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CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS FROM MOROCCAN AROMATIC HERBS

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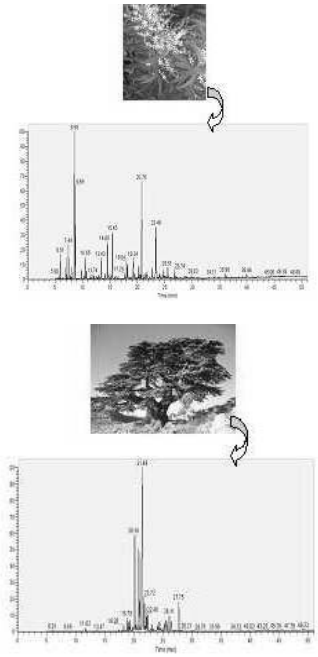
The aim of this study was to evaluate the antioxidant and antibacterial potential of essential oils (EOs) of two Moroccan aromatic herbs essential oils: *Lippia citriodora* and *Cedrus atlantica.* The composition of these species was analyzed by GC/MS and 54 compounds were identified. The essential oil extracted from *L. citriodora* contained, as main components citral (19.07%), cuparene (12.33%), 1-butenylidene-cyclohexane (9.4%), eucalyptol (7.9%), spathulenol (7.55%), β-cyclocitral (6.54%) and caryophhyllene oxide (6.27%) and the major constituents of the *Cedrus atlantica* oil were β-himachalène (29.4%), α-longipinene (20.75%), β-chamigrene (14.39%), longifolene (V4) (11.61%) and α-himachalène (5.1%). The oils were also subjected to screening for their possible antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the reducing power assays. The antibacterial activity of these essential oils has been evaluated against two bacteria (*Staphylococcus aureus* and *Escherichia coli*) with significant importance for food industry and we determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each essential oil. The tested essential oils showed a variable degree of antimicrobial activity. These results suggest that the essential oil from *Lippia citriodora* has potential to be used as a natural antioxidant and antimicrobial agent in food processing.



INTRODUCTION

In recent years, an upsurge of interest in the use of natural substances as phytomedicines has resulted in a more thorough investigation of plant resources. By enhancing awareness about the side effect of chemical preservatives, public demand and scientific interest in the use of these natural antimicrobials and antioxidants for food preservation is increasing rapidly. Aromatic and medicinal plants have acquired particular attention in the field of intensive research on the natural

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antioxidant and antimicrobial compounds. They constitute a constant source of active reagents against pathogen germs. Essential oils (EOs) are aromatic oily liquids obtained from different plant parts and widely used as food flavours.[1] Volatile oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties.[2,3]

Lippia citriodora Kunth (Verbenaceae) are largely used as herbal tea for their aromatic, digestive and antispasmodic properties.[4] Traditionally they are utilized as remedies for gastrointestinal and respiratory problems. Some species have shown antimalarial, antiviral and cytostatic properties. It is believed that their essential oils and phenolic compounds are responsible for specific biological properties attributed to the genus.[5]

The Atlas cedar (*Cedrus atlantica*) is an endemic species of the North African mountains from Morocco and Algeria. The essential oil from this plant has been shown to possess anti-inflammatory, antiviral, antifungal, and antimicrobial activities.[6-9]

Currently, there is a lack of information concerning the volatile constituents of selected aromatic herbs essential oils. However, there is no published report on antioxidant activity of *Lippia citriodora* and *Cedrus atlantica* oils. In this context, the aim of this study was to study the chemical composition of *L. citriodora* and *C. atlantica* essential oil from Morocco, and to evaluate the antioxidant activity and antibacterial potential of essential oils against two food-borne pathogenic bacteria. *Escherichia coli* (Gram-negative), *Staphylococcus aureus* (Gram-positive). The present study provides a theoretical basis for the potential application of essential oil from the studied aromatic herbal to be used as a natural resource of antioxidant and antimicrobial agents in food industry.

EXPERIMENTAL

L. citriodora aerial parts were collected during the months of May and June 2012 in the north-eastern part of Morocco. The cones of *C. atlantica* Endl (Pinaceae) were collected from the region of Ifrane, Middle Atlas, Morocco, in July 2012. All plants were air dried for 1 month in the absence of light at room temperature and then stored in sealed paper bags.

Essential oil extraction

The aerial parts of the plant, including inflorescence and cones, were cut into small pieces. Portions (100 g) of each plant material were hydrodistilled for 3 h in a Clevenger type apparatus to isolate the essential oil (yield for *L. citriodora* 0.83% v/w, and for *C. atlantica* 0.62% v/w, respectively). The essential oils (EOs) were subjected to GC-MS-analysis.

Gas chromatography/mass spectrometry (GC/MS) analysis

The components of the EO were analyzed using a Thermo Polaris Q, an ion-trap GC/MS Equipped with a Trace GC and a triple auto sampler. Components were separated with capillary column (DB-5ms Prepared by Agilent Technology USA) having 30 m length, 0.250 mm internal diameter and 0.25 μm thickness. Ionization of the sample components was performed in the EI mode (70 eV). A vaporization injector operating in the split mode (1:50) at 250 °C, with 1 ml/min; temperature programme: 5°C/min from 50°C to 280°C; then at 280°C the sample was kept for 5 minutes more. Mass spectra were acquired in the range of 40 to 650 m/z. Components of the essential oil were identified by comparing the mass spectra obtained with those of standard mass spectra from the NIST library (NIST 05). Relative concentration of the components was calculated from the peak areas of the total ion chromatograms.

Free radical-scavenging activity: DPPH test

The free radical scavenging activity of the EOs was studied by DPPH method – based on the decrease of the 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) maximum absorbance at 519 nm in the antioxidant presence.[10] The decreasing of the DPPH radical absorption by the action of antioxidants could be used for measuring the antioxidative activity. For our experience, we found that 40 min as being the required time to complete the reaction. Ascorbic acid and α-Tocopherol (100μg/mL) (Sigma–Aldrich) were used as a synthetic reference.

The antioxidant activity (radical scavenging activity) was calculated using the formula:

$$\% \text{ inhibition} = [(A_0 - A_s)/A_0] \times 100 \quad (1)$$

where: A_0 = blank absorbance; A_s = sample absorbance.

Reducing power

The reductive potential of the oils was determined according to the method of Oyaizu (1986) with slight modifications.[11] Briefly, 2.5 mL of different methanolic concentrations of the essential oils were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide ($K_3Fe(CN)_6$; 1%). The mixture was incubated at 50 °C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid were added, and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride. Finally the absorbance was measured at 700 nm against a blank by using UV spectrophotometer (Thermo Scientific Evolution 260 Bio). α-Tocopherol was used as a control. The assay was carried out in triplicate.

Determination of antibacterial activity

Two species of bacteria, one Gram negative bacteria (*Escherichia coli* O157 ATCC 1659) and one Gram positive bacteria (*Staphylococcus aureus* ATCC 13565) were used as indicator microorganisms to detect the antimicrobial activity. All strains mentioned above were obtained as actively growing cultures from the microbial culture collection of the Microbiology Laboratory, Institute of Agronomy and Veterinary Hassan (IAV HASSAN II), Rabat, Morocco.

Disc diffusion assay

A 3.8% Mueller-Hinton solution was prepared, 9 ml of the solution was poured aseptically in sterile Petri dish and left to cool and solidify on the bench. Another solution of 0.8% agar-agar was prepared, 6ml was taken from the solution in sterile tube and inoculated with 60 µl of the cell suspension (final concentration: 10^6 UFC/ml). A cylinder of 8 mm diameter was deposed on the basal solid layer on which the second solution was added and allowed to cool and solidify in turn. Then the cylinder was removed and filled with 50 µl of pure essential oil. The Petri dish was incubated at a temperature of 37°C for 24h. All zones of inhibition of growth form a diameter which is measured in millimeters. All tests were performed in duplicate.[12]

Minimum inhibitory concentration (MIC) of plant extracts and essential oils

A solution of 3% TSB was prepared, to which is added a solution of Agar-agar 0.15%. A different concentration of essential oil was prepared by macrodilution. We need 10 test tubes. The first tube is filled with 2 ml of the prepared solution and the other 9 tubes with 1ml. Then 20 µl of pure essential oil was added in the first tube, chained with a dilution and a concentration was obtained from 0.0039% to 1%. After that, each tube was inoculated with 10 µl of bacterial suspension (final concentration 10^6 UFC/ml). A witness sample was prepared without essential oil. All tubes were incubated for 18 h at 37°C. The tests were performed three times. MICs were defined as the lowest concentration of compound that inhibits bacteria after 24 h.[12]

Minimum Bactericidal concentration (MIB) of plant extracts and essential oils

A 4% TSA solution was prepared; 15 ml of the solution was aseptically poured into sterile Petri dishes to allow its cooling and solidification. 100 µl of cleared solution was taken from the CMI tubes and poured in the TSA layer. The solution was then left for incubation at 37°C, for 24 hr. The CMB is determined as the lowest concentration, for which there is no bacterial growth on solid medium.[12]

Statistical Analysis: The measurements were performed in triplicate and for statistical processing Excel 2007 was used, standard deviation (STDV) was < 10%.

RESULTS AND DISCUSSION

The constituents identified by GC–MS analysis, in order of elution and the quantitative data are presented in Table 1. 54 compounds were overall identified in all studied herbals. The essential oil of *L. citriodora*, of which 98.4% of the composition was determined, contained as main components citral (19.07%), cuparene (12.33%), 1-butenylidenecyclohexane (9.4%), eucalyptol (7.9%), spathulenol (7.55%), β-cyclocitral (6.54%) and caryophhyllene oxide (6.27%), (S)-cis-verbenol (4.6%), caryophyllene (3.6%) and α-campholenal (3.38%). The chemical composition of the essential oil of *L. citriodora* from Morocco was different from that observed from Algerian or Argentinean plant materials,[13,14] variations in the chemical composition should be attributed to varieties rather than bioclimatic conditions.

The GC/MS analysis of *Cedrus atlantica* essential oil revealed the presence of 18 compounds, representing 99.52% of the total composition. The main components of *C. atlantica* essential oil were β-himachalène (29.4%), α-longipinene (20.75%), β-chamigrene (14.39%), longifolene (V4) (11.61%), and α-himachalène (5.1%). The essential oils of *Cedrus atlantica* collected the region of Ifrane (Morocco) proved to have a different concentration than those of other plants studied in Turkey, which the major constituents were α-pinene (24.78%), abieta-7,13-diene (16.67%), abieta-8,11,13-triene (6.85%), manool (5.83%), terpinen-4-ol (3.74%) and α-terpineol (3.42%).[15]

Table 1

Chemical composition of essential oils of *Lippia citriodora* and *Cedrus atlantica* from Morocco

No	RT (min)	Compound	*L. citriodora*	*C. atlantica*
			Concentration (% peak area)	
1.	5.55	1,6-Heptadien-4-ol	0.13	
2.	5.8	1-Isopropyl-4-methylbicyclo la(3.1.0) hex-2-ene	0.28	
3.	6.46	3-Carene	0.16	
4.	7.16	α-Pinene	1.37	
5.	7.32	2-ethyl-2-Hexen-1-ol	0.17	
6.	7.43	α-Campholenal	3.38	
7.	7.73	Propanoic acid 2 2-dimethyl-, butyl ester	0.45	
8.	7.86	Izovaleric acid, isobutylester	0.01	
9.	8.09	Oxalic acid, allyl pentylester	1.04	
10.	8.55	1-Butenylidenecyclohexane	9.4	
11.	8.68	Eucalyptol (1,8-Cineole)	7.9	

Table 1 (continued)

12.	9.79	5-Isopropyl-2-methyl bicyclo(3.1.0)hexan-2-ol	1.2	
13.	10.49	Propanoic acid 2 2-dimethyl-, pentylester	1.6	
14.	11.3	p-Mentha-2,8-dienol	0.4	
15.	11.63	4- Methyl -1-methyl-1-cyclohexene		0.5
16.	12.95	4-Menth-1-en-4-ol	0.48	
17.	13.43	4-Menth-1-en-8-ol	1.54	
18.	14.13	p-Mentha-1,8-dien-7-ol	0.75	
19.	14.59	(S)-cis-Verbenol	4.6	
20.	15.16	p-Menth-1-en-3-one	0.43	
21.	15.44	β-Cylocitral	6.54	
22.	16.03	Iso-geraniol	0.59	
23.	16.49	4α-(1-Hydroxy-ethyl)-hexahydrobenzo[1,3]dioxin-4-one	0.36	
24.	17.75	1,2-Epoxy-p-menth-8-ene	0.46	
25.	18.04	α-Cubebene	1.84	
26.	18.13	Norbornane, 7,7-dimethyl-2-methylene-	1.31	
27.	18.26	Isolongifolene 4 5-dihydro		0.83
28.	18.75	Citral	19.07	
29.	18.79	1,1,3α-Trimethyl-7-methylenedecahydro-1H-cyclopropa[a]naphthalene		1.55
30.	19.05	4β-H,5α-Eremorphila-1(10)11-dien-2-one		1.24
31.	19.21	Di-epi-a-Cedrene		0.6
32.	19.24	Caryophyllene	3.6	
33.	19.36	Aristalene		1.48
34.	19.43	Cedrene	0.95	
35.	20.16	β-Chamigrene		14.39
36.	20.28	Guaia-1(5),11-diene	0.17	
37.	20.77	Cuparene	12.33	
38.	20.85	Longifolene-(V4)		11.61
39.	21.49	α-Logipinene		20.75
40.	21.72	Cadina-3,9-diene		3.11
41.	22.04	Isolongifolene, 4,5,9,10-dehydro-		2.43
42.	22.4	α-Calacorene		2.13
43.	23.01	α-Himachalene		5.1
44.	23.18	3-Isobutyl-4,5-dimethyl-3H-isobenzofuran-1-one		0.85
45.	23.24	Spathulenol	7.55	
46.	23.45	Caryophyllene oxide	6.27	
47.	23.53	Terrenin		
48.	24.1	Allo-Aromadendrene oxide-1	0.82	
49.	24.44	Isoledene		1.15
50.	24.79	γ-Muurolene	1.22	
51.	25.46	Cubenol		1.26
52.	25.78	Tumerone		1.14
53.	26.77	Z,Z,Z-4,6,9-Nanadecatriene		
54.	27.33	β-Himachalene		29.4

Compounds, identified on the basis of comparison with MS database spectra. RT: retention time.

Antioxidant activities of the essential oils from *Lippia citriodora* and *Cedrus atlantica* were tested by the DPPH radical scavenging and the reducing power assays.

The DPPH radical scavenging is a commonly used method to evaluate the ability of plant extracts to scavenge free radicals generated from DPPH reagent. The DPPH radical scavenging activities of the two essential oils and of the reference substance (ascorbic acid and α-tocopherol) are shown in (Fig. 1).

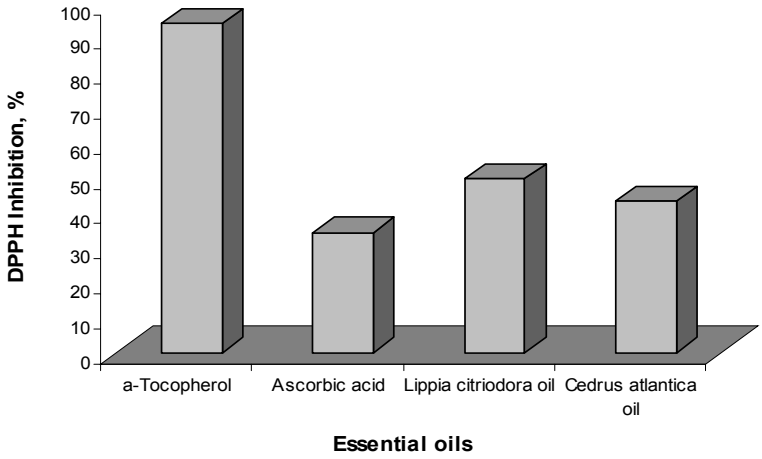

Fig. 1 – Free radical scavenging activity of the essential oils of *L. citriodora* and *C. atlantica* and standards by DPPH method.

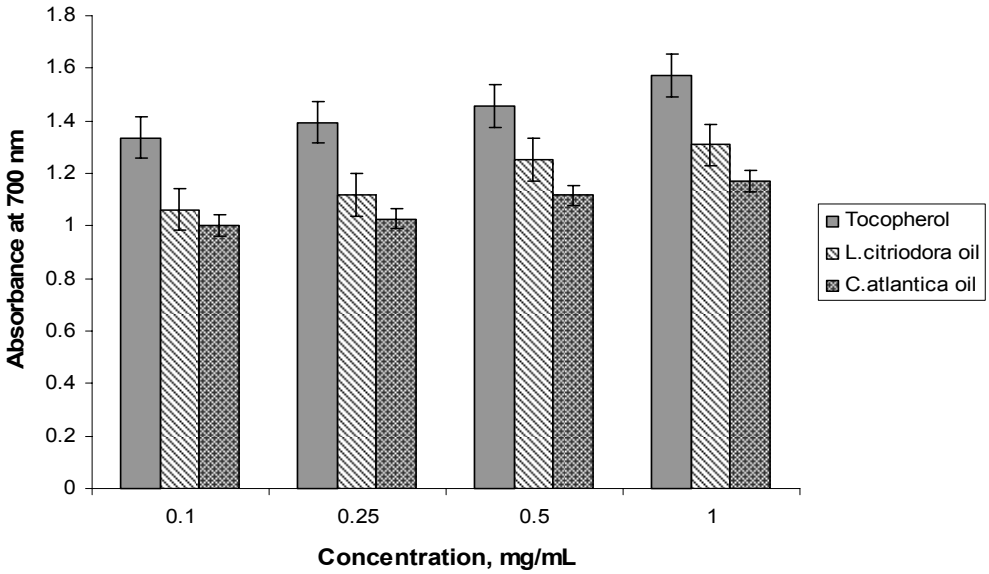

Fig. 2 – Antioxidant capacities of *Lippia citriodora* and *Cedrus atlantica* essential oils, using ferric reducing power method.

The bioactivities of essential oils are dependent upon their major components. Therefore, activity of *L. citriodora* and *C. atlantica* essential oils could be attributed to the high contents of monoterpenes and sesquiterpenes. In addition, Tepe et al. reported that the essential oils which contain monoterpene hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes have greater antioxidant properties.[16]

The reducing power of a compound serves as a significant indicator of its antioxidant activity. It has been widely accepted that the higher the absorbance at 700 nm, the greater is the reducing power.

In this study, the ability of the essential oil, and α-tocopherol as a positive control, to reduce Fe^{3+} to Fe^{2+} was determined. As can be seen in Fig. 2, the reducing capacity of the essential oils and α-tocopherol increased with increasing concentration, and as anticipated, the reducing power of the essential oils was inferior to α-tocopherol, which is known to be a strong reducing agent. The antioxidant activity measured with the ferric reducing power assay (Fig. 2) revealed similar results to those obtained with the DPPH technique. The highest antioxidant activities were obtained for *L. citriodora* oil, which also indicated their potential as electron donors to scavenge free radicals.

The characterized essential oils obtained from plants growing in Morocco were challenged against common Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria associated with food poisoning. The objective was to evaluate their antibacterial properties by the presence or absence of grown inhibition zones and zone diameter, MBC and MIC values. Results are summarized in Table 2.

Table 2

Zones of growth inhibition (mm), minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of selected plant oils

Essential oils	Bacteria	Diametre of inhibition (mm)	MIC	MBC
Lippia citriodora	*E. coli*	22.0±0.0	0.500	>1
	S. aureus	17.0±0.0	1	>1
Cedrus atlantica	*E. coli*	NI	>1	>1
	S. aureus	NI	>1	>1

NI: no inhibited.
MIC: minimum inhibitory concentration (as % v/v).
MBC: minimum bactericidal concentration (as % v/v).

Our results showed that essential oil from was the most active against *E. coli* when tested by disk diffusion, with 22.0 mm the mean diameter of the inhibition zone, but exhibited a noticeable antibacterial effect against both studied bacteria. In the case of *E. coli*, the essential oil provoked a mean halo diameter of 22.0 ± 0.3 mm and in the case of *S. aureus* a mean inhibition halo of 17.0 ± 0.1 mm. *Cedrus atlantica* EO did not the inhibit any of the two tested bacteria. It can be observed that *E. coli,* which is a cause of serious food poisoning and occasionally death and as well as *S. aureus*, which produce enterotoxin A and beta-haemolysin involved in food poisoning incident were inhibited by *L. citriodora* oil at 0.5% (v/v). The antibacterial activity of the essential oils of *L. citriodora* against studied bacteria may be due to the presence of citral, β-cylocitral, cuparene, spathulenol, caryophyllene oxide and caryophyllene, since the antimicrobial properties of caryophyllene and caryophyllene oxide found in *Salvia sclarea* were also observed by Ulubelen et al.[17] Literature survey also revealed that terpenes and cuparene sesquiterpenes possess antimicrobial and antioxidant activity, which are consistent with our present studies.[18] Although the mechanism of action of terpenes is not fully understood, it is thought to involve membrane disruption by the lipophilic compounds.[19] It is interesting to point out that the bacteria demonstrating the biggest inhibition zones by diffusion method are not always the ones that present the lowest MIC values. In fact, the diameter of the growth inhibition zone is affected by the oil solubility and volatility.[19]

Our results suggested that monoterpenes and sesquiterpenes in the essential oil from *L. citriodora* be the main bioactive constituents for the antimicrobial and antioxidant activities.

CONCLUSIONS

The present study provides information of the chemical composition, antioxidant and antimicrobial activity of the EOs of *L. citriodora* and *C. atlantica* obtained from plants growing in Morocco. In addition, it reports for the first time on the antioxidant activity of *Lippia citriodora* and *Cedrus atlantica* essential oils. The results obtained in this study support the possible use of *L. citriodora,* essential oils in the food industry, where bacterial pathogens cause severe destruction by hampering the quality of food and consumer demand as well as for using as a source of natural antioxidants.

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