

SYNERGIC AND ANTIBIOFILM EFFECTS OF MELAMPODIUM DIVARICATUM L. (ASTERACEAE) ESSENTIAL OIL UPON BACTERIA ASSOCIATED WITH DENTAL CARIES

Raquel Regina Duarte Moreira*; Caio Humberto Perego*; José Ricardo Soares de Oliveira**; Tháís da Silva Moraes***; Carlos Henrique Gomes Martins***.

*Departamento de Princípios Ativos Naturais e Toxicologia, Faculdade de Ciências Farmacêuticas UNESP.

**Centro Universitário de Araraquara -UNIARA.

***Laboratório de Pesquisa em Microbiologia Aplicada- LaPeMA, Universidade.

*Autor para correspondência e-mail: raquelrioclaro@gmail.com

PALAVRAS-CHAVE

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RESUMO: *Melampodium divaricatum* é uma espécie herbácea, que ocorre naturalmente nas regiões Nordeste do Brasil e é conhecida por seu valor medicinal e pela ação contra patógenos orais. O objetivo deste estudo foi verificar o efeito sinérgico e a atividade contra a formação de biofilme do OE de *M. divaricatum* contra bactérias associadas à cárie dentária. O método checkerboard foi utilizado para investigar a eficácia antimicrobiana in vitro da combinação de dicloridrato de clorexidina com o óleo essencial de *M. divaricatum*. O ensaio da placa de microtitulação foi utilizado para determinar a Concentração Inibitória Mínima de Biofilme (MICB₅₀) de óleo essencial contra as bactérias. Foi observado efeito sinérgico e atividade contra a formação de biofilme do óleo essencial (OE) de *M. divaricatum* contra as bactérias associadas à cárie dentária. Os efeitos combinados de OE com clorexidina foram aditivos para *L. casei*, antagônicos a *S. mutans* e indiferentes a *S. sobrinuns* e *S. mitis*. A atividade do antibiótico revelou valores significativos de MICB₅₀ (200 a 400 µg/mL). Estes resultados sugerem que o OE de *M. divaricatum* é um produto natural promissor para o desenvolvimento de novas estratégias terapêuticas para combater bactérias cariogênicas.

EFEITO SINÉRGICO E ANTIBIOFILME DO ÓLEO ESSENCIAL DE MELAMPODIUM DIVARICATUM L. (ASTERACEAE) SOBRE BACTÉRIAS ASSOCIADAS A CÁRIE DENTÁRIA

ABSTRACT: *Melampodium divaricatum* is an herbaceous species, naturally occurring in the Northeast regions of Brazil and is known for its medicinal value and the action against oral pathogens. The objective of this study was to verify the synergistic effect and activity against biofilm formation of the EO from *M. divaricatum* against bacteria associated with dental caries. The checkerboard method was used to investigate the in vitro antimicrobial efficacy of the combination of Chlorhexidine dichlorohydrate with *M. divaricatum* EO. The microtitration plate assay was used for determination the Minimum Inhibitory Concentration of Biofilm (MICB₅₀) of essential oil against the bacteria. The synergistic effect and activity against biofilm formation of the essential oil (EO) from *M. divaricatum* against bacteria associated with dental caries were observed. The combination effects of EO with chlorhexidine were additive to *L. casei*, antagonistic to *S. mutans* and indifferent to *S. sobrinuns* and *S. mitis*. The antibiofilm activity revealed significant results MICB₅₀ values (200 to 400 µg/mL). This report suggests that the EO is a promising natural product to develop novel therapeutic strategies to fight against cariogenic bacteria.

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INTRODUCTION

In the microbiota of the oral cavity about 700 different species of bacteria occur. Maintaining homeostasis is essential for maintaining oral health (ARWEILER & NETUSCHIL, 2016). Dental caries is a worldwide public health problem, it is estimated that half of the population is affected with the disease, which makes caries the most prevalent oral disease among the diseases that affect the oral cavity (WHO, 2017). Several plant derivatives, including essential oils have been evaluate against planktonic cells of cariogenic bacteria (CREVELIN, *et al*, 2015; BADARJÍ, *et al*, 2016; ABRÃO, *et al*, 2018; ZHANG, *et al*, 2021). But the cariogenic bacteria may form a complex community structure, that can adhere to the teeth surface forming biofilms. The biofilms firmly adhered to a solid surface covered by an extracellular polysaccharide matrix, and are able to adhere to teeth and causing pathological alterations in oral cavity (JAKUBOVICS, *et al*, 2021).

The mechanical removal of the biofilm is an important factor to prevention of caries, but no sufficient. So, is necessary associate a chemical product to control of the biofilm formation (FURIGA, *et al*, 2008). Chlorhexidine is the most effective antiplaque agent used currently, but its use for a long time can cause undesirable side effects such as taste change, greenish brown coloration of the teeth, mucosal peeling and stone formation, in addition to the development of antimicrobial resistance of the oral flora (CHOO, *et al*, 2001). That is why it is necessary and important to develop new therapeutic alternatives that have biological properties capable of combating this disease safely.

Therefore, the cariogenic bacteria biofilm is usually very resistant to antimicrobials currently used in oral hygiene (ALBERTSSON, *et al*, 2013). So, investigated the anti-biofilm of cariogenic bacteria has attracted the interest of research groups. And, the essential oils, have been actives against a wide variety of the biofilm of oral pathogens (BERSAN, *et al*, 2014; GALVÃO, *et al*, 2012; KOMMEREIN, *et al*, 2021).

M. divaricatum is an herbaceous species, naturally occurring in the Northeast regions of Brazil, locally known as “falsa-calêndula”, and “flor-de-ouro” (AGRA, *et al*, 2008; LORENZI, 2002), appreciated in the local traditional medicine by its diaphoretic and diuretic proprieties, as well as, for treatment of leucorrhea (AGRA, *et al*, 2008). The previous studies revealed the sesquiterpenes (*E*)-caryophyllene, germacrene D, and bicyclogermacrene as their major oil (MOREIRA, *et al*, 2014). The antimicrobial activity of the EO and principal compounds from *M. divaricatum* aerial parts was related for Pelissari, *et al*. (2010). Currenty, Moreira, *et al*. (2014), demonstred that EO, showed active against *Streptococcus sobrinus*, *Lactobacillus casei*, *S. mutans* and *S. mitis* with MIC values equal 90, 30, 20 and 18 µg/mL, respectively.

The objective of this study was to verify the synergistic effect and activity against biofilm formation of the EO from *M. divaricatum* against bacteria associated with dental caries, and which, already presented potential activity against those oral pathogens, in previous study of Moreira, *et al* (2014).

MATERIAL AND METHODS

Plant Material: The essential oil used for all the experiments was previously prepared and fully characterized. Details on the oil preparation and composition were previously reported Moreira *et al* (2014).

Bacteria strains: The bacteria from the American Type Culture Collection were employed: *Streptococcus mutans* (ATCC 25175), *Streptococcus sobrinuns* (ATCC 33478), *Streptococcus mitis* (ATCC 49456) and *Lactobacillus casei* (ATCC 11578).

Synergistic effect: The checkerboard assays was used to investigate the *in vitro* antimicrobial efficacy of the combination of Chlorhexidine dichlorohydrate with *M. divaricatum* EO, according to the protocol previously described by Lewis, *et al*. (2002). The synergistic tests were performed in triplicate, with cell suspension of the 5×10^5 CFU/mL for all tested bacteria. Fractional inhibitory concentration (FIC) index values were calculated on the basis of the equation previously established in the literature, so as to evaluate the synergistic effect between Chlorhexidine dichlorohydrate and EO. Synergistic was define as an FIC

of ≤ 0.5 , and additivity was defined as an FIC of >0.5 but <1 . Indifference was defined as an FIC of ≥ 1 but <4 , whereas antagonism was defined as an FIC of ≥ 4 (LEWIS, *et al*, 2002).

Determination of Minimum Inhibitory Concentration of Biofilm (MICB₅₀): The plate assay was used for determination the Minimum Inhibitory Concentration of Biofilm (MICB₅₀) of essential oil against the bacteria (WEI, *et al*, 2006). The final concentration of EO ranged from 0.195 to 400 $\mu\text{g/mL}$. Chlorhexidine dichlorohydrate (Sigma) at concentrations between 0.115 and 59 $\mu\text{g/mL}$ was assessed as negative control; the bacterial strains in the absence of the antibacterial agent were used as positive control.

The cell suspension was added at concentrations varying from 1×10^6 CFU/mL. After incubation at 37 °C at 24 h/5-10% CO₂. Following incubation the culture supernatants from each well were then decanted, and planktonic cells were removed by washing with PBS, pH 7.2. The biofilm was fixed with methanol for 15 min and air dried at room temperature. It was then stained with 0.2% (w/v) crystal violet (Sigma) for 20 min and rinsed thoroughly with water until the control wells became colorless. Biofilm formation was quantified by the addition of 200 mL of 33% acetic acid to each crystal violet-stained well. The plate was shaken at room temperature for 30 min, and the absorbance at 595 nm using a microplate reader (ASYS, Eugendorf). The percentage of inhibition was calculated using the equation $(1 - A_{595} \text{ of the test} / A_{595} \text{ of non-treated control}) \times 100$ (WEI, *et al*, 2006). This procedure was carried out in triplicate. Selection of the best inoculum concentration and incubation time for the antibiofilm activity assay was accomplished by standardizing biofilm formation (data not shown).

RESULTS AND DISCUSSION

Benefits for the anti-periodontium and anticariogenic action are frequently reported, derived from the use of EO, with the control of biofilm formation. According to literature data, anticariogenic products that contain EO in their formulations have a very high level of antimicrobial potential. Therefore, many of these products demonstrate antibiofilm activity by different mechanisms, such as inhibition of biofilm proliferation, interference with biofilm colonization and/or broad spectrum of antiseptic activity. So, the search for antimicrobials containing EO has increased over the years and the possibility of their being used in oral hygiene product formulations (KIANI, *et al*, 2017; DOBLER, *et al*, 2020).

The MIC values of EO alone and in combinations with chlorhexidine dichlorohydrate *in vitro* checkerboard interactions are shown in Table 1.

Table 1 - Synergistic activity of the *M. divaricatum* essential oil and chlorhexidine dichlorohydrate against dental caries bacteria.

Microorganisms	MIC ($\mu\text{g/mL}$) Alone		MIC ($\mu\text{g/mL}$) Combination		FIC		FICI	Outcome
	(1)	(2)	(1)	(2)	(1)	(2)		
<i>S. mutans</i>	20	0.92	80	0.23	4.0	0.25	4.25	Antagonic
<i>S. sobrinus</i>	90	1.84	90	0.23	1.0	0.125	1.125	Indifferent
<i>S. mitis</i>	18	14.75	18	29.5	1.0	2.0	3.0	Indifferent
<i>L. casei</i>	30	3.69	15	0.23	0.5	0.06	0.6	Additive

(1) Essential oil of *Melampodium divaricatum*. (2) Chlorhexidine dichlorohydrate.

Source: Prepared by the authors.

The activity of EO with chlorhexidine dichlorohydrate against the test oral pathogens recorded additive effect to *L. casei* (IFIC index 0.6), antagonistic to *S. mutans* (IFIC index 4.25) and indifferent to *S. sobrinus* (IFIC index 1.125) and *S. mitis* (IFIC index 3.0).

The MICB₅₀ values for the EO and chlorhexidine dichlorohydrate are shown in Table 2. The MICB₅₀ values were of 200 µg/mL for *S. mutans*, *S. sobrinus* and *L. casei* and 400 µg/mL for *S. mitis*. Compared with planktonic cells, the biofilm is less sensitive to the antimicrobial compound. Literature reports have described that biofilms tend to be 10 to 1.000 times more resistant to antimicrobial agents as compared with the planktonic state (MAH & O'TOOLE, 2001). The results of the present study evidencing that EO exhibits significant inhibition of the biofilm formation by 50% or even more, and the average increase in MICB₅₀ in relation to MIC was twofold.

Table 2 - Comparative results of antibacterial activity Minimum Inhibitory Concentration/ Minimum Inhibitory Concentration of Biofilm (MIC/MBC₅₀) of essential oil from *M. divaricatum* against dental caries bacteria.

Microorganisms	Essential oil of <i>M. divaricatum</i>		Chlorhexidine dichlorohydrate	
	MIC*	MIBC ₅₀ (µg/mL)	MIC*	MIBC ₅₀ (µg/mL)
<i>S. mutans</i>	20	200	0.92	14.75
<i>S. sobrinus</i>	90	200	1.84	29.50
<i>S. mitis</i>	18	400	14.75	29.50
<i>L. casei</i>	30	200	3.69	14.75

* values obtained in the synergism test.

Source: Prepared by the authors.

The behavior of the biofilm differs markedly from the behavior of the planktonic state of the same bacterium, because bacterial biofilms respond distinctly depending on the growth phase, antimicrobial concentration, and period of exposure to the antibacterial agent (WEI, *et al*, 2006).

To date, no study has been found in the literature that investigated the antibiofilm action of *Melampodium divaricatum* against cariogenic bacteria, which makes it impossible to compare the data obtained in this study with other authors. However, this activity may be related to its chemical composition, which in previous studies revealed the sesquiterpenes (*E*)-caryophyllene, germacrene D, and bicyclogermacrene as their major oil (MOREIRA, *et al*, 2014). Several studies have shown interesting results regarding the antimicrobial and anti-inflammatory activities of *Melampodium divaricatum* EO, particularly those concerning a antimicrobial activity and the growth inhibition oral pathogens (PELLISSARI, *et al*, 2010; MOREIRA, *et al*, 2014).

In the study by Moreira *et al*, (2014) the main components of the extract of *M. divaricatum* were isolated and did not show promising antibacterial activity against cariogenic bacteria. This observation suggests that the antimicrobial action of EO's is through a possible synergistic action between all components of the oil and not due to the action of any specific constituent. This same hypothesis is also raised by other authors who investigate the antibacterial action of other EO's and needs to be further investigated (ACÍ-

MOVIĆ *et al*, 2020). In the scientific literature, similar reports are found, such as the promising action of *Stachys koelzii* against *P. intermedia* more promising than its isolated compounds (RAMAK 2018).

Currently, there is a trend towards the prescription of periodontal products containing EO for biofilm control, especially when mechanical control is insufficient. (FINE, *et al*, 1996; TELES, *et al*, 2009).

Teles *et al*, (2009), reported that adjunct use of essential oil mouthrinses and triclosan/copolymer dentifrices can result in additional reductions in plaque and gingivitis, particularly in hard-to-reach areas such as interproximal spaces. Antimicrobial agents such as essential oils are capable of affecting bacteria growing in supragingival biofilms and disrupt preexisting plaque. The use of essential oil mouthrinses and dentifrices containing triclosan/copolymer might affect the subgingival microbiota through the disruption of the contiguous supragingival plaque.

Studies have emphasized the clinical effectiveness in reducing biofilm and gingivitis promoted by EO introduced in oral hygiene products, an action that is very similar to that obtained with chlorhexidine. there are also reports that the concomitant use with EO does not promote extrinsic stains on the teeth, nor other undesirable effects, when compared to chlorhexidine mouthrinses. Another study evaluating the effect of EO contained in toothpastes on the composition of BD highlighted that its use did not affect the balance of the oral microflora, nor did it allow the emergence of opportunistic pathogens (HENZ, & BARON, 2009).

Nogueira *et al*, (2006) evaluated the genotoxic activity of *M. divaricatum* extract and antigenotoxic activity against agents inducing DNA damage, through assays with Salmonella typhimurium, where the extract was not mutagenic against the strains evaluated by the authors (S. typhimurium TA100, TA98, TA97a and TA102) and decreased the mutagenicity of the evaluated damage-inducing agents (flatoxin B1, benzo(a)pyrene and daunomycin). These results corroborate those found in the present study, and may guarantee potential safety for use in mouth care products that act against cariogenic microorganisms used.

This is the first study that sought to assess the antibiofilm activity of EO and its synergistic effect of *M. divaricatum* with chlorhexidine against cariogenic bacteria. Additional studies should be carried out to increase the understanding of the mechanisms of action of EO *Melampodium divaricatum* in the oral cavity and the possibility of its incorporation in topical gel formulations or mouthwashes, for its use as an anticariogenic agent.

CONCLUSION

Hence, essential oil of *M. divaricatum* deserves further investigation in the search for novel prototypes and promising biomolecules to treat the infections caused for bacteria associated with dental caries.

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