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Antimicrobial Activity of the Thio-Cyclized *Lippia citriodora* Leaf Essential Oil Cultivated in Algeria

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Abstract: The essential oil of *Lippia citriodora* extracted by hydrodistillation from dry leaves with a yield of 0.3 % which characterization by TLC, UV-Vis, and IRTF analyses make it possible to distinguish the presence of aldehydes functions within the chemical composition. The GC-MS has permitted to confirm and to identify the neral and the geranial in appreciable proportions in the chemical composition of *Lippia citriodora* essential oil. The thionation of the carbonyl compounds of the essential oil has led to the transformation of the carbonyl compounds into corresponding thiones. The UV-Vis spectroscopy and the FT-IR spectra have shown the disappearance of the aldehyde function and its replacement by thione and thiol functions in solution by tautomery. The GC-MS has permitted to identify the formation of unsaturated cyclic compounds such as the 2-Isopropyl-5-methyl-cyclohexa-2,5-dienethione, the 6-Isopropyl-3-methyl-cyclohexa-2,4-dienethione and the 5-Isopropenyl-2-methyl-cyclohex-2-enethione, as well as an aromatic compound namely the thiothymol (2-Isopropyl-5-methyl-benzenethiol).The antibacterial and especially antifungal activity of the essential oil of *Lippia citriodora* has been greatly improved with the replacement of the oxygen by the sulphur and therefore the increase of the hydrophobic character and the volatility of the chemical composition of the oil.

Key words: *Lippia citriodora*, essential oil, aldehyde, thionation, antimicrobial activity.

Introduction

The essential oils are known for their biological activities [1-3] and in particular, their microbiological activities [4,5], but these activities are limited to certain microbial strains because of the qualitative and quantitative variability of the chemical composition of the essential oils [6,7]. Recently, thiocarbonyl derivatives isolated from marine algae have been identified for their antibacterial actions [8] and also the thionation of the essential oils of *Artemisia Herba alba* and *Ruta montana* consisting mainly of ketones has revealed highers antimicrobial actions [9]. However, the yields of the essential oils are very low [10, 11] and especially for some plants such as *Lippia citriodora* [12-14] due to the resistance of several strains and their limited antimicrobial actions [15]. Among the essential oils which chemical composition is comprised of aldehydes, they are those extracted from the genus *Lippia* as *Lippia citriodora* species. The works carried out on this species have shown that the essential oil composed mainly of the two iso-

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mers neral and geranial which antimicrobial activity is very limited even at high dose. The antibacterial and antifungal activities of the essential oil of *Lippia citriodora* have been studied in this work in order to strengthen its activity and decrease the resistance of the microbial strains towards the modified oil with the introduction of the sulphur atom instead of the oxygenation atom inducing a decrease in the polarity and an increase in the hydrophobic character and the volatility. The thioaldehydes are generally unstable due to the presence of the hydrogen sterically demanding [16] adjacent to the thiocarbonyl group leading to new cyclization products.

Materials and methods

Plant material

The leaves of *Lippia citriodora* were collected in June 2016 from the Miliana region (latitude: 36°17 24"N, Longitude: 2°12 36"E), Ain Defla (Algeria). The plant was identified by the botanist Mr M. Benissad of the EL Hamma botanical garden in Algiers (Algeria) and at the botanical laboratory of the department of agronomy of the University of Blida (Algeria).

Chemical products

P_2S_5 (99 %), $NaHCO_3$ (99 %), CH_2Cl_2 (99 %), DMSO (99 %) were purchased from Sigma Aldrich, Biochem, Panreac and Panreac respectively.

Preparation of the oil

The extraction of the essential oil was conducted by hydrodistillation with a Clevenger apparatus with 100 g dried leaves of *Lippia citriodora* for 3 h.

The obtained essential oil was dried with anhydrous sodium sulphate for its analysis and subsequent uses.

Thionation of the oil

The previously dried essential oil was dissolved in 30 ml of CH_2Cl_2, 0.7 g of P_2S_5 and 0.146 g of sodium bicarbonate are added to the previous solution. The mixture was heated under reflux in a water bath. After cooling, the solvent was removed by a rotary evaporator and the product was recovered in sealed tubes for further works.

Thin layer chromatography

Glass plates (20x20 cm) covered with silica gel 60GF254 from Merck with 0.5 mm thickness of stationary phase are used. The plates have been activated in oven at 120ºC before use. The eluents used for essentials oils separation were hexane/ethyl acetate mixture in proportions (9.5: 0.5/ v:v).

UV-Vis analysis

The UV-Vis spectra were recorded on a Shimadzu UV-1800 double beam spectrophotometer, controlled by a micro-computer with a 10 mm cell.

FT-IR analysis

The FT-IR spectrums were recorded with a Jasco Brand Fourier transform spectrometer.

GC-MS analysis

The gas chromatography-mass spectrometry (GC-MS) analyses were carried out using a Shimadzu HP 6890 gas chromatograph system equipped with a mass selective detector (GC-MSD) quadripole TQ8030 equiped with an injector operating in split-splitless mode and an Rtx-5MS capillary column (30 m x 0.25 mm i.d, film thickness 0.25 mm). Helium was used as a carrier gas at a flow rate of 1 mL/min. The ionization was performed with electronic impact and a filament intensity of 70 eV. The mass spectrometry analysis (MSD) was performed in Scan mode from 35 to 550 amu. The Methanol was used as a solvent and was eluted with a time delay of 3 min. Source and interface temperatures were 200°C and 250°C, respectively.

The analysis of the essential oil of *Lippia citriodora* was performed in split mode, at a temperature of 250°C and the injected volume was 1 µL. The oven temperature was held at 40°C and the heating program was 40 to 250°C at a rate of 6°C/min.

The essential oil of thionated *Lippia citriodora* was analysed in splitless mode, at a temperature of 250°C and an injected volume of 1 µL. The

oven temperature was held at 60°C. The oven temperature was programmed from 60 to 250°C at a rate of 2°C/min and a temperature plateau at 250°C for 10 min.

The identification of the constituents of the essential oil of *Lippia citriodora* before and after thionation was carried out by comparing the mass-spectra obtained with those of the database (Wiley8.LIB) and with the literature for the known compounds. Especially the new cyclization products were identified from their mass spectral peaks, both parents and fragments.

The chemical composition of the various constituents of the two essential oils was obtained from the areas under peaks. The individual components were determined quantitatively by computerized peak area measurement.

Antimicrobial activity tests

The antimicrobial activity was performed on selected bacterial and fungal strains from the hygiene department of Blida/Algeria.

The microorganisms used are: Gram-positive bacteria [*Staphylococcus aureus* ATCC 6538], Gram-negative bacteria [*Escherichia coli* ATCC 25922] and the yeast [*Candida albicans* ATCC 10231].

The sensitivity of the microbial strains was tested towards an antibiotic [ciprofloxacin (ATB); 0.1 mg/disc] and antifungal [Metronidazol (ATF 1); 0.25 mg/disc] according to the method of diffusion in solid media.

The essential oils of *Lippia citriodora* before and after thionation were used directly and solubilized in dimethyl sulphoxide (DMSO) at concentrations of 5, 10 and 30 %. Samples of 10 µl of each concentration were deposited under aseptic conditions on sterile Whatman paper discs. A disc impregnated with the DMSO represents a negative test.

The evaluation of the antimicrobial activity of the essential oil of *Lippia citriodora* before and after thionation was carried out with two bacterial strains and a fungal strain referenced by the method of diffusion on agar medium called also aromatogram. Discs of 9 mm diameter soaked with pure essential oil of *Lippia citriodora* and modified at different concentrations were deposited into Petri dishes containing a previously inoculated medium. After incubation at 37°C for 24 hours for bacteria and 48 hours for yeast, the results were conducted by measuring inhibition diameters.

Results and discussion

The obtained essential oil is pale yellow with a pleasant lemon smell. Its density is 0.902 g/ml at 20°C and its refractive index and rotary power are 1.491 and -21.6 at 20°C, respectively. The characterization by thin-layer chromatography using silica gel of the essential oil of *Lippia citriodora* using a mobile phase consisting of a hexane/ethyl acetate mixture (9.5/0.5) allowed after UV analysis at 254 nm to distinguish the presence of 2 spots with 0.25 and 0.31 frontal ratios.

Lippia citriodora essential oil was obtained with a yield of 0.28 %. This result is comparable to those obtained (0.36 %) in Blida and (0.29 %) in Kabylie (Algeria) [17].

The UV-Vis analysis of the essential oil of *Lippia citriodora* (Figure 1) solubilized in methanol has allowed to identify two peaks in the ultraviolet region located respectively at 223 and 232 nm which are characteristic of the $\pi \rightarrow \pi^*$ electronic transitions of an alkene double bond and $n \rightarrow \pi^*$ of an aldehyde carbonyl function.

As shown in Figure 2, the FT-IR spectra indicate the presence of all the chemical functions of the various constituents of the essential oil of *Lippia citriodora*. The wide band located at 3454.76 cm^{-1} is attributed to the alcohol function. A very low intensity peak located at 3020 cm^{-1} specific to the vibration of the C-H bond of an aldehyde. The set of peaks between 2955.87 cm^{-1} and 2864.60 cm^{-1} are characteristic of the C-H bond of the methyl and methylene groups. However the shoulder localized around 1720 cm^{-1} and the important peak at 1672. 60 cm^{-1} are attributed respectively to the vibrations of the C=O bond of ketone and aldehydes.

The results obtained by UV-Vis and IRTF spectroscopic techniques for *Lippia citriodora* essential oil were completed by GC-MS analysis. The analysis (Figure 3) indicated the presence of monoterpenes (57.51 %) and sesquiterpenes

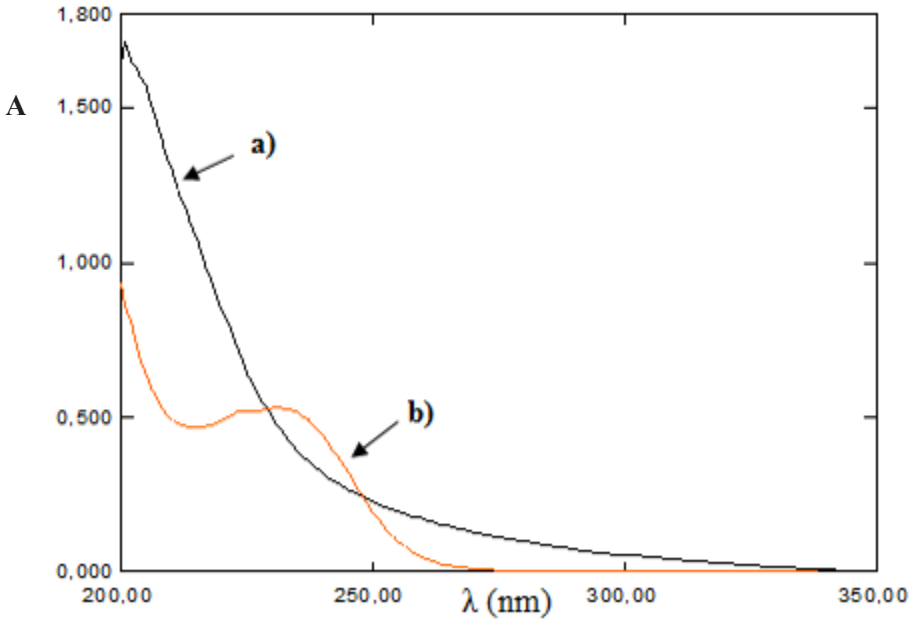

Figure 1. UV-Vis spectra of the essential oil of *Lippia citriodora* in methanol:a) Pure ; b) After thionation

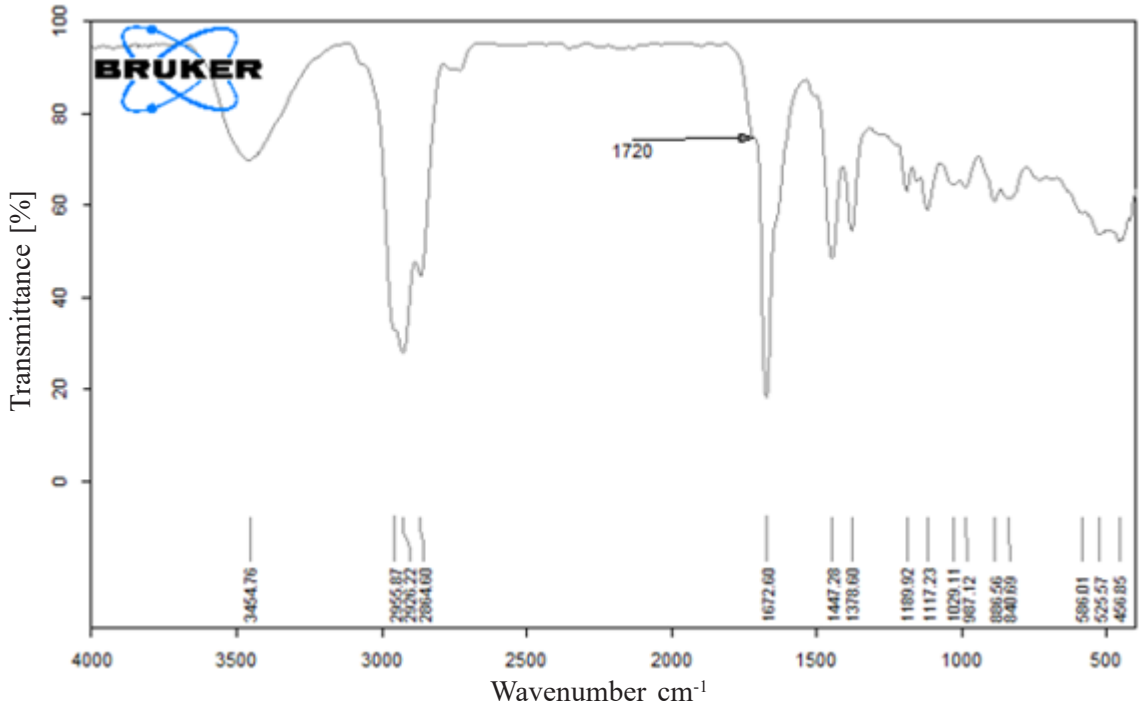

Figure 2. FT-IR spectra of the essential oil of *Lippia citriodora*

(40.70 %).The results summarized in Table 1 confirm the presence of terpenic alcohols (terpinen-4-ol (0.28 %), α-terpineol (0.35 %)), monoterpenic hydrocarbons (D-limonene (8.70 %)), terpenic aldehydes (neral (20.62 %) and geranial (27.55 %)), terpenic ketone (carvone (0.01 %)), sesquiterpenic hydrocarbons (δ-cadinene (2.62 %) and curcumene (13.62 %)) and oxygenated sesquiterpenics (Spathulenol (11.14 %), caryophyllene-oxyde (12.31 %) and Nerolidol (1.01 %)).

The thionation of the essential oil of *Lippia citriodora* which chemical composition is given

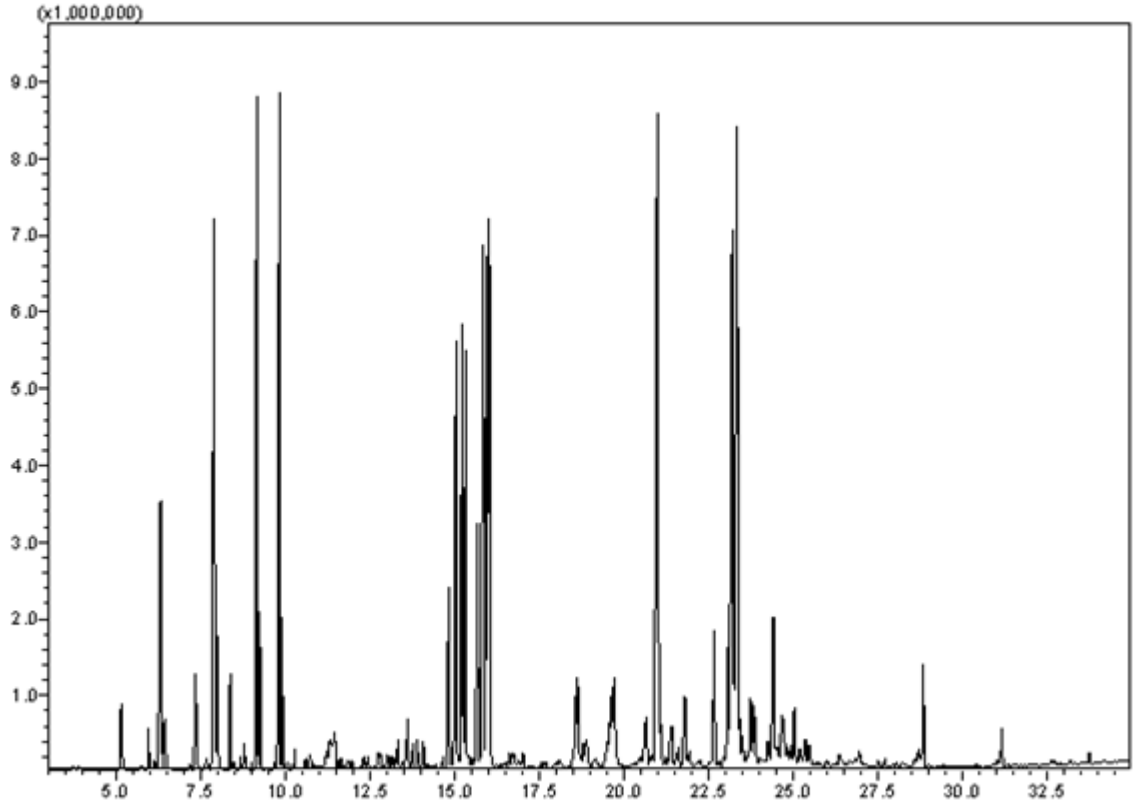

Figure 3. GC-MS chromatogram of the essential oil of *Lippia citriodora*

Table 1. Chemical composition of the essential oil of *Lippia citriodora*

Compounds	tr (min)	Area (%)
D-Limonene	7.89	8.70
1,8-Cineol	8.00	1.69
Terpinen-4-ol	13.01	0.28
α-Terpineol	14.07	0.35
Neral	15.04	20.62
Carvone	15.12	0.01
Geranial	16.04	27.55
δ-Cadinene	18.63	2.62
Curcumene	21.00	13.62
Spathulenol	23.20	11.14
Caryophyllene oxide	23.34	12.31
Nerolidol	25.05	1.01

in table 2 provided a product of semi-solid consistency of orange color with a very strong pleasant smell and sulphurated. An appreciable yield after the 5 h reaction was obtained and an analysis by TLC in the same conditions was carried out. The two spots which appeared after total disappearance of the two spots of the essential oil of *Lippia citriodora* have frontal ratios of 0.81 and 0.89, respectively. The UV-Vis spectra recorded after thionation in methanol (Figure 1-b) was totally different from the starting oil and it clearly shows the disappearance of the specific band of the carbonyl function and the increase of that located at 223 nm.

The transformation of the carbonyl compounds of the essential oil of *Lippia citriodora* into sulphur compounds led to a radical change in the FT-IR spectra (Figure 4) characterized by the disappearance of the C=O vibration peak and its replacement by the C=S vibration of the thione function located at 1204.78 cm^{-1} and 988.30 cm^{-1}. In addition, the vibration of the C=C bond became more important, the band relative to the S-H thiol bond located at 2550 cm^{-1} and the chelation band at 2317.65 cm^{-1} are clearly visible on the spectra. The GC-MS chromatogram of the thionation product of *Lippia citriodora* essential oil (Figure 5) confirms the results relating to the chromophores and the chemical functions which are characteristic of the chemical structures of the modified essential oil. The non-carbonyl compounds namely the neral, the geranial and the carvone were converted by cyclization to enethiol and thione (Figure 6) as well as the thionation of carvone to 5-

Table 2. Chemical composition of the essential oil of *Lippia citriodora* after thionation

Compounds	tr (min)	Area (%)
D-Limonene	10.02	0.89
1,8-Cineol	10.18	1.77
5-Isopropenyl-2-methyl-cyclohex-2-enethione	20.67	2.87
2-Isopropyl-5-methyl-cyclohexa-2,5-dienethione	22.59	3.50
6-Isopropyl-3-methyl-cyclohexa-2,4-dienethione	24.99	10.88
2-Isopropyl-5-methyl-benzenethiol	27.13	7.98
δ-Muurololene	29.04	1.51
δ-Cadinene	31.24	1.38
Curcumene	37.53	14.05
Spathulenol	43.50	39.48
Caryophyllene oxide	43.66	12.53
Nerolidol	47.51	3.16

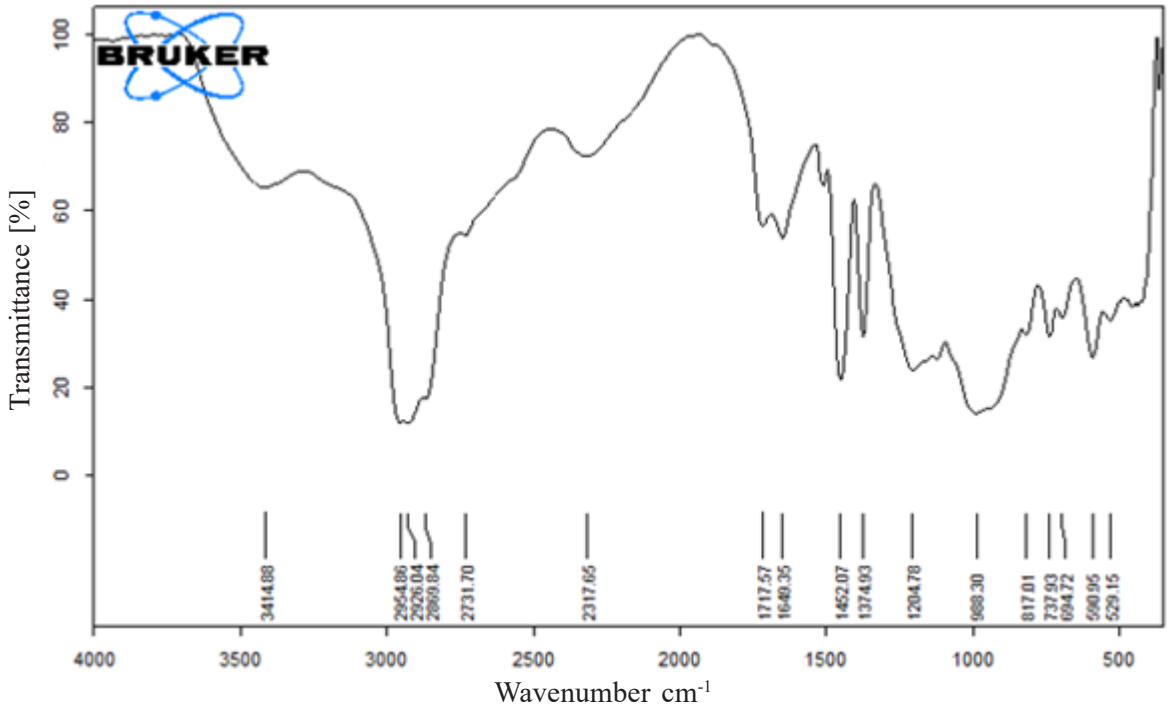

Figure 4. FT-IR thionation spectra of the essential oil of *Lippia citriodora*

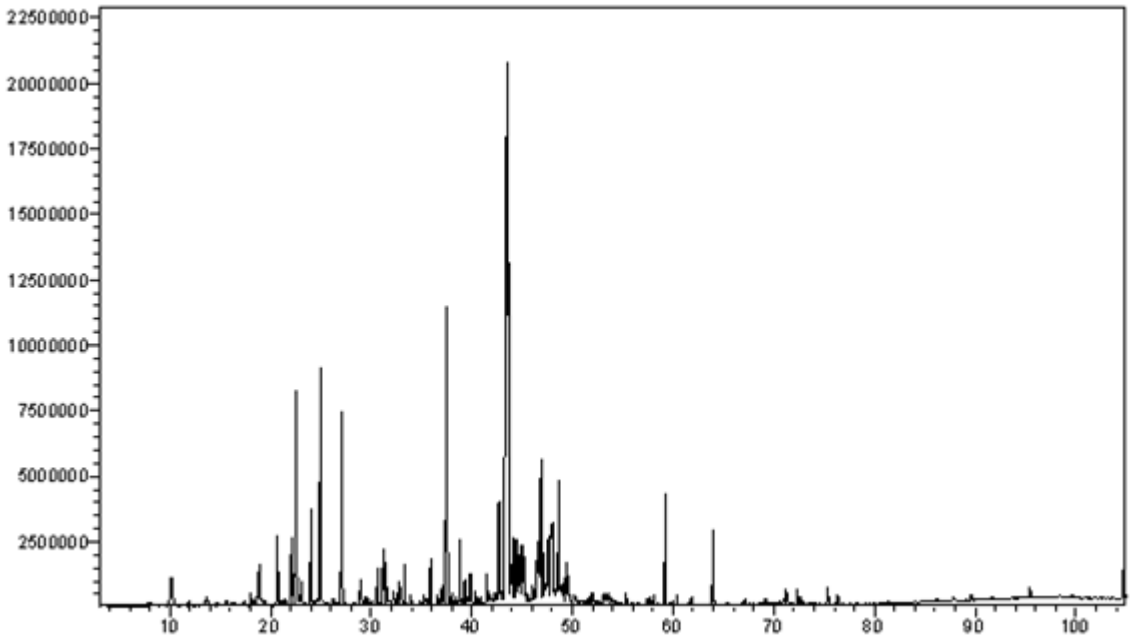

Figure 5. GC-MS thionation chromatogram of the essential oil of *Lippia citriodora*

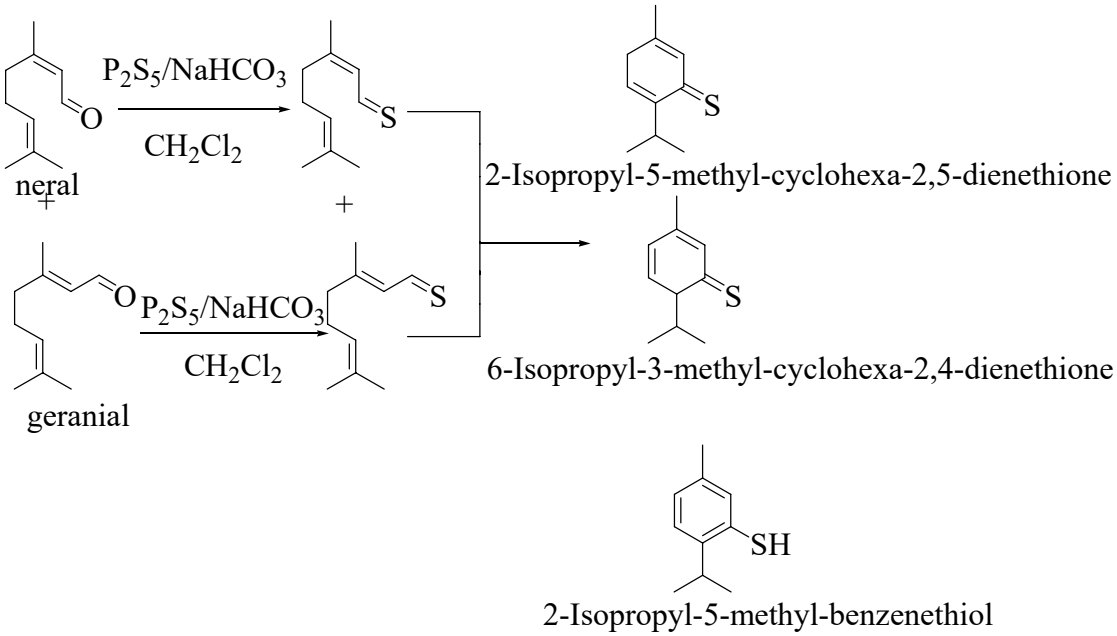

Figure 6. Thionation reactions of neral and geranial

Isopropenyl-2-methyl-cyclohex-2-enethione (carvothione) according to the following reactions (Figure 7). It should be added that in solution the thione function is transformed into a thiole function by irreversible tautomerism [9] according to Figure 8.

The essential oil of Lippia citriodora has a very important antibacterial action towards the tested strains and in comparison with the antibiotic ciproflaxine and the antifungal metronidazole for all selected doses (as shown in Table 3). However, the different germs did not exhibit the same behaviour, the strain *Escherichia coli* (Figure 9-a) was more sensitive than the strain *Staphylococcus aureus* (Figure 9-b)), whereas the most important inhibition areas were observed with *Candida albicans* fungal strain (Figure 9-c). This antibacterial activity and particularly the antifun-

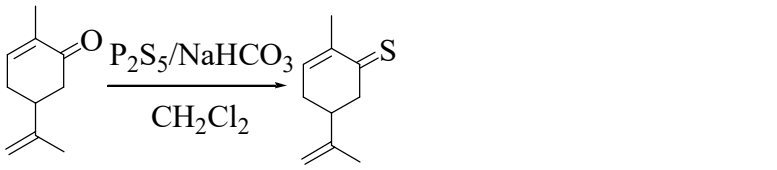

Figure 7. Thionation reaction of carvone

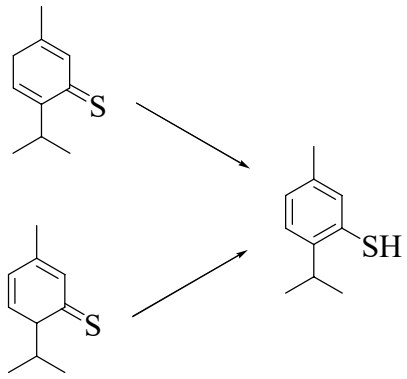

Figure 8. Tautomerisation reaction of thione-enethiol

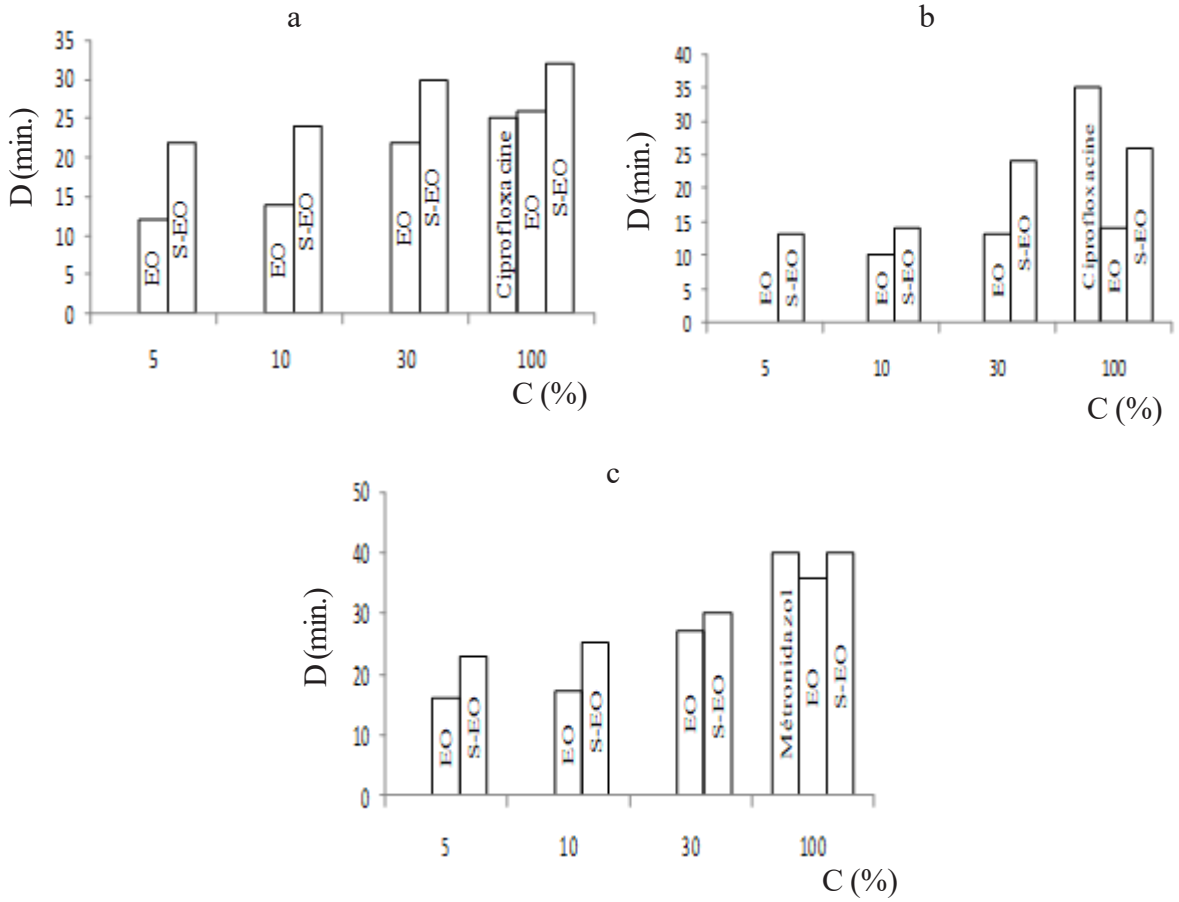

Figure 9. Histograms of the inhibitions diameters according to the concentration of the essential oil before and after thionation: a) *Escherichia coli*, b) *Staphylococcus aureus*, c) *Candida albicans*

Table 3 Results of antimicrobial activity tests of essential oils and thio-cyclizated essential oils (mm)*

Microbial strains Concentrationof EO (%)		*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
Ciprofloxacine		25	35	-
Metronidazol		-	-	40
EO	5	12	-	16
	10	14	10	17
	30	22	13	27
	100	26	14	36
S-EO	5	22	13	23
	10	24	14	25
	30	30	24	30
	100	32	26	40

* Tests with pure solvent (DMSO) were negative

gal activity were attributed to the quantitative presence of oxygenated monoterpenes and sesquiterpenes [18] in addition to the effect of synergetic interactions between the various constituents of *Lippia citriodora* essential oil [19].

The antimicrobial activity has been greatly improved due to the change of the chemical composition of the essential oil of *Lippia citriodora* via the thionation reaction by the formation of new sulphurated compounds with thiols and thiones functions. The bacterial strains have become more sensitive after chemical modification of the essential oil. However, the sensitivity of *Candida albicans* has greatly been improved and has reached an inhibition diameter identical to the commercial antifungal metronidazole in addition to the convergence of the results obtained with the antimicrobial action of sulphurated compounds [20,21].

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