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CHEMICAL COMPOSITIONS, INSECTICIDAL AND ANTIMICROBIAL ACTIVITIES OF TWO MOROCCAN ESSENTIAL OILS OF *CITRUS LIMONUM* AND *SYZYGIUM AROMATICUM*

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Abstract

In this study, the chemical composition, antimicrobial and insecticidal activities of *Citrus limonum* and *Syzygium aromaticum* essential oils were executed. The GC-FID/MS analysis allowed for the identification of 16 and 11 volatiles, comprising 66.6 and 88.6% of the oils, respectively. The major components of the *Citrus limonum* oil were neral (13.6 %), neryl acetate (10.8 %) and α-pinene (9.5 %), and for *Syzygium aromaticum* oil were 1,1,4,8-tetramethylcis, cis, 4,7,10-cycloundecatriene (27.7 %), caryophyllene oxide (24.3 %), eugenol (17.6 %). The results of the antimicrobial activity, evaluated against 8 selected bacteria and fungus, was found moderate, with the activity of the two oils present remarkable activities against all strains. Moreover, the insecticidal tests against *Sitophilus granarius* at different temperatures showed a very important (most values of LD_{50} approach 0). All good activities mentioned were probably due to the major constituents of each essential oil.

Keywords: *Essential oils, Citrus limonum, Syzygium aromaticum, Chemical analysis; antimicrobial activity; insecticidal activity.*

Introduction

In recent years, serious problem affects the quality of food, so these are subjected, during the culture period, to the aggressions of biotic origin (pests and microorganisms) which cause significant losses as well as a consequent fall in the agronomic and organoleptic qualities. many researchers have focused on this problem for to find adequate solutions [1].

The use of chemical products, mainly pyrethroids and organophosphates, is the most common method used to protect crops [2]. Due to their toxicity, these products are applied by spraying the soil and/or walls prior to the replacement of the litter for the next production cycle, in order to avoid direct contact with animals [3-4]. However, this method only offers partial control since misuse of chemical products has resulted in the loss of Feld efficacy and the development of resistant pests and microorganism populations. Consequently, natural products such as plant extracts, particularly essential oils (EOs) of medicinal and aromatic plants, have recently received increased attention, so its have used as alternative management practices, which are often characterized by low toxicity towards mammalians and high biodegradability, high effective as a contact and fumigant insecticides and as repellents against insect pests [5]. Since EOs are volatile, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites (monoterpenes, sesquiterpenes and phenylpropanoid compounds), they are complex mixtures, their bioactivity can result from various interactions among the constituents [6]. Likewise, many essential oils exhibited superior activity as natural oils or as an artificial mixture of all the major constituents compared to that of major individual constituent. Except insecticidal and antimicrobial activities in food sciences, EOs are known to possess remarkable pharmaceutical and therapeutic potentials. we also note, three modes of action of EOs on the insect pest have been found [7-8]. They include acting on the nervous system of insects, suppression and interference of normal growth, development, metamorphosis, and reproduction of insects, as well as inhibition of mitochondrial membrane respiratory enzymes or regulation of oxygen consumption and the amount of carbon dioxide released in insects [9].

The present study was to evaluate the insecticidal activity of two EOs obtained from *Citrus limonum* (Rutaceae) and *Syzygium aromaticum* (Myrtaceae) against adults of *Sitophilus granarius* and their antimicrobial potential against bacteria transmitted by this insect and against their external microbial load. The physical-chemical proprieties and chemical composition of the EOs was also determined.

Methods

Collection of plants

The two species of aromatic and medicinal plants, *Citrus limonum* and *Syzygium aromaticum*, representing the Rutaceae and Myrtaceae families respectively, were collected in Morocco (5 Kgs each), in the regions of Beni Mellal and Marrakech, respectively. These species have been verified by Dr. T. Ainane a botanist at the EST-Khenifra - Morocco. Voucher specimens were deposited in the herbarium of the Department of environmental engineering, EST-Khenifra, University of Sultan Moulay Slimane, Morocco (under numbers S2017TA5 for *Citrus limonum* and S2017TA6 *Syzygium aromaticum*).

Extraction

The EOs were obtained by hydrodistillation, for each species of the togetherness of aerial part (stems, leaves and flowers) in fractions of 250 g for 3 hours using a Clevenger-type extractor (it was repeated 8 times). Matter less dense than water is collected by simple decanting and dried on anhydrous sodium sulphate before analysis. EOs were stored in the dark in sealed tubes in the refrigerator at 4°C for later use in the various tests.

Physical-chemical properties

Yield " Y" of the essential oil is the ratio of the weight w of the extracted essential oil to the weight W of the vegetable matter. It is expressed in ml distillate for 100 g of dry matter (w/W %).

$$Y(\%) = \frac{w}{W} \times 100$$

The relative density of 20 °C, noted " d^{20} " is the ratio, expressed as a decimal number, by the quotient of the mass of a certain volume of essential oil to 20 °C. The density of the water at the same temperature [10]. A pycnometer of 50 ml is used to determine this parameter. The density is expressed by the following relationship:

$$d^{20} = \frac{m_2 - m_0}{m_1 - m_0}$$

m_0: mass of the empty pycnometer.

m_1: mass of the pycnometer filled with water.

m_2: mass of the pycnometer filled with essential oil.

Measurements of pH were made using a pH meter according [11].

The refractive index " n " is a dimensionless quantity characteristic of a medium, describing the behaviour of light in that medium. The refractive index was measured at 20 ° Cusing an Abbe Convex Refractive Index Refractometer between: 1.3000 and 1.7200 [12].

Determination of chemical compositions

Analyses of essential oils were carried out by gas chromatography coupled with mass spectrometry (GC/MS) (National Center for Scientific and Technical Researchs, Rabat, Morocco).

The instrument of analysis, GC/MS used is of type (Hewlett Packard 5971A). The determination of the relative proportions of different molecules is obtained by gas chromatography coupled with flame ionization (GC/FID). Analyses by GC/MS and GC/FID are carried out under identical conditions. The GC/MS was made on a column DB-5 (5% of phenyl methyl siloxane) whose dimensions are: length: 30 m ; diameter: 250 µm ; film thickness 0.32 microns. The temperature program applied was 40 °C during 5 min of 40 at 200 °C à 3 °C/min and then maintained at 200 °C during 5 min.. The carrier gas was helium (Pressure: 49.9 kPa ; Flow: 1 ml/min). The mass spectrometer source at a temperature of 230 °C and the mass range is swept by 50 à 350 amu [13].

Insecticidal activity:

Sitophilus granarius, also known as the wheat weevil, is a species of coleopteran insects of the Curculionidae family with a quasi-cosmopolitan distribution. The larvae are white and apodic and develop inside the grains. Outside, adults are small, uniform (5 mm in length), dark-brown and that are unable to fly [14].

The insects of the species *Sitophilus granarius* were reared respectively on wheat, tender in plastic boxes of one litre capacity, transparent and wire mesh. The whole is placed in enclosures whose temperature is 30 °C and the relative humidity is 70 % [15].

The insecticide test procedure used in this study is as follows: in petri dishes (experimental chambers) containing ten insects, the essential oils: Citrus limonum and Syzygium aromaticum were tested at increasing concentrations of : C1= 0.01 µL/cm^3, C2=0.02 µL/cm^3 and C3=0.03 µL/cm^3 and at different temperatures: T1=25 °C, T2=30 °C and T3=35 °C. The essential oils were placed in steel cylinders with a constant depth of 0.5 cm and diameters of D1=1 cm, D2=2 cm and D3=3 cm. The assembly is introduced into a fumigation chamber included in the experimental enclosure (semi-ventilated). Repetitions were carried out in triplicate for each trial to minimise errors with an oil-free control. The number of dead insects was recorded as a function of time after 1 day.

Adjusted mortality in treated insects is expressed according to the formula:

$$M\% = \left(\frac{M_I - M_C}{100 - M_C}\right) \times 100$$

With:

M% : Mortality corrected;
M_I : Mortality observed in insects;
M_C : Mortality observed in controls.

The determination of the lethal dose of 50% LD50 is determined by linear interpolation on curves giving the percentage of mortality as a function of the logarithm of the concentration tested.

Antimicrobial Activities:

Antimicrobial property evaluations of two essential oils: *Citrus limonum*, and *Syzygium aromaticum* were performed against Gram-positive bacteria such as : *Staphylococcus aureus* (CECT 976), *Enterococcus faecium* (CECT 4932), *Listeria monocytogenes* (CECT 911) and *Bacillus subtilis* (CECT 4071), Gram negative bacteria such as: *Escherichia* *coli* (CECT 431), *Yersinia enterocolitica* (CECT 4315) and *Pseudomonas aeruginosa* and the yeast *Candida albicans.*

the antimicrobial activity of EOs were carried out using disc diffusion and agar well diffusion method. The MHA and SDA plates were prepared for bacterial and fungal strains respectively. Each of discs which are cut in to 5 mm in diameter from whatman filter paper, then discs were immensely soaking with respective various oils for 24 h. After that each EO aseptically in to the agar surface in a plate. Agar well diffusion method followed by Maadane et al. [16]. Wells of 8 mm were punched in the respective medium using sterile cork borer. The wells were dispensed with respective oils (18; 20 ml). All the plates were incubated for 24 h at 37 °C for bacteria and 72 h at 25±2 °C for fungi respectively. The measurement of zone of inhibition was taken from around the well. Each experiment was carried out by triplicate.

The MIC of various EOs was determined by micro-dilution method with the help of CLSI guidelines (CLSI, 2014). The MIC was carried out, by preparing the EO in 100 ml of concentrations were diluted in the range of 3.125, 6.25, 12.50, 25.00, 50.00 and 100.00 µl/ml. From the wells where there was no change in colour and therefore no growth, aliquots of 10 µL from each well are transferred and inoculated onto Muller-Hinton agar (MHA) and then incubated during 18 h at the right temperatures for each germ. The MBC is the smallest concentration where there is no subculture [17].

Results & Discussion

Physical-chemical proprieties

All the physical-chemical properties of two essential oils such as *Citrus limonum* and *Syzygium aromaticum* have been determined. The values obtained are given in tables 1 and 2. Analysis of these data leads of two EOs *Citrus limonum* and *Syzygium aromaticum* to the following conclusions:

- The average yields of the essential oils studied are: 0.69 (±0.08) w/W% and 1.31 (±0.21) w/W%, respectively.
- The densities have values around 0.9 they're smaller that 1 which proves that oils are less dense than water.
- The pH of two EOs is acidic.
- The values of the refractive indices obtained are respectively comprised between 1.47 and 1.52.

Analysis of EOs :

The analysis of the results of the chemical composition carried out by GC/MS of two EOs of *Citrus limonum* and *Syzygium aromaticum* are mentioned in the Table 3 and Table 4, respectively.

The EO of *Citrus limonum* has main components: Neral (13.6 %), neryl acetate (10.8 %), α-pinene (9.5 %), geranyl isovalerate (6.8 %), cis-trance limonene oxide (6.7 %), the β-bisabolene (4.8 %), the trans-α-bergamotene (3.4 %), limonene diol (3.2 %) and linalool (2.2 %). A comparison of our results with previous work gives a total difference in chemical composition. Results from Bertuzzi et al. [18] indicate that limonene (71.9 %) is the main constituent of the EO of Citrus limonum. Another study by Himed et al. [19] indicates that the identification of the components of the EO on the peel of *Citrus limonum* (Lisbon - Portugal) shows an interesting content of limonene (64.9 %) followed by the β-pinene (7.8 %) and alpha-terpinene (5.5 %). On the other hand, according to the study by Moufida & Marzouk [20], Geranial (3.7 %) is the lead compound.

The analysis of the results mentioned in Table 4 of the chemical composition of *Syzygium aromaticum* essential oil carried out by GC/MS, shows that: The main majority compounds identified are: 1,1,4,8-tetramethylcis, cis, 4,7,10-cycloundecatriene (27.7 %), caryophyllene oxide (24.3 %), eugenol (17.6 %).The δ-cadinene (4.5 %), caryophyllene (3.2 %), humulene epoxide II (2.9 %), tarragol (1.8 %), aromandendrene (1.3 %) and 1-(3,4-methylenedioxyphenyl)propan-1-ol (1.0 %) have acceptable levels. The content of the remaining 36 constituents is less than 1 %. With regard to composition, previous work shows that the essential oil of *Syzygium aromaticum* contains eugenol as the main compound, and other compounds such as eugenyl acetate, β-caryophyllene, myrcene and α-terpinene [21-22].

Insecticidal activities

The results of the insecticidal tests of the essential oils *Citrus limonum* and *Syzygium aromaticum* against Sitophilus granarius are shown in Table 5 as 50% lethal doses (LD_{50}) depending on the parameters studied such as: cylinder diameter D and the incubation temperature T. The study of the insecticidal activity of these essential oils on an organism harmful to stored products *Sitophilus granarius* confirmed that the essential oils studied have a significant insecticidal activity. The activity of the essential oils tested varies widely depending on the nature of the essential oil and the factors used. In addition, the insecticidal activities of the essential oils studied are probably due to the major constituents of each essential oil, thus their synergistic properties. Depending on the lethal dose of 50 % (LD_{50}), it is concluded that the essential oils of *Citrus limonum* have significant insecticidal activities compared to *Syzygium aromaticum*, particularly, at room temperature. From a global, the factors studied such as cylinder diameter and temperature influence insecticidal activity, hence the increase in cylinder diameter and the increase in temperature favours good activity. All these tests carried out can confirm that the treatment of foodstuffs with essential oil from aromatic and medicinal plants can be very effective in controlling pests of stored foodstuffs. These EOs contain molecules that exhibit interesting products.

Antimicrobial Activities

The results of the antibacterial evaluations of all methods applied to the two essential oils *Citrus limonum* and *Syzygium aromaticum* are expressed in terms of diameters (Φ) of inhibition zones measured around the discs, the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and the report (MBC/MIC). All the values obtained are shown in Table 6. From the results obtained, it can be concluded that both essential oils have interesting antimicrobial activities against *Staphylococcus aureus*, *Enterococcus faecium*, *Listeria monocytogenes*, *Bacillus subtilis*, *Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *Candida albicans*. Diameters of inhibition zones Φ obtained from the disc diffusion method vary from 7.11 à 18.01 mm the minimum inhibitory concentrations MIC vary from 31.6 à 56.21 µl/ml and minimum bactericidal concentrations MBC vary from 43.52 à 87.21 µl/ml. Mostly, the essential oil of *Citrus limonum* has remarkable activities with respect to all the strains studied and also the essential oil of *Syzygium aromaticum* has significant activities with respect to all the strains studied, except with respect to *Peudomonas aeruginosa*, it has a medium activity. On the other hand, according tothe MBC/MIC report, the bactericidal effect was observed in the two EOs against all bacteria, except the EO of *Syzygium aromaticum* which was a bacterostatic effect against *Bacillus subtilis*. In general, it is also established in many works that the activity of an essential oil is related to the majority compounds and the possible synergistic effects between the constituents [23-24].

Conclusions

During the present study, the work carried out gave us a general point of view on the valuation of these essential oils, from where we conducted a chemical analysis which proved the presence of the majority bioactive compounds such as: Neral, neryl acetate, α-pinene for *Citrus limonum* and 1,1,4,8-tetramethylcis,cis,4,7,10-cycloundecatriene, caryophyllene oxide, eugenol for *Syzygium aromaticum.* In the other hand, biological study intended for the two EOs, the results obtained showed that the two essential oils have interesting prospects, and can be the subject of several biological applications particularly in the agricultural field and this by the conservation of seeds against biotic aggressions (insect pests and microbial infections). This approach can help to reduce the quantity of insecticides applied, and subsequently reduce the negative impact of synthetic agents, such as residues, resistance and environmental pollution. At the end of this work, it seems important to continue the search for essential oils from medicinal plants and also other extracts and purified molecules. It is also recommended to study other *in vitro* and *in vivo* activities for agricultural purposes.

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Table 1. Physical-chemical properties of EOs.

EOs	$Y(\%)$	d^{20}	pH	n
Citrus limonum	0.69 ± 0.08	0.9084 ± 0.0002	4.96 ± 0.84	1.4791 ± 0.0005
Syzygium aromaticum	1.31 ± 0.21	0.9011 ± 0.0002	5.07 ± 0.75	1.5347 ± 0.0005

Table 2. Organoleptic properties of EOs.

EOs	Aspect	Color	Odour
Citrus limon	Mobile liquid	Yellow	Gentle and tonic
Syzygium aromaticum	Clear, fluid and mobile liquid	Dark yellow	Spicy

Table 3. Chemical composition of the essential oil of *Citrus limonum*.

Peak	R_T	RI	RI(lit.)	Compounds	C (%)
1	3.2	976	1033	(-)-α-Pinene	9.5
2	3.5	1113	1125	Linalool	2.2
3	3.7	1135	1148	Cis-limonene oxide	2.4
4	3.8	1184	1175	Trans-Limonene oxide,	4.3
5	4.5	1194	1191	Terpinen-4-ol	1.3
6	4.6	1206	1207	2-Methylbicyclo[4.3.0]non-1(6)-ene	2.9
7	5.5	1662	1268	Neral	13.6
8	7.4	1294	1289	Limonene-diol	3.2
9	7.5	1298	1302	1,5-dimethyl-7-oxabicyclo[4.1.0]heptane	1.5
10	7.8	1357	1351	Neryl acetate	10.8
11	8.0	1388	1397	Geranyl isovalerate	6.8
12	8.3	1438	1424	(Z,E)-α-Farnesene	1.5
13	8.6	1486	1477	Trans-α-Bergamotene	3.4
14	9.8	1509	1508	Β-Bisabolene	4.8
15	10.6	1568	1571	Caryophyllene oxide	1.4
16	19.2	1619	1621	Acetic acid, 1,3,7-trimethylocta-2 ,6-dienyl ester	1.3
Grouped compounds					
Monoterpene hydrocarbons					9.5
Oxygen-containing monoterpenes					24.2
Sesquiterpene hydrocarbons					9.7
Oxygen-containing sesquiterpenes					23.5
Total					66.6

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T**able 4.** Chemical composition of the essential oil of *Syzygium aromaticum.*

Peak	R_T	RI	RI(lit.)	Compounds	C (%)
1	7.5	1358	1366	Eugenol	17.6
2	8.3	1394	1391	Caryophyllene	3.2
3	8.8	1402	1405	1,1,4,8-tetramethyl-cis,cis,4,7,10-cycloundecatriene	27.7
4	9.2	1429	1423	Aromandendrene	1.3
5	9.9	1451	1458	Trans-Calamenene	2.8
6	10.0	1484	1487	Δ-Cadinene	4.5
7	10.3	1521	1526	2-Pentadecen-4-yne, (Z)-	1.5
8	10.8	1578	1583	Caryophyllene oxide	24.3
9	11.0	1598	1594	1-(3,4-Methylenedioxyphenyl)propane-1-ol	1.0
10	11.1	1624	1622	Humulene epoxide ii	2.9
11	22.6	1662	1668	Estragole	1.8
Grouped compounds					
Oxygen-containing monoterpenes					17.6
Sesquiterpene hydrocarbons					45.7
Oxygen-containing sesquiterpenes					25.3
Total					88.6

Table 5. LD_{50} Insecticide activities of different EOs.

Diameter	Temperature	*Citrus limonum*	*Syzygium aromaticum*
D1 = 1 cm	**T1 = 25 °C**	0.015 ± 0.005	>>
	T2 = 30 °C	0.01 ± 0.005	0.955 ± 0.005
	T3 =35 °C	0.008 ± 0.005	0.072 ± 0.005
D1 = 2 cm	**T1 = 25 °C**	0.008 ± 0.005	>>
	T2 = 30 °C	0.009 ± 0.005	>>
	T3 =35 °C	0.006 ± 0.005	0.028 ± 0.005
D1 = 3 cm	**T1 = 25 °C**	0.009 ± 0.005	0.570 ± 0.005
	T2 = 30 °C	0.006 ± 0.005	0.501 ± 0.005
	T3 =35 °C	0.003 ± 0.005	0.020 ± 0.005

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Table 6. Parameters of the antibacterial activity of EOs.

Strains		*Citrus limon*	*Syzygium aromaticum*
Staphylococcus aureus	**Φ (cm)**	19.02 ± 0.85	21.6 ± 1.01
	MIC (µl/ml)	33.42	31.6
	MBC (µl/ml)	43.52	56.6
	MBC / MIC	1.30	1.79
Enterococcus faecium	**Φ (cm)**	19.21 ± 1.20	20.07 ± 0.84
	MIC (µl/ml)	36.41	33.07
	MBC (µl/ml)	56.33	63.07
	MBC / MIC	1.55	1.91
Listeria monocytogenes	**Φ (cm)**	18.01 ± 1.14	16.61 ± 1.14
	MIC (µl/ml)	49.01	48.1
	MBC (µl/ml)	77.32	68.1
	MBC / MIC	1.58	1.42
Bacillus subtilis	**Φ (cm)**	15.5 ± 0.94	15.1 ± 1.11
	MIC (µl/ml)	43.45	35.1
	MBC (µl/ml)	66.76	73.1
	MBC / MIC	1.54	2.08
Escherichia coli	**Φ (cm)**	16.71 ± 1.54	17.1 ± 1.40
	MIC (µl/ml)	49.81	47.1
	MBC (µl/ml)	66.44	76.1
	MBC / MIC	1.33	1.62
Yersinia enterocolitica	**Φ (cm)**	17.21 ± 1.44	16.17 ± 1.04
	MIC (µl/ml)	46.61	44.47
	MBC (µl/ml)	71.53	74.71
	MBC / MIC	1.53	1.68
Peudomonas aeruginosa	**Φ (cm)**	15.31 ± 1.61	7.11 ± 0.21
	MIC (µl/ml)	45.1	47.11
	MBC (µl/ml)	75.64	83.11
	MBC / MIC	1.68	1.76
Candida albicans	**Φ (cm)**	16.3 ± 0.37	16.21 ± 1.11
	MIC (µl/ml)	53.23	56.21
	MBC (µl/ml)	83.33	87.21
	MBC / MIC	1.57	1.55