Evaluation of antimicrobial activity of the association of crude extracts of *Schinus terebinthifolius* Raddi and *Lippia sidoides* Cham. against clinical isolates of *Staphylococcus aureus*

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In search of new therapeutic alternatives to multidrug-resistant bacteria from plant extracts, this study aimed to evaluate the antimicrobial activity of ethanol extracts from *Schinus terebinthifolius* Raddi and *Lippia sidoides* Cham., alone and in associations with antibiotics, against clinical isolates of *Staphylococcus aureus* of 16 different sites. In qualitative evaluation, commercial discs of oxacillin, erythromycin and clindamycin were used and disks containing the crude extracts alone and in combination. In the characterization of the strains for resistance it has been observed that seven were resistant to oxacillin, seven D-test positive and six resistant to clindamycin and erythromycin. Associated extracts were less efficient than the association *Lippia sidoides* Cham. and antibiotics, except for resistant microorganisms. When antibiotics were associated with *Schinus terebinthifolius* Raddi extract most resistant isolates have become sensitive. When combined, the extracts obtained the best results, increasing the initial activity.

**Keywords:** *Lippia sidoides* Cham; *Schinus terebinthifolius* Raddi; *Staphylococcus aureus*; synergism.

1. Introduction

The medicinal plants have traditionally been used for treating various diseases. Its applications are vast and range from combat cancer to the pathogenic micro-organisms. The plants, besides their use in folk medicine for therapeutic purposes, have contributed over the years to obtain various drugs, widely used in the clinic [1].

Knowledge about medicinal plants symbolize often the only therapeutic resource of many communities and ethnic groups. It can be considered as a medicinal plant that plant administered in any form and by any route to the man, exercising some sort of pharmacological action. The use of plants in the treatment and cure of diseases is as old as the human species [2, 3].

The evaluation of the therapeutic potential of medicinal plants and some of its constituents, such as flavonoids, alkaloids, triterpenes, sesquiterpenes, tannins, lignins, has been the subject of incessant studies, which have been proven pharmacological actions through preclinical testing. Many of these substances have great potential to be taked as medicinal agents. In Brazil, the use of medicinal plants was mainly disseminated by indigenous culture. Being a country with is rich in diversity, whose territory has seven major biomes being designated as Amazon forest, “cerrado”, atlantic forest, “pantanal”, “caatinga”, “pampa” and mangrove, is a rich source of therapeutic products [4].

The main northeastern Brazil ecosystem is the biome of the “caatinga”, an indigenous word that means open forest, so named because of their appearance during the dry season. It is exclusively Brazilian and consists of extensive semi-arid plains and plants in the surrounding area that form an integral part of the culture of these people. In this region, many plants such as rosemary, mastic, olive, “quixaba”, pomegranate, are known for their phytotherapeutic activities [5].

The “aroêira-vermelha”, *Schinus terebinthifolius* Raddi, is a kind of family *Anacardiaceae*, native to Brazil. It is a deciduous tree, whose size varies according to the region where it is located [6]. It is used to treat wounds and ulcers of the skin and mucous membranes against infections of the respiratory system, the digestive system, genitourinary tract, hemoptysis and metrorrhagia [7]. Although more frequent along the Brazilian coast from Ceará to the south, the *Schinus terebinthifolius* is inside, as evidenced by the historical works of use from the Amazon and Minas Gerais, among other regions [8]. Presents medicinal properties; the parts used are: bark, leaves and fruit. It is astringent, antidiarrheal, de purative, diuretic and febrifuge. The bark used for fever, hemoptysis and uterine diseases in general, extracts an oil used against tumors and diseases of the cornea. To the fruit and its essential oil is assigned antimicrobial activity against Gram-positive bacteria and anti-inflammatory for enzyme inhibition. This anti-inflammatory action has specific inhibitory character and is directly related to triterpenoids present in the fruit. The aerial parts of the plant show antioxidant properties [9, 10]. Studies with *Schinus terebinthifolius* extracts revealed action against *Enterococcus faecalis* [11] e *Staphylococcus aureus* [12, 13].

*Lippia sidoides* Cham., popularly known as rosemary pepper, is a shrub native to semi-arid region of northeastern Brazil, widely used in folk medicine as an antiseptic for local use on the skin and mucous membranes. The popular use
has been supported by several studies that demonstrate its potential as a natural antimicrobial agent, alternatively to synthetic drugs [14].

Rosemary Peppermint belongs to the Verbenaceae family is a deciduous shrub, erect, much branched and brittle, 2-3 m tall, own vegetation of the Brazilian semi-arid northeast. The leaves are very aromatic and spicy, petiolate, 2-3 cm long. The flowers are small, whitish, gathered in cobs of short axis on the armpits of leaves. It can be multiplied by cutting using preferably thinner branches [15].

The extract of Lippia sidoides Cham. has a strong action against fungi, bacteria, such as Staphylococcus aureus, Streptococcus mutans, responsible for dental caries; Corynebacterium xerosis, which causes smelly armpits and feet; Candida albicans, found in infections of the mouth and vaginal discharge; Trichophyton rubrum and Trichophyton interdigitale mycoses agents on the skin. It presents molluscicidal action against the snail Biomphalaria glabrata, schistosomiasis host, and action larvicide against Aedes aegypti, which transmits “dengue” [16].

The Ministry of Health defined hospital infection as that acquired after admission of the patient and whose manifestation occurred during hospitalization or immediately after discharge. The occurrence of hospital infection determines an increase in length of stay (four days on average), the hospitalization costs and mortality rates in the population affected [17]. Two pathogens are often cited as responsible for hospital infections, they are Staphylococcus aureus and Pseudomonas aeruginosa [18].

Staphylococcus are Gram-positive cocci and catalase, with approximately 0.5 to 1.5 m in diameter, real estate, not sporulated and generally not encapsulated. The genus Staphylococcus has 33 kinds, and 17 of them can be isolated from human biological samples. The species of major medical interest, especially in nosocomial environment is S. aureus, which is often associated with various infections in humans [19, 20].

The distribution of S. aureus is very large, since this bacterium is able to significantly resist desiccation and cold, can remain viable for long periods in particles of dust. This micro-organism can be found in the environment of circulation of the human, and the man himself is its main reservoir, besides being present in various parts of the body such as nasal cavity, throat, intestines and skin [21]. Nasal colonization is devoid of symptoms, that is, the individual does not develop infection. This asymptomatic colonization has great clinical importance, since, with the colonized nostrils, the individual contaminates their own hands and becomes bacteria transfer vehicle in the mechanism infection by contact. According to some studies, the nasal entainment also contributes to transmission of the bacteria by air dissemination [22]. This microorganism is frequently isolated from infected surgical wounds, which may represent foci for the development of systemic infections. Staphylococcal pneumonia is usually seen in the elderly. Nosocomial pneumonia produced by S. aureus occurs in cases of chronic obstructive pulmonary disease (COPD), aspiration and intubation, and the underlying malignant diseases are recognized as risk factors for the development of bacteremia [21, 23].

The efficiency of the dissemination of S. aureus is due in part to the versatility of this micro-organism. The ability to quickly adapt to different environments often hostile due to pH, moisture, osmolality or nutrient deficiency, enables not only the colonization of man and the environment around them, creating reservoirs of cells capable to colonize others [24]. The analysis of S. aureus invasion mechanism shows that, at first, this bacterium adheres to the skin or mucosa to then break the epithelial barriers, compromising structural intercellular bonds such as ankylos and desmosomes. After the invasion of the epithelium, the micro-organism uses various strategies to allow their survival and proliferation in the host organism. These strategies are related to complement opsonization, phagocytosis neutralization and inhibition of humoral and cellular immune responses [25]. The high infection potential is not restricted to its ease of propagation and dissemination in the tissue, but also the production of molecules with high pathogenicity, which include enzymes and toxins. The beta-lactamases, coagulases, hyaluronidases and catalases are some of the enzymes produced for this purpose. Besides these, the bacterium also produces DNAse, lipases, proteases and esterases. Among the toxins produced by these pathogens include the following: alpha, beta and gamma toxins, leukocidin, the esfoliatina, which is often associated with various infections in humans [19, 20].

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In search of new therapeutic alternatives to multi-resistant bacteria from plant extracts, this study aimed to evaluate qualitatively and quantitatively the antimicrobial activity of ethanol crude extracts of Lippia sidoides Cham. and Schinus terebinthifolius Raddi, separately and in combination, against clinical isolates of Staphylococcus aureus characterized for sensitivity and resistance to antibiotics of clinical use.

2. Material and methods

2.1 Obtaining samples of vegetables

Samples of leaves of Lippia sidoides Cham. was collected at the Agronomic Institute of Pernambuco - IPA. The identification was carried out and the excixata of the species is deposited in the Herbarium of the Biological Sciences Center of the Federal University of Pernambuco (UFPE), under number 70207. The sample of Schinus terebinthifolius Raddi shell was obtained by woodsmen of the Metropolitan Region Recife. The samples were dried at 45 °C until complete dryness, and processed in a Wiley mill to obtain a powder.
2.2 Obtaining plant extracts

Taken 100 g of dry plant sample and added 200 mL of PA Ethanol for three consecutive times. Every addition of solvent made, the set (plant-solvent) was taken to the shaker table for 2 hours to optimize the removal of the active ingredients. After each extraction, the material was filtered on filter paper and in the end the total was evaporated to dryness in a rotavaporator to 45 °C with 50 RPM.

2.3 Micro-organisms

*Staphylococcus aureus* isolates used were from the Department of Antibiotics Collection of Federal University of Pernambuco and are presented in Table 1.

Table 1 Clinical isolates of *Staphylococcus aureus*, registration number in the Culture Collection of the Department of Antibiotics and site of isolation.

<table>
<thead>
<tr>
<th>Number</th>
<th>Micro-organisms</th>
<th>Isolation site</th>
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</thead>
<tbody>
<tr>
<td>UFPEDA 01</td>
<td><em>Staphylococcus aureus</em></td>
<td>Urine</td>
</tr>
<tr>
<td>UFPEDA 670</td>
<td><em>Staphylococcus aureus</em> (A18)</td>
<td>Urine</td>
</tr>
<tr>
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<td><em>Staphylococcus aureus</em> (A19)</td>
<td>Blood</td>
</tr>
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<td><em>Staphylococcus aureus</em> (A20)</td>
<td>Catheter tip</td>
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<td><em>Staphylococcus aureus</em> (A39)</td>
<td>Secretion of Ulcer</td>
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<td>UFPEDA 700</td>
<td><em>Staphylococcus aureus</em> (A48)</td>
<td>Urine</td>
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<td>UFPEDA 707</td>
<td><em>Staphylococcus aureus</em> (A55)</td>
<td>Tracheal secretion</td>
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<tr>
<td>UFPEDA 711</td>
<td><em>Staphylococcus aureus</em> (A59)</td>
<td>Surgical Wound Secretion</td>
</tr>
<tr>
<td>UFPEDA 718</td>
<td><em>Staphylococcus aureus</em> (A66)</td>
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<td><em>Staphylococcus aureus</em> (A67)</td>
<td>Surgical Wound Secretion</td>
</tr>
<tr>
<td>UFPEDA 725</td>
<td><em>Staphylococcus aureus</em> (5563)</td>
<td>Catheter tip</td>
</tr>
<tr>
<td>UFPEDA 728</td>
<td><em>Staphylococcus aureus</em> (5201)</td>
<td>Oropharynx</td>
</tr>
<tr>
<td>UFPEDA 729</td>
<td><em>Staphylococcus aureus</em> (5199)</td>
<td>Nasal secretion</td>
</tr>
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<td><em>Staphylococcus aureus</em> (5660)</td>
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</tr>
<tr>
<td>UFPEDA 731</td>
<td><em>Staphylococcus aureus</em> (5107)</td>
<td>Surgical Wound Secretion</td>
</tr>
<tr>
<td>UFPEDA 732</td>
<td><em>Staphylococcus aureus</em> (179)</td>
<td>Bone fragment</td>
</tr>
</tbody>
</table>

2.4 Evaluation of Antimicrobial Activity

2.4.1 Agar diffusion technique

It was applied the defined and standardized technique for Clinical Laboratory Standards Institute (CLSI), to confirm the existence of activity [28]. The micro-organisms used had 18-24 hours of growth, and were suspended in physiological saline with a concentration equivalent to the standard 0.5 McFarland. The solutions of the extracts used for this test were made with concentrations of 200,000 μg/mL. Paper discs were soaked with 10 μL of solutions were made only controls soaked in ethanol, and dried in an oven of 30 °C for 20 minutes. Oxacillin, Erythromycin and Clindamycin discs were used as standards and for characterizing micro-organisms. It was also evaluated the combination of the extracts tested with each other and with the antibiotic disks following the same procedure: soaking the commercial discs in 10 μL of solutions and drying them in an oven of 30 °C for 20 minutes. The reading test was performed after 18-24 hours of incubation in an oven of 35 °C. The whole experiment was done in triplicate.

2.4.2 Broth Microdilution test

The technique described by CLSI was used [29]. The solutions of the extracts were prepared in penicillin sterile vials with cover at the following concentrations: Mother Solution 1 (SM1): 20 mg of crude extract *Lippia sidoides* Cham. + 1mL of ethanol (20,000 μg / mL); Mother solution 2 (SM2): 20 mg of crude extract of *Schinus terebinthifolius* Raddi + 1mL ethanol (20,000 μg / mL); Mother Solution 3 (SM3): 1mL of the SM1 + 1mL of the SM2 (40,000 μg / mL); Mother Solution 4 (SM4): 0.4 mL of SM1 (40%) + 0.6 mL of SM2 (60%) (20,000 μg / mL); Mother Solution 5 (SM5): 0.6 mL of SM1 (60%) + 0.4 mL of SM2 (40%) (20,000 μg / mL). The microplates with 96 wells were prepared so that each well had at the end volume of 100 μL and different concentrations of the extracts, ranging from 2000 μg / ml to 3.9 μg / mL. Later they were inoculated 10 μL of suspensions of micro-organisms. After the period of bacterial incubation, 20 μL of a 0.01% resazurin solution were added to each well, and reincubated on bacteriological incubator at 35 °C for two hours [30]. The wells that acquire a pink color indicates the presence of viable cells, while in wells where no change in dye color is interpreted as absence of viable cells, indicating inhibition of cell growth by the extract. The MIC (Minimum Inhibitory Concentration) refers to the last blue line well in direction left - right. For confirmation of bacterial death, MBC (Minimum bactericidal concentration), withdrew 1μL of controls wells and all situations in

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which there was no apparent bacterial growth demonstrated by resazurin and plated the contents in petri dishes containing agar Müller Hinton, being the same incubated for 24 hours at 35 °C. The absence of bacterial growth indicates the MBC.

3. Results and discussion

3.1 Agar Diffusion Test

Resistance to oxacillin was observed in seven strains (671, 719, 725, 728, 729, 731 and 732). The induced resistance to clindamycin (Test D +) was also observed in seven other strains (670, 691, 700, 718, 719, 729 and 730). And only six strains (671, 707, 725, 728, 731 and 732) were resistant to Clindamycin and Erythromycin. Resistance to oxacillin and induced resistance to clindamycin was observed in 43.75% of analyzed strains while the resistance to Clindamycin and Erythromycin was simultaneously observed in 37.50%.

The ethanolic extract of *Lippia sidoides* showed inhibition halos with results ranging between 13 and 31 mm diameter facing the analyzed strains. In a previous study Oliveira et al. (2006) [31] evaluated the activity of the essential oil of *Lippia sidoides* in inhibiting the growth of *Staphylococcus aureus* strains and inhibition halos was observed between 15 and 21 mm diameter. And in the study, Silva et al. (2010) [16] using methanolic extract of leaves *Lippia sidoides* against twenty isolates of *S. aureus* have been observed by measuring inhibition zones between 9 and 27 mm diameter. These results confirm those obtained.

The results of *Schinus terebinthifolius* extract were kept between 19 and 28 mm in research done of the essential oil of *S. terebinthifolius* were observed inhibition halos of 21 mm in diameter and between 14 and 20 mm against *S. aureus*, and these results similar to those obtained in this study [32, 33].

The *Lippia sidoides* Cham. extracts and *Schinus terebinthifolius* Raddi presented, to all strains, best activity when combined with halo between 27 and 40 mm while statements separately show halos between 13 and 31 mm in diameter (Fig. 1).

![Fig 1. Comparison of diffusion test results agar extracts of *Lippia sidoides* Cham. and *Schinus terebinthifolius* Raddi, isolated and associated manner.](image)

It was also observed (Fig. 2a), that associations of *L. sidoides* Cham. extract with *S. terebinthifolius* Raddi were less efficient than *L. sidoides* Cham combination with antibiotics selected except for the micro-organisms were resistant to thereof (671, 725, 728, 731, 732). Being then the association of *L. sidoides* Cham. with antibiotics the most effective at 68.75% of cases. The association of *Lippia sidoides* Cham. with *Schinus terebinthifolius* Raddi achieved better results against the strains analyzed than *Schinus terebinthifolius* association with oxacillin and erythromycin. *Schinus terebinthifolius* associated with Clindamycin was more effective against six of the strains studied (37.5%), with two results equal to *Lippia sidoides* Cham. association with *Schinus terebinthifolius* Raddi (Fig. 2b).

Other authors, making several associations between antibiotics and plant extracts, they found values approximate the association of *Lippia sidoides* Cham. with antibiotics and above *Schinus terebinthifolius* association with antibiotics, such as Braga et al. (2005) [34] with 73% of synergistic interactions of the methanol extract of *Punica granatum* with six antibiotics (chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin) against clinical isolates of *S. aureus* MRSA and *S. aureus* MSSA; Aiyegoro et al. (2009) [35] with 61.7% of synergism among the combinations tested of *Helichrysum longifolium* extract with six antibiotics (penicillin G, amoxicillin, chloramphenicol, oxytetracycline, erythromycin and ciprofloxacin) against various Gram-negative and Gram-positive.

When only compared the associations made with antibiotics, in association with *S. terebinthifolius* all strains resistant to oxacillin and clindamycin have become sensitive, two resistant to erythromycin become sensitive and four had intermediate resistance. *L. sidoides* associated presented two sensitive isolates to oxacillin and one to the Clindamycin and Erythromycin.
3.2 Microdilution test Broth

The ethanolic extract of *Lippia sidoides* Cham. showed MIC ranging between 125 and 500 μg / mL and MBC between 125 and 1000 μg/mL (Table 2).

**Table 2** Minimum inhibitory concentrations (MIC) and minimum bactericidal (CMB) of ethanolic extracts of *Lippia sidoides* Cham. and *Schinus terebinthifolius* Raddi and their associations.

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>Crude extract of <em>Lippia sidoides</em> Cham.</th>
<th>Crude extract of <em>Schinus terebinthifolius</em> Raddi</th>
<th>Association 100:100</th>
<th>Association 40:60</th>
<th>Association 60:40</th>
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<td></td>
<td>CMI μg/mL</td>
<td>CMB μg/mL</td>
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</table>

The *Schinus terebinthifolius* Raddi extract showed variable results MIC also between 125 and 500 μg / mL and MBC between 500 and 2000 μg / ml against the tested clinical isolates (Table 2). Tonial (2010) [36] studying the antimicrobial activity of methanolic extract of *Schinus terebinthifolius* Raddi obtained MIC 2300 μg / mL for *Staphylococcus aureus*, being a lower result than achieved in this study. Dourado (2012) [33], evaluating the essential oil antimicrobial activity of *Schinus terebinthifolius* Raddi against *Staphylococcus aureus*, found MIC 10.92 μg / mL and MBC 21.84 μg / mL, more efficient results than those observed in this study.

Comparing the MIC results between the two extracts studied in ten of the strains evaluated the MIC was the same for both extracts. Of the remaining six, four showed better results for the *Lippia sidoides* Cham. extract and two to *Schinus terebinthifolius* Raddi. As to the results of the MBC, it was observed that the *Lippia sidoides* extract Cham. was more effective against all strains tested (Table 2).

The association 100:100, *Lippia sidoides* Cham. and *Schinus terebinthifolius* Raddi presented MIC ranging between 125 and 500 μg / mL. While the association 40:60 presented results between 125 and 1000 μg / mL. While the association 60:40 achieved the best results, with MIC variable between 62.5 and 250 μg / mL (Tab. 2). It was found that the association 60:40 introduced more effective results of MIC for all strains tested, with two strains equal to the result of the association 100:100 and two at the 40:60 association.

The assessment of MBC, it can be observed in Table 2 results between 250 and 1000 μg / ml for associating 100:100, between 250 and 2000 μg / ml and 40:60 for the association between 125 and 1.000 μg / ml for association 60:40. When comparing the results show that with respect to the MBC parameter, the association 60:40 is also more efficient than the other two evaluated, and the best results in all strains tested.
4. Conclusions

The ethanolic extract of Schinus terebinthifolius Raddi was more efficient compared to the micro-organisms tested for the ethanolic extract of Lippia sidoides Cham. in the agar diffusion test. In combination, the extracts have enhanced antimicrobial activity against all strains of Staphylococcus aureus tested. The associated extracts were less efficient than the association Lippia sidoides Cham. and antibiotics, except for micro-organisms that were resistant, the association held between S. terebinthifolius Raddi and antibiotics performed better when compared with the combination of L. sidoides Cham. extract and antibiotics for resistant micro-organisms.

In the evaluation of Minimum Inhibitory Concentration, the extracts obtained similar results in 62.5% of the strains. The Lippia sidoides Cham. extract obtained more efficient results of MBC in all tested strains. The association 60:40 Lippia sidoides / Schinus terebinthifolius presented the most effective results of MIC and MBC for all strains tested.

Medicinal plants can be an important adjunct of antibacterial drugs already known in the treatment of infectious diseases and control of bacterial resistance.

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