Phytochemical screening, antioxidant and antibacterial activities of extracts prepared from different tissues of *Schinus terebinthifolius* Raddi that occurs in the coast of Bahia, Brazil

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ABSTRACT

Background: *Schinus terebinthifolius* is widely used in traditional medicine by Brazilian quilombola and indigenous communities for treatment of several diseases. Extracts from different tissues are being used to produce creams to treat cervicitis and cervicovaginitis. However, most studies are limited to the assessment of the essential oils and extracts obtained from the leaves. **Objective:** The aim was to evaluate antioxidant and antibacterial activities, to assess the phytochemical profile and to quantify total phenolic compounds of various extracts prepared from *S. terebinthifolius* grown in the coast of Bahia, Brazil. **Materials and Methods:** Extracts were obtained by hot continuous extraction (soxhlet) and by maceration. Quantification of phenolic compounds was performed using the Folin-Ciocalteu method and antioxidant properties were assessed by 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. Phytochemical screening was performed as described by in the literature and antibacterial activity against *Enterococcus faecalis* (ATCC 29212) was determined by the microdilution broth assay. **Results:** Extraction method greatly affected the metabolite profile of the extracts. Antioxidant activity varied between 21.92% and 85.76%, while total phenols ranged between 5.44 and 309.03 mg EAG/g of extract. Leaf extract obtained with soxhlet showed minimum inhibitory concentration (MIC) of 15.62 μg/mL, while stem extract obtained by maceration was able to inhibit the growth of *E. faecalis* at 62.5 μg/mL. Stem bark extracts showed a MIC of 500 μg/mL for both extraction methods, while no inhibition was observed for fruit extracts. **Conclusion:** In general, total phenolic content, antioxidant and antibacterial activities were higher in samples obtained by soxhlet. Our results provide important clues in order to identify alternative sources of bioactive compounds that can be used to develop new drugs.

Key words: Aroeira vermelha, bioactive compounds, Brazilian species, medicinal properties, quilombola communities

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INTRODUCTION

The World Health Organization has recommended scientific certification and popular use of medicinal plants in the treatment of several diseases and as starting material for the discovery of new drugs. This includes the development of synthetic and semi-synthetic drugs based on metabolites form plants, animals and microorganisms that exhibit some biological activity.[1-3] A successful approach towards the identification and utilization of medicinal plants in the treatment of diseases is the project named "Farmácia Viva" (Living Pharmacy) developed by the Federal University of Ceará in Brazil. In this project, several plant-based drugs were produced from species which had their efficacy scientifically verified. Some examples include vaginal creams produced from *Schinus terebinthifolius* extracts to be used in the treatment of

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cervicitis and cervicovaginitis and *Maytenus ilicifolia* used to treat gastritis and gastric ulcer.[4]

Africans and indigenous communities were responsible for the cultural and biological knowledge-base of useful plants in Brazil. Quilombola transplanted an African botanical classification system and introduced native Brazilian medicinal plants into their own culture.[5] *S. terebinthifolius* is endemic to South America and it is particularly found in Brazil, Paraguay and Argentina. Its pioneer characteristics and ability to adapt to various environmental conditions has allowed this to occur in various habitats.[6] Essential oil extracted from the seeds is rich in mono and sesquiterpenes which confers several biological activities to the oil.[7-10] The resin produced by *S. terebinthifolius* is used in the treatment of rheumatism and buboes.[11] Stem bark and leaves are also used against many diseases of the urinary tract system, in the treatment of menstrual disorders and inflammations in general.[6,12] In rural areas of Brazil, including quilombola and indigenous communities, medicinal plants are often the only source of treatment available and *S. terebinthifolius* is widely used.[5,13-16]

Recently we have observed an increasing interest to identify extracts can be used to treat infections by species of the genus *Enterococcus*, due to its great clinical importance. *Enterococcus faecalis* is the most common species of this genus to cause infection which has acquired over the years natural resistance to several known antimicrobial agents.[17-19] Species form this genus are commonly found in mammal's intestines and in the female genital tract. *E. faecalis* and *Enterococcus faecium* are the main cause of infections from this genus in humans.[20] Therefore, based on the traditional use of this species and the importance of discovering and characterizing plant-based bio-products that could help to improve the quality of life, especially of poor communities, we set out an experiment to assess the antibacterial activity of *S. terebinthifolius* extracts against *E. faecalis*.

Oxidative stress results from an imbalance between the generation free radicals and the endogenous action of antioxidant defense systems, which is divided into enzymatic and nonenzymatic systems. Nonenzymatic antioxidant defense system is composed by a range of compounds such as α-tocopherol (Vitamin E), β-carotene, sodium ascorbate (Vitamin C) and phenolic compounds which are widely found in medicinal plants.[21-23] The main function of the antioxidant defense system is to inhibit or reduce damages caused by free radicals and reactive oxygen species. These damages are related to a number of chronic diseases, including cancer and some cardiovascular and neurodegenerative diseases.[24] Phenolic compounds are derived from secondary plant metabolism and are essential for plant growth and reproduction. Flavonoids, xanthones, phenolic acids, tannins and tocopherols are the most common natural source of phenolic antioxidants.[25-29]

Although several studies describe the antioxidant activity of *S. terebinthifolius*, the vast majority is limited to the study of essential oils[9,30] and extracts obtained from the leaves.[30-32] Considering the importance of phenolic compounds demonstrated by several studies and in order to identify new sources of compounds with high phenolic content and thus high antioxidant activity, we performed a phytochemical screening, including determination of total phenolic compounds, as well as measured the antioxidant activity of *S. terebinthifolius* extracts obtained from fruits, stem bark, stem and leaves by different extraction methods.

MATERIALS AND METHODS

Biological material

Schinus terebinthifolius fruits, stem, stem bark and leaves were collected in Mata de São João (12.57°S 38.00°W), Bahia, Brazil. This region presents a tropical climate with annual average temperatures of 24°C. The rainy season happens between April and June with annual average rainfall of 1800 mm. We decided to work with *S. terebinthifolius* because this species is used by quilombola and poor communities of that region to treat various diseases. Samples of 14 different specimens were collected on the 16th of February 2010, which correspond to the end of the summer season. Samples were stored at 4°C prior to the analysis. Later, samples were dried in an oven at 105°C for 24 h until constant weight was reached. In this work, the sample called stem corresponds to the branches and petioles of the leaves. Fruits presented a globular shape and red color. Only mature fruits with no visible signals of damage were collected.

Preparation of extracts

Extracts were obtained from two different methods: Continuous extraction using a soxhlet apparatus and maceration as described previously,[23,33] with some minor modifications. For the extraction in soxhlet, approximately 15 g of dried and ground plant material was used. Samples were subjected to continuous extraction using 300 mL of ethanol, and the system remained under heating for 8 h which corresponded to 15 cycles. For the extraction by maceration, approximately 15 g of dried and ground plant material was extracted for 72 h using 300 mL of ethanol, at room temperature. Then, extracts were collected, and the solvent removed under reduced pressure at 40°C using a rotary evaporator (4000 Laborota echo). Samples remained in the exhaust hood at room temperature until all residual solvent evaporate, and the extracts dried completely.

Phytochemical screening

Phytochemical screening of the extracts was performed as described by in the literature with minor modifications.[34,35]

(a) Saponins: Approximately 1 mg of each extract was diluted in 1 mL of distilled water and vortexed. Presence of saponins in the extracts is shown by the formation of abundant and persistent foam. (b) Phenols and tannins: Three drops of ferric chloride (10%, in ethanol) were added to 2 mL of each extract (1 mg/mL, in ethanol). Color of the extracts varying between blue and red was considered indicative of the presence of phenols. The formation of a dark blue precipitate indicates the presence of hydrolysable tannins and the formation of a green precipitate the presence of condensed tannins. (c) Anthocyanins and anthocyanidins, (d) flavones, flavonols and xanthones and (e) chalcones aurones and flavonols: For each extract, the pH of a 2 mL aliquot (1 mg/mL, in ethanol) was adjusted to 3, 8.5 and 11. For aliquots which the pH was adjusted to 3 the appearance of red color was an indication for the presence of anthocyanins, anthocyanidins, chalcones and aurones (c and e). For aliquots which the pH was adjusted to 8.5 the appearance of purple color confirmed the presence of anthocyanins and anthocyanidins (c) in the aliquots that have previously showed red color at pH 3. Aliquots at pH 11 were considered positive for the presence of anthocyanins and anthocyanidins present when showed a purple-blue color or were considered positive for the presence of flavones, flavonols and xanthones (d) when showed a yellow color. Chalcones and aurones (e) were positive when red purple color was formed and the presence of only flavonols assigned when an orange-red color was observed. (f) Free steroids and free tetracyclic triterpenes: About 1 mg of each extract was dissolved using 1 mL of chloroform. Then, 1 mL of acetic anhydride was added and then 3 drops of concentrated sulfuric acid was slowly added. The emergence of evanescent blue color then permanent green was indicative of the presence of free steroids and a brownish red for free pentacyclic triterpenes.

Quantification of total phenols

Quantification of total phenolic compounds was performed using the Folin-Ciocalteu method, with some modifications.[23] Briefly, 100 μL aliquot of each extract (1 mg/mL in ethanol) was mixed with 500 μL of Folin-Ciocalteu reagent and 6 mL of distilled water. After 60 s, 2 mL of 15% Na_2CO_3 were added to the mixture and vortexed for 30 s. Finally, the volume was adjusted to 10 mL with distilled water. Samples were kept in the dark for 2 h. After this period the absorbance was measured using a ultraviolet (UV)-Vis spectrophotometer (850M Analyser) at 750 nm, using as blank a solution containing methanol and all reagents except the extracts. The total phenolic content was determined by interpolating the absorbance of the samples against a linear calibration curve constructed with standard gallic acid (0–600 μg/mL). Analyses were performed in triplicate.

Antioxidant activity

Antioxidant properties of the extracts were assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described previously,[23,33] with some modifications. The consumption of the DPPH free radical was monitored by measuring the decrease in absorbance of the tested solutions of the extracts in different concentrations using a UV-Vis spectrophotometer in 515 nm wavelength, using gallic acid as the standard.

Reaction mixtures were prepared by adding 1 mL of 120 mM DPPH solution to 1 mL of extracts at concentration of 20 μg/mL, which result in a solution, content concentration of extract of 10 μg/mL, and concentration the DPPH equal to 60 mM. Similarly, 1 mL of the DPPH solution (120 mM) was added to 1 mL of the solution of gallic acid. Gallic acid concentrations ranged from 0.2 to 2.4 mg/mL. The reaction mixtures were incubated for 30 min at 25°C in the dark. Then, the absorbance was read at 515 nm, using ethanol as blank.

The percentage of antioxidant activity was obtained by the following equation:

$$AAT(\%) = [Abs_{DPPH} - (Abs_{sample} - Abs_{blank})] \times 100 / Abs_{DPPH}$$

where Abs_{DPPH} is the initial absorbance (60 mM) solution of DPPH and Abs_{sample} is the absorbance of the reaction mixture. Analyses were performed in triplicate.

Antibacterial activity

Antibacterial activity of *S. terebinthifolius* extracts against gram-positive *E. faecalis* (ATCC 29212) was determined by the microdilution broth assay in 96-wells plates as described by Ribeiro,[36] with some modifications. Extracts tested concentrations ranged between 3.9 and 500 μg/mL. Dimethylsulfoxide in water (20% v/v) was used as a negative control and 1% chlorhexidine gluconate was used as a positive control. The inhibitory effect of the extracts on bacteria growth was assessed after 24 h of incubation by visual analysis of the growth in each well. All analysis was performed in triplicate.

Statistical analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences Statistics® (International

Business Machines - IBM) and Microsoft® Excel 2010 programs (Microsoft). Analysis of variance was used to identify statistically significant differences between the samples ($P < 0.05$) followed by Tukey's multiple comparison tests. The analysis results are presented as the mean of replicates ± standard deviations.

RESULTS

Extraction yield

Initially, we evaluated the effect of two extraction methods on the yield of the extracts. Extracts obtained with soxhlet showed lower yields than those obtained by maceration [Table 1]. The largest differences were found in samples of stem and stem bark, which were 3.8 and 3.4-fold higher, respectively, when the samples were extracted by maceration. Differences in yield of extracts obtained from fruits and leaves were less accentuated. Fruits and leaf extracts were only 1.13 and 1.43-fold higher, respectively, when samples were extracted by maceration in ethanol [Table 1].

Antioxidant activity

In vitro antioxidant activity of the extracts was assessed to identify potential sources of substances possibly useful against the deleterious effects of free radicals. All tested samples showed antioxidant activity, presented as percentage of consumption of the radical DPPH, which ranged between 60.37% and 85.76% for extracts obtained by soxhlet and between 21.92% and 82.60% for those prepared by maceration [Figure 1a]. Comparison between extracts obtained from the same tissues, but by different extraction methods, suggests that the antioxidant activity is enhanced for samples obtained by soxhlet. Remarkable differences in antioxidant activity attributed to the extraction methods were observed for leaf samples: Antioxidant activity of extracts obtained by soxhlet was nearly three times higher than it was found for extracts obtained by maceration. Extracts prepared from the stem bark showed the highest consumption of the radical DPPH: Antioxidant activity of the extracts obtained with soxhlet and maceration was 85.76 and 82.60%, respectively. For the stem samples, antioxidant activity was 80.30 and 60.37% for extract obtained by soxhlet and maceration, respectively [Figure 1a].

Table 1: Yield of the extracts* obtained from fruits, stem, stem bark and leaves of *S. terebinthifolius*

Extraction method	Tissue			
	Fruits	Stem bark	Stem	Leaves
Soxhlet	20.17±1.73	5.91±0.56	4.16±0.30	18.23±1.76
Maceration	22.87±1.80	20.14±1.86	15.74±1.12	26.00±2.47

*Data are expressed in percentage (mean±SD). SD: Standard deviation; *S. terebinthifolius: Schinus terebinthifolius*

Total phenolic content

Total phenolic content of the extracts obtained by soxhlet ranged between 5.44 and 309.03 mg EAG/g of extract, while for extracts obtained by maceration varied between 73.90 and 228.51 mg EAG/g of extract [Figure 1b]. Soxhlet extraction was more effective in extracting phenolic compounds than maceration for all tissues, except for fruits. Remarkable differences in total phenolic content attributed to the extraction methods were observed for fruits, stem and stem samples. For the fruits, total phenolic content of the extract obtained by maceration was nearly 20 times higher than it was found for extracts obtained by soxhlet. However, for the stem and stem bark samples total phenolic content of the extract obtained by soxhlet was nearly two times higher than it was found for extracts obtained by maceration. Extracts prepared from the stem bark showed the highest total phenolic content 309.03 and 228.51 mg EAG/g of extract for the samples obtained by soxhlet and maceration, respectively. Nearly no difference was found for leaves extracts: Total phenolic content

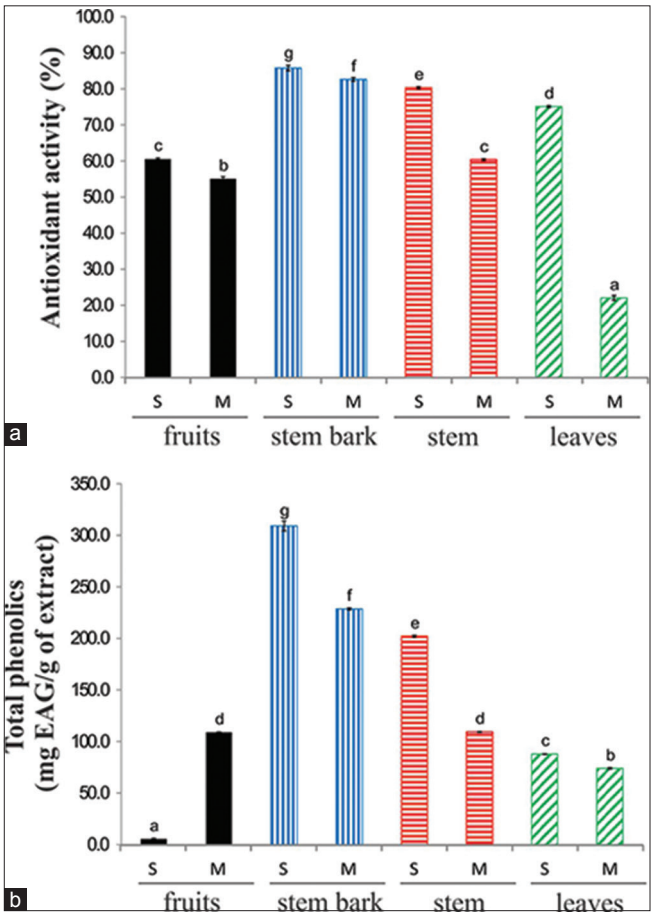

Figure 1: (a) Antioxidant activity (%) and (b) total phenolic content (mg EAG/g of extract) of the extracts obtained from fruits, stem, stem bark and leaves of *Schinus terebinthifolius* by soxhlet (S) and maceration (M). Different letters on the bars indicate significant differences ($P < 0.05$)

87.70 and 73.90 mg EAG/g for the extracts obtained by soxhlet and maceration, respectively. Samples of stem and stem bark showed the highest total phenolic content and antioxidant potential, while the leaves and fruits extracts showed relatively high values of antioxidant activity, but the content of total phenolic content did not show the same pattern [Figure 1b].

Phytochemical screening

Free steroids were only detected in the samples obtained by maceration while pentacyclic triterpenes were mostly found in the extracts obtained by soxhlet. This already suggests that the extraction method plays an important role in the metabolite profile of the extracts. Saponins were detected in all fruits and stem bark samples, but they were not detected in extracts obtained from the leaves. Among stem samples, saponins were only found in the extracts obtained by soxhlet. Flavonols were detected in the stem bark extract obtained by maceration while chalcones and aurones were only identified in the stem bark extract obtained by soxhlet. Anthocyanins, anthocyanidins, flavones, and xanthones were detected in extracts from fruits, leaves and stem, but were not found in extracts of the stem bark. Curiously, these extracts contained rather anthocyanins, anthocyanidins or flavones and xanthones, but never both group of compounds. Phenolic compounds were detected in all extracts. None of the methods effectively extracted condensed tannins [Table 2].

Antibacterial activity

Leaf extract obtained by soxhlet showed the highest inhibitory activity on the growth of *E. faecalis*. This extract showed the minimum inhibitory concentration (MIC) equal to 15.62 μg/mL. The stem extract obtained by maceration was able to inhibit the growth of bacteria *E. faecalis*, but at higher concentration (62.5 μg/mL). Interestingly, leaf extract obtained by maceration and stem extract obtained by soxhlet showed no activity (MIC > 500 μg/mL). Stem bark extracts showed a MIC value equal to 500 μg/mL for both methods of extraction, while no inhibition of the bacterial growth was observed for the extracts obtained from the fruits [Table 3].

DISCUSSION

Brazil possesses an extremely rich plant biodiversity, which encompasses potentially useful species in a wide range of applications, including agriculture, pharmaceutical, cosmetics, textiles and food industries.[37] This has led to a growing interest of research groups seeking to scientifically validate the therapeutic potential of native species.[36,38,39] Brazil has more than 3.000 quilombolas communities and only a few ethnobotanical studies have been conducted with these groups to provide information about their use of medicinal plants.[14-16]

Medicinal plants display large amounts of antioxidants compounds such as Vitamins C and E, and carotenoids. However, the antioxidant properties of a given extract are mainly due to the presence of phenolic compounds, such as flavonoids and phenolic acids.[40-44] Antioxidant activity of extracts prepared in ethanol, dichloromethane, as well as of the essential oils obtained from leaves of *S. terebinthifolius* have been reported.[6,9,30,32,45] It has been reported that extracts prepared with ethanol show higher antioxidant potential, therefore justifying the use of ethanol as the extraction solvent in this study. Essential oils and the dichloromethane extracts showed antioxidant activity of 75.2 and 72.7%, respectively, at a concentration of 400 μg/mL.[30] Considering that the concentration of the extracts used in this study for the evaluation of the antioxidant activity was 10 μg/mL, results described in this work demonstrate greater antioxidant potential than those reported in the literature.

In a qualitative assay, Ceruks *et al.*[11] reports the evaluation of antiradical potential of phenolic compounds isolated

Table 2: Phytochemical profile of the extracts obtained from fruits, stem, stem bark and leaves of *S. terebinthifolius*

Class of metabolites	Maceration (M)				Soxhlet (S)			
	Fruits	Leaves	Stem	Stem bark	Fruits	Leaves	Stem	Stem bark
Saponins	+	−	−	+	+	−	+	+
Phenolic compounds	+	+	+	+	+	+	+	+
Hydrolysable tannins	+	−	+	+	−	+	+	+
Condensed tannins	−	−	−	−	−	−	−	−
Anthocyanins and anthocyanidins	+	−	+	−	−	+	−	−
Flavones and xanthones	−	+	−	−	+	−	+	−
Chalcones and aurones	−	−	−	−	−	−	−	+
Flavonols	−	−	−	+	−	−	−	−
Free steroids	−	+	+	+	−	−	−	−
Free pentacyclic triterpenes	+	−	−	−	+	+	+	+

S. terebinthifolius: Schinus terebinthifolius

Table 3: Antibacterial activity of the extracts obtained from fruits, stem, stem bark and leaves of *S. terebinthifolius* against *E. faecalis* (ATCC 29212)

Extraction method	Minimum inhibitory concentration* (µg/mL)			
	Fruits	Stem bark	Stem	Leaves
Soxhlet	>500	500	>500	15.62
Maceração	>500	500	62.5	>500

*Chlorhexidine gluconate 1% was used as positive control. *S. terebinthifolius: Schinus terebinthifolius; E. faecalis: Enterococcus faecalis*

from the leaves of *S. terebinthifolius*, suggesting that the isolated compounds (three flavonoids and two gallic acid esters) are likely to be responsible for the antiradical potential determined in the extracts. Natural compounds are responsible for the protective effect against oxidative damages that plants are subjected to. These oxidative damages can be generated by many factors, but phenolic compounds are often associated with the protective effect of plant. Therefore, to determine the content of phenolic compounds in plant is an important step in the identification of possible sources of bioactive compounds. All extracts evaluated in this study showed high levels of phenolic compounds compared to data available in the literature for other species.[39,46-48] Higher levels of phenolic compounds were observed in samples obtained by soxhlet, therefore justifying their higher antioxidant activity. Continuous extraction via soxhlet seems to provide more promising extracts for studies aimed at identifying new sources of bioactive compounds.

Despite the fact that phenolic compounds contribute immensely to the antioxidant potential of the extracts, other compounds rather than phenols show high antioxidant activity such as ascorbic acid, carotenoids and fat-soluble vitamins.[49] Triterpenes and biflavonoids are the most abundant compounds present in the Anacardiaceae family, but some other compounds such as phenols and cinnamic acid derivatives have been identified.[50] It has been reported that ethanolic extracts obtained from the barks of *S. terebinthifolius* contained of phenols, triterpenes and anthraquinones. However, flavones, xanthones, flavonoids, free steroids, triterpenes and anthraquinones were found when extracts were prepared in hexane. On the other hand, ethanolic extracts of the leaves showed positive results for phenols, flavones, flavonoids, xanthones, anthocyanidins, flavanones, and free steroids.[51]

High incidence of drug-resistant pathogens has increased the attention on several medicinal plants and their metabolites for antimicrobial properties. A recent paper has reviewed the state-of-the-art of the research on antibacterial agents from native Brazilian plant species related to *E. faecalis* infections.[52] In addition, seven plant extracts obtained from Brazilian Amazon rain forest and Atlantic forest were found to be effective against *E. faecalis*.[17] Extracts of *Salvadora persica* prepared with ethanol and chloroform showed antimicrobial activity against *E faecalis* and *Candida albicans* with concentrations ranging from 125 to 1000 µg/mL, while in our study extracts showed antimicrobial activity against *E faecalis* with concentrations ranging from 15.62 to 500 µg/mL.[53] This highlights the potential of *S. terebinthifolius* as an important source of new antimicrobial compounds.

In this study, we reported the antimicrobial activity of several extracts of *S. terebinthifolius* against *E faecalis*. Extracts obtained from leaves of *S. terebinthifolius* had great antimicrobial activity when prepared by soxhlet. This is in agreement with the results presented by Gundidza *et al.*,[10] which examined the antimicrobial activity extracts from the leaves of this species, demonstrating that the biological activity is related to compounds present in the obtained extracts. Our results show that *S. terebinthifolius* has a great potential as source of bioactive compounds, not only in the leaves, but also in different tissues. Further studies are recommended for evaluation of these extract as an effective antibacterial agents.

CONCLUSION

We have demonstrated that extracts obtained from fruits, stem bark, stems and leaves of *S. terebinthifolius* display a variety of secondary metabolites, including high content of phenolic compounds, which may be responsible for the detected antioxidant activity. We also showed that the extraction method influences the metabolite profile of the samples, both qualitatively and quantitatively. Additionally, we demonstrated that maceration is more efficiently extracting free steroids, while soxhlet would be the choice for studies aiming at obtaining increased levels free pentacyclic triterpenes. However, based on our antioxidant and antibacterial results we suggest that extraction via soxhlet provides more promising results for studies aimed at identifying new sources of bioactive compounds.

The mechanism underlying bacterial growth inhibition is a complex trait, which may involve synergistic effects of the metabolites present in plant extracts. Due to the importance of finding new sources of antibacterial compounds, along with the increasing antibiotic resistance, our results stimulate further studies to evaluate the antioxidant and antimicrobial activity of compounds isolated from *S. terebinthifolius*, particularly of the extract obtained from the leaves by continuous extraction in soxhlet.

Most importantly, our results add important information regarding the validation of the traditional use of *S.*

terebinthifolius, which has been used in the form of lotions, gels and soaps by rural, indigenous and quilombola communities due to its antimicrobial properties.

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