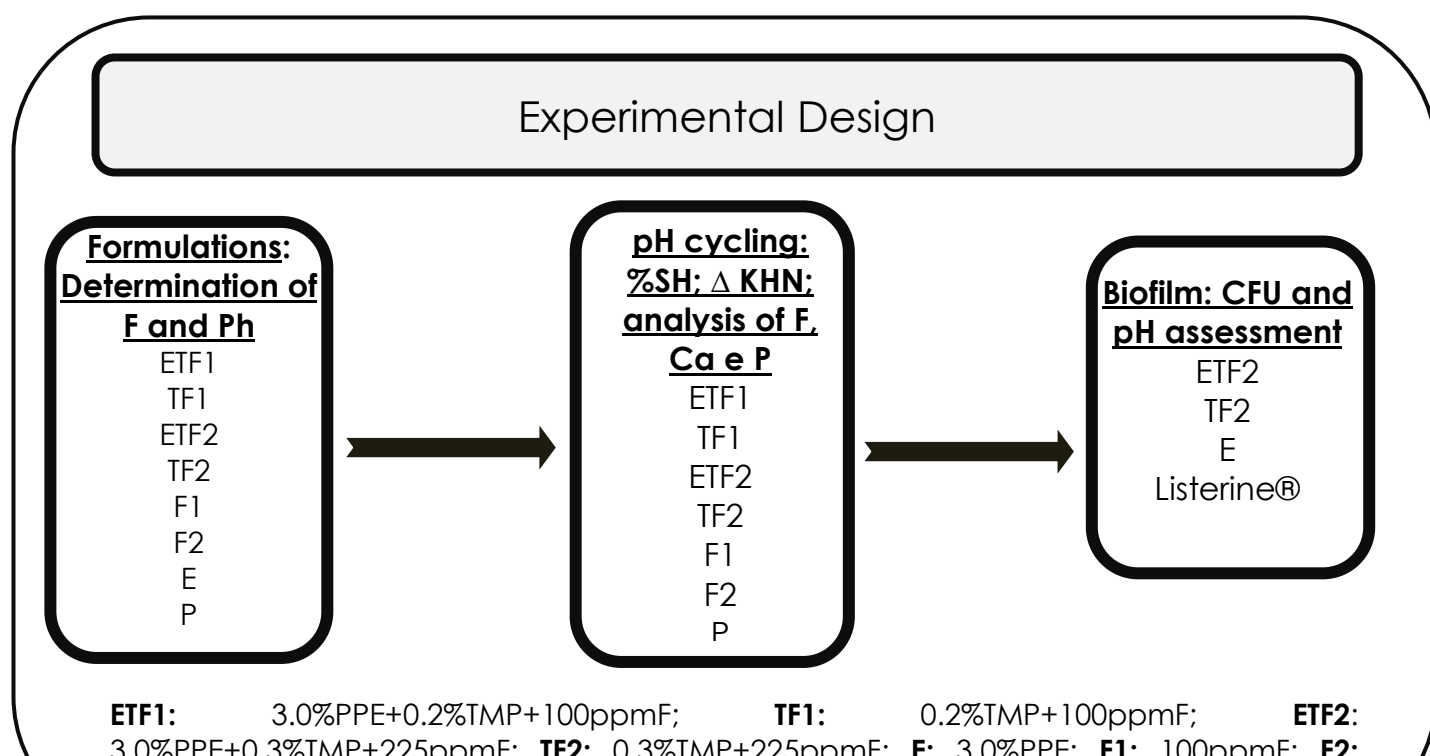
TABLE 4: Percentage of reduction of *C. albicans* and *S. mutans* dual-biofilms formed for 24 hours and exposed to different treatments for 1 or 10 minutes.

Biofilm-24h	% Biofilm reduction			
	<i>Candida albicans</i>		<i>Streptococcus mutans</i>	
Groups/Treatment (min)	1	10	1	10

ETF2	26	36	12	36
TF2	17	32	11	28
E	20	32	25	28
Positive control	42	-	53	56
Negative control	-	-	-	-

TABLE 5: pH values of *C. albicans* and *S. mutans* dual-biofilms formed for 24 hours and exposed to different treatments for 1 or 10 minutes.

Biofilm-24h	pH	
	<i>Candida albicans</i> and <i>Streptococcus mutans</i>	
Groups/Treatment (min)	1	10
ETF2	5.15	5.20
TF2	5.10	5.09
E	5.28	5.28
Positive control	5.67	5.51
Negative control	5.45	5.51



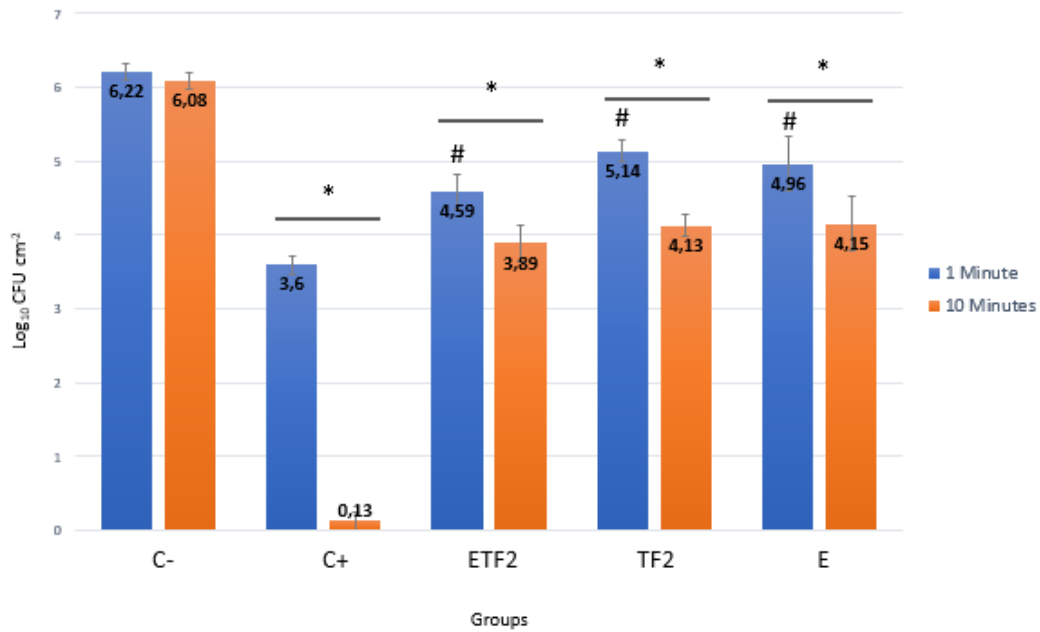
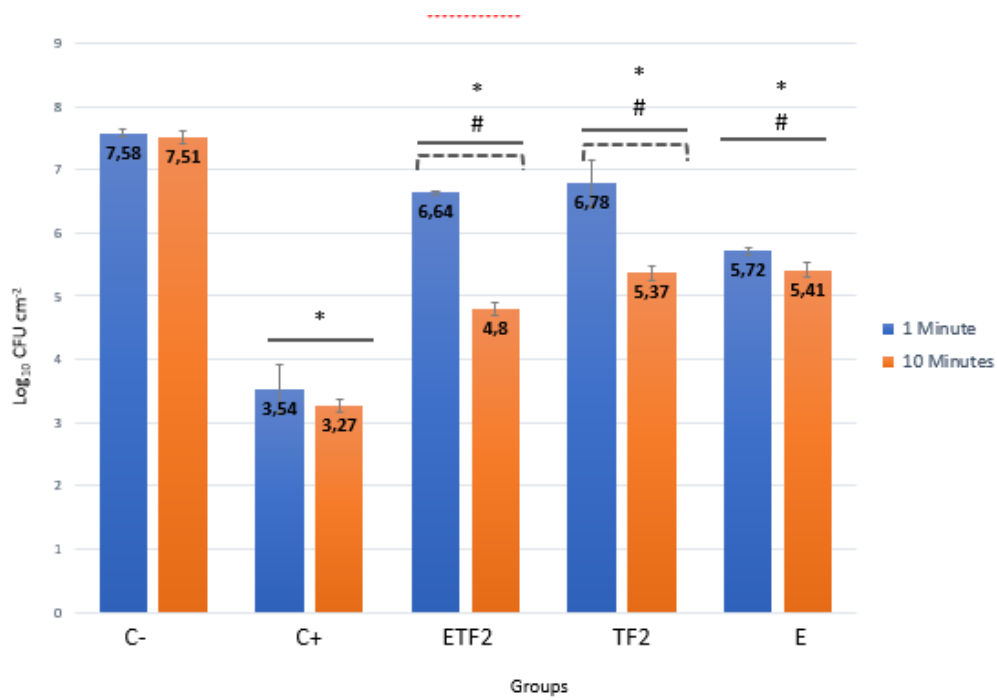


FIGURE 1 Workflow of experimental design according to the analysis carried out in the present study.

FIGURE2: *C. albicans* biofilm treated for 1 or 10 minutes

Effect of treatment with solutions for 1 and 10 minutes on *C. albicans* biofilm cells. The data represented the mean \pm SD from five groups. * $p < .05$ versus negative control; # ETF2 (1 min), TF2 (1min) e E (1 min) versus C+ (1 min).

FIGURE 3: *S. mutans* biofilm treated for 1 or 10 minutes



Effect of treatment with solutions for one and 10 minutes on *S. mutans* biofilm cells. The data represented the mean \pm SD from five groups. * $p < .05$ versus negative control; # ETF2, TF2 and E versus positive control; --- ETF2 (1 min) versus ETF2 (10 min), and TF2 (1 min) versus TF2 (10 min).

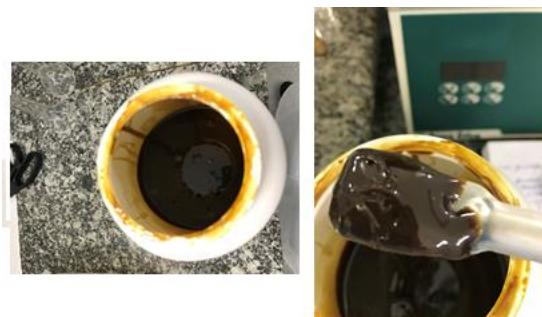
Anexos

1. Obtenção do extrato da casca de romã

A parceria com a Apis Flora (Incl. Coml. Ltda, Ribeirão Preto, SP), sob a supervisão da farmacêutica e pesquisadora responsável pela empresa Dra Andresa Aparecida Berretta e Silva, permitiu o desenvolvimento das formulações para enxaguatório tendo como base extrato da casca de romã. Foi realizada a compra via empresa Apis Flora da casca desidratada da romã, já triturada, e esterilizada da empresa Santosflora Comércio de Ervas, que é distribuidora, importadora e exportadora de ervas e apresenta certificado da *Food and Drug Administration* (FDA).

Inicialmente o processo de obtenção do extrato da casca da romã proposto seria de maceração e percolação em etanol. Contudo, como o pó da casca da romã apresentou-se extremamente fino, ele impediu a filtração pelo percolador, o que inviabilizou seu uso para o processo de extração. Assim, seguiu-se com a extração utilizando-se somente a maceração com etanol 70° GL na proporção de 1:3, por 24 horas. Essa mistura foi então filtrada a vácuo, separando-se o extrato bruto e a borra. Na borra, adicionou-se novamente etanol 70° GL, e o processo de maceração foi novamente realizado. Esse processo foi repetido por 5 vezes até que não se observasse mais a presença de sólidos solúveis no extrato (“dry matter”), indicando possível esgotamento da droga pelo processo extrator. Essas 5 frações foram misturadas e concentradas em um rotoevaporador a vácuo até obtenção do extrato mole (Fig. 1).

Figura 1. Extrato mole (bruto) da casca da romã após a rotoevaporação.



Em seguida, o extrato mole foi pesado e diluído em água deionizada a fim de se obter concentrações de 1%, 3%, 6% e 12% e se verificar a capacidade de diluição do extrato mole nessas concentrações (Fig. 2a). Em seguida, todas as concentrações foram centrifugadas por 30 minutos a 8000 rpm (Fig. 2b) e, em todas elas, observou-se a formação de precipitado de extrato (Fig. 2c).

Figura 2. a) extrato mole pesado e após diluição em água deionizada; b) soluções nas diferentes concentrações para serem centrifugadas; c) extrato após centrifugação.



Em vista desses resultados, decidiu-se substituir a água por uma solução contendo o copolímero em bloco Poloxamer 407, composto que fica solúvel na forma de unímeros mas que se modifica para micelas, sendo capaz de tornar solúvel compostos que são insolúveis na água (Dumortier et al. 2006). Paralelamente, também se utilizou o co-solvente propilenoglicol buscando-se também evitar a precipitação do extrato. Nesse caso, o extrato mole foi ressuscitado a quente (aproximadamente 75° C) em propilenoglicol de forma a se obter um extrato bruto a 35% m/m de sólidos. O extrato resultante foi então centrifugado por 30 minutos a 3000 rpm e o sobrenadante foi separado. Caracterizou-se o extrato quanto ao teor de sólidos (secagem em estufa) e padronizou-se o mesmo em % de sólidos (35%).

2. Teste microbiológico para seleção da concentração do extrato nas formulações

para enxaguatório

Tempo de morte celular

Com os extratos a 1%, 3%, 6% e 12% diluídos em propilenoglicol, realizou-se o ensaio de tempo de morte celular dos microrganismos alvo (*C. albicans* ATCC 10231) e *S. mutans* ATCC 35668) buscando-se a concentração mais efetiva para a preparação da formulação para enxaguatório. Contudo, na concentração de 12% não foi possível realizar a diluição do extrato, independente do solvente selecionado. Os tempos de contato dos extratos com os microrganismos foram de: 0,5, 1, 2, 3, 4, 5 e 10 min. Os ensaios foram realizados em triplicata em três ocasiões diferentes e estão descritos na tabela a seguir (Tabela 1).

Tabela 1. Médias e desvios-padrão das células de *Candida albicans* e *Streptococcus mutans* nos diferentes tempos de avaliação (T0-T10) após serem submetidas aos extratos da casca de romã diluídos em propilenoglicol nas diferentes concentrações (1, 3 e 6%).

<i>Candida albicans</i>								
EXTRATO 1%	Tempo (min)							
	T0	T0,5	T1	T2	T3	T4	T5	T10
Média de cada triplicata	6,26	4,94	6,18	5,53	5,29	5,50	5,12	5,39
(log ₁₀ CFU/cm ²)	6,49	5,26	5,01	5,38	5,35	5,55	4,71	5,72
	6,44	5,29	7,09	5,56	5,29	5,32	5,29	5,68
Média	6,40	5,17	6,10	5,49	5,31	5,46	5,04	5,60
DV	0,09	0,15	0,72	0,07	0,03	0,09	0,22	0,14
<i>Streptococcus mutans</i>								
EXTRATO 3%	Tempo (min)							
	T0	T0,5	T1	T2	T3	T4	T5	T10
Média de cada triplicata	5,72	5,44	5,50	5,63	4,82	5,42	5,14	5,72
(log ₁₀ CFU/cm ²)	5,77	5,20	5,22	5,68	5,63	5,74	5,44	5,83
	5,84	5,34	5,28	5,71	5,67	5,67	3,46	5,80
Média	5,77	5,33	5,33	5,68	5,37	5,61	4,68	5,78
DV	0,04	0,08	0,11	0,03	0,37	0,13	0,81	0,04
EXTRATO 6%	Tempo (min)							
	T0	T0,5	T1	T2	T3	T4	T5	T10
Média de cada triplicata	5,97	5,66	5,59	5,84	5,01	5,77	5,72	5,86
(log ₁₀ CFU/cm ²)	5,72	5,60	5,69	5,71	5,28	5,92	5,12	6,28
	5,90	5,56	5,09	5,79	5,56	5,80	5,42	5,63
Média	5,87	5,33	5,46	5,78	5,28	5,83	5,42	5,92
DV	0,09	0,04	0,24	0,05	0,19	0,06	0,20	0,24
EXTRATO 1%	Tempo (min)							
	T0	T0,5	T1	T2	T3	T4	T5	T10
Média de cada triplicata	8,49	8,00	8,65	8,56	8,34	8,48	8,50	8,58
(log ₁₀ CFU/cm ²)	8,65	8,48	8,07	8,37	8,68	8,48	8,65	8,61
	8,54	8,59	8,60	8,58	8,54	8,31	8,44	8,59
Média	8,56	8,35	8,44	8,50	8,52	8,42	8,53	8,59
DV	0,06	0,24	0,25	0,09	0,12	0,07	0,08	0,01
EXTRATO 3%	Tempo (min)							
Média de cada	8,57	8,65	8,39	8,69	4,09	7,98	8,26	7,97

triplicata	8,67	8,42	8,39	7,28	4,18	8,03	7,94	8,00
(log ₁₀ CFU/cm ²)	8,38	8,52	8,52	8,83	8,18	8,04	8,26	7,77
Média	8,54	8,53	8,43	8,27	5,49	8,02	8,15	7,91
DV	0,11	0,08	0,05	0,66	1,80	0,02	0,14	0,10
EXTRATO 6%								
Média de cada	8,53	8,41	8,18	7,55	7,94	7,83	7,90	7,44
triplicata	8,16	8,09	8,24	7,71	8,18	7,85	7,88	7,46
(log ₁₀ CFU/cm ²)	8,53	8,43	8,18	7,80	7,95	7,84	8,01	7,53
Média	8,41	8,31	8,20	7,69	8,03	7,84	7,93	7,48
DV	0,16	0,15	0,03	0,09	0,11	0,01	0,05	0,03

Com base em dados prévios obtidos em ensaios antimicrobianos realizados com extrato da casca de romã no laboratório de microbiologia da Apis Flora e por não ter ocorrido diferença entre as concentrações de 1, 3 e 6% considerando-se o tempo de aplicação de um enxaguatório de 1 minuto, selecionou-se a concentração de 3% para o desenvolvimento da formulação inicial para enxaguatório. Já as concentrações de TMP e fluoreto foram baseadas no trabalho de Favretto et al. 2013, ou seja, 0,2% de TMP com 100 ppm de F e 0,3% de TMP com 225 ppm de F.

Microdiluição em caldo: mínima concentração inibitória (MIC)

Seguindo-se a sugestão dada pelo parecerista da FAPESP, realizou-se também ensaios da microdiluição em caldo para se obter as mínimas concentrações inibitórias do extrato da casca de romã contra células planctônicas de *Candida albicans* e *Streptococcus mutans*, seguindo-se as normas estabelecidas pela *Clinical and Laboratory Standards Institute* (CLSI, Documentos M27-A2 e M07-A9). Assim, para esse ensaio um novo extrato com uma concentração de 25% (250 mg/ml) diluído em propilenoglicol e água foi preparado. O extrato foi efetivo contra ambos microrganismos, com valores de MIC de 0,97 e 1,95 mg/ml respectivamente para *C. albicans* e *S. mutans*.

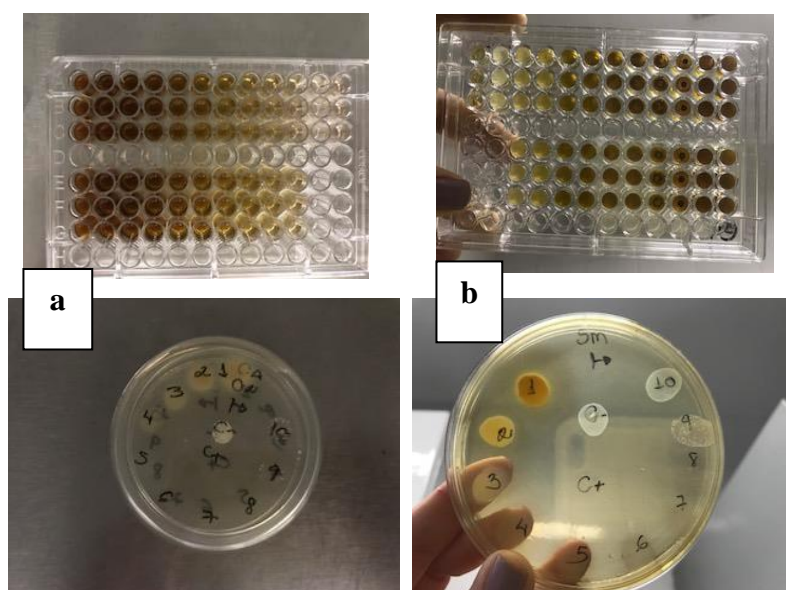


Figura 3. Ensaio microdiluição em caldo e atividade fungicida/bactericida do extrato da casca de romã: para *C. albicans* (a) e para *S. mutans* (b).

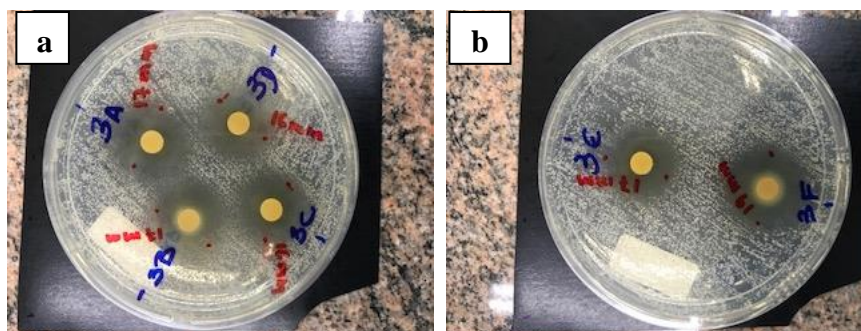
3. Desenvolvimento da formulação líquida composta por extrato da casca da romã, TMP e F

A partir dos resultados prévios dos testes microbiológicos com os extratos da casca da romã, a concentração de 3% de extrato foi selecionada para o preparo das formulações de enxaguatório bucal. Foram desenvolvidas 6 formulações líquidas e testou-se o halo de inibição para *C. albicans* ATCC 10231 de cada uma delas, como mostra a Tabela 2 e Figura 4.

Tabela 2. Composição das formulações líquidas e medidas dos halos de inibição para *C. albicans*.

Formulação	Composição	Halo de inibição (mm) para <i>C. albicans</i>
A	Com poloxamer e propilenoglicol	17
B	Sem poloxamer	17
C	Sem flúor	16
D	Sem TMP	16
E	Sem F e TMP	17
F	Substituindo poloxamer por goma xantana e goma arábica	19

Figura 4. a: Halo de inibição para *C. albicans* das formulações A-B-C-D. b: Halo de inibição contra *C. albicans* das formulações E-F.



Com esse ensaio, observou-se que os compostos adicionados ao extrato para o preparo das formulações não prejudicaram a atividade antimicrobiana do extrato da casca da romã. No entanto, ao passar pelo processo de centrifugação para avaliar a estabilidade dessas formulações, foi possível observar uma pequena sedimentação de sólidos. Isso provavelmente se deve as características do pó da casca de romã ser muito fino e possivelmente passar através do filtro selecionado para o processo de extração.

Assim, um novo extrato foi preparado a partir da extração com etanol a 70% v / v por maceração e filtração. Em resumo, foi adicionado 1 kg de casca de romã em pó a um Erlenmeyer contendo 2,5 L de uma solução de etanol (70% v / v) por aproximadamente 12 horas para sedimentação do pó. A seguir, a solução foi filtrada com um filtro 0,22 e o extrato obtido foi concentrado em um evaporador rotativo sob pressão reduzida e temperatura controlada de 40-60°C (extrato bruto). Após a evaporação do solvente, o extrato bruto foi resolubilizado em propilenoglicol. Em seguida as 6 formulações (A a F, Tabela 2) foram novamente preparadas (Tabela 3). Todas passaram pelo processo de centrifugação durante 30 minutos e observou-se que não houve a deposição de sólidos, demonstrando, assim, uma estabilidade da formulação.

Tabela 3. Composição das novas formulações feitas com extrato da casca de romã a 3% (m/m).

Matéria-prima/função	Quantidade (g)					
	A'	B'	C'	D'	E'	F'
Extrato glicólico da casca de romã	10,40	10,40	-	-	10,40	-
Estabilizante	0,50	0,50	0,50	0,50	0,50	0,50
Conservante microbiológico	0,10	0,10	0,10	0,10	0,10	0,10
Quelante	0,01	0,01	0,01	0,01	0,01	0,01
Fluoreto de sódio	0,02	0,05	0,05	0,02	-	-
TMP	0,20	0,30	0,30	0,20	-	-
Edulcorante I	7,50	7,50	7,50	7,50	7,50	7,50
Edulcorante II	10,00	10,00	10,00	10,00	10,00	10,00
Água purificada q.s.p	100	100	100	100	100	100

Teste de Folin-Denis para determinar as concentrações de totais fenólicos nas formulações

A tabela 4 ilustra os ensaios em triplicata para cada formulação desenvolvida com extrato da casca de romã a 3% e a quantificação de fenóis totais por meio do teste colorimétrico de Folin-Denis. Como esperado, foi possível detectar quantidades de fenóis apenas nas formulações contendo romã (extrato glicólico, Formulação A, B e E).

Tabela 04. Concentração de compostos fenólicos no extrato glicólico de romã e nos enxagatatórios obtidos (n=3).

Amostras avaliadas	Fenóis totais expressos em ácido gálico (mg/mg) (média±SD)
Extrato Glicólico de Romã (30%p/v)	114,98 ± 3,55
Formulação A	11,48 ± 0,22
Formulação B	11,59 ± 0,55
Formulação C	0,54 ± 0,07
Formulação D	0,53 ± 0,07
Formulação E	11,56 ± 0,01
Formulação F	0,49 ± 0,06

4. Microdiluição em caldo: mínima concentração inibitória (MIC) das formulações

Foram realizados novos ensaios de microdiluição em caldo para determinação das MICs para *C. albicans* e *S. mutans* de todas as formulações. Não houve concentração bacteriostática/bactericida ou fungistática/fungicida para as formulações C, D e F, uma vez que essas amostras não apresentam o ativo do extrato da casca de romã. Já para as formulações A', B' e E' e para o extrato houve atividade antimicrobiana como é demonstrado na Tabela 5.

Tabela 5. Valores de MIC (MBC/MFC) para *C. albicans* e *S. mutans*.

	MBC/MFC baseados nas concentrações de sólidos da casca de romã (mg/mL) presentes nas formulações	
	<i>S. mutans</i>	<i>C. albicans</i>
Formulação A'	-	Poço 3: 4 mg/mL
Formulação B'	-	Poço 3: 4 mg/mL
Formulação E'	-	Poço 3: 4 mg/mL
Extrato	Poço 6: 5,12 mg/mL	Poço 6: 5,12 mg/mL

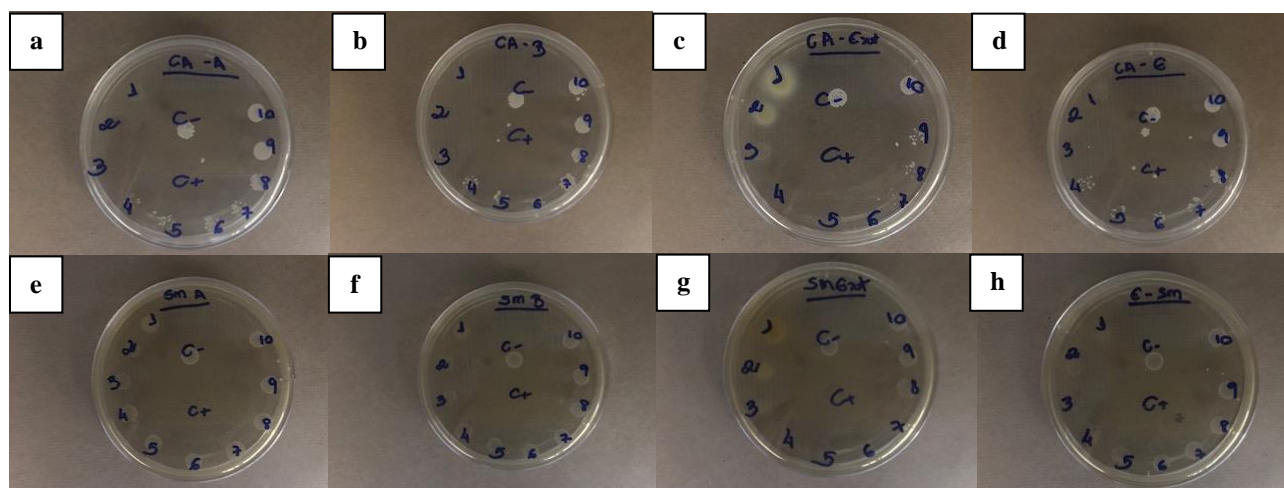


Figura 5. Plaqueamento dos MICs das formulações para *C.albicans*: a) Formulação A'; b) Formulação B'; c) Extrato; d) Formulação E'. Plaqueamento dos MICs das formulações para *S.mutans*: e) Formulação A'; f) Formulação B'; g) Extrato; h) Formulação E'.

Além disso, foi realizado um teste piloto de ciclagem do enxaguatório em seis blocos de dentes bovinos. Os blocos foram imersos em uma solução desmineralizadora

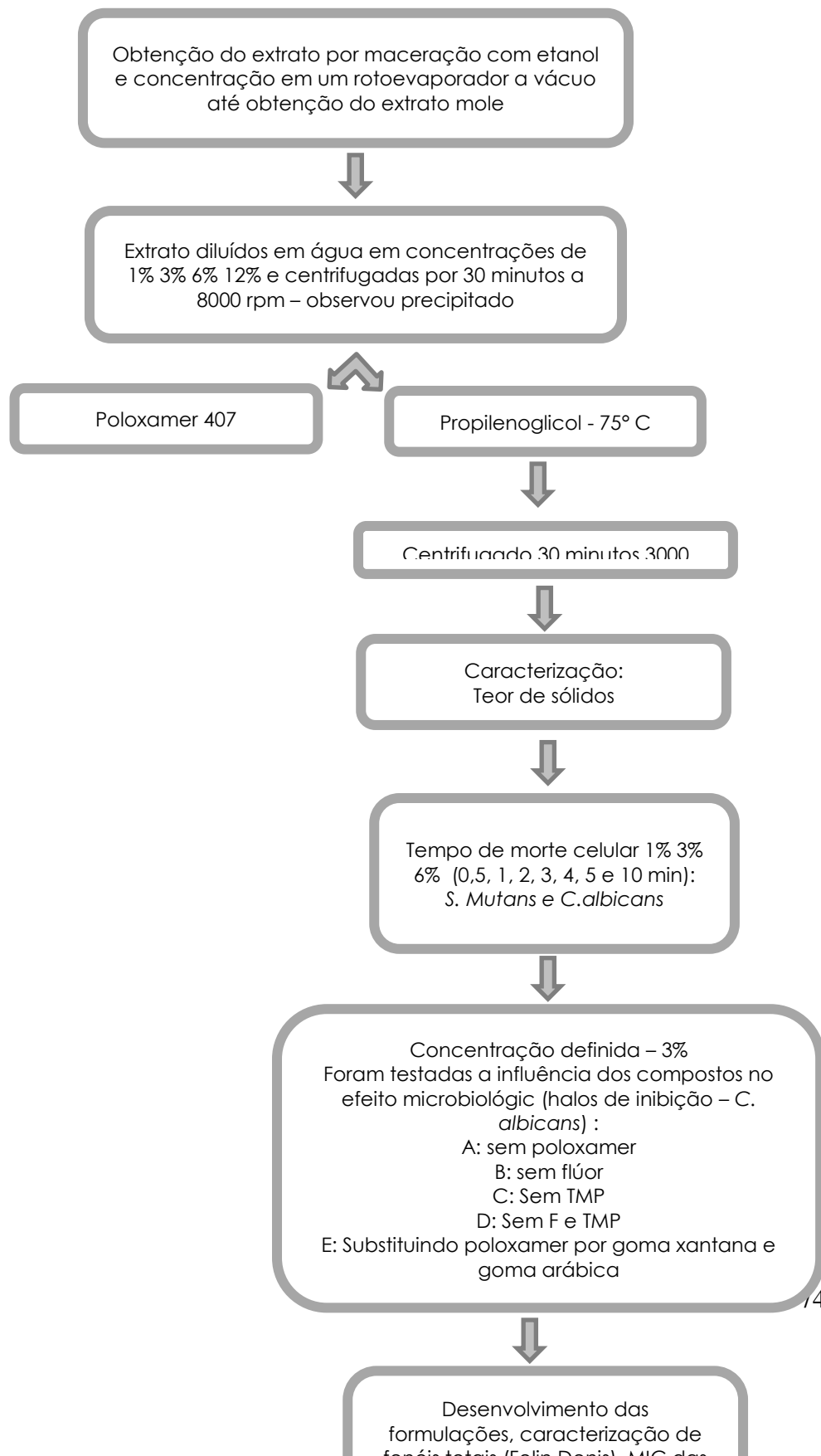
por 18 horas e em uma solução remineralizadora por 8 horas. Após 18 h e, solução desmineralizadora, os blocos foram removidos, lavados com água deionizada e imersos na formulação para enxaguatório por 1 minuto. Foram, então, novamente lavados e imersos em solução remineralizadora. Passados as 8 horas em solução remineralizadora, os blocos foram submetidos novamente ao tratamento com a formulação por 1 minuto. Esse protocolo foi realizado por 5 dias, e a partir do quinto dia o bloco ficou imerso por dois dias na solução remineralizadora, não havendo contato com o enxaguatório nesse período.

Na microscopia eletrônica de varredura (Figura 6) foi possível observar a deposição do TMP sobre a superfície do esmalte após ocorrer o desafio cariogênico, o que nos assegurou a dar continuidade com a presente formulação



Figura 6. Superfície do esmalte do dente bovino após o tratamento com a formulação A' realizado durante a ciclagem de pH. As setas indicam a presença de TMP depositado sobre a superfície do esmalte.

Passo a passo para o desenvolvimento das formulações:





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