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Antimicrobial and anti-adherent potential of the ethanolic extract of *Praxelis clematidea* (Griseb.) R.M.King & Robinson on pathogens found in the oral cavity

Potencial antimicrobiano e anti-aderente do extrato etanólico de *Praxelis clematidea* (Griseb.) R.M.King & Robinson em patógenos encontrados na cavidade oral

Potencial antimicrobiano y anti-adherente del extracto etanólico de *Praxelis clematidea* (Griseb.) R.M.King & Robinson sobre patógenos encontrados en la cavidad oral

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Abstract

With the evolution of bacterial resistance over conventional antimicrobials and the development of new antimicrobials, the search for new compounds of natural origin has intensified, since plants with therapeutic potential constitute a source of new biologically active compounds, encouraging the development of new therapeutic options. The objective of this study was to evaluate the antibacterial and anti-adherent activity of the ethanolic extract of *Praxelis clematidea* (Griseb.) R.M. King & Robinson (*Asteraceae*) on strains of *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterococcus faecalis*. Tests were carried out to determine the MIC and MBC by means of broth microdilution in 96-well plates, and to determine the MICA, a technique with inclined tubes in the presence of 5% sucrose was used. It was observed that the ethanolic extract of *P. clematidea* has strong bactericidal activity on *K. pneumoniae*, and moderate bactericidal activity on *S. pneumoniae* and *E. faecalis*. Being able to inhibit adherence to *K.*

pneumoniae strains. Thus, the ethanolic extract of *P. clematidea* proves to be effective as an antimicrobial in the control and prevention of infections by *S. pneumoniae*, *K. pneumoniae* and *E. faecalis*. It also has an effective anti-adherent effect on *K. pneumoniae* strains.

Keywords: *Praxelis clematidea*; Antibacterials; Bacteria.

Resumo

Com a evolução da resistência bacteriana sobre os antimicrobianos convencionais e o desenvolvimento de novos antimicrobianos, a procura por novos compostos de origem natural tem se intensificado, uma vez que plantas que apresentam potencial terapêutico constituem uma fonte de novos compostos biologicamente ativos, incentivando o desenvolvimento de novas opções terapêuticas. O objetivo deste estudo foi avaliar a atividade antibacteriana e antiaderente do extrato etanólico de *Praxelis clematidea* (Griseb.) R.M. King & Robinson (*Asteraceae*) sobre cepas de *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa* e *Enterococcus faecalis*. Foram realizados os ensaios para determinação da CIM e CBM por meio de técnica de microdiluição em caldo em placas de 96 poços, e para determinação da CIMA foi utilizada técnica com tubos inclinados na presença de 5% de sacarose. Observou-se que o extrato etanólico de *P. clematidea* apresenta forte atividade bactericida sobre *K. pneumoniae*, e moderada atividade bactericida sobre *S. pneumoniae e E. faecalis.* Sendo capaz de inibir a aderência das cepas de *K. pneumoniae.* Com isso, o extrato etanólico de *P. clematidea* demonstra ser eficaz como antimicrobiano no controle e prevenção de infecções por *S. pneumoniae, K. pneumoniae* e *E. faecalis.* Também apresentando eficaz efeito antiaderente sobre as cepas de *K. pneumoniae.*

Palavras-chave: *Praxelis clematidea;* Antibacterianos; Bactérias.

Resumen

Con la evolución de la resistencia bacteriana sobre los antimicrobianos convencionales y el desarrollo de nuevos antimicrobianos, se ha intensificado la búsqueda de nuevos compuestos de origen natural, ya que las plantas con potencial terapéutico son fuente de nuevos compuestos biológicamente activos, favoreciendo el desarrollo de nuevas opciones terapéuticas. El objetivo de este estudio fue evaluar la actividad antibacteriana y antiadherente del extracto etanólico de *Praxelis clematidea* (Griseb.) R.M. King & Robinson (*Asteraceae*) sobre cepas de *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa* y *Enterococcus faecalis*. Se realizaron pruebas para determinar la CMI y CBM mediante microdilución en caldo en placas de 96 pocillos, y para determinar la

CIMA se utilizó una técnica con tubos inclinados en presencia de sacarosa al 5%. Se observó que el extracto etanólico de *P. clematidea* tiene una fuerte actividad bactericida sobre *K. pneumoniae* y una moderada actividad bactericida sobre *S. pneumoniae* y *E. faecalis*. Ser capaz de inhibir la adherencia a cepas de *K. pneumoniae*. Así, el extracto etanólico de *P. clematidea* demuestra ser eficaz como antimicrobiano en el control y prevención de infecciones por *S. pneumoniae, K. pneumoniae* y *E. faecalis*. También tiene un efecto antiadherente eficaz sobre las cepas de *K. pneumoniae*.

Palabras clave: *Praxelis clematidea;* Antibacterianos; Bacterias.

1. Introduction

Changes in the oral environment can cause pathologies of the oral cavity and affect related systemic conditions, influencing the reduction of the immunological competence of an already compromised patient (Parahitiyawa et al., 2010; Zarco, Vess & Ginsburg, 2012).

Dental infections are significantly associated with the occurrence and activity of systemic pathologies such as diabetes (Chapple & Genco, 2013; Dorocka-Bobkowska et al., 2010; Preshaw et al., 2012; Teeuw, Gerdes & Loos, 2010), cardiovascular disease (Janket, Baird, Chuang & Jones, 2003), atherosclerosis (Asai et al., 2015; Friedlander, Sung, Chung & Garrett, 2010), rheumatoid arthritis (Smit et al., 2012), renal dysfunction (Iwasaki et al., 2012), pneumonia (Tada & Miura, 2012), nosocomial pneumonia (Kalil et al., 2016), multiple sclerosis and other systemic immune problems (Somma, Castagnola, Bollino & Marigo, 2010).

Still, given the relevance of maintaining oral health in the prevention of systemic pathologies of high social impact (Asai et al., 2015), it is observed with high and varied frequency different microorganisms exogenous to the oral cavity. Among these microorganisms, stands out the *Staphylococcus aureus*, considered the dominant oral pathogen in ICU patients and in chronic elderly patients (Ortega et al., 2015), *Streptococcus pneumoniae*, identified as the most prevalent etiologic agent in infections associated with oral microbiota alteration (Awano et al., 2008; Kadioglu, Weiser, Paton & Andrew, 2008), *Klebsiella pneumoniae*, with a high prevalence in the oral cavity of stroke patients (Lam, McMillan, Samaranayake, Li & McGrath, 2013; Malik et al., 2018), the presence of *Pseudomonas aeruginosa* in the oral microbiota has been associated with the development of respiratory infections in hospitalized and immunosuppressed patients (Persson et al., 2008; Raghavendran, Mylotte & Scannapieco, 2007), and *Enterococcus faecalis* had its

opportunistic growth identified in the oral microbiota of patients undergoing radiotherapy (Almståhl, Finizia, Carlén, Fagerberg-Mohlin & Alstad, 2018).

In order to combat multiresistant pathogens, *Praxelis clematidea*, a species of plant belonging to the Eupatorieae tribe of the Asteraceae family (Hsu, Peng & Wang, 2006), has been suggested as a possible alternative due to its chemical compositions and biological activities (Gasparetto, Campos, Budela & Pontarolo, 2010). Phytochemical studies with *P. clematidea* have isolated and identified compounds originating from secondary metabolism of the plant that provide biological activity, such as antiparasitic, antimicrobial, antiviral and antifungal (Maia et al., 2011; Oliveira-Filho et al., 2013b; Sarto & Zanusso-Júnior, 2014).

Due to the significant relationship between these microorganisms and the alteration of the oral microbiota associated with systemic pathologies, the current evolution of bacterial resistance and the search for new microbial agents, especially of natural origin, the objective of this study is concentrated on the antimicrobial evaluation of the extract *P. clematidea* on strains of *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterococcus faecalis*.

2. Methodology

2.1 Test substance

The substance evaluated in the study was the ethanolic extract of *Praxelis clematidea* (Griseb.) R.M. King & Robinson (*Asteraceae*). To carry out the pharmacological tests, the dried and pulverized vegetable material was macerated with 95% ethanol (EtOH), followed by several extraction processes in an interval of 72 hours. The ethanolic solution was filtered, followed by evaporation of the solvent with the assistance of a rota-evaporator at an average temperature of 50 °C. Thus, the crude ethanolic extract obtained from *P. clematidea* was solubilized in a mixture of MeOH:H2O (3:7) through a mechanical stirrer for 60 minutes, obtaining the hydroalcoholic solution (Maia et al., 2011).

2.2 Bacterial strains and culture media

The microbial strains selected for this study were: *Staphylococcus aureus* (IAL-2093, ATCC-29213), *Streptococcus pneumoniae* (IAL-1746), *Klebisiella pneumoniae* (IAL-1920,

ATCC-13883), *Pseudomonas aeruginosa* (IAL-1874, ATCC-9027) *e Enterococcus faecalis* (IAL-2394, ATCC-29212), from the Nucleus for Collection of Microorganisms of the Adolfo Lutz Institute (IAL). All strains were maintained on Mueller Hinton Agar medium (MHA) at a temperature of 4 °C, and used for the 24-hour replication assays in MHA incubated at 35 °C. In the study of antimicrobial activity, a bacterial inoculum of approximately 1.5 x 108 CFU/mL was used, standardized according to the turbidity of the 0.5 McFarland scale (Cleeland & Squires, 1991; Hadacek & Greger, 2000).

2.3 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the ethanolic extract was determined by the broth microdilution technique (Cleeland & Squires, 1991; Hadacek & Greger, 2000). 96 sterile orifice plates with a lid were used. In each hole of the plate, 100 µL of the double-concentrated Mueller Hinton broth liquid medium was added. Then, 100 µL of the ethanolic solution at the initial concentration of 2048 µg/mL (also doubly concentrated), was dispensed in the wells of the first line of the plate. And by means of a two-fold serial dilution, concentrations of 1024, 512, 256, 128, 64 and 32 µg/mL were obtained, in order to have the highest concentration in the first row of the plate and the lowest concentration in the last. Finally, 10 µL of the inoculum with approximately 1.5 x 108 CFU/mL of bacterial species was added to the cavities, where each column of the plate refers to a specific strain of bacteria.

At the same time, positive control was performed with the chlorhexidine gluconate at 0.12% antimicrobial. A microorganism control was carried out by placing 100 µL of the same doubly concentrated Mueller Hinton broth, 100 µL of sterile distilled water and 10 µL of the inoculum of each species in the wells. In order to verify the absence of interference in the results by the solvents used in the preparation of the solution, in this case MeOH:H2O (3:7), a control was carried out in which 100 µL of the doubly concentrated broth, 100 µL of MeOH:H2O (3:7) and 10µL of the bacterial suspension were placed in the wells. A sterility control of the medium was also performed, where 200 µL of Mueller Hinton broth was placed in an orifice without the suspension of bacteria.

The plates were aseptically closed and incubated at 35 °C for 24 hours for further reading, using resazurin as an indicator of microbiological growth. The MIC for the extract was defined as the lowest concentration capable of visually inhibiting the bacterial growth

seen in the orifices when compared to the control growth. The experiments were carried out in duplicate.

2.4 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of the extract was also determined for bacterial strains. After the MIC reading, 20 µL aliquots were removed from each well of the microtiter plate that did not show bacterial growth, and transferred to wells of a new microtiter plate containing 100 µL of Mueller Hinton broth, devoid of any antimicrobials. The inoculated plates were aseptically closed and incubated at 35 °C, and MBCs were recorded after 48 h. MBC was defined as the lowest concentration of the ethanolic extract that resulted in visible inhibition of microorganism growth (Guerra, Mendes, De Oliveira, Da costa & Coutinho, 2008; Ncube, Afolayan & Okoh, 2008).

2.5 Determination of Minimum Inhibitory Concentration of Adherence (MICA)

The minimum inhibitory concentration of adherence (MICA) of the ethanolic solution was determined in the presence of 5% sucrose, according to Gebara, Zardetto e Mayer (1996), using corresponding concentrations of pure ethanolic extract until dilution 1: 1024. Starting the bacterial growth, the strain of *Klebsiella pneumoniae* was grown at 37 ºC in Mueller Hinton broth (DIFCO, Michigan, EUA), then 0.9 mL of the subculture was distributed in test tubes and after 0.1 mL of the solution corresponding to the dilutions of the ethanolic extract were added. Incubation was performed at 37 ºC for 24 hours with tubes tilted at 30º. The reading was performed by visual observation of the bacterium's adherence to the tube walls, after staining with fuchsin (Albuquerque et al., 2013). The test was performed in duplicate. The same procedure was performed for the positive control, chlorhexidine digluconate at 0.12% (Periogard®, Colgate- Palmolive Company, New York, EUA). MICA was defined as the lowest concentration of the agent in contact with sucrose that prevented adherence to the glass tube.

3. Results e Discussion

Table 1 shows the inhibitory activity of the ethanolic extract of *P. clematidea* on the strains of *K. pneumoniae, S. pneumoniae* and *E. faecalis*. Observing that the ethanolic extract

was able to inhibit the growth of *K. pneumoniae* at a concentration of 512 µg/mL, *S. pneumoniae* and *E. faecalis* at a concentration of 1024 µg/mL.

Table 1. Minimum Inhibitory Concentration in µg/mL of the ethanolic extract of *Praxelis clematidea* (Griseb.) R.M. King & Robinson.

Praxelis clematidea **(Griseb.) R.M. King & Robinson**					
	P. aeruginosa	*S. pneumoniae*	*S. aureus*	*E. faecalis*	*K. pneumoniae*
1024 µg/mL	-	+	-	+	+
512 µg/mL	-	-	-	-	+
256 µg/mL	-	-	-	-	-
128 µg/mL	-	-	-	-	-
64 µg/mL	-	-	-	-	-
Negative control	-	-	-	-	-
Positive control	+	+	+	+	+
(+) Inhibition (-) Without inhibition					Own authorship

Source: Authors.

In Table 2, it can be seen that the minimum bactericidal concentration (CBM) of the ethanolic extract of *P. clematidea* showed similar results to the minimum inhibitory concentration, demonstrating growth inhibition of *K. pneumoniae* at a concentration of 512 µg/mL, *S. pneumoniae* and *E. faecalis* at a concentration of 1024 µg/mL.

The potential for antimicrobial activity determined by the MIC should be classified as follows: strong activity when MIC values up to 500 µg/mL, moderate activity when MIC values between 600 and 1500 µg/mL, weak activity when compounds show MIC above 1500 µg/mL (Sartoratto et al., 2004).

For a compound to be considered bactericidal, the CBM must be equal to or twice as high as the MIC, while for the compound to be considered bacteriostatic, the CBM must be greater than twice the MIC (Hafidh et al., 2011).

Thus, the results obtained in the MIC show that although the ethanolic extract does not have antimicrobial activity on the strains of *P. aeruginosa* and *S. aureus*, it presents strong bactericidal activity on the strains of *K. pneumoniae*, and moderate bactericidal activity on the strains of *S. pneumoniae* and *E. faecalis.*

Table 2. Minimum Bactericidal Concentration in µg/mL of the ethanolic extract of *Praxelis clematidea* (Griseb.) R.M. King & Robinson.

Praxelis clematidea **(Griseb.) R.M. King & Robinson**					
	P. aeruginosa	*S. pneumoniae*	*S. aureus*	*E. faecalis*	*K. pneumoniae*
1024 µg/mL	-	+	-	+	+
512 µg/mL	-	-	-	-	+
256 µg/mL	-	-	-	-	-
128 µg/mL	-	-	-	-	-
64 µg/mL	-	-	-	-	-
Negative control	-	-	-	-	-
Positive control	+	+	+	+	+
(+) Inhibition (-) Without inhibition					Own authorship

Source: Authors.

The evaluation of the minimum inhibitory concentration of adherence of the ethanolic extract of *P. clematidea* was tested on the strains of *K. pneumoniae*, since it showed strong antimicrobial activity. As described in table 3, the ethanolic extract of *P. clematidea* showed a minimum inhibitory concentration of adherence of 1:1, and was compared to the antimicrobial chlorhexidine digluconate at 0.12%, which was able to inhibit the adherence with a concentration of 1:8. That is, although the ethanolic extract is able to inhibit microbial adherence, its minimum concentration was eight times higher when compared to the standard antimicrobial.

Table 3. Minimum Inhibitory Concentration of Adherence in µg/mL of the ethanolic extract of *Praxelis clematidea* (Griseb.) R.M. King & Robinson and chlorhexidine digluconate at 0.12% on *Klebsiella pneumoniae* strains.

Praxelis clematidea **(Griseb.) R.M. King & Robinson**								
µg/mL	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	-	+	+	+	+	+	+	+
Chlorhexidine digluconate at 0.12%								
µg/mL	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	-	-	-	-	+	+	+	+

(-) No adhesion to the tube wall (+) Adhesion to the tube wall Own authorship

Source: Authors.

Due to the novelty about the evaluation at the antimicrobial activity of the ethanolic extract of *P. clematidea* on the strains of *S. pneumoniae*, *K. pneumoniae* and *E. faecalis*, it was not possible to compare the results presented with the scientific literature. However, in a study on the antimicrobial potential of an isolated component of *P. clematidea*, the flavonoid 5,7,4'-trimethoxyflavone, significant antibacterial activity was reported on the strains of *S. aureus* and *P. aeruginosa* (Oliveira et al., 2013b). The divergence between the results can be justified by the diluted concentration of the flavonoid in the ethanolic extract, or by the possibility that other secondary metabolites present in the extract could interfere with the flavonoid's antimicrobial activity. Since other studies evaluating phytochemical preparations with a high flavonoid content also reported the antibacterial activity (Pavithra, Sreevidya & Verma, 2009; Zhao et al., 2011).

In phytochemical studies with ethanolic extract of *P. clematidea*, six flavonoids were isolated (Maia et al., 2011). This class is becoming increasingly an object of investigation, and many studies have isolated and identified flavonoids that have antifungal, antiviral and antibacterial activities. In addition, studies have shown synergy between active flavonoids and between flavonoids and conventional chemotherapeutic agents (Cushnie & Lamb, 2005; Mota et al., 2009).

Although previous reports deny the antimicrobial effect of ethanolic extract on gram positive or gram negative bacteria (Oliveira et al., 2013a), this study revealed the extract potential of inhibiting both bacterial classes, since the strong bactericidal activity of the extract was observed on strains of *K. pneumoniae*, and moderate bactericidal activity on the strains of *S. pneumoniae*, and *E. faecalis*.

With the evolution of bacterial resistance over conventional antimicrobials and the development of new antimicrobials, the search for new compounds of natural origin has intensified, since plants with therapeutic potential constitute a source of new biologically active compounds, encouraging the development of new therapeutic options (Samuelsson & Bohlin, 2017). Thus, it is important to emphasize the relevance of carrying out further studies that evaluate the biological activities of compounds isolated from *P. clematidea*, as well as further development of toxicity and mutagenicity studies of the ethanolic extract and isolated compounds of *P. clematidea* in comparison with the chlorhexidine digluconate at 0.12% is necessary, aiming to identify the best relationship of efficacy, safety and biocompatibility

4. Final Considerations

P. clematidea demonstrated topical antimicrobial activity on some of the systemic bacterial pathogens frequently found in the oral cavity. Presenting strong bactericidal activity on *K. pneumoniae*, and moderate bactericidal activity on *S. pneumoniae* and *E. faecalis*. Being able to inhibit the adherence of *K. pneumoniae* strains.

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