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Antibiofilm activity of glycolic plant extracts on *Klebsiella pneumoniae* clinical isolates

Atividade antibiofilme de extratos glicólicos de plantas em isolados clínicos de *Klebsiella pneumoniae*

Actividad antibiofilm de extractos glicólicos de plantas en aislados clínicos de *Klebsiella pneumoniae*

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Abstract
Objective: Evaluate the anti-biofilm activity of *Gymnema sylvestre*, *Hamamelis virginiana*, *Juglans regia*, *Persea americana*, *Pfaffia paniculata*, *Rosmarinus officinalis*, *Stryphnodendron barbatiman* and *Thymus vulgaris* extracts in 3 MDR strains and 1 ATCC (4352) of *Klebsiella pneumoniae*. Methods: First were made a screening with broth microdilution test, protocol M7-A9, according to CLSI. Extracts that presented values for Minimal Microbicidal

Concentration (MMC) were selected for biofilms tests on 96 wells plates. Treatments used concentrations of 25 and 50 mg/mL, after biofilms were measured by biomass and MTT tests and statistically analyzed by ANOVA and Tukey test ($p<0.05$). Results: All extracts showed MIC for all *K. pneumoniae* strains studied, with values ranging from 12.5-100 mg/mL. Biomass of the strains ATCC and MDR strain (400381) of *K. pneumoniae* obtained reductions of 37.7 and 44.3% with *P. paniculata* and *R. officinalis* extracts. The isolate 386546 obtained a reduction of 29.7% ($p<0.05$) under *R. officinalis* action. Conclusion: Among the extracts studied, those that were most effective in the anti-biofilm action were *J. regia*, *P. paniculata* and *R. officinalis*. Therefore, these extracts could act as bactericidal agents against *K. pneumoniae* MDR.
Keywords: *Klebsiella pneumoniae*; Anti-biofilm; Phytotherapy; Bacteria; Resistance; Microbiology.

Resumo
Objetivo: Avaliar a atividade antibiofilme dos extratos de *Gymnema sylvestre*, *Hamamelis virginiana*, *Juglans regia*, *Persea americana*, *Pfaffia paniculata*, *Rosmarinus officinalis*, *Stryphnodendron barbatiman* e *Thymus vulgaris* em 3 cepas clínicas e 1 ATCC (4352) de *Klebsiella pneumoniae*. Métodos: Primeiramente foi feita uma triagem com teste de microdiluição em caldo, protocolo M7-A9, conforme CLSI. Os extratos que apresentaram valores de Concentração Microbicida Mínima (CMM) foram selecionados para testes de biofilmes em placas de 96 poços. Os tratamentos utilizaram concentrações de 25 e 50 mg/mL, após os biofilmes foram mensurados pelos testes de biomassa (Cristal violeta) e MTT, e analisados estatisticamente por ANOVA e teste de *Tukey* ($p <0,05$). Resultados: Todos os extratos apresentaram CIM para todas as cepas de *K. pneumoniae* estudadas, com valores variando de 12,5-100 mg/mL. A biomassa das cepas ATCC e MDR (400381) de *K. pneumoniae* obteve reduções de 37,7 e 44,3% com os extratos de *P. paniculata* e *R. officinalis*. O isolado 386546 obteve redução de 29,7% ($p <0,05$) sob ação de *R. officinalis*. Conclusão: Dentre os extratos estudados, os mais eficazes na ação anti-biofilme foram *J. regia*, *P. paniculata* e *R. officinalis*. Portanto, esses extratos poderiam atuar como agentes bactericidas contra *K. pneumoniae* MDR.
Palavras-chave: *Klebsiella pneumoniae*; Antibiofilme; Fitoterapia; Bactéria; Resistência; Microbiologia.

Resumen
Objetivo: Evaluar la actividad anti-biofilm de extractos de *Gymnema sylvestre*, *Hamamelis virginiana*, *Juglans regia*, *Persea americana*, *Pfaffia paniculata*, *Rosmarinus officinalis*, *Stryphnodendron barbatiman* y *Thymus vulgaris* en 3 cepas MDR y 1 ATCC (4352) de *Klebsiella pneumoniae*. Métodos: Primero se realizó un cribado con caldo de prueba de microdilución, protocolo M7-A9, según CLSI. Los extractos que presentaban valores para la concentración microbicida mínima (CMM) se seleccionaron para las pruebas de biopelículas en placas de 96 pocillos. Los tratamientos utilizaron concentraciones de 25 y 50 mg/mL, luego de que se midieran las biopelículas mediante pruebas de biomassa (cristal violeta) y MTT, y se analizaran estadísticamente mediante ANOVA y prueba de *Tukey* ($p<0.05$). Resultados: Todos los extractos mostraron CMM para todas las cepas de *K. pneumoniae* estudiadas, con valores que variaron de 12.5-100 mg/mL. La biomasa de las cepas ATCC y MDR (400381) de *K. pneumoniae* obtuvo reducciones de 37,7 y 44,3% con extractos de *P. paniculata* y *R. officinalis*. El aislado 386546 obtuvo una reducción del 29,7% ($p <0,05$) bajo la acción de *R. officinalis*. Conclusión: Entre los extractos estudiados, los más eficaces en la acción anti-biofilm fueron *J. regia*, *P. paniculata* y *R. officinalis*. Por tanto, estos extractos podrían actuar como agentes bactericidas frente a *K. pneumoniae* MDR.
Palabras clave: *Klebsiella pneumoniae*; Antibiofilm; Fitoterapia; Bacteria; Resistencia; Microbiología.

1. Introduction

Klebsiella pneumoniae is a Gram-negative bacillus in the family of enterobacteria. It is an opportunistic pathogen, present in 70% of hospital infections, and causes mainly pulmonary and urinary tract infections (Bowers et al., 2016; Rahim et al., 2016). *K. pneumoniae* may be prevalent as an infectious agent in patients with urinary catheters and may develop bacterial biofilms in these devices (Djeribi, Bouchloukh, Jouenne, & Menaa, 2012; Ramstedt et al., 2019). This virulence mechanism is fundamental in many bacterial infections, as it can resist the host's defense system and antimicrobial treatments (Djeribi et al., 2012; Vuotto, Longo, Balice, Donelli, & Varaldo, 2014). In addition to the ability to form biofilms, the species has other virulence factors, such as polysaccharide capsules, siderophore, urease, the presence of fimbriae and resistance to antibiotics, in which they help in the maintenance and resistance of infections (Bowers et al., 2016; Clegg & Murphy, 2016; Rahim et al., 2016).

Several classes of antimicrobials are commonly used to treat infections caused by *K. pneumoniae*, but excessive and inappropriate use can favor the increase in microbial resistance (Aslam et al., 2018; Goossens, 2009; Osman, Hassan, Orabi, & Abdelhafez, 2014). In hospital settings, where the use of these drugs is frequent, multidrug-resistant (MDR) bacteria have been a problem (Aslam et al., 2018). *K. pneumoniae* has an easier time developing resistance when compared to other bacteria.

Resistance to most conventional antibiotics is due to the emergence of strains producing broad-spectrum β-lactamase (ESBL) and carbapenemase producers (Cai et al., 2012). In view of this, the World Health Organization (WHO) declared in 2017 that *K. pneumoniae* is among the critical resistant bacteria that need research to develop new treatment alternatives (WHO, 2017). The consequences of antimicrobial resistance caused by *K. pneumoniae* are serious and can lead to increased morbidity, mortality or even longer hospital stays. The growth of MDR bacteria limits and makes therapeutic options more and more difficult, thus emerging the need to search for new drug alternatives (Li & Webster, 2018; Pacios et al., 2020).

Among the therapies studied, for the control of resistant strains, phytotherapy gained notability. It is known that medicinal plants are rich in phytochemicals with diverse biological activities. Many studies have demonstrated the antimicrobial action of different plant extracts against bacteria and fungi (de Oliveira et al., 2017; De Zoysa, Rathnayake, Hewawasam, & Wijayaratne, 2019; Elansary et al., 2018; Hadadi, Nematzadeh, & Ghahari, 2020). However, there are still many gaps, whether due to plant species or even the use of extracts against MDR strains (Farooqui et al., 2015; Khalil, Fikry, & Salama, 2020). Considering the world scenario of microbial resistance and the wide variety of plant extracts with potential to be explored, the present study evaluated the antimicrobial action of *Gymnema sylvestre* (Gimena), *Hamamelis vir-giniana* L. (Hamamelis), *Juglans regia* L. (Walnut), *Persea americana* (Avocado), *Pfaffia paniculata* (Brazilian Ginseng), *Rosmarinus officinalis* L. (Rosemary), *Stryphnodendron barbatiman* (Barbatiman) and *Thymus vulgaris* (Thyme) in planktonic forms and biofilms of *Klebsiella pneumoniae* MDR strains.

2. Methodology

2.1 Extracts

Glycolic extracts of *Gymnema sylvestre* (Gimena), *Hamamelis virginiana* L. (Hamamelis), *Juglans regia* L. (Walnut), *Persea americana* (Avocado), *Pfaffia paniculata* (Brazilian Ginseng), *Rosmarinus officinalis* L. (Rosemary), *Stryphnodendron barbatiman* (Barbatiman) and *Thymus vulgaris* (Thyme) were purchased from Mapric (SP, Brazil) at a concentration of 200 mg/mL (20%), eluted in propyleneglycol.

2.2 *K. pneumoniae* ATCC and multidrug-resistant strains

Antibacterial activity was tested on four *K. pneumoniae* strains, one ATCC 4352 strain (ATCC - American Type Culture Collection) and three MDR strains (367725, 386546, 400381) provided by Bioclin Laboratory, belonging to Policlin medical group - São José dos Campos, São Paulo, Brazil. The resistance of clinical isolates was identified using the autoSCAN 4 automated system (Beckman Coulter, Brea, CA, USA) (Delgado-Gardea et al., 2016). The resistance profile of clinical isolates is shown in Appendice - Table A1.

2.3 Glycolic extracts MIC and MMC

MIC (Minimum Inhibitory Concentration) and MMC (Minimum Microbicidal Concentration) values of the extracts were determined by Broth Microdilution Method, protocol M7-A9 (2012), according to CLSI. First, suspensions of *K. pneumoniae* strains were prepared in sterile saline solution (NaCl 0.9%) and turbidity was adjusted to 10^6 CFU/mL in a spectrophotometer (Micronal, São Paulo, Brazil). The test was performed in 96-well plates (TPP, Trasadingen, Switzerland), where each extract was serially diluted in 10 wells with 100 μL/well of *Muller Hilton* Broth (Himedia, Mumbai, India), obtaining concentrations from 100 to 0.19 mg/mL. Next, 100 μL of standardized microorganism suspension was added to all the wells and the plate was incubated in at 37°C for 24 h. After, MIC was determined in the last well of the microplate where no turbidity was observed. For MMC determination, aliquots were removed from the microplates and seeded on Brain Heart Infusion (BHI) agar (Himedia, Mumbai, India) and were determined in the lowest concentration where no growth of colonies was observed.

2.4 Antimicrobial action against monotypic biofilms

After the screening promoted by the broth microdilution test, only the extracts that obtained MMC into the interval tested were selected for monotypic biofilm tests: *H. virginiana*, *J. regia*, *P. americana*, *P. paniculata*, and *R. officinalis*.

Initially, the *K. pneumoniae* strains were cultured in BHI broth (Himedia, Mumbai, India) at 37 °C/24 h. After incubation, the microorganism suspension was centrifuged at 2000 rpm/10 min (MPW-350, Warsaw, Poland) and washed twice with 0.9% saline solution for the removal of the microorganisms metabolites. The turbidity of the suspensions was adjusted in a spectrophotometer (Micronal) at a concentration of 10^7 CFU/mL. The microorganism suspension was distributed into 96-well plates with N=10 for each group, then 100 μL/well of BHI broth (Himedia, Mumbai, India) was added and the plate was incubated at 37 °C for 48h, under constant stirring (75 rpm).

2.5 Treatment

In the control group, 200 μL/well of 0.9% saline was applied and in the experimental groups, 200 μL/well of each extract (*H. virginiana*, *J. regia*, *P. americana*, *P. paniculata*, and *R. officinalis*) at concentrations of 25 and 50 mg/mL were applied, with 10 replicates for each group. After 5 min of treatment, the extracts were removed and the wells were washed twice with 0.9% saline solution. In order to evaluate the action of the extracts on the biofilm, the following tests were performed: measurement of the biomass by violet crystal and evaluation of the metabolic activity of the microorganisms by MTT (3- (4,5-Dimethylthiazol-2- yl) -2,5-Diphenyltetrazolium bromide.

2.6 Measurement of biomass by the Crystal violet test

After the treatments, 200 μL/well of methanol were added and after 20 min, for biofilm fixation, it was removed and the plate was incubated at 37 °C for 24 h for drying. After incubation, 200 μL/well of violet crystal 1% (V/V) were added for 5 min and then the dye was removed and the wells washed twice with acetic acid 33% (Synth, Diadema, Brazil) and sterile saline solution (0.9% NaCl). The plate was read at 570 nm by the microplate reader (Lonza Biotek ELX808LBS, Winooski, Vermont) and the optical densities were converted into biofilm biomass (Skogman et al., 2012; Marcos-Zambrano et al., 2014).

2.7 Metabolic activity evaluation

Two hundred microliters of MTT (3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide) solution were added to each well of the plate. The plate was incubated, protected from light, for 1 h at 37 °C. The solution was removed and 200 μL of dimethylsulfoxide (DMSO) was added to the plate, which was incubated again, at 37 °C for 10 min and then placed in the shaker under constant shaking for another 10 min. After this process, the optical densities were read in a microplate reader at 570 nm and converted into a percentage of metabolic activity of the microbial cells.

2.8 Statistical analysis

Data were statistically analyzed by ANOVA, complemented by the *Tukey* test, in order to verify the differences among the groups with $p<0.05$, with GraphPad Prism 5.0 software

3. Results

All extracts showed MIC for all *K. pneumoniae* strains studied, at different concentrations (table 1). For *K. pneumoniae* ATCC 4352, *H. virginiana* obtained growth inhibition with 12.5 mg/mL, *P. americana* and *R. officinalis* with 25 mg/mL, *P. paniculata* with 50 mg/mL and *S. barbatiman*, *G. sylvestre*, *J. regia*, and *T. vulgaris* with 100 mg/mL (Table 1). For KPC 367725

strain, the MIC of *G. sylvestre*, *J. regia*, *S. barbatiman* and *T. vulgaris* extracts obtained was 100 mg/mL, *H. virginiana*, *P. americana*, and *R. officinalis* showed inhibition with 25 mg/mL and *P. paniculata* with 50 mg/mL.

For KPC strain 386546, *H. virginiana* showed MIC with 12.5 mg/mL, *J. regia*, *P. americana*, *P. paniculata*, and *R. officinalis* with 25 mg/mL, *S. barbatiman*, and *T. vulgaris* with 50 mg/mL and *G. sylvestre* with 100 mg/mL. KPC 400381 strain was inhibited by *H. virginiana* with 12.5 mg/mL, *J. regia*, *P. americana* and *R. officinalis* with 25 mg/mL, *G. sylvestre*, *J. regia* and *S. barbatiman* with 50 mg/mL and *T. vulgaris* with 100 mg/mL.

Regarding the microbicidal activity, *G. sylvestre*, *S. barbatiman*, and *T. vulgaris* extracts did not present MMC values for any strain of *K. pneumoniae* and the *J. regia* showed only two microbicidal actions (ATCC 4352 and KPC 367725) of the four strains analyzed. On the other hand, *H. virginiana*, *P. americana*, *P. paniculata*, and *R. officinalis* extracts were able to eliminate all strains, in different concentrations (Table 1).

For the ATCC 4352 strain, *H. virginiana* and *R. officinalis* extracts obtained MMC with 50 mg/mL, *P. americana* extract with 25 mg/mL and *P. paniculata* with 100 mg/mL. For strain 367725, *H. virginiana*, P americana, and *R. officinalis* extracts presented MMC with 50 mg/mL and *P. paniculata* extract with 100 mg/mL. For strain 386546, *H. virginiana* obtained MMC with 12.5 mg/mL, *J. regia*, *P. americana* and *R. officinalis* with 25 mg/mL and *P. paniculata* with 100 mg/mL. For strain 400381, *J. regia*, *P. americana*, and *R. officinalis* extracts showed microbicidal activity with 25 mg/mL, *H. virginiana* with 50 mg/mL extract and *P. paniculata* with 100 mg/mL.

With the screening promoted by the broth microdilution test, it was defined that extracts with MMC for all *K. pneumoniae* strains would be selected for biofilm tests. The concentrations of the extracts applied in monotypic biofilms also derived from the results of the broth microdilution test.

Table 1: Broth microdilution test performed with *Klebsiella pneumoniae* strains.

	ATCC 4352		**KPC 367725**		**KPC 386546**		**KPC 400381**	
Glycolic extracts	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**
G. sylvestre (gimena)	100	Abs	100	abs	100	abs	50	abs
H. virginiana L. (witch hazel)	12,5	50	25	50	12,5	12,5	12,5	50
J. regia L. (walnut)	100	Abs	100	abs	25	25	25	25
P. americana (avocado)	25	25	25	50	25	25	25	25
P. paniculata (brazilian ginseng)	50	100	50	100	25	100	50	100
R. officinalis L. (rosemary)	25	50	25	50	25	25	25	25
S. barbatiman (barbatiman)	100	Abs	100	abs	50	abs	50	abs
T. vulgaris (thyme)	100	Abs	100	abs	50	abs	100	abs

Legends: values express in mg/mL; Abs - Absent; MIC – Minimum Inhibitory Concentration; MMC – Minimum Microbicidal Concentration; absent – No values for MMC were found.
Source: Autors.

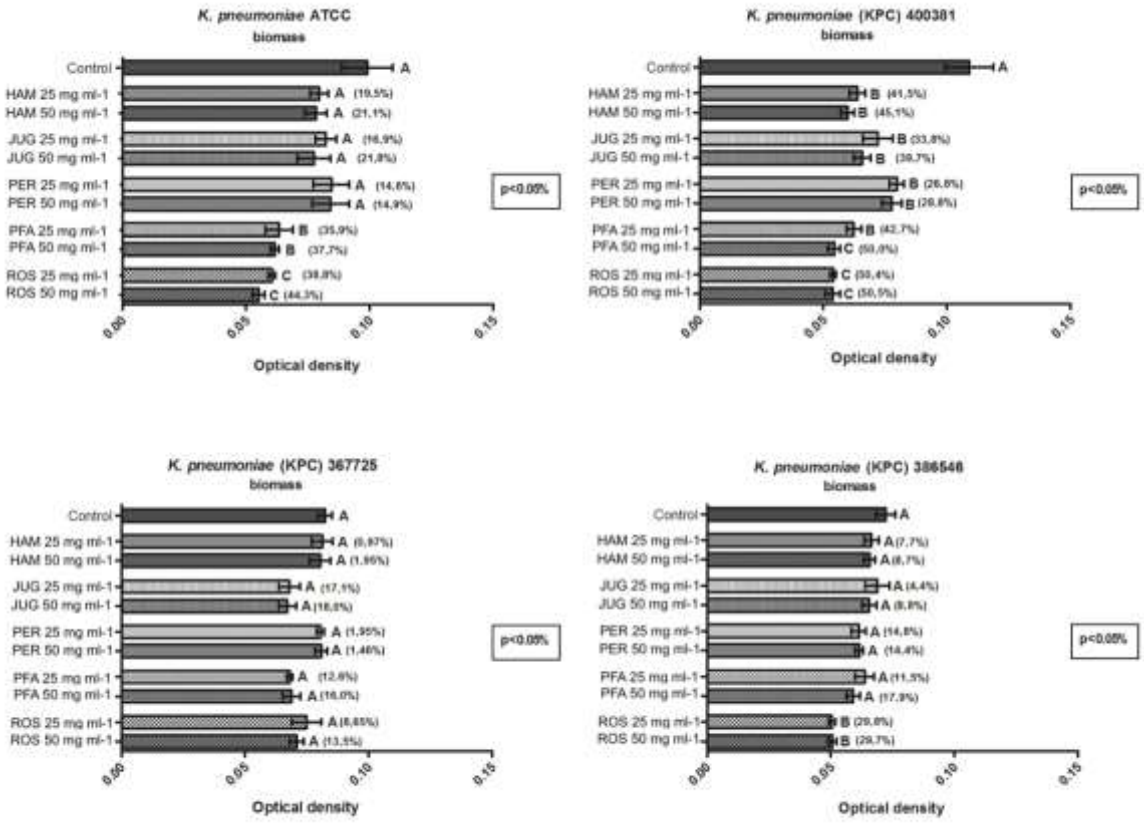
3.1 Biomass reduction

For the ATCC strain, only the *P. paniculata* and *R. officinalis* extracts presented statistically significant reductions ($p<0.05$) when compared to the control. For *P. paniculata* at 25 and 50 mg/mL there were reductions of 35.9% and 37.7%, respectively, and for *R. officinalis* at 25 mg/mL and 50 mg/mL, the reductions were 38.8 and 44.3%, respectively (Figure 1).

The five extracts tested in the clinical strain 400381 obtained statistically significant reductions ($p<0.05$) compared to the growth of the control group. *P. americana* extract obtained reductions of 26.8 (25 mg/mL) and 28.8% (50 mg/mL), *H. virginiana* of 41.5 (25 mg/mL) and 45.1% (50 mg/mL) , *J. regia* of 33.8 (25 mg/mL) and 39.7% (50 mg/mL) , *P. paniculata* of 42.7 (25 mg/mL) and 50% (50 mg/mL). *R. officinalis* extract promoted the greatest reductions ($p<0.05$), 50.4 (25 mg/mL) and 50.5% (50 mg/mL) (Figure 1).

For KPC strain 386546, only the extract of *R. officinalis* presented statistically significant reductions ($p<0.05$) of the biomass, compared to the control group. These reductions were 29.8% for the concentration of 25 mg/mL and 29.7% for 50 g/mL. For the strain KPC 367725, no extract showed a statistically significant reduction of the biomass ($p<0.05$) (Figure 1).

Figure 1 – Biomass reduction after extracts action on *Klebsiella pneumoniae* strains.



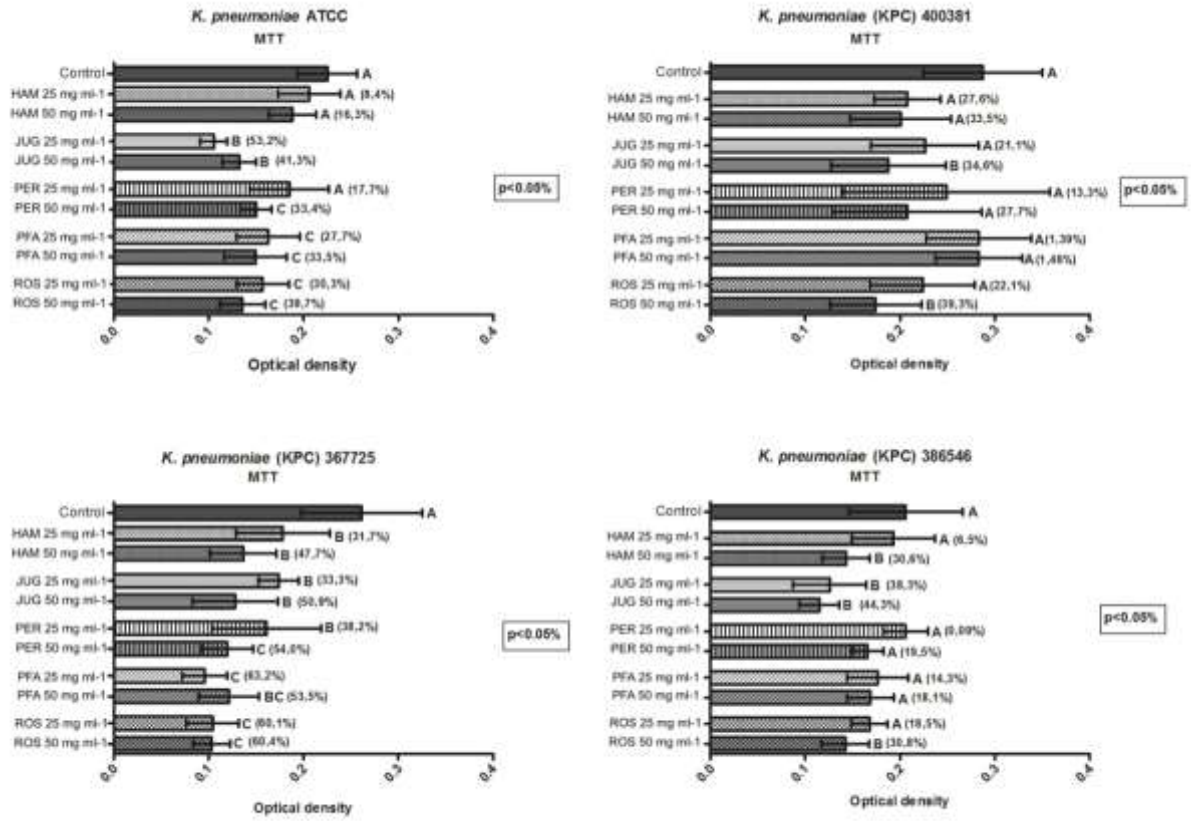
Legend: HAM- Glycolic extract of *Hamamelis virginiana*; JUG - *Juglans regia* glycolic extract; PER - Glycolic extract of *Persea americana*; PFA - Glycolic extract of *Pfaffia paniculate*; ROS - Glycolic extract of *Rosmarinus officinalis*. Different letters (A, B and C) indicate statistical significant difference (ANOVA, Tukey, $p<0.05$). *K.pneumoniae* ATCC ($p=0.0001$); KPC 400381 ($p=0.0001$); KPC 367725 ($p=0.0018$); KPC 386546 ($p=0.0001$).
Source: Authors.

3.2 Reduction of metabolic activity of strains

For *K. pneumoniae* ATCC strain, *J. regia* promoted the greatest reductions in the metabolic activity: 53.2% at 25 mg/mL and 41.3% at 50 mg/mL, statistically different from other groups ($p<0.05$). *R. officinalis* and *P. paniculata* extracts also showed significant reductions when compared to the control ($p<0.05$): 30.3 and 39.7% for *R. officinalis* and 27.7 and 33.5% for *P. paniculata*. Only *P. americana* extract at 25 mg/mL and *H. virginiana* extract at 25 and 50 mg/mL did not present statistical significant reduction ($p<0.05$) in metabolic activity.

Regarding KPC 367725, all 5 extracts showed a significant reduction in metabolic activity. The extracts of *R. officinalis* (25 and 50 mg/mL), *P. paniculata* (25 mg/mL) and *P. americana* (50 mg/mL) showed the greatest reductions. They were statistically similar to each other ($p<0.05$) but different from the control group ($p<0.05$). For the KPC strain 386546, only extracts of *J. regia* (25 and 50 mg/mL), *H. virginiana* (50 mg/mL) and *R. officinalis* (50 mg/mL) showed a statistically significant reduction in metabolic activity ($p<0.05$). For KPC 400381 strain, only extracts of *R. officinalis* and *J. regia*, both at 50 mg/mL presented a statistically significant reduction when compared to the control group ($p<0.05$) (Figure 2).

Figure 2 – Metabolic activity reduction after extracts action on strains of *Klebsiella pneumoniae*.



Legend: HAM- Glycolic extract of *Hamamelis virginiana*; JUG - Glycolic extract of *Juglans regia*; PER - Glycolic extract of *Persea americana*; PFA - Glycolic extract of *Pfaffia paniculata*; ROS – Glycolic extract of *Rosmarinus officinalis*. Different letters (A, B and C) indicate statistical significant difference (ANOVA, Tukey, $p<0.05$). *K.pneumoniae* ATCC ($p=0.0001$); KPC 400381 ($p=0.0001$); KPC 367725 ($p=0.0001$); KPC 386546 ($p=0.0001$).
Source: Authors.

4. Discusssion

Bacterial resistance to conventional antimicrobials increases progressively, representing a global threat (Roca et al., 2015). The emergence of MDR pathogens searches for new treatment alternatives essential (Li & Webster, 2018; Pacios et al.,

2020). Therefore, phytotherapy has been studied for its antimicrobial action, as it produces several bioactive compounds with therapeutic properties and has shown promising results against MDR bacteria (Assis et al., 2018; Elisha, Botha, McGaw, & Eloff, 2017; Gadisa et al., 2019). Based on the above, this study evaluated the antimicrobial activity of *Gymnema sylvestre*, *Hamamelis virginiana*, *Juglans regia*, *Persea americana*, *Pfaffia paniculata*, *Rosmarinus officinalis*, *Stryphnodendron barbatiman* and *Thymus vulgaris* against multidrug resistance strains *Klebsiella pneumoniae*.

In this study, *G. sylvestre* glycolic obtained MIC at 100 mg/mL in *K. pneumoniae* ATCC and two clinical strains, but no MMC value was found, indicating a low antimicrobial potential. Corroborating with our results, Selvi et al. (2007) used the agar diffusion test and found the low antimicrobial activity of *G. sylvestre* in *K. pneumoniae* (Selvi, Devi, Chinnaswamy, Giji, & Sharmila, 2007). The ethanolic extract indicated moderate inhibition of the mentioned species and the aqueous extract showed no inhibition. The absence of antimicrobial activity of the aqueous extract of *G. sylvestre* against *K. pneumoniae* (MTCC 530) was also observed by Arora and Sood et al. (2017), in which they found that the strain was insensitive even when tested at a concentration of 30% of the extract (Arora & Sood, 2017).

In relation to the Barbatiman extract (*Stryphnodendron barbatiman*, *S. adstringens* or *S. obovatum*), there are few reports in the literature that have evaluated its antimicrobial action. Ishida et al. (2009) reported the antifungal action of the extract on *Cryptococcus neoformans* (Ishida, Rozental, de Mello, & Nakamura, 2009). The action of Barbatiman extract was also demonstrated against common microorganisms of the oral microbiota, in this study Pereira et al. (2011) observed that the mentioned extract can be an adjunctive method of prophylaxis and treatment of oral infectious conditions of the oral cavity (E. M. Pereira et al., 2011). De Freitas et al. (2018) reported that tannins obtained from the stem bark of the species have the potential for topical treatment of vaginal candidiasis and suggest that it may be an alternative for the treatment of infections caused by *Candida* spp. resistant to antifungals (de Freitas et al., 2018). Studies in the literature involving Barbatiman extract and microorganisms are scarce, and so far no reports of their action on *K. pneumoniae* strains have been found. Thus, the present study is a pioneer in the identification of antimicrobial activity against the growth of this species, thus, our findings demonstrate MIC of 50-100 mg/mL, showing that this extract can be a promising alternative against standard strains and *K. pneumoniae* MDR.

Fournomiti et al. (2015) assessed the antimicrobial action of *Thymus vulgaris* essential oil on clinical strains of *K. pneumoniae* and the results showed mean MIC values of 11.34 mg/mL, being different from the results of the present study in which clinical strains of *K pneumoniae* were inhibited with 50 mg/mL and the ATCC strain with 100 mg/mL (Fournomiti et al., 2015). However, in the present study, the glycolic extract was used, which has advantages in relation to the water solubility of the extract, when compared to essential oil. On the other hand, our results are consistent with the study by Van Vuuren et al. (2009), who evaluated the essential oil of *T. vulgaris* in strains of *K. pneumoniae* NTCC 9633 (National Collection of Type Cultures), with MIC in 40 mg/mL (van Vuuren, Suliman, & Viljoen, 2009), similar to our findings.

In the present study, extracts of *G. sylvestre*, *S. barbatiman*, and *T. vulgaris* showed bacteriostatic action, with different MIC values, these results may open new paths for pioneering studies with herbal medicines, which aim to interact with different therapies using the capacity inhibitory effect of these extracts in combination with conventional antimicrobial drugs (Hong et al., 2016; Kuok et al., 2017; Olajuyigbe & Afolayan, 2012, 2013; Silva et al., 2019).

The extracts of *H. virginiana*, *J. regia*, *P. americana*, *P. paniculata*, and *R. officinalis* showed better antimicrobial results, with microbicidal concentrations (MMC) for all tested strains. The broth microdilution test indicated 50 mg/mL MMC for glycolic extract of *H. virginiana* in strains KPC 367725, 400381 and ATCC 4352, with replications of biofilm ranging from 41% to 47%. Despite being considered antiseptic; few studies have evaluated the antimicrobial action of *H. virginiana*. Mouchrek et al. (2015) evaluated the anti-biofilm action of several commercial types of mouthwash and, among the tested substances, *H.*

virginiana demonstrated a reduction in dental biofilm, however, only the clinical aspect of biofilms was evaluated, that is, there was no identification of microbial species that suffered reductions (Mouchrek Junior et al., 2015).

Pereira et al. (2008) found that two different aqueous extracts of *J. regia* showed antimicrobial action against clinical isolates of *K. pneumoniae* from urinary infections with MIC values of 100 mg/mL (J. Pereira et al., 2008), corroborating the results obtained in the present study for strains KPC 367725 and ATCC 4352, except for the fact that, in our study, the glycolic extract was used. Rather et al. (2012) evaluated the action of *J. regia* essential oil in *K. pneumoniae* strains, using the agar diffusion technique and the verified antimicrobial action (Rather et al., 2012). Regarding the action of antibiofilm, De Paula Ramos et al. (2016) evaluated the potential of the glycolic extract of *J. regia* in the ATCC strain of *K. pneumoniae* (4352) and found that the biomass reduction was not statistically significant ($p < 0.05$) compared to the control group (Paula-Ramos et al., 2016), confirming the results of this study. The present study also demonstrates that the clinical strain 400381 was sensitive to the action of the extract of *J. regia*, with reductions of 33.8 and 39.7% of the biofilm, showing a strong potential for the use of antimicrobials in these resistant multi-drug strains, with promising results.

Few studies have evaluated the action of *P. americana* extract on strains of *K. pneumoniae*. Idris et al. (2009) found that extracts of *P. americana* based on petroleum ether, ethyl acetate, chloroform and methanol obtained similar MIC values against clinical strains of *K. pneumoniae*, with growth inhibition at a concentration of 30 mg/mL (Idris, S, GI, & CE, 2009). In the present study, it was possible to observe the microbicidal activity of the extract of *P. americana* against all strains of *K. pneumoniae*.

Although studies on the anti-inflammatory role of *P. paniculate* are common (Costa et al., 2015; da Silva et al., 2015), studies on its antimicrobial activity are rare. In the present study, it was found that the glycolic extract of *P. paniculata* has an important antimicrobial action in resistant multidrug clinical strains of *K. pneumoniae*, corroborating with De Paula Ramos et al. (2016), who evaluated a clinical strain (Paula-Ramos et al., 2016). It can be seen that the sensitivity to the extract varies according to the strain, with MIC and MMC values ranging from 12.5 to 25 mg/mL (Paula-Ramos et al., 2016) and from 50 to 100 mg/mL in the present study. The results found in the biofilm tests follow the same direction as those found by De Paula Ramos (2016), who indicated reductions in biomass and metabolic activity of 55.7 and 72.3%, respectively, with the use of the extract. *P. paniculata* at 200 mg/mL in strain ATCC 4352 (Paula-Ramos et al., 2016). In the present study, lower concentrations of the extract were applied, 25 and 50 mg/mL, with reductions of 35.9 and 37.7% for biomass and 42.7 and 50% for metabolic activity, respectively.

Prabuseenivasan et al. (2006) used the essential oil of *R. officinalis* and the indicated antimicrobial action of the herb. By testing the zone of inhibition, they found a 27.5 mm zone of inhibition for the growth of the ATCC 15380 strain of *K. pneumoniae*, indicating its high sensitivity. These authors also indicated that even using an ATCC strain of *K. pneumoniae*, few extracts obtained antimicrobial activity, only 9 of the 21 extracts tested showed positive results (Prabuseenivasan, Jayakumar, & Ignacimuthu, 2006). The question of *K. pneumoniae* resistance can also be seen in the present study, since 3 of the 8 extracts tested did not have great antimicrobial potential. However, it is important to emphasize that 5 extracts showed a promising antimicrobial potential, even when applied to biofilms of strains resistant to conventional antibiotics. These results corroborate the development of new antimicrobial drugs, in addition to pointing out new directions for research involving the active principles of the extracts under study.

5. Conclusion

In conclusion, the glycolic extracts of *H. virginiana*, *J. regia*, *P. americana*, *P. paniculata* and *R. officinalis* showed antimicrobial action, resulting in concentrations with microbicidal capacity and reductions in the *K. pneumoniae* MDR biofilms.

The extracts of *G. sylvestre*, *S. barbatiman*, and *T. vulgaris*, despite not presenting microbicidal concentrations, provided inhibitory concentrations in resistant strains of *K. pneumoniae*, which can be explored as adjuvant therapy with the use of antibiotics. In addition to the antimicrobial action on planktonic strains, the extracts also demonstrated anti-biofilm action. Among the extracts studied, those that were most effective in the anti-biofilm action were *J. regia*, *P. paniculata* and *R. officinalis*, promoting significant reductions in biomass and metabolic activity. In view of this, our findings revealed that these extracts investigated have antimicrobial and anti-biofilm activity, and may be a promising therapeutic alternative for *K. pneumoniae* MDR

Declarations

Ethical Statement: Clinical isolates of *K. pneumoniae* were obtained from a hospital in São José dos Campos, São Paulo, Brazil, where the samples are collected directly from the patient. We asked the hospital staff for permission to obtain access to laboratory strains, being cautious about all the ethical and legal precepts that govern this type of subject, where after hospital approval, we obtained access only the samples already collected and analyzed from micro-organisms that belong to the sample repertoire of the laboratory, not getting direct contact with the patient, due to this the approval of the ethics committee is not applied.

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