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Antimicrobial activity of commercial bee honey (*Apis mellifera*) on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

.

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RESUMEN

Los patógenos multirresistentes (PMR) representan una amenaza creciente para la salud pública, y se prevé que podrán causar hasta 10 millones de muertes anuales en 2050 en todo el mundo. La miel, como alternativa a los antibióticos convencionales, tiene capacidad antimicrobiana gracias a componentes dependientes e independientes del peróxido de hidrógeno. Este estudio investiga este potencial antimicrobiano con miel de *Apis mellifera* frente a *Staphylococcus aureus* y *Pseudomonas aeruginosa*, ofreciendo una alternativa más fácil de extraer que otras mieles. Mediante la prueba de difusión de Kirby-Bauer, ensayos de MIC y MBC, y ensayos de erradicación de biopelículas adaptados a un método de microdilución en damero, evaluamos mieles de diferentes fuentes florales y orígenes. Los resultados indicaron que la miel de aguacate de Pichincha inhibió significativamente el crecimiento planctónico de *S. aureus* ATCC 25923, *Staphylococcus aureus* resistente a la meticilina (SARM) 333 y *P. aeruginosa* P28, superando la eficacia de la miel de eucalipto de Cotopaxi y Pichincha. Los tratamientos combinados de miel y antibióticos demostraron una mayor actividad antimicrobiana, pero las mieles de eucalipto demostraron mejores resultados en la erradicación de biopelículas de *S. aureus* ATCC 25923. Estos resultados apoyan el potencial de la miel como agente antimicrobiano natural, en consonancia con otras investigaciones sobre tratamientos alternativos. Futuros estudios deberían explorar más a fondo la sinergia entre distintas mieles y antibióticos. Además, examinar más los mecanismos dependientes e independientes del peróxido en las mejores miles evidenciando el rol del peróxido de hidrógeno en la erradicación de biopelículas. Finalmente, los resultados subrayan la necesidad de disponer de miel de alta calidad y origen conocido para maximizar los resultados terapéuticos.

Palabras clave: miel, *Apis mellifera*, resistencia antimicrobiana, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, peróxido de hidrógeno, CIM, CBM, biopelícula, sinergia antibiótica.

ABSTRACT

Multidrug-resistant (MDR) pathogens pose a growing public health threat, projected to cause up to 10 million deaths annually by 2050 worldwide. Honey as an alternative from conventional antibiotics, provides antimicrobial capacity due to hydrogen peroxide-dependent and hydrogen peroxide-independent mechanisms. This study investigates this antimicrobial potential with honey from *Apis mellifera* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, offering an alternative more easily obtainable than other honeys. Through Kirby-Bauer difusión test, MIC and MBC assays, and biofilm eradication assays adapted to a checkerboard microdilution method, we evaluated honeys from different floral sources and origins. Results indicated that avocado honey from Pichincha significantly inhibited the planktonic growth of *S. aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) 333, and *P. aeruginosa* P28, surpassing the efficacy of eucalyptus honey from Cotopaxi and Pichincha. Combined treatments of honey and antibiotics demonstrated enhanced antimicrobial activity, where the best outcomes in eradicating *S. aureus* ATCC 25923 biofilm were obtained with eucalyptus honey samples and antibiotics. These findings support the potential of honey as a natural antimicrobial agent, aligning with other research on alternative treatments. Future studies should further explore the synergy between different types of honey and antibiotics. Additionally, examining the peroxide-dependent and independent mechanisms of the best honey candidates will elucidate the role of hydrogen peroxide in biofilm eradication. The results underscore the need for high-quality honey of known origin to maximize therapeutic outcomes.

Keywords: honey, *Apis mellifera*, antimicrobial resistance, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, hydrogen peroxide, MIC, MBC, biofilm, antibiotic synergy

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INTRODUCTION

Multidrug resistance (MDR) pathogens are on the rise, while the development of new drugs to combat this problem is stagnating and accelerating the public health risk to about 10 million deaths a year by 2050 as reported by the World Health Organization (WHO, 2019). The exact definition of this issue is the ability of microorganisms to adapt and survive treatments designed to either inhibit their growth or kill them (Baquero et al., 2021). This growing issue is due to the overuse and misuse of antimicrobial drugs, the lack of new antimicrobial development, and the ability of microbes to rapidly adapt and develop resistance mechanisms (Chang et al., 2015). For this reason, new treatments and alternative solutions are being explored to prevent MDR pathogens from taking more lives.

Over the last few years, many alternative solutions have been investigated such as bee honey, which possesses antimicrobial activity against a wide range of pathogens including Gram-positive and Gram-negative bacteria as well as fungi (Machado et al., 2023). The antimicrobial activity of honey is due to the synergistic action of several components, such as phenolic compounds, antimicrobial agents (bioactive peptides and/or carbohydrates), enzymes, acidity, and osmolarity (Alvarez-Suarez et al., 2010; Proaño et al., 2021). The main antimicrobial compounds can be divided into hydrogen peroxide-dependent and hydrogen peroxide-independent components, such as phenolic compounds (phenolic acids, flavones, and flavonols) (Alvarez-Suarez et al., 2014). This concentration of phenolic compounds in honey varies according to floral origin, climate, and storage conditions, which overall will change the antimicrobial properties of its own (Machado et al., 2023).

Bee honey can be used as a natural antimicrobial agent for the treatment of topical and systemic infections (Alvarez-Suarez et al., 2010). Numerous species of bees produce an enormous diversity of honey with antimicrobial activity such as manuka honey, therefore we

are interested in evaluating the potential antimicrobial activity of commercial honey bees (*Apis mellifera*), which are easier to obtain in a greater amount of honey and process in further applications (Cabezas-Mera et al., 2023). It is important to use high-quality honey of known origin to obtain the best therapeutic results and, in the present study, we selected to evaluate the antimicrobial activity of commercial eucalyptus and avocado kinds of honey from *Apis mellifera.* Bee honey is a promising alternative to traditional treatments (antibiotics or antifungics) for the treatment of MDR and biofilm-associated infections (Cremers et al., 2020). Further research is needed to identify the numerous antimicrobial compounds, to understand their mechanisms of action, and to develop new clinical applications (Valdés-Silverio et al., 2018).

Research Question

What is the antimicrobial potential of commercial bee (*Apis mellifera*) honeys against *Staphylococcus aureus* and *Pseudomonas aeruginosa*?

Objectives

- Perform a general screening to determine the antimicrobial potential of honey from different origins against skin pathogens.
- Compare the antimicrobial activity of honey from different floral origins of *Apis mellifera* against *P. aeruginosa* and *S. aureus*.
- Determine if there is a synergistic effect and the relationship between the antibiotic plus the floral origin of honey against *S. aureus* and *P. aeruginosa* biofilms.

METHODOLOGY

Honey Samples

The study used a total of 67 honey samples belonging to *Apis mellifera* from three different provinces in Ecuador, more exactly Pichincha, Cotopaxi, and Imbabura. Samples were collected from apiaries directly from the artisanal hives of *Apis mellifera* in the general pot honey production zones. The honey samples were collected in sterilized plastic containers and stored at 4–6 ◦C in the dark until further preparation using the same methodology as described by Villacrés-Granda et al. (2021). For the anti-microbiological capacity studies developed here, the collected samples were first weighted and stock solutions of each sample were prepared at 50% and 80% (w/v) diluted in water and then further filtered in 0.22-µm filters to ensure no other microorganism was affecting the results. For all experimental assays, the honey concentration was set up to 20% (w/v), where previous studies showed significant antimicrobial activity among honey samples through minimal inhibitory concentration (MIC) and biofilm inhibition/eradication assays (Proaño et al., 2021; Villacrés-Granda et al., 2021). In addition, a solution of artificial honey lacking H_2O_2 was made as a honey-negative control to compare if there are additional substrates that increase antimicrobial effects, which is a normal product of glucose oxidation consisting of 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose, and 33.5 g glucose in 17 mL of deionized water; this was also evaluated from 20% (w/v) diluted in water. Finally, after the screening evaluation step (see next subsection), four honey mixes were made to boost the antimicrobial capabilities, this was made using samples from the same provinces and floral origin.

Initial Screening

For selecting the best honey candidates and reducing the viable honey samples for further essays an initial screening was made on Mueller-Hinton agar (MHA). The screening was done on six skin-associated pathogen strains, more exactly *S. aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) 333, *P. aeruginosa* ATCC 27833, *P. aeruginosa* P28, *Candida albicans* ATCC 10231, and *C. albicans* INSPI. Filter paper disks of 10-mm diameter were used to put 30 µL the honey solution at a concentration of 83%. For the agar plates, a 0.5 McFarland turbidity standard ($1x10^8$ colony-forming units (CFU)/mL) as reference for the fresh bacterial colonies in saline solution was used to cultivate on each agar plate. Then after the filter paper absorbed the honey each disk was placed on the previously cultivated agar plate and the plates were then incubated for 20-24 hours at 37°C, as previously described (Cabezas-Mera et al., 2024). After the process results were measured depending on the formation of a halo around the honey disk and compared to the positive control antibiotic and the Clinical Laboratory Standards Institute (CLSI) disk diffusion parameters (CLSI, 2022).

Minimum inhibitory concentration and minimum biocidal concentration

The antimicrobial activity of honey extract treatments was evaluated in the present study. The microdilution method for minimum inhibitory concentration (MIC) assays was performed, as described by Wiegand and colleagues (Wiegand et al., 2008), under the considerations established in the CLSI guidelines (CLSI, 2022; Macia et al., 2014). The following final concentrations of honey were tested at 20, 15, 10, 5, and 1% (w/v). Briefly, 10 µL of the alternative treatments at concentrated solutions and 190 µL of Mueller-Hinton broth (MHB) plus bacteria with a final concentration of $1x10^5$ CFU/mL were placed in a 96-well plate and incubated at 37°C for 20-24 hours. Additionally, 200 µL of MHB medium plus

bacteria was placed as a positive control and 200 µL of the medium as a negative control (sterility control). The results were measured using the Biotek Instruments ELx808IU spectrophotometer at 570 nm (OD570) and the lowest concentration of each treatment without bacterial growth was classified as MIC (Macià et al., 2014). Finally, the minimum bactericidal concentration (MBC) was identified by removing 50 µL of each treatment into the 96-well plate and then placing 50 µL of resazurin (0.015%) incubating the 96-well plate at 37°C for 1-2 hours until a complete color change was observed in the positive control (i.e., resazurin blue to pink color), as previously described by Elshikh and colleagues (2016). The MBC value was determined when the blue resazurin color remained unchanged indicating no metabolism and microbial death. All tests were performed at least six times and with two replicates.

Biofilm eradication assays

In the biofilm eradication assays, 190 µL of a microbial suspension in MHB at 10^8 CFU/mL was introduced into 96-well flat bottom plates from Tecan Group Ltd. (Mannedorf, Switzerland) (Fernandez-Soto et al., 2023). The plates were then placed in an incubator at 37 ºC under aerobic conditions for 24 h to obtain mature and well-formed biofilms. Then, the MHB was carefully removed, a washing step using phosphate-buffered saline (PBS, at pH 7.4) was conducted, and 190 µL of fresh MHB containing honey samples at different concentrations (30, 25, 20, 15, and 10% at w/v) was added within wells of the 96-well plate. Then the 96-well plate was incubated again for 24 h at 37 ºC under aerobic conditions (Fernandez-Soto et al., 2023). At the end of and eradication assays, all plates were washed three times with 190 µL of PBS after the media was removed to eliminate planktonic cells from biofilm samples. Each biofilm sample was then suspended in PBS and vigorously agitated with a micropipette tip to dissolve the biofilm samples into 190 µL of PBS. Biofilm formation was measured using spectrophotometry in the ELISA Elx808 spectrophotometer (BioTek, Winooski, GU, USA) at

an optical density of 570 nm. Finally, the percentage of biofilm eradication was determined as previously outlined (Fernandez-Soto et al., 2023; Patel et al., 2021; Sornsenee et al., 2021). All assays contained positive controls involving microbial growth in media only and reference controls consisting of media with microbial growth plus a standard antimicrobial treatment (an antibiotic or antifungal agent). Additionally, a well-containing medium devoid of any bacterial inoculum was employed to serve as a sterility control (negative control). All assays were conducted with triplicate controls/samples across a minimum of two independent experiments.

Checkboard Microdilution assay

The checkboard microdilution assay was performed as described b0079 Bellio et al (2021), using the adaptations of Liu et al. (2018). Briefly, two separated sterilized 96-well plates were used for the preparation of antibiotic (treatment A) and honey (treatment B). In the first plate, 200 µL of antibiotic 2x larger than the highest to be tested dilution was set up on row A. Then, 100 µL was taken to row B and diluted with 100 µL of medium, two more serial dilutions were made in the rows below; this was done from columns 1-4. For the second 96-well plate, 200 µL of honey 2x larger than the highest to be tested dilution was dispensed on column 3 followed by moving 100 µL to column 2 and diluting with 100 µL of medium. One more serial dilution was made to column 1, this was done from row A-E (Bellio et al., 2021). Finally, from columns 2-4 antibiotic 96-well plate, 100 µL of antibiotic was taken and dispensed to columns 1-3 so treatments would be mixed on the appropriate concentrations. The plate should end up with twelve concentrations of honey at 40, 20, and 10% combined with 4, 2, 1, and 0.5 µg/mL of standard antimicrobial treatment (ciprofloxacin and gentamicin) considering the planktonic MIC proportions used in Liu et al (2018). Taking into consideration that column 1 and row E were used for controls of antibiotic and honey treatments, respectively, without combining them, an additional volume of 100 µL of medium was needed to dilute the

controls in the total volume of 200 µL before passing to eradication 96-well plates. Lastly, the 200 µL of mixed treatments and treatment controls were used for the biofilm eradication assay previously prepared and described.

Statistical analyses

All data of the present study were further evaluated by statistical analyses. Due to the non-normal distribution of the data set, a non-parametric test was applied, more exactly the Wilcoxon nonparametric test was used for pairwise comparison between control and treated samples (honey or antimicrobial agent by itself or combined) in biofilm eradication assays. Statistical analyses were realized in R studio version 4.0 (https://www.rstudio.com/products/rstudio/download/) using several R packages ("ggpubr", "rstatixs", "openxlsx" and the "tidyverse" set of packages) (Kassambara, 2021; Wickham et al., 2019). Finally, all p-values <0.05 were considered significant.

RESULTS

Initial Disk Diffusion Assay Screening

The initial screening evaluation of the antimicrobial activity of the honey group set was realized through disk diffusion assay (see Appendix A). As shown in Table 1, the honey samples showed different degrees of initial inhibition halos against *S. aureus* ATCC 25923, MRSA 333, *P. aeruginosa* ATCC 27833, *P. aeruginosa* P28, *C. albicans* ATCC 10231, and *C. albicans* INSPI. Several honey samples revealed significant growth inhibition, while certain honey samples evidenced visible inhibition of bacteria/fungi growth even without well-defined inhibition halos. Considering these results, four honey mixes were made to boost the antimicrobial capabilities (see Appendix A), selecting samples from the same provinces and floral origin as shown in Table 2. Also, the best results were obtained against bacteria and further evaluation focused on the antibacterial activity against against *S. aureus* ATCC 25923, MRSA 333, *P. aeruginosa* ATCC 27833, and *P. aeruginosa* P28.

Minimum inhibitory concentration and minimum biocidal concentration against skin pathogens

Honey inhibition and biocidal capabilities are shown in the plots with green and purple bars of Figures 1–4 (see Appendix B), respectively. The most effective honey mix was the mix (3) of avocado from Pichincha evidencing MBC values at 10%, 15%, and 20% of honey dilution against *S. aureus* ATCC 25923, MRSA 333, and *P. aeruginosa* P28, respectively. The planktonic growth inhibition values for these MIC values were 88%, 93%, and 94%, respectively (see Appendix B). Other positive results were obtained through honey mixes of eucalyptus from Pichincha and Cotopaxi against *P. aeruginosa* ATCC 27833, both evidencing MIC values on 20% diluted honey with 52% and 51% of planktonic growth inhibition respectively, as shown in Figure 1. Due to time constraints, further evaluation of combined

treatments focused on the biofilm eradication of reference bacterial strains, more exactly *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27833.

Biofilm eradication by checkboard microdilution essay

The highest biofilm eradication values with honey are shown in red boxes of Figures 5–8 (see Appendix B). Regarding honey and antibiotic treatment controls on biofilm eradication assays, *P. aeruginosa* ATCC 27833 demonstrated the highest eradication value of 59% with avocado honey from Pichincha at 20% (w/v), while antibiotic (ciprofloxacin) at 4 µg/mL evidenced the highest eradication value of 52%. When both treatments were combined, eucalyptus honey from Pichincha 10% (w/v) plus gentamicin at 4 µg/mL showed its highest eradication value of 54% against *P. aeruginosa* ATCC 27833, whereas eucalyptus honey from Cotopaxi at 20% (w/v) plus gentamicin at 2 µg/mL evidenced its highest eradication value of 48%. Meanwhile, avocado honey from Pichincha showed its best efficiency at 20% (w/v) plus gentamicin at 4 µg/mL yielding 60% biofilm eradication, and, finally, tura honey from Imbabura at 40% (w/v) plus gentamicin at 2 µg/mL eradicated 66% of *P. aeruginosa* ATCC 27833 biofilm. Concerning honey and antibiotic controls against *S. aureus* ATCC 25923, the highest biofilm eradication value was 50% with eucalyptus honey from Cotopaxi at 40% (w/v) and gentamicin at 2 µg/mL showing a 50% of biofilm eradication. When both treatments were combined, eucalyptus honey from Pichincha at 40% (w/v) plus gentamicin at 4 µg/mL achieved the highest biofilm eradication value of 50%. Moreover, eucalyptus honey from Cotopaxi at 40% (w/v) plus gentamicin at 4 µg/mL demonstrated the highest biofilm eradication value of 74%. Finally, avocado honey from Pichincha at 40% (w/v) plus gentamicin at 1 µg/mL demonstrated the best *S. aureus* ATCC 25923 biofilm eradication of 44%, and tura honey from Imbabura at 40% (w/v) plus gentamicin at 4 µg/mL showed its highest biofilm eradication of 58%.

DISCUSSION

Several honey types have been studied for their antimicrobial activity and potential applications in medical treatments (Alvarez-Suarez et al., 2010). However, the current "medical grade" honey is hard to obtain and produce on a higher scale, which makes *Apis mellifera* honey a more economical and reliable alternative when compared to other kinds of honey. The term "medical grade honey" is normally described as a "licensed medical device either incorporated into sterile dressings or sterilized in tubes", being applied to honey that has shown inhibitory properties against the growth of numerous microorganisms *in vitro* and is also able to eradicate pathogens that colonized wounds such as manuka honey (Jenkins & Cooper, 2012). The antimicrobial properties of honey are based on hydrogen peroxide-dependent and independent mechanisms. Peroxide of hydrogen damages microbial DNA, while peroxide-independent mechanisms exert osmotic pressure and decrease pH thus inhibiting microbial growth (García-Tenesaca et al., 2017; Machado et al., 2023; Proaño et al., 2021). Previous studies have also demonstrated their high planktonic cell inhibition with avocado honey showing a low MIC of 20% (w/v) against *Escherichia coli* CECT 515 and a low MIC of 5% (w/v) against *S. aureus* CECT 86 (Combarros-Fuertes, M. Estevinho, et al., 2020). These studies are in agreement with the best results obtained against *P. aeruginosa* P28 and *S. aureus* ATCC 25923 in the present study through MIC values at 20 and 10% (w/v) with bacterial growth inhibition values of 94 and 88% (Figures 2 and 3), respectively. Regarding MDR pathogens, previous studies evaluating manuka honey reported a MIC value of <12.5% (v/v) against MRSA ATCC 43300 (Sherlock et al., 2010), while honey with different flowering origins exhibited MIC values in the range of 10-25% (w/v) against *P. aeruginosa* clinical (Combarros-Fuertes, Fresno, et al., 2020). Our results also showed MIC values of 15% (w/v) against MRSA 333 with 93% planktonic cell inhibition (Figure 4), demonstrating that avocado

honey can reach similar MIC values against MRSA; also MIC values of 20% (w/v) against *P. aeruginosa* P28 with 94% planktonic cell inhibition (Figure 2) as in previous studies, despite it was not applied at medical grade purification. These results with avocado honey could be attributed to the high levels of peroxide, as reported by García-Tenesaca et al. (2017). However, it is also important to mention the osmotic pressure action of the artificial honey control evidencing its partial contribution to the antimicrobial activity of the studied honey (Tables 3 and 4).

Even though antibiofilm inhibition activity has been reported for eucalyptus and avocado honeys, its antibiofilm activity can be improved by combining the honey treatments with antibiotics to reach higher levels of biofilm eradication (Cabezas-Mera et al., 2023; García-Tenesaca et al., 2017; Proaño et al., 2021). García-Tenesaca and colleagues reported that their avocado honey samples at 20% (w/v) inhibited 60% of biofilm formation against *K. pneumoniae* KPC 609803 and other eucalyptus honey at 20% (w/v) inhibited only 20% against the same pathogen strain (García-Tenesaca et al., 2017). Although García-Tenesaca only evaluated *K.pneunomiae* KPC 609803 biofilm inhibition, the same trend was observed with our avocado honey samples at 20% (w/v) on Gram-negative biofilms, where we reported 59% biofilm eradication against *P. aeruginosa* ATCC 27833. Meanwhile, Lu et al. (2019) evaluated manuka honey at 16-32% (w/v) to eradicate *P. aeruginosa* ATCC 15692 and *P. aeruginosa* UCBPP-PA14 biofilms showing significant biofilm disruption and similar to our results. Furthermore, this study revealed the best synergy of treatments with honey avocado (Pichincha) at 20% (w/v) and ciprofloxacin at 4 µg/mL achieving 60% biofilm eradication of *P. aeruginosa* ATCC 27833. The present combined treatment showed similar results as reported by Yasir and colleagues, where the alternative combined treatment of 125 µg/mL of antimicrobial peptide (Mel4) and ciprofloxacin at 0.5 µg/mL was able to disrupt 61% of pre-formed biofilms of *P. aeruginosa* ATCC 27853 (Yasir et al., 2020).

On the other hand, our eucalyptus honey sample at 40% (w/v) by itself showed 60% biofilm eradication of *S. aureus* ATCC 25923. This antibiofilm activity is in agreement with García-Tenesaca and colleagues that their eucalyptus and avocado honeys at 20% (w/v) were able to inhibit 40 and 65% biofilm formation of *S. aureus* (García-Tenesaca et al., 2017), respectively. Another study reported a disruption of preformed *S. aureus* ATCC 25923 biofilm with manuka honey at 16%–32% (w/v) (Lu et al., 2014), agreeing with the results obtained in the present study. However, the best-combined treatment outcomes in the present study were obtained with eucalyptus honey samples and antibiotics. More exactly, the best synergy outcome was obtained with eucalyptus honey from Cotopaxi at 40% (w/v) and gentamicin at 2 µg/mL eradicating 74% of *S. aureus* ATCC 25923 biofilms (Figure 8). When compared to the literature, similar results were reported by the synergic effect of manuka honey at 8% (w/v) and rifampicin at 0.02 µg/mL inhibiting 85% of *S. aureus* biofilms (Liu et al., 2018). Although the present study evidenced similar synergic effects on the biofilm eradication of *S. aureus*, the amount of antibiotic and honey did not demonstrate a synergic effect as high as reported by manuka honey. However, it is important to mention that Liu and colleagues only reported 15% of *S. aureus* biofilm eradication when applying manuka honey at 8% (w/v) and gentamicin at 0.625 µg/mL, which is much lower than the value of 74% biofilm eradication on *S. aureus* ATCC 25923 achieved in the present study. So, further studies must evaluate the combination of the present honey mix combinations with additional antibiotics (such as rifampicin) to increment the synergic effect of combined treatment and elevate biofilm eradication percentages. Finally, this study had several limitations such as the low number of combined treatments tested, the lack of other applied methodologies to evaluate biofilm eradication (such as colony-forming unit (CFU) counting and LIVE/DEAD assays), and the number of skin-related pathogens studied. Therefore, additional analyses must be realized and a more complex evaluation should be performed in future studies.

CONCLUSIONS

The present study demonstrated the antimicrobial activity of *Apis mellifera* honey from different floral origins against *P. aeruginosa* and *S. aureus*. Preliminary evaluation through MIC assays showed better planktonic growth inhibition by avocado honey from Pichincha against *S. aureus* ATCC 25923, MRSA 333, and *P. aeruginosa* P28 when compared to eucalyptus honey from Cotopaxi and Pichincha. When analyzing honey and antibiotic combinations, both honey samples showed a tendency to improve their antimicrobial activity when compared by themselves, but the best results in eradicating *S. aureus* ATCC 25923 biofilm were obtained with eucalyptus honey. The results obtained in the present study are in agreement with the results of other alternative and combined treatments reported in the literature. Future studies are necessary to further evaluate the synergy between honey and antibiotics by applying different microbial methodologies (such as CFU counting and LIVE/DEAD assays) and evaluating the synergy with a larger range of treatment concentrations, as well as other types of antibiotics and a greater number of skin-related pathogens. Finally, the peroxide-dependent and independent mechanisms of the best-combined treatments should be analyzed to determine how significant the peroxide hydrogen action is involved in the biofilm eradication of each pathogen.

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APPENDIX A: TABLES

Table 1. Inhibition halo obtained in the initial disk diffusion essay against six skin-related pathogens.

Sample #	Microorganism	Inhibition Type	Assay 1 (mm)	Assay 2 (mm)	Average (mm)	SD	SD %	Positive Control (mm)	Antibiotic
4.3	*S. aureus* ATCC 25923	Complete	12	13	12.5	0.707	5.66	29	CTX
AG4	*S. aureus* ATCC 25923	Complete	14	14	14	0.000	0.00	30	CTX
AG1	*S. aureus* ATCC 25923	Complete	13	15	14	1.414	10.10	27	CTX
AG3	*S. aureus* ATCC 25923	Complete	12	11	11.5	0.707	6.15	27	CTX
T101	*S. aureus* ATCC 25923	Complete	14	11	12.5	2.121	16.97	30	CTX
AG1	MRSA 333	Complete	11	10.5	10.75	0.354	3.29	30	C
AG3	MRSA 333	Complete	12	16	14	2.828	20.20	30	C
AG4	MRSA 333	Complete	13	14	13.5	0.707	5.24	30	C
4.1	*P. aeruginosa* ATCC 27833	Complete	12	11	11.5	0.707	6.15	33	MEM
3.4	*P. aeruginosa* ATCC 27833	Complete	13	15	14	1.414	10.10	30	C
3.5	*P. aeruginosa* ATCC 27833	Complete	12	10.5	11.25	1.061	9.43	30	C
T102	*P. aeruginosa* ATCC 27833	Complete	15	14	14.5	0.707	4.88	30	C
4.4	*P. aeruginosa* ATCC 27833	Complete	13	14	13.5	0.707	5.24	30	C
AG1	*P. aeruginosa* P28	Complete	12	12	12	0.000	0.00	17	CTX
AG3	*P. aeruginosa* P28	Complete	13	12	12.5	0.707	5.66	17	CTX
AG4	*P. aeruginosa* P28	Complete	11	11	11	0.000	0.00	15	CTX
AG5	*P. aeruginosa* P28	Complete	12	12	12	0.000	0.00	15	CTX
AG3	*C. albicans ATCC*	Complete	11	11	11	0.000	0.00	24	FLU
AG4	*C. albicans* INSPI	Complete	11	10.5	10.75	0.354	3.29	15	FLU

Legend- Evaluation of disk diffusion assay of honey mixes against six dermal pathogens *S. aureus ATCC 25923, MRSA 333, P. aeruginosa ATCC 27833, P. aeruginosa P28, Candida albicans ATCC 10231, and C. albicans INSPI*. For data evaluation, values were compared to antibiotics as a positive control. The antibiotic/antifungal abbreviations are as follows: C=Ciprofloxacin, MEM=Meropenem, CTX=Cefotaxime, FLU=Fluconazole.

Table 2. Honey mix composition

Code	Flowering and province	Floral species	Number of samples
Mix 1	Eucalyptus (Pichincha)	*Eucalyptus* spp.	17
Mix 2	Eucalyptus (Cotopaxi)	*Eucalyptus* spp.	21
Mix 3	Avocado (Pichincha)	*Persea americana*	5
Mix 4	Tura (Imbabura)	*Calliandra trinervia*	6

Table 3. Summary of the results obtained in Minimum Inhibitory Concentration (MIC) assays in each alternative treatment against reference skin-related pathogens.

Microorganisms	**Honey sample**	**Concentration (%)**	**Bacterial Growth (%)**	**Bacterial Growth inhibition (%)**	**Standard deviation (%)**	***p- value C+***		***p-value C-***	
P. aeruginosa ATCC 27833	C+	0	100	0	12.03%				
	Artificial	20	56.13	43.87	7.59%	0.033	*		
		15	63.68	36.32	10.81%	0.148	ns		
		10	69.34	30.66	4.89%	0.288	ns		
		5	72.21	27.79	4.18%	0.71	ns		
		2.5	75.38	24.62	3.33%	1	ns		
		1	76.67	23.33	1.55%	1	ns		
	Mix 1	20	48.22	51.78	7.56%	<0.0001	****	0.0005	***
		15	68.99	31.01	14.56%	0.00004	****	0.1672	ns
		10	61.39	38.61	6.37%	0.0005	***	<0,0001	****
		5	73.92	26.08	6.48%	0.00001	****	0.4348	ns
		2.5	71.85	28.15	2.17%	0.0002	***	0.0006	***
		1	72.95	27.05	2.87%	0.00008	****	<0,0001	****
	Mix 2	20	48.85	51.15	8.60%	<0.0001	****	0.0035	**
		15	54.13	45.87	3.55%	0.0001	***	<0,0001	****
		10	63.24	36.76	4.84%	0.000003	****	0.0004	***
		5	72.78	27.22	5.74%	0.00008	****	0.7673	ns
		2.5	69.86	30.14	2.85%	0.00008	****	<0,0001	****
		1	71.18	28.82	3.02%	0.00008	****	<0,0001	****
	Mix 3	20	51.78	48.22	7.29%	0.00002	****	0.0337	*
		15	59.75	40.25	13.74%	0.00004	****	0.3337	ns
		10	69.46	30.54	7.21%	0.0003	***	>0,999	ns

Microorganisms	**Honey sample**	**Concentration (%)**	**Bacterial Growth (%)**	**Bacterial Growth inhibition (%)**	**Standard deviation (%)**	***p- value C+***		***p-value C-***	
		5	74.75	25.25	2.60%	0.0009	***	0.0729	ns
		2.5	72.63	27.37	3.24%	0.0001	**	0.0144	*
		1	85.76	14.24	2.77%	0.007	**	<0,0001	****
	Mix 4	20	51.67	48.33	5.88%	0.0001	***	0.0168	*
		15	54.54	45.46	7.76%	0.00001	****	0.0016	**
		10	62.99	37.01	5.12%	0.0001	***	0.0002	***
		5	75.45	24.55	2.18%	0.001	**	0.0161	*
		2.5	72.31	27.69	3.35%	0.00002	****	0.0133	*
		1	73.2	26.8	4.23%	0.00003	****	0.0017	**
S. aureus ATCC 25923	C+	0	100	0	11.26%				
	Artificial	20	59	41	8.26%				
		15	63.34	36.66	2.69%	<0,0001	****		
		10	69.91	30.09	7.12%	<0,0001	****		
		5	76.08	23.92	3.54%	<0,0001	****		
		2.5	74.99	25.01	1.82%	<0,0001	****		
		1	75.81	24.19	6.38%	<0,0001	****		
	Mix 1	20	82.46	17.54	27.00%	0.0001	***	0.0212	*
		15	66.79	33.21	7.81%	0.000002	****	0.1176	ns
		10	68.72	31.28	2.70%	0.0001	****	0.8358	ns
		5	86.16	13.84	5.18%	0.0001	***	<0,0001	****
		2.5	87.49	12.51	4.28%	0.0004	***	<0.0001	****
		1	90.118	9.882	6.56%	0.0051	**	<0,0001	****
	Mix 2	20	57.91	42.09	6.06%	0.000006	****	0.4111	ns
		15	82.63	17.37	19.24%	0.0004	***	<0,0001	****

Microorganisms	**Honey sample**	**Concentration (%)**	**Bacterial Growth (%)**	**Bacterial Growth inhibition (%)**	**Standard deviation (%)**	***p- value C+***		***p-value C-***	
		10	77.42	22.58	13.49%	0.0001	***	0.0665	ns
		5	80.83	19.17	7.61%	0.00002	****	0.0407	*
		2.5	85.55	14.45	7.48%	0.0004	***	0.001	***
		1	85.03	14.97	6.21%	0.0001	***	0.0002	***
	Mix 3	20	13.22	86.78	1.59%	<0.0001	****	<0,0001	****
		15	12.53	87.47	5.56%	<0.0001	****	<0,0001	****
		10	11.68	88.32	2.90%	<0.0001	****	<0,0001	****
		5	94.488	5.512	10.72%	0.1	ns	<0,0001	****
		2.5	91.071	8.929	10.84%	0.03	*	<0,0001	****
		1	90.809	9.191	8.62%	0.01	*	<0,0001	****
	Mix 4	20	56.59	43.41	3.68%	0.0001	***	0.1966	ns
		15	66.31	33.69	12.84%	0.000006	****	0.9803	ns
		10	81.22	18.78	5.64%	0.0000004	****	0.0001	***
		5	86.86	13.14	5.96%	0.000006	****	<0,0001	****
		2.5	87.32	12.68	6.86%	0.0000005	****	<0,0001	****
		1	89.23	10.77	6.04%	0.000001	****	<0,0001	****

Legend – Evaluation of Minimum inhibitory concentration of honey mixes against S. aureus ATCC 25923 and P. aeruginosa ATCC 27833. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of bacterial growth and growth inhibition for each treatment and standard deviation values were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. The p values are shown in the following format: * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant.

Table 4. Summary of the results obtained in Minimum Inhibitory Concentration (MIC) assays in each alternative treatment against multiresistant dermal pathogens.

Microorganisms	**Honey sample**	**Concentration (%)**	**Bacterial Growth (%)**	**Bacterial Growth inhibition (%)**	**Standard deviation (%)**	***p- value C+***		***p-value C-***	
MRSA 333	C+	0	100	0	8.47%				
	Artificial	20	83.56	16.44	10.39%	0.1071	ns		
		15	84.58	15.42	12.82%	0.1091	ns		
		10	93.6	6.4	4.54%	0.2141	ns		
		5	88.85	11.15	5.77%	0.2141	ns		
		2.5	100	0	5.38%	0.6747	ns		
	Mix 1	20	89.958	10.042	10.22%	0.0002	***	0.8785	ns
		15	74.09	25.91	13.44%	0.0002	***	0.1304	ns
		10	130	-30	7.52%	0.0096	**	0.0002	***
		5	100	0	4.80%	0.0002	***	0.0002	***
		2.5	100	0	6.10%	0.0002	***	0.0002	***
	Mix 2	20	70.1	29.9	0.1435	<0.0001	****	0.0152	*
		15	100	0	0.09904	0.5259	ns	0.0003	***
		10	100	0	4.45%	0.0002	***	0.0002	***
		5	100	0	4.98%	0.0002	***	0.0002	***
		2.5	100	0	3.01%	0.0002	***	0.0002	***
	Mix 3	20	7.61	92.39	1.84%	<0.0001	****	<0.0001	****
		15	7.188	92.812	5.47%	<0.0001	****	<0.0001	****
		10	78.95	21.05	5.35%	<0.0001	****	0.0002	***
		5	86.23	13.77	4.25%	<0.0001	****	0.4418	ns
		2.5	82.02	17.98	7.04%	0.002	**	0.004	**

Microorganisms	**Honey sample**	**Concentration (%)**	**Bacterial Growth (%)**	**Bacterial Growth inhibition (%)**	**Standard deviation (%)**	***p- value C+***		***p-value C-***	
	Mix 4	20	80.34	19.66	5.19%	0.0007	***	0.4351	ns
		15	70.19	29.81	12.56%	<0.0001	****	0.0207	*
		10	81.59	18.41	6.95%	0.0001	***	0.0012	**
		5	93.66	6.34	4.18%	<0.0001	****	0.0464	*
		2.5	91.05	8.95	6.09%	<0.0001	****	0.0042	**
P. aeruginosa P28	C+	0	100	0	1.42%				
	Artificial	20	83.39	16.61	2.22%	<0.0001	****		
		15	83.77	16.23	8.52%	<0.0001	****		
		10	96.01	3.99	0.53%	<0.0001	****		
		5	97.49	2.51	1.24%	<0.0001	****		
		2.5	97.12	2.88	1.88%	0.0004	***		
	Mix 1	20	96.74	3.26	1.38%	<0.0001	****	0.0002	***
		15	94.41	5.59	2.62%	<0.0001	****	0.0011	**
		10	95.37	4.63	1.04%	<0.0001	****	0.0457	*
		5	86.79	13.21	2.72%	<0.0001	****	<0.0001	****
		2.5	76.86	23.14	2.39%	<0.0001	****	<0.0001	****
	Mix 2	20	81.11	18.89	1.34%	<0.0001	****	0.0104	*
		15	93.55	6.45	0.74%	<0.0001	****	0.0002	***
		10	97.08	2.92	1.44%	<0.0001	****	0.1193	ns
		5	94.65	5.35	1.00%	<0.0001	****	0.0012	**
		2.5	96.14	3.86	1.46%	<0.0001	****	0.2786	ns
	Mix 3	20	5.792	94.208	3.32%	<0.0001	****	<0.0001	****
		15	89	11	3.62%	<0.0001	****	0.1049	ns

Microorganisms	Honey sample	Concentration (%)	Bacterial Growth (%)	Bacterial Growth inhibition (%)	Standard deviation (%)	*p- value C+*		*p-value C-*	
		10	100	0	0.97%	<0.0001	****	<0.0001	****
		5	100	0	2.48%	0.3865	ns	0.0064	**
		2.5	100	0	1.30%	0.261	ns	0.0019	**
	Mix 4	20	80.46	19.54	5.15%	<0.0001	****	0.1949	ns
		15	91.51	8.49	1.75%	<0.0001	****	0.0078	**
		10	95.66	4.34	3.53%	<0.0001	****	0.4382	ns
		5	95.86	4.14	2.55%	<0.0001	****	0.0927	ns
		2.5	93.61	6.39	2.66%	<0.0001	****	0.0077	**

Legend – Evaluation of Minimum inhibitory concentration of honey mixes against MRSA 333 and *P. aeruginosa* P28. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of bacterial growth and growth inhibition for each treatment and standard deviation values were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. The p values are shown in the following format: * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant.

APPENDIX B: FIGURES

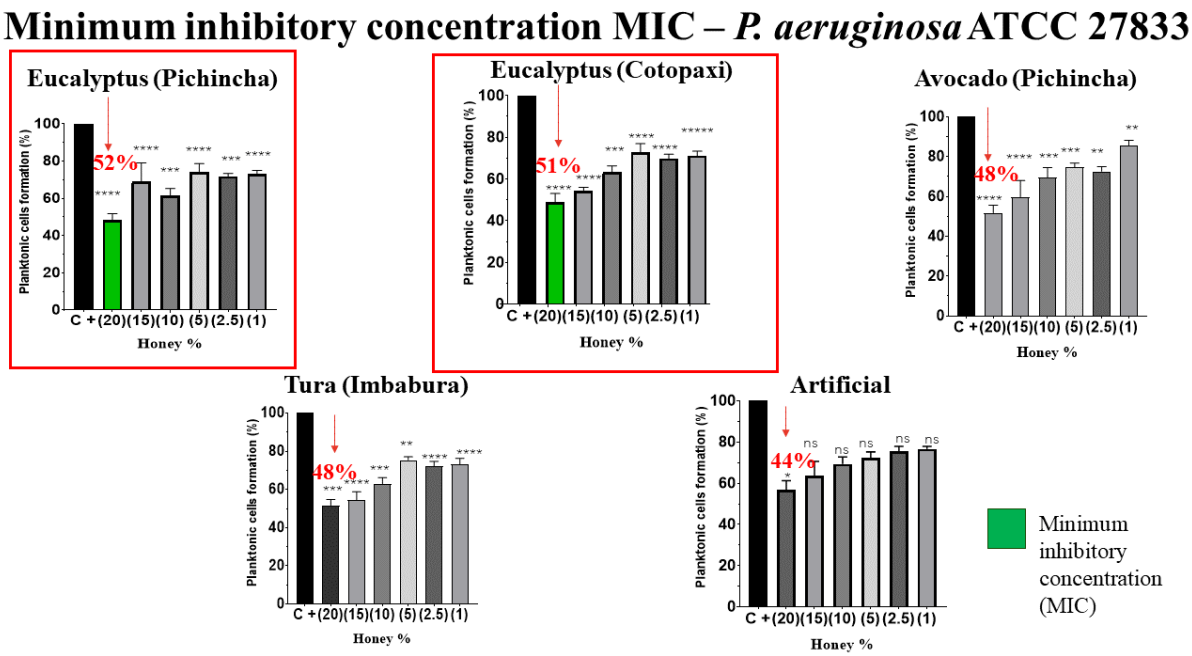

Figure 1. MIC & MBC of four honey mixes for *P. aeruginosa* ATCC 27833

Legend- Evaluation of Minimum inhibitory concentration of honey mixes against *P. aeruginosa* ATCC 27833. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of bacterial growth and growth inhibition for each treatment and standard deviation values were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. The p values are shown in the following format: * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant. The highest bacterial inhibition of each treatment is shown in red percentages.

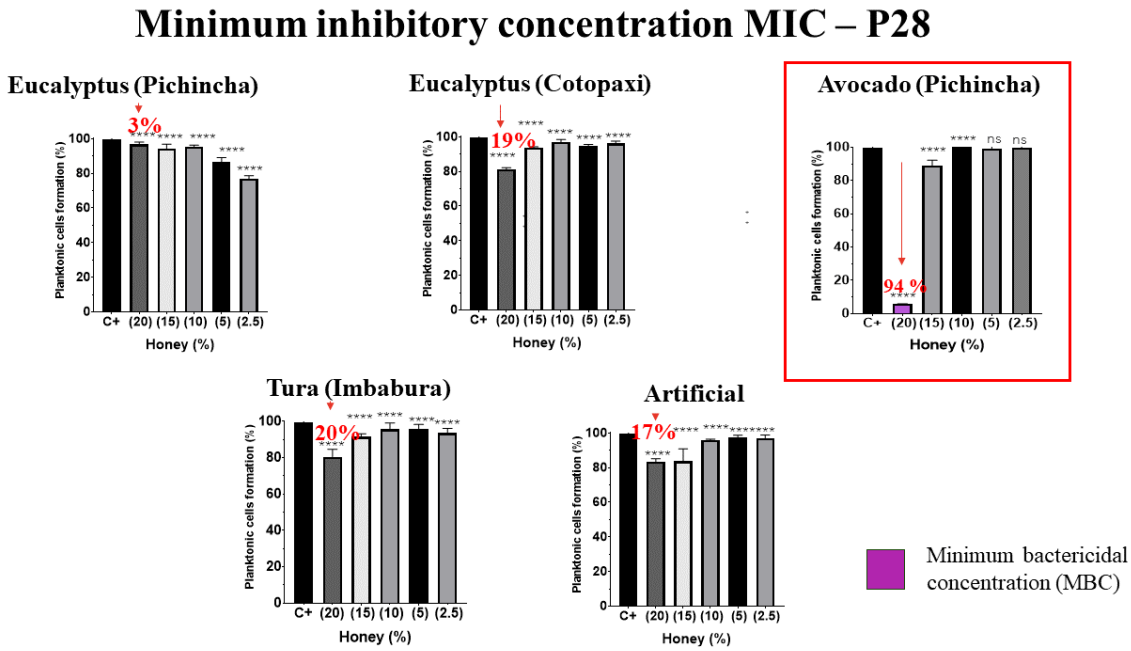

Figure 2. MIC & MBC of four honey mixes for *P. aeruginosa* P28

Legend- Evaluation of Minimum inhibitory concentration of honey mixes against *P. aeruginosa* P28. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of bacterial growth and growth inhibition for each treatment and standard deviation values were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. The p values are shown in the following format: * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant. The highest bacterial inhibition of each treatment is shown in red percentages.

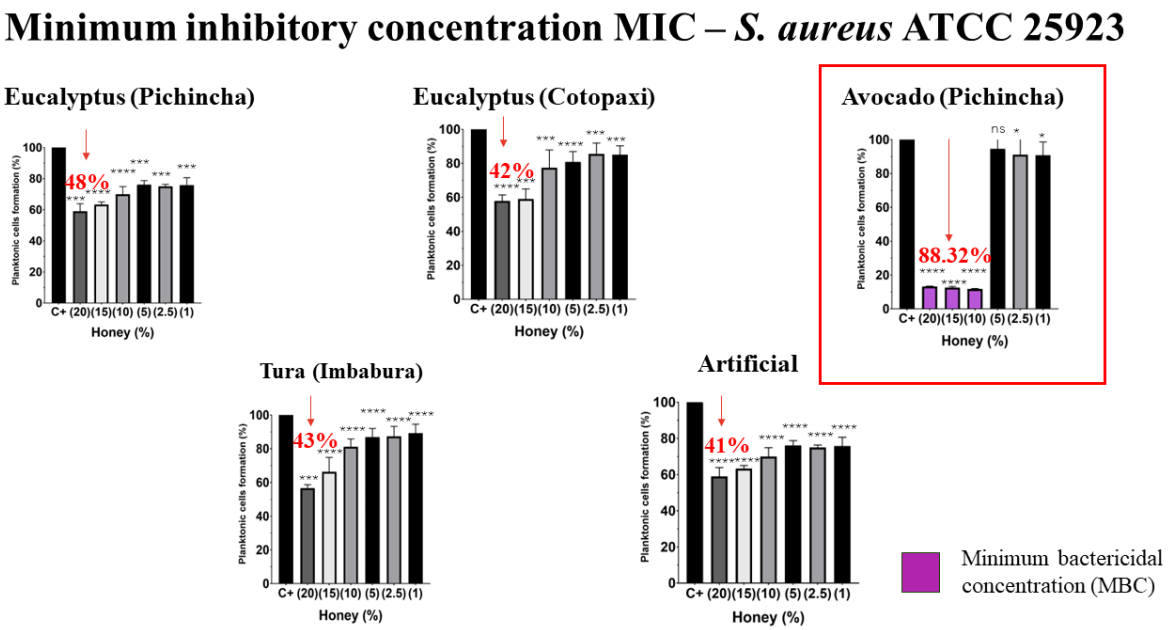

Figure 3. MIC & MBC of four honey mixes for *S. aureus* ATCC 25923

Legend- Evaluation of Minimum inhibitory concentration of honey mixes against *S. aureus* ATCC 25923. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of bacterial growth and growth inhibition for each treatment and standard deviation values were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. The p values are shown in the following format: * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant. The highest bacterial inhibition of each treatment is shown in red percentages.

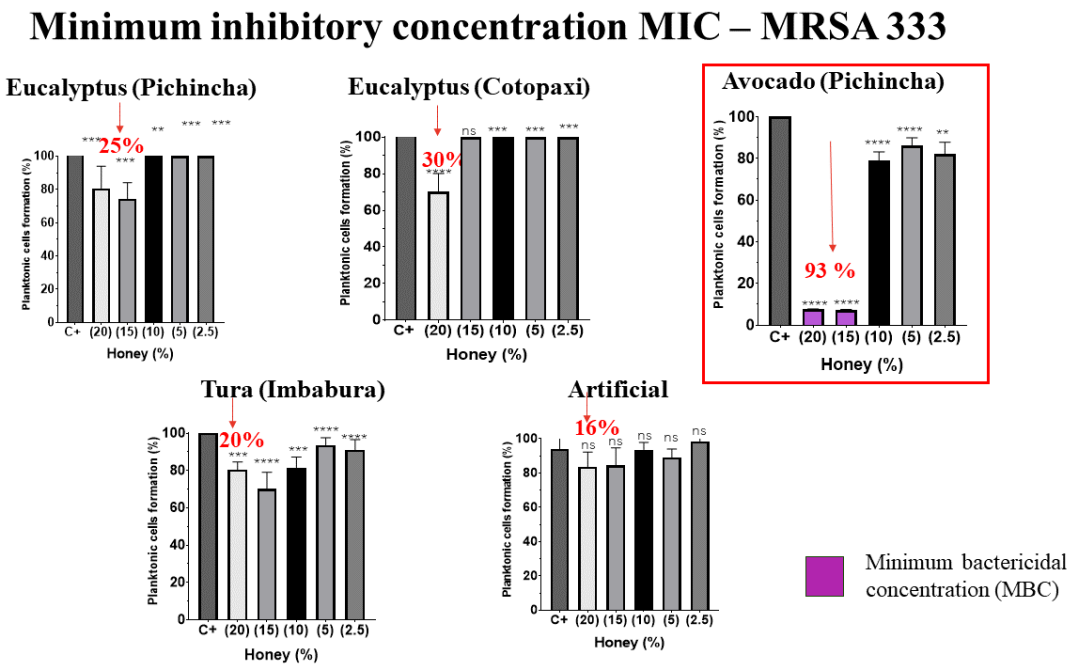

Figure 4. MIC & MBC of four honey mixes for MRSA 333

Legend- Evaluation of Minimum inhibitory concentration of honey mixes against MRSA 333. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of bacterial growth and growth inhibition for each treatment and standard deviation values were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. The p values are shown in the following format: * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant. The highest bacterial inhibition of each treatment is shown in red percentages.

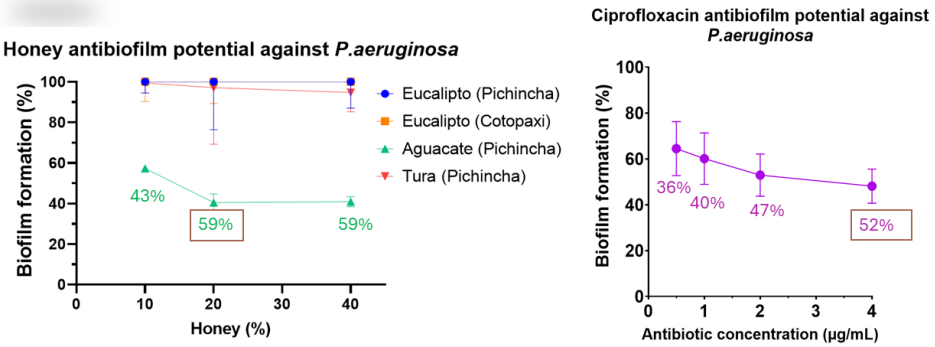

Figure 5. Evaluation of Biofilm eradication of honey mixes and ciprofloxacin isolated against *P. aeruginosa* ATCC 27833.

Legend – Evaluation of Biofilm eradication of honey mixes and ciprofloxacin isolated against *P. aeruginosa* ATCC 27833. The percentage of Biofilm inhibition and growth inhibition for each treatment and standard deviation values were obtained. The highest biofilm inhibition of the best treatment is shown in a red box.

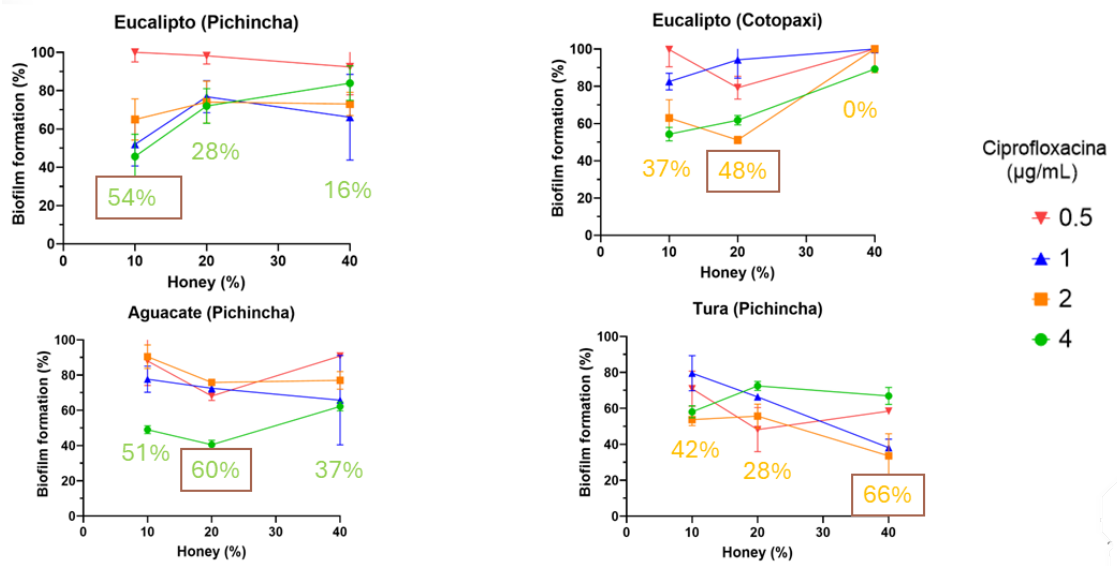

Figure 6. Evaluation of Biofilm eradication of honey mixes and ciprofloxacin combined against *P. aeruginosa* ATCC 27833

Legend – Evaluation of Biofilm eradication of honey mixes and ciprofloxacin combined against *P. aeruginosa* ATCC 27833. The percentage of Biofilm inhibition and growth inhibition for each treatment and standard deviation values were obtained. The highest biofilm inhibition of the best treatment is shown in a red box.

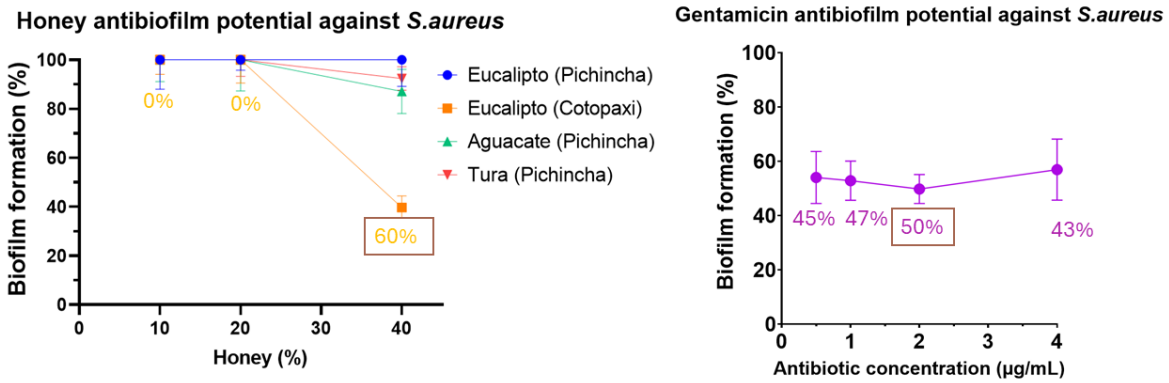

Figure 7. Evaluation of Biofilm eradication of honey mixes and gentamicin isolated against *S. aureus* ATCC 25923.

Legend – Evaluation of Biofilm eradication of honey mixes and gentamicin isolated against *S. aureus* ATCC 25923. The percentage of Biofilm inhibition and growth inhibition for each treatment and standard deviation values were obtained. The highest biofilm inhibition of the best treatment is shown in a red box.

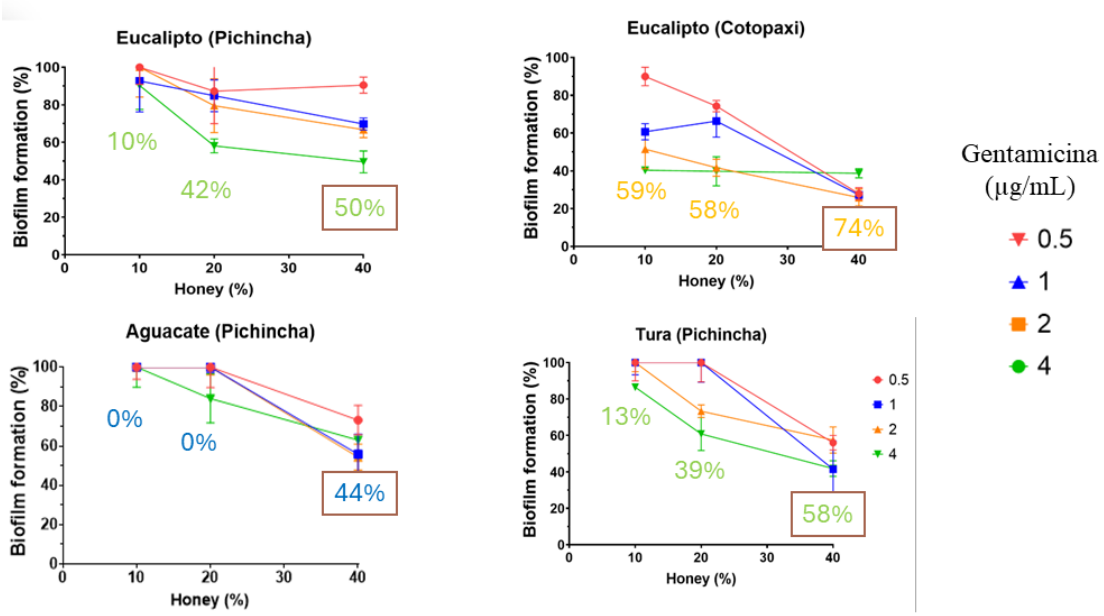

Figure 8. Evaluation of Biofilm eradication of honey mixes and gentamicin combined against *S. aureus* ATCC 25923.

Legend – Evaluation of Biofilm eradication of honey mixes and gentamicin combined against *S. aureus* ATCC 25923. The percentage of Biofilm inhibition and growth inhibition for each treatment and standard deviation values were obtained. The highest biofilm inhibition of the best treatment is shown in a red box.