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Volatile Composition and Biological Activity of Key Lime *Citrus aurantifolia* Essential Oil

Federica Spadaro[a,b], Rosaria Costa[c], Clara Circosta[a] and Francesco Occhiuto[a] *

[a]*Pharmaco-Biological Department*, *School of Pharmacy*, *University of Messina*, *Messina – Italy*
[b]*Fondazione Prof. A. Imbesi*, *Messina*, *Italy*
[c]*Dipartimento Farmaco-Chimico*, *School of Pharmacy*, *University of Messina*, *Messina*, *Italy*

focchiuto@unime.it

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The essential oil of *Citrus aurantifolia* (Christm) Swingle fruits (limes) was studied for its potential spasmolytic effects in relation to its chemical composition. The essential oil, extracted by hydrodistillation (HD), was analyzed by GC-FID and GC-MS. The antispasmodic activity was evaluated on isolated rabbit jejunum, aorta and uterus. The results indicated that the essential oil of *C. aurantifolia* possesses important spasmolytic properties, which are likely to be due to its major constituents, limonene (58.4%), β-pinene (15.4%), γ-terpinene (8.5%), and citral (4.4%).

Keywords: *Citrus aurantifolia* (Christm) Swingle, Rutaceae, Hydrodistillation, GC-FID, GC-MS, Spasmolytic activity, Lime Mexicana.

Lime {*Citrus aurantifolia* (Christm) Swingle, Rutaceae} is a polyembryonic species cultivated across the globe, mostly in hot subtropical or tropical regions, such as southern Florida, India, Mexico, Egypt and the West Indies [1]. The juice and volatile oil are the major commercial products of lime [2]. Lime volatile oil consists mainly of limonene, α-terpineol, terpinen-4-ol, 1,4-cineole, 1,8-cineole, β-pinene, *p*-cymene, β- bisabolene, citral (geranial and neral) [3]. The oil is used in food, cosmetics, and as a flavoring agent in foods and medicines. Various *Citrus* species, including lime, were recently investigated for their insecticidal effect [4,5]. Lime also possesses other pharmacological activities, such as antioxidant, antiproliferative and hypotensive [6,7].

This study was conducted to analyze the chemical composition of the volatile oil from lime fruits using gas chromatographic techniques. In addition, the spasmolytic activity of the oil was evaluated on isolated smooth muscle; this is the first such study on the volatile oil of lime.

Effects on isolated rabbit jejunum: The essential oil of *C. aurantifolia* caused inhibition of spontaneous contractions and a slight decrease in muscle tone of rabbit jejunum preparations. The inhibitory effect was concentration-dependent for doses ranging between 2-10 μg/ mL (Table 1), with an average effective concentration (EC_{50}) of 4.60 ± 0.6 μg/mL (mean ± SEM, n = 6). These inhibitory effects on spontaneous motility are also manifested in the presence of atropine, and in conditions in which the motility is essentially myogenic. The contraction of smooth muscle preparations, including rabbit jejunum, is dependent on an increase in cytoplasmic free [Ca^{2+}] ion concentration, which activates the contractile elements.

Effects on isolated rabbit aorta: In the rabbit aorta preparation, a K^+ concentration of 80 mM is known to cause smooth muscle contractions through the opening of voltage dependent Ca^{2+} channels, thus allowing an influx of extracellular calcium, causing a contractile effect. In this preparation, the essential oil caused relaxation of K^+- induced contraction, as demonstrated by a right-ward shift in the dose-response curve constructed in a K^+-rich medium (Figure 1), suggesting that in the spasmolytic effect of the essential oil, activation of the Ca^{2+} channel is involved.

Table 1: Effects of lime essential oil (2-10 μg/mL) and nifedipine (0.01-1 μg/mL, used as positive control) on amplitude and frequency of spontaneous contractions of isolated rabbit jejunum. Data are expressed as mean ± SEM (n = 6).

		Spontaneous contractions	
Treatment	Concentration μg/mL	Amplitude inhibition (%)	Frequency inhibition (%)
Essential oil	2	10 ± 1.2	8 ± 1.3
	4	35 ± 2.1*	15 ± 2.2
	8	68 ± 3.0*	47 ± 2.8*
Nifedipine	0.01	3.5 ± 1.1	6.3 ± 1.3
	0.1	65.5 ± 2.4*	57.0 ± 3.0*
	1	89.0 ± 4.2*	77.8 ± 4.1*

* $P<0.05$ with respect to basal values.

Effects on isolated rabbit uterus: On spontaneously contracting uterus preparations, the addition of increasing concentrations of essential oil (2-10 μg/mL), similar to the effect observed on rabbit jejunum, caused uterine muscle relaxation. The releasing effect is characterized by reduced amplitude of contractions and a small reduction of muscle tone baseline. The strongest effect was observed at 10.0 μg/mL concentration that causes a reversible stop of the organ activity. After washing, the organ made an immediate and complete recovery of contractile activity. In quiescent uterine preparations, the essential oil (10 μg/mL), when added prior to the reference agonist drugs (adrenaline, acetylcholine), partially antagonized their maximal contractile response (Figure 2), suggesting a non-specific effect.

GC analysis: Peak identification and quantitative data for the essential oil are reported in Table 2. Data are presented either as raw FID peak area percent or as peak area percent corrected with the use of response factors, following a procedure already consolidated in previous works [8,9]. Table 2 also reports the retention indices, both experimental and literature values. Precision of the method has been

Table 2: GC composition of key lime essential oil. Values are average of three repetitions.

Peak no.	Compound	[a]LRI_{exp}	[b]LRI_{lit}	[c]A%	[d]RF	[e]g/100 g
1	α-Thujene	928	927	0.6	1.0	0.6
2	α-Pinene	936	933	2.0	1.0	2.0
3	Camphene	952	953	0.07	1.0	0.07
4	Sabinene	976	972	1.8	1.0	1.7
5	β-Pinene	982	978	12.6	1.0	12.3
6	Myrcene	992	991	1.3	1.0	1.3
7	Octanal	1006	1006	0.01	1.3	0.01
8	α-Phellandrene	1010	1007	0.05	1.0	0.05
9	α-Terpinene	1020	1018	0.4	1.0	0.3
10	*p*-Cymene	1028	1025	0.1	1.0	0.1
11	Limonene	1034	1030	53.8	1.0	52.7
12	(*E*)-β-Ocimene	1050	1046	0.2	1.0	0.2
13	γ-Terpinene	1063	1058	16.5	1.0	16.2
14	*cis*-Sabinene hydrate	1074	1069	0.05	1.3	0.06
15	Terpinolene	1090	1086	0.6	1.0	0.6
16	Linalool	1102	1101	0.3	1.3	0.4
17	*trans*-Sabinene hydrate	1105	1098	0.05	1.3	0.06
18	Nonanal	1107	1107	0.02	1.3	0.03
19	*exo*-Isocitral	1145	1144	0.01	1.3	0.02
20	Camphor	1150	1149	0.01	1.3	0.01
21	Citronellal	1154	1152	0.08	1.3	0.1
22	Borneol	1176	1173	0.01	1.3	0.01
23	*cis*-Pinocamphone	1180	1172	0.01	1.3	0.01
24	Isogeranial	1182	1179	0.06	1.3	0.07
25	Terpinen-4-ol	1184	1180	0.2	1.3	0.2
26	α-Terpineol	1198	1195	0.4	1.3	0.5
27	Decanal	1208	1208	0.03	1.3	0.04
28	Nerol	1228	1229	0.3	1.3	0.4
29	Citronellol	1229	1232	0.09	1.3	0.1
30	Neral	1240	1238	1.1	1.3	1.4
31	Geraniol	1254	1255	0.4	1.3	0.5
32	Geranial	1272	1268	1.4	1.3	1.8
33	Perillaldehyde	1280	1278	0.02	1.3	0.03
34	Bornyl acetate	1287	1285	0.01	1.6	0.02
35	δ-Elemene	1338	1335	0.05	1.0	0.05
36	Citronellyl acetate	1350	1353	0.02	1.6	0.03
37	Neryl acetate	1360	1361	0.7	1.6	1.1
38	Geranyl acetate	1379	1380	0.7	1.6	1.1
39	β-Elemene	1393	1391	0.02	1.0	0.02
40	Dodecanal	1411	1410	0.02	1.3	0.02
41	*cis*-α-Bergamotene	1416	1416	0.06	1.0	0.06
42	(*E*)-Caryophyllene	1424	1424	0.4	1.0	0.4
43	*trans*-α-Bergamotene	1437	1434	0.9	1.0	0.9
44	(*Z*)-β-Farnesene	1440	1439	0.05	1.0	0.04
45	(*E*)-β-Farnesene	1454	1452	0.07	1.0	0.07
46	α-Humulene	1460	1454	0.04	1.0	0.04
47	β-Santalene	1463	1459	0.03	1.0	0.03
48	γ-Curcumene	1480	1482	0.02	1.0	0.02
49	Germacrene D	1485	1480	0.06	1.0	0.06
50	(*Z*)-α-Bisabolene	1503	1503	0.1	1.0	0.1
51	(*E*,*E*)-α-Farnesene	1507	1505	0.2	1.0	0.2
52	β-Bisabolene	1512	1508	1.3	1.0	1.3
53	(*E*)-α-Bisabolene	1543	1540	0.03	1.0	0.03
54	Germacrene B	1564	1568	0.1	1.0	0.1
55	*epi*-α-Bisabolol	1691	1688	0.02	1.3	0.03
	Total			99.6		100.00
	Monoterpene hydrocarbons			89.3		88.4
	Sesquiterpene hydrocarbons			3.6		3.4
	Oxygenated monoterpenes			6.0		8.0
	Oxygenated sesquiterpenes			0.02		0.03
	Monoterpene aldehydes			2.7		3.5
	Monoterpene alcohols			1.8		2.3
	Monoterpene ketones			0.02		0.02
	Monoterpene esters			1.4		2.2
	Sesquiterpene alcohols			0.02		0.03
	Aliphatic aldehydes			0.08		0.1

[a] Linear Retention Index from SLB-5MS column measured against C_7-C_{30} *n*-alkanes; [b] From ref. [15] and [16]; [c] Percentage FID peak area. Values are means of triplicate analyses; [d] FID response factor; [e] Peak percent normalized by FID response factor.

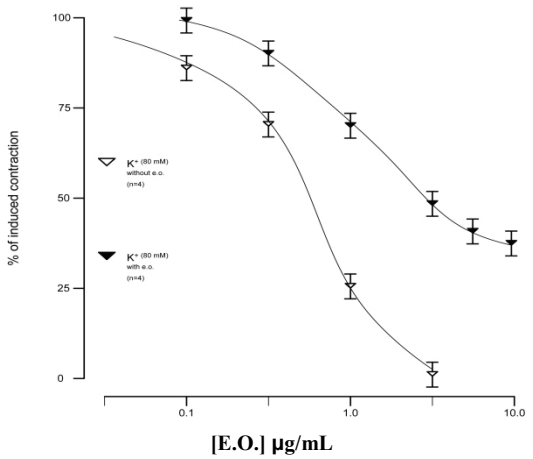

Figure 1: Effect of the essential oil of *Citrus aurantifolia* against contractions induced by high potassium concentration in rabbit aortic ring preparations

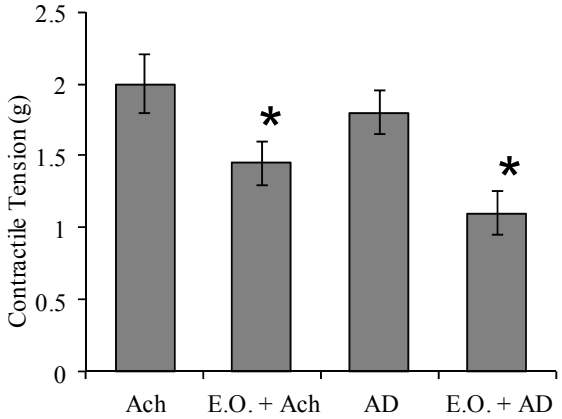

Figure 2: Effects of lime essential oil on the maximum force of contraction induced by acetylcholine (ACh) and adrenaline (AD) on isolated quiescent rabbit uterus preparation. Each column represents the mean ± ES of three experiments. * P <0.05.

evaluated by measuring the RSD%, relative to three replicates, and it was assessed as <5%. As can be seen from the Table, 55 compounds were determined accounting for about 99.5% of the whole oil. Monoterpenes were the predominant fraction, followed by sesquiterpene hydrocarbons. In addition to the terpenoid compounds, a small amount of aliphatic aldehydes was found. Among the monoterpene hydrocarbons, limonene, β-pinene and γ-terpinene were present in the highest amount, whereas oxygenated monoterpenes were mainly represented by citral (neral and geranial), nerol and geraniol, and neryl and geranyl acetate. Sesquiterpenes mainly consisted of *trans*-α-bergamotene and β-bisabolene, with only an oxygenated derivative present at trace level (*epi*-α-bisabolol).

Data obtained are generally in accord with those already published for distilled lime oils [10,11]. However, in one previous case, the composition differed from that here presented [12]. However, in this study by Afolayan and Asekun, lime fruits were from South Africa and from very ripe to rotten stage. An unusual composition was recorded (only 25% identified), characterized by the absence of terpene hydrocarbons and α-terpineol, and with terpinen-4-ol as the highest peak (11.7 vs. 2.7%, respectively). Also, various long chain linear hydrocarbons and fatty acids were detected, seemingly artifacts.

It is worth emphasizing that the distillation procedure that was applied in this study, which basically consisted of the hydrodistillation of fruit peels, was different from the conventional

procedure adopted in industry. In fact, industrial production is based on expression of the whole fruits, leading a juice-oil emulsion, which is subjected to distillation to obtain the oil [13].

Distilled lime oils differ greatly from the corresponding cold pressed (or expressed) oils, both in terms of organoleptic properties and volatile composition. During distillation, acid catalyzed reactions take place, such as bicyclic hydrocarbon rearrangements that form alcohols (i.e. α-terpineol, terpinen-4-ol, borneol) and hydrocarbons (i.e., γ-terpinene, α-terpinene). Also, distilled products lack the non volatile residue, composed of waxes, coumarins and psoralens. In general, the formation of such stable molecules from the distillation process is a desirable event, since the sharp, fresh and terpene-like flavor of key lime oil makes it one of the most important flavoring agents in the drinks and sweets industries.

The results obtained show that the essential oil of *C. aurantifolia* possesses important spasmolytic properties. In the preparations with spontaneous motility, this spasmolytic activity is shown by a progressive reduction in amplitude of contractions and muscle tone, while in the quiescent preparations the miolytic activity of the essential oil is manifested as the ability to prevent, even if partially, physiological modulator (neurogenic or humoral)-induced spasms. It was observed that the miolytic activity of the essential oil had a little influence on the frequency of contractions, a fact evident also after atropinisation of the tissue and which is strong in respect of the effects of spasmogenic KCl. From this finding, it can be assumed that in the determinism of this activity mechanisms interfering with the kinetics of calcium in miocell take place.

Experimental

Plant material and animals: *Citrus aurantifolia* (Christm) Swingle (Rutaceae), cultivar Mexicana (Lime mexicana) fruits were gathered in November, 2011 (intermediate maturation) from Reggio Calabria (farm G. Corigliano, Bovalino, Italy). The fruits were hand-peeled, separating the external part of the fruits (flavedo), with a yield of 29%, w/w, of lime peel over the whole fruit. Fresh plant material was employed for all extractions.

Rabbits (New Zealand), weighing 1.7-2.0 Kg of both genders, were housed under standard laboratory conditions with free access to food and water. Animal care, environmental conditions and use followed the guidelines of the Council of European Communities. The experimental procedures were approved by the Bioethical Committee of the Italian National Health Institute.

Hydrodistillation: The procedure used was that described in the European Pharmacopoeia [14]. Fresh lime peel (200 g) was added with 500 mL water and submitted to hydrodistillation for 3 h (until no more essential oil was obtained) using a Clevenger-type apparatus. The essential oil was collected, dried over anhydrous sodium sulfate and stored at 4°C until used.

GC-FID analysis: A Shimadzu GC2010 apparatus was used equipped with a flame ionization detector, a split/splitless injector and an AOC-20i series auto-injector. Capillary column: SLB-5MS, 30 m x 0.25 mm I.D. x 0.25 μm film thickness (Supelco, Milan, Italy); column temperature, 50–250°C (10 min) at 3°C/min; injector temperature: 250°C; detector temperature: 280°C; carrier gas, He at 99.5 kPa (30.0 cm/s); injection mode: split; split ratio, 1:100; injected volume, 1.0 μL of diluted oil. Data handling utilized *GCsolution* software.

GC/MS analysis: Samples were analyzed by GC-MS (EI) on a GCMS-QP2010 system equipped with a FFNSC database [15] and Adams library [16]; GC conditions: capillary column and temperature program as in GC-FID analysis; carrier gas: He, delivered at a constant pressure of 30.6 kPa (30.1 cm/s); injector temperature, 250°C; injection mode, split; split ratio, 1:50. MS scan conditions: source temperature, 200°C; interface temperature, 250°C; E energy, 70eV; mass scan range, 40-400 amu. Data handled through use of *GCMSsolution* software.

Isolated rabbit jejunum preparation: Rabbits, fasted for 24 h, were sacrificed by cervical dislocation. The abdomen was opened and 2 cm segments of jejunum were removed and suspended in 20 mL tissue baths containing Tyrode's solution, maintained at 37°C and aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The composition of the Tyrode's solution in mM was: 2.68 KCl, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.55 (pH 7.4). The intestinal responses were recorded isotonically using a transducer (HSE F30, 372 chicks - Hugo Sachs Elektronik, Harvard Apparatus GmbH-Germany). The data were digitally recorded and collected by data acquisition software HSE-ACAD W (Hugo Sachs Elektronik, Harvard Apparatus GmbH-Germany) and displayed on a computer monitor. Each preparation was allowed to equilibrate for at least 30 min before the addition of the essential oil

Isolated rabbit aortic preparation: Thoracic aortic rings of rabbits, 2-3 mm in diameter, were individually mounted in 20 mL tissue baths containing Kreb's solution thermostatted at 37°C and aerated with a mixture of O_2 (95%) and CO_2 (5%). The composition of the Kreb's solution was (mM): NaCl 118.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.3, $MgSO_4$ 1.2 and glucose 11.7 (pH 7.4). In each preparation, after an equilibration period of 1 h, the contractile response was induced with KCl (80 mM). Changes in isometric tension of the rings were measured using an isometric transducer (HSE F30, 372 chicks - Hugo Sachs Elektronik, Harvard Apparatus GmbH-Germany).

Isolated rabbit uterus preparation: Isolated uterus preparations from virgin female rabbits were used. Miometrial segments, 1.5 cm in length, were isolated and mounted in 20 mL tissue baths containing Tyrode solution sufficiently oxygenated and thermostatted at 37°C. The tone and the contractile activity of the organ were recorded by means of an isotonic transducer (HSE F30, 372 chicks - Hugo Sachs Elektronik, Harvard Apparatus GmbH-Germany). All experiments were conducted with preparations of the uterus in spontaneous contractile activity, in order to evaluate the effects of cumulative concentrations of the essential oil on the basal tone, amplitude and frequency of contractions. In some experiments, the spontaneous contractile activity of the preparation was inhibited by reducing the temperature of the bath of the organ to 30-32°C and changing the nutrient solution of Tyrode as follows: NaCl 9g, 0.42g KCl, $CaCl_2 \cdot 2H_2O$ 0.08g, 0.5g $NaHCO_3$, H_2O to 1 L, pure O_2, pH 7. This was in order to assess whether the pre-treatment of the preparation with the essential oil influences the maximal contractile response induced by certain agonists (epinephrine 1.0 μg/mL, acetylcholine 1.0 μg/mL).

Statistical analysis: statistical comparison was carried out by one-way analysis of variance (ANOVA) and Dunnet's test. The data represent means ± SEM, and values of $P < 0.05$ were considered statistically significant. This number of replicates allowed to generate a mean and SEM for each experiment. Each experiment was assessed in triplicate.

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