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RESEARCH ARTICLE

SUSCEPTIBILITY OF THE DIFFERENT STAGES OF THE MEDFLY *CERATITIS CAPITATA* WIEDEMANN (DIPTERA: TEPHRITIDAE) TO THE EXTRACTS OF *VIOLA ODORATA* AND *EUCALYPTUS CAMALDEULENSIS*

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ABSTRACT

The medfly (*Ceratitis capitata*) is a polyphagous serious fruit pests spreaded in Africa and worldwide. The medfly infests several plant species and causes economic losses in many crops. The problems of using undesirable chemical insecticides against medfly have forced the scientists to look for more safe pesticides. Therefore, two plant extracts, *Viola odorata* and *Eucalyptus camaldeulensis*, were tested against the adult and immature stages of *C. capitata*. Each extract composition was analyzed using GC-MS. Terpenoids were identified as the major constituents in *V. odorata* extract, while *E. camaldeulensis* extract contains heterocyclic organic compounds together with terpenoids. The toxicity of both extracts on *C. capitata* was addressed using the contact and spray application methods. The contact treatment method showed higher toxicity than the spray method. In the meantime, the pupae were more susceptible to *V. odorata* extract than the full grown larvae; while *E. camaldeulensis* extract showed higher toxicity on larvae. Both extracts affected adult flies with higher toxicity in *V. odorata*-treated flies. The pupal deformations were recorded in the plant extracts-treated *C. capitata*. The malformations of pupae included emergence of only head and thorax of the flies and keeping the rest of the body inside the puparia, decoloration of the puparium, and no emergence of the flies at all. Furthermore, the *V. odorata* extract induced adult antennal and wings abnormalities. Both extracts degraded within a short period of time in the soil. In conclusion, the *V. odorata* and *E. camaldeulensis* extracts might act as powerful insecticides against *C. capitata*.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is regarded as one of the most serious pests as it is able to infest and destroy a broad range of horticultural fruits and vegetables[1]. Plant protection authorities almost all over the world severely restrict the transfer of possible pest-infested horticultural products[2,3]. This economic barrier pushed governments to launch multiple research projects dealing with the control of such pest. Egypt started using multiple control methods to manage this

pest at the adult phase to minimize these economic losses and high expenses. In past years, the control of this pest depended mainly on using the chemical pesticides, which have several disadvantages such as residual effects on environmental components (food, air, water)[4], negative actions on the health of both human and animals[5], and adverse effects on non-target organisms[6]. Furthermore, target insects can tolerate their effects *via* creation of insecticide resistance. As the hazards and disadvantages of using these compounds were growing on the environment and human, the need to find out new safe control strategies has become necessity and obligation. Many studies were directed to test the ability of using biological control agents from different taxa to decrease the pest population[7-12]. Furthermore, great attention has focused on using green pesticides[13] to become a new trend in controlling this pest because of its safety to the environment. These compounds have no harmful residues and high biodegradation rate in the environment[14,15]. In addition, they have a wide range of action modes, including toxicity, repellency, antifeedant, and regulation of insect development[16]. Furthermore, these products are not limited by the development of insecticide resistance as they are containing several bioactive compounds, which interfere with several physiological and developmental processes in insects' bodies[17-19]. It should be taken in consideration that not all botanical pesticides are safe for humans and mammals[20]. Some green pesticides may have few disadvantages including their variable efficacy, and low toxicity and persistence against target pests, which is partly due to the rapid breakdown of bioactive compounds through photo-degradation and rains.

Eucalyptus genus (Family Myrtaceae) is a native plant to Australia and has more than 700 species distributed around the world. The presence of essential oil in this plant supplies defense character to *Eucalytpus* leaves against herbivorous insects and attack by other harmful pests[21]. The genus *Viola* lies in the family Violaceae and it has about 500 species. *Viola tricolor, Viola arvensis*, and *Viola odorata* are reported as medicinal plants[22]. *V. odorata* L. has the common name of sweet violet and is considered as a herb with stout root stocks. The species is locally present in Europe, North Africa, and Western Asia; it can be also growing in the temperate and sub-temperate areas. The plant has been grown for corrosion control in the Java Mountains and as medicinal active herb in Cuba[22]. It is used mainly for therapeutic intents and perfume production. *V. odorata* has also repellent effects against insects, for instance, the essential oil of *V. odorata* repelled the yellow fever mosquito, *Aedes aegypti*; the malaria vector, *Anopheles stephensi*; and the filariasis and encephalitis vector, *Culex quinquefasciatus* against human skin[23].

In this study two plant leaves extracts, *Eucalyptus camaldeulensis* Dehnh (Myrtacae) and *V. odorata* Linn (Violaceae), were tested against the medfly "*C. capitata*" developmental stages. Additionally, pupal malformation and adult ultrastructural changes following treatment of *C. capitata* with these extracts has been observed and recorded. The main purpose of the study is not to compare the toxicity of both extracts against *C. capitata*, but it is to address the toxicity of each extract alone. This study aimed to figure out a new vision about the possibility of disrupting the life cycle of *C. capitata* by controlling different stages using the tested plant extracts and indicating the extent of which the extracts will persist effectively in the agroecosystem. Therefore, both extracts were analyzed chemically using GC-MS to identify the major bioactive components in each extract.

MATERIAL AND METHODS

Insect rearing

The Mediterranean fruit fly, *C. capitata* (Wiedemann) adults were obtained from the rearing lab in Horticultural Insect

Research Department (HIRD), Plant Protection Research Institute (PPRI), Agricultural Research Center (ARC), Giza, Egypt. The colony was reared in the lab for two years. Flies were fed on sugar and protein hydrolysate in a ratio 3:1, respectively. Produced eggs were collected daily and the hatched larvae were reared on artificial medium as previously described[24]. Larvae were allowed to complete three larval instars and full-grown larvae were collected for pupation. The collected larvae placed on fine sand to complete its pupation within nine days. A day before flies' emergence, pupae were sieved gently and located in screen cage measuring 30 cm × 30 cm × 30 cm allowing the emerged flies to feed, mate, and produce eggs for new generation.

Preparation of plant extracts for toxicity assays in larvae and pupae

V. odorata leaves dough was obtained from Hashem Ikhwan Company, Cairo (Egypt). Crude natural acetone extract of *V. odorata* leaves dough was carried out according to the method described by Aslam *et al*.[25]. Briefly, 1.0 g of leaves was dissolved in 100 mL acetone and used to form a concentration of 1×10^4 ppm (weight/volume) as initial concentration. Serial successive concentrations were prepared; 0.25×10^3, 0.5×10^3, 1.0×10^3, 2.0×10^3, and 3.0×10^3 ppm. The concentrations were preserved at −20ºC till use (about two weeks).

E. camaldeulensis leaves were collected from Al-Orman botanical garden, Dokki, Giza (Egypt). The leaves were carefully washed to remove dirt then they were naturally air-dried for 30 days in a shad place. After complete dryness of the leaves, they were grinded by electric grinder to a fine powder. The leaf powder was extracted by methanol solvent using Soxhlet apparatus. Briefly, 70 g of leaf powder were extracted with 400 mL of methanol solvent at 67ºC[26]. The extraction was continued for 48 hours and was preserved in refrigerator at 4ºC until use.

Chemical analysis of plant extracts

GC-MS analysis of the two extracts was achieved in analytical chemistry unit, Faculty of science, Assiut University, Assiut (Egypt). Temperature of injector was set at 250ºC and the temperature of oven initiated at 100ºC (hold time: 10 minutes) and passing by four ramps, the final temperature was set at 280ºC (on hold time: 2 minutes), and then the split flow was adjusted at 20 mL/minute, carrier flow = 1.0 mL/minute. The analysis, based on GC-MS retention time, was cited by MAINLIB and RepLib library to identify the bioactive compounds present in both extracts. The start mass was at 40 atomic mass unit (amu) and end mass was at 800 amu.

Application of plant extracts on larvae and pupae of *C. capitata*

The plant extracts of *V. odorata* and *E. camaldeulensis* were used against three different ages of *C. capitata*. Full-grown larvae and pupae aged one and eight-days old were treated by spray and contact treatment methods using five different concentrations from both extracts; 0.25×10^3, 0.5×10^3, 1.0×10^3, 2.0×10^3 and 3.0×10^3 ppm. In spray treatment method, ten freshly harvested individuals of full-grown larvae and pupae of both ages were collected, washed, dried with tissue paper and prepared for the experiment. Both the pupae and the full-grown larvae were sprayed with 1.0 mL of the extract using small sprayer (15 mL) for 30 seconds. The treated insects for each concentration were allowed to develop in non-treated fine sand placed in Petri plate measured (9 cm). Each treatment was replicated three times.

On the other side, contact treatment method was conducted using 10 g of sterilized fine sand, which were distributed in Petri plate (9 cm) and well mixed with 2 mL of each concentration, separately. Ten full-grown larvae and pupae of both ages were placed in each replicate and allowed to burry either by themselves (in

larvae) or by us (in pupae) in sand. Each treatment was replicated three times.

Positive control treatments were performed using the solvent alone (acetone in *V. odorata* and methanol *in E. camaldeulensis*) and the negative control treatments were using water alone. Number of emerged flies was recorded and flies that could not emerge or partially emerged from pupae were considered dead flies. Additionally, detected malformations in *C. capitata* pupae were also recorded.

Application of plant extracts on *C. capitata* adults

In a small cage, 2 mm length wick was saturated with 1.0 mL of diluted extract (concentrations were 5×10^3, 7×10^3, 10×10^3, 20×10^3 and 30×10^3 ppm for each extract) and hanged in the cage by a wire. Three groups of 5-6 old day *C. capitata* adult flies were selected randomly from the rearing cage. Each group, consisting of male-female pairs was placed in a small cage where food source and water were available. After 24 hours, mortality was recorded, and LC_{50} and LC_{90} toxicity values were determined.

Scanning electron microscopy of *C. capitata* adult antennae

Because of the high activity of *V. odorata* leaves extract, LC_{30} was used to study the ultrastructural changes of the antennae using JEOL 5400LV scanning electron microscope (SEM, JEOL Ltd., Musashino, Japan). Identification of the antennal sensilla were taken at magnifications ranging between 150 and 3500. The selection of this part for ultrastructural studies is based on the fact that *C. capitata*, as many herbivorous insects, depends on the olfaction to find the host plant. Thus, SEM was performed on the antennal sensilla (chemoreceptors).

Persistence of plant extracts in the agroecosystem

Eight concentrations of both plant extracts were prepared (1×10^3, 2×10^3, 3×10^3, 5×10^3, 7×10^3, 10×10^3, 20×10^3, and 30×10^3 ppm). Each concentration (2 mL) was sprayed on 10 g of sterilized sand covering ten pupae in a 9 cm dish. This procedure was repeated daily from zero time (as initial spraying) for 3 days using one-day old pupae. Each treatment was replicated three times. After 10-14 days (average emergence time of *C. capitata*), mortality was recorded and the dissipation curve of each extract was achieved.

Statistical analysis

Mortality rates were adjusted using Abbot's Formula. Statistical analysis were conducted using SPSS statistical software. Probit analysis was used for calculating LC_{50} and LC_{90} values.

RESULTS

Chemical analysis of *V. odorata* and *E. camaldeulensis* extracts

GC-MS analysis of *V. odorata* revealed 32 compounds representing 64% of the extract total content (Table 1). Most of the compounds were categorized under three groups of terpenoids namely, monoterpines, tetraterpines, and sesquiterpines. Monoterpenes represented the major constituents present in *V. odorata* extract and included α-linalool (15.81%); eucalyptol (1.99%), terpineol (1.99%), estragole (1.87%), eugenole (0.91%), camphor (0.14%), D-limonene (0.10%), α-pinene (0.10%), and bornylacetate (0.31%).

Tetraterpenoids included lycopene (0.97%), rhodopin (0.99%), and asta-xanthin (0.29%), while sesquiterpenes were represented by cmurrolene (0.78%), azulene (0.26%), cubenol (0.19%), tucadinuol (1.33%), and murrolene (0.78%). Other secondary metabolites were also detected including glycine (amino acid, 6.19%), fatty acids (1.88% hexadecanoic acid, 0.13% methyl esteroleic acid, docosahexaenoic acid, propanoic acids, 0.13% laricic acid). Milbemycin B was also detected by GC-MS analysis with lower content (0.10%). This compound was commercially used as insecticide. Ascorbic acid (vitamin C, 6.47%) and 0.13% ethyl iso-allocholate (antifungal agent) were also detected.

Table 1: GC-MS analysis of *Viola odorata* L. leaves acetone extract.

Number	RT	Compound Name	MF	MW	Area (%)	Library
1	6.73	a-Pinene	$C_{10}H_{16}$	260	0.10	MAINLIB
2	7.37	D-Limonene	$C_{10}H_{16}$	136	0.10	RepLib
3	7.49	Eucalyptol (1,8-Cineole)	$C_{10}H_{18}O$	154	1.99	RepLib
4	7.49	Trifluoroacetyl-α-terpineol	$C_{12}H_{17}F_3O_2$	250	1.99	MAINLIB
5	7.98	Linalool oxide	$C_{10}H_{18}O_2$	170	0.16	RepLib
6	8.22	α-Linalool	$C_{10}H_{18}O$	154	15.81	MAINLIB
7	9.18	Ethyl iso-allocholate (antifungal)	$C_{26}H_{44}O_5$	436	0.13	MAINLIB
8	9.37	1,7,7-Trimethyl-, (1S) (Camphor)	$C_{10}H_{16}O$	152	0.14	RepLib
9	9.72	p-Menth-1-en-4-ol	$C_{10}H_{18}O$	154	0.14	RepLib
10	9.88	Estragole	$C_{10}H_{12}O$	148	1.87	RepLib
11	11.06	Agaricic acid (Laricic acid)	$C_{22}H_{40}O_7$	416	0.13	MAINLIB
12	11.28	Bornyl acetate	$C_{12}H_{20}O_2$	196	0.31	MAINLIB
13	12.32	Eugenole	$C_{10}H_{12}O_2$	164	0.91	MAINLIB
14	15.41	Azulene	$C_{15}H_{24}$	204	0.26	RepLib
15	15.59	Naphthalene	$C_{15}H_{24}$	204	0.78	RepLib
16	17.36	5H-Cyclopropa[3,4]benz[1,2-e]azulen-5-one,9,9a-bis(acetyloxy)-1,1a,1b,2,4a,7a,7b,8,9,9a-decahydro-2,4a,7b-trihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl	$C_{26}H_{34}O_{10}$	506	0.11	MAINLIB
17	17.71	Cubenol	$C_{15}H_{26}O$	222	0.19	MAINLIB
18	18.14	tau-Cadinol	$C_{15}H_{26}O$	222	1.33	MAINLIB
19	18.73	Carboxaldehyde	$C_{23}H_{32}O$	324	0.39	MANILIB
20	21.31	Milbemycin B	$C_{33}H_{47}ClO_7$	590	0.10	MAINLIB
21	22.94	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.88	MAINLIB
22	23.59	l-(+)-Ascorbic acid	$C_{38}H_{68}O_8$	652	6.47	MAINLIB
23	23.97	Oleic acid	$C_{18}H_{34}O_2$	282	0.13	RepLib
24	28.89	Ethyl linoleate	$C_{20}H_{36}O_2$	352	1.20	MAINLIB
25	29.67	Linolenin	$C_{21}H_{36}O_4$	352	0.14	MAINLIB
26	32.28	Docosahexaenoic acid	$C_{69}H_{98}O_6$	1022	0.13	MAINLIB
27	33.17	Glycine	$C_{36}H_{69}NO_6Si_3$	695	6.19	MAINLIB
28	34.74	Docosahexaenoic acid	$C_{69}H_{68}O_6$	1022	0.14	RepLib
29	41.77	1,1',2,2'-tetrahydro-1,1'-dimethoxy-lycopene	$C_{42}H_{64}O_2$		0.97	MAINLIB
30	42.0	Propanoic acid	$C_{27}H_{42}O_4$	430	1.21	MAINLIB
31	50.37	Astaxanthin (Terpene)	$C_{40}H_{52}O_4$	596	0.29	MAILIB
32	53.11	Rhodopin (Carotenoid)	$C_{40}H_{58}O$	554	0.99	MAILIB
Total					64.0%	

RT: retention time, MF: molecular formula, MW: molecular weight.

GC-MS analysis of *E. camaldeulensis* extract indicated 29 compounds representing 73.17% of the extract total content (Table 2). The major components were terpenes including p-cymene (1.46%), limonene (1.16%), eucalyptol (1.54%), camphor (0.28%), menth-1en-4-ol (2.08%), and spathulenol (7.03%), in addition to phytol (6.08%) that is a cyclic diterpene alcohol. Furan, which is a heterocyclic organic compound, was present with 14.81% together with its derivative furfural

(1.09%). Glycerin, which has antibacterial and antiviral properties, was present with 0.42%. Pyrrogalol (10.6%), which is an organic compound belonging to phenol family, was also detected. Other components were less than 1.0% and were not reported previously to have insecticidal effect.

Table 2: GC-MS analysis of *Eucalyptus camaldeulensis* leaves methanol extract.

Number	RT	Compound Name	MF	MW	Area (%)	Library
1	5.78	Furfural	$C_5H_4O_2$	96	1.09	MAINLIB
2	10.40	Glycerin	$C_3H_8O_3$	92	0.42	MAINLIB
3	13.28	p-Cymene	$C_{10}H_{14}$	134	1.27	RepLib
4	13.44	Limonene	$C_{10}H_{16}$	136	1.16	RepLib
5	13.62	Eucalyptol (1,8 Cineol)	$C_{10}H_{18}O$	154	1.54	RepLib
6	17.33	Camphor	$C_{10}H_{10}O$	152	0.28	RepLib
7	18.71	p-Menth-1en-4-ol	$C_{10}H_{18}O$	154	2.08	RepLib
8	19.55	Furan	$C_8H_{14}O$	126	14.81	RepLib
9	23.10	p-Cymene-7-ol	$C_{10}H_{14}O$	150	0.19	RepLib
10	23.31	Ascaridol epoxide	$C_{10}H_{16}O_3$	184	0.57	MAINLIB
11	27.61	Pyrogallol	$C_6H_6O_3$	126	10.69	RepLib
12	32.11	Erucic acid	$C_{22}H_{42}O_2$	338	0.29	MAINLIB
13	33.97	D-Allose	$C_6H_{12}O_6$	180	3.35	MAINLIB
14	35.29	α-Guaienen	$C_{15}H_{20}$	204	0.47	MAINLIB
15	36.92	D-Mannose	$C_6H_{12}O_6$	180	0.28	MAINLIB
16	38.22	Spathulenol	$C_{15}H_{24}O$	220	7.03	MAINLIB
17	38.87	Globulol	$C_{15}H_{26}O$	222	0.81	MAINLIB
18	43.35	Farensol	$C_{15}H_{26}O$	222	6.01	RepLib
19	44.48	Caryophyllene oxide	$C_{15}H_{24}O$	222	0.53	MAINLIB
20	44.67	Virdiflorol	$C_{23}H_{38}O_2$	346	0.88	MAINLIB
21	45.50	Longipinocarvone	$C_{15}H_{22}O$	218	0.95	MAINLIB
22	46.59	2H-Pyran	$C_{22}H_{40}O_2$	336	1.24	MAINLIB
23	49.97	Gallic acid	$C_8H_8O_5$	184	1.58	MAINLIB
24	51.87	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.71	RepLib
25	53.78	4-Methylesculetin	$C_{16}H_8O_4$	192	1.55	MAINLIB
26	56.95	Phytol	$C_{20}H_{40}O$	296	6.08	RepLib
27	57.73	Linolenin	$C_{18}H_{30}O_2$	278	0.68	RepLib
28	65.63	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.18	RepLib
29	65.73	Oleic acid	$C_{39}H_{76}O_3$	592	0.70	MAINLIB
Total					73.17%	

RT: retention time, MF: molecular formula, MW: molecular weight.

Toxicity of *V. odorata* leaves extract to different immature stages of *C. capitata*

The toxicity values of *V. odorata* on *C. capitata* full grown larvae, one-day old pupae, and eight-day old pupae using spray and contact treatments were calculated to determine the active concentrations against each developmental stage. LC_{50} and LC_{90} values indicated that different ages of pupae were more susceptible to *V. odorata* extract than full grown larvae showing lower toxicity values (Table 3). Furthermore, data retrieved from LC_{90} values showed that full grown larvae (LC_{90} equal to 2.6×10^5 ppm in larvae treated by contact method compared to 3.6×10^5 ppm in those treated by spray method) and one-day pupae (LC_{90} equal to 6.9×10^2 ppm in pupae treated by contact method compared to 1.5×10^3 ppm in those treated by spray method) had higher susceptibility to *V. odorata* leaves extract when treated

by contact rather than spray method. On the contrary, this pattern was not recorded in the eight-day old pupae, which did not show clear preference to either methods where the toxicity was slightly different between contact and spray methods in eight-day old larvae (LC_{90} equal to 1.6×10^3 ppm in pupae treated by contact method compared to 1.8×10^3 ppm in those treated by spray method) as shown in Table "3".

Table 3: Toxicity values in ppm of *Viola odorata* leaves extract on some immature developmental stages of *Ceratitis capitata* using spray and contact methods.

Stage	Method	LC_{50} (ppm)	Confidence limit		LC_{90} (ppm)	Confidence limit*		Slope ± SE**	$\chi2$	P
			Lower	Upper		Lower	Upper			
Full-grown larvae	Spray	1.5×10^4	5.7×10^3	6.7×10^5	3.6×10^5	4.2×10^4	1.2×10^9	0.95±0.33	4.13	0.247
	Contact	3.6×10^4	2.5×10^4	7.1×10^4	2.6×10^5	1.2×10^5	1.5×10^6	1.51±0.32	2.52	0.471
One-day old pupae	Spray	2.7×10^2	1.7×10^2	3.7×10^2	1.5×10^3	1.1×10^3	2.5×10^3	1.71±0.38	7.14	0.070
	Contact	2.4×10^2	1.7×10^2	3.6×10^2	6.9×10^2	5.5×10^2	1.1×10^3	2.49±0.67	1.66	0.198
Eight-day old pupae	Spray	6.3×10^2	5.3×10^2	7.3×10^2	1.8×10^3	1.4×10^3	2.3×10^3	2.82±0.29	5.87	0.118
	Contact	6.2×10^2	3.1×10^2	1.03×10^3	1.6×10^3	1.3×10^3	5.1×10^3	3.10±0.29	11.78	0.008

*95% lower and upper fiducial limit, **SE: standard error.

Toxicity of *E. camaldeulensis* leaves extract to different immature stages of *C. capitata*

Contact treatment method appeared to be more effective than spray method at each concentration on full-grown larvae, one-day old pupae, and eight-day old pupae (Table 4). Based on data presented in Table "4", the calculated LC_{90} values by contact method were 19.0×10^3, 5.5×10^4, and 7.3×10^4, while in spray method the values were 57.4×10^3, 1.5×10^6, and 2.3×10^6 for full grown larvae, one-day old pupae, and eight-day old pupae, respectively. Furthermore, the obtained data are impressive that larvae were more susceptible to *E. camaldeulensis* than pupae of both ages, as they required lower concentrations of the extract to achieve LC_{90} values.

Table 4: Toxicity values in ppm of *Eucalyptus camaldeulensis* extract on some immature developmental stages of *Ceratitis capitata* by contact and spray treatment methods.

Stage	Method	LC_{50} (ppm)	Confidence limit		LC_{90} (ppm)	Confidence limit*		Slope ± SE**	$\chi2$	P
			Lower	Upper		Lower	Upper			
Full-grown larvae	Spray	6.8×10^2	2.0×10^2	2.2×10^3	57.4×10^3	17.3×10^3	1.9×10^5	0.68±0.27	0.31	0.958
	Contact	1.2×10^2	0.29×10^2	5.2×10^2	19.0×10^3	4.5×10^3	8.0×10^4	0.59±0.32	0.06	0.996
One day-old pupae	Spray	4.4×10^3	8.5×10^2	22.2×10^3	1.5×10^6	29.4×10^4	7.6×10^6	0.51±0.36	1.77	0.622
	Contact	5.8×10^2	1.7×10^2	2.0×10^3	5.5×10^4	15.9×10^3	1.9×10^5	0.66±0.27	0.40	0.940
Eight day-old pupae	Spray	2.1×10^4	4.5×10^3	9.4×10^4	2.3×10^6	5.0×10^5	10.5×10^6	0.61±0.34	5.07	0.167
	Contact	1.4×10^3	5.1×10^2	4.0×10^3	7.3×10^4	26.3×10^3	2.0×10^5	0.80±0.23	4.29	0.232

*95% lower and upper fiducial limit, **SE: standard error.

***C. capitata* pupal malformations due to the treatments with *V. odorata* and *E. camaldeulensis* extracts**

Pupal malformation was not recorded in larval treatments developed into pupae. Malformations of pupae treated with sweet violet extracts were noted in both one-day old treated pupae and eight-day old pupae. The pupal malformations were observed and summarized in three different categories. The first category of malformation was characterized by emergence of only head and thorax of flies and keeping the rest of the body inside the puparia (Figure 1B). The second category of malformation was characterized by decoloration of the puparium (Figure 1C)**,** while the third category showed no emergence of the flies at all (Figure 1D).

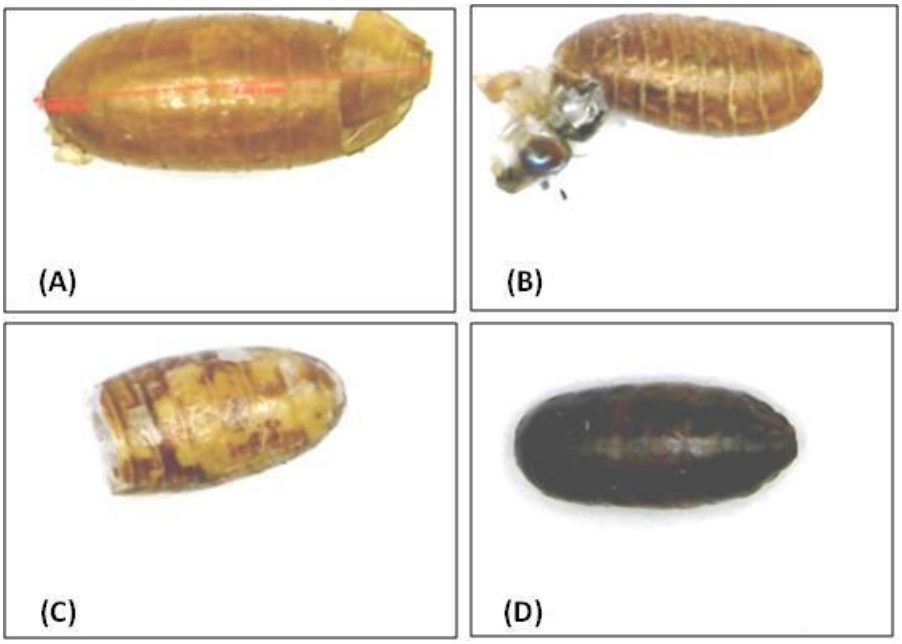

Figure 1: Different forms of pupal abnormalities resulting from exposing *Ceratitis capitata* pupae to different concentrations of sweet violet dough extracts, *V. odorata*. (A) Normal capsule of pupa (control), (B) emergence of malformed adult (head and thorax), (C) capsule malformed, and (D) pupal malformation without emergency (black pupa).

On the other hand, *E. camaldeulensis* extract-treated *C. capitata* pupae recorded several forms of malformations. In Figure "2", the recorded malformations indicated flies emergence failure and consequently disruption of *C. capitata* lifecycle. The first form of pupal malformations was characterized by clear elongation of pupal capsule with failure to emerge as adult (Figure 2B). Another form of pupal malformation was present in the form of undeveloped fly within pupal cocoon (Figure 2C). Some pupae appeared with opened capsule with only head of adults could be seen (Figure 2D). On the other hand, emerged adults showed more clear malformations than pupae, which appeared in the form of abnormal adult with unwinged thorax, stunting back legs, and abnormal abdomen (Figure 2E), malformed adult with undeveloped body parts (Figure 2F), abnormal adult showing deformities in all abdomen (Figure 2G), and malformed adult with a one wrinkled wing and abnormal abdomen (Figure 2H).

Toxicity of plant extracts to *C. capitata* adults

E. camaldeulensis showed slight effect on adults of *C. capitata* with LC_{90} value of 1.07×10^{12} ppm (Table 5). Furthermore, *V. odorata* extract appeared to be more

effective due to the lower LC_{90} value, which recorded 2.61×10^8 ppm. The higher activity of *V. odorata* than *E. camaldeulensis* on adults may be linked to the chemical analysis of the chemical constituents of both extracts, which will be discussed in detail in the discussion part.

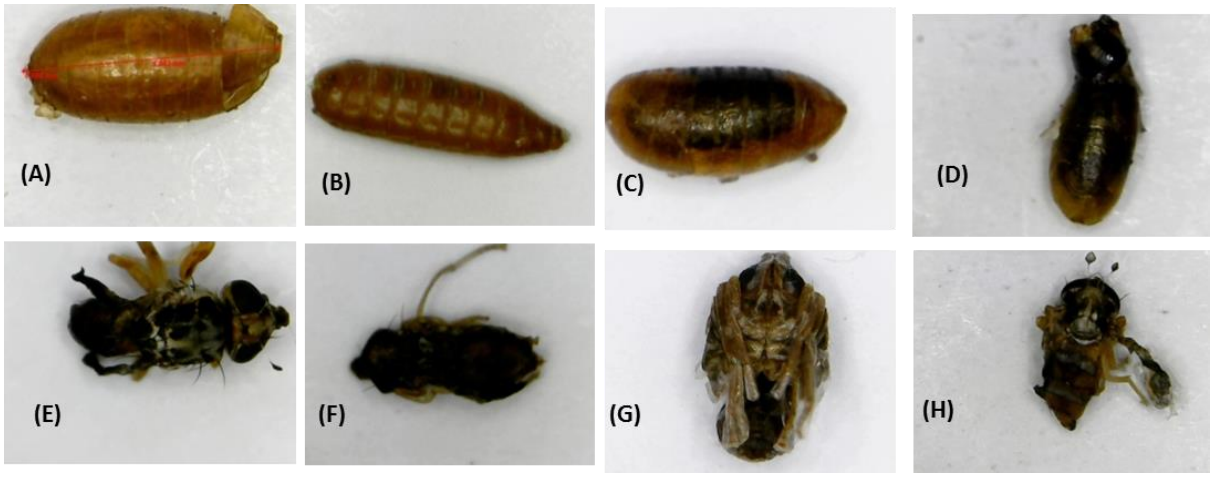

Figure 2: Different forms of pupal abnormalities resulting from exposing *Ceratitis capitata* pupae to different concentrations of methanol extracted *Eucalyptus camaldeulensis* indicating (A) empty capsule of healthy control pupa, (B) elongated pupa that fail to emerge, (C) pupa with undeveloped fly, (D) opened pupal capsule that fail to emerge appearing head of adult, (E) abnormal adult with unwinged thorax, stunting back legs, and abnormal abdomen, (F) malformed adult with undeveloped body parts, (G) abnormal adult showing deformities in all abdomen, and (H) malformed adult with a one wrinkled wing and abnormal abdomen.

Table 5: Toxicity (in ppm) of the extract of *Viola odorata* and *Eucalyptus camaldeulensis* on *Ceratitis capitata* adult flies.

Treatment	LC_{50}	Confidence limit		LC_{90}	Confidence limit*		Slope ± SE**	$\chi2$	P
		Lower	Upper		Lower	Upper			
V. odorata	5.5×10^4	6.5×10^3	4.7×10^5	2.6×10^8	3.1×10^7	2.2×10^9	0.38±0.48	1.96	0.582
E. camaldeulensis	1.1×10^8	1.8×10^5	6.5×10^{10}	1.1×10^{12}	1.8×10^9	6.2×10^{14}	0.18±1.41	7.58	0.057

*95% lower and upper fiducial limit, **SE: standard error.

Ultrastructural alterations of adult C. capitata exposed to V. odorata extract

Scanning electron micrographs were taken in different magnifications to view the ultrastructural changes following exposure of adult *C. capitata* to *V. odorata* extract (Figure 3). Selection of *V. odorata* for this study was preferred based on the fact of higher effect of this extract on adults compared to *E. camaldeulensis* extract. Comparison between control and treatment specimens, showed visual change in the shape of funicular sensilla, which appeared sharp in its shape in control treatment (Figure 3A), while the shape was characterized by curvature at the tip of the sensilla in *V. odorata* treated adults (Figure 3B).

Persistence of plant extracts in soil

The relationship between pupal percentages of mortality and plant extract residues in soil; *V. odorata* and *E. camaldeulensis* mixed with soil indicated the reduction of pupal mortality in a time dependent manner suggesting disintegration of the

plant extracts with time (data not shown) and the presence of plant extract in the sand had disappeared in comparison to control (acetone with *V. odorata* and ethanol as solvent with *E. camaldeulensis*).

According to the dissipation curve presented in Figure "4A and B" for both extracts, functional redundancy were very short (after 3 days) and then both plant extracts lost many of their components in the environment. Overall, the effectiveness of the treatment decreases with time and the extracts lost most of their activity within few days after spreading in the soil.

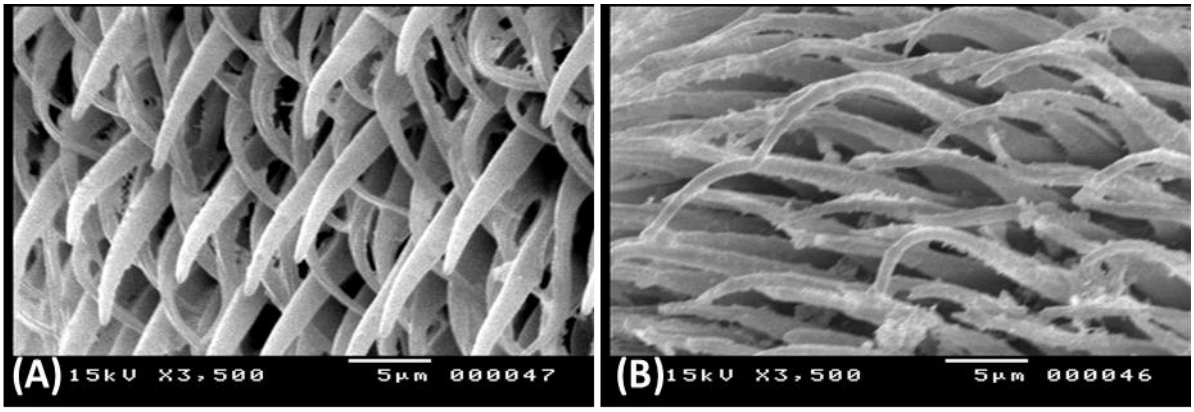

Figure 3: Scanning electron microscopy of antennae of control and *Viola odorata* treated *Ceratitis capitata* flies. (A) Control treatment showing normal funicular sensilla and (B) treated sample with the leaves extract of *V. odorata* showing curvy funicular sensilla.

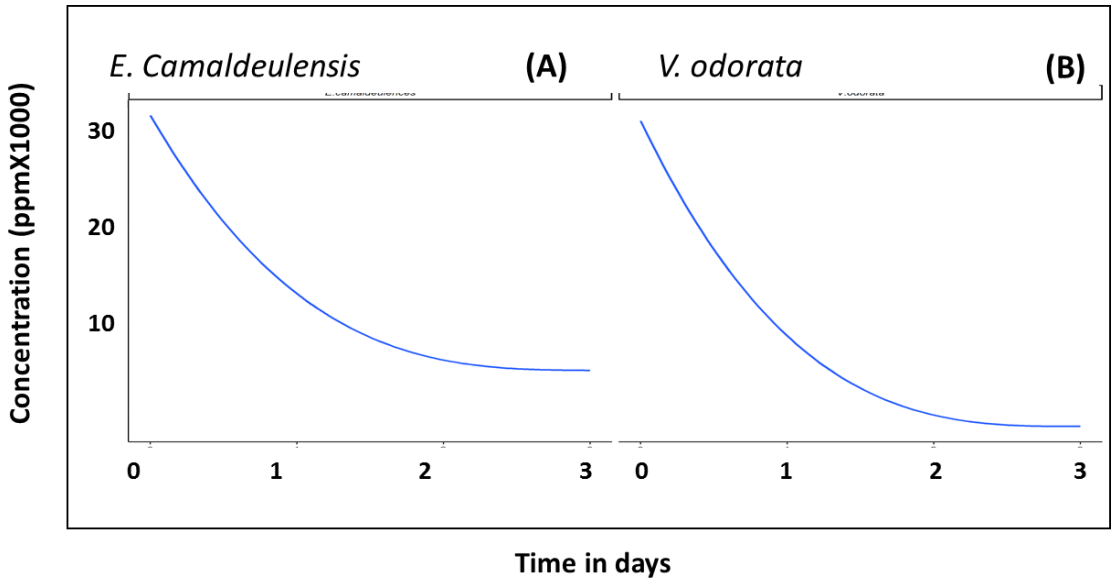

Figure 4: Dissipation curve in the soil of two plant extracts. (A) *Eucalyptus camaldeulensis* and (B) *Viola odorata*.

DISCUSSION

Keeping economic plants safe from the negative effects of harmful insects is a major goal for researchers and public authorities. This is limited by the impact of insect control method on human life and the ecosystem. This limitation pushed researchers to look for alternative methods for insect control such as nanotechnology-based pesticides[27], plant extract-based pesticides[28], and insect pathogens derived bio-pesticides[29]. Like most other plant

extracts and essential oils, both plant extracts showed a characteristic chemical composition by GC-MS. *V. odorata* contained 3 major groups of compounds having previous application in pest management including monoterpenes, tetra-terpenes, and sesquiterpenes. On the contrary, *E. camaldeulensis* showed different pattern of chemical content. The major component was furan (14.81%), which is a heterocyclic organic compound. The second components was pyrrogalol (10.6%), which is an organic compound belonging to phenol family. Spathulenol (7.03%), and phytol (6.08%) that is a cyclic diterpene alcohol, and acid gulebeulol (6.01%) were also identified. Minor components represented in D-allose (3.35%) and p-menth-1-en-4-ol (2.08%). Ascorbic acid, eucalyptol (1,8 Cineol), gallic acid, P-menth-1-en4-ol, p-cymene, 2H-pyran, D-limonene, and oleic acid represented slightly more than 1%. Keeping identical pattern for plant extracts chemical identification by GC-MS is a difficult process as it is highly affected by the extraction method. Several extraction methods are used to isolate essential oils and extracts from plant tissues. Based on this fact, the chemical profile and the stereo chemical pattern of each extract vary based on the method of extraction[13,30]. Generally, both plant extract contained several forms of terpenoids and insecticidal components, which explain their toxic effects on different stages of *C. capitata*.

The current study focused on the use of two plant extracts as potential control methods for different developmental stages of *C. capitata* using two application methods, the spray and contact methods. The pattern of mortality in larval stage of *C. capitata* using the plant extract of *V. odorata* and *E. camaldeulensis* showed a clear preference of contact treatment method rather than spray method. Generally, contact method provides better coverage of the pesticide rather than spraying[31,32]. Additionally, contact method provide wet condition for bioactive compounds present in the plant extract. The wet condition increases the activity of the compounds leading to higher insecticidal activity[33,34].

Our results recommended higher toxicity to *V. odorata* extract on pupae rather than larvae and on the other side it showed that *E. camaldeulensis* extract is more toxic to larvae than pupae. Generally, plant extracts and essential oils vary in their effects against different stages of insects. For instance, the essential oil of *Ricinus communis* has higher insecticidal effect against larvae of *Aedes aegypti* rather than pupae[33]. The higher toxicity of *E. camaldeulensis* over *V. odorata* on larvae may be linked to the chemical composition of this extract. GC-MS analysis of both plant extracts revealed a percentage of 14.8 % furan (a heterocyclic organic compound) and furfural (a furan aldehyde, 1.09%), which were restricted only to *E. camaldeulensis*. Furan derivatives are used as agrochemical bio-regulators against larval stages of many insects. *Culex quinquefasciatus* and other species of mosquito larvae treated with furan derivatives suffered increased mortality compared to control[34]. Furyl triazine was proved to have detrimental effects on larval survival when applied to adult females of house flies, *Musca domestica*, where it interferes with the normal development as females deposit eggs and the eggs hatch, but the larvae fail to pupate[35].

V. odorata was more toxic to pupae than larvae. Keeping into consideration that the extract of *V. odorata* has more percentage of terpenes than *E. camaldeulensis* may explain this phenomenon. To understand the mode of action, it should be pointed to two notices; the first is the plant products containing lipophilic hydrocarbon mono-terpenes causing biochemical dysfunction and mortality to insects[36-37]. Consequently, the present work suggested the extracts of *V. odorata* and *E. camaldeulensis* leaves changed the structure of the Mediterranean fruit fly puparium by lipophilicity property *via* terpenes that may be attributed to

disruption of lipids of the epicuticle and to penetration inside the puparium causing death to pupae. Terpenes are considered as good penetration enhancing compounds towards the cuticle of insects. The second notice following penetration, other insecticidal compounds disturb the physiological processes leading to the death of the insect. Different deformations of pupae (one-day and eight-day old) were recorded during this study. These abnormalities might resulted from obstruction of physiological functions by some of the chemical compounds present in *V. odorata* and *E. camaldeulensis* leaves extracts. Of the most suspected compounds causing such physiological alteration are the secondary metabolites. The secondary metabolites refer to plant compounds that are not essential for a cell to survive, but play a role in the interaction of the cell with its surroundings. They may act as safe guard of crops from biotic or abiotic stresses and may be considered as natural defense factors of plants against herbivores[37,38].

Our results reported that *V. odorata* is more toxic than *E. camaldeulensis* against adult *C. capitata* as it has smaller LC_{50} and LC_{90} values. This success probably is caused by the presence of specific chemical constituents in a plant extract than another, specifically monoterpenoids. As mentioned for pupae, terpenes present in higher percentages in *V. odorata* allows the penetration of the cuticle of adults. Following penetration, monoterpenoids may act on various targets in insects and mammals, especially on the nervous system, including ɤ-aminobuteric acid (GABA)-gated chloride channels, octopamine receptors of octopamine and triamine, acetylcholine esterase, nicotinic acetylcholine receptors (nAChR), sodium channels, and possibly other targets[39,40]. Majority of monoterpenoids such as linalool showed high inhibition to acetylcholine esterase[40].

Studying the ultrastructural changes of the antennae in adult *C. capitata* following treatment with *V. odorata* extract visualized abnormal appearance of curvy funicular sensilla. The leaf extract of *Cannabis sativa* caused a drastic manipulation in the morphological appearance of sensilla trichoidea of *Chironomous samoensis*[41]. Generally, flies of *C. capitata* respond to odors depends mainly on the receptors present on antennae, which allow the detection of active constituents of natural odors efficiently and with high sensitivity[42]. Changes in the shape and appearance of antennal sensilla in *V. odorata* treated *C. capitata* may cause a delay in olfaction process leading to failure to avoid toxic actions of this extract on different stages of *C. capitata*. Whatever mode of action of these two plant extracts, its compounds may work together by synergism to delay the physiological transformation and causing death of larvae, pupae and adults either by spray or contact treatment methods.

The dissipation curve of both plant extracts suggested both extracts lost most of their activities within few days after application. Pesticide dissipation rate after application is a useful tool for the assessment of its residual levels. Residue dissipation curves can be used to estimate the time required to reach residues levels below maximum residual levels (MRLs)[43-45]. It is the first time that a dissipation curve had been created for these extracts. Temperature and solar radiations, rain, wind, and volatilization are major factors affecting biodegradation. Isman[46] has argued that the extensive use of natural pesticides requires research directed at the practical application of such products under complex agro-ecological conditions, particularly understanding how different plant-derived pesticides perform when applied to different crops under different growing conditions. Van der Linden *et al.*[47] proposed a procedure for the assessment of persistence in the soil. The system considered three protection goals: (1) protection of soil functions relevant to agricultural production, (2) protection of the structure of agro-ecosystems, and (3) protection of the structure of soil ecosystems in general. The two plant extracts used in

this study appeared to degrade fast in the soil, which means that it can be used as one of the safe alternatives in new control trends.

Generally, many plant species have toxic effects on medfly, but very few are studied. In the present study, the acetone-based extract of *V. odorata* has been used, to the first time to the best of our knowledge, as a natural product in the control of the immature stages and adults of *C. capitata*. *V. odorata* L. was used to evaluate toxicity against common pistachio psylla adult, *Agonoscena pistacia*[48], and repellency against some species of mosquitoes[49]. Based on the toxicity of *V. odorata* leaves extract and the role of their components against medfly immature stages and flies, it seems that more studies of this plant extract or of the proportions of its terpene groups might be of vital importance in the future to control the Mediterranean fruit fly. There is a need to produce the terpenes-containing compounds as a daily spray in soil especially at high peaks of the pest during September to December with sweet violet dough extract. It should be used as a bio-pesticide against immature stages and flies of *C. capitata*, as it is locally abundant, inexpensive to prepare, biodegradable over time that has no effect of pesticide residues and keep the environmental balance safe. On the other side, *E. camaldeulensis* is recommended against pupal stages of *C. capitata*, as represented by the low lethal concentration values.

Generally, contact treatment method was superior in its toxicity against *C. capitata* than spray treatment method. Further, *V. odorata* was potent against larvae and adult flies, while *E. camaldeulensis* extract was superior against pupae. *V. odorata* extract is highly recommended against adult stages of *C. capitata*. Both extracts caused morphological malformations in pupae and adult *C. capitata*. In conclusion, this study addressed the toxic effects and alterations in *C. capitata* different stages following treatment with *V. odorata* and *E. camaldeulensis* plant extracts. The application method, the age of the stage, and the selected extract affected the potency against the fly.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

NAS, SMA, and AEM conceived the idea and designed the experiments. SMA collected data. SMA and AMAI achieved the statistical analysis. AMAM and AMAI wrote and revised the manuscript. All authors approved the paper.

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حساسية الأطوار المختلفة لذبابة فاكهة البحر الأبيض المتوسط (DIPTERA: TEPHRITIDAE) *CERATITIS CAPITATA* WIEDEMANN لمستخلصي نباتات البنفسج *VIOLA ODORATA* والكافور *EUCALYPTUS CAMALDEULENSIS*

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تُعتبر ذبابة فاكهة البحر الأبيض المتوسط "*Ceratitis capitata*" من الآفات الخطيرة المنتشرة في إفريقيا وفي جميع أنحاء العالم. وتصيب ذبابة فاكهة البحر الأبيض المتوسط العديد من أنواع النباتات وتسبب خسائر اقتصادية في العديد من المحاصيل. وقد استحثت مشاكل استخدام المبيدات الحشرية الكيميائية غير المرغوب فيها ضد ذبابة الفاكهة العلماء على البحث عن مبيدات حشرية أكثر أمانًا. لذلك تم في هذه الدراسة اختبار مستخلصين نباتيين "زهرة البنفسج *Viola odorata* Linn. ونبات الكافور *Eucalyptus camaldeulensis* Dehnh." ضد المراحل البالغة وغير البالغة من ذبابة الفاكهة. كما تم تحليل كل مستخلص باستخدام تقنية "GC-MS". وتم تحديد التربينويدات على أنها المكونات الرئيسية في مستخلص زهرة البنفسج، بينما احتوى مستخلص نبات الكافور على مركبات عضوية حلقية غير متجانسة مع التربينويدات. وتم تحديد سُمية كلا المستخلصين على ذبابة الفاكهة باستخدام طرق التلامس والرش. وأظهرت طريقة المعاملة بالتلامس سُمية أعلى من طريقة الرش. وكانت الشرانق أكثر تأثرًا بمستخلص زهرة البنفسج من اليرقات الكاملة، بينما أظهر مستخلص نبات الكافور سُمية أعلى على اليرقات. وقد أثر كلا المستخلصين على الذباب البالغ، وكانت السُمية أعلى في الذباب المعامل بمستخلص زهرة البنفسج. وتم تسجيل تشوهات العذارى في ذبابة الفاكهة المعاملة بمستخلصات النباتات بما في ذلك ظهور الرأس والصدر فقط من الذباب وإبقاء باقي الجسم داخل الشرانق، ووجود شرانق ذات ألوان باهتة، وعدم خروج الحشرات البالغة من الشرانق على الإطلاق. علاوة على ذلك، تسبب مستخلص نبات البنفسج في حدوث تشوهات في قرون الاستشعار والأجنحة عند البالغين. وقد تحلل في التربة كلا المستخلصين خلال فترة زمنية قصيرة. والخلاصة أنه قد تعمل مستخلصات زهرة البنفسج ونبات الكافور كمبيدات حشرية قوية ضد ذبابة الفاكهة.