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Photodynamic Therapy With Propolis: Antibacterial Effects on *Staphylococcus aureus, Streptococcus mutans* and Escherichia coli Analysed by Atomic Force Microscopy

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Abstract

Introduction: Photodynamic therapy (PDT) is a process that uses a light source (e.g. laser), oxygen molecules and a photosensitizing agent. PDT aims to act against pathogens, including those resistant to antimicrobials. The association of PDT with natural drugs, such as Propolis, has not been widely studied.

Methods: Therefore, this study aimed to evaluate the antimicrobial effect of PDT *in vitro* by using Propolis as a photosensitizing agent. For this purpose, the dry Propolis extract was used as a photosensitizer and a low-power laser (Photon Laser III model) was irradiated onto the microwells for 90 seconds. Gram-positive and Gram-negative bacterial strains were used in the tests at a concentration of 5×10^5 CFU/mL. Initially, the antibacterial activity of the photosensitizers without laser action was determined by using a serial microdilution method before the experiment with a laser. After the incubation of the plates in a bacteriological oven, resazurin (0.1%) was added and the minimum inhibitory concentration (MIC) was determined. Alterations in the morphology of the bacteria were analysed by using atomic force microscopy (AFM).

Results: Bacteria were sensitive to Propolis with MICs ranging from 13.75 to 0.85 mg/mL, but no susceptibility was observed for methylene blue without laser application. A change was observed for MIC values of Propolis against *Staphylococcus aureus* after irradiation, which decreased from 1.71 mg/mL to 0.85 mg/mL. However, this behaviour was not observed in *Escherichia coli*, the only gram-negative strain used. In addition, AFM images revealed alterations in the size of one of the bacteria tested.

Conclusion: The Propolis is more active against gram-positive bacteria and PDT improved its activity against one of the strains tested.

Keywords: Photodynamic therapy; Propolis; Antimicrobial activity.

Introduction

Photodynamic therapy (PDT) is characterized by the use of a photosensitizer agent that produces reactive oxygen species (ROS) as a result of laser application and oxygen molecules.[1,2] More specifically, the therapeutic effect of PDT is achieved with the formation of singlet oxygen and free radicals after laser irradiation, which results in activity against a great number of pathogens, including antimicrobial-resistant microorganisms.[3]

PDT can be used for a vast number of applications, such as the treatment of malignant tumours and fungal infections, and tissue repair and healing, and it can also be used as an antimicrobial agent. In cancer treatment, for example, the use of PDT has the advantage of targeting the damaged tissue only, thus preserving the surrounding cells, attenuating inflammatory factors and increasing apoptosis.[4,5]

Colon cancer noticeably requires new treatment alternatives, particularly in cases of metastasis. In this regard, PDT has been considered an applicable method.

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Thus, the development of multifunctional photosynthetic approaches involving double or triple therapies could be an option to minimize such characteristics.[6] Some advantages can be mentioned regarding the use of PDT for antimicrobial applications, including the fact that bacterial cell death can occur faster, and consequently, the use of chemical agents at high concentrations for a long period of time is no longer required.[7]

In this context, natural products have already proven to be effective for the treatment of some diseases since adverse effects on normal cells are milder than those observed with other drugs.[8] In light of this, Propolis has been extensively studied as it presents several properties of interest for the scientific community. The most important properties of Propolis include antioxidant, antimicrobial, anti-parasitic, cytotoxic (including tumour cell apoptosis and antiproliferative effects)[8] and immune-stimulating activities.[9,10]

Several studies have highlighted the antimicrobial potential of Propolis. Literature shows that Propolis has an effect against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Lactobacillus acidophilus,* and *Enterococcus faecalis*.[10,11] In addition to these strains, *Streptococcus mutans* as well as *Staphylococcus epidermidis*, *Escherichia coli, and Salmonella typhimurium* have also shown susceptibility to this product.[12]

Given the extensive evidence of the antimicrobial activity of Propolis, along with the applicability of PDT in the medical area, this association was used as an investigation model in the present study. Thus, our aim was to evaluate the antimicrobial effect of PDT by using Propolis as a photosensitizing agent.

Materials and Methods

Photosensitizer

The dried alcoholic extract of Propolis (22%) was used as a photosensitizer. The Propolis extract was obtained in rural areas located in the state of Minas Gerais and kindly supplied by Mel Milagres LTDA (CNPJ 00870513000153).

Laser

In PDT, a low-power laser with an adjusted wavelength and energy is required. Photon Laser III was used with Propolis and dyes for the photosensitizing process. This equipment has an active laser medium of gallium arsenide and aluminium (GaAlAs) and operates at 660 nm, which is the wavelength corresponding to the high absorption length for these photosensitizers.

The laser power was set to 100 mW, which was measured before each experiment. The area of the plate to be irradiated was previously determined and the laser was applied on a continuous basis for 90 seconds, with the laser aperture over the microwells. The irradiation was carried out within a laminar airflow cabinet and in the dark.

Preparation of Microorganisms and Determination of Minimum Inhibitory Concentration

Bacterial strains (*S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *S. mutans* ATCC 25175) were previously cultured in a bacteriological incubator according to the protocol suggested by the Clinical and Laboratory Standards Institute in 2015. From the grown colonies, standardized suspensions were prepared in sterile saline solution (NaCl 0.85% w/v) to obtain absorbance between 0.08 and 0.13 at a wavelength of 625 nm, corresponding to the McFarland scale (1-2 × 10^8 CFU/mL) and to the recommendations by the CLSI in 2015.

Before preparing the bacterial inoculum, the microorganism suspension was diluted in Mueller-Hinton broth or TSB (*S. mutans* ATCC 25175) to obtain a bacterial concentration of 5 × 10^5 CFU/mL. Minimum inhibitory concentration (MIC) values for the photosensitizer (i.e. Propolis), the laser (90 seconds) and the photosensitizer associated with the laser (90 seconds) were determined according to the CLSI.

Initially, the antibacterial activity of the photosensitizers without the laser action was determined. For this purpose, these substances were submitted to two-fold serial dilutions in which the concentrations of Propolis varied from 110 to 0.05 mg/mL and of methylene blue varied from 0.01 to 0.000004%. The experiment was performed in triplicate and the microplates were placed in a bacteriological incubator at 37 ºC for 24 hours. After this period, the results were observed with the addition of resazurin (0.1%), followed by incubation at 37 ºC for 20 minutes. The dye colour turns from blue to pink based on the microorganism viability, and MIC is defined as being the lowest concentration allowing the suppression of microbial growth.

Analysis of the Antibacterial Effect by Using Atomic Force Microscopy

Atomic force microscopy (AFM) was used for imaging, and the sub-MIC concentrations in the treatments with Propolis, Propolis + laser and bacteria growth control were used to observe possible alterations in the bacterial cell morphology. To this end, the same procedure performed before MIC determination was reproduced with an inoculum of 1 × 10^5 CFU/mL. For this analysis, the samples were treated as previously described.[13]

The images were produced with the aid of an atomic force microscope (TT-AFM Workshop, USA) operating in the intermittent-contact mode, approximately 248 kHz and using TAP300-G10 (TED PELLA, Inc) probes. The software Gwyddion 2.45 was used to treat the images obtained. Multiple areas of each sample were examined in order to analyse the mean height of the treated and untreated bacteria. Statistical analysis of the results was performed with the software GraphPad Prism 7.01, in which the mean height of the strains was determined by using one-way ANOVA with Tukey post-test (n = 22

samples). Statistical significance was considered for $P<0.05$ and the results were expressed as mean ± minimum significant difference (MSD) .

Results

Determination of Minimum Inhibitory Concentration

The *in vitro* sensitivity of the bacterial strains *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) and *S. mutans* (ATCC 25175) was assessed by determining the MIC in the different groups. In this sense, the MIC was considered as being the lowest concentration of the photosensitizer capable of inhibiting bacterial growth. The results were interpreted by inspecting the wells visually. It was observed that the values of MIC varied from 0.85 to 1.71 mg/mL, as shown in Table 1.

Without the application of the laser, methylene blue had no activity against the selected bacteria at the concentrations tested. The laser-treated group had no activity against bacteria. On the other hand, Propolis showed antibacterial activity against all the strains used, where *S. aureus* (ATCC 29213) and *S. mutans* (ATCC 25175) were the most susceptible species with a MIC of 1.71 mg/mL.

After the determination of MIC for the agents without laser irradiation, the same assay was performed to determine the effect of the laser on this activity. It was verified that the MIC of Propolis against *S. aureus* (ATCC 29213) changed after laser irradiation for 90 seconds. This behaviour, however, was not observed in the other strains *E. coli* (ATCC 25922) and *S. mutans* (ATCC 25175). These results are shown in Table 2.

In Table 2, we can observe that the MIC found in methylene blue against *S. aureus* after 90-second irradiation was 0.005%. On the other hand, *E. coli* showed no activity change after laser irradiation as there was no interference with the bacterium development. The same finding was observed for *S. mutans*. The action of laser irradiation alone was also investigated and the results showed that it had no antibacterial activity against the strains used in this study.

Table 1. The Minimum Inhibitory Concentration of the Photosensitizer Towards the Tested Bacteria.

Bacterial Strains	Methylene Blue	Propolis
S. aureus (ATCC 29213)	-	1.71
E. coli (ATCC 25922)	-	13.75
S. mutans (ATCC 25175)	-	1.71

- No inhibitory activity was detected.
Results are expressed in mg/mL.

Table 2. The minimum inhibitory concentration of the photosensitizing agents against the bacteria after laser irradiation. Results are expressed in mg/mL.

Bacterial Strains	Methylene Blue and LASER	Propolis and LASER
Staphylococcus aureus	0.005	0.85
Escherichia coli	-	13.75
Streptococcus mutans	-	1.71

- No inhibitory activity was detected

Atomic Force Microscopy Analysis

Once the association between Propolis and laser irradiation was capable of inhibiting the growth of *S. aureus* (ATCC 29213), this strain was selected for AFM imaging. For that purpose, the contents of the microwells corresponding to the sub-MIC concentrations of Propolis and MIC of Propolis + laser were submitted to AFM analysis, including growth in the control group.

As shown in Figure 1, the growth of *S. aureus* (ATCC 29213) compared to the control group (Figure 1A) presented a behaviour characterizing this strain as gram-positive bacteria. On the other hand, bacteria treated with Propolis showed similar morphology, but it is possible to observe in Figure 1B that this group had an increased height (1.5 µm) compared to the control group (1.13 µm). The bacteria treated with Propolis + laser (Figure 1C) presented a similar behaviour, although the height found in this group was 1.4 µm. In the face of this information, one can conclude that both treatments (i.e. Propolis alone and Propolis + laser) were able to interfere with the bacteria morphology as there was a change in their size, as shown in Figure 1D.

These observations can also be evidenced by the topographic images of the treatment groups, which demonstrated changes.

Discussion

Propolis has been extensively investigated as it exhibits several properties of interest in the scientific community. Its potential has been primarily described in relation to its antioxidant, antimicrobial and cytotoxic activities.[14] In this study, the antibacterial activity of Propolis was reiterated with an effect on the three bacterial strains used. Based on the MIC determination, it was possible to observe that Propolis inhibited bacterial growth at concentrations varying from 13.75 to 0.85 mg/mL.

In addition, *S. aureus* also showed sensitivity to Propolis in this study. These results are corroborated by the literature, demonstrating that Propolis has a good antibacterial activity against this species.[13] Similarly, the use of three methicillin-resistant species and one sensible species demonstrated that green Propolis was able to inhibit the growth of these bacteria.[14] In fact, the Propolis used in this study was also able to inhibit *S. mutans* growth. These findings are in agreement with the effects observed elsewhere, showing that this product can also inhibit the growth of *S. mutans*.[10]

These results might be associated with the presence of flavonoids and other aromatic compounds in the extract, which act on the cell wall of gram-positive bacteria.[13] However, methylene blue did not inhibit the growth

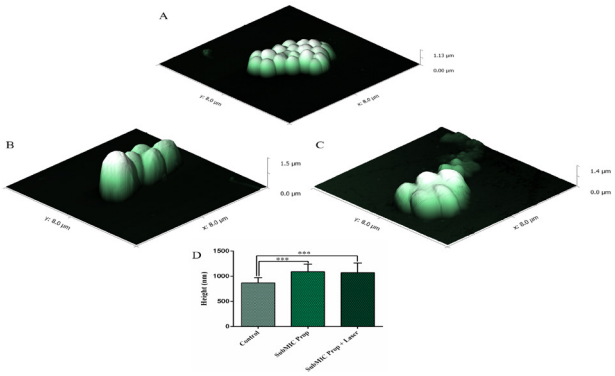

Figure 1. Three-Dimensional AFM Images of *S. aureus* (ATCC 29213). (A) Control, with bacteria treated with Propolis at subMIC concentration; (B) bacteria treated with Propolis; (C) bacteria treated with Propolis + laser at MIC concentration. All images show scale bars (X and Y) of 8 µm length at a resolution of 512 pixels;(D) Bar graph shows mean heights with results expressed as mean ± MSD and $P<0.0001$ (***) which was considered statistically significant.

of the above-mentioned strains, even at the highest concentration used. This finding is also in agreement with the literature as antibacterial activity was found in methylene blue after laser application.[15,16] In addition, the use of the isolated laser also had no activity against bacteria since the photosensitizer is an essential element of PDT. In this context, for the laser wavelength to be absorbed it is necessary to have the photosensitizer, which accumulates in pathological tissues, leading to the destruction of the inappropriate cells.[17]

Based on the results obtained, the influence of the laser on the antibacterial activity of Propolis was investigated. It was observed that laser application only improved the antibacterial activity of Propolis against *S. aureus* (ATCC 29213), as well as that of methylene blue against the same strain. Therefore, one can conclude that the light source was able to influence the activity of the photosensitizing agent against *S. aureus* (ATCC 29213).

Staphylococcus aureus is frequently associated with several human infections, including cutaneous (e.g. simple folliculitis, impetigo, boils and carbuncles) and surgical wound infections.[18] Therefore, the effect obtained by associating Propolis and the laser demonstrates that this method is efficient in inhibiting the growth of this important bacterial species involved in several infectious processes.

No changes in MIC were observed for the activity of Propolis against *S. mutans* (ATCC 25175) after laser application, which maintained the values previously found. The same behaviour was observed in *E. coli* (ATCC 25922) as this species was more resistant to the activity of Propolis, not only when the extract was used alone, but also when it was associated with the laser.

The antibacterial activity of Propolis against *E. coli* has already been reported elsewhere,[12] although the methods used were different from those applied in this study. However, the literature reports the effect of this product against this species. Due to their cell wall structure, which is more complex than the one found in gram-positive bacteria, gram-negative species are usually more resistant to the action of antibiotics. In fact, antibiotics may not be able to go beyond the lipid bilayer, which can explain the lower susceptibility of this species to Propolis.[19]

This resistance presented by gram-negative bacteria to the action of Propolis has been attributed to the high lipid content and complexity of the cell wall formed by the presence of differentiated lipopolysaccharide substances, which ensures bacterial antigenicity and pathogenicity.[19]

In addition, to make an antimicrobial PDT, it is essential that the PS penetrate the cell walls of bacteria and enter the plasma membrane or cytoplasm; however, as gram-negative bacteria, such as *E. coli*, limit the simple diffusion of the PS in the cytosol due to its membrane barriers, it is more difficult to obtain a potential PS to mediate a PDT of gram-negative bacteria.[20] The result found in this study is in agreement with the literature that demonstrates that Gram-negative bacteria are more resistant to PDT.[21,22]

As the effect of Propolis on *S. aureus* improved after laser application for 90 seconds, this species was selected for AFM analysis in order to assess the morphological alterations that could have occurred after exposure to the treatments. For this purpose, the treatments with Propolis alone and Propolis with a laser were considered.

Figures 1 and 2 allow the observation of changes in bacterial size compared to the control group. The group treated with only Propolis showed significantly higher height than the control group. Similarly, the group treated with Propolis + laser also showed statistically significant changes compared to the control group, with a mean height of 1.4 µm. These results show that the antibacterial effect of Propolis on *S. aureus* was achieved by altering the morphology of the bacteria, that is to say changing their size.

In this study, the morphological alterations in the same species promoted by the ethanolic extract of Propolis in the function of time have also been observed by using AFM analysis.[20] These analyses revealed that after four

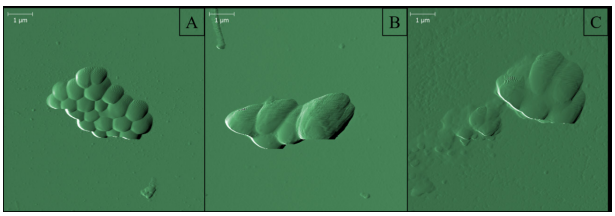

Figure 2. Two-Dimensional AFM Images of *S. aureus* (ATCC 29213). (A) Control, with bacteria treated with Propolis at subMIC concentration; (B) bacteria treated with Propolis; (C) bacteria treated with Propolis+ laser at MIC concentration. All images show scale bars (X and Y) of 8 µm length at a resolution of 512 pixels.

hours of exposure to the product, the bacteria showed slight differences in their size. After 12 hours in contact with the extract, the microorganisms exhibited increased volume, which, according to the authors, may have occurred due to the loss of morphological characteristics and the presence of lysis.[23]

After treatment with Propolis extract, *S. aureus* strains were isolated from bovine mastitis for analysis using the same method[24] which evidenced the occurrence of morphological and size modifications. It is worth mentioning that the alterations promoted by both treatments performed in the present study indicate that Propolis has an effect on the size of the bacteria. It is also important to highlight that these analyses were carried out over 24 hours of contact with Propolis alone and Propolis with laser application. The Propolis used in this study is a commercial product and thus its composition is different from that of extracts used in the above-mentioned works.

The obtained results revealed that Propolis can be used as a photosensitizing agent in PDT for the treatment of infectious agents, including *S. aureus* (ATCC 29213), which presented susceptibility to the method used here. According to the results obtained with AFM, it was also possible to infer that the antibacterial effect of this product is associated with changes in bacterial size.

These observations point to the necessity of performing more studies with gram-positive bacteria and gram-negative species in order to demonstrate the mechanism behind such alterations. In addition, the method tested in the present study should be further investigated to demonstrate the effectiveness of natural products like Propolis for the treatment of diseases contending other infectious agents.

Conclusion

Based on the results reported here, it is possible to conclude that Propolis is more active against Gram-positive bacteria and PDT improved its activity against one of the strains tested. It was also possible to observe that *S. aureus* (ATCC 29213) was susceptible to the applied method, whereas (*E. coli* 25922) was the most resistant strain. This resistance was observed in both treatments, that say Propolis alone and Propolis associated with the application of the laser.

Ethical Considerations

Not applicable.

Conflict of Interests

The authors declare no conflict of interest.

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