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Chemical study and evaluation of insectical properties of African *Lippia citriodora* essential oil

Fatouma Mohamed Abdoul-latif[1*], Ayoub Ainane[2], Talal Mohamed Abdoul-latif and Tarik Ainane[2]

ABSTRACT

The main objective of this study is to highlight the chemical composition, the insecticidal and the antibacterial activities of essential oil of *Lippia citriodora* growing spontaneously in central Morocco, particularly in Beni Mellal. After extracting the essential oil by a clevenger type distiller from the aerial parts, all constituents were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). Their chemical composition of chemotype revealed the presence of 45 compounds in the essential oil, which has Geranial as the major constituent with a content of approximately (35.45%), followed by Neral (26.11%) and Limonene (14.06%), with a total percentage of (75.62%). The insecticidal activities were carried out against three weevils: *Sitophilus granarius, Sitophilus oryzae* and *Sitophilus zeamais,* the results of this test to show a very important activity during 24 hours of the treatments confirmed by the values of LD_{50} and LD_{90} which tend towards 0, or this activity was probably due to the major constituents. The bacterial power was studied in vitro against gram-positive bacteria: *Bacillus cereus* (CECT 193) and *Listeria monocytogenes* (CECT 4032), gram-negative bacteria: *Yersinia enterocolitica* (CECT 9144), *Pseudomonas aeruginosa* (CECT 116) and *Staphylococcus aureus* (CECT 239), the results of this tests made it possible to quantitatively assess the bacterial power by looking for minimum MIC inhibitory concentrations and minimum MBC bactericidal concentrations, which have a varying activity depending on the strain tested.

Keywords: Essential oil; *Lippia citriodora*, Chemical constituents; Insecticidal activity; Bacterial activities.

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INTRODUCTION

In all civilizations and on all continents, Human use of medicinal and aromatic plants (MAP) has always been to cure and fight disease (Chaachouay *et al*., 2019). Therapeutic use of plants today, despite advances in pharmacology is very common in some countries, particularly in the developing countries. The biogeographically position of Morocco plays an important role in providing rich biodiversity, which constitutes a true reserve for the aromatic genetic plants and medicinal plants, estimated at approximately 4500 species, 940 genera, and 135 families. The Atlas and the Rif mountainous regions were the regions of endemism (Kane *et al*., 2019). Moroccan biodiversity is characterized by a very notable endemism allowing it to occupy a decent position among the Mediterranean countries, which have a traditional aptitude based on medicinal and aromatic plants (Soultan *et al*., 2019). The essential oils are volatile liquid fractions, mostly obtained by extraction, they contain the substances aroma and which are important in cosmetics, food and pharmaceuticals (Senthilkumar *et al*., 2019). There are different classification criteria for essential oils and we can cite consistency, origin and chemical nature of the main components (Yousefi *et al.,* 2019; Sararit and Auamcharoen, 2020).

Lemon verbena, Aloysia citriodoral or formerly *Lippia citriodora*, is a woody plant of the Verbenaceae family, native to South America, particularly Chile. Verbena is a perennial shrub that grows quickly and can reach under the best conditions 2 to 3 meters in height (Atkins, 2004). Its stems, straight branched into narrow and slender branches, bear entire, lanceolate leaves, a little rough, deciduous in winter. In the heart of summer, from July to September, long clusters appear at the ends of the stems which bring together numerous small flowers, white, purple or reddish (Quirantes-Piné *et al*., 2013). The *Lippia citriodora* was introduced in Europe at the end of the 17th century around 1784 it is cultivated for its very fragrant leaves owing to the lemon aroma released which are used for culinary preparations, herbal tea preparations, or some and liqueurs, thus popular uses in the treatment of spasms, asthma, colds, gas, colic, diarrheic, fever, insomnia, indigestion, and anxiety, and by therefore it is renowned for its antipyretic, sedative, antispasmodic and digestive properties (Amin *et al*., 2018; Fitsiou *et al*., 2018). Verbena has become a plant deeply rooted in Africa and in the Moroccan pharmacopoeia and culinary art. It is widely cultivated, particularly in the regions of Marrakech, Agadir and Beni Mellal, in family gardens and in agricultural fields. Its crops were first established in the early 1960s and today it occupies several hectares for production exported to international markets (Salhi *et al*., 2019).

The insecticidal activities were carried out against three weevils: *Sitophilus granarius*, *Sitophilus oryza* and *Sitophilus zeamais.* The insects studied are beetles of the family of Curculionidae, the adults of *Sitophilus granaries* are small, uniformly dark brown beetles about 5 mm in length, unable to fly. These larvae, white and legless, develop inside the grains (Ainane *et al*., 2019a; Ak, 2019; Mantzoukas *et al*., 2019). The rice weevil (*Sitophilus oryza*) are brown with small sizes (2.5 to 4 mm). The elytra are marked with four characteristic reddish yellow spots. In adulthood, the rice weevil is identical to the corn weevil (*Sitophilus zeamais*), and a dissection is needed to distinguish the two species. These larvae, white and legless, develop inside the grains (Ainane *et al*., 2019a; Ak, 2019; Mantzoukas *et al*., 2019). *Sitophilus zeamais* (corn weevil) corn weevils are identical to rice weevils (*Sitophilus oryza*), and dissection is required to separate the two species. Adults are reddish-brown with four yellow to red spots on elytra and have a characteristic long rostrum (Ainane *et al*., 2019a; Ak, 2019; Mantzoukas *et al*., 2019).

The objective of the present investigation on the study of the essential oil composition of *Lippia citriodora* and their insecticide and antibacterial properties *in vitro*. The bacterial power was studied *in vitro* against gram-positive bacteria: *Bacillus subtilis* (CECT 498), *Bacillus cereus* (CECT 193), *Micrococcus luteus* (CECT 243) and *Listeria monocytogenes* (CECT 4032), gram-negative bacteria: *Yersinia enterocolitica* (CECT 9144), *Escherichia coli* (CECT 516), *Pseudomonas aeruginosa* (CECT 116) and *Staphylococcus aureus* (CECT 239), The bioactivity test was carried out against insects of stored foodstuffs and eight phytopathogenic bacteria, responsible for replicated diseases in agricultural crops.

MATERIAL AND METHODS

Collection of plants

Lippia citriodora is an aromatic and medicinal plant belonging to the Verbenaceae family. The aerial part of this species was collected in the region of Beni Mellal-Khenifra of Morocco (Middle Atlas 32°58’22.9’’N 5°39’38.7’’W). A botanist at the Forest Research Center of Khenifra (Morocco) verified the studied species. The herbs were air-dried in the room and then were milled into 80 mesh powder before hydrodistillation.

Essential oil extraction

The essential oil from the aerial parts (leaves, stems and flowers) of *Lippia citriodora* was obtained by hydrodistillation in fractions of 500 g for 4h using a Clevenger type extractor (500ml clevenger device for essential oil extraction Type: TF-500 in borosilicate glass. The length of the condenser: 400mm with an extraction capacity of 5ml) The water vapor saturated with essential oil to undergo a condensation operation and then to be collected in a separating funnel. The fractions less dense than water were decanted and dried over anhydrous sodium sulfate (Na_2SO_4). The essential oil obtained was stored in the refrigerator at a temperature of 4°C for subsequent use in the various tests (Ainane *et al*., 2019a).

Determination of chemical compositions

The essential oil analysis was performed by gas chromatography coupled with mass spectrometry (GC/MS) (in National Center for Scientific and Technical Research, Rabat, Morocco). The instrument of analysis, GC/MS used is of type (Hewlett Packard 5971A). The determination of the relative proportions of the different constituents is obtained by quantitative analyzes: gas chromatography coupled with flame ionization (GC/FID).

The analyzes are carried out under the same conditions. The GC/MS was carried out on a DB-5 type column, the dimensions of which are: diameter: 250 µm; length: 30 m; film thickness 0.2 mm. The temperature program applied is as follows: 280°C; detector temperature: 300°C; oven temperature: 50°C (1 min), 50 to 150°C (3 °C/min) and from 150°C to 250°C (5 °C/min) and in isothermal (250°C) for 5 min. The carrier gas is helium with a flow rate of 1 mL /min (Ainane *et al*., 2019b).

The insecticidal activity Insecticide tests:

The insects were reared on wheat, rice and maize, tender in 1 liter plastic boxes, pellucid and screened. The set is placed in speakers whose temperature is 30°C ± 1°C and the relative humidity is 70% (Germinara *et al*., 2017). The insecticidal tests used in this study are as follows: in experimental chambers containing ten insects of each species (*Sitophilus granarius*, *Sitophilus oryza* and *Sitophilus zeamais*), the sensitivity of essential oil is tested in increasing concentrations of 0.01 µl/cm³, 0.015 µl/cm³, 0.02 µl/cm³, 0.025 µl/cm³ and 0.03 µl/cm³ respectively on Whatman paper number one. The whole is introduced into the experimental fumigation chamber (semi-ventilated). Three replicates were performed for each concentration and each species to minimize errors. The Mortality is recorded as a function of time after a day and corrected mortality is expressed by Abbot's formula (Abbott *et al*., 1925).

The determination of the lethal dose of 50% (LD_{50}) and 90% (LD_{90}) is determined by linear interpolation on curves giving the percentage of mortality as a function of the logarithm of the concentration tested.

Antibacterial Activities

The evaluation of the antibacterial properties of essential oils *of Lippia citriodora* was carried out against gram-positive bacteria: *Bacillus cereus* (CECT 193) and *Listeria monocytogenes* (CECT 4032), gram-negative bacteria: *Yersinia enterocolitica* (CECT 9144), *Pseudomonas aeruginosa* (CECT 116) and *Staphylococcus aureus* (CECT 239) by two methods. The antibacterial activity of *Lippia citriodora* EO by first method was performed using the diffusion method: (disk diffusion and agar well diffusion method) and for the bacterial strains, MHA and SDA plates were prepared respectively. The discs were cut to a diameter of 5 mm from Whatman filter paper, then the respective discs were immensely soaked in oil for a period of 24 hours. then *Lippia citriodora* EO aseptically into the surface of the agar in a plate (Bennamara & Abourriche, 2020). The second method is microdilution method followed by Maadane *et al*. (2017). The wells were distributed with the respective oil (18; 20 ml) in the plate (96 wells). All the plates of were incubated for 24 h at 30 °C or 37 °C depending on the strain of bacteria. The zone of inhibition was measured around the well. Each experiment was performed in triplicate. MIC of various *Lippia citriodora* OE was determined by the microdilution method using CLSI guidelines. MIC was performed by preparing *Lippia citriodora* EO at 100 ml. Concentrations were diluted within the range of 3.125, 6.25, 12.50, 25.00, 50.00 and 100.00 µL/mL. From the wells where there has been no color change and therefore no growth, 10 µL aliquots of each well are transferred and inoculated onto Muller-Hinton agar (MHA) then incubated for 18 h with good temperatures for each germ. MBC is the smallest concentration where there is no subculture.

RESULTS AND DISCUSSIONS

Analysis of *Lippia citriodora* EO

The analysis of the results of the chemical composition carried out by GC/MS of *Lippia citriodora* OE is mentioned in Table 1.

The chromatographic analysis by GC/MS revealed the presence of 45 compounds in the essential oil, which has Geranial as the major constituent with a content of approximately (35.45%), followed by Neral (26.11%) and Limonene (14.06%), with a total percentage of (75.62%). The content of the rest of the constituents is low.

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

Pic	RT	Compound	%
1	3.12	α-pinène	0.55
2	6.06	β-pinène	0.17
3	6.75	Sabinene	0.54
4	7.49	Myrcene	0.45
5	8.29	Limonene	14.06
6	8.26	β-Ocimene	0.50
7	8.79	1,8-cineol	0.45
8	8.93	β-bourbonene	0.01
9	8.99	Linalool	0.35
10	9.24	Nonanal	0.02
11	9.30	Verbenol	1.43
12	9.39	cis-citral	1.01
13	9.51	Terpinen-4-ol	0.05
14	9.80	α-Copaene	0.01
15	9.98	β-Copaene	0.01
16	9.98	Nerol	1.05
17	10.22	Neral	26.11
18	10.26	Geraniol	1.65
19	10.30	Geranial	35.45
20	10.40	Bicyclogermacrene	0.86
21	10.49	Furfural	0.01
22	10.80	Bergamotene	0.15
23	10.85	3-carène	0.01
24	10.97	α-Cedrene	0.35
25	11.11	β-Acoradiene	0.19
26	11.26	β-Caryophyllene	2.38
27	11.53	Caryophyllene oxide	0.83
28	11.58	Santonin	0.01
29	11.70	Fluoranthene	0.01
30	12.20	α-Cubebene	0.01
31	13.00	α-Curcumene	1.91
32	13.16	Geranyl propanoate	0.26
33	20.92	Zingiberene	0.86
34	23.36	Cembrene	0.01
35	24.68	Aromandendrene	0.01
36	25.53	Nerolidol	1.03
37	25.87	carvacrol	1.65
38	26.00	spathulenol	3.64
39	26.13	α-cadinol	0.66
40	26.58	Farnesol	0.02
41	27.44	α-Cadinene	0.19
42	28.34	β-Cadinene	0.12
43	29.43	γ-Cadinene	0.45
44	29.62	γ-Atlantone	0.01
45	29.89	Sitosterol	0.01

The chemical composition of *Lippia citriodora* essential oil has been studied by several authors (Oukerrou *et al*., 2017; Farahmandfar *et al*., 2018 ; Kaskoos, 2019). The genus of plants *Lippia* exhibits rich biological diversity, in particular a genetic variety which makes it possible to synthesize different constituents of essential oils in cultivated plants around the world (Tohidi *et al*., 2019). The essential oil composition obtained from plant stock to keep the same environmental conditions (Aissi *et al*., 2016).

Insecticidal activities

The results of the insecticidal tests of *Lippia citriodora* essential oil against *Sitophilus granarius*, *Sitophilus oryzae* and *Sitophilus zeamais*, are presented in Table 2 in the form of lethal doses at 50% and 90% (LD_{50} and LD_{90}).

The study of the insecticidal activity of essential oil of *Lippia citriodora* confirmed that the insecticidal activity is important and varies widely depending on the insect used. In addition, this activity is probably due to the major constituents.

According to the lethal dose of 50% and 90% (LD_{50} and LD_{90}), it is concluded that the essential oil of *Lippia citriodora* exhibits significant insecticidal activity and is effective under more precise conditions.

Table 2. LD_{50} and LD_{90} of insecticidal activities of *Lippia citriodora* EO.

Species	LD_{50}	LD_{90}
Sitophilus granarius	0.014 ± 0.003	0.029± 0.004
Sitophilus oryzae	0.010± 0.002	0.024± 0.005
Sitophilus zeamais	0.010± 0.002	0.025± 0.005

Antimicrobial Activities

The results o of the antibacterial evaluations of all the methods applied to the essential oil of *Lippia citriodora* in terms of: diameters (Φ) of the zones of inhibition measured around the discs, the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC)) and the report (MBC / MIC). All the values obtained are expose in Table 3.

The results obtained show that the essential oil of *Lippia citriodora* has an interesting antibacterial activity; acceptable and favorable against all the bacteria of gram positive bacteria: *Bacillus cereus* and *Listeria monocytogenes*, while Gram-negative bacteria: gram-negative bacteria: *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* exhibit moderate to weak antibacterial activity.

The diameters of the zones of inhibition Φ obtained by the disk diffusion method vary from 2.50 to 12.65 mm, the minimum inhibitory concentrations of MIC vary from 468.52 to 1224.21 µL/mL and the minimum concentrations of MBC bactericides range from 625.14 to 1224.21 µL/mL. Similarly results showing high antimicrobial activity of essential oil extracts of *Lippia citriodora* have been previously reported in the following references (Kizil *et al*., 2018; Benoua *et al*., 2019)**.** However, they were only limited to disk diffusion or well diffusion assays and no MIC and MBC values were reported. The antimicrobial activity of the essential oil could be attributed to the main constituent, although possible antagonistic effects should not be excluded (Yamani *et al*., 2016 ; Ryu *et al*., 2018; Solomou *et al*., 2020)**.** These effects need to be further investigated using model methods.

The plant species that were the subject of our study, namely *Lippia citriodora*, is frequently used in traditional Moroccan pharmacopoeia. The results of insecticidal activities show a very important activity during 24 hrs of the treatments confirmed. The antibacterial activities of these essences were tested on 8 bacteria belonging to 6 families: *Bacillaceae*, *Listeriaceae*, *Pseudomonadaceae* and *Staphylococcaceae*. These bacteria are responsible for several kinds of ailments in Morocco.

Table 3. Parameters of the antibacterial activity of *Lippia citriodora* EO.

Strains	Parameters	Values
Bacillus subtilis	Φ (cm)	11.32 ± 0.93
	MIC (µl/ml)	693.33± 3.05
	MBC (µl/ml)	921.04± 5.98
	MBC / MIC	1.32
Listeria monocytogenes	Φ (cm)	6.02 ± 1.22
	MIC (µl/ml)	910.44 ± 5.38
	MBC (µl/ml)	1004.85 ± 8.20
	MBC / MIC	1.10
Yersinia enterocolitica	Φ (cm)	5.81 ± 1.50
	MIC (µl/ml)	705.71 ± 4.54
	MBC (µl/ml)	804.50± 4.24
	MBC / MIC	1.14
Staphylococcus aureus	Φ (cm)	7.32± 0.85
	MIC (µl/ml)	468.52± 2.87
	MBC (µl/ml)	625.14± 3.28
	MBC / MIC	1.33
Pseudomonas aeruginosa	Φ (cm)	6.74 ± 0.63
	MIC (µl/ml)	571.25 ± 3.07
	MBC (µl/ml)	862.58 ± 4.98
	MBC / MIC	1.51

The essential oil of Lippia citriodora to reveal the most important bacterial activities. These effects are certainly attributable to the presence of compounds such as: Geranial (35.45%), Neral (26.11%) and Limonene (14.06%), with a total percentage of (75.62%). This shows that the Moroccan flora can constitute an important reserve of interesting plant species, which can be used in several fields such as pharmaceutical and agro-food industries.

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Fatouma Mohamed Abdoul-Latif[1*], Ayoub Ainane[2], Talal Mohamed Abdoul-Latif[1], Tarik Ainane[2]

[1]Medicinal Research Institute, Center for Research and Study of Djibouti, BP 486, Djibouti.

[2]Superior School of Technology,University of Sultan MoulaySlimane, PB 170, Khenifra 54000 Morocco.

[*]**Corresponding author**

E-mail: fatouma_abdoulatif@yahoo.fr