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Research Article

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Evaluation of the Cytotoxic, Antimicrobial and Antioxidant Activity of the Plant Especies *Tabebuia roseo-alba* (Ridl) Sand

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ABSTRACT

The objective of this research was to evaluate the biological potential of the plant species Tabebuia roseo-alba (Ridl.) Sand. It was a quantitative study of in vitro an experimental type in which the extracts of the species Tabebuia roseo-alba were submitted to phytochemical prospection, to the cytotoxic activity by means of the methyltetrazolium colorimetric assay (MTT), to the evaluation of the antimicrobial activity by means of the broth microdilution assay to determine the minimum inhibitory concentration (MIC), and to the scavenging capacity of the free radical 2,2 diphenyl-1-picrylhydrazyl (DPPH). In the phytochemical analysis, the presence of secondary metabolites flavonoids, free steroids, triterpenes, saponins, alkaloids, anthraquinones and anthrones were evidenced. Cytotoxicity was observed at the concentration of 500 µg/mL for all samples, and at the concentration of 100 µg/mL the proliferation of the macrophages used in the test was observed. The ethanolic extract of the stem and the methanolic fractions of the stem and the hexane fractions of the stem bark presented antimicrobial activity against the bacterial strains Staphylococcus epidermidis and Escherichia coli. In the antioxidant assay, the tested samples showed no satisfactory activity. Therefore, the plant species Tabebuia roseo-alba demonstrated biological potential in the tests carried out in this study. Thus, other studies are suggested with the aim of enhencing the activities found in this research.

Keywords: *Tabebuia roseo-alba*; Medicinal plants; Antimicrobian activity

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INTRODUCION

Cutaneous infections involve a large diversity of etiologic agents and multiple pathogenetic mechanisms, because once the skin is injured, microorganisms that are normally present on the surface of the skin gain access to the underlying tissues [1]. Human attempts to interfere in the treatment of wounds date back to antiquity, showing that since then humans already recognized the importance of protecting wounds in order to avoid complications and repercussions in the form of local or general damages for the carrier thereof [2]. Even today, wound healing process remains a major health area, particularly in surgical practice, at a time when the indiscriminate use of antimicrobials has been detrimental to the treatment of hospitalized patients, with repercussions on the increasing outbreak of antimicrobial resistant microorganisms [3]. As a result of this, the search for new antimicrobial substances from natural products has increased the interest of pharmaceutical companies and researchers in several areas on species used in folk medicine [4]. Medicinal plants can be defined as those that contain biological activity with one or more active principles useful to

human health. Two types of medicines arose from these plants: phytotherapic drugs, originated exclusively of the integral botanical material, and the phytopharmaceutical drugs, obtained by the isolation of the active principle of plant extract [5]. The *Tabebuia roseo-alba* (Ridl) Sand, known as *ipê-branco*, belongs to the family Bignoniaceae and is an economically important species due to its ornamental use and its ethnopharmacological components [6]. Previous research has shown that leaves and stem bark of this plant showed antimicrobial activity against microorganisms associated with nosocomial infections [7]. A previous study evaluated the antimicrobial activity of *Tabebuia roseo-alba* by means of an *in vitro* agar perforation test against eight microbial species among gram-positive, gram-negative and fungus bacteria. The hexane extract of the leaf and the ethanolic extract of the stem presented inhibitory activity on the microbial growth against the strains *S. aureus, S. epidermidis, E. faecalis* and *K. pneumoniae* [8].
Due to the importance of researching new pharmacological substances present in plant species, the present study aimed to perform the phytochemical profile of the ethanolic extracts and fractions of *Tabebuia roseo-alba* (Ridl.) Sand stem bark and leaves, and to evaluate the antibacterial, antioxidant and cytotoxic *in vitro* activity of these extracts.

EXPERIMENTAL SECTION

This was an experimental *in vitro* study with the vegetal species *Tabebuia roseo-alba* (Ridl.) Sand (*ipê-branco*), in which the ethanolic extracts and fractions of the leaves, stem bark and stems were used. This research was conducted at the Research Laboratory in Wound Care (LpTF in Portuguese) at the Federal University of Alagoas.

Plant Material

The plant species *Tabebuia roseo-alba* (Ridl.) Sand was collected in the municipality of Coruripe-AL. The plant material was identified by the botanist Rosangela Pereira Lyra Lemos and a sample is deposited in the herbarium of the Environment Institute of Alagoas (IMA-AL), under the registration MAC 46880.

Preparation of Extracts

The plant material of the leaves, stem bark and stem were dried at room temperature for 7 days; the ethanolic extracts were obtained by steeping in 98% ethanol (EtOH) and then by concentrating them on a rotary evaporator at a maximum temperature of 40°C and subsequent drying at room temperature.

Fractionation of Extracts

Fractionation of the extracts of the species in question was carried out with a 100 mg aliquot of ethanolic extracts, in which they were partitioned in a vacuum filter column, using silica gel as a stationary phase, and hexane, chloroform ($CHCl_3$), ethyl acetate (AcOEt) and methanol (MeOH) as mobile phase, following this order of polarity [9]. The solutions obtained were concentrated in a rotary evaporator, resulting in 4 phases: hexane phase, chloroform phase, ethyl acetate phase and methanolic phase. Thereafter, the wet materials were placed in suitable and dried vials at room temperature.

Phytochemical Analysis

Phytochemical prospecting of the species *Tabebuia roseo-alba* was done according to the methodology of Matos [9] with the purpose of identifying the classes of the components present in the vegetal species, thus allowing a possible comparison with the literature data. The qualitative and semi-quantitative tests included phenolic and tannin tests (reaction with ferric chloride), anthocyanins, anthocyanidins, flavanoids, leucoantocyanidins, catechin, flavanones (pH variation test with sodium hydroxide and hydrochloric acid), flavonols, flavanonois, xanthones (effervescence test with granular magnesium and hydrochloric acid), steroids and triterpenes (Liebermann-Burchard test), saponins (foam test and precipitation test), alkaloids (identification with Dragendorff), anthraquinones, anthrones and coumarins (test with UV light). To perform the tests, 30 mg of the extracts were removed and solubilized in 30 mL of ethanol, placed in test tubes and numbered.

In vitro Biological Assays

Broth microdilution technique:

The crude ethanolic extracts and fractions of the stem and stem bark of the *Tabebuia roseo-alba* species were tested against Gram-positive bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 31488) and *Enteroccocus faecalis* (ATCC 29212); and Gram-negative: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 31488), *Klebsiella pneumoniae* (ATCC 31488), *Salmonella enterica* (ATCC 1307), and the fungus *Candida albicans* (ATCC 24433).The protocol followed was based on the Clinical Laboratory Standards Institute [10]. The solubilization of the samples was done by mixing 0.01 g/mL of the crude ethanolic extract and the plant fractions, added to 1 mL of Cremophor or 2% Dimethylsulfoxide (DMSO) solution and diluted in 4.9 mL of Mueller-Hinton Broth, obtaining a 2,000 μg/mL concentration. All plate wells received 100 μL sterile Mueller Hinton Broth; 100 μL of the samples were placed in columns 1 to 9 of line A. Column 10 was destined for Growth Control, in which only the microbial inoculum was added; column 11 was destined for the Negative Control, with Cremophor or 2% DMSO and column 12 for the Plate Sterility Control (SC), in which only the

Mueller-Hinton Broth was used. After this process, 100 µL of the contents of each hole in line A was homogenized and transferred to line B, thus repeating the process until line H, discarding the excess. The bacteria samples were solubilized in a 1.5×10^8 UFC/mL solution, with concentration according to the standard of 0.5 of the McFarland scale, and subsequently re-diluted in a ratio of 1:10 (v/v). Each well received 5 µl of bacterial inoculum with final concentration of 10^4 UFC/mL. The plates with the bacteria were stored in a bacteriological oven at 35°C for growth for 18 hours. After this time, 20 µL of 5% 2,3,5-Triphenyl Tetrazolium Chloride (TTC) was added to each well, and the plates were reincubated for another 3 hours. The wells that showed reddish staining indicated bacterial growth, while those that maintained original staining indicated inhibition of bacterial growth.

Cell viability assay using the methyltetrazolium colorimetric assay (MTT):

This assay was performed based on the methodology of Mosmann [10], which evaluates mitochondrial cell activity by reducing MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide), which has yellowish coloration, in formazan crystals, which has dark-blue coloration, through the enzyme succinate dehydrogenase found in the active mitochondria. Thus, the darker the staining gets at the end of the reaction, the greater the cell viability.

J774 lineage macrophages cultured with 10 mL of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) in CO_2 oven were used in the *in vitro* test. Cells were counted and adjusted in RPMI medium supplemented with 10% FBS. These macrophages were plated in a 96-well plate (1.5×10^5/well) and kept in CO_2 oven for adherence of viable cells at the bottom of the wells for one hour. After this period, the samples were diluted at concentrations of 500 and 100µg/ml and then incubated for 48 hours with the macrophages adhered and kept in an oven at 5% CO_2. Control wells contained cells cultured only with culture medium or cells cultured in the presence of the substance diluent (0.1% DMSO). One hour before MTT was added, three wells were lysed with 2 µl of Triton 100X for comparison of cell death. After the incubation period, the supernatant was discarded and each well received 100 µL of the MTT solution (5 mg/10 mL) and then re-incubated for one hour in an oven at 37°C and 5% CO_2. Soon after, the supernatant was removed and 100 µL of DMSO was added and a reading was performed in spectrophotometer at 550 nm. The cell viability of the cultures treated with the substances was compared to the standard of death obtained in the control cultures.

Evaluations of the Free Radical Scavenging Capacity

Qualitative assays:

The evaluation of the scavenging capacity of free radicals against the synthetic radical DPPH was carried out according to the methodology described by Soler-Rivas [11].

The samples were then dissolved in spectroscopic methanol and then loaded onto 60 F_{254} silica gel chromate plates and eluted in previously defined solvent systems. After elution, the plates were dried at room temperature and, on the right side of the plate, at a height equivalent to one half, (+) - catechin (1 mg/mol in MeOH and $CHCl_3$) was applied as a control. Then, the chromate plates were submerged for 10 seconds in methanolic solution of the DPPH radical (0.4 mM). After drying at room temperature, the appearance of yellow spots under a purple background in the positions of the retention factors of the substances, when compared to (+) - catechin, suggests a possible activity.

Quantitative assays:

In this assay, only the samples that showed positive results in the qualitative assay were quantitatively evaluated against the DPPH radical using the methodology described by Brand-Willams et al. [12] and Sánchez-Moreno et al. [13]. After obtaining the calibration curve, aliquots of 0.1 mL of each sample concentration (200 to 25 µg/mL) were individually placed in polystyrene cuvettes and 0.9 mL of the DPPH (100 µmol/L) radical solution. Then, the solutions were protected from the presence of light, homogenized with the aid of tips and the readings were made at the time of 0, 15, 30, 45 and 60 minutes. The absorbances were recorded against MeOH (HPLC grade) as white and ascorbic acid and BHT (2,6-di-tert-butyl-4-methylphenol), used as positive standards [12].

Determination of IC_{50}

In order to obtain the IC_{50}, the mathematical equation $Y = a + b.x$ and the absorbance values obtained by the mean in the 60-minute time were used. Together with the angular and linear coefficients of the line of the calibration curve, it was possible to determine x, which corresponds to the DPPH concentration remaining in the medium after reaction with the samples. The percentage of remaining DPPH (% DPPHrem) in the reaction medium was obtained according to the equation described by Sánchez-Moreno et al. [13].

In order to determine the IC_{50}, concentration of each sample required to decrease the concentration of the DPPH radical by 50%, plotted by linear regression in the MicrocalOrigin Pro 7.0 program, a curve from the % DPPHrem versus the concentrations of the samples evaluated (25 to 200 µg/ml) and for controls (100 to 5µg/ml). After obtaining the curve and, and replacing in the mathematical equation $Y = a + b.x$ the values obtained from its linear and angular coefficients (*a* and *b*) and Y by 50, the value of *x*, which is the $IC_{50,}$ will be known. In this sense, the higher the DPPH consumption per one sample, the lower its IC_{50} and the greater its ability to scavenge free radicals. In order to investigate whether the differences between the averages obtained were random or significant, the Tukey test was applied by using analysis of variance (ANOVA), assuming the probability of error at the level of 5% ($p<0.05$).

RESULTS AND DISCUSSION

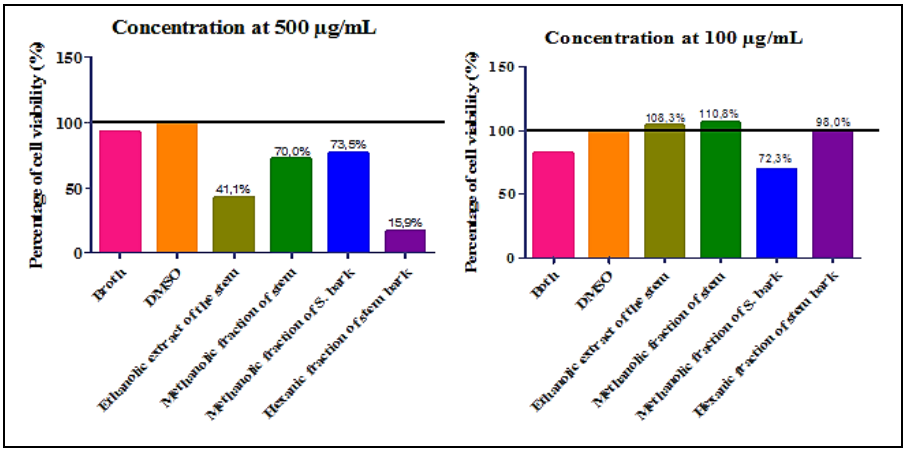
The presence of the following constituents: leucocyanidines, catechins, flavones, flavonoids, flavanones, xanthones, free steroids, triterpenes, saponins, alkaloids, anthraquinones and anthrones was observed through the phytochemical analysis. Plants of this family present a diversity of classes of chemical constituents. A recent study carried out the phytochemical analysis of *Tabebuia serratifolia* leaves and obtained positive results for reducing sugars, organic acids, alkaloids, depsides and depsidones, foamy saponins, phenols and tannins [14]. Another study [15] detected the presence of tannins, flavonoids, terpenes, coumarins and steroids in the species *Jacaranda cuspidifolia*, belonging to the Bignoniaceae family. The antimicrobial activity of the plant extracts was evaluated by determining a small amount of the substance required to inhibit the growth of the test microorganism, which is known as the minimum inhibitory concentration [16]. The ethanolic extract of the stem and its methanolic fraction of the stem of the species *Tabebuia roseo-alba* showed moderate and weak antimicrobial inhibitory activity against Gram-positive *S. epidermidis* (500 µg/mL) and Gram-negative *E. coli* (62.5 µg/mL) bacteria (Table 1).

Table 1: Minimum inhibitory concentration (MIC) of the samples tested. Brazil, 2016

Minimal Inhibitory Concentration (µg/mL)					
Microorganism		**Ethanol Extract of Stem**	**Methanolic Fraction of Stem**	**Methanolic Fraction of Stem Bark**	**Hexane Fractions of Stem**
Gram-Positive	*S. epidermidis*	500	500	-	1000
Gram-Negative	*E. coli*	1000	1000	62.5	-

Source: Authors

When compared with a study carried out with the ethanolic extract of the leaves of *Tabebuia avellanedae*, the results are similar, since this research presented inhibitory activity against bacterial strains *S. aureus* and *S. epidermidis* considered moderate and weak with MIC ≥ 312.5 µg/mL and 625 µg/mL [17]. Another study carried out with *T. chrysantha* extracts proved that the leaf methanolic extract inhibited the growth of *S. aureus* at 125 µg/mL, but did not inhibit the growth of *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa* [18]. These data differ from those found in the present study, since the methanolic fraction of the stem bark was considered as presenting active antimicrobial activity against the strain *Escherichia coli*, with a MIC of 62.5 µg/mL, in which inhibition was demonstrated with a MIC of 62.5 µg/mL. According to the literature, the presence of flavones, flavonoids, alkaloids and steroids in the studied species possibly justifies its antimicrobial activity [19,20]. In the evaluation of the cytotoxic activity, all the extracts at 500 µg/mL were toxic. However, in the concentration of 100 µg/mL, the methanolic fraction of the stem bark presented cell viability of 75.2% when compared to the control DMSO (Figure 1).



Source: Authors

Figure 1: Cytotoxic activity of crude ethanolic extract and fractions of the plant species *T. roseo-alba*. Brazil, 2016

Another study showed that the hexane and chloroform extracts of the plant species *Tabebuia serratifolia* showed higher cytotoxic activity at 400 and 800 µg/mL, respectively, whereas at the concentration of 100 µg/mL these samples showed no cytotoxicity [21,22]. These data corroborate with the present study, evidencing that the higher the concentration, the greater the cytotoxicity. The methanolic fraction of stem bark, when submitted to the cytotoxicity assay, demonstrated cell toxicity at all concentrations studied. These data show that the antimicrobial activity of this sample could be related to the cytotoxicity found in the MTT assay, and that, possibly, the presence of alkaloids in this fraction justifies its cytotoxicity [22]. The ethanolic extract of the stem and the methanolic fraction of the stem were not active in the broth microdilution assay, but in the cell viability assay these samples induced proliferation of the test cells, presenting a percentage of 8.33 and 10.88 greater than the control. A research has shown that the alkaloids present in medicinal plants did not present toxic effect on the proliferation of fibroblasts, but increased the migration of these cells, being responsible for the cicatrization process [23]. On the other hand, a study attributed the induction of cell proliferation of spleen cells to steroids, xanthones and flavonoids present in plant species [24]. Another activity

evaluated with the studied species was the free radical scavenging capacity of the samples by means of the DPPH assay, whose values higher than 200 μg/mL are considered without antioxidant activity (Table 2).
A study showed that the species *Tabebuia ochracea* and *Tabebuia rosea-alba* showed antioxidant activity, and that the fractions of these species showed antiradical activity [25]. These data diverge from the results of the present study, considering that the stem extract and the methanolic fractions of the stem, hexane and methanolic fractions of the stem bark showed no antioxidant activity.

Table 2: Mean of the absorbance of the samples tested. Brazil, 2016

Sample	IC_{50} (μg/mL) ± SD
Ethanolic extract of the stem	302.77 ± 8.43
Methanolic fraction of stem bark	1559.02 ± 2.79
Methanolic fraction of the stem	259.98 ± 0.97
Hexanic fraction of stem bark	1108.95 ± 0.96

Source: Authors.

CONCLUSION

The classes of secondary metabolites alkaloids, steroids, xanthones, flavonoids, anthraquinones and terpenes were identified in the species *Tabebuia roseo-alba* of Alagoas State. Regarding the antimicrobial activity, the best MICs were 62.5 μg/mL against *E. coli* originated from the methanolic fraction of stem bark and 500 μg/mL against *S. epidermidis* originated from the stem ethanol extract and its fraction. Thus, this plant species proved to have antimicrobial potential. As for cytotoxicity, the studied plant was potentially cytotoxic. In regard to the antioxidant activity, the ethanolic extracts and their respective fractions did not present antiradical activity.

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