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RESEARCH ARTICLE OPEN ACCESS

EVALUATION OF ANTIMICROBIAL ACTIVITY AND TOXICITY OF THE PROPOLIS AND CHRYSIN AND P-COUMARIC ACID COMPOUNDS

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ABSTRACT

Introduction: In recent years, research involving natural products has been the target of many investigations in search of new drugs to help treat infections. **Objective**: In the present study, we used *in vitro* assays to evaluate the antimicrobial activity by microdilution and to determine the minimum inhibitory concentration (MIC) of the green propolis extract and two of its isolated compounds - p-coumaric acid and chrysin. **Methods**: These substances were assessed in isolation and associated with each other and standard antimicrobials against *Escherichia coli* and *Staphylococcus aureus* ATCC strains. Cytolytic and hemolytic assays were also performed to verify the cytotoxicity of these compounds. **Results**: The results showed that propolis and p-coumaric acid presented antimicrobial activity against the *Staphylococcus aureus* ATCC strain, registering MIC of 190 µg/mL and MIC of 250 µg/mL, respectively.As for the toxicity evaluation, both propolis and isolated compounds did not present a cytolytic effect against the leukocytes or nor hemolytic against the positive human "O" blood cells. **Conclusion**: It can be verified that propolis and p-coumaric acidpresented good potential as an antimicrobial agent and with low toxicity, being a possible alternative in the treatment of infections caused by *Staphylococcus aureus.*

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INTRODUCTION

Antibiotic therapy is indispensable for infection treatments, and several antibiotics are used to avoid late systemic response's consequences. However, to achieve effectiveness, the appropriate therapeutic serum levels of the medicinal products, their absorption, bioavailability and the metabolic pathways involved to avoid toxic concentrations must be evaluated. The indiscriminate use of antimicrobials, however, has led to the emergence of multi-resistant strains revealing a severe worldwide public health problem. The World Health Organization (WHO) has classified antibiotic resistance as one of the three major threats to human health (Mancuso *et al*., 2021). Although there is a remarkable diversity of antimicrobials, it is necessary to seek new alternatives for the treatment and the control of different infections to minimize their deleterious consequences. Currently, an important strategy to overcome this resistance is the use of combination drugs. Other studies associate extracts and natural compounds with standard antimicrobials and some of these products have shown significant synergistic response against invading microorganisms. Thus, experimental therapies with natural products have been the subject of research to understand the pathophysiology of some types of diseases and reduce clinical manifestations in patients (Enioutina *et al*., 2020). From the ethnobotanical point of view, Brazil is considered one of the wealthiest countries. Besides having a great cultural diversity, it has several types of traditional communities and ethnic groups, such as the indigenous ones. Thesecommunities come over time using natural products like medicines, tonics, sedatives, diuretics, carminatives, and others. The combination of culture and rituals of some of these communities eventually contributed to folk medicine (Mohammadhosseini*et al*., 2017). Nature with its biological diversity provides these types of products that have been shown to be effective in the treatment of infections caused by viruses, bacteria, and fungi, and also as

therapeutic support for multiple organs and systems of the body (Bittner *et al*., 2021; Llivisaca-Contreras *et al*., 2021). In this way, propolis has become one of the natural products that have been the object of research and has shown to have other great therapeutic properties, among them antifungal, antibacterial, antiviral, antitumor, tissue repair, skin disorders, and antimutagenic activity (Ibrahim and Alqurashi, 2022; Luo *et al*., 2022; Nainu *et al*., 2021; Onur *et al*., 2022, Salatino *et al*., 2021). Theconfirmation of its therapeutic potential has stimulated the scientific community to isolate its compounds and evaluate their bioactivities increasingly. Among its chemical compounds, indeed the phenolic compounds (flavonoids and phenolic acid) have long been the focus of attention since they are the main responsible for the biological activities of the extract (Ahuja *et al*., 2021; Nichitoi, 2021; Salatino *et al*., 2021). Although there are several studies in the literature showing the antimicrobial activities of propolis, few studies have evaluated these activities on isolated principles such as, for example, chrysin and p-coumaric acid, and still less on the combination of these compounds with traditional drugs against specific ATCCs of gram-positive and gram-negative bacteria. Given this context, there was interest in doing this study to verify the activity of these combined and isolated compounds, which may be of great value in combating infections and the clinical complications arising from them.

MATERIALS ANDMETHODS

CHEMICALREAGENTS: In all experiments, reagents and compounds with an analytical grade of purity came from several commercial sources. All solutions were prepared with Milli-Q water (Millipore Reagent Water System USA) and sterilized by filtration on a0.22 µm Millex GV filter. Muller Hinton agar and broth (MH) culture media were obtained from Biomerieux (Marcy l 'Etoile, France). The resazurin dye was purchased from Sigma-Aldrich (Spruce Street, St. Louis, USA) as an indicator of microbial growth.The compounds chrysin, p-coumaric acid, and the antimicrobials, oxacillin, and amikacin were from Sigma-Aldrich®. The ethanolic extract of green propolis was donated gently by Prof. Dr. Niraldo Paulino, director of the company Medical Lex Ltda, under production originated in the company Pharmanectar Ltda (lot CT # 0706).

EXTRACT PREPARATION: The propolis was collected from a beehive near the city of Caeté-MG, Brazil, being produced by percolation/maceration process and mixed with a solution containing 96GL ethyl alcohol. This mixture was left for seven days under constant stirring for 10 min, once daily. After this period, it was obtained by evaporation of the alcohol from the solution through an extractor (Soxlhet) of plant compounds and natural products. After evaporation of the solvent, the resulting dry matter was dissolved in 10% ethanolic solution to obtain the final concentration of 100 mg/ml.

BIOLOGICAL TOXICITYTESTS: These are laboratory tests used to evaluate the toxicity of substances under specific and controlled experimental conditions. According to the International Standard Organization, ISO 10993, the *in vitro* cytotoxicity test should be the first test to be performed. This test can demonstrate the biocompatibility of the product and define its basal cytotoxicity, providing valuable information about the toxic potential of the product (Koulaouzidou*et al*., 1999). For this, hemolytic and cytolytic assays were performed according to a previously standardized technique, with some adaptations (Migliolo *et al*., 2012).

Hemolytic assay (Determination of hemolyticpercentage): The hemolytic activity of the compounds was determined by the degree of hemolysis against the 1% (v/v) O positive human blood red blood cell solution.

Preparation of the 1% (v/v) red blood cellsolution: A 5.0 mL volume of healthy human donor positive human blood collected in EDTA tube was centrifuged at 3000 RPM for 5 min for plasma withdrawal followed by three centrifugations and successive washes with 0, 9% (w/v), Na + 154 mEq/L; Cl-154 mEq/L, osmolarity 308 mOsm/L, pH 4.5 to 7.0 (saline solution -Fresenius®). After the last centrifugation, all supernatant was removed. To prepare 10 mL of stock solution of 1% erythrocytes, 100 µL of the red cell concentrate was added and added to 9900 µL of 0.9% NaCl solution (Migliolo*et al*., 2012).

Preparation of the compounds: propolis, chrysin, p-coumaric acid andtest: From the propolis stock solution at 100,000 µg/dL, a working solution at the concentration of 2000 µg/mL was prepared with saline, the chrysin was dissolved in 1% DMSO solution (10 µL of DMSO PA plus 900 µL of saline) and p-coumaric acid was prepared using 10% ethanolic solution (100 µL of ethanol PA plus 900 µL of saline), both with a final concentration of 2000 µg/mL. Three hundred microliters of triplicates of each compound were withdrawn and transferred to hemolysis tubes. Successive 1:2 dilutions with saline were performed, and then 300 µL of the 1% (v/v) red blood cell suspension was added. The concentrations of propolis and the two active components ranged from 2000 µg/mL to 3.90 µg/mL. The samples were incubated for one hour at room temperature and then centrifuged at 3000 RPM for 2 min. 150 µL of the supernatants were then removed and transferred to 96-well flat bottom microplates for absorbance reading at 405 nm (Multiskan® FC reader, Thermo Scientific, USA). For the 100% hemolysis reference samples,150 µL of the 1% (v/v) red cell suspension and 150 µL of 1% (v/v) Triton X-100 were used and for reference 0% hemolysis, 150 µL of the 1% (v/v) red blood cell suspension and 150 µL of saline. All assays were performed in triplicate. The value of the hemolytic percentage was obtained after adjusting for logarithmic regression (Migliolo*etal*., 2012).

Cytolytic assay (flowcytometry): Analyzes of the changes caused by the compounds on white blood cells (leukocytes) were performed on the Cell-Dyn 3700 hematological counter (Abbot). This technology analyzes leukocytes on two separate channels, WOC (optical) and WIC (impedance).

Preparation of whole blood: A volume of 8.0 mL of healthy donor positive O blood collected in EDTA tube was centrifuged at 3000 RPM for 5 min for plasma withdrawal followed by three successive washes with physiological saline solution (Fresenius Brand). After each washing and centrifugation, the plasma phase was removed, and the final volume filled with physiological saline so that at the end of the three washes the initial volume of 8.0 mL was maintained. The plasma phase was removed with the purpose of removing possible factors that could interfere with the activity of the active components and their effects on the cell lines (Migliolo*et al*., 2012).

Preparation of solutions in whole blood and assay: The propolis solution was prepared with 4000 µg/mL saline from a stock solution at 100 mg/dL. Chrysin was dissolved in 1% DMSO solution (10 µL of DMSO PA plus 900 µL of saline), and p-coumaric acid was prepared using 10% ethanolic solution (100 µL of ethanol PA plus 900 µL of saline), both with a final concentration of 2000 µg/mL Initially, 1.0 ml triplicates were withdrawn from each solution and placed in test tubes. Then, 1.0 mL of positive "O" whole blood washed with a physiological solution was added to each tube, thereby making up a final volumeof2.0 mL of solution and the final concentration of 1000 µg per mL for the two active compounds and of 2000 µg/mL for propolis. Immediately after the addition of blood, the first reading (T0) of the triplicates was performed. After 30 min (T30) and after 120 min (T120) incubation at room temperature, they were again analyzed on CellDyn 3700 (Flow Cytometric Automated Hematology Analyzer, Abbott). The total leukocyte number readings at each incubation time were recorded by the mean of the triplicates (Migliolo *et al*., 2012).

Preparation of negative and positive controls: The negative control, reference to the absence of leukocyte destruction, was prepared in triplicate, adding 1.0 mL of washed whole blood to 1.0 mL of saline solution. The positive control, reference of leukocyte destruction, was prepared in triplicate, adding 1.0 mL of whole blood washed with a physiological solution to1.0 mL of Triton X-100 solution (200 µL of Triton X-100 10% (v/v) plus 1800 µL saline) (Migliolo*et al*., 2012).

ANTIMICROBIALASSAYS

Preparation of solutions: The propolis extract was dissolved in 10% ethanolic solution (100 µL of ethanol PA plus 900 µL of water) to obtain a final concentration of 100,000 µg/mL. Chrysin was dissolved in 1% DMSO solution (10 µL of DMSO PA plus 900 µL of water), and p-coumaric acid was prepared using 10% ethanolic solution (100 µL PA ethanol plus 900 µL water) both were at a final concentration of 1000 µg/mL. The stock solutions of the antimicrobials, oxacillin, and amikacin were prepared with sterile water at a concentration of 2000 µg/mL. For the performance of the assays, the antimicrobial solutions were diluted 1:10 immediately before the microtiter to 200µg/ml.To evaluate the effect of the combination of the isolated compounds, a 1:1 (v/v) solution was prepared by mixing the solutions of these compounds with a solution at 500 µg/mL for each compound. For the evaluation of this same combined effect of the antimicrobial compounds, solutions were prepared by mixing theantimicrobial solution with each of the solutions of propolis, chrysin, p-coumaric acid, and the association of the latter two compounds. All solvents and diluents used in the preparation of the compounds for carrying out the assays were sterilized by filtration on a 0.22 µm Millex GV filter.

Antibacterial assays: The reference strains used in the experiments, Gram-positive - *Staphylococcus aureus* (ATCC 29213) and Gram-negative - *Escherichia coli* (ATCC 25922), were kindly provided by the Laboratory of Microbiology of the University Hospital of Brasilia, Brazil.

Preparation of the inoculums: For the preparation of the bacterial inoculum, some fresh colonies after 24 hours of incubation at 37° C in Muller Hinton agar were withdrawn and suspended in 5.0 ml of sterile distilled water in test tubes until a suspension of 0.5 on the scale of McFarland containing approximately 1-5 x 10^8 CFU/ml. The reading was performed on the VITEK® 2 DensiCheck turbidity meter (BioMérieux®,France).This suspension was diluted 1:100 and then 1:20 (20 mL containing 0.5 - 2.5 x 10^5 CFU/mL). To complete the preparation of the bacterial inoculum, 100 µL of20mg/mL aqueous resazurin solution was added to the 20 mL bacterial suspension in order to reveal bacterial growth by means of a change in coloration of blue (absence of growth) to pink (presence of growth) (Liu *et al*., 2007).

Microdilutionassay: For the microdilution test, sterile flat bottom 96-well plates were used. From each solution of the isolated substances and their associations, and of the antibiotics (amikacin and oxacillin), aliquots were withdrawn in 200 µl triplicate and serial 1:2 dilutions were carried out in Muller Hinton broth, then 100 µL of bacterial inoculum containing approximately 0,5 - 2.5 x 10^5 CFU/mL in all wells, except in the absence/growth control wells to which 100 µL of the resazurin solution (25 µL at 20 mg/mL of resazurin plus 5 mL sterile water). The final platelet concentrations ranged from 50000 µg/mL to 24 µg/mL for propolis extract, for chrysin and 500 µg/mL at 0.25 µg/mL p-coumaric acid, and 250 µg/mL to 0.125 µg/mL for the association of chroma plus p-coumaric acid and antibiotics (amikacin and oxacillin) from 100 µg/mL to 0.048 µg/mL. Plates were incubated for 24h at 37° C.

Although they were used to prepare the compounds at the concentration recommended by the Clinical Laboratory Standards Institute (CLSI), assays with 1% DMSO and 10% ethanol solutions were performed to confirm the interference. For control of absence of growth/sterility, Muller Hinton broth (MH) plus test compound was used, and for the full growth, control was used, MH broth plus bacterial inoculum. After incubation, the reading was performed by visualization of the color change of the wells. The minimum inhibitory concentration (MIC) for each compound was interpreted as the lowest concentration that maintained the initial color (blue), indicating the absence of growth (Liu*et al*., 2007).

RESULTS

HEMOLYTIC TESTS OF PROPOLIS, CHRYSIN AND P-COUMARIC ACID ONERYTHROCYTES: Figure 1 shows the hemolytic effects on erythrocytes after addition of propolis, chrysin and p-coumaric acid and reading of the supernatants by the Multiskan® FC reader, Thermo Scientific, USA. Expressed in numbers, one can verify the hemolytic percentage of each compound in decreasing concentrations ranging from 2000 to 3.90 µg/mL, compared to 1% erythrocyte suspension, when compared to positive (C+) controls - presenting 100% of hemolysis, and negative (C-) absence of hemolysis. In general, the three compounds had a hemolytic effect only at the highest concentrations, which were higher than the concentrations that had an inhibitory effect on bacterial growth (MIC) in antimicrobialassays. In figure 1A it can be observed that there was the only hemolysis at concentrations higher than 500 µg/mL, demonstrating, therefore, to be a product with low toxicity. In figure 1B, the hemolytic effect of chrysin occurred only in the two highest concentrations, whereas in those smaller than 500 µg/mL there was no such activity. In Figure 1C, p-coumaric acid at concentrations below 250 µg/mL, no hemolytic activity was observed against erythrocyte suspension, demonstrating that this compound shows no hemolytic activity at the concentration capable of inhibiting the bacterial growth found in our study, a relevant fact about the toxicity of the product for therapeutic use.

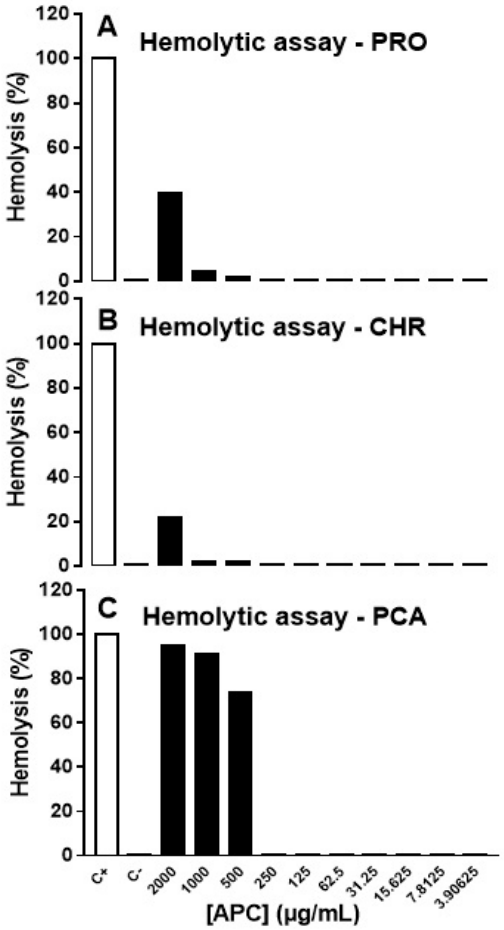

Figure 1. Percentage of hemolysis of human erythrocytes (O+) caused by propolis (1A), chrysin (1B) and p-coumaric acid (1C). As control of absence of hemolysis (C-), the red cell suspension was incubated with NaCl solution 0.9% (w/v), Na + 154 mEq/L; Cl-154 mEq/L, osmolarity 308 mOsm/L, pH 4.5 to 7.0 (Fresenius saline), and as a 100% hemolysis (C+) control, the red blood cell suspension was incubated with Triton X- 100% concentration (v/v). The values represented are expressed by the means of the triplicates. PRO = propolis, CRI = chrysin, APC = p-coumaric acid

CYTOLYTHIC TESTS OF PROPOLIS, CHRYSIN, AND P-COUMARIC ACID ON TOTALLEUKOCYTES: Figure 2 shows the cytolytic effects on total leukocytes in whole blood prepared samples after addition of the final concentration of 2000 µg/mL, chrysin and p- coumaric acid at the final concentration of 1000 µg/mL. The readings were performed on the Cell-Dyn 3700 hematology equipment (Abbot). Leukocyte counts averaged at initial time (T0) were 2,600 leukocytes/mm^3 for the positive control samples (treated with Triton X), 2,800 leukocytes/mm^3 for the negative control (saline) samples, 2,790 leukocytes/mm^3 for the samples with propolis addition, 2,790 leukocytes/mm^3 with chrysin, 2,800 leukocytes/mm^3 with p-coumaric acid. In the time 30 minutes (T30), the counts were 12 leukocytes/mm³ for the positive control, 2,780 leukocytes/mm³ for the negative control, 2,780 leukocytes/mm³ for propolis, 2,770 leukocytes/mm³ for the chrysanthemum, 2,780 leukocytes/mm³ for p-coumaric acid. A mean of 12 leukocytes/mm^3 for the positive, 2,770leukocytes/mm^3 for negative control, 2,730 leukocytes/mm^3 for propolis, 2,700 leukocytes/mm³ for chrysin, 2,740 leukocytes/mm³ for p-coumaric acid. The destruction of leukocytes in the positive control (treated with Triton X 100 detergent) was noted. At the first count (T0), there was a small decrease in leukocytes when compared to the leukocyte count of the negative control. In the readings, T30 and T120 the leukocytes were practically destroyed. The averages of the leukocyte count of the samples treated with propolis and those treated with p-coumaric acid in the three incubation and reading times revealed that both did not present significant variations when compared with the negative control readings and their respective initial readings (T0). In the samples treated with the chrysin, there was a small reduction in the leukocyte count when compared to the negative control group and also with the initial count (T0) of the chrysin itself.However, this variation is not considered significant since it is within the acceptable reading variation coefficient of the equipment, which is up to ± 5%.

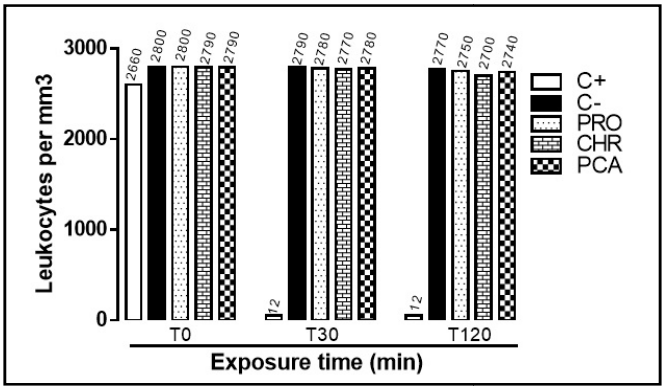

Figure 2. Graphical analysis of flow cytometry showing total leukocyte count in whole blood at 0, 30, and 120 minutes under the action of propolis (blue color) at the concentration of 2000 µg/mL, chrysin (orange) and p (Triton X-100 10% v/v, represented by the light blue color), both at the concentration of 1000 µg/mL, compared to the negative controls (physiological solution, represented by yellow color) and positive

Figure 3 shows the percentage of leukocyte destruction in the prepared whole blood samples after the addition of propolis, chrysin, and p-coumaric acid at time 0, 30, and 120 minutes. In the final reading (T120), the percentage of total leukocyte destruction in samples treated with p-coumaric acid was 1.1% when compared to the negative control initial reading (T0). However, when compared with the negative control at the same final reading time (T120) the percentage wasequal to the negative control. By doing the same analysis with chrysin, the percentage of leukocyte destruction was 2.5% in the final reading (T120) when compared to the negative control (T0) and 1.4% when compared to the control negative at the same time as the final reading (T120). The positive control reached the maximum of destruction (99.5%), already in the second reading (T30). In figure 4 the histograms of the leukocyte count of the samples and the controls can be visually verified in the three incubation times. The Cell-Dyn 3700 hematology counter was used for the cell count. This equipment analyzes the leukocytes in two reading channels, one optical (WOC) and the other impedance (WIC).

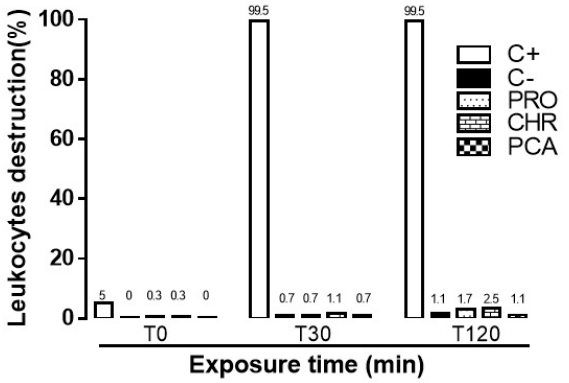

Figure 3. Percentage of leukocyte destruction after exposure of whole blood at 0, 30, and 120 minutes under the action of propolis (blue) at the concentration of 2000 µg/mL and chroma (orange) and p-coumaric acid (gray color) at the concentration of 1000 µg/mL, compared to the negative (physiological solution, represented by yellow color) and positive controls (Triton X-100 10% (v/v), represented by light blue color

In optical counting, a laser beam strikes the cells aligned by a continuous flow and undergoes dispersion, this scattered light is captured at four different angles (0º, evaluates the size, 10º, evaluates cellular complexity, polarized 90º, measures the surface cellular and evaluates the internal structure and 90º depolarized, measures some types of granules). The electrical impedance count, leukocytes are quantified and measured by transient changes in resistance between two electrodes caused by the passage of cells through an opening in the counting chamber. Each cell that passed changed the amplitude and generated a pulse that was proportional to the volume of the particle that produced it. The result was reported in numbers and employing histograms. Figure 4A shows the histograms of the leukocyte counts at zero time that only the positive control showed a small reduction in the number of leukocytes (represented by the colored dots plotted in the histogram) when compared to the other treatments.

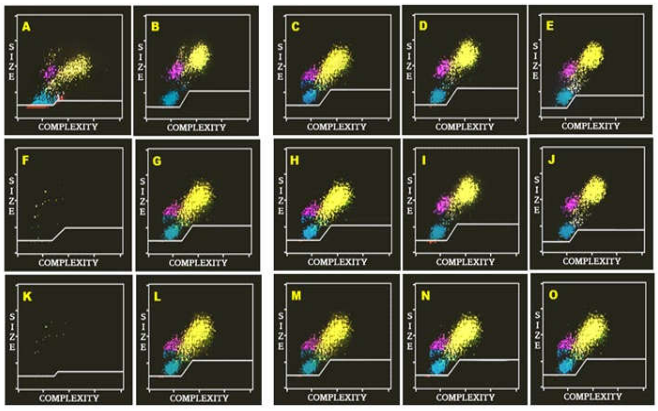

Figure 4. Total leukocyte count in blood samples at times: zero (SUPERIOR panels), 30 min (INTERMEDIARY panels) and 120 min (INFERIOR panels). Readings were performed at each time, according to the type of treatment: Positive controls (A, F and K), Negative controls (B, G, L), 2000 µg/mL of propolis extract (C, H, M)), 1000 µg/ml chrysin (D, I, N), or p-coumaric acid (E, J, O). In the histogram, leukocytes are represented by colored dots: yellow (neutrophils), blue (lymphocytes), purple (monocytes), green (eosinophils), and white (basophils).Each point corresponds to a single cell type being plotted as it passed through the cell detector of the flow cytometer

In Figure 4B shows the count after thirty minutes, the colored spots (leukocytes) of the positive control practically disappeared, indicating almost the destruction of the leukocyte, which was already expected, in view of being the positive control of destruction; while the colored spots representing the leukocytes in the remaining histograms remained similar to those at time zero. In Figure 4C it can be seen visually that the histograms of the samples and the controls practically did not undergo modification when compared to the time of 30 min.

Antibacterial Assays

Sensitivity tests showed distinct activities of gram-positive and gram-negative microorganisms, compared to the compounds tested. First, the microdilution readings were performed against *Staphylococcus aureus* strains ATCC 29213 for the evaluation of the antimicrobial activity of propolis (PRO), chrysin (CRI), p-coumaric acid (APC), chrysin + p-coumaric acid (CRI + APC), and oxacillin (OXA) in isolation and associated with the compounds. Bold depicts concentrations that were able to inhibit bacterial growth, minimum inhibitory concentration (MIC). The results reveal that p-coumaric acid (APC) was able to inhibit bacterial growth in the concentration of 250 µg/mL. Chrysin (CRI) had no inhibition of growth activity, even at the highest concentration tested, which was 500 µg/mL. The minimum inhibitory concentration of the chrysin associated with p-coumaric acid (CRI + APC) was 250 µg/mL. Propolis (PRO) had a minimum inhibitory concentration (MIC) of 190 µg/mL. Oxacillin, both isolated and associated with the compounds, presented the same result of 0.39 µg/mL. According to table M07 of the Clinical and Laboratory Standards Institute (CLSI), the minimum inhibitory concentration of this antibiotic for the strain used should be between 0.12 and 0.5µg/mL. In the second phase, microdilution readings were carried out against *Escherichia coli* strains ATCC 25922 for the evaluation of the antimicrobial activity of propolis(PRO), chrysin (CRI), p-coumaric acid (APC), more acidic chrysin + p-coumaric acid (CRI + APC), amikacin (AMI) and combinations of the compounds with amikacin. Bold depicts concentrations that were able to inhibit bacterial growth, minimum inhibitory concentration (MIC). The results of the antimicrobial activities of the isolated compounds show that they did not present activity, even in the highest concentrations tested. The MIC of propolis was more significant than 50,000 µg/mL, of chrysin and p-coumaric acid was higher than 500 µg/mL, of the association of chrysin plus p-coumaric acid (CRI + APC) was higher than 250 µg/mL. Isolated amikacin (MAI) inhibited growth at the concentration of1.56 µg/mL, the same value found in the associations between the compounds and amikacin. According to table M07 of the Institute of Clinical and Laboratory Standards (CLSI), the minimum inhibitory concentration (MIC) of this antimicroial against the strain used should be between 0.5 and 4.0 µg/mL.

DISCUSSION

The activity of the compounds in the inhibition of the microbial growth of propolis and p-coumaric acid showed activity against *Staphylococcus aureus*, with MIC of 190 µg/mL and MIC of 250 µg/mL, respectively. However, they showed no activity against the gram-negative strain of *Escherichia coli* in the highest concentration tested. Also, the addition of chrysin did not potentiate or reduce p-coumaric acid activity, since the MIC of 250 µg/mL of p-coumaric acid alone was the same as that of the combination. The antimicrobial activity of the combination between the compounds and oxacillin against *Staphylococcus aureus* showed that there was no increase or reduction of the activity of this antimicrobial since the MIC of0.39 µg/mL of the combination was the same as that of the antibiotic tested isolated form. Likewise, in the evaluation of the antimicrobial activity of the association between these same compounds with amikacin against *Escherichia coli*, it was observed that there was no increase or reduction of the activity of this antimicrobial since the MIC of 1.56 µg/mL of the association was the same as the antibiotic alone. Considering that chemically this propolis extract is a heterogeneous mixture of natural compounds, and many of them have already been identified (Salatino *et al*., 2021), it is possible that many of the data found here may also be correlated to: alcohols, higher hydrocarbons, aromatic acids , higher fatty acids typical of waxes and their esters, flavanols, flavanones, flavones, ketones, dihydrochalcones and chalcones, terpenoid steroids, sugars, amino acids, lignans, minerals (magnesium, sodium, potassium, barium, cadmium strontium, lead, copper, manganese-calcium, vanadium, silicon, aluminum, chromium, titanium, silver, molybdenum, cobalt and vitamins A, B1, B2, B6, C and E (Calegari *et al*., 2017).

Thus, the present study is in agreement with the literature regarding the greater antimicrobial activity of propolis against gram-positive and little activity against gram-negative. The data obtained are similar to those found by Al-Ani et al. (2018), that working with ethanolic extract of propolis of German origin found MIC of 1.2 mg/mL for gram-positive bacteria *Staphylococcus aureus*. As also seen in the publication by Ristivojević *et al*. (2016) that evaluating the antimicrobial activity of 53 propolis samples found that they had higher activity against gram-positive, the *Staphylococcus aureus* MICs of the samples ranged from 0.4 mg/mL to 14.5 mg/mLThe lack of activity of propolis against *Escherichia coli* does not rule out the possibility that the propolis extract has inhibitory activity against this microorganism. This fact can be observed in the study by Ristivojević *et al*. (2016) that using a sample of various propolis extracts showed moderate activity in three out of fifty-three of these samples against *Escherichia coli*. According to scientific literature, regardless of the type of extract, the antimicrobial activity of propolis is more evidence against gram-positive bacteria (Nichitoi *et al*., 2021). The lower sensitivity of gram-negative occurs probably due to the difference in the chemical constitution of the cell wall. Although the gram-negative bacteria have a less rigid wall than the gram-positive ones and have a lower amount of peptidoglycan, they are chemically more complex andhave a higher lipid content and are therefore less susceptible to propolis (Nichitoi *et al*., 2021; Marcucci *et al*., 2001). This variation of the activity of the extract, in front of the microorganisms, can also be justified because of the different types of propolis existing in Brazil and the world. Bankova *et al*. (2014), for example, evaluated samples of propolis from different regions of Europe and the Middle East. They found that the higher the concentration of phenolic compounds the more potent the bacterial activity, confirming that the qualitative and quantitative differences of the samples can directly influence the action potential. Besides, the chemical composition of the resin may be linked because this may vary according to the season, climate, relief, region, botany existing around the hive, and even with the bee collector species (Bankova*et al*., 1998). As for the results obtained in our study with p-coumaric acid, these were similar to those found by Zhao *et al*. (2015), which evaluated the activity of this compound against *Staphylococcus aureus*, obtained a MIC of 625 µg/mL, whereas for *Escherichia coli* no inhibitory effect was observed. This same lack of concentration activity tested in our study against *Escherichia coli* was evidenced by Alves *et al*. (2013), which only observed inhibitory activity from 1000 µg/mL.

In relation to the chrysalis flavonoid, it did not show activity against S*taphylococcus aureus* and *Escherichia coli* strains, even at the highest concentration tested (500 µg/mL). This result corroborates the study by Alves *et al*. (2013), who did not observe chyme inhibitory activity against these two pathogens up to the concentration of 1000 µg/mL and also the work of Wang *et al*. (2011), which evaluated the activity of this same compound against somebacterialstrains of *Staphylococcus aureus*, showed no interference in bacterial growth up to the concentration of 1024 µg/mL. Others also support the results obtained here from our research group, in which the green propolis extract was investigated in the behavioral and cognitive alterations resulting from the systemic infection. This work demonstrated that experimentally induced sepsis in rats caused changes in behavior (anxiety, depression) and cognition. However, in the groups of animals treated with propolis extract and in those treated with antibiotics (clindamycin 25 mg/kg + gentamicin3 mg/kg), a reduction in deleterious effects and restoration of responses to basal levels were observed. The following tests evaluated these alterations: open field(locomotion), elevated cross maze (anxiety), forced swimming

(depression/stress) and step-down inhibitory avoidance. The results obtained in this research showed that the animals treated with the propolis extract obtained responses similar to those treated with antibiotics, suggesting that one of the reasons for the reduction of deleterious and neuroprotective effects was due to the antimicrobial activity of propolis (Soares *et al*., 2017). Considering the investigation of toxicity and evaluation of the antimicrobial activity of propolis, chrysin and p-coumaric acid extract against the strains ATCCs of *Escherichia coli* and *Staphylococcus aureus*, it was concluded that there was a higher activity of the propolis extract against gram-positive, where this extract, together with chrysin and p-coumaric acid, proved to be products with minimal toxicity. In spite of having low toxicity, the flavonoid chrysin showed no activity against strains of *Staphylococcus aureus* and *Escherichia coli*, even in the highest concentration tested. The p-coumaric acid, on the other hand, presented goodpotential as an antimicrobial agent and with low toxicity, and, therefore, could be a possible alternative in the treatment of infections caused by *Staphylococcus aureus*. Considering the results obtained, it is a fact that we cannot ignore the knowledge and practices of traditional peoples in the analysis, study, and research for the use of plants for medicinal and therapeutic purposes. Thanks to this knowledge, scientific and technological innovations involving many products from nature were possible. Considering the numerous investments of the pharmaceutical industries in search for increasingly useful substances to treat infections, despite the need for further pharmacokinetic studies in animals and humans, the p-coumaric acid might be a potential candidate to reduce or at least to alleviate the deleterious consequences of infectious processes.

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