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Synergistic antibiotic activity of volatile compounds from the essential oil of *Lippia sidoides* and thymol

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ABSTRACT

Lippia sidoides Cham. (Verbenaceae) is used in the folk medicine as topical antiseptic in skin and mucous membranes and its therapeutic effect is attributed to the thymol presence. The objective of this work was to verify the chemical composition and antibiotic modifying activity of the essential oil extracted from the leaves of *L. sidoides* and its major component thymol. The essential oil was obtained by hydrodistillation and analyzed by GC/MS. The synergistic activity was evaluated using gaseous contact method. The essential oil was obtained (yield of 1.06%) and the GC/MS analysis identified the main constituents: thymol (84.9%) and p-cymene (5.33%). The antibiotic modifying activity was verified using the minimal inhibitory dose method and gaseous contact. It verified the interference of essential oil and thymol against all tested aminoglycosides. There were no statistical differences between the activity of the essential oil and thymol against *Pseudomonas aeruginosa*, indicating this to be the responsible composition for such activity. However, the oil was shown more effective when compared to the thymol against *Staphylococcus aureus*. The essential oil of *L. sidoides* and its major component thymol influence the activity of aminoglycosides and may be used as adjuvant in antibiotic therapy against respiratory tract bacterial pathogens.

1. Introduction

Staphylococcus aureus and *Pseudomonas aeruginosa* plow the most common bacterium isolated from the sputum [1,2] and that usually colonize or infect the upper respiratory tract [3]. Several works tell that those bacterial species are resistant to most of the antimicrobial used at the clinic treatment [4,5].

With the growing incidence of infections resistant to antibiotics, an arsenal of either new agents of the supplementation of current antibiotics was needed. According to Daferera et al. [6], the use of essential oils as antimicrobial agents offers a low risk of development of microbial resistance, because of the presence of different compounds present in the oil. This antimicrobial activity can be related to different action mechanisms, avoiding the adaptation of the microorganisms. So, essential oils assume important role in the combat to the development of microbial resistance [7].

The bush *Lippia sidoides* Cham. (Verbenaceae), popularly known, in Brazil, as "alecrim pimenta", is native of the semiarid areas of the Northeastern Brazilian region, and used in the traditional medicine as antiseptic [8]. The antimicrobial activity of *L. sidoides* is well known: the activity of the essential oil was evaluated against *Candida albicans* and *Streptococcus mutans* [9,10]. Further, essential oil has shown anti-inflammatory, antioxidant and gastroprotective effects, associated with low toxicity [11,12]. The therapeutic effect of *L. sidoides* is attributed

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mainly to the presence of the main component, the thymol, substance with improved antimicrobial activity [13]. However, all reports of antimicrobial activity are related to the direct contact between the essential oil or thymol and microorganisms.

In this paper, we report the antibacterial and antibiotic modifying activity of the essential oil extracted from the leaves of *L. sidoides* Cham. and thymol using gaseous contact.

2. Methods

2.1. Plant material

Leaves of *L. sidoides* Cham. were collected in August, 2010, from the Small Aromatic and Medicinal Plants Garden of the Laboratory of Natural Products Research (LPPN) at Regional University of Cariri (URCA), county of Crato, Ceará State, Brazil. A voucher specimen was deposited in the Herbarium Caririense Dárdano de Andrade Lima with the voucher number 3038.

2.2. Drug

Thymol was obtained from Sigma Chemical Corp. (St. Louis, MO, USA). The antibiotic disks were obtained from LABORCLIN (Brazil).

2.3. Extraction of the essential oil

Samples of *L. sidoides* fresh leaves (140 g) were triturated and submitted to hydrodistillation process, in a Clevenger-type apparatus for 2 h. The collected essential oil was subsequently dried by anhydrous sodium sulfate (Na_2SO_4), and stored under refrigeration at <10 °C until it is analyzed and tested.

2.4. Analysis of the essential oil by GC–MS

Analysis by CG/MS of the essential oil was carried out on a Hewlett-Packard Model 5971 GC/MS using a non-polar DB-1 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 m film thickness); carrier gas helium, flow rate 0.8 mL/min and with split mode. The injector temperature and detector temperature were 250 °C and 200 °C, respectively. The column temperature was programmed from 35 °C to 180 °C at 4 °C/min and then 180 °C to 250 °C at 10 °C/min. Mass spectra were recorded from 30–450 m/z. Individual components were identified by matching their 70 eV mass spectra with those of the spectrometer data base using the Wiley L-built library and two other computer libraries MS searches using retention indices as a pre-selection routine, as well as by visual comparison of the fragmentation pattern with those reported in the literature [14,15].

2.5. Antimicrobial activity using gaseous contact

Antibacterial activity of the essential oil from *L. sidoides* (EOLS) and thymol was assayed using gaseous contact [16]. The tests were performed in triplicate using concentrations of 50, 25, 12, 6 and 3% of EOLS or thymol dissolved in DMSO. *Staphylococcus aureus* ATCC12624 and *Pseudomonas aeruginosa* ATCC15442 were used as bacteria patterns. Brain Heart Infusion Broth (BHI) was used for bacterial growth (24 h, 37 °C). 10^5 UFC/mL of the inoculum from overnight culture of each bacterial species was inoculated in Mueller Hinton Petri dishes (90 mm diameter) by streaking method. Disks containing gentamicin (10 μg), amikacin (10 μg) and neomycin (10 μg) were used to determine changes in diameter of the zone of inhibition of studied strains.

Minimal inhibitory dose (MID) was defined as the dose minimum of EOLS per unit space required to suppress the growth of microorganism in a closed system. The MID values were expressed as weight per unit volume (mg/L air), where the solution with 50 μg equals 1 mg/L air [17]. After this, 50 μL of each dilution was added to the upper Petri dish and incubated for 24 h at room temperature. Petri dishes without the essential oil or thymol and with DMSO were used as positive and negative controls, respectively.

2.6. Statistical analysis

The results were expressed as means ± standard error of mean (S.E.M.) and variance analysis (ANOVA) statistical significance was determined by one way analysis of variance (ANOVA) followed by Tukey's test, with the level of significance set at $p<0.05$ using the program *GraphPad Prism 5.0*.

3. Results and discussion

The leaves of *L. sidoides* provided an oil yield of 1.06%. The chemical composition of the essential oil obtained by hydrodistillation is presented in Table 1. A total of 7 different components were identified by GC–MS analysis. Retention indices of all the components were determined by the Kovats method. The constituents of the oils were identified by comparison of their mass spectra with those of mass spectral libraries (NIST and Wiley). The main components were thymol (84.9%), ethyl-methyl-carvacrol (5.33%) and p-cymene (3.01%) totalizing 93.24%. In some studies, the concentration of thymol in the essential oil of the leaves can vary among 34.2 the 95.1% [11,18].

In Table 2, the antibacterial activity by gaseous contact is shown. In this table, it was demonstrated that *S. aureus* is more susceptible (MID of 0.0625 mg/L air). Nostro et al. and Oliveira et al. [18,19] showed the antibacterial activity against *S. aureus* of the *L. sidoides* essential oil EOLS and thymol by direct contact method. Other studies show that the antimicrobial activity of the essential oils has been attributed to the small terpenoids and a composition of phenolic

Table 1
Chemical components of *Lippia sidoides* fresh leaves essential oil.

Components	Tr (min)	IK[a]	(%)
p-cymene	4.2	1020	5.33
1,8-cineol	4.4	1031	1.68
γ-terpinene	5.0	1060	1.32
Ethyl-methyl-carvacrol	9.7	1164	3.01
Thymol	11.8	1288	84.9
Carvacrol	12.9	1292	0.41
β-caryophyllene	15.1	1418	1.17
Total identified			97.82

[a] Relative retention indices experimental: n-alkanes were used as reference points in the calculation of relative retention indices.

Table 2
Antibacterial activity of the volatile compounds of essential oil of *L. sidoides* and thymol by gaseous contact.

Bacteria	MID (mg/L air)					
	1	0.5	0.25	0.125	0.0625	0.03125
S. aureus ATCC 12624	−	−	−	−	+	+
P. aeruginosa ATCC 15442	+	+	+	+	+	+

Note: +: growth observed; −: no growth observed; MID: minimal inhibitory dose.

compounds such as thymol, carvone, carvacrol, menthol and murolene, components that also exhibit antifungal activity [19,20]. This result is in accordance with previous reports indicating that Gram-negative bacteria are more resistant to essential oils than Gram-positive [21–23].

In Table 3, the antibiotic activity of aminoglycosides against *S. aureus* was enhanced in the presence of the essential oil (EOLS) and thymol. The result was demonstrated with EOLS, being observed with an increase of 429.41, 349, 256.82 and 21.53% in the antibiotic activity of gentamycin at concentrations of 50, 25, 12 and 6%, respectively. We also observed an enhancement of the antibiotic activity when amikacin and neomycin were combined with the volatile compounds of the essential oil.

However, thymol was less effective against *S. aureus* when compared with EOLS, despite the increase observed. Table 3 shows an enhancement of 155.11, 152.54 and 79.46% in the antibiotic activity of amikacin at concentrations of 50, 25 and 12% when combined with the volatile thymol. The antibiotic activity of neomycin was enhanced with the same method. This results show that other compounds present in essential oil are important for development of antimicrobial activity. The strains were susceptible to all antibiotics tested in accordance of standards set by NCCLS [24]. There were statistical differences between capacity of EOLS or thymol in increasing the activity of aminoglycosides tested.

Table 4 shows a modification of the antibiotic activity of the volatile constituents of EOLS and thymol against *P. aeruginosa*. In most of the results, there were not statistical differences between EOLS and thymol for the increase of the activity of the tested aminoglycosides. The antibiotic activity of all antibiotics was enhanced by EOLS and thymol.

The aim of synergy studies is to determine the scientific reasons about the better activity of many herbal extract drugs and essential oils when compared to single constituents [25]. EOLS

Table 3
Modification of the antibiotic activity of antibiotics by the volatile constituents of *L. sidoides* essential oil (EOLS) and thymol using gaseous contact against *S. aureus* ATCC 12624.

Samples	TER	N. TRE	DMSO	50%	25%	12%	6%
EOLS	AMI	26 ± 0.0^{f}	26 ± 0.0^{f}	$\geq 90 \pm 0.0^{a}$	77.66 ± 1.15^{c}	34 ± 0.0^{d}	27.33 ± 1.15^{f}
	Increase	–	–	≥246.15	198.70	30.77	5.11
	GEN	17 ± 0.0^{b}	17 ± 0.0^{b}	$\geq 90 \pm 0.0^{a}$	76.33 ± 1.15^{c}	60.66 ± 1.15^{g}	20.66 ± 0.57^{h}
	Increase	–	–	≥429.41	349.0	256.82	21.53
	NEO	18 ± 0.0^{a}	18 ± 0.0^{a}	76 ± 0.0^{d}	73 ± 0.0^{e}	56.66 ± 0.57^{b}	25.66 ± 0.57^{c}
	Increase	–	–	322.22	305.55	214.77	42.55
Thymol	AMI	26 ± 0.0^{f}	26 ± 0.0^{f}	66.33 ± 1.15^{b}	65.66 ± 0.57^{b}	46.66 ± 1.15^{e}	27 ± 0.0^{f}
	Increase	–	–	155.11	152.54	79.46	3.84
	GEN	17 ± 0.0^{b}	17 ± 0.0^{b}	55 ± 0.0^{c}	49.66 ± 0.57^{a}	45.66 ± 1.15^{f}	23 ± 0.0^{d}
	Increase	–	–	223.53	192.12	168.59	35.29
	NEO	18 ± 0.0^{a}	18 ± 0.0^{a}	57.0 ± 0.0^{f}	56 ± 0.0^{f}	49.66 ± 1.15^{c}	24 ± 0.0^{b}
	Increase	–	–	216.66	211.11	175.89	33.33

Averages followed by the same letters, in the column, don't differ significantly among themselves ($n = 3$, $p < 0.05$ — Tukey's test). Averages followed by different letters, in the line, differ significantly when compared among themselves and respective controls ($n = 3$, $p < 0.05$ — Tukey's test). The results are expressed as means ± standard error. Amikacin (AMI), gentamicin (GEN), neomycin (NEO); TRE: treatment; N.TRE: no treatment.

Table 4
Modification of the antibiotic activity of the volatile constituents of *L. sidoides* essential oil (EOLS) and thymol by gaseous contact on *P. aeruginosa* ATCC 15442.

Samples	TRE	N. TRE	DMSO	50%	25%	12%	6%
EOLS	AMI	20 ± 0.0^{d}	20 ± 0.0^{d}	28.66 ± 1.15^{b}	27.33 ± 1.15^{ab}	25 ± 0.0^{ac}	20 ± 0.0^{d}
	Increase	–	–	43.30	36.65	25	–
	GEN	15 ± 0.0^{e}	15 ± 0.0^{e}	19.66 ± 1.56^{f}	19.33 ± 1.15^{f}	18 ± 0.0^{f}	15 ± 0.0^{e}
	Increase	–	–	31.06	28.86	20	–
	NEO	15 ± 0.0^{e}	15 ± 0.0^{e}	19.66 ± 1.56^{f}	19 ± 1.0^{f}	15 ± 1.0^{e}	15 ± 0.0^{e}
	Increase	–	–	31.06	26.66	–	–
Thymol	AMI	20 ± 0.0^{d}	20 ± 0.0^{d}	26.66 ± 1.15^{a}	26.33 ± 0.57^{a}	26.33 ± 0.57^{a}	26 ± 0.0^{a}
	Increase	–	–	33.3	31.5	31.5	30
	GEN	15 ± 0.0^{e}	15 ± 0.0^{e}	20 ± 1.73^{f}	19.33 ± 1.15^{f}	18.66 ± 1.15^{f}	18.66 ± 1.15^{f}
	Increase	–	–	33.33	28.86	24.4	24.4
	NEO	15 ± 0.0^{e}	15 ± 0.0^{e}	19.66 ± 0.57^{f}	18 ± 0.0^{f}	15.66 ± 1.15^{e}	15 ± 0.0^{e}
	Increase	–	–	31.06	20	4.4	–

Averages followed by the same letters, in the column, don't differ significantly among themselves ($n = 3$, $p < 0.05$ — Tukey's test). Averages followed by different letters, in the line, differ significantly when compared among themselves and respective controls ($n = 3$, $p < 0.05$ — Tukey's test). The results are expressed as means ± standard error. Amikacin (AMI), gentamycin (GEN), neomycin (NEO); TRE: treatment; N.TRE: no treatment.

contains in its chemical composition other compounds, associated with thymol, showing antimicrobial activity such as carvacrol, p-cymene, β-caryophyllene and 1,8-cineol [26–29]. These compounds may act synergistically increasing the antimicrobial activity of essential oil against Gram-positive bacteria. However, thymol alone presented an antibacterial activity similar to the EOLS against *P. aeruginosa*, with no statistical difference when compared to EOLS.

The mechanisms of the essential oils can interact with the antibiotics involving different interactions among the present compositions in the oil and the bacterial membrane. According to Burt [30], the thymol and the carvacrol, present also in the essential oil of *Origanum vulgare*, could cause distortions in the physical structure of the cell due to their effect against the plasmic membrane, modifying its permeability; denaturing essential enzymes; modifying the proton motive forces due changes in the pH and electric potential and enhancing the intake of antibiotics [31]. Besides, they can alter the activity of the calcium channels and promote the extrusion of some important ions [32].

Impairment of bacterial energy systems may also be a mechanism of action due the direct or gaseous combination of compounds and essential oils with the microorganisms [33,34]. Gram-negative bacteria are surrounded by an additional membrane, which provide a hydrophilic surface and functions as a permeability barrier for many external hydrophobic agents, this barrier is due to the presence of lipopolysaccharide (LPS) [35,36]. Membrane permeabilizers, as thymol, could interact and disorganize the anionic LPS thereby sensitizing the bacteria to antibiotics [31,37].

Some studies also reported the modulatory activity of antibiotics by gaseous contact. The essential oils of *Zanthoxylum articulatum*, *Vanillosmopsis arborea*, *Lippia microphylla* and *Croton zehntneri* were capable to interact with aminoglycosides and quinolone antibiotics, promoting the increase of their activity against *S. aureus* and *P. aeruginosa* [16,38–42]. However, our results are more expressive when compared with these works. Furthermore, in our study, the major component of essential oil was tested with the aim to identify the compound or compounds responsible for the observed activities.

4. Conclusions

The results obtained in that study suggest that the volatile components of the essential oil of *L. sidoides* and its major component thymol may suppress the growth of bacterial pathogens of respiratory infections, as cystic fibrosis, and they can be adjuvant in the patients' treatment. Other studies are necessary to verify the bioavailability and the possible toxicity of that association with aminoglycosides using *in vivo* models and elucidate the action mechanisms involved in that interaction.

Conflict of interest

No conflict of interest to declare.

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