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PRÓPOLIS VERMELHA COMO OPÇÃO À AMOXICILINA PARA REDUÇÃO DO COMPLEXO VERMELHO EM MODELO DE BIOFILME SUBGENGIVAL COMPLEXO *IN VITRO*

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, intitulada "PRÓPOLIS VERMELHA COMO OPÇÃO À AMOXICILINA PARA REDUÇÃO DO COMPLEXO VERMELHO EM MODELO DE BIOFILME SUBGENGIVAL COMPLEXO *IN VITRO*" em sessão pública realizada em 28 de agosto de 2020 considerou o candidato Kadmo Azevedo de Figueiredo aprovado.

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“Aqueles que se sentem satisfeitos sentam-se e nada fazem. Os insatisfeitos são os únicos benfeitores do mundo.”

(Walter S. Landor)

RESUMO

A própolis vermelha brasileira é antimicrobiano de amplo espectro, com efeito em bactérias Gram-positivas e negativas. Uma vez que o biofime periodonto patogênico é o principal agente etiológico da periodontite, torna-se relevante a busca por novos antimicrobianos naturais que possam atuar como coadjuvantes no tratamento da doença periodontal. Este estudo investigou os efeitos do extrato de Própolis Vermelha Brasileiro (EBPVB) em biofilmes subgengivais multiespécies com sete dias de formação. Culturas de biofilme mistas contendo 31 espécies associadas à saúde ou doença periodontal foram cultivadas por seis dias em um dispositivo de Calgary. Em seguida, os biofilmes maduros foram tratados por 24 h com EBPVB em diferentes concentrações (200-1600 µg/mL), amoxicilina (AMOXI) a 54 µg/mL (controle positivo) ou veículo (controle negativo). A atividade metabólica do biofilme foi determinada por colorimetria e as contagens/proporções bacterianas foram determinadas por hibridação DNA-DNA. Os dados foram analisados pelos testes de Kruskal –Wallis, seguido do post-hoc de Dunn. O tratamento com EBPVB em 1600, 800 e 400 µg/mL reduziram a atividade metabólica do biofilme em 56%, 56% e 57%, respectivamente, em comparação com a redução de 65% obtida com o AMOXI. A contagem total média de células foi significativamente reduzida em todos os grupos teste (~ 50-55%). Proporções mais baixas de espécies do complexo vermelho, verde e amarelo foram observadas no tratamento com EBPVB (400 µg/mL) e AMOXI, mas somente o AMOXI reduziu as proporções de espécies de *Actinomyces*. Em conclusão, o EBPVB foi tão eficaz quanto o AMOXI na redução da contagem e da proporção de patógenos de biofilme multiespécie maduro (formado por sete dias) e não afetou os níveis das espécies de *Actinomyces* compatíveis com o hospedeiro. Esses dados sugerem que a EBPVB pode ser uma alternativa ao AMOXI como adjuvante na terapia periodontal. Estudos *in vivo* são necessários para validar esses resultados.

Palavras-chave: própolis; antimicrobiano; Doença periodontal; amoxicilina

ABSTRACT

Brazilian red propolis is a broad-spectrum antimicrobial, with an effect on Gram-positive and negative bacteria. Since perioidontopathogenic biofime is the main etiological agent of periodontitis, the search for new natural antimicrobials that can act as adjuvants in the treatment of periodontal disease becomes relevant. This study investigated the effects of Brazilian Red Propolis (BRP) extract on seven-day-old multispecies subgingival biofilms. Mixed biofilm cultures containing 31 species associated with periodontal health or disease were grown for six days on a Calgary device. Then, mature biofilms were treated for 24 h with BRP extract at different concentrations (200–1600 µg/mL), amoxicillin (AMOXI) at 54 µg/mL (positive control) or vehicle (negative control). Biofilm metabolic activity was determined by colorimetry, and bacterial counts/proportions were determined by DNA–DNA hybridization. Data were analyzed by Kruskal–Wallis and Dunn's tests. Treatment with BRP at 1600, 800 and 400 µg/mL reduced biofilm metabolic activity by 56%, 56% and 57%, respectively, as compared to 65% reduction obtained with AMOXI. Mean total cell counts were significantly reduced in all test groups (~50–55%). Lower proportions of red, green and yellow complex species were observed upon treatment with BRP (400 µg/mL) and AMOXI, but only AMOXI reduced the proportions of *Actinomyces* species. In conclusion, BRP extract was as effective as AMOXI in killing seven-day-old multispecies biofilm pathogens and did not affect the levels of the host-compatible *Actinomyces* species. These data suggest that BRP may be an alternative to AMOXI as an adjunct in periodontal therapy. In vivo studies are needed to validate these results.

Keywords: propolis; antimicrobial; periodontal disease; amoxicillin

SUMÁRIO

1 INTRODUÇÃO

As doenças periodontais caracterizam-se por processos infecciosos que afetam os tecidos de proteção e sustentação do dente, possuindo como fator etiológico microrganismos específicos que se encontram presentes no biofilme bacteriano e que geram uma reação inflamatória nos tecidos periodontais. (MEISEL e KOCHER, 2005). A doença periodontal está relacionada à disbiose do biofilme subgengival devido à presença periodontopatógenos, principalmente *Porphyromonas gingivalis*, *Tannerela forsythia*, *Treponema denticola* e *Aggregatibacter actinomycetemcomitans*. Esses microrganismos expõem os tecidos periodontais a seus produtos ou componentes, como lipossacarídeos e fímbrias, provocando uma resposta inflamatória exacerbada, a qual pode iniciar a destruição tecidual observada nessa doença (HAJISHENGALLIS *et al.*, 2011). Em geral, as doenças do periodonto atingem de 20 a 50% da população mundial, causando impacto na qualidade de vida das pessoas. (DURHAM *et al.*, 2013). Via de regra, o processo inflamatório é caracterizado por uma resposta de defesa do organismo provocada por estímulos nocivos e a extensão desse processo é regulada por fatores pró e anti-inflamatórios (SILVA e MACEDO, 2011). Os leucócitos residentes e as células do endotélio se responsabilizam pela resposta inicial ao biofilme bacteriano, sendo a gengivite considerada como a lesão inicial, na qual acontecem alterações vasculares com formação de exsudato inflamatório que se dá pela desarmonia entre as bactérias e a defesa do hospedeiro. Nesta fase os sinais clínicos apresentam-se com alteração da cor da gengiva, sangramento e edema. (CEKICI *et al.*, 2013). A periodontite é uma patologia infecciosa oral frequente, relacionada a bactérias patogênicas do biofilme do dente causando uma resposta imune inflamatória do hospedeiro, provocando a destruição dos tecidos periodontais circundante, sendo assim capaz de provocar a perca do elemento dentário.(COLOMBO *et al.*, 2015).

O tratamento das doenças periodontais consiste no controle dos microrganismos periodontopatogênicos que se encontram no biofilme dentário, com a finalidade de diminuir ou eliminar a inflamação periodontal. (POURABBAS *et al*., 2014). Sendo assim, é preciso que se tenha um rígido controle mediante da eliminação dos fatores que auxiliam seu acúmulo e da descontaminação da superfície radicular, adquirida através da raspagem e alisamento corono-radicular (QUIRYNEN *et al*., 2005, CARVALHO, 2014).

A raspagem e alisamento radicular (RAR) dentre os métodos não cirúrgicos utilizados na odontologia possibilita diversas melhorias significativas no controle clínico da doença periodontal, no entanto somente a terapia mecânica pode não alcançar uma total eficácia frente

aos patógenos periodontais em bolsa profunda, havendo a necessidade do uso de outras opções de tratamento que ajudem na diminuição das bactérias mais persistentes que se encontram aderidas ao periodonto. (AKRAN, 2018).

Diversas alternativas de tratamento associadas a RAR estão disponíveis como a introdução da antibioticoterapia de uso local ou sistêmica como também a terapia foto dinâmica (TFD) (MEDEIROS, LINS e LEMOS, 2017). A combinação de metronidazol sistêmico (MTZ) e amoxicilina (AMX) com RAR demonstrou ser um tratamento periodontal eficaz. No entanto, algumas questões essenciais associadas ao uso desses antibióticos permanecem sem resposta, como o tempo ideal de administração durante o curso do tratamento periodontal. Embora esses agentes sejam frequentemente prescritos após a fase de cicatrização do procedimento, existe plausibilidade biológica para apoiar seu uso em conjunto com o tratamento mecânico (FERES et al 2013 e BORGES *et a.,l* 2017). Fritoli et al. (2015) em sua revisão sistemática sugeriu algum benefício do uso de antibiótico durante a fase ativa do tratamento periodontal, embora mais estudos clínicos controlados ainda sejam necessários. Por outro lado, alguns estudos mostraram que, pacientes que foram submetidos a antibioticoterapia sistêmica podem apresentar alguns inconvenientes em curto prazo, como superinfecção e distúrbios gastrointestinais (HAFFAJEE, SOCRANSKY e GUNSOLLEY, 2003 e TEUGHLS *et al.* 2020) e surgimento de bactérias resistentes ao tratamento (BAQUERO e NEGRI, 1997; FERES 2008 e KOUIDHI, QURASHI e CHAIEB, 2015).

Os produtos naturais têm atraído o interesse de pesquisadores e da população mundial devido à evidência científica recente de suas propriedades farmacológicas benéficas para a humanidade e também devido a possibilidade da descoberta de novos possíveis medicamentos (NEWMAN e CRAGG, 2016; HARVEY, EDRADA-EBEL e QUINN, 2015)

A própolis tem sido amplamente utilizada pela população em geral há décadas e as evidências científicas disponíveis comprovam o seu efeito anticariogênico (HAYACIBARA, *et al.*, 2005), antimicrobiano (BUENO-SILVA *et al.*, 2017), anti-biofilme (BUENO-SILVA *et al.*, 2013), anti-inflamatório (MARCUCCI *et al.*, 2001), anti-oxidante (AHN *et al.*, 2004), e anticarcinogênico (GALIOTTI *et al.*, 2018).

Como o Brasil é um país com uma vegetação diversa ao longo de sua grande extensão, vários tipos de própolis podem ser encontrados. Assim, mais de 500 amostras de própolis foram coletadas ao longo de todos os estados das regiões Sudeste, Sul, Centro-Oeste e alguns estados do Nordeste. Devido à biodiversidade geográfica, vegetal e química, a própolis brasileira foi classificada inicialmente em 12 grupos com base em seu perfil químico complexo, determinado

pela aparência e cor dos extratos, espectro de absorção UV-visível, cromatografia em camada fina de alta eficiência e cromatografia líquida de alta eficiência (PARK, ALENCAR e AGUIAR, 2005). Esta grande variedade de tipos de própolis é um fato único no mundo e não ocorre em outros países da América, Ásia e Europa.

Em 2008, a própolis popularmente conhecida como própolis vermelha, encontrada no litoral de Maceió, Alagoas, foi considerada o 13º grupo de acordo com a classificação proposta anteriormente. A própolis vermelha de Alagoas tem como origem botânica a *Dalbergia ecastophyllum*, planta pertencente à família Leguminosae, mesma família da soja e feijão. Vários de seus compostos isolados são comumente encontrados em plantas da família das leguminosas, que pertence à origem botânica da própolis vermelha (SILVA *et al.*, 2008). Assim, esta própolis vem se destacando recentemente devido às suas propriedades biológicas e compostos isolados.

A própolis brasileira chamada de vermelha apresentou composição química distinta das demais própolis brasileiras, revelando-se com uma natureza mais polar o que facilita sua dissolução em veículos aquosos e revelou a presença de diversas isoflavonas, compostos com reconhecidas propriedades benéficas para os seres humanos. (ALENCAR *et a.l,* 2007; PICCINELLI *et al.,* 2011; BUENO-SILVA *et al.*, 2013a). A estrutura da maioria desses compostos nunca foi relatada nos outros 12 tipos de própolis brasileiras, o que torna a PVB única e especial em relação a sua composição química e suas diversas atividades biológicas (FREIRE, ALENCAR e ROSALEN, 2016). A composição química da própolis também pode variar de acordo com a estação do ano em que é produzida (BUENO-SILVA *et al.*, 2017a).

Os compostos fenólicos e os flavonóides são considerados as principais classes de compostos bioativos da própolis vermelha brasileira. Dentre estes compostos, destacam-se a formononetina, isoliquiritigenina, neovestitol e vestitol. A formononetina é um fitoestrógeno que é um composto polifenólico não esteroidal com atividade biológica demonstrada como por exemplo atividade anti-inflamatória, antioxidante, analgésica e anti-proliferativa (MA *et al.*, 2013; CAVENDISH *et al.*, 2015; WANG *et al.*, 2018). A isoliquiritigenina é uma chalcona que já teve suas propriedades anti-inflamatórias e antiproliferativas claramente demonstradas na literatura (CHEN *et al.*, 2018; ZHANG *et al.*, 2018)

O neovestitol, 7',4'-di-hidroxi-2-methoxyisoflavona, fórmula molecular $C_{16}H_{16}O_4$, foi isolado pela primeira vez em 1976 a partir de uma planta leguminosa africana (INGHAM, 1976). Ele permaneceu desapercebido até alguns anos atrás, quando estudos químicos revelaram sua presença na própolis vermelha cubana (PICCINELLI *et al.*, 2011). Já o vestitol,

(3S)-3-(2-hidroxi-4-metoxifenil)-3,4-di-hidro-2H-cromen-7-ol, fórmula molecular $C_{16}H_{16}O_4$ é uma isoflavona, presente em plantas da família das leguminosas identificada também em amostras de própolis vermelha cubana (CUESTA-RUBIO *et al.*, 2007).

Diversas atividades biológicas/farmacológicas da própolis vermelha recebem muita atenção na literatura mundial (FREIRES, ALENCAR e ROSALEN., 2016). Assim, pode-se destacar os principais efeitos da própolis vermelha e seus compostos bioativos relacionados a odontologia como por exemplo propriedade antimicrobiana, anti-cárie e anti-inflamatória. A combinação de neovestitol/vestitol que também apresenta atividade anticárie também inibiu o crescimento de *P. gingivalis* e *A. actinomicetemcomitans* (BUENO-SILVA *et al*., 2013) em culturas planctônicas, demonstrando seu potencial em inibir o desenvolvimento do biofilme periodontal.

Em relação a atividade anti-inflamatória, o extrato bruto da própolis vermelha brasileira, cujos compostos majoritários são o neovestitol e vestitol, exerce efeito anti-inflamatório sobre macrófagos por meio da inibição da liberação de óxido nítrico e redução da liberação de IL-12, GM-CSF, IFN-Ɣ, IL-1β, IL-4 e TGF-β. Os mecanismos moleculares anti-inflamatórios da BRP parecem ser devidos à regulação negativa de genes envolvidos em a)resposta do tipo Toll-like (TLR); b)enzimas óxido nítrico sintetase (todos os tipos), responsáveis pela produção de óxido nítrico e; c)vias de sinalização NFkB e MAPK como por exemplo os genes *Tirap*, *Pdk1*, *Pak1*, *Nfkb1*, *Mtcp1*, *Gsk3b*, *Fos*, *Elk1*, *Il1β*, *Il1f9* (BUENO-SILVA *et al.*, 2015; 2017B). O neovestitol inibiu a produção de óxido nítrico sem afetar a viabilidade dos macrófagos e reduziu os níveis de citocinas inflamatórias como por exemplo GM-CSF, IFN-c, IL-1b, IL-4, TNF-alfa e IL-6, enquanto aumentou a produção de IL-10, citocina regulatória da resposta inflamatória. O neovestitol também diminuiu a transcrição de genes envolvidos na produção de óxido nítrico, nas vias de sinalização NF-kB, da IL-1b e do TNF-alfa (BUENO-SILVA *et al*., 2017C).

Além do efeito em macrófagos, tanto a PVB quanto o neovestitol e vestitol isolados inibiram a migração de neutrófilos para cavidade peritoneal de camundongos na dose de 10 mg/Kg de peso do animal (BUENO-SILVA *et al*., 2013a, 2016a). O vestitol mostrou atividade inibitória na migração de neutrófilos induzida por diferentes estímulos inflamatórios. Sua atividade está relacionada com a diminuição da liberação das quimiocinas CXCL1/KC e CXCL2/MIP-2 por macrófagos residentes, resultando em diminuição do rolamento e adesão de leucócitos na microcirculação mesentérica. Além disso, foi demonstrado que parte da atividade redutora da migração de neutrófilos do vestitol está relacionada à diminuição da quimiotaxia via bloqueio do influxo de cálcio nestas células (FRANCHIN *et al*., 2016).

O extrato hidroalcoólico da BRP também foi efetivo na melhora dos eventos biológicos associados à cicatrização da queimadura dérmica in vivo (infiltrado inflamatório, deposição de colágeno, taxa de epitelização) quando incorporados em filmes de curativo à base de colágeno, com resultados melhores que os da própolis verde. Em conjunto, esses achados corroboram o potencial uso de PVB na oclusão de feridas e reparo tecidual (ALMEIDA *et al*., 2013).

Portanto, já há evidências científicas para justificar o desenvolvimento de produtos odontológicos a base de própolis vermelha como substitutos ou auxiliares de tratamentos coadjuvantes existente hoje, como no caso da antibioticoterapia sistêmica, sendo necessários estudos bem delineados visando a comparação e comprovação da eficácia desse produto e posteriormente estudos clínicos para confirmar tais resultados e observar a ausência de efeitos tóxicos da própolis vermelha.

2. OBJETIVO

O objetivo deste estudo foi avaliar o efeito do extrato bruto da própolis vermelha (EBPV) sobre modelo de biofilme subgengival multiespécie *in vitro*.

2.1. Objetivos específicos

Avaliar o efeito antimicrobiano das diferentes concentrações (1600; 800; 400 e 200 µg/ml) do EBPV;

Comparar a ação inibitória da atividade metabólica do biofilme tratado com o EBPV (1600; 800; 400 e 200 µg/ml) com o grupo tratado com Amoxicilina 54 µg/mL e com o grupo veículo controle;

Comparar a composição microbiana dos biofilmes tratados com o EBPV com o biofilme tratado com o veículo controle.

3. ARTIGO CIENTÍFICO

O artigo a seguir foi publicado no periódico Antibiotics, IF: 3.898 e pode ser acessado no endereço https://www.mdpi.com/2079-6382/9/8/432.

Brazilian Red Propolis Is as Effective as Amoxicillin in Controlling Red-Complex of Multispecies Subgingival Mature Biofilm *In Vitro*

Abstract: This study investigated the effects of Brazilian Red Propolis (BRP) extract on seven-day-old multispecies subgingival biofilms. Mixed biofilm cultures containing 31 species associated with periodontal health or disease were grown for six days on a Calgary device. Then, mature biofilms were treated for 24 h with BRP extract at different concentrations (200–1600 µg/mL), amoxicillin (AMOXI) at 54 µg/mL (positive control) or vehicle (negative control). Biofilm metabolic activity was determined by colorimetry, and bacterial counts/proportions were determined by DNA–DNA hybridization. Data were analyzed by Kruskal–Wallis and Dunn's tests. Treatment with BRP at 1600, 800 and 400 µg/mL reduced biofilm metabolic activity by 56%, 56% and 57%, respectively, as compared to 65% reduction obtained with AMOXI. Mean total cell counts were significantly reduced in all test groups (~50–55%). Lower proportions of red, green and yellow complex species were observed upon treatment with BRP (400 µg/mL) and AMOXI, but only AMOXI reduced the proportions of *Actinomyces* species. In conclusion, BRP extract was as effective as AMOXI in killing seven-day-old multispecies biofilm pathogens and did not affect the levels of the host-compatible *Actinomyces* species. These data suggest that BRP may be an alternative to AMOXI as an adjunct in periodontal therapy. In vivo studies are needed to validate these results.

Keywords: propolis; antimicrobial; periodontal disease; amoxicillin

1. Introduction

The main etiological factor of periodontal disease is a dysbiosis of the oral subgingival biofilm associated with the presence of periodontopathogens, mainly *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*. These microorganisms release metabolic byproducts or components (e.g., lipopolysaccharides and fimbriae) into periodontal tissues, causing an exacerbated inflammatory response which leads to tissue destruction [1]. Periodontal diseases are estimated to affect between 20 and 50% of the world population, with a negative impact on the individual's health-related quality of life [2].

The treatment of periodontal diseases consists of controlling the occurrence of periodontopathogenic microorganisms to reduce or eliminate tissue inflammation. Mechanical removal of subgingival biofilms by means of scaling and root planning (SRP) significantly improves all periodontal clinical parameters. [3]. However, the mechanical therapy alone may not be fully effective to reverse the major dysbiosis associated with periodontitis, requiring the use of systemic antibiotics. Systemic administration of amoxicillin (AMOXI) was shown to be an effective adjunctive treatment in the management of periodontitis [4,5]. In addition, the combination of AMOXI and metronidazole (MTZ) with SRP has proven an effective approach [6–8]. However, some essential questions associated with the use of these antibiotics remain unanswered, such as potential systemic side effects and the risk of selecting resistant strains [9].

Natural products, such as propolis, have attracted the interest of researchers and laypeople worldwide due to the recent scientific evidence of their beneficial pharmacological properties [10,11]. Brazilian Red Propolis (BRP) has a unique chemical composition that differs from that of other types of Brazilian propolis. BRP contains mostly polar compounds, which facilitates its dissolution in aqueous vehicles and sustains the presence of several isoflavones—compounds with documented beneficial properties for humans [12]. The main relevant pharmacological actions of BRP include antimicrobial [13–15], anti-inflammatory [16–18], anti-tumoral [19], healing [20], antioxidant and antiparasitic properties [15].

Recently, our research group reported that BRP extract is effective in reducing multispecies subgingival biofilm formation [13], but its effects on mature biofilms remain to be determined. Disruption of mature biofilms by antimicrobial agents is challenging and

commonly ineffective since mature biofilm cultures are more virulent than newly formed ones or than planktonic cells [21,22]. Thus, in this study, we investigated the inhibitory effects of BRP extract on mature multispecies subgingival biofilms comparatively to AMOXI.

2. Results

Figure 1 shows the effects of BRP extract and controls on the metabolic activity of multispecies biofilm cells. Treatment with BRP extract at 1600, 800 and 400 µg/mL significantly reduced biofilm metabolic activity by 56%, 56% and 57%, respectively, as compared to the vehicle control ($p < 0.05$). However, there was no significant difference in metabolic activity between cultures treated with BRP at 200 µg/mL and the vehicle ($p > 0.05$).

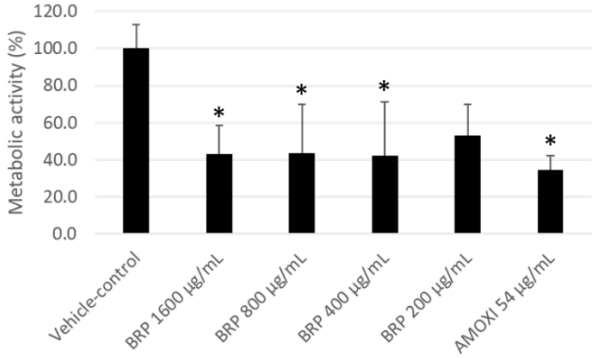

Figure 1. Metabolic activity of multispecies biofilm cultures treated with the dilution vehicle (negative control), Brazilian Red Propolis (BRP) ethanolic extract (1600, 800, 400 and 200 µg/mL) and amoxicillin (AMOXI) (54 µg/mL). Vehicle-treated biofilms were considered as with 100% metabolic activity. (*) indicates significant differences when compared to the control group (Kruskal–Wallis followed by Dunn's post-hoc test, $p \leq 0.05$).

Since the extract concentration of 200 µg/mL did not reduce biofilm metabolic activity, we did not include this treatment group in the checkerboard DNA–DNA hybridization analysis. Figure 2 shows the mean total counts of biofilm cells treated with BRP (1600, 800 and 400 µg/mL) and AMOXI (54 µg/mL). All treatment groups were different from the vehicle control ($p < 0.05$), with no significant difference between them ($p > 0.05$). Interestingly, no significant difference in total cell counts was observed between biofilm cultures treated with BRP extract, regardless of the tested concentration, and those treated with the standard drug AMOXI ($p > 0.05$).

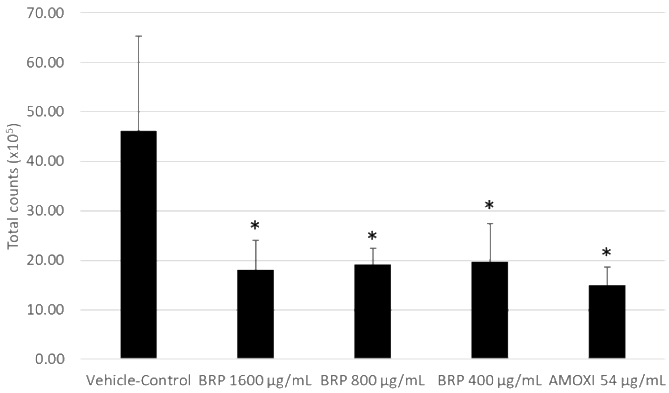

Figure 2. Total bacterial counts (× 10^5) in biofilms treated with the BRP ethanolic extract at 1600, 800 and 400 µg/mL and amoxicillin (AMOXI) at 54 µg/mL. (*) indicates a significant difference when compared to the control group (Kruskal–Wallis followed by Dunn´s post-hoc test, $p \leq 0.05$).

As there was no statistical difference between the three tested concentrations of BRP extract, the lowest effective concentration was selected for further analysis. Figure 3 shows that treatment with BRP (400 µg/mL) and AMOXI (54 µg/mL) significantly reduced the proportions of red-complex (disease-associated), green- and yellow-complex (health-associated) bacterial strains and that only AMOXI decreased the proportions of *Actinomyces* species, as compared to the control group ($p < 0.05$).

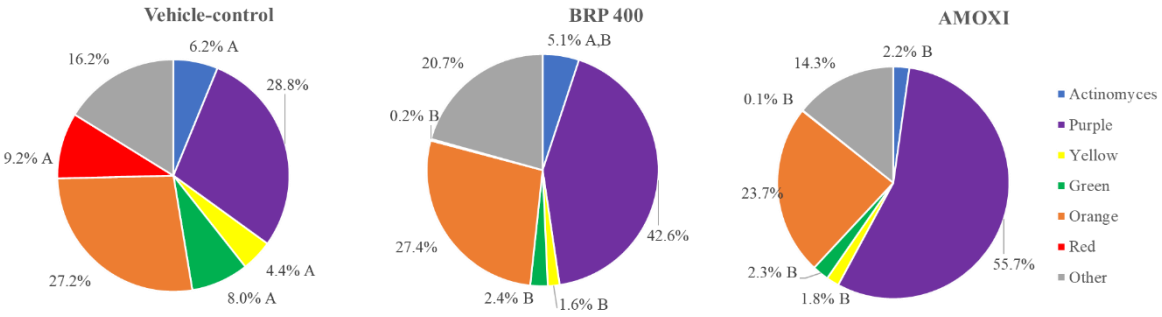

Figure 3. The effects of Brazilian Red Propolis (BRP) extract, amoxicillin (AMOXI) and vehicle control on the proportion of bacterial complexes. The colors represent different microbial complexes as described by Socransky et al., 1998. The data were analyzed by Kruskal–Wallis followed by Dunn's post-hoc test. Different letters indicate significant differences between groups within the same bacterial complex ($p \leq 0.05$).

Figure 4 shows the mean total counts of each bacterial strain in treated biofilms. Treatment with BRP (400 µg/mL) and AMOXI (54 µg/mL) reduced the mean counts of eight different species (*Actinomyces gerencseriae*, *Capnocytophaga ochracea*, *Capnocytophaga gingivalis*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Streptococcus anginosus* and *Streptococcus mutans*) when compared to vehicle-treated biofilms

($p < 0.05$). *Streptococcus sanguinis* counts were significantly reduced only in BRP-treated biofilms, whereas *Streptococcus oralis*, *Streptococcus gordonii*, *Actinomyces israelii* and *Fusobacterium nucleatum vincentii* counts were reduced only in AMOXI-treated biofilms, as compared to the control group ($p < 0.05$).

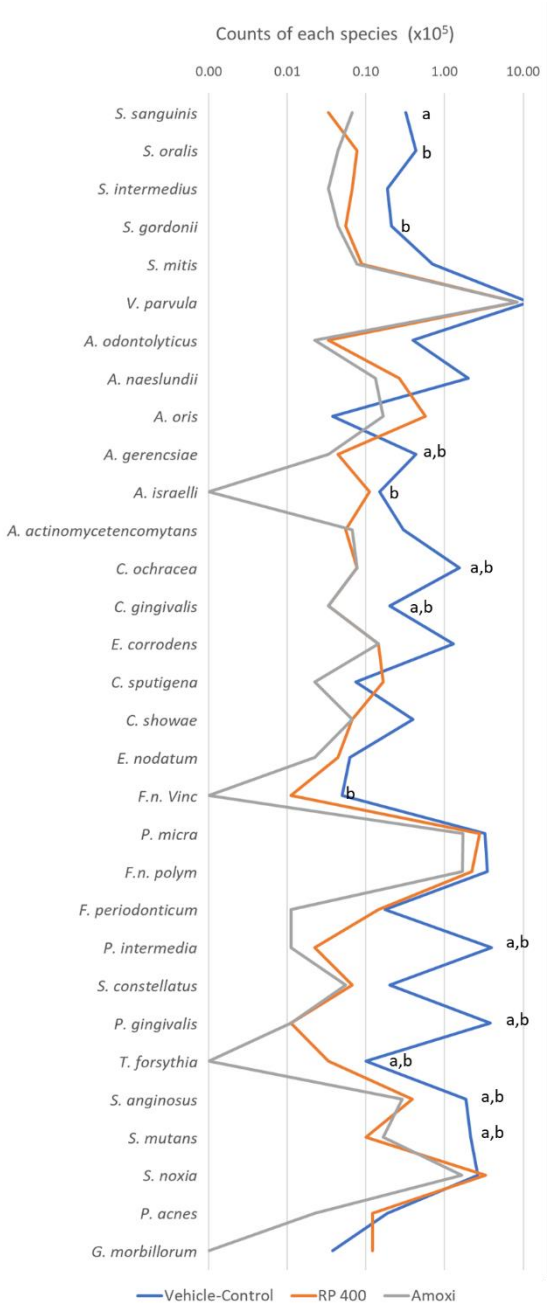

Figure 4. Mean total counts of bacterial strains in biofilms treated with the vehicle control, BRP extract (400 µg/mL) and amoxicillin (AMOXI) (54 µg/mL). The data were analyzed by Kruskal–Wallis followed by Dunn's post-hoc test ($p < 0.05$). Letter "a" indicates statistically significant differences between BRP (400 µg/mL) and the vehicle-control but no difference between BRP (400 µg/mL) and AMOXI; letter "b" indicates statistically significant differences between AMOXI and the vehicle-control but no difference between AMOXI and BRP (400 µg/mL).

3. Discussion

Our study showed that BRP extract was effective in reducing the metabolic activity and total cell counts of red-complex strains in mature biofilms similarly to AMOXI. Moreover, both substances significantly reduced the mean counts of eight bacterial species, including well-known periodontal pathogens, such as *P. gingivalis*, *T. forsythia* and *P. intermedia*, while the proportions of the host compatible *Actinomyces* species were only reduced by AMOXI.

Subgingival biofilms associated with periodontitis are classically categorized into color-coded complexes, according to their role in periodontal health or disease. The red complex is composed by microorganisms associated with disease; the orange complex is associated with the health–disease transition, while the yellow, purple and green complexes as well as *Actinomyces* species are associated with a healthy periodontal condition [23]. It has been well established that pathogens in the red and orange complexes may trigger and/or sustain the dysbiosis state in biofilms associated with periodontitis, thereby leading to a persistent, exacerbated and damaging inflammatory response [1]. Thus, the effects of BRP in reducing the proportion of red-complex pathogens, such as *P. gingivalis* and *T. forsythia*, similarly to a potent antibiotic such as AMOXI, was considered a remarkable result.

A recent study showed that BRP extract was able to reduce the proportions of red and orange bacterial complexes at the concentration of 1600 μg/mL, while chlorhexidine reduced only those from the red complex [13]. The differences between our findings and those of Miranda et al. [13] can be explained by the treatment regimen used in our studies. While Miranda et al. [13] performed two 1 min daily treatments during biofilm formation, we grew biofilm cultures for six days to a mature state and then treated them for 24 h. This protocol was selected to mimic the systemic use of antibiotics, which are normally administered for one/two week(s). Although mature biofilms are more resistant to antimicrobials than immature biofilms, the effective concentration of BRP extract for a 24 h treatment (400 μg/mL) was four-fold lower than that used for daily treatments (1600 μg/mL) [13]. Once the contact period of the BRP extract with biofilm cells was longer in our study, we reasoned that testing a lower effective concentration of the extract would be more appropriate, particularly because our findings showed that increasing BRP extract concentration above 400 μg/mL does not augment the inhibitory effects of the extract against mature biofilms.

An intriguing result observed in our study was that AMOXI-treated biofilms showed lower proportions of beneficial *Actinomyces* species. Interestingly, a previous clinical study

showed a considerable decrease in the proportions of *Actinomyces* species up to one year after AMOXI administration in a group of adults with periodontitis. This may be considered an undesired effect of AMOXI since species of the genus *Actinomyces* have been closely related to rebiosis [4,6,24].

The combination of AMOXI and MTZ with SRP has been well studied and has shown clinically relevant benefits for the treatment of severe periodontitis and killing of subgingival pathogens [6]. However, another study using the same *in vitro* subgingival biofilm model showed that administration of AMOXI alone or AMOXI plus MTZ for a period of 24 h has similar effects on biofilm metabolic activity [25]. Therefore, in the present study, we used only AMOXI as a positive control. The concentration of AMOXI (54 μg/mL) used in our study was the most effective one from a range of concentrations previously tested in a 24 h treatment [25]. Consistent with the findings reported by Soares et al., our study showed a very similar effectiveness of AMOXI in reducing biofilm metabolic activity by 65%.

While systemic antibiotics are clinically effective, there have been reports of side effects [7] and, more importantly, a significant increase in antibiotic resistance rates worldwide [26]. Altogether, this scenario has encouraged researchers to constantly seek novel candidates for co-adjuvant periodontal therapy, such as propolis, probiotics and resolvins [27–30]. The interplay between microbial dysbiosis and inflammation on the onset and progression of periodontal diseases has been a topic of debate, and apparently, BRP extract seems to have effectivity on both aspects. It has been recently shown in vitro and in vivo that BRP can modulate the inflammatory process by inhibiting the Toll-like response and the NF-κB pathway, an important inflammatory mediator [16–18,31]. In addition, our findings and others reported elsewhere [13] indicate that BRP extract has an excellent killing effect against periodontal pathogens during biofilm formation. These data suggest that BRP holds concomitant anti-inflammatory and antibacterial properties. Its complex chemical composition, including the presence of formonetin, medicarpin, neovestitol and vestitol, may explain the major antimicrobial and anti-inflammatory properties displayed by the extract [14,32].

Isolating and purifying fractions from natural products is a relevant and novel strategy in drug discovery and development. Working with crude extracts may prove a challenging task due to their complex chemical composition and the common presence of compounds with unknown polarity and solubility [33]. A fraction containing both neovestitol and vestitol, which are compounds isolated from BRP, showed significant activity against *Streptococcus mutans* biofilms [34]. Thus, the neovestitol/vestitol fraction of BRP should be further tested in

subgingival biofilm models. Additionally, these compounds were shown to have anti-inflammatory properties when tested isolated. Both neovestitol and vestitol were reported to modulate the NF-κB pathway in lipopolysaccharide (LPS)-activated macrophages [35,36] and decreased neutrophil migration, rolling and adhesion, by reducing the expression of ICAM-1 in an in vivo LPS-induced acute peritonitis model [37,38]. Lastly, a recent study also showed that these compounds downregulated the expression of proteins commonly used as targets for cancer treatment [39].

Despite the body of evidence showing the beneficial properties of BRP, its clinical use still warrants further research. Since most of the published articles on this topic report laboratorial or animal studies, the safety and possible toxicity of BRP in humans remain to be determined.

4. Materials and Methods

4.1. Preparation of BRP Extract

BRP samples were collected in a private farm in the city of Maceio, Alagoas State, northeastern Brazil. The samples were scraped off the boxes in which they were transported, which also contained *Apis mellifera* bees. Any residues of bee wax or other materials were removed. The chemical analysis of BRP samples was previously performed by our research group [16]. High-performance liquid chromatography revealed the main compounds found in BRP samples were formononetin, vestitol, neovestitol, quercetin, liquiritigenin and isoliquiritigenin [16], which is in accordance with the literature [14,15,31]. Briefly, the BRP ethanolic extract was obtained by adding 25 g of red propolis into 200 mL of 80% ethanol/ 20% water (*v/v*) under continuous mixing for 45 min. Next, the suspension was filtered with qualitative filter paper (80 g) to remove any possible impurity, the solvent was evaporated using a rotary evaporator equipment, and the BRP ethanolic extract was obtained, with a yield of 73% (from 100 g of red propolis, 73 g of BRP ethanolic extract was obtained). The extract was kept at 4 °C and protected from light to prevent stability loss. The BRP ethanolic extract was re-diluted in 80% ethanol to obtain concentrations of 3200, 1600, 800 and 400 µg/mL. Prior to biofilm treatments, the BRP extracts were diluted 1:1 with the culture media, with final treatment concentrations as 1600, 800, 400 and 200 µg/mL. The final ethanol concentration in the extract used in the treatment was 3.2%.

4.2. In Vitro Multispecies Biofilm Model

In vitro multispecies biofilm cultures were prepared as described by Miranda et al. [13], with some modifications. The bacterial species included in the biofilm model are listed in Table 1.

Table 1. List of bacterial species cultured in multispecies biofilms. The strains were categorized into the microbial complexes described by Socransky et al. [25].

Multispecies Biofilm Strains
Actinomyces complex
Actinomyces naeslundii ATCC 12104
Actinomyces oris ATCC 43146
Actinomyces gerencseriae ATCC 23840
Actinomyces israelii ATCC 12102
Purple complex
Veillonella parvula ATCC 10790
Actinomyces odontolyticus ATCC 17929
Yellow complex
Streptococcus sanguinis ATCC 10556
Streptococcus oralis ATCC 35037
Streptococcus intermedius ATCC 27335
Streptococcus gordonii ATCC 10558
Streptococcus mitis ATCC 49456
Green complex
Aggregatibacter actinomycetemcomitans ATCC 29523
Capnocytophaga ochracea ATCC 33596
Capnocytophaga gingivalis ATCC 33624
Eikenella corrodens ATCC 23834
Capnocytophaga sputigena ATCC 33612
Orange complex
Campylobacter showae ATCC 51146
Eubacterium nodatum ATCC 33099
Fusobacterium nucleatum vincentii ATCC 49256
Parvimonas micra ATCC 33270
Fusobacterium nucleatum polymorphum ATCC 10953
Fusobacterium periodonticum ATCC 33693
Prevotella intermedia ATCC 25611
Streptococcus constellatus ATCC 27823
Red complex
Porphyromonas gingivalis ATCC 33277
Tannerella forsythia ATCC 43037
Other
Streptococcus anginosus ATCC 33397
Streptococcus mutans ATCC 25175
Selenomonas noxia ATCC 43541
Propionibacterium acnes ATCC 11827

Gemella morbillorum ATCC 27824

Tryptone soy agar with 5% sheep blood (Probac, São Paulo, Brazil) was used to grow most species under anaerobic conditions, 85% nitrogen, 10% carbon dioxide and 5% hydrogen, while *Eubacterium nodatum* were cultured on fastidious anaerobic agar with 5% sheep blood. *Porphyromonas gingivalis* was grown on tryptone soy agar containing yeast extract enriched with 1% hemin, 5% menadione and 5% sheep blood. *Tannerella forsythia* was grown on tryptone soy agar containing yeast extract enriched with 1% hemin, 5% menadione, 5% sheep blood and 1% *N*-acetylmuramic acid. All species were allowed to grow on agar plates for 24 h and then transferred to glass tubes containing BHI culture medium (Becton Dickinson, Sparks, MD) supplemented with 1% hemin. After 24 h growing on conical tubes, the optical density (OD) was adjusted for the inoculum to have about 10^8 cells/mL of each species. A dilution of individual cell suspensions was performed, and 100 µL aliquots containing 10^6 cells from each species were added to 11,700 µL of BHI broth complemented with 1% hemin and 5% sheep blood to obtain an inoculum of 15 mL.

The multispecies biofilm model was developed using a Calgary biofilm device (CBD) in a 96-well plate (Nunc; Thermo Scientific, Roskilde, Denmark). A 150 µL aliquot of each inoculum was added to the wells and corresponded to ~1×10^4 cells of each bacterial strain, except for *P. gingivalis* and *Prevotella intermedia*, whose inocula were adjusted to 2×10^4 cells. A lid containing polystyrene pins was used to seal the 96-well plate (Nunc TSP system; Thermo Scientific, Roskilde, Denmark). Coated plates were incubated at 37°C under anaerobic conditions. On day three, the spent medium (BHI broth with 1% hemin and 5% sheep blood) was replaced, and biofilm cultures were kept at 37°C under anaerobic conditions for an additional four days to obtain seven-day-old biofilms [13].

4.3. Treatments with BRP Extract

Biofilm cultures were treated with the BRP extract for 24 h after six days of biofilm development. Biofilm-coated CBD pins were transferred to 96-well plates containing culture media plus the BRP ethanolic extract (1600, 800, 400 or 200 µg/mL), dilution vehicle (negative control) and AMOXI at 54 µg/mL (positive control). All groups were diluted with culture media at a 1:1 ratio. The vehicle control was the same solution used to dissolve the BRP extract and consisted of 6.4% ethanol in 10% phosphate buffer (v/v, final concentration: 3.2%). After treatment, the pins were washed with PBS and submitted to biological tests, as following described.

4.4. Quantification of Biofilm Metabolic Activity

The effects of BRP extract and controls on the metabolic activity of multispecies biofilm cells were measured in a spectrophotometric assay with 2,3,5-triphenyltetrazolium chloride (TTC) (catalog No. 17779; Fluka analytical). TTC is used to differentiate between metabolically active and inactive cells. TCC white substrate is enzymatically reduced to red formazan 1,3,5-triphenyl by live cells due to the activity of several dehydrogenases. The change in substrate color is an indirect measure of bacterial metabolic activity.

To measure the metabolic activity of biofilm cells, the pins were transferred to 96-well plates with 200 μL/well of fresh BHI medium supplemented with 1% hemin and 0.1% TTC solution. The plates were incubated under anaerobic conditions for 8 h at 37 °C. TTC reduction to red formazan was read at 485 nm in a spectrophotometer [13].

4.5. Checkerboard DNA–DNA Hybridization

The pins coated with seven-day-old biofilms from each group were transferred to Eppendorf tubes containing 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 7.6)); then, 100 μL of 0.5 M NaOH was added to each tube. The tubes containing the pins and the final solution were boiled for 10 min, and the solution was neutralized by adding 0.8 mL of 5 M ammonium acetate. The samples were individually analyzed for the presence and count of the 31 bacterial species using the DNA–DNA hybridization technique, as previously described (Socransky et al. 1994; Mestnik et al. 2010). Briefly, following sample lysis, the DNA was placed onto a nylon membrane using a Minislot device (Immunetics, Cambridge, USA) and fixed onto the membrane at 120 °C for 20 min. Next, the membrane was placed in a Miniblotter 45 (Immunetics). Digoxigenin-labelled whole genomic DNA probes of the 31 bacterial species were hybridized in each lane of the Miniblotter. Following hybridization, the membranes were washed, and DNA probes were detected using a specific antibody to digoxigenin conjugated with phosphatase alkaline. The signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, USA), and the data were obtained in Typhoon Trio Plus program (Molecular Dynamics, Sunnyvale, USA). Two lanes in each membrane contained the standards with 1×10^5 and 1×10^6 cells of each strain. The signals were converted into absolute counts via comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero. The measurements of the experimental groups were compared against those of the negative and positive controls. Counts below the method detection limit (1×10^4) were considered zero [40].

4.6. Statistical Analysis

The metabolic activity data were analyzed by Kruskal–Wallis followed by Dunn's post-hoc test, whereas the microbial composition (checkerboard DNA–DNA hybridization) data were analyzed using Kruskal–Wallis followed by Dunn's post-hoc test. A 5% significance level was considered in all statistical tests.

5. Conclusions

Collectively, our findings showed that BRP (at a minimal concentration of 400 μg/mL) extract was as effective as 54 μg/mL of AMOXI in killing seven-day-old multispecies biofilm pathogens and did not affect beneficial *Actinomyces* spp. growth. This suggests that BRP may be an alternative to AMOXI as an adjunct in periodontal therapy, but *in vivo* studies are needed to validate these results.

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4. CONCLUSÃO

O extrato bruto da própolis vermelha brasileira (no mínimo a 400 μg/mL) foi tão eficaz quanto a amoxicilina em inibir o biofilme subgengival multiespécie maduro por meio de um modelo *in vitro*. Ademais, a própolis também beneficiou a composição deste biofilme, favorecendo microrganismos associados a saúde periodontal. Apesar destes resultados laboratoriais promissores, futuros estudos clínicos são necessários para verificação/confirmação destes achados.

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