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BIOFILM PRODUCTION AND ANTIBACTERIAL ACTIVITY OF THE ESSENTIAL OIL OF MENTHA PIPERITA AGAINST STAPHYLOCOCCUS AUREUS ISOLATED FROM PATIENTS WITH CHRONIC RHINOSINUSITIS

PRODUÇÃO DE BIOFILME E ATIVIDADE ANTIBACTERIANA DO ÓLEO ESSENCIAL DE MENTHA PIPERITA CONTRA STAPHYLOCOCCUS AUREUS ISOLADO DE PACIENTES COM RINOSSINUSITE CRÔNICA

PRODUCCIÓN DE BIOFILM Y ACTIVIDAD ANTIBACTERIANA DEL ACEITE ESENCIAL DE MENTHA PIPERITA CONTRA STAPHYLOCOCCUS AUREUS AISLADO DE PACIENTES CON RINOSINUSITIS CRÓNICA

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ABSTRACT
Background: Chronic Rhinosinusitis (CRS) is an inflammatory disease of the mucosa of the nasal cavity and paranasal sinuses. The Staphylococcus aureus (S. aureus) are frequently found in patients with CRS and biofilms from this species are often associated with recalcitrant or recurrent disease. Objective: To verify the formation of biofilm in S. aureus carried by patients with Chronic Rhinosinusitis with Nasosinusal Polyposis (CRSwNP) and to perform in vitro tests to evaluate the antimicrobial capacity of the essential oil of Mentha piperita (M. piperita). Methods: Foram analisadas 14 amostras de S. aureus isolados oriundos de pacientes com CRSwNP, atendidos no ambulatório de otorrinolaringologia do Regional Hospital de Presidente Prudente- SP. Samples were seeded on blood agar and subjected to Gram, catalase and coagulase stains. To verify the production of biofilm, we used the methods of adherence in tubes of borosilicate and Congo red agar, as well as, the antimicrobial activity of the essential oil of M. piperita was evaluated through the technique of Minimum Inhibitory Concentration (MIC). Results: The results indicated that the analyzed S. aureus has the ability to form a biofilm and that the essential oil of M. piperita is effective in inhibiting the growth of this bacterium according to the concentrations used. Conclusion: In view of the results obtained, we concluded that the S. aureus isolated from patients with CRSwNP have the ability to form a biofilm and that the essential oil of M. piperita has antimicrobial potential on the tested strains. More studies must be carried out in order to prove its use as an alternative to combat infections and control the spread of this bacterium.

KEYWORDS: Sinusitis. Nasal Polyp. Biofilms. Mentha piperita. Staphylococcus aureus.

RESUMO
Introdução: A Rinossinusite Crônica (RSC) é uma doença inflamatória da mucosa da cavidade nasal e seios paranasais. Os *Staphylococcus aureus (S. aureus)* são frequentemente encontrados em pacientes com RSC e os biofilmes dessa espécie estão frequentemente associados a doenças

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JOURNAL HEALTH AND TECHNOLOGY - JHT
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recalcitrantes ou recorrentes. Objetivo: Verificar a formação de biofilme em *S. aureus* carreados por pacientes com Rinossinusite Crônica com Polipose Nasossinusal (RSCcPN) e realizar testes *in vitro* para avaliar a capacidade antimicrobiana do óleo essencial de *Mentha piperita (M. piperita)*. Métodos: Foram analisadas 14 amostras de *S. aureus* isolados oriundos de pacientes com RSCcPN, atendidos no ambulatório de otorrinolaringologia do Hospital Regional de Presidente Prudente- SP. As amostras foram semeadas em ágar sangue e submetidas a coloração de Gram, catalase e coagulase. Para verificar a produção de biofilme, utilizamos os métodos de aderência em tubo de borossilicato e ágar vermelho congo, assim como, foi avaliado a atividade antimicrobiana do óleo essencial de *M. piperita* através da técnica de Concentração Inibitória Mínima (CIM). Resultados: Os resultados indicaram que os *S. aureus* analisados possuem capacidade de formar biofilme e que óleo essencial de *M. piperita* é eficaz para inibir o crescimento dessa bactéria conforme as concentrações utilizadas. Conclusão: Diante dos resultados obtidos, concluímos que os *S. aureus* isolados de pacientes com RSCcPN possuem a capacidade de formar biofilme e que o óleo essencial de *M. piperita* possui potencial antimicrobiano sobre as cepas testadas. Mais estudos devem ser realizados a fim de comprovar a sua utilização como uma alternativa no combate as infecções e no controle da disseminação desta bactéria.

PALAVRAS-CHAVE: Sinusite. Pólipos Nasais. Biofilmes. *Mentha piperita. Staphylococcus aureus*.

RESUMEN
Introducción: La Rinosinusitis Crónica (RSC) es una enfermedad inflamatoria de la mucosa de la cavidad nasal y senos paranasales. Staphylococcus aureus (S. aureus) se encuentra con frecuencia en pacientes con CRS y las biopelículas de esta especie a menudo se asocian con enfermedad recalcitrante o recurrente. Objetivo: Verificar la formación de biofilm en S. aureus portados por pacientes con Rinosinusitis Crónica con Poliposis Nasosinusal (RSCcNP) y realizar ensayos in vitro para evaluar la capacidad antimicrobiana del aceite esencial de Mentha piperita (M. piperita). Métodos: Se analizaron 14 muestras de S. aureus aisladas de pacientes con CRSwNP, atendidos en el ambulatorio de otorrinolaringología del Hospital Regional de Presidente Prudente-SP. Las muestras se sembraron en agar sangre y se sometieron a tinción de Gram, catalasa y coagulasa. Para verificar la producción de biopelícula se utilizaron los métodos de adherencia en tubo de borosilicato y agar rojo congo, así como también se evaluó la actividad antimicrobiana del aceite esencial de M. piperita mediante la técnica de Concentración Mínima Inhibitoria (MIC). Resultados: Los resultados indicaron que los S. aureus analizados tienen la capacidad de formar una biopelícula y que el aceite esencial de M. piperita es eficaz para inhibir el crecimiento de esta bacteria según las concentraciones utilizadas. Conclusión: A la vista de los resultados obtenidos, concluimos que los S. aureus aislados de pacientes con CRSwNP tienen la capacidad de formar una biopelícula y que el aceite esencial de M. piperita tiene potencial antimicrobiano sobre las cepas probadas. Se deben realizar más estudios para comprobar su uso como alternativa para combatir infecciones y controlar la propagación de esta bacteria.

PALABRAS CLAVE: Sinusitis. Pólipos Nasales. Biopelículas. Mentha piperita. Staphylococcus aureus

1- INTRODUCTION

Chronic Rhinosinusitis (CRS) is an inflammatory disease of the mucosa of the nasal cavity and paranasal sinuses, lasting longer than 12 weeks. CRS is phenotypically classified as CRS with nasal polyps (CRScPN) or CRS without nasal polyps (CRSsPN) [1]. Infection and persistence of Staphylococcus aureus are associated with CRS, and may be particularly relevant in the form of nasal polyps (CRSwNP) [2].

Despite the great diversity of antibacterial drugs that act on various microorganisms that are pathogenic to humans, alternative medicine has been seeking natural means for the treatment of infections caused by bacteria. With this, there is a growing interest in the therapeutic use of natural

products, specifically those derived from plants. The literature reports the antibacterial action of the essential oil of Mentha piperita, both for Gram positive and Gram negative bacteria [3].

Thus, this study has the objective of verifying the formation of biofilm in S. aureus carried by patients with CRScPN and to carry out in vitro tests with different concentrations of the essential oil of M. piperita (peppermint), thus enabling a possible alternative for the control of infections caused by these bacteria.

2- METHOD

Samples

Fourteen samples of *S. aureus* from the bacteria bank of the Universidade do Oeste Paulista were analyzed. Samples were isolated from patients attended at the ENT outpatient clinic of the Hospital Regional de Presidente Prudente/SP with a previous diagnosis of CRSwNP (clinical, tomographic and endoscopic diagnosis) and with surgical indication for removal of the nasal polyp, between September 2018 and August 2019.

The study was approved by the Research Ethics Committee through Plataforma Brasil (Protocol: CAAE 91934418.3.0000.5515) and all participants signed and provided a free and informed consent form for the use of samples in research.

Identification of Staphylococcus aureus

To guarantee the purity of the samples received, the S. aureus isolates were spread on blood agar plates, stained using the Gram method and submitted to catalase and coagulase tests. For sowing, Baird Parker agar was used and incubated for 24 hours at 37°C.

Study of biofilm production by the borosilicate tube adhesion method

To research the phenotypic production of biofilm, we used the borosilicate tube adherence method described by Christensen [4], which consists of inoculating isolated colonies of *S. aureus* in tubes containing 2.0 ml of Trypticase Soy Broth (TSB) and Incubate at 37°C for 48 hours without shaking. Subsequently, the contents were discarded in 1.0 ml aliquots of 0.4% trypan blue aqueous solution and added to each tube. After gentle agitation, to ensure the coloring of the material adhered to the inner surface of the tubes, the dye was discarded. The positive result was indicated by the presence of a layer of colored material, adhered to the inner wall of the tubes. The presence of a colored ring only on the liquid-air contact surface was not considered a positive result.

To control the technique, international reference strains were used, including non-biofilm producing *S. aureus* ATCC 33591 (negative control) and biofilm producing S. aureus ATCC 29213 (positive control).

JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

Study of biofilm production by Congo red agar method

The phenotypic characterization of biofilm production was studied by culturing samples of S. aureus on Congo Red Agar (CRA) plates proposed by Arciola et al. (2001). CRA plates (0.8g of Congo red dye and 36 g of sucrose for 1 liter of Brain and Heart Infusion - BHI) were inoculated and incubated aerobically for 24 hours at 37ºC and subsequently "overnight" at room temperature. In CRA, biofilm-producing species form black colonies, while non-producing species form red colonies.

Antibacterial activity of essential oil in vitro

The minimum inhibitory concentration (MIC) of the essential oil of M. piperita was determined by the broth microdilution test [5]. For this purpose, 14 bacterial isolates included in the study and two standard strains ATCC 33591 (non-biofilm producer) and ATCC 29213 (biofilm producer) were tested. We use 96-well polystyrene plates and each well will receive known amounts of essential oil in Tween 80 in order to obtain 5 different concentrations: 50; 40; 30; 20 and 10%.

The dilutions were obtained through a heterogeneous mixture of EO and distilled water after a lot of agitation and used momentarily. MIC was defined as the lowest concentration of essential oil to inhibit bacterial growth (without color change) and MIC90% was subsequently calculated. For the positive control of bacterial viability, we used the BHI broth + microbial inoculum (5 µl) and for the sterility control, only the BHI broth.

3- RESULTS

The 14 samples from the bacteria bank at Universidade do Oeste Paulista were submitted to two culture growth methods to confirm the species. The first culture method used was Blood Agar, where the samples were seeded through an inoculation loop and subsequently placed in an oven at 37°C for a period of 24 hours. Then, the selected plates were seeded on Mannitol Agar and incubated for a period of 24 hours at 37°C in a bacteriological oven, confirming the growth of *S. aureus*.

After confirming the isolates, biofilm identification techniques were performed using Congo red BHI medium and the adherence method in borosilicate tubes using trypan blue dye. Petri dishes containing BHI + congo red were seeded with samples through the sterile swab. The plates were placed in an oven at a temperature of 37°C for a period of 24 hours. The following day, the reading was performed, which found 13 samples positive for Biofilm growth (where one strain was ATCC 29213 – used as a positive control) (Table 1).

JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

Table 1. Results of the biofilm production research by the BHI + congo red phenotypic method and by the tube adherence method using the trypan blue dye.

Sample	BHI Agar + Congo Red	Trypan Blue
1- N	-	-
4 – O	+	+
5 – O	+	+
5 - N2	+	+
18 – O	+	+
18 – N	+	+
19 – N	+	+
19 – O	+	+
25 – O	+	-
28 – O	+	-
28 – N	+	+
29 – N	+	+
33 – O	+	+
46 – O	-	+
ATCC 33591	negative control	
ATCC 29213	positive control	

Source: The authors, 2023

To prepare the microdilution technique, the concentrated essential oil of *M. piperita* was diluted in concentrations of 50; 40; 30; 20 and 10% in Tween 80. The bacteriological samples were suspended in BHI broth, preserving the pre-established concentrations of essential oil, the procedure was carried out in a 96-well microplate, bottom in U. After preparing the samples, they were incubated at 37°C for a period of 24 hours, in a bacteriological oven, resulting in the data in table 2.

JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

Table 2. CIM test results.

	Concentrations of *Mentha piperita* (%)				
Samples	50	40	30	20	10
1-N	-	-	-	-	-
4-O	-	-	-	-	-
5-N2	-	-	-	-	-
5-O	-	-	-	-	-
18-N	-	-	-	-	-
18-O	-	-	-	-	-
19-N	-	-	-	-	-
19-O	-	-	-	-	-
25-O	-	-	-	-	-
28-N	-	-	-	-	-
28-O	-	-	-	-	-
29-N	-	-	-	-	-
33-O	-	-	-	-	-
46-O	-	-	-	-	-
ATCC 29213	-	-	-	-	-
ATCC 33591	-	-	-	-	-

Source: The authors, 2023

Subtitle: + Presence of bacterial growth and - Absence of bacterial growth

4- DISCUSSION

To evaluate the antibacterial activity of M. piperita oil in different concentrations against S. aureus isolated from patients with chronic rhinosinusitis and nasosinusal polyposis, the diffusion method in agar wells was used, where we evaluated growth inhibition in different concentrations. The results found demonstrate that in all concentrations, the essential oil of M. piperita was effective in eliminating the *S. aureus* tested, including the standard samples (ATCC 29213 and ATCC 33591).

The bacteria *S. aureus* belongs to the human microbiota, however, it can cause several diseases including infections from mild to a higher degree, such as pneumonia, meningitis, among others [3].

Pigeon [6] describes that gram-positive bacteria are more sensitive to essential oils than gram-negative bacteria. This refers to the structural complexity of the cell wall of gram-negative bacteria, which have an external membrane made up of lipopolysaccharides that surrounds the cell wall, providing more resistance and hindering the diffusion and dissemination of the antibacterial action.

Biofilm growth occurs through a combination of cell division and recruitment of other microorganisms. In the case of chronic rhinosinusitis, bacteria are the organizers of biofilms, contributing

JOURNAL HEALTH AND TECHNOLOGY - JHT
ISSN 2764-7625

to greater resistance to antibiotics. According to Souza et al., bacteria belonging to biofilms can resist antibiotic doses about 1000 times higher than if they exist in planktonic form.

Biofilms are organized in such a way that bacteria acquire several advantages related to their planktonic forms, mainly in protection against the immune system and the action of antibiotics, in addition, they are organized in such a way that they maintain the adequate physical and chemical environment to survive. and grow [7].

In this research, we found that there was formation of biofilm. By the Congo red agar method, 13 samples were positive for the growth of biofilm associated with *S. aureus*. In the method of verifying the production of biofilm using the technique of adherence to a borosilicate tube, the production of biofilm was observed on the wall of 12 tubes. In both methods, the presence of biofilm formation associated with S. aureus occurred. According to Bezerra [8], the high rate of enterotoxin-secreting S. aureus cultured from the nasal cavity and the presence of *S. aureus* as a biofilm in patients with CRSwNP, demonstrates that S. aureus plays an important role in the development of the disease.

The successive search for new antimicrobial agents boosted numerous studies focused on the antimicrobial effect of phytoconstituents extracted from a wide range of plants that had already been used for a long time [9].

Nonato [10] concluded in his research that from the concentration of 8 µl/mL of essential oil of *M. piperita*, there is no visible microbial growth of the bacteria *S. aureus*, according to him, the essential oil of *M. piperita* at low concentrations it already has a bactericidal effect. Ferreira [11] also concluded in his research that both the essential oil of *M. piperita* and the hydroalcoholic extract of *M. piperita* have in vitro antibacterial activity for the bacterial strain of S. aureus.

According to Valeriano [12], *M. piperita* stands out for having antibacterial, antiviral and antifungal activity, and this activity is mainly associated with the major compound's menthol, menthone, methyl acetate and iso-menthone.

The possible antibacterial action of the essential oil of *M. piperita* can be an alternative potential to create new drug formulas based on this substance, as natural plant compounds cause less risk to the human body [10].

5- CONSIDERATIONS

Biofilm formation was observed in the analyzed samples. The ability to form a biofilm is an important factor in CRSwP, as it is associated with a more severe disease, difficult to treat, with greater postoperative recurrence, in addition to contributing to damage to the nasal mucosa and increasing the number of inflammatory cells in the nose. tissue.

The results obtained in this research also demonstrated that the essential oil of *M. piperita* has a high inhibitory potential on the strains of *S. aureus* tested, and can be used as an alternative to fight

infections and control the spread of this bacteria. It is a positive result that expands the existing antimicrobial therapeutic options on the market today.

REFERENCES

[1] Kim D, Assiri AM, Kim JH. Recent Trends in Bacteriology of Adult Patients with Chronic Rhinosinusitis. Journal of Clinical Medicine. 2019;8:1889. https://doi.org/10.3390/JCM8111889.

[2] Poddighe D, Vangelista L. *Staphylococcus aureus* Infection and Persistence in Chronic Rhinosinusitis: Focus on Leukocidin ED. Toxins (Basel) 2020;12:678. https://doi.org/10.3390/TOXINS12110678.

[3] Santos AL, Santos DO, Freitas CC de, Ferreira BLA, Afonso IF, Rodrigues CR, *et al*. *Staphylococcus aureus*: visitando uma cepa de importância hospitalar. J Bras Patol Med Lab 2007;43:413–23. https://doi.org/10.1590/S1676-24442007000600005.

[4] Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, *et al*. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985;22:996. https://doi.org/10.1128/JCM.22.6.996-1006.1985.

[5] CLSI C and LSI. M100-performance standards for antimicrobial susceptibility testing. Clinical and Laboratory. 2018;28.

[6] Pombo JCP, Ribeiro ER, Pinto R de L, Silva BJM da. Efeito antimicrobiano e sinergístico de óleos essenciais sobre bactérias contaminantes de alimentos. Segurança Alimentar e Nutricional 2018;25:108–17. https://doi.org/10.20396/SAN.V25I2.8651785.

[7] Capistrano ECJ. Avaliação da associação entre biofilmes bacterianos, bactérias intracelulares e superantígenos estafilocócicos em pacientes com rinossinusite crônica. [Tese de Doutorado]; São Paulo: Universidade de São Paulo; 2018. https://doi.org/10.11606/T.17.2018.TDE-10042018-141217.

[8] Bezerra TFP. O papel do biofilme na rinossinusite crônica com polipose nasossinusal. [Tese de Doutorado]; São Paulo: Universidade de São Paulo; 2012. https://doi.org/10.11606/T.5.2012.TDE-01082012-135039.

[9] Alves LA, Freires I de A, Castro R de D. Efeito antibacteriano de óleos essenciais sobre bactérias formadoras do biofilme dentário. Revista Brasileira de Ciências Da Saúde. 2011;14:57–62. https://doi.org/10.4034/RBCS.2010.14.02.07.

[10] Nonato I da S. Efeito antibacteriano do óleo essencial de *Mentha Piperita L.* sobre as bactérias Sthaphylococcus aures e Escherichia coli. [Dissertação]; Governador Mangabeira: Faculdade Maria Milza; 2018.

[11] Ferreira H. Avaliação da atividade antibacteriana de extratos de plantas medicinais: Significância sanitária em região de tríplice fronteira. [Tese de Doutorado]; São Paulo: Universidade de São Paulo; 2018.

[12] Valeriano C, Piccoli RH, Cardoso MG, Alves E. Atividade antimicrobiana de óleos essenciais em bactérias patogênicas de origem alimentar. Revista Brasileira de Plantas Medicinais. 2012;14:57–67. https://doi.org/10.1590/S1516-05722012000100009.