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Article

New Insights into Cutaneous Asepsis: Synergism between *Pfaffia* and Rosemary Extracts

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Abstract: (1) Background: In hospitals, medical and dental clinics, antiseptics or disinfectants play an essential role in the control of nosocomial infections. This study aimed to evaluate *R. officinalis* and *P. paniculata* glycolic extracts regarding: (I) their antimicrobial action on planktonic and biofilm (monotypic and cutaneous biofilm model—*S. aureus*, *S. epidermidis* and *C. acnes*); and (II) their cytotoxicity on human keratinocytes (HaCaT). (2) Methods: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed (CLSI protocol M7-A6 and M11-A8). MTT analysis was used to evaluate the antibiofilm activity of the extracts on biofilms and their cytotoxicity on human keratinocytes. (3) Results: The combined glycolic extracts MIX A (75% *P. paniculata* + 25% *R. officinalis*); MIX B (50% *P. paniculata* + 50% *R. officinalis*); and MIX C (25% *P. paniculata* + 75% *R. officinalis*) promoted MBC values by 50 mg/mL on *S. aureus*, absent on *S. epidermidis*, and ranged 6.25–50 mg/mL for *C. acnes*. The cutaneous biofilm model was reduced more than 90%. In addition, it showed biocompatibility with human keratinocytes, resulting in percentages of viability greater than 50%. (4) Conclusions: The combination of extracts promoted antimicrobial action on planktonic cultures, and monotypic and heterotypic biofilms of skin pathogens. Additionally, these extracts are biocompatible against human keratinocytes.

Keywords: skin infections; herbal medicine; *Staphylococcus aureus*; *Cutibacterium acnes*; *Staphylococcus epidermidis*

1. Introduction

Nosocomial infections, also referred to as healthcare-associated infections, represent a significant burden and safety issue for patients in developing countries. The overall prevalence of healthcare-associated infections in developing countries is up to 15.5%, while it reaches 34.1% for critical care patients [1–4]. Several Gram-positive bacteria, resident human

flora, have been identified as opportunistic bacteria responsible for healthcare-associated infections. Among these bacteria, three appear as very important: (i) *Staphylococcus aureus*, (ii) *Staphylococcus epidermidis*, and (iii) *Cutibacterium acnes* also known as *Propionibacterium acnes* [5,6]. The *Staphylococcus* genus stands out due to the number of clinical isolates resistant to oxacillin, exhibiting a frightening 81.33% rate of resistance [1].

In hospitals and medical and dental clinics, the appropriate use of gloves is crucial for preventing the transmission of infections from the patient to the medical staff and vice versa. Regardless of the material of the glove used, glove perforations are common in the clinical setting. It has been documented that glove perforation rates during surgery can be as high as 30% [7]. Therefore, antiseptics or disinfectants play an essential role in the control of nosocomial infections, in which antiseptics of skin sites that will receive some type of invasive procedures, including surgical incisions and catheter insertions, are of great importance because of their antimicrobial action, preventing infections of deeper sites [8,9]. Chlorhexidine digluconate (CHX) is a widely used potent disinfectant; however, its continuous use can impose selective pressure on bacteria, promoting the creation of adaptive resistance of these microorganisms. The lack of response of nosocomial pathogens to conventional treatments has become one of the most serious global public health threats of the 21st century, as antimicrobial resistance promotes high rates of mortality and morbidity [10–12].

In this scenario, there is a need to search for new, effective, safe, and low-cost alternatives. Phytotherapy is a field of alternative medicine that uses plants to treat diverse diseases, and it is becoming more common in modern medicine because of its antimicrobial action and biocompatibility; for example, *Rosmarinus officinalis*, which is popularly known as rosemary, has phytochemicals that have several pharmacological effects, including antimicrobial, anti-inflammatory, and antioxidant effects [5,13–15]. Additionally, *Pfafia paniculata*, which is a plant native to the Neotropics known as Brazilian ginseng, has antimicrobial, anti-inflammatory, anticancer and regenerative properties [16].

Therefore, the aim of this study was to evaluate *R. officinalis* and *P. paniculata* glycolic extracts regarding (I) their antimicrobial action on planktonic and biofilm (monotypic and heterotypic) cultures of *S. aureus*, *S. epidermidis*, and *C. acnes*; and (II) their cytotoxicity on human keratinocytes (HaCaT) seeking the development of new drugs to combat nosocomial infections.

2. Results

2.1. Minimum Inhibitory, Minimum Bactericidal, and Fractional Inhibitory Concentrations of *P. paniculata* and *R. officinalis*

Table 1 presents the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and fractional inhibitory concentration (FIC) of *P. paniculate*, *R. officinalis*, and the combined extract on planktonic cultures of *S. aureus*, *S. epidermidis*, and *C. acnes*. The MIC values of all the extracts tested were in the range of 25 to 50 mg/mL in *S. aureus* and *S. epidermidis*, but in the range of 0.195 to 6.25 mg/mL in *C. acnes*. On the other hand, the minimum bactericidal concentration (MBC) values ranged from absent to 50 mg/mL in *S. aureus*. It was absent in *S. epidermidis* and ranged from 6.25 to 50 mg/mL in *C. acnes*. The FIC values of Mix A, B, and C were indifferent in *S. aureus* and were antagonistic in *S. epidermidis*. However, the FIC values of Mix A and B were synergistic, and Mix C was indifferent in *C. acnes*.

Table 1. MIC, MBC, and FIC values in mg/mL of *P. paniculata*, *R. officinalis*, and the combined extract on planktonic cultures of *S. aureus*, *S. epidermidis*, and *C. acnes*. (*P. paniculata* and *R. officinalis* 75% + 25% (Mix A), *P. paniculata* and *R. officinalis* 50% + 50% (Mix B), *P. paniculata* and *R. officinalis* 25% + 75% (Mix C), indifferent (IND), antagonistic (ANT), synergistic (SYN), not applicable (-)).

| Extract | <i>S. aureus</i> | | | <i>S. epidermidis</i> | | | <i>C. acnes</i> | | |
|-----------------------|------------------|--------|-----|-----------------------|--------|-----|-----------------|------|-----|
| | MIC | MBC | FIC | MIC | MBC | FIC | MIC | MBC | FIC |
| <i>P. paniculata</i> | 25 | 25 | - | 25 | Absent | - | 6.25 | 50 | - |
| Mix A | 25 | 25 | IND | 50 | Absent | ANT | 3.12 | 25 | SYN |
| Mix B | 50 | 50 | IND | 50 | Absent | ANT | 3.12 | 25 | SYN |
| Mix C | 50 | 50 | IND | 50 | Absent | ANT | 6.25 | 6.25 | IND |
| <i>R. officinalis</i> | 50 | Absent | - | 25 | Absent | - | 0.195 | 6.25 | - |

2.2. Biofilm Viability Measured by MTT Analysis

The percentages of *S. aureus* and *S. epidermidis* biofilm treated during 24 h by several mixtures of *P. paniculata* and *R. officinalis* by MTT analysis is presented in Table 2. On *S. epidermidis*, all the extracts were effective in the reduction of biofilm and had a statistically significant difference compared to the control group ($p < 0.0001$). On *S. aureus*, all the extracts were effective in biofilm reduction, except for *P. paniculata* and Mix A 50 mg/mL. The groups Mix A, Mix B, and Mix C 100 mg/mL were the most effective in biofilm reduction, with a statistically significant difference compared to CHX 0.12%.

Table 2. Evolution of *S. aureus* and *S. epidermidis* biofilm treated during 24 h by several mix of *P. paniculata* and *R. officinalis* by MTT analysis. (Brain Heart Infusion (BHI), *P. paniculata* and *R. officinalis* 75% + 25% (Mix A), *P. paniculata* and *R. officinalis* 50% + 50% (Mix B), *P. paniculata* and *R. officinalis* 25% + 75% (Mix C), chlorhexidine digluconate 0.12% (CHX 0.12%), (-) reduction on biofilm, (+) growing of biofilm, no significant (ns), $p < 0.0332$ (*), $p < 0.0002$ (***), $p < 0.0001$ (****). For statistical analyses all treatments were compared with BHI group).

| Groups | <i>S. aureus</i> | | <i>S. epidermidis</i> | |
|---------------------------------|------------------|----------------|-----------------------|----------------|
| | % Evolution | <i>p</i> Value | % Evolution | <i>p</i> Value |
| Negative control (BHI) | +100 | | +100 | |
| <i>P. paniculata</i> 50 mg/mL | -19.20 | ns | -45.87 | **** |
| <i>P. paniculata</i> 100 mg/mL | -62.64 | **** | -76.64 | **** |
| <i>R. officinalis</i> 50 mg/mL | -46.63 | *** | -56.24 | **** |
| <i>R. officinalis</i> 100 mg/mL | -78.56 | **** | -80.11 | **** |
| Mix A 50 mg/mL | -18.46 | ns | -79.13 | **** |
| Mix A 100 mg/mL | -79.10 | **** | -79.13 | **** |
| Mix B 50 mg/mL | -34.95 | * | -78.38 | **** |
| Mix B 100 mg/mL | -82.68 | **** | -79.48 | **** |
| Mix C 50 mg/mL | -78.53 | **** | -77.65 | **** |
| Mix C 100 mg/mL | -80.77 | **** | -72.71 | **** |
| Positive control (CHX 0.12%) | -42.3 | **** | -74.9 | **** |

The reduction of *C. acnes* biofilm and heterotypic biofilms (in %) by MTT analysis after treatment by the groups for 24 h is shown in Table 3. On *C. acnes*, Mix A 50 and 100 mg/mL were the most effective in biofilm reduction, being statistically as effective as CHX 0.12%. On the cutaneous biofilm model (heterotypic biofilms), all the tested extracts were as effective as CHX 0.12% and had a statistically significant difference compared to

the Brain-Heart-Infusion (BHI) control group ($p < 0.0001$). The biofilm reduction varied from 88.73% to 93.50% with the tested extracts.

Table 3. Evolution of *C. acnes* biofilm and heterotypic biofilms (in %) by MTT analysis after treatment by the groups for 24 h. (Brain Heart Infusion (BHI), *P. paniculata* and *R. officinalis* 75% + 25% (Mix A), *P. paniculata* and *R. officinalis* 50% + 50% (Mix B), *P. paniculata* and *R. officinalis* 25% + 75% (Mix C), chlorhexidine digluconate 0.12% (CHX 0.12%), (–) reduction on biofilm, (+) growing of biofilm, no significant (ns), $p < 0.0332$ (*), $p < 0.0021$ (**), $p < 0.0002$ (***), $p < 0.0001$ (****). For statistical analyses all treatments were compared with BHI group).

| Groups | <i>C. acnes</i> | | Heterotypic Biofilms | |
|---------------------------------|-----------------|----------------|----------------------|----------------|
| | % Evolution | <i>p</i> Value | % Evolution | <i>p</i> Value |
| Negative control (BHI) | +100 | | +100 | |
| <i>P. paniculata</i> 50 mg/mL | +260.60 | **** | –88.73 | **** |
| <i>P. paniculata</i> 100 mg/mL | +51.32 | * | –91.78 | **** |
| <i>R. officinalis</i> 50 mg/mL | +17.97 | ns | –90.90 | **** |
| <i>R. officinalis</i> 100 mg/mL | +48.32 | ns | –92.82 | **** |
| Mix A 50 mg/mL | –46.33 | ns | –93.46 | **** |
| Mix A 100 mg/mL | –56.36 | * | –93.77 | **** |
| Mix B 50 mg/mL | +68.84 | *** | –92.77 | **** |
| Mix B 100 mg/mL | –36.26 | ns | –93.50 | **** |
| Mix C 50 mg/mL | –36.26 | ns | –92.78 | **** |
| Mix C 100 mg/mL | –47.31 | ns | –91.78 | **** |
| Positive control (CHX 0.12%) | –50.5 | ** | –85.8 | **** |

2.3. Scanning Electron Microscopy Analysis

It was found that Mix A promoted a great structural reduction of the heterotypic biofilm, leaving only a few cocci deposited on the polystyrene disk. On the other hand, Mix B obtained a greater number of cocci adhering to the surface, visually similar to that obtained by the 0.06% CHX group (positive control). Mix C exhibited high microbial growth (Figure 1).

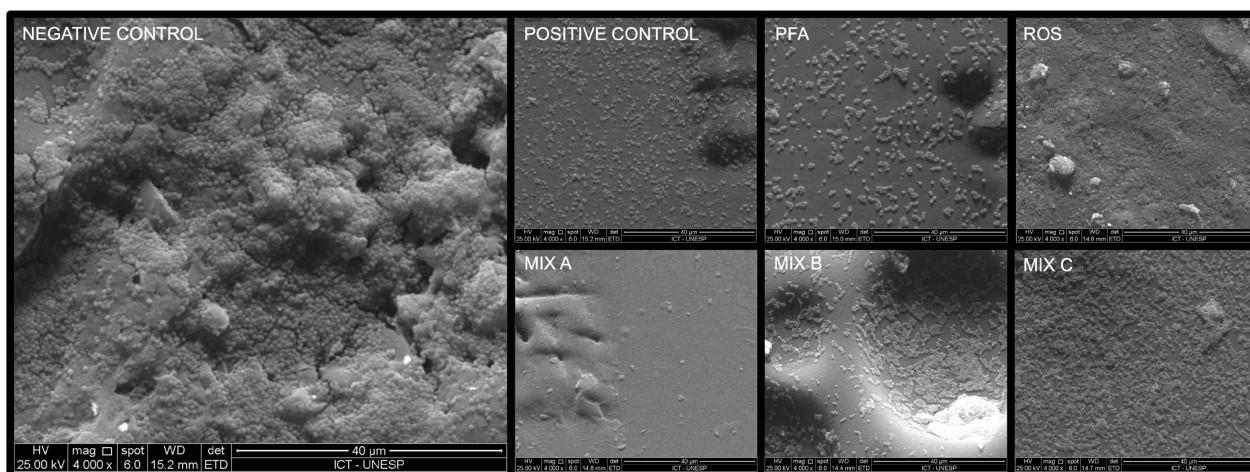


Figure 1. The microbial biofilms in scanning electron microscopy. (Brain heart infusion broth (Negative control), chlorhexidine digluconate 0.06% (Positive control), *P. paniculata* (PFA), *R. officinalis* (ROS), *P. paniculata* and *R. officinalis* 75% + 25% (Mix A), *P. paniculata* and *R. officinalis* 50% + 50% (Mix B), *P. paniculata* and *R. officinalis* 25% + 75% (Mix C). The micrographs were taken at a magnification of 4000×).

2.4. Cell Viability Measured by MTT Analysis

The application of the extracts on HaCaT resulted in percentages of viability greater than 50%. There was a statistically significant difference between the all the groups and the DMEM + 10% FBS control group ($p < 0.05$). In addition, the *R. officinalis* 100 mg/mL group was as biocompatible as the CHX group ($p < 0.05$). The CHX group promoted a 14.6% cell viability applied for 24 h, resulting in greater cytotoxicity with a statistically significant difference compared to all the tested extracts ($p < 0.05$) (Table 4).

Table 4. Cellular viability of human keratinocytes (HaCaT) (in %) by MTT analysis after treatment by the groups for 24 h. (*P. paniculata* and *R. officinalis* 75% + 25% (Mix A), *P. paniculata* and *R. officinalis* 50% + 50% (Mix B), *P. paniculata* and *R. officinalis* 25% + 75% (Mix C), chlorhexidine digluconate 0.12% (CHX 0.12%), $p < 0.0001$ (***). For statistical analyses all treatments were compared with negative control group).

| Groups | % Viability | <i>p</i> Value |
|-----------------------------------|-------------|----------------|
| Negative control (culture medium) | 100 | |
| <i>P. paniculata</i> 50 mg/mL | 70.3 | **** |
| <i>P. paniculata</i> 100 mg/mL | 60.1 | **** |
| <i>R. officinalis</i> 50 mg/mL | 91.3 | **** |
| <i>R. officinalis</i> 100 mg/mL | 60.1 | **** |
| Mix A 50 mg/mL | 70.8 | **** |
| Mix A 100 mg/mL | 59.7 | **** |
| Mix B 50 mg/mL | 68.8 | **** |
| Mix B 100 mg/mL | 59.7 | **** |
| Mix C 50 mg/mL | 65.9 | **** |
| Mix C 100 mg/mL | 59.1 | **** |
| Positive control (CHX 0.06%) | 14.6 | **** |

3. Discussion

Anaerobic bacteria normally reside on human skin, in the oral cavity, and in the upper respiratory tract, and their removal during surgical procedures is a challenge. In the literature, Foster et al. [17] and Dörfel et al. [18] have proved that bacteria such as *C. acnes* and *S. aureus* have presented high levels of resistance to CHX, so the creation of new antiseptics for prolonged preparations of human skin with agents capable of penetrating deeper is necessary to access mainly *C. acnes*, which may be located within the hair follicles and the pilosebaceous glands. This is the main bacterium responsible for causing surgical site infections, which is why we emphasize that the present work has identified new therapeutic options, where the mixture of extracts was able to promote reductions in the resistance structure of microorganisms (biofilm) with similar and even better results than the conventional antiseptic (CHX) in in vitro tests.

The antimicrobial and antibiofilm actions of the glycolic extracts of *R. officinalis* L., *P. paniculata*, and their combinations were tested in this study on planktonic and biofilm (monotypic and heterotypic) cultures of *S. aureus*, *S. epidermidis*, and *C. acnes*, where it was found that these extracts are effective. In addition, the same extracts were biocompatible with HaCaT without a statistically significant difference compared to the control group (DMEM + 10% FBS). Therefore, the null hypothesis of this study must be rejected.

To the best of our knowledge, this is the first study that evaluated the combined antimicrobial and antibiofilm actions of the glycolic extracts of *R. officinalis* L. and *P. paniculata* on *S. aureus* cultures, in which 100 mg/mL Mix B and Mix C resulted in a biofilm reduction of 82.68 and 80.77%, respectively. In the literature, Manilal et al. [19] evaluated the antimicrobial actions of the ethanolic, methanolic, and aceto-ethylic extracts of 100 mg/mL

R. officinalis in planktonic and biofilm cultures of methicillin-resistant *S. aureus*, where the biofilm reduction varied from 71.4 to 85.7%. In the present study, the biofilm reduction of *S. aureus* cultures was of 78.56% with 100 mg/mL *R. officinalis* glycolic extract. In addition, the antimicrobial action of the glycolic extract of *R. officinalis* was tested on monomicrobial biofilms of *Candida albicans*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, and *Pseudomonas aeruginosa* and on polymicrobial biofilms composed of *C. albicans* with each bacterium, where it was found that the extract at the concentration of 200 mg/mL was effective in reducing both monotypic and heterotypic biofilms [20].

In another study, the antimicrobial action of *R. officinalis* was tested on 29 aerobic and anaerobic bacteria and yeasts in the agar dilution test in which the extract was found to be effective on a variety of microorganisms, indicating its topic use for dermatologic disorders [21]. In this context, the essential oil of *R. officinalis* was effective on *C. acnes* by reducing the length, width, and height of the biofilm and altering the morphology of *C. acnes*, resulting in its death [22]. In the present study, *R. officinalis* when combined with *P. paniculata* in Mix B and Mix C was as effective as 0.12% CHX on *C. acnes*.

Moreover, the antimicrobial action of the aqueous extract of *R. officinalis* was effective over 34 aerobic and anaerobic bacteria, including *S. epidermidis*. In addition, the same study suggested the combination of *R. officinalis* aqueous extract with other herbal extracts like *Salvia rosmarinus*, *Salvia lavandulifolia*, and *Origan compactum* [23]. In the present study, the glycolic extract of *R. officinalis* at 100 mg/mL was the most effective group on *S. epidermidis*.

The glycolic extract of *P. paniculata* demonstrated antimicrobial action, promoting a great reduction in the heterotypic biofilm of *S. aureus*, *S. epidermidis*, and *C. acnes*, where the extract promoted a reduction of 91.78% of the biofilm structure that mimics the human skin microbiota. To the best of our knowledge, there are no similar studies in the literature to compare the results. However, Ramos et al. [16] demonstrated that the extract of *P. paniculata* promotes antimicrobial action against *Klebsiella pneumoniae* by reducing planktonic and biofilm cultures of the microorganism.

The application of the extracts on HaCaT resulted in percentages of viability greater than 50%. There was a statistically significant difference between the all the groups and the DMEM + 10% FBS control group ($p < 0.05$). In addition, the *R. officinalis* 100 mg/mL group was as biocompatible as the CHX group ($p < 0.05$). To the best of our knowledge, there are no studies that have evaluated the biocompatibility of *R. officinalis* L. and *P. paniculata* glycolic extracts on human keratinocytes; however, the biocompatibility of *R. officinalis* L. has been proven on a variety of human cells [15,24].

Finally, the isolated and combined extracts of *P. paniculata* and *R. officinalis* had antimicrobial effects on the planktonic and biofilm cultures of microorganisms from the skin microbiota, highlighting that the combinations of extracts promoted greater antimicrobial efficacy on monotypic and heterotypic biofilms, presenting similarly to, or even superiorly to, those obtained with CHX. It is also important to highlight that the three mixtures of extracts (Mix A, Mix B, and Mix C) promoted reductions of more than 90% on heterotypic biofilms of *S. aureus*, *S. epidermidis*, and *C. acnes*. This proves that it is a possible therapeutic option, exhibiting an antimicrobial effect on the human epithelial microbiota with low cytotoxic activity on human keratinocytes, thus being a new alternative to be explored in the asepsis of human skin.

4. Materials and Methods

4.1. Bacterial Strains and Plants Extracts

The reference strains of American Type Culture Collection of *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), and *Cutibacterium acnes* (ATCC 6919) were used in microbiological analysis. A bacterial inoculum of each strain was prepared and adjusted by a spectrophotometer (Visible Spectrophotometer V-5000, Shanghai Metash Instruments Co., Ltd., Shanghai, China) in saline solution (NaCl 0.9%) (LGC Biotechnology®, Cotia, São Paulo, Brazil) at 1×10^6 CFU/mL for MIC test and 1×10^8 CFU/mL for biofilm test.

The extract of *Pfaffia paniculata* (Mapric Greentech company[®], São Paulo, Brazil) was collected from the plant roots, and the extract of *Rosmarinus officinalis* (Mapric Greentech Company[®], São Paulo, Brazil) was collected from the plant leaves. Both extracts were obtained commercially, diluted in propylene glycol in concentration of 20% (200 mg/mL).

4.2. Determination of Minimum Inhibitory and Minimum Bactericidal Concentration

Clinical and Laboratory Standards Institute (CLSI) protocol M7-A6 was followed to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using the microdilution broth method. The extracts *P. paniculata*; *R. officinalis*; 75% *P. paniculata* + 25% *R. officinalis* (Mix A); 50% *P. paniculata* + 50% *R. officinalis* (Mix B); and 25% *P. paniculata* + 75% *R. officinalis* (Mix C) were used in these analyses. In 96-well plates (TPP, Zollstrasse, Switzerland), 100 µL of Mueller Hinton Broth (Himedia[®], Mumbai, India) was applied in each well, for a total of $n = 10$ for each group. Then, ten serial dilutions of 100 µL of each extract in the first well of the corresponding line of each plate were performed. The inoculum was prepared using microbial cultures with 24 h of incubation, using the following parameters: $\lambda = 490$ nm (*S. aureus* and *S. epidermidis*) with optical density (OD) = 0.374 ± 0.020 for both bacteria and $\lambda = 600$ nm and an optical density (OD) = 0.100 ± 0.020 for *C. acnes*. Then, 100 µL/well of each inoculum was added, respecting a different plate for each bacterial strain, for a total of 3 plates. All plates were incubated at 37 °C for 24 h (in anaerobic conditions for *C. acnes*). The MIC of each extract was determined considering the lowest concentration extract well that inhibited 50% of bacterial growth. For MBC determination, 10 µL of each well was inoculated in BHI agar (Himedia[®], Mumbai, India) and incubated for 48 h at 37 °C (in anaerobic conditions for *C. acnes*). The lowest seeded concentration extract that did not show growth on solid medium was determined as the MBC.

In addition, the FIC was calculated using the formula $FIC_{index} = FIC_A + FIC_B = (\text{MIC of extract A in combination} / \text{MIC of extract A alone}) + (\text{MIC of extract B in combination} / \text{MIC of extract B alone})$.

4.3. Monotypic and Cutaneous Model Biofilm

The bacterial inoculums were prepared again, following the same parameters described above, but using a concentration of 10^8 CFU/mL, to form monotypic and heterotypic biofilms (cutaneous model biofilm). For monotypic biofilm, 100 µL of each respected bacterial inoculum and 100 µL of BHI broth (Himedia, Mumbai, India) were added to form biofilms in 3 days in 96-well plates (TPP, Zurich, Switzerland), using 8 wells from each line ($n = 8$) at 37 °C (in anaerobic conditions for *C. acnes*). For cutaneous biofilm model, 33.3 µL of each of the respective bacterial inoculum, totaling 100 µL of the three inoculums, and 100 µL of BHI broth (Himedia[®], Mumbai, India) were added to form biofilms in 3 days in 96-well plates (TPP, Zollstrasse, Switzerland) using 8 wells from each line ($n = 8$) at 37 °C. The BHI broth (Himedia[®], Mumbai, India) was changed every 24 h. Later, the biofilms were treated with two concentrations (50 and 100 mg/mL) of *R. officinalis*, *P. paniculata*, Mix A, Mix B, and Mix X for 24 h. In this analysis, BHI (Himedia[®], Mumbai, India) and 0.12% CHX (Colgate-Palmolive[®], New York, NY, USA) were used as control groups. Subsequently, the wells' contents were discarded, and the biofilms were washed with sterile saline solution (LGC Biotechnology[®], Cotia, São Paulo, Brazil) 3 times to remove dead cells.

4.4. Bacterial Viability by MTT Analysis

After the treatments, 100 µL of MTT solution 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (CAS n^o: 298-93-1, purity: 97.5%, Sigma-Aldrich[®], St. Louis, MO, USA) was dispensed into each well of the plates, with subsequent incubation for 1 h at 37 °C (in anaerobic conditions for *C. acnes* monotypic and heterotypic biofilms). Then, MTT solution was discarded, and 100 µL of dimethyl sulfoxide (DMSO) (CAS n^o: 67-68-5, purity: 99.9%, Sigma-Aldrich[®], St. Louis, MO, USA) was added. The plates were incubated again for 10 min at 37 °C (in anaerobic conditions for *C. acnes* monotypic and heterotypic

biofilms) and agitated by Shaker (Miulab[®], Micro plate shaker MIX-1500, Hangzhou, China) for 10 min. Lastly, the plates were read at 570 nm on spectrophotometer (Lonza Biotek[®], ELX808LBS, Winooski, VT, USA) to obtain the optical densities (OD) that were later converted into percentage of viability using the formula:

$$\% \text{ Viability} = (\text{OD Treated Group} \times 100) / \text{Mean OD Control Group}$$

4.5. Antimicrobial Analysis by Scanning Electron Microscopy

This analysis was carried out over heterotypic biofilms, which formed again as explained above, over polystyrene discs measuring 1 cm in radius and 0.5 cm in thickness deposited within 24 well plates (TPP, Zurich, Switzerland) ($n = 2$ for each experimental group). The treatments were applied for 24 h, and then the extracts were discarded and the biofilms were washed with sterile saline solution 0.9% (LGC Biotechnology[®], Cotia, São Paulo, Brazil) with subsequent fixation with methanol (CAS n^o: 67-56-1, purity: 99.8% Synth[®], Diadema, Brazil) for 1 h. Then, the samples were gradually dehydrated with ethanol (CAS n^o: 64-17-5, purity: 99.5%, Synth[®], Diadema, Brazil) 10%, 25%, 50%, 75%, and 100% concentrations, and incubated at 37 °C for 24 h. The polystyrene discs were placed in aluminum stubs and covered with gold for 120 s at 40 mA (Quorum Technologies[®], Emitech—SC7620, Kent, UK). Finally, the biofilms were analyzed by scanning electron microscope (FEI—Inspect S50, Hillsboro, OR, USA) in 4000 \times .

4.6. Human Keratinocytes Viability by MTT Analysis

The cells (HaCaT) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (LGC Biotechnology, Cotia, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, New York, NY, USA), incubated at 37 °C, with atmospheric humidity, with 5% CO₂ using cell culture flasks (TPP, Zurich, Switzerland). After reaching subconfluence, which is characterized by 70% occupation of the cell culture flask, the cells were removed from the floor using trypsin (Sigma-Aldrich[®], St. Louis, MO, USA) and sent for cell counting. The cytotoxicity was evaluated using a concentration of 4×10^4 cell/well in 96-well plates (TPP, Zurich, Switzerland) incubated for 24 h for cellular adherence. The cells were treated with two concentrations (100 and 50 mg/mL) of *P. paniculata*, *R. officinalis*, Mix A, Mix B, and Mix C for 24 h. DMEM (LGC Biotechnology, Cotia, São Paulo, Brazil) + 10% FBS (Invitrogen, New York, USA) and 0.06% CHX (Colgate-Palmolive[®], New York, NY, USA) were used as control groups. Then, 200 μ L of MTT solution (CAS n^o:298-93-1, purity: 97.5%, Sigma-Aldrich[®], St. Louis, MO, USA) was added to each well and the plates were incubated for 1 h at 37 °C covered from light. Then, the solution was removed and 200 μ L of DMSO (CAS n^o: 67-68-5, purity: 99.9%, Sigma-Aldrich[®], St. Louis, MO, USA) was added for 10 min at 37 °C and agitated by shaker (Miulab[®], Micro plate shaker MIX-1500, Hangzhou, China) for 10 min. Lastly, the plates were read at 570 nm on spectrophotometer (Lonza Biotek[®], ELX808LBS, Winooski, VT, USA) to obtain the optical densities that were later converted into percentage of viability using the formula:

$$\% \text{ Viability} = (\text{OD Treated Group} \times 100) / \text{Mean OD Control Group}$$

4.7. Statistical Analysis

Data were normally distributed by the D'Agostino, Shapiro-Wilk and Kolmogorov-Smirnov tests. They were statistically analyzed by the one-way ANOVA method complemented by the Tukey test, with a significance levels: $p < 0.0332$ (*), $p < 0.0021$ (**), $p < 0.0002$ (***), $p < 0.0001$ (****). Statistical analysis was carried out using GraphPad Prism 9.0 software.

5. Conclusions

The results of this study demonstrated that the combined *P. paniculata* and *R. officinalis* glycolic extracts have antimicrobial and antibiofilm effects on *S. aureus*, *S. epidermidis*, and *C. acnes*. In addition, these extracts are biocompatible against human keratinocytes.

Therefore, these extracts represent an important and promising natural alternative in the fight against healthcare-associated infections.

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