

Full Length Research Paper

Evaluation of the potential modulator of bacterial resistance, acute toxicity and chemical composition of *Schinopsis brasiliensis* Engl.

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In Brazil's semi arid region, *Schinopsis brasiliensis* Engl. (Anacardiaceae) is used in traditional medicine, but little is known about its chemical, pharmacological and toxicological properties. In this study, the phytochemical and biological potential of the extract of *S. brasiliensis* (EESb) was evaluated. The main secondary metabolites in EESb were quantified by spectroscopy in the visible region. The minimum inhibitory concentration (MIC) of the extract, of antibiotics alone and of antibiotics combined with the extract were determined using microdilution method, and the acute toxicity was determined by *in vivo* tests. In microbiological testing, the EESb showed good antimicrobial activity against *Staphylococcus aureus* and, when combined with synthetic antibiotics, potentiated its activity by reducing the MIC values. *In vivo* studies showed low acute toxicity in rats treated with EESb. These results demonstrate the richness of the phenolic compounds in *S. brasiliensis* and its effectiveness as an antibacterial and in modulating activities of bacterial resistance. The probable low toxicity was also demonstrated.

Key words: Ethnopharmacology, phenolic compounds, modifying antibiotic activity, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

Brazil is known throughout the world for its biodiversity and complex cultural heterogeneity, which was influenced

by indigenous, European, and African cultures in the colonial period (Elisabetsky and Wannmacher, 1993;

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Albuquerque et al., 2007). The direct relationship between the population and the local flora has generated a rich system of knowledge about its therapeutic properties. This ethnopharmacological knowledge is a great starting place for researchers in search of new drugs (Fabricant and Farnsworth, 2001; Balunas and Kinghorn, 2005).

In recent years, the use of plant extracts for therapeutic purposes has become a growing research interest (Oliveira and Petrovick, 2010). These extracts consist of a complex of plant metabolites whose therapeutic effectiveness is related to the phytochemical interaction wherein molecules perform additional tasks. Thus, it is not surprising that within plant mixtures, secondary metabolites may be more biologically active than the individual components (Lila and Raskin, 2005; Wagner, 2011). Among the Brazilian medicinal plants, *Schinopsis brasiliensis* Engl. (Anacardiaceae), a thorny tree popularly known as braúna, baraúna, braúna-parda and braúna-do-sertão can be highlighted (Lorenzi, 1992). It is one of the most important medicinal plants for communities located in the Caatinga in Northeastern Brazil where people use its bark in various mixtures designed to treat flu symptoms, colds, diarrhea, dysentery, fractures, general inflammations, general infections and sexual impotence (Albuquerque et al., 2005, 2007, 2011; Silva and Albuquerque, 2005; Albuquerque and Oliveira, 2007; Almeida et al., 2006, 2010).

Despite the importance and widespread use of *S. brasiliensis* in folk medicine, there are few experimental studies that seek to evaluate its pharmacological and phytochemical properties. The presence of a considerable concentration of phenolic compounds such as tannins and flavonoids as well as antioxidant and antimicrobial properties has been reported (Saraiva et al., 2011, 2013). A new alkyl phenol, methyl 6-eicosanyl-2-hydroxy-4-methoxybenzoate and an unusual steroid 5a,8a-epidioxyergosta-6,22-dien-3-ol have been isolated from this plant (Cardoso et al., 2005). Because of its current high use value for the Caatinga communities and its potential to be framed in various categories of ethnobotanical use, *S. brasiliensis* the Brazilian Institute for the Environment's (IBAMA) list of species was threatened with extinction (Lucena et al., 2007). Knowing the phytochemical and pharmacological properties of *S. brasiliensis* may, in addition to finding new bioactive molecules, provide subsidies for the conservation and sustainable use of this species. Thus, this study aims to evaluate the phytochemical properties, modulating activity of the bacterial resistance, and the acute toxicity of the extract of *S. brasiliensis*.

MATERIALS AND METHODS

Plant material and ethanolic extraction

The bark of five different specimens of *S. brasiliensis* Engl,

(Anacardiaceae), were collected in the semiarid region of the Paraíba state, with a voucher specimen prepared and deposited in the herbarium Professor Jayme Coelho de Moraes (Herbarium Code EAN), Federal University of Paraíba, under the number EAN-14049. The plant material was dried in a circulating air oven at $40 \pm 1^\circ\text{C}$ and crushed into slicer with a particle size of 10 mesh. The powdered plant particles (100 g) were extracted exhaustively with 96% ethanol by percolation, and subsequently the concentration conducted on a rotary evaporator.

Drugs

Penicillin, ampicillin, erythromycin, amoxicillin, levofloxacin, cephalothin and gentamicin were acquired from a local pharmacy. All drugs were dissolved in sterile 0.9% saline solution.

Phytochemical tests

Determination of total polyphenols

The total polyphenol content of plant extracts was measured by Folin-Ciocalteu method (Chandra and Mejia, 2004). The extract were dissolved in distilled water to obtain a final concentration of $200 \mu\text{g ml}^{-1}$. From each solution, a 1 ml aliquot was added to 1 ml of 1 mol L^{-1} Folin-Ciocalteu reagent (Sigma-Aldrich). This mixture remained undisturbed for 2 min before the addition of 2 ml of 20% (w/v) Na_2CO_3 solution and left undisturbed for 10 min. Thereafter, the reading was performed Spectrophotometer Shimadzu, at 757 nm. The calibration curve was obtained with a stock solution of gallic acid (Sigma-Aldrich) ($1000 \mu\text{g ml}^{-1}$), from which dilutions were made at concentrations between 1 and $40 \mu\text{g ml}^{-1}$.

Determination of total flavonoids

The total flavonoids were determined by the method described by Meda et al. (2005). The extracts were diluted with methanol at $1000 \mu\text{g ml}^{-1}$. To the 5 ml of each test solution, was added the same volume of 2% (w/v) AlCl_3 solution in methanol. This mixture remained undisturbed for 10 min before the Ultraviolet (UV) spectrophotometric reading at 415 nm wavelength. The total flavonoids were determined by the calibration curve using quercetin (Sigma-Aldrich) as standard at concentrations between 2 and $30 \mu\text{g ml}^{-1}$.

Determination of condensed tannins

The content of condensed tannins was verified through the method described by Makkar and Becker (1993) wherein 0.25 ml of the sample was added to 1.5 ml vanillin (Sigma-Aldrich) dissolved in methanol (4% w/v) and subsequently in 0.75 ml of concentrated hydrochloric acid (HCl) (37%). After the HCl addition, the tube content was shaken in water bath at 30°C for 3 to 4 seconds before being read on a spectrophotometer at a 500 nm wavelength. Catechin (Sigma-Aldrich) was used as standard at concentrations between 10 and $100 \mu\text{g ml}^{-1}$.

Determination of saponins

The quantification of total saponins followed the method described by Makkar et al. (2007). First, 250 μl of an 8% vanillin solution in ethanol was added to a 250 μl extract solution in 80% methanol; then 2.5 ml of 72% sulfuric acid were added. The tubes were incubated at 60°C in a water bath for 10 min, and then transferred

Table 1. Total content of phytochemical constituents of the ethanolic extract of *S. brasiliensis*.

Phytochemical constituents	Concentration
Total polyphenols	24.51±0.65 ¹
Total flavonoids	14.43±0.44 ²
Condensed tannins	26.31±0.95 ³
Saponins	69.87±0.95 ⁴

¹Gallic acid equivalent (GAE); ²Quercetin equivalent (QE); ³Catechin equivalent (CE); ⁴Disogenin equivalent (DE).

to an ice bath to rest for 4 min. The absorbance reading at 544 nm was performed against a blank consisting of the vanillin solution, 80% methanol and sulfuric acid. The calibration curve was obtained from a disogenin (Sigma-Aldrich) solution at concentrations between 100 and 500 µg ml⁻¹.

Microbiological assays

It were used clinical isolates *Staphylococcus aureus* resistant to ampicillin, ciprofloxacin, cephalixin, erythromycin, penicillin and amoxicillin and *Escherichia coli* resistant to amoxicillin, cephalothin, levofloxacin, chloramphenicol, tetracycline and gentamicin. The minimum inhibitory concentration (MIC) was determined by a microdilution method in 96-well plates using Mueller-Hinton broth (CLSI, 2012). Colonies of microorganisms were suspended in a 0.9% saline solution, and by a spectrophotometric method at 625 nm, the suspension adjusted to a final concentration of 5 × 10⁶CFU mL⁻¹. Serial dilutions of the extract in the range of 1000 to 2.4 µg mL⁻¹ and antibiotics in the range of 2500 to 2.4 µg mL⁻¹ were performed. Dimethyl sulfoxide (DMSO) 10% was included as a negative control. The plates were incubated at 37±1°C for 24 h. Bacterial growth was indicated by addition of 20 µL of 0.01% aqueous resazurin solution (Sigma-Aldrich) with incubation at 37±1°C for 2 h. MIC values were identified as the lowest concentration in which no bacterial growth is visible. Evaluation of extracts as modulators of antibiotic resistance was performed according to Coutinho et al. (2010). The MIC of the antibiotic was determined in presence and absence of sub-inhibitory concentrations (MIC/8) of EESb. Plates were incubated as described earlier and each assay was performed in triplicate.

Acute toxicity

Wistar rats (131 to 258 g) were used in this research. They were maintained under standardized conditions of light (light–dark cycle of 12 h), relative humidity (60% ± 15%) and temperature (25 ± 2°C) with free access to food and water. The animals were acclimatized to laboratory conditions before the commencement of the experiment. All experiments were approved by the Ethics Committee on Animal Research CCTR/UFCG under register no.196/2014. The animals were kept in groups of five per cage. Prior to testing, the animals were fasted for 12 hours. The extract was administered by gavage at a concentration of 2000 mg kg⁻¹, and the animals were maintained under the aforementioned conditions, being observed within 30 min and at 1, 2 and 4 h after the extract administration to observe the emergence of toxic signs (Almeida et al., 1999). At 24, 48 and 72h after the administration of the extract, deaths were recorded and the consumption of water

and food were measured and the animal was weighed. A control group was treated with a saline solution, the same solution used to dilute the extract. After the period of observation, the surviving animals were dissected, including the resection and weighing of organs (liver, kidneys, heart, lungs and spleen), which were also examined macroscopically.

Statistical analysis

The microbiological test results were expressed as geometric means. Two-way analysis of variance followed by Bonferroni post-test was applied. For acute toxicity test, the results were expressed as the mean ± standard deviation (SD) and subjected to an analysis of variance (ANOVA) followed by Dunnett's post-test or a Tukey-Kramer test. The confidence limit was 95% (p < 0.05). The statistical software used was GraphPad Prism-5[®], version 2007.

RESULTS

Phytochemical tests

The total content of the major groups of secondary metabolites present in the extract of *S. brasiliensis* are shown in Table 1. The values were expressed as gallic acid equivalents (GAE) per gram of the crude extract to phenolic compounds, quercetin equivalents (QE) for flavonoids, catechin equivalent (CE) for the condensed tannin and disogenin equivalent (DE) to saponins.

Microbiological assays

The EESb showed efficacy against four strains tested with the lowest MIC values acquired on *S. aureus* strains, SA ATCC 25923 and SA16 strains, sensitive to 7.8 and 15.6 µg ml⁻¹ respectively. However, over the strains of *E. coli*, EESb showed no significant activity (MIC > 1000 µg ml⁻¹). In the assays of modulation of resistance, the EESb showed the best results on strains of *E. coli*, significantly reducing (P<0.001) the MIC of all antibiotics tested. The most pronounced decrease was of cephalothin, whose MIC was reduced from 49.6 to 12.4 µg ml⁻¹. For *S. aureus*, the combination of extract that provided significant results (p < 0.001) was with erythromycin; the MIC decreased from 1000.0 to 793.7 µg ml⁻¹ (Figures 1 and 2).

Acute toxicity

In the acute toxicity test, the rats used showed no behavioral changes after EESb administration at 2000 mg Kg⁻¹ v.o. Feed intake and water remained stable in the control group and the group treated with the extract. However, when macroscopically analyzing the viscera of the animals in the group treated with the extract compared with the control group, the results point to possible hepatotoxic and nephrotoxic action of EESb, considering the weights of the kidneys and liver were

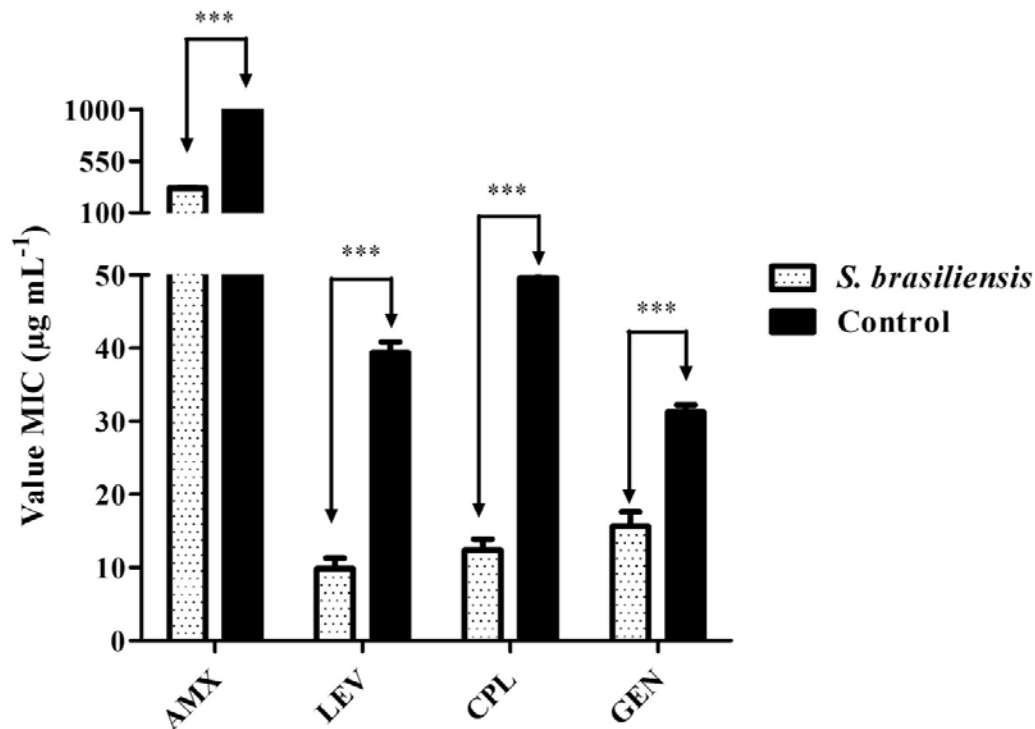


Figure 1. Modification of the antibiotic activity of extract of *S. brasiliensis* against resistant strain of *E. coli*. AMX – Amoxicillin; LEV – Levofloxacin; CPL – Cephalotin; GEN – Gentamicin; *** - Statistically significant with P value <0.001 ; ns - not statistically significant value with $P > 0.05$.

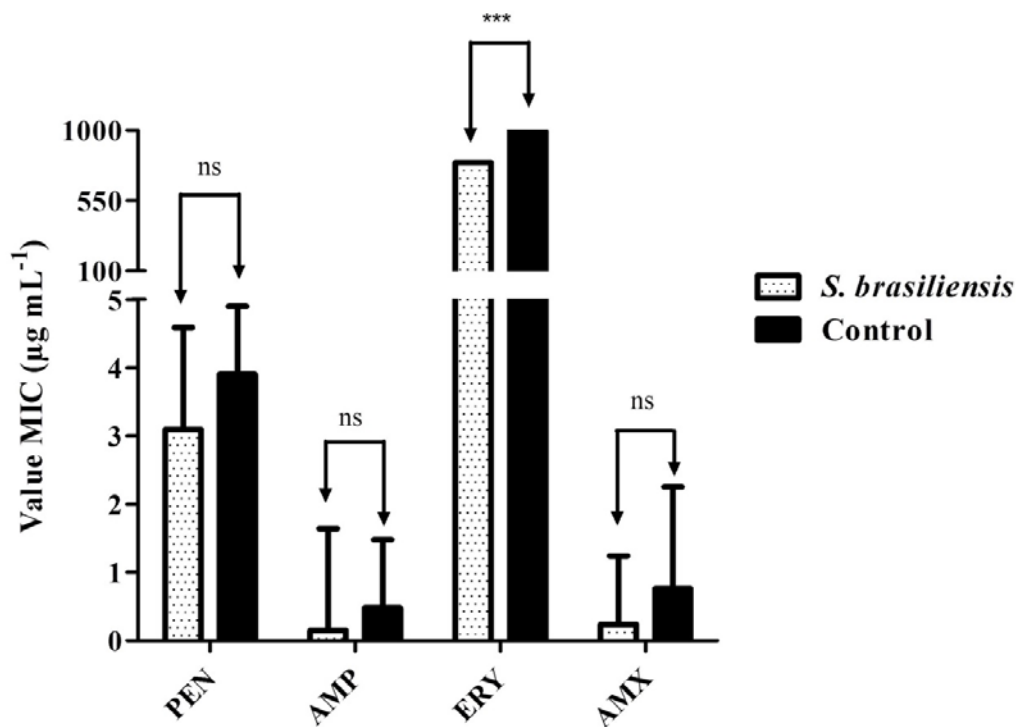


Figure 2. Modification of the antibiotic activity of extract of *S. brasiliensis* against resistant strain of *S. aureus*. PEN – Penicillin; AMP – Ampicillin; ERY – Erythromycin; AMX – Amoxicillin; *** - Statistically significant with P value <0.001 ; ns - not statistically significant value with $P > 0.05$.

significantly decreased ($P < 0.05$). Such toxic effect can be proven only by histopathology. During the observation of the animals after the administration of the extracts, there were no deaths; it was not possible to determine the mean lethal dose (LD_{50}).

DISCUSSION

Phytochemical tests

The concentrations of some substances in the methanol extract of EESb has already been measured by Saraiva et al. (2011), who found a higher concentration of polyphenols (825.65 ± 40.99 mg of tannic acid equivalent per gram of extract) and lower flavonoid concentration (11.29 ± 0.94 mg of rutin equivalent per gram of extract). This difference may be related to the solvent used in the production of extracts; while the aforementioned authors used methanol, this study used ethanol. Other aspects that may also have influenced the difference in the concentration of secondary metabolites in both studies are the collection site of plant material and intrinsic factors such as the stage of plant development and tissues differentiation (Chaves et al., 2013; Mirdehghan and Rahemi, 2007; Ncube et al., 2011).

Microbiological assays

Rios and Recio (2005), suggest that in order to have clinical relevance, the MIC of crude extracts must not be higher than $1000 \mu\text{g ml}^{-1}$. Considering this parameter, the EESb showed efficacy against both strains of *S. aureus*. Extracts of *S. brasiliensis* showed the antimicrobial activity reported in the literature. Chaves et al. (2011) when testing the hydroalcoholic extract, Saraiva et al. (2011) the methanol extract and Saraiva et al. (2013) different proportions of different fractions of methanolic extract showed efficacy against gram positive, gram negative and fungal strains through an agar diffusion test. However, the impossibility of measuring the amount of extract spread on the agar affects the ease of using this method to accurately determine the MIC (Hadacek and Greger, 2000).

The use of plant extracts associated with other natural or synthetic antibiotics is well documented in the literature. This association has shown that some plant extracts seem to work synergistically with the antibiotic used, resulting in better antimicrobial activity against several multidrug-resistant strains (Sato et al., 2004; Aqil et al., 2006; Coutinho et al., 2009a,b, 2010; Ncube et al., 2012; Figueiredo et al., 2013).

The mechanisms related to the synergistic effects of combining antibiotics and extracts relate primarily to factors such as the multiplicity of targets on which the mixture of bioactive compounds can act, including enzymes, receptors, ion channels, transport proteins,

DNA/RNA, antibodies and many others, as well as the suppression of bacterial resistance mechanisms for compounds present in the extract (Imming et al., 2006; Schmidt et al., 2008; Wagner and Ulrich-Merzenich, 2009).

Both the antimicrobial activity and the synergistic effect may be related to the richness of *S. brasiliensis* regarding polyphenolic compounds and saponins since some of these compounds have already been cited as exhibiting such activities (Cowan, 1999; Gibbons, 2005, 2008). Tannins can prejudice the metabolism of bacteria, inhibiting enzymes, oxidative phosphorylation and the electron transport system and neutralizing microbial adhesins and proteins in the cell envelope (Cowan, 1999; Scalbert, 1991). In turn, flavonoids and saponins can destabilize bacterial membranes, breaking the barrier function and forming pores respectively; both of which cause intramembranous leakage of materials (Ikigai et al., 1993; Francis et al., 2002; Cushnie and Lamb, 2005; Sung and Lee, 2008).

Acute toxicity

The low toxicity of extracts of *S. brasiliensis* suggested in this assay has been demonstrated by studies on plants of the same family. For example, Ayoka et al. (2006) evaluated extracts of *Spondias mombin* L., and Konan et al. (2007) assessed a hydroalcoholic extract of *Anacardium occidentale* L. and observed the absence of toxic symptoms or the death of animals. It is suggested that further tests should be performed to confirm the toxicological profile of the plant

Conclusion

The tests performed demonstrated that the extract obtained from the bark of *S. brasiliensis* is an effective antimicrobial, and with the capacity to modulate bacterial resistance activity and may be considered to possess low toxicity. This study represents the first report of these biological activities on the quantitative phytochemistry of ethanol extract of *S. brasiliensis*, and the results that justify the ethnopharmacological use of this plant in Brazilian traditional medicine against certain types of infections. However, further studies are needed in order to understand the mechanism of antimicrobial and modulating action and the toxicological profile of the plant.

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Conflicts of interests

The authors declare that they have no competing interests.

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