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Fluctuations of chemical composition of essential oil and Antimicrobial of Lemon verbena (Lippia Citriodora) during growth stages in syria

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Abstract: Lemon verbena was cultivated in Syria and used in folk and Arabian medicine. Essential oil of fresh leaves is extracted, determined and analyzed with gas chromatography/ mass spectrometry (GC-MS) whereas no one determined the composition of Syrian origin before. Leaves is harvested in three stages of growth: in May, which approves the highest rate of growth, July, mid-season, and October, full bloom. The major component were citral (geranial and neral), limonene, β-caryophyllene, limonene, Germacrene D, Ar-curcumene, γ-elemene, 1,8-cineol,cedrene and β-caryophyllene oxide. The study at growth stages showed significant differences in the percentages of the main constituents especially citral, Ar-Curcumene, β-caryophyllene oxide and limonene. geranial and neral range from 17 to 19.4% and from 19.3 to 25.6 %, respectively. In addition limonene ranges from 3.3 to 6.07% and β-caryophyllene oxide from 8.74 to 17.8%, while Ar-Curcumene 4.95% to9.85%.Moreover it's the first time that E-nuciferol appears along with components. Antimicrobial activity of the essential oil of Lemon verbena were studied by the disk diffusion method. Whereas E.coli has more sensitive than Staph. aureus and S. typhimurium in order and its increase in July because of increasing the citral percentage.
Keywords: Lemon Verbena (Lippia Citriodora), Essential oil, Antimicrobial Activity , Growth stage , GC-MS.

1 - Introduction:

The scientific name of Lemon verbena is Lippie citriodora belongs to Verbenaceae . The genus Lippia includes approximately 200 species of herbs, shrubs and small trees which spread widely in South America, Central America and tropical Africa [1 ,2] . It's native in South America in Chile ,Argentina , Peru and Uruguay has been transported to Europe in the end of 17th century, spreading in southern Europe, especially Italy and Spain, and in South Africa, particularly Morocco. Lemon verbena used in folk medicine for the treatment of asthma, spasms, cold, fever, flatulence, colic, diarrhea , indigestion, insomnia, and anxiety [3,4,5], It is also recommended as analgesic, anti-inflammatory and antipyretic [1] It is believed that these properties due to its essential oils and polyphenols [6,7]. and its sedative and analgesic properties due to the presence of verbascoside, which is present as one of the most important and major polyphenols (phenyl propanoids) in lemon verbena tea, was isolated by methanol extraction, followed by partitioning with ethyl acetate, and identified as being acteoside [8]. As reported in treatment of Alzheimer's disease due to the presence of Luteolin 7-diglucuronideas the major flavonoid compound [9,10] ,it is also used in perfume industry , food additives and flavoring beverages [1].

Many lists in the composition of the essential oil of Lemon verbena have developed which differ from each due to the different geographical, climatic environment and harvest time. The main ingredients in these

lists are citral (neral and geranial), limonene and 1,8 cineole [7,11,12,13,14,15,16]. also Insecticidal, antioxidant[17,18,19] and antibacterial properties are studied especially when applied to the following pathogens : Escherichia coli, Mycobacterium tuberculosis, Staphylococcus aureus, Helicobacter pylori, Pseudomonas aeruginosa and Candida albicans [20,21,22,23,24,25].

Lemon verbena was cultivated in Syria and used in folk and Arabian medicine . but no one determined the component of essential oil of Syrian origin, most people prefer to prepare Lemon verbena tea from fresh leaves during the year .So the aim of this research is to compare and determine the composition of essential oils from fresh Lemon verbena leave's during of growth stages by Gas-mass chromatography , and to study antibacterial activity of its essential oil .

2. Materials and Methods:

2.1. Plant material:

Lemon verbena shrubs were collected from gota in Damascus and transferred to Experimental garden of Faculty of Science in Damascus university. Leafs were harvested in three month : may , which approves the highest rate of growth where shrubs length is 50 cm nearly, July, mid-season, and October, full bloom. The harvest time at 7:30 AM from days 21-30 for each month .Then leaves was prepared for extraction after harvest.

2.2. Extraction of essential oils :

The fresh leaves of Lippa citriodora were is subjected to hydro distillation using Apparatus for the determination of essential oils in vegetable drugs European Pharmacopoeia (2007) [26]. Extraction times were performed for 3 hours with three replications. The extracted essential oils were dried over anhydrous sodium sulphate (99%, Merck)and stored in sealed vials at low temperature(-20°C) prior to GC-MS analysis.

2.3. GC-MS analysis :

The essential oil were analyzed using a Agilent 7890 A GC equipped with DB-1 capillary columns (30 m X 0.25 mm , 0.25 µm film thickness) and a mass spectrometer Agilent 5972Cas a detector. The carrier gas was helium, at a flow rate of 1 ml/min. The column temperature was performed at 60 ^{o}C and hold for 4 min then gradually increased from 60 ^{o}C to 64^{o}C with a rate of 1^{o}C/min ,from 64° C to 155°C at 2.5 °C/min and from 155° C to 250°C at 5 °C/min. The injector were set at 250 ^{o}C. One microliter of the sample was injected and split ratio 1:80 [27].For MS detection, an electron ionization system was used with ionization energy of 70 eV and detector temperature 230° C. Identification of the compounds based on the comparison of their relative retention time and mass spectra with those of NIST and Wiley libraries data of the GC-MS system .

2.4.Antimicrobal activity assay:

The fresh oil was tested for their antibacterial activities by disk diffusion method which described in[28]with some modifications. the following bacteria were used: Escherichia coli, Salmonella typhimurium and Staphylococcus aureus achieved from department of microbiology in Damascus University. These were maintained in 4 ^{o}C on nutrient agar plate .Different dilutions of the oils (100 %, 50 %, 25%, 12.5%) were made with DMSO dimethyl sulfoxide (≥99.9%, Sigma). Mullar Hinton agar (HIMEDIA, India) was prepared by dissolving of 38g/L in water. The sterile nutrient agar was inoculated with microbial cells (200µl of microbial cell suspension in 20 ml agar medium) and poured into sterile petri dishes (0.5 McFarland for each plate).Sterile filter paper discs of 6mm diameter were impregnated with 4µl of the extract solution and placed on the surface of the inoculated agar plates. Then, the plates were incubated overnight (24h) at 37°C.The diameters of inhibition zones were measured in millimeters. The inhibition zones with diameter≥12mm were considered as having Antibacterial activity. Tests were carried out in triplicate.

3. Results and discussion:

The analysis of essential oil from fresh leaves revealed a complex mixture of compound and summarized in table1. Whereas 41 compounds in May were identified representing 97.76% of the total oil by GC-MS. Geranial (25.6%), neral (19.4%), Germacrene D (5.91%), γ-Elemene (5.84%), Ar-Curcumene (4.95%), α-Cedrene (3.17%),β-Caryophyllene (5.89%), and β-Caryophyllene oxide (8.74%) representing 79.5% of the total oil.1,8-Cineol (1.11%), Geranyl acetate (1.4%), α-Zingiberene(1.78%) were detected in minor percentages.

Table 1. Composition of essential oil of lemon verbena during growth stages

No.	compound	RT	Composition (%) in May	Composition (%) in July	Composition (%) in October
1	α-Thujene	4.712	tr	tr	tr
2	1R-α-Pinene	4.888	0.14±0.01	0.21±0.07	0.24±0.06
3	Sabinen	6.022	-	0.75±0.18	0.96±0.26
4	6-methy-5-Hepten-2-one	6.054	0.99±0.37	-	-
5	L-β-pinene	6.767	0.08±0.01	tr	0.1±0.03
6	β-Cymene	7.950	tr	-	tr
7	1,8-Cineol	8.195	1.11±0.43	1.68±0.15	2.12±0.55
8	Limonene	8.345	3.3±1.33	6.07±0.92	5.96± 1.69
9	β-trans-Ocimene	9.382	1.62±0.3	0.39±0.14	0.5±0.1
10	cis-β-Terpineol	10.062	0.14±0.01	0.19±0.03	0.26±0.05
11	β-Pinene oxide	11.304	0.16±0.03	0.16±0.02	0.17±0.02
12	β-Linalool	11.946	0.27±0.03	0.17±0.03	0.25±0.04
13	cis-Limonene oxide	13.221	0.19±0.01	0.16±0.01	0.17±0.04
14	trans-Limonene oxide	13.481	-	0.18±0.02	0.18±0.04
15	trans-Chrysanthemal	13.778	0.38±0.21	0.26±0.14	0.41±0.07
16	β-Citronellal	14.365	0.15±0.02	tr	tr
17	Berbenol (d-Verbenol)	14.988	0.29±0.03	0.22±0.09	0.31±0.13
18	4-Terpineol	15.760	-	0.14±0.01	0.12±0.01
19	(S)-cis-Verbenol	15.955	0.42±0.03	0.4±0.09	0.4±0.1
20	α-Terpineol	16.545	0.54±0.04	1.19±0.13	1.28±0.18
21	D-(+)-Carvone	18.689	-	0.25±0.05	0.2±0.03
22	Neral (β-Citral)	18.959	19.4±2.86	13.9±0.73	17±0.5
23	p-Menth-1en-3-one	19.255	-	0.28±0.05	0.17±0.05
24	nerol	19.602	0.51±0.01	tr	0.61±0.01
25	Geranial (α-Citral)	20.632	25.6±2.6	19.3±1.46	22.2±1.04
26	Geraniol	21.237	0.5±0.17	tr	0.75±0.17
27	α-Copaene	26.944	0.5±0.17	0.64±0.1	0.61±0.12
28	Geranyl acetate	27.06	1.4±0.07	0.97±0.05	1.15±0.1
29	β-Bourbonene	27.241	0.35±0.08	0.62±0.11	0.49±0.06
30	O-Methyleugenol	27.596	-	0.29±0.01	-
31	β-Caryophyllene	28.888	5.89±0.71	5.15±1.17	3.56±0.72
32	β-Cubebene	29.428	0.35±0.32	0.13±0.01	tr
33	α-Humulene	30.531	0.41±0.02	0.39±0.13	0.26±0.06
34	Allo-Aromadendrene	30.852	0.48±0.16	0.75±0.05	0.64±0.01
35	α-Longipinene	31.479	0.13±0.01	0.23±0.01	-
36	Geranyl n-propionate	31.682	0.28±0.08	0.35±0.01	0.24±0.01
37	Germacrene D	31.866	5.91±0.01	3.15±1.17	2.35±0.98

38	Ar-Curcumene	32.149	4.95±0.28	9.85±1.71	9.64±0.13
39	γ-Elemene	32.627	5.84±0.36	1.85±0.81	2.43±1.13
40	α-Zingiberene	32.976	1.78±0.30	1.4±0.4	0.87±0.38
41	γ-Cadinene	33.510	0.18±0.06	0.33±0.05	0.28±0.03
42	α-Cedrene	33.730	3.17±0.18	2.48±0.87	3.00±0.43
43	(+)-δ-Cadinol	34.053	0.69±0.19	0.4±0.3	0.17±0.11
44	Spathulenol	36.532	-	0.15±0.03	-
45	β-Caryophyllene oxide	36.236	8.74±0.7	17.8±2.31	15.7±0.49
46	Dihydrocurcumene	36.556	tr	1.12±0.19	-
47	E-Nuciferol	37.204	-	0.96±0.22	1.37±0.2
48	Isoaromadendrene epoxide	39.194	tr	0.39±0.53	-
49	tau.-Cadinol	39.361	0.92±0.01	2.62±0.67	2.63±0.55
	Total		97.76	97.92	99.75

-Results were expressed as mean ± SD (n= 3). tr : indicates a compound precentage ≤ 0.05

47 compounds in July (mid-season) , which comprised 97.92% of the total oil. Geranial (19.3%), neral (13.9%), limonene (6.7%), Germacrene D (3.15%),Ar-Curcumene (9.85%), β-Caryophyllene (5.15%) and β-Caryophyllene oxide (17.8%) were the most abundant components, constituting 75.85% of the total oil. α-Cedrene (2.48%), γ-Elemene (1.58%),1,8-Cineol (1.68%), α-Zingiberene (1.4%), tau.-Cadinol (2.62%) were detected in minor percentages.

But in October (full bloom), 43 compounds were identified representing 99.75% of the total oil. Geranial (22.2%), neral (17%), limonene (5.96%), ,Ar-Curcumene (9.64%), β-caryophyllene(3.56%), α-cedrene (3%) and β-caryophyllene oxide (15.7%) were the most abundant components, constituting 77.06% of the total oil. Germacrene D (2.35%), γ-elemene (1.58%) , 1,8-Cineol (2.12%), E-nuciferol (1.37%), geranyl acetate (1.15%), tau.-Cadinol (2.63%) were detected in minor percentages

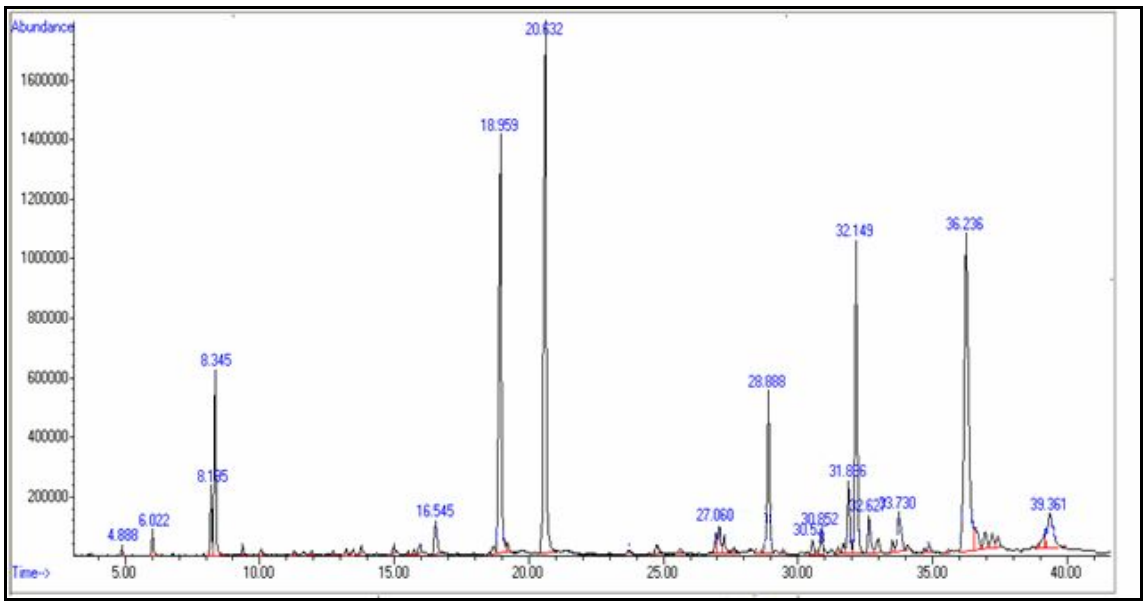

Figure 1. GC-MS chromatogram of essential oil in July

According to the literature, limonene is the component found to occur in higher quantities in essential oils of the genus lippia, followed by: p-cymene, α -pinene, camphor, β -caryophyllene, linalool and thymol in a decreasing order [1,2].Nevertheless the repeated reference to the presence of some major compounds namely, neral, geranial, limonene and 1,8-cineole. In our results did not show the presence of p-cymene, camphor and thymol. However p-cymene, α –pinene and linalool show up in low percentages which were mentioned in previous studies on Lemon verbena[7,11,12,14, 15,16].The majer component were limonene, citral (geranial

and neral), β-caryophyllene, Germacrene D, Ar-curcumene, γ-elemene,1,8-Cineol, α-cedrene and β-caryophyllene oxide. But a higher percentage of β-caryophyllene oxide, α-cedrene and tau.-cadinol was shown in comparison with that of the last literatures. Moreover it's the first time to observe the E-nuciferol among the components of Syrian origin. The percentage of citral followed the order (In May > In October > In July), geraniol is higher than neral in all stages, which corresponding with previous studies[7,12,29].

As concluded from table 2, that May has the highest percentage of oxygenated monoterpenes and hydrocarbons sesquiterpenes. On the other hand hydrocarbons monoterpenes have the highest percentage in October while oxygenated sesquiterpenes are the highest in July.

Table 2. percentage Composition of Compound group in the essential oil of lemon verbena during growth stages

Compound group	May	July	October
Hydrocarbons Monoterpenes	5.14	7.42	7.76
Oxygenated monoterpenes	51.34	45.43	47.61
HydrocarbonsSesquiterpenes	29.94	22.18	24.51
Oxygenated Sesquiterpenes	10.35	22.89	19.87

The different environmental and geographical conditions play a role in the components difference of the essential oil compared with previous studies [7, 11,12,16]. On the other hand, harvest time, extraction method and analysis conditions are important factors influence of which cannot be ignored [13,18,29].

Growth stages also have influence on the composition of oil in terms of genetic, ontogenetic, biotic and abiotic agents of each stage, moreover process of transition from the vegetative phase to the flowering phase is critical and complicated [30].In addition, the plant may suffer from Fungi and natural enemies which attack it during flowering phase [29].

As for antimicrobial activity, Table 3 shows diameter of inhibition zone plus diameter of the disc. Results reveal that the essential oil of Lemon verbena was very effective against all of microorganism used.

Table 3. antimicrobial activity of Lemon verbena in agar diffusion method

Month	Org.	Essential oil Con.											
		100%			50%			25%			12.5		
May	**E.coli**	25.3	±	1.15	19	±	2.89	16	±	1.53	12.5	±	1
	Staph. aureus	21	±	1	17	±	2.52	13.5	±	1.53	10	±	0.58
	S. typhimurium	19	±	0.5	14	±	0.58	10	±	1.15	8	±	0
July	**E.coli**	19.3	±	1.53	15.5	±	2.52	11.33	±	0.58	9.3	±	0.58
	Staph. aureus	16	±	1.53	13	±	1	10.5	±	0.58	8	±	0
	S. typhimurium	14	±	2.08	11	±	0.58	9.5	±	1.15	8	±	0
October	**E.coli**	21	±	3.61	14.5	±	0.71	11	±	0	8.7	±	0.58
	Staph. aureus	19.5	±	2.65	15	±	2.31	11.5	±	2.08	8	±	1.15
	S. typhimurium	16	±	2.03	12.3	±	0.58	10	±	0	8.3	±	0.58

Results were expressed as mean ± SD (n= 3), Inhibition zones including the diameter of the paper disc (6 mm).

The antimicrobial activity of lemon verbena due to essential oil components especially β-Citral ,α-Citral , 1,8-Cineol , Limonene , β-Caryophyllene oxide[31,32,33,34]which increase upon the increasing of concentration of essential oil .

E.coli was The most sensitive then Staph. aureus and S. typhimurium in order . Antimicrobial activity is the largest in May then October and July for investigated bacteria . It's observed that the high concentration of citral is in May followed by October and July , whereas the high concentration of β-caryophyllene oxide and

limonene was in July while Ar-curcumene in October. So citral has the highest effect of essential oil components of lemon verbena . As result there is correlation between citral percentage and antimicrobial activity.

4. Conclusions :

- The study of the essential oil from fresh leaves of L. citriodora of Syrian origin showed the presence of geranial, neral, limonene, β-Caryophyllene, Germacrene D, Ar-Curcumene, γ-Elemene,α-Cedrene, and β-Caryophyllene oxide as major components .
- The study at growth stages showed significant differences showed in the percentages of the main constituents especially citral, β-Caryophyllene oxide and limonene.
- The E-Nuciferol appears in the components for the first times .
- It's found out that lemon verbena oil has antimicrobial activity against Escherichia coli, Salmonella typhimuriumand Staphylococcus aureus especially which is extracted in May .

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