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Chemical composition, antimicrobial and insecticidal activities of the tunisian C*itrus aurantium* essential oils

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Abstract: The chemical composition of volatile fractions from leaves, flowers and peels of *Citrus aurantium* growing in Tunisia obtained by hydrodistillation were analysed using GC and GC-MS. Furthermore, the isolated essential oils were evaluated for their *in vitro* antimicrobial activity against eight bacteria, eight phytopathogenic and nine human pathogenic fungi. The essential oils from peels and its main compound limonene have been found to possess strong contact toxicity against four storage-grain insects. The highest mortality rate was observed when the essential oil was applied against *Cryptolestes ferrugineus*, *Liposcelis bostrychophila* and *Tribolium castaneum*.

Keywords: antimicrobial activity; *Citrus aurantium*; essential oil; insecticidal activity; limonene; volatile fractions

Plants provide a multitude flavours and fragrances that found many applications in everyday life. According to different authors, approximately 3000 plants species contain essential oils, among which only 300 are commercially important. Essential oils and some of their constituents are used not only in pharmaceutical products for their therapeutic activities, but also in agriculture, as food preserves and additives for human or animal use, in cosmetics and perfumes, and other industrial fields. In many cases, they serve as plant defence mechanisms against predation by microorganisms, insects, and herbivores (Bakkali *et al.* 2008). Complex composition of the essential oils and variety of chemical structures of their constituents are responsible for a wide range of biological activities, many of which are increasing interest in the fields of human and animal health. Particularly, many essential oils and their constituents have been traditionally used for their biological activities, which were known since ancient times.

Citrus aurantium (*Rutaceae*), commonly known as a bitter orange tree is an ever green tree of 2–2.5 m high, having white perfumed flowers and orange fruits, cultivated in tropical and subtropical zones.

The *Citrus* genus marks its presence in daily life in the fields of traditional medicine, cosmetics, food

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and perfumery industry. *C. aurantium* is among the species most frequently used for both traditional and modern medicinal purposes in the treatment of various diseases (Moraes *et al.* 2009; Stohs *et al.* 2011). Its zests are generally treated as agro-waste and are also considered as a potential source of valuable secondary metabolites. Immature peels are used to treat indigestion and other intestinal diseases (Deyhim *et al.* 2006; Kim *et al.* 2008). Peels and flowers of *C. aurantium* essential oils are frequently used in food as perfumes; also reported to be rich in flavonoids, coumarins, triterpenes and vitamins (Haggag *et al.* 1999) and to have interesting antimicrobial and antifungal effects. Fruits of bitter orange tree are also used for their sedative, relaxant, anxiolytic and antispasmodic properties (Carvalho & Costa 2002; Stange *et al.* 2002; Arias & Ramon-Laca 2005; Haaz *et al.* 2006 ; Pultrini *et al.* 2006; Sokovic & Vangriensven 2006).

In recent years, many studies have been focused to study chemical composition of the essential oils from various parts of *Citrus aurantium* growing in Pakistain (Kamal *et al.* 2011), Brazil (Wolffenbuttel *et al.* 2015; Amorim *et al.* 2016), Morocco (Ouedrhiria *et al.* 2015), Iran (Khakpourk *et al.* 2014; Azhdarzadeh *et al.* 2016), Croatia (Radan *et al.* 2018), northern Tunisia (Trabelsi *et al.* 2014), India (Periyanayagam *et al.* 2014) and Algeria (Abderrazak *et al.* 2014; Djenane 2015). In addition, it has been reported that essential oils of *C. aurantium* were a source of bioactive compounds, they have been recognized as antimicrobial (Trabelsi *et al.* 2014; Ouedrhiria *et al.* 2015; Azhdarzadeh *et al.* 2016), antioxidant (Trabelsi *et al.* 2014; Ouedrhiria *et al.* 2015; Radan *et al.* 2018), anti-inflammatory (Amorim *et al.* 2016) and anti-anxiety agent (Khakpour *et al.* 2014).

As a contribution to the valorization of Tunisian aromatic and medicinal plants (Zardi-Bergaoui *et al.* 2007; El Ayeb-Zakham *et al.* 2017; Hichri *et al.* 2017, 2018; Jlizi *et al.* 2018) our research has been devoted to the study of chemical composition, to the evaluation of antimicrobial activity of essential oils isolated from the flowers, leaves and peels of the Tunisian *C. aurantium* and to identify the effect of the essential oil extracted from peels and its main compound limonene on storage-grain insects (Zarrada *et al.* 2015) by fumigant toxicity and repulsive effect in order to determine the LC_{50} et LC_{90} and understand the type of relationship between this volatile fraction and its principal constituent.

MATERIAL AND METHODS

Plant material. *Citrus aurantium* was harvested from the region of Monastir (Tunisia) at a flowering stage. Identification was performed according to the Tunisian Flora in the Laboratory of Genetics Biodiversity and Valorisation of Bio resources (LR11ES41), at the Higher Institute of Biotechnology, the University of Monastir (Tunisia). A voucher specimen (C.T.-14) was deposited in the same laboratory. The plant was dried in a ventilated area. Fresh flowers, leaves and peels were separated and cut in little pieces and weighed before the isolation process of the volatile compounds.

Preparation of the essential oils. Flowers, leaves and peels were subjected to hydrodistillation for 5 h using a using a standard apparatus recommended in the European Pharmacopoeia. The obtained essential oils were collected by decantation, dried over anhydrous Na_2SO_4, filtered and stored at 4°C until analysis.

Gas chromatography analysis. Gas chromatograph: HP 5890-series II equipped with flame ionization detector (FID), HP-5 (30 m × 0.25 mm i.d., 0.25 µm film thickness) and the HP-Innowax column (polyethylene glycol column, 0.25 mm internal diameter, 30 m length and 0.25 µm film in thickness) fused silica capillary column, carrier gas nitrogen (1.2 ml per min). The oven temperature was programmed from 50°C (1 min) to 280°C at 5°C per minute. The injector and the detector temperatures were 250 and 280°C, respectively. Volume injected: 0.1 µl of 1% hexane solution. The identification of the components was performed by comparison of their retention times with those of pure authentic samples and by mean of their retention indices relative to the series of *n*-hydrocarbons.

Gas chromatography-mass spectrometry analysis. GC/EIMS analyses were performed with the Varian CP-3800 gas-chromatograph equipped with the HP-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and the Varian Saturn 2000 ions trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and 240°C respectively; oven temperature programmed from 60°C to 240°C at 3°C per min; carrier gas helium at 1 ml/min; injection of 0.2 µl (10% hexane solution); split ratio 1:30. The identification of the constituents was based on comparison to retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, on computer match-

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ing against commercial and an internal library mass spectra built up from pure substances, components of known oils and MS literature data (Davies 1990; Adams 1995). Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using methanol as *CI* ionizing.

Biological activity

Antimicrobial assay. The isolated essential oils were controlled for their antibacterial activity against eight pathogenic bacteria (Table 1) collected from Pasteur Institute 106510, by using the agar disc diffusion method (Bagamboula *et al.* 2004). The nutrient agar (*NA*) medium cooled at 45°C was supplemented with a bacterial suspension (10^6 CFU/ml) and poured into Petri dishes. After solidification, sterile Whatman paper discs (diameter 6 mm) were placed to the surface of the cultivation medium and 7 µl of the tested essential oil was dropped onto each disc and cultivated at 37°C for 24 hours. The experiment was replicated twice.

Antifungal assay. Tested essential oils were monitored for their antifungal activity towards nine phytopathogenic (Table 2) and fifteen human fungi (Table 3) collected from the Laboratory of Phytopathology (Sousse, Tunisia), respectively, using the disc diffusion method (Hamza *et al.* 2006). A conidial suspension of tested fungi was prepared (10^4–10^5 CFU/ml) and added on the potato dextrose agar (PDA)medium (for phytopathogenic strains) or on the Sabouraud Dextrose agar (for human fungi) cooled at 45°C and supplemented with streptomycin sulfate (300 mg/ml), and poured uniformly into Petri dishes (diameter 90 mm). Sterilized paper discs (6 mm, Whatman No. 1 filter paper) were impregnated with 7 µl of the tested essential oil and placed on inoculated plates whereas the negative control plates had no product added to the filter paper at 25°C for 4 days. The test was performed in triplicate.

Insecticidal activity

Insects rearing. Insect adults were obtained from laboratory cultures, maintained in a growth chamber in total obscurity at 28 ± 1°C and 60% of relative humidity (*RH*) in the Laboratory of entomology of The Regional Center of Research in Horticulture and Organic Agriculture of Chott-Mariem (CRRHAB).

Fumigant assay. This bioassay was determined using the closer container method, which consists

Table 1. Percentages of major compounds (≥ 10%) of the flower, leave, and peel essential oils of *C. aurantium* growing in different countries

Countries	Compounds (%)								
	limonene			linalool			linalyl acetate		
	F	*L*	*P*	*F*	*L*	*P*	*F*	*L*	*P*
Mediterranean countries									
Tunisia (Zaghouan)	–	–	96.90	–	64.10	–	–	–	–
Tunisia (Nabeul)	–	–	–	–	62.40	–	–	–	–
Tunisia (Monastir)	–	–	73.60	41.82	37.24	–	13.75	–	–
Egypt	–	–	96.50	–	–	–	–	–	–
Greece	–	–	94.70	–	58.20	–	–	12.40	–
Corsica	–	–	91.80	–	26.30	–	–	35.00	–
Italy	–	–	93.40	–	–	–	–	–	–
Turkey (Antalya)	–	–	94.40	–	–	–	–	–	–
Turkey (Marmaris)	–	–	93.70	–	–	–	–	–	–
Others countries									
Iran	–	62.00	–	–	–	–	–	–	–
Brazil	–	–	93.00	–	24.10	–	–	36.70	–
Cuba	–	–	86.20	–	–	–	–	–	–
Mauritius	–	–	–	–	66.10	–	–	–	–

F – flower; *L* – leave; *P* – peel

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Table 2. Antibacterial activity of *Citrus aurantium* essential oils at the concentration of 7 µl/disc

Bacteria	Peels	Leaves	Flowers	Gentamicin
	IZ (mm)			
Staphylococcus epidermidis CIP 106510	9	9	9	21
Pseudomonas aeruginosa ATCC27853	7	6	6	30
Staphylococcus aureus ATCC 25923	9	9	13	33
Micrococcus luteus NCIMB 8166	9	16	10	27
Escherichia coli ATCC35218	6	10	11	26
Salmonella enterica subsp. *Enterica* ser. Thyphymerium LT2	6	9	10	21
Listeria monocytogenese ATCC19115	8	9	11	37
Enterococcus feacium 29212	6	13	15	26

IZ – inhibition zone; *IZ* < 6 – no activity

Table 3. Antifungal activity of *Citrus aurantium* essential oils to human isolates at the concentration of 7 µl/disc

Reference number	Origin	Yeasts	Peels	Leaves	Flowers	Amphotericin
			IZ (mm)			
1	ATCC	*Candida parapsilosis* ATCC 22019	7	21	17	10
2	ATCC	*Candida albicans* ATCC 90028	6	8	7	11
1596	sputum	*Candida albicans*	–	–	–	20
1605	finger perionyxis	*Candida albicans*	7	18	20	23
1613	bronchial washing	*Candida albicans*	8	28	22	24
1623	sputum	*Candida tropicalis*	7	17	15	20
1628	axillairy intertrigo	*Candida albicans*	9	25	17	19
1630	inter-toes intertrigo	*Candida albicans*	8	25	20	21
1644	inch perionyxis	*Candida tropicalis*	–	20	14	24
1672	vaginal smears	*Candida albicans*	8	12	13	22
E613	vaginal smears	*Candida glabrata*	7	13	11	22
E617	urine	*Candida glabrata*	9	15	14	22
1625	back lesion	*Candida parapsilosis*	9	10	10	23
1627	inch perionyxis	*Candida parapsilosis*	8	10	9	23
1581	axillary intertrigo	*Candida parapsilosis*	7	15	10	22

IZ – inhibition zone; *IZ* < 6 – no activity

in isolating groups of ten insects, and then, fixing paper discs, treated by different doses of oil (25, 50, 100 and 200 µl/l), on the top of containers. Five replications were made both for tested oils and control. The mortality of insects (%) was observed and determined after 3, 6 and 24 h of treatment. Insects are considered as dead when no leg or antennal movements were observed.

Repellency test. Whatman filter papers (diameter 9 cm) were cut in two equal haves. Test solutions were prepared by dissolving 1, 2, 4 and 8 µl of essential oil in 200 µl of acetone. Each solution was applied to half a filter-paper disc as uniformly as possible with a micropipette. The other half of the filter paper was treated with acetone only. Treated and untreated halves were attached to their opposites using adhesive tape and placed in Petri dishes. Twenty insects were released separately to the center of each filter paper disc. Five replications were used for each dose. Observations on the number of insects present on both treated and untreated halves were recorded after 2 hours. This experimental method was described by Jilani and Saxena (1990) with some modifications. Percentage repellency (*PR*) was calculated by following Equation 1:

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$$PR = [(Nc - Nt)/(Nc + Nt)] \times 100 \quad (1)$$

where: *Nc* – number of insects present in the untreated area after the exposure interval; *Nt* – number of insects present on the treated area after the exposure interval

Mean repellency values were assigned to repellency classes from 0 to 5, where: 0 – < 0.1%; 1 – 0.1–20.0%; 2 – 20.1–40.0%; 3 – 40.1–60.0%; 4 – 60.1–80.0%; 5 – 80.1–100.0% repellency.

Statistical analysis. The data were corrected using the Abbott's formula (Abbott 1925) for the mortalities then subjected to the Probit analysis using SPSS (SPSS 2011) to estimate LC_{50} and LC_{90} the values of each of the essential oils against four insects (Finney 1971). Means were separated at the 5% significance level by using the Duncan's test.

RESULTS AND DISCUSSION

Oil yield. The oil yields for the three parts of *C. aurantium* were 0.12, 0.40 and 1.35% attributed to flowers, leaves and peels, respectively.

Chemistry analysis. The components of the studied essential oils of *C. aurantium* collected in January from Jemmel (Tunisia) are shown in Table 4. The different constituents of each sample were identified by GC and GC-MS. We noticed that the isolated oils from flowers and leaves essential oils were particularly rich in oxygenated monoterpene (59.02–69.21%) represented by linalool (41.82–37.24%) and linalyl acetate (13.75–7.87%), followed by hydrocarbon monoterpenes (24.61–32.28%). The most important abundant hydrocarbon monoterpenes were

Table 4. Chemical composition of *Citrus aurantium* flowers, leaves and peels essential oils

Sample	Compound	*RI*[a]	*RI*[b]	Leaves	Flowers	Peels
1	tricylene	1015	930	≤ 0.1	≤ 0.1	≤ 0.1
2	α-thujene	1020	940	10.65	6.15	≤ 0.1
3	α-pinene	1027	935	0.68	0.77	≤ 0.1
4	α-fenchene	1066	954	1.21	≤ 0.1	≤ 0.1
5	camphene	1071	955	1.92	1.93	≤ 0.1
6	β-pinene	1113	979	9.68	9.21	0.9
7	sabinene	1124	976	≤ 0.1	≤ 0.1	0.18
8	δ-3-carene	1144	1012	≤ 0.1	≤ 0.1	0.33
9	myrcene	1161	991	≤ 0.1	≤ 0.1	0.9
10	α-phellandrene	1168	1005	0.24	≤ 0.1	≤ 0.1
11	α-terpinene	1186	1019	0.80	0.72	≤ 0.1
12	limonene	1196	1032	6.52	5.03	73.6
13	β-phellandrene	1206	1032	≤ 0.1	≤ 0.1	≤ 0.1
14	1,8-cineole	1213	1033	0.40	≤ 0.1	≤ 0.1
15	(*z*)-β-ocimene	1235	1040	≤ 0.1	0.36	≤ 0.1
16	γ-terpinene	1245	1063	≤ 0.1	≤ 0.1	≤ 0.1
17	(*E*)-β-ocimene	1250	1048	≤ 0.1	0.11	1.12
18	*o*-cymene	1260	1022	≤ 0.1	≤ 0.1	≤ 0.1
19	*p*-cymene	1269	1025	≤ 0.1	≤ 0.1	≤ 0.1
20	hexyl acetate	1280	1015	≤ 0.1	≤ 0.1	≤ 0.1
21	terpinolene	1286	1089	≤ 0.1	≤ 0.1	0.15
22	6-methyl-5-hepten-2-one	1337	986	≤ 0.1	≤ 0.1	≤ 0.1
23	nonanal	1391	1105	0.25	≤ 0.1	≤ 0.1
24	α-*p*-dimethylstyrene	1437	1087	≤ 0.1	≤ 0.1	≤ 0.1
25	*cis*-linalool oxide	1141	1078	0.59	0.28	≤ 0.1
26	α-cubebene	1459	1351	0.11	≤ 0.1	≤ 0.1
27	*trans*-linalool oxide	1462	1093	3.88	0.17	≤ 0.1
28	citronellal	1464	1160	0.21	≤ 0.1	≤ 0.1
29	δ-elemene	1465	1336	1.61	≤ 0.1	≤ 0.1

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Table 4. To be continued

Sample	Compound	RI^a	RI^b	Leaves	Flowers	Peels
30	α-copaene	1489	1378	≤ 0.1	≤ 0.1	0.6
31	linalool	1548	1101	37.24	41.82	4.8
32	linalyl acetate	1554	1262	7.87	13.75	1.6
33	*cis*-sabinene hydrate	1556	1097	≤ 0.1	≤ 0.1	≤ 0.1
34	*trans-p*-menth-2-en-1-ol	1564	1140	3.10	≤ 0.1	≤ 0.1
35	*trans*-β-bergamotene	1583	1432	0.16	≤ 0.1	≤ 0.1
36	β-elemene	1587	1389	0.21	≤ 0.1	≤ 0.1
37	β-caryophyllene	1595	1427	1.00	0.45	0.55
38	linalyl propionate	1596	1322	≤ 0.1	≤ 0.1	≤ 0.1
39	terpinen-4-ol	1600	1179	≤ 0.1	0.4	0.07
40	γ-elemene	1623	1492	2.62	0.29	1.21
41	*cis-p*-menth-2-en-1-ol	1633	1121	≤ 0.1	≤ 0.1	≤ 0.1
42	citonellyl acetate	1657	1360	≤ 0.1	≤ 0.1	≤ 0.1
43	(*E*)-β-farnesene	1665	1458	0.10	≤ 0.1	≤ 0.1
44	α-humulene	1668	1461	≤ 0.1	≤ 0.1	0.37
45	neral	1671	1248	3.40	4.8	3.26
46	α-terpineol	1693	1190	≤ 0.1	1.26	0.12
47	α-terpinyl acetate	1695	1353	≤ 0.1	2.57	1.38
48	neryl acetate	1720	1368	≤ 0.1	1.01	0.51
49	geranial	1724	1278	0.19	0.28	≤ 0.1
50	β-bisabolene	1729	1520	≤ 0.1	≤ 0.1	0.17
51	bicyclogermacrene	1734	1495	≤ 0.1	≤ 0.1	≤ 0.1
52	(*E,E*)-α-farnesene	1749	1496	≤ 0.1	≤ 0.1	2.53
53	geranyl acetate	1750	1384	1.70	2.49	≤ 0.1
54	δ-cadinene	1755	1523	0.16	≤ 0.1	0.87
55	nerol	1790	1233	≤ 0.1	0.34	0.83
56	2-phenylethyl acetate	1826	1258	≤ 0.1	≤ 0.1	≤ 0.1
57	geraniol	1843	1257	≤ 0.1	2.46	≤ 0.1
58	jasmone	1914	1388	0.16	≤ 0.1	≤ 0.1
59	caryophyllene oxide	1976	1588	≤ 0.1	≤ 0.1	≤ 0.1
60	(*E*)-nerolidol	2030	1566	≤ 0.1	1.12	0.4
61	(*E,Z*)-farnesyl acetate	2196	1818	0.13	0.24	0.56
62	methyl anthranilate	2203	1362	0.12	0.29	0.63
63	(*E,Z*)-farnesol	2276	1743	≤ 0.1	≤ 0.1	0.58
64	indole	2423	1303	0.5	0.1	≤ 0.1
Monoterpenes				91.30	93.82	89.32
Monoterpenes hydrocarbons				32.28	24.61	77.44
Monoterpenes oxygenated				59.02	69.21	11.88
Sesquiterpenes				6.28	4.7	8.8
Sesquiterpenes hydrocarbons				6.13	3.47	7.57
Sesquiterpenes hydrocarbons				0.15	1.23	1.23
Divers				1.19	0.74	1.4
Total identified				98.78	99.26	99.32

a – polar column; *b* – non-polar column; *RI* – retention indices on polar and non-polar columns

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found to be α-thujene (6.15–10.65%) and β-pinene (9.21–9.68%). Peels essential oil was dominated by-limonene (monoterpene hydrocarbon) (73.60%). While oxygenated monoterepenes presented only 11.68% of the total oil.

A comparative study of the chemical composition of essential oils of *Citrus aurantium* growing in Tunisia (Hosni *et al.* 2010, 2013; Ellouze & Abderrabba 2014), Greece (Sarrou *et al.* 2013), Corsica (Boussaada *et al.* 2007), Italy (Dugo *et al.* 2011), Iran (Gholivand & Piryaei 2013), Brazil (Boussaada *et al.* 2007), Egypt (Dugo *et al.* 2011), Turkey (Kirbaslar & Kirbaslar 2003), Cuba (Pino & Rosado 2000) and Mauritius (Gurib-Fakim & Demarne 1995) showed a natural differences in chemical composition due to the season or seasonality in which the plant species was collected, the degree of maturity of the plant species, the geographical location of planting (latitude, longitude, altitude, relative humidity, soil chemicals, and winds), and the management of plant species (Tirado *et al.* 1995; Ellouze *et al.* 2012; Sarrou *et al.* 2013).

Table 1 represented major components of flower essential oil (FEO), leave essential oil (LEO) and peel essential oil (PEO) of *Citrus aurantium* growing in ten countries; it indicates difference in percentage and chemical composition. Linalool (41.82%) and linalyl acetate (13.75%) are more abundant in the FEO harvest in Tunisia (Monastir) but the proportion of these components is limited (≥ 10%) in Greece, Corsica, Italy, Iran, Brazil, Egypt, Turkey, Cuba and Mauritius. It appears that LEO is characterized by linalool as a chemo-type, it is found at its highest percent in Tunisia (Zaghouan 64.10%, Nabeul 62.40%), Greece (58.20%) and slightly more abundant in Mauritius (66.10%). In Iran, the same essential oil was dominated by limonene (62.00%). The LEO is distinguished by its high content in linaly acetate from Corsica (35.00%) and Brazil (36.70%) and low content in linalool (26.30 and 24.10%, respectively). On the other hand, limonene was the chemotype of the majority of PEO, except Tunisia (Nabeul) and Iran which is the major component of the LEO (62.00%). Its highest amount was in Tunisia (Zaghouan; 96.60%), Egypt (96.50%), Greece (94.70%), Italy (93.40%), Turkey (93.00%), Corsica (91.80 %), and in Cuba (86.20 %) and less in the region of Monastir in Tunisia (73.60%).

Thus, the three essential oils isolated from flowers, leaves and peels of *C. aurantium*, were rich in linalyl acetate, linalool and limonene with different yields.

Antibacterial activity of essential oil. According to the results given in Table 2, leaves and flowers essential oils displayed good antibacterial activity against *Micrococcus luteus* NCIMB 8166 (inhibition zone (*IZ)* – 16 and 10 mm, respectively), *Staphylococcus aureus* ATCC 25923 (IZ – 9 and 13 mm, respectively) and *Enterococcus feacium* 29212 (*IZ* – 13 and 15 mm, respectively) at a concentration of 7 µl/disc. While they appeared to be slightly active against the other tested bacteria, *Escherichia coli* ATCC35218, *Salmonella enterica* subsp. *enteritidis* ser. Thyphymerium LT2 and *Listeria monocytogenese* ATCC19115.

Peels essential oil displayed a limited activity against the tested bacteria but it was the only essential oil that inhibits *Pseudomonas aeruginosa* ATCC27853 (*IZ* – 9 mm). The later was found to be resistant against the leaves and flowers essential oils extracted from *C. aurantium*. This result confirms the resistance of this strain against various antibiotics and biocides (Mann *et al.* 2000). The antimicrobial activity seems to be due to the favourable and unfavourable effect of the

Table 5. Antifungal activity of *Citrus aurantium* essential oils at the concentration of 7 µl/disc

Fungi	Peels	Leaves	Flowers	Carbendazine
	IZ (mm)			
Aspergillus sp.	8	22	18	17
Alternaria sp.	8	–	12	–
Penicillium sp.	7	8	–	24
Botrytis cinerea	–	–	–	24
Fusarium sambucium	–	–	–	–
Fusarium solani	–	–	–	–
Trichoderma harzianum T_1	7	9	–	20
Trichoderma viride T	10	62	–	20
Fusarium oxysporum F_{33}	8	8	–	17

IZ < 6 – no activity

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components of the volatile fractions which proves the relation between the chemical structure and the activity.

Antifungal activity of essential oil. The isolated essential oils were found to be more effective towards human fungi than bacteria (Table 3). We noticed that the flowers and leaves essential oils were found to be more active than peels essential oil against all tested *Candida* strains except *C. albicans* 1596, which showed resistance towards all tested essential oils at the used concentration (7 µl/disc). *C. albicans* 1613 (origin: bronchial washing) showed high sensitivity against essential oils from leaves and flowers (*IZ* – 28 and 22 mm, respectively).

Regarding to antifungal activity against nine phytopathogenic fungi of isolated essential oils from the peels, leaves and flowers from *C. aurantium*, it was found that most of samples did not demonstrate required effect (Table 5). However, we noticed that only the essential oils from leaves and flowers showed detectable activity against *Aspergillus* sp. (*IZ* – 22 and 18 mm, respectively). Essential oil of leaves showed exceptional inhibition towards *Trichoderma viride* T_v (*IZ* – 62 mm).

From above results, it was found to be difficult to correlate the found antimicrobial activity with a specific compound identified in tested essential oils. Nevertheless, we found overall that essential oils of leaves and flowers presenting a comparable chemical composition act in most cases almost in the same way on tested microorganisms. The literature indicated that there is a relationship between major compounds in the essential oil and antimicrobial effect (Farag *et al.* 1989; Deans & Svoboda 1990). In addition, it is also known that the synergistic or antagonistic phenomena of a minor compound have to be considered (Burt 2004). The antimicrobial activity of our essential oils can be explained by the significant variety of phytochemicals.

Fumigant toxicity. Values with the same letter are not significantly different at $P < 0.05$ (Table 6). For *Sitophilus graniarum*, *Tribolium castaneum* and *Liposcelis bostrychophila*, we varied the concentration of dose of air between 50–200 µl/l. For the fourth pets *Cryptolestes ferrugineus* which were characterized by high sensitivity, we used concentration from 5 µl/l to 50 µl/l. We chose these particular insects because

Table 6. Mortality of four insect species after 24 h exposure to different concentrations of essential oil (*EO*) and limonene (Lim)

Species	Dose (µl/l air)	Mortality (%)		DL_{50} (%)		DL_{90} (%)	
		EO	Lim	*EO*	Lim	*EO*	Lim
Tribolium castaneum	0	0^{a}	0^{a}	64.78	64.78	103.55	103.55
	25	12^{b}	14^{b}				
	50	16^{b}	28^{c}				
	100	68^{c}	88^{d}				
	200	88^{e}	98^{d}				
Liposcelis bostrychophila	0	0^{a}	0^{a}	23.11	57.89	40.04	86.79
	25	2^{a}	2^{a}				
	50	34^{b}	48^{b}				
	100	86^{c}	94^{c}				
	200	100^{d}	100^{d}				
Sitophilus graniarum	0	0^{a}	0^{a}	101.50	103.77	181.27	184.11
	25	6^{ab}	20^{b}				
	50	6^{ab}	26^{b}				
	100	22^{b}	30^{b}				
	200	80^{c}	98^{c}				
Cryptolestes ferrugineus	0	0^{a}	0^{a}	20.62	20.62	35.35	35.35
	5	2^{a}	2^{a}				
	12.5	38^{b}	48^{b}				
	25	54^{c}	52^{b}				
	50	96^{d}	100^{c}				

Values in a column followed by the same letter are not significantly different ($P < 0.05$) by the Duncan's test

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they were available in the Laboratory of Entomology (Sousse, Tunisia). Many anterior works have reported on the effectiveness of plant essential oils against insects, especially stored-products insects.

The essential oil extracted from peels of *C. aurantium* and the main compound of this volatile fraction were active towards four insects and caused mortality: a dose of 200 µl/l air was required to obtain over 80% of mortalities for essential oil and limonene of *S. graniarum*, *T. castaneum* and *L. bostrychophila*. But to realize over 96% mortality for the essential oil and its major compound used in this study, we needed a concentration of 50 µl/l air with *C. ferrugineus*.

At the concentration of 25 µl/l of air, essential oil and limonene were not toxic enough to cause mortality of *L. bostrychophila*; we notice the low percentage (2%). At the same concentration, limonene killed 20% of *S. graniarum* which is more toxic than essential oil (6%). The main component and its essential oil caused half of the mortality rate at a dose of 25 µl/l air of *C. ferrugineus*.

S. graniarum resisted to toxicity caused by limonene and essential oil at the concentration of 100 µl/l of air, we notice a percentage of mortality rate which does not exceed 30%. Results showed that 100 µl/l of air, toxic enough to obtain a rate of over 68% mortality for *T. castaneum* and *L. bostrychophila*. But the higher rate was realized in the presence of limonene and *L. bostrychophila* was more sensitive to toxicity than *T. castaneum*.

Lethal concentrations (LC_{50} and LC_{90}). LC_{50} and LC_{90} were calculated using the probit analysis (Finney 1971). These doses correspond to the dose required for the 50% mortality rate and the 90% for the tested insects.

To confirm the classification of these four stored product pests in a resistance or sensitivity with regard to the essential oil extracted from the best of *C. aurantium* and limonene, we need to calculate lethal concentrations (LC_{50} and LC_{90}).

By examining the Figure 1 below, we deducted that *C. ferrugineus* pests are less resistant to essential oil (*EO*) since they kill only 50% of the population of insects with the concentration of 20.62 µl/l air. They caused the mortality of half *L. bostrychophila* population at the concentration of 57.89 µl/l air; 50% of the *T. castaneum* population was killed at a concentration of 64.78 µl/l air. *S. graniarum* were less sensible to the *EO* at the concentration of 103.77 µl/l air. We obtain the same classification of sensitivity of four insects to limonene. These classifications were confirmed by calculation of LC_{90}.

Repellent activity. Repellent effect was studied against *T. castaneum*. The histogram below shows that repulsivity varies between 56 and 82%, 54 and 84% after 1 h and between 62–76% and 64–86% after 2 h for limonene and EO (Table 7).

This repellent activity shows that the effect of limonene after 2 h was concentration-independent. We found the same class of repellency, but this effect after 1 h was corrected to the concentration (except for 50 and 100 µl/l air) and by following the class of repellency.

Observations were made each 2 h, at concentrations 25, 50, and 100 µl/l air have shown that *EO* kept the same class of repellency which changed only when we increased the concentration to 200 µl/l air. But after 1 h this effect was corrected to the concentration (except 50 and 100 µl/l air) and by following the class of repellency.

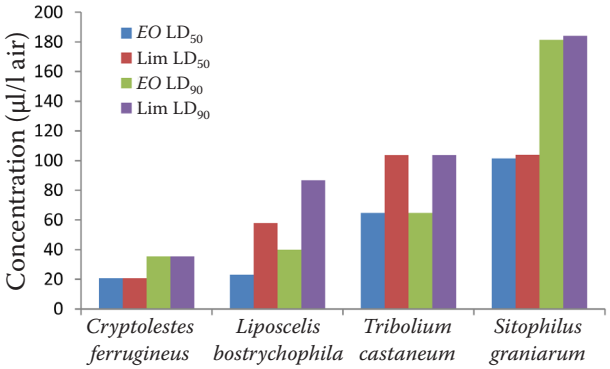

Figure 1. LC_{50} and LC_{90} of essential oil (*EO*) and limonene (Lim) for four insects

Table 7. Repellency index (*RI*) for *Tribolium castaneum* insect after 1 and 2 h exposure to different concentrations of the essential oil (*EO*) and limonene (Lim)

Dose (µl/l air)	*RI* (%)			
	1 h		2 h	
	EO	Lim	*EO*	Lim
25	54^3	56^3	72^3	62^3
50	64^4	60^4	66^4	62^3
100	70^4	68^4	64^4	74^4
200	84^5	82^5	86^5	76^5

CONCLUSIONS

The chemical composition of leaves, flowers and peels essential oils of *Citrus aurantium* from Tunisia witch screened for their antimicrobial activity, only those from leaves and flowers displayed good antimicrobial activity against *Micrococcus luteus*,

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Staphylococcus aureus, *Enterococcus feacium* ans *Candida* strains. *C. albicans* 1613 (origin: bronchial washing), were reported. Essential oil of leaves showed exceptional inhibition towards the phytopathogenic fungi *Trichoderma viride* T_v (*IZ* – 62 mm). Only the oil from peels and its' main compound (limonene) were evaluated for their insecticidal effects against four storage-grain insects. Peels essential oil showed higher fumigant toxicity than limonene at different concentrations of air against grain storage but both volatiles have a similar class of resistance.

The results allowed classify the insects used in an increasing scale of sensitivity – *Cryptolestes ferrugineus*, *Liposcelis bostrychophila* and *Tribolium castaneum*. *Sitophilus graniarum* was more resistant against toxicity caused by limonene and essential oil. On the other hand, the study of the repulsiveness effect of essential oil and limonene against *Tribolium castaneum* showed that these two volatiles have a similar class of resistivity.

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