

Evaluation of *Citrus limonum* (Sapindales: Rutaceae) L. essential oil as protectant against the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae)

S. Guettal, S. Tine, F. Tine-Djebbar* and N. Soltani¹

Laboratory of Water and Environment, LarbiTebessi University, Tébessa, Algeria

E. Mail :fouzia.djebbar@univ-tebessa.dz

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ABSTRACT

We studied the fumigant toxicity and repellent properties of essential oil of *Citrus limonum* (Sapindales: Rutaceae L.) against *Sitophilus granarius* (L.) adults. The chemical composition of this essential oil by gas chromatography-mass spectrometry (GC-MS) showed that it contains 50 compounds and the major compounds were: Limonene (19.81 %), α -Citral (17.16 %) and Neryl acetate (16.23 %). The essential oil showed fumigant toxicity, with dose-response relationship and repellent activity against *S.granarius*.

Keywords: *Citrus limonum*, essential oil, fumigant toxicity, GCMS, granary weevil, protectant, repellent, *Sitophilus granarius*.

INTRODUCTION

The food security is major challenge for human beings for the ever-increasing world population (20). The Post-harvest losses by stored insect pests are 9% in developed countries and 20% in developing countries (52). Insect pests are important because the favourable environmental conditions promote their development (1,21), resulting in weight loss, decreased germination and loss of grain quality (43). Thirty-nine species of pests, attacks the stored grains and grain products (55). These are controlled with chemical insecticides (methyl bromide or phosphine) but their use has led to insecticide resistance, resurgence of pest species, environmental pollution, toxic hazards to humans and other non-target organisms (61). Hence, research is being done to use essential oils of aromatic plants to protect the stored grains from the insect pests (38), as it is difficult for insects to develop resistance to essential oil's because of their complicated mechanism (31). Plant parts with insecticidal properties are used as insecticides as these are safe for the humans and environment (55). Many plant families (*Rutaceae*, *Meliaceae*, *Asteraceae*, *Labiataeae*, *Piperaceae*, *Verbenaceae* and *Annonaceae*) are used as botanical pesticides (30).

The genus *Citrus* (Rutaceae family) has several species (*C. limonum*, *C. sinensis*, *C. medica*, *C. nobelis*, *C. aurantifolia* and *C. aurantium*) in Algeria (63) and numerous studies have investigated the chemical composition and insecticidal activity of *Citrus* species (6,13,22,46). *Citrus limonum* (Figure 1) has antimicrobial, antioxidant, antifeedant and antifungal activity (7,8,17) and its insecticidal effects have been reported on coleopteran species (50). The grain weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) (Figure 2), is primary pest of stored grain products and causes major loss in stored grains (45,53), due to insect secretions at high infestation levels in storage (66) and thereby deteriorated the bread quality of wheat (37). This study aimed (i). to determine the

*Correspondence author,¹Laboratory of Applied Animal Biology, University Badji Mokhtar, Annaba, Algeria.

chemical composition of essential oil (EO) of *Citrus limonum* (Sapindales: Rutaceae), (ii). to examine its insecticidal and repellent activity and (iii). to develop it as alternate to synthetic insecticide against *S. granarius*.



Figure 1. Leaves of *Citrus limonum*.



Figure 2. *Sitophilus granarius* (L, 1758) (GX 45).

MATERIALS AND METHODS

(i). Study site: The study was done during January-March 2017 in semi-arid Tébessa, Eastern Algeria (35°24'15"N, 8°07'27"E; elevation: 867 m a.s.l.), Total Annual Precipitation: 296 mm, Highest maximum temperature: 32.3°C and minimum temperature: 5.4 °C.

(ii). Insects rearing: *S. granarius* (Coleoptera: Curculionidae) colonies (1-7 days old) were maintained in our laboratory in Larbi Tebessi University, Tébessa without exposure to insecticides. These were reared in plastic containers on sterilized wheat grains. Insect rearing and all experiments were done in BOD Incubator [26 ± 1 °C and 65 ± 5 % R.H.]. In all studies newly emerged adults (of both sex) were used.

(iii). Plant Material and Essential oil extraction: *Citrus limonum* L. plants were harvested during January - March 2017, from the Tébessa area. The plant parts were washed with tap water, to remove soil and other surface contaminants. After shade drying in laboratory (27 ± 2 °C) for 5 days, the plant material was cut into small pieces(1cm). Fifty

g air-dried aerial parts were hydro-distilled with 500 ml distilled water for 3 h using a Clevenger-type apparatus as per method in the British Pharmacopoeia (11). The obtained oil was collected, dried over anhydrous sodium sulphate and stored in screw capped glass vials at 4 °C in refrigerator till used. The oil yield was calculated based on the dry weight of samples (15).

(iv). Gas chromatography and gas chromatography-mass spectrometry identification: The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of essential oil of *C. limonum* was done with an HP Agilent 6890 plus gas chromatograph (GC) equipped with a HP-5MS column (length: 30 m × internal diameter: 0.25 mm, and 0.25 mm film thickness). The column oven temperature was set at 60 °C for 8 min, and then increased to 250 °C at the rate of 2 °C/min. The injector and detector temperatures were kept at 250 and 270 °C, respectively. Carrier gas was helium, the flow rate in the column was 0.5 ml/min, and the split ratio was set to 50:1 with injection of 0.2 µl of oil sample. The GC/MS analysis was performed with a Quadrupole mass spectrometer that operated at 70 eV. Constituent's identification was based on comparison of retention times with those of corresponding reference standards using the NIST and WILEY libraries (34). Percentage compositions of essential oil were calculated according to the area of the chromatographic peaks.

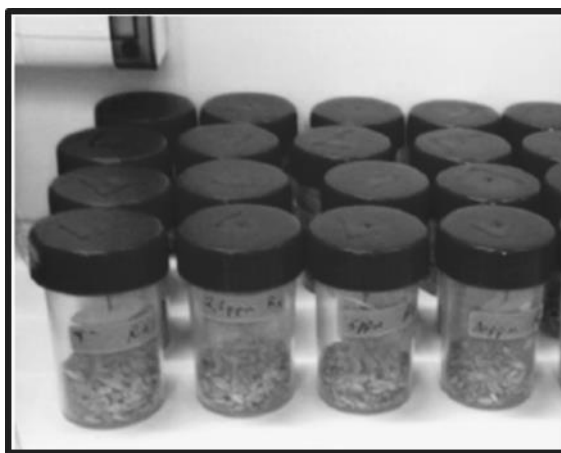


Figure 3. Fumigant toxicity study Jars

(v). Fumigant Bioassay

The fumigant toxicity of *C. limonum* essential oil on *S. granarius* was tested in Glass Jars (60 mL) using method of Huang *et al.* (28) with some modifications. In each Jar 10 adults (both sexes, male or female, 1 to 7 d-old) were released. Whatman filter paper No. 2 disks were cut to 2 cm dia and attached with thread to the under surface of glass vial screw caps (Fig. 3). The hanging filter papers were impregnated with essential oil concentrations: 20, 40, 80, 100, 200 and 400 µL/L air. Control insects were kept in Jars without essential oil. Each dose was replicated five times. After 24, 48 and 72 h from the start of exposure, numbers of dead and alive insects were counted. Those insects unable to

move their heads, antennae and body were considered dead. Lethal concentrations (LC₁₀, LC₂₅ and LC₅₀) at 95 % confidence limits (95 % FL) were determined.

(vi). Repellent activity

The repellent effects of essential oil were evaluated against adults of *S. granaries* using the method of McDonald *et al.* (47). The filter paper discs of 9 cm dia (cut into two equal parts) were used. Four doses (1, 2, 4 and 8 µl/ml) of essential oil diluted with acetone were prepared. Then, 0.5 mL of each solution was spread evenly over one-half of the disc. After 15 min (time required for complete evaporation of solvent), the two halves of discs were glued together using adhesive tape. The filter paper disc was placed in box. A batch of 10 adult insects was placed in the centre of each disk. The number of insects on the part of filter paper treated with essential oil (P) and the number of those present on the control (C) part were recorded after 15 and 30 min.

The percentage of repulsion (RP) was calculated as under:

$$PR = [(P-C) / (P+C)] \times 100$$

Where, PR: Percentage of repulsion, P: Number of adult insects in treatment area, C: Number of adult insects in control area

To categorise the repellent effects of essential oil, the method of Tapondjou *et al.* (64) was used. Five groups were formed based on the mean percent repellency (PR):

Class 0: PR = 0-0.1 %; **Class I:** PR = 0.1-20 %; **Class II:** PR = 20.1-40 %; **Class III:** PR = 40.1-60 %; **Class IV:** PR = 60.1-80 %, and **Class V:** PR = 80.1-100 %.

The Essential oil repellent Index (RI) was calculated as under:

$$RI = 2G / (G+P).$$

Where, RI: Repellent Index, G: Number of adult insects in treatment area, P: Number of adult insects in control area.

For each calculated RI (Repellent Index), the mean and standard deviations were determined and explained below:

Mean (< 1-SD). Essential oil concentration has repellent property.

Mean (> 1+SD). Essential oil concentration has attractant property.

Mean (falls between 1-SD and 1+SD). Essential oil concentration is neutral.

RESULTS AND DISCUSSION

Extraction yield and chemical analysis

The results of steam distillation showed that the yield of extracted essential oil from *C. limonum* was 0.94 % (dry weight basis). The chemical composition of *C. limonum* EO is given in Table 1 and Fig. 4. Gas chromatography and mass spectrometry of essential oil revealed 50 compounds with 3-main components: Limonene (19.81 %), Z-citral (13.58 %) and Geranyl acetate (16.23 %).

Table 1. Chemical composition of *C. limonum* oil: retention time (RT) and different constituents concentration (%).

Time	RT	Compounds	Area (%)
MONOTERPENES			59.10
Monoterpene hydrocarbons			29.98
1	10.648	α - Pinene	0.33
2	13.381	β -Pinene	6.97
3	14.296	β -Myrcene	0.64
4	15.106	L-phellandrene	0.06
5	15.487	Δ . 3-Carene	0.31
6	17.116	Δ L-Limonene	19.81
7	17.569	β -Trans-S-Ocimene	0.26
8	18.306	1,3,6,-Octariene	1.28
9	18.923	γ -Terpinolene	0.13
10	21.005	α - Terpinolene	0.11
11	19.564	Cis-Sabinene Hydrate	0.04
12	41.396	Camphene/ α -Terpene	0.04
Oxygenated Monoterpene			29.12
13	22.090	Linalool	1.43
14	24.292	Cis- Limonene oxide	0.05
15	24.620	Trans- Limonene oxide	0.06
16	27.439	Terpinene-4-ol	0.21
17	25.217	Citronella	2.27
18	28.480	α -Terpineol	0.45
19	29.560	Decanal	0.07
20	31.801	Nerol	6.03
21	33.608	Geraniol	1.98
22	40.818	Geranyl acetate	16.23
23	46.023	Geranyl butyrate	0.07
24	48.447	Bicyclogermacrene	0.20
25	59.368	α - Bisabolol	0.07
SESQUITERPENES			3.03
Hydrocarbon Sesquiterpene			2.51
26	43.777	Trans-Caryophyllene	1.63
27	44.770	α - Bergamotene	0.23
28	45.806	α -Humulene	0.14
29	50.086	Δ .Cadinene	0.04
30	49.252	β -Bisabolene	0.40
31	57.648	α - Cadinol	0.07
Oxygenated Sesquiterpene			0.52
32	36.611	Undecanal	0.05
33	52.539	Farnesol	0.08
34	53.267	Spathulenol	0.19
35	53.570	Caryophyllene oxide	0.11
36	56.713	Isospathulenol	0.02
37	59.368	α -Bisabolol	0.07
Others			16.25
38	6.706	Xexanal	0.05
39	14.094	6-Methyl-5-Hepten-2-One	0.48
40	22.321	PelargonicAldehyd	0.23
41	25.217	Methanone,Dicyclopropyl	0.09
42	26.673	CycloHexane,ethenyl	0.34
43	27.979	Cyclo Hexane Vinyl	0.46

44	32.591	Z-Citral	13.58
45	34.679	Acetic acid geraniol ester	0.19
46	34.765	Geranaldehyde	0.10
47	37.734	Geranic acid methylester	0.11
48	39.710	Citronellyl propionate	0.53
49	41.204	Trans crotomide	0.05
50	46.871	Pyrrolidine	0.04
		Total	78.33

RT: Retention time

The steam distillation showed that the *C. limonum* yield of essential oil of was 1.56 ± 0.15 % (dry weight basis). This yield was higher than other regions in Algeria [0.7 % in Tizi-Ouzou (17) and 1 % in Skikda (10)]. The higher oil content in our study may be due to the arid and dry conditions in Tebessa region, the higher yields are obtained in dry weather. However, the EO yields also depends on the citrus species 1- 3 % (23,24). The essential oil yields of Citrus silt ranges from 0.7 to 0.9 %, these variations may be due to (i). Harvest period, it influences the quantity and quality. (ii). Hydro-distilled part of plant viz., flowers, peels or young and old leaves (36). Besides the geographical area, its climate, genetics of plant, plant organ used, the degree of freshness, the drying period, the method of extraction used, etc. directly impacts the EOs yields (65).

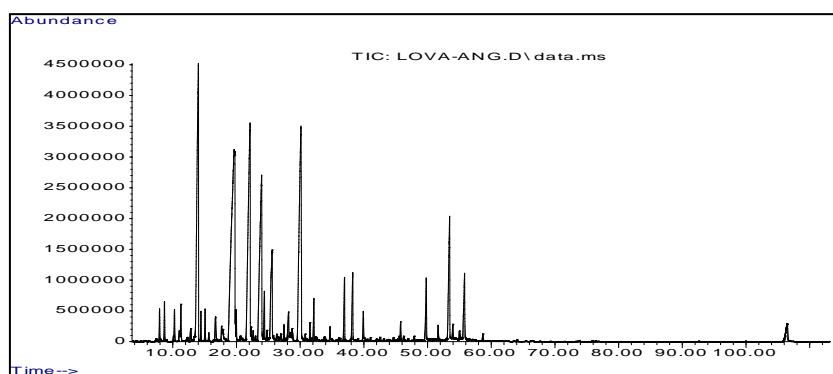


Figure 4. GC-MS chromatogram for essential oils obtained from *C. limonum* (Abundance as function the time in min).

The qualitative and quantitative analysis by GCMS of *C. limonum* EO identified 50 compounds, which represent total of 78.33 %. The Limonene (19.81 %) was major compound. Our results of the chemical profile of essential oil of *Citrus limon* agree with those of Vekiari *et al.* (65), who reported that the Lemon species are characterized by high concentration of β -Pinene (21.2 %), γ -terpinene (17.4 %), α -pinene (9.8%). Boughendjioua & Djeddi (10) which found that EO of *C. limon* grown in Collo (Skikda city) contains 53 compounds which represent: 99.938 %, the major compounds were: Limonene (61.64 %), β -Pinene (13.85 %), γ -Terpinene (9.95 %). It is worth noting that there is a great variation in the chemical composition of Citrus oils due to differences in origin, genetic background, season, climate, age, ripening stage and method of extraction, etc. (16,19).

Citrus EOs contains 85-99 % volatile and 1-15 % non-volatile components (59). The volatile compounds are mixture of monoterpene (Limonene) and sesquiterpene

hydrocarbons and their oxygenated derivatives including: aldehydes (Citral), ketones, acids, alcohols (Linalool) and esters (4,40). Our results are in agreement with Salim-ur-Rehman *et al.* (60); his EOs mainly contained the monoterpene compounds (97 %), while other compounds (alcohols, aldehydes and esters) were only 1.80 % and 2.20 %.

Senatore *et al.* (62) reported that the qualitative and quantitative variations in the chemical composition of EOs, depended on one or combination of three factors: genetic, age and the plant environment. Besides the plant, the phenological state of plant, the season, the climate and other factors also affects the essential oils composition even within species (33,57). Several researchers have found different chemotypes of citrus EOs such as β -pinene, limonene, linalool, linalyl acetate, citral or citronellal (25). The major constituents of *C. limonum* essential oil from different regions are summarized in Table 2.

Table 2. The major constituents of volatile oil in *C. limonum* in different countries.

Major constituents	Country	References
Limonene (56.30 %), β -pinene (8.81 %), γ -terpinene (6.42 %), α -citral (4.96 %), β -citral (3.83 %), α -terpineol (3.38 %).	Egypt	1
Limonene (51.40 %), β -pinene (17.04 %), and γ -terpinene (13.46 %).	Algeria	18
Limonene (54.6 %), γ -terpinene (19.1 %) and β -pinene (14.5 %).	Italy	9
Limonene (61.4 %), β -pinene (13.1 %) and γ -terpinene (11.3 %).	Iran	28
Limonene (73.25 %), α -pinene (8.44 %), α -terpinene (6.21 %) and geraniol (2.53 %).	Iran	67

Fumigant toxicity

The mortality of *S. granarius* after exposure to different concentrations of the tested essential oil is given in Figure 3 and 4. The highest mortality (27.50; 75 and 92.50 %) of *S. granarius* occurred at 400 μ L/litre air concentration of *C. limonum* Essential oil at 24, 48 and 72 h after treatment, respectively. We calculated LC₁₀, LC₂₅ and LC₅₀ values of EO and their fiducial limits (Table 3).

Table 3. Toxicity of *C. limonum* essential oil against the adults of *S. granarius*.

Time (h)	Hill slope	R ²	Lethal concentrations (μ L/liter air)		
			LC ₁₀ (95 % FL)	LC ₂₅ (95 % FL)	LC ₅₀ (95 % FL)
24	0.64	0.94	52.91 (25.00 - 87.80)	293.76 (214.10 - 505.20)	1630 (830.10 - 6866)
48	0.99	0.98	17.63 (11.08 - 27.20)	53.21 (39.62 - 68.01)	160.69 (134.60 - 195.50)
72	1.01	0.89	12.07 (0.90 - 38.35)	35.73 (8.80 - 72.80)	105.80 (60.70 - 184.50)

Essential oils are mixtures of volatile hydrocarbons with variety of functional groups and their insecticidal constituents are mainly monoterpenoids (14). They have acute fumigant and contact toxicity (44), repellent activity (25), anti-feedant activity (26,29) and are inhibitory to the development and growth of insects (35). The toxicity of essential oils extracted from aromatic plants has been studied on stored insects (12,50).

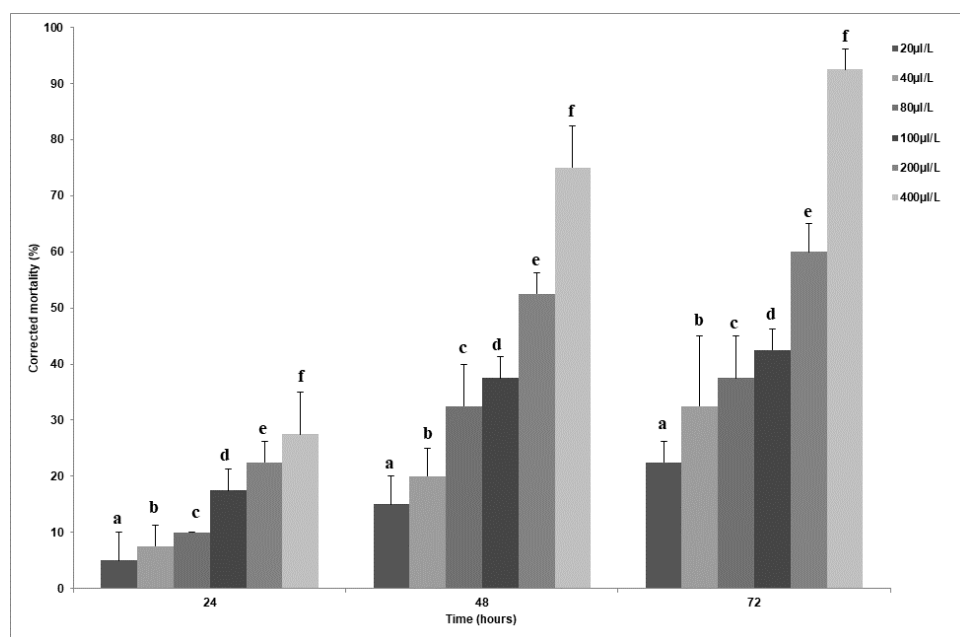


Figure 5. Efficacy of *C. limonum* EO applied on adult of *S. granarius*: corrected mortality (mean \pm SE, n = 5 replicates, each containing 10 adults).

Our results indicate that *C. limonum* plant EO was insecticidal against adults of *S. granarius*. Results of fumigant toxicity showed that as the concentration and exposure time increased, the mortality also increased. It varies with plant derived material, insect species, exposure time (41) and developmental stage (40). Essential oil can be inhaled, ingested or skin absorbed by insects (51). The fumigant toxicity of plant essential oils has been shown against pests of stored products (58). Due to the high volatility of the monoterpenes and their lipophilic properties, they have fumigant effects on the stored product insects (56). Similar results were found with application of five Citrus essential oils against *Tribolium confusum* (12) and with volatile monoterpenes and aromatic compounds against adults of *Sitophilus granarius* (42). Abou-Taleb *et al.* (3) reported that twenty EOs isolated from the Egyptian plants including the *C. limon* have fumigant potential similar to our study. The EOs of *Citrus limon*, *Citrus aurantifolia*, *Citrus paradisi* and *Citrus sinensis* are highly toxic with LC₅₀ values (9.89 mg/L air, 29.37 mg/L air, 24.13 mg/L air and 19.67 mg/L air respectively), against *Sitophilus oryzae* adults (2). Furthermore, the oil of *Origanum vulgare* is toxic to adults of *T. castaneum* with an LC₅₀ of 9.97 mg/L air (3). Comparing the fumigant toxicity of plant oils (Anis seed, Camphor, Citronella, Eucalyptus, Geranium, Lavender and Lemon) against the adults of *Sitophilus oryzae*, at 10 and 50 µL concentrations for 24, 48 and 72 h, they showed that Lemon oil was most toxic and their respective LD₅₀ values were 58.86, 44.90 and 40.38 µL (31).

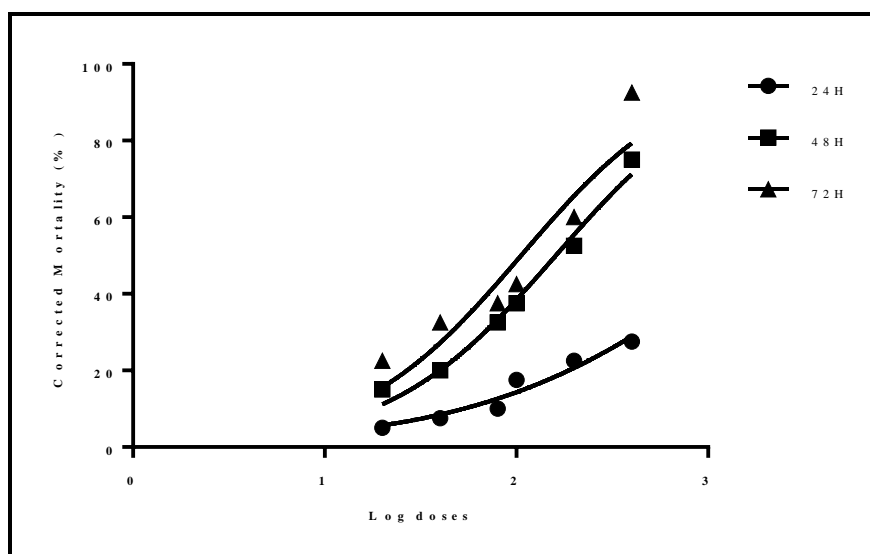


Figure 6. Efficacy of *C. limonum* EO against adult of *S. granarius* as function the exposure time (h)

Repellent activity

The repellent activity in insects is defence mechanism against toxins secreted from the plants. The repellency (%) of *C. limonum* EO at 1, 2, 4 and 8 $\mu\text{L}/\text{mL}$ concentrations against adult of *S. granarius* at different periods after treatment are presented in table 4. Our results revealed an increase in the repellency (%) depending on the exposure period and concentration. The maximum repellency rate (70 %) was recorded at dose of 8 $\mu\text{L}/\text{mL}$ after 30min. According to our results, *C. limonum* plant belonged to the repellent class IV.

Table 4. Repellent index (RI) and repulsion percentage (RP) of different concentrations of *C. limonum* EO against adults of *S. granarius*.

<i>C. limonum</i> E.O.Conc. ($\mu\text{l}/\text{ml}$)	Periods (min)	RI	SD	1-SD	1+SD	Effect	RP (%)
1	15	0.70	0.11	0.88	1.11	Repellent	30 \pm 10 II
	30	0.65	0.10	0.90	1.10	Repellent	35 \pm 7.5 II
2	15	0.55	0.10	0.90	1.10	Repellent	45 \pm 7.5 III
	30	0.50	0.11	0.88	1.11	Repellent	50 \pm 10 III
4	15	0.45	0.10	0.90	1.10	Repellent	55 \pm 7.5 III
	30	0.40	0.16	0.83	1.16	Repellent	60 \pm 10 III
8	15	0.35	0.10	0.90	1.10	Repellent	65 \pm 7.5 IV
	30	0.30	0.11	0.88	1.11	Repellent	70 \pm 10 IV

E.O.: Essential Oil, RI: Repellent Index, RP: Repulsion Percentage.

The repellent effects of volatile essential oils make them an attractive candidate to control the stored grain pests in the traditional postharvest storage system (49). The present study revealed the effective repellent activity of *C. limonum* EO against *S. granaries* and

all tested concentrations showed repellent activity. Our results are in agreement with previous works done to evaluate the different plants extracts against stored insect pests. Akhtar *et al.* (5) showed repellency (%) of 70.33, 68.4, 58.43 and 64.26 % from *Psidium guajava* (leaves), *C. reticulata* (fruit peel), *C. sinensis* (fruit peel) and *C. limon* (fruit peel) respectively, against *Sitophilus oryzae*. Repellent activity is linked to the presence of monoterpenes and sesquiterpenes that killed the insects by inhibiting AChE activity in the nervous system (27). Several studies have recorded the repellent activity of essential oils against stored product pests (9,50,54). Besides, the essential oils of *Schzygium aromaticum* (L.), *Aegle marmelos* (L.), *Coriandrum sativum* (L.) and *Citrus reticulata* (B.) are highly repellent to *S. oryzae* and *T. castaneum* with marked effects on *S. oryzae* (48).

All above findings support the results of our present study. The repellency against the adult stored-grain insect pests may be due to the suffocation and inhibition of different biosynthetic processes of the insect metabolism (18). However, the mode of action of *C. limonum* oil is yet to be confirmed.

CONCLUSIONS

The EO of *C. limonum* exhibited fumigant toxicity and strong repellent activity against *S. granarius* adults confirming its potential as natural alternative to synthetic fumigants to control the stored-product pests. Hence, it may be used to flush out insect infestations from empty stores before fresh grain is stored. Thus the *Citrus* genus offers scope to develop potential insecticide and repellent formulations for stored grain pests. Hence in-depth studies are needed (i). to isolate and identify the active substances, (ii). to study their physiological impact on other insects and (iii). potential uses to manage coleopterons insects in grains storage especially *Sitophilus granarius*.

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