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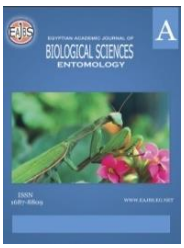

Larvicidal and Biochemical Effects of Some Essential Oils and Bee Products Against *Culex pipiens* Larvae *(Diptera: Culicidae)*

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ABSTRACT

The use of plant-based insecticides is safer for both humans and the environment. Any insecticide has a negative impact on the growth and metabolic rate of the insect. The toxicity of two bee products (bee venom and propolis) and the commercial oils of garlic, marjoram and anise were tested against the late 3rd larval instar of *Culex pipiens*. After 24 h of treatment, the three oils showed larvicidal activity with LC_{50} of 1323.81, 840.71 and 577.53 ppm for garlic, marjoram and anise, respectively. The metabolic rate change within the treated larvae was estimated through the determination of total proteins, carbohydrates and lipids. Garlic oil significantly reduced protein content from 33.8± 0.98 to 30.4 ± 0.4 mg/g. Marjoram and garlic oils significantly reduced carbohydrate content from 15.76 ± 0.7 to 11.43 ± 0.66 and 11.2 ± 0.7 mg/g, respectively. Anise oil significantly increased lipid content from 3.11 ± 0.21 to 3.57 ± 0.14 mg/g.

INTRODUCTION

Mosquitoes are annoying insects that cause serious problems for humans. they are carriers of many diseases like lymphatic filariasis, chikungunya, dengue, malaria, Japanese encephalitis and dirofilariasis, (Hubálek, 2008; Lebl *et al*., 2015; Govindarajan *et al*., 2016). *Culex* spp. spread widely in Egypt and they are vectors of many pathogens such as *Wuchereria bancrofti* and West Nile and Rift Valley fever viruses (Hanafi *et al*., 2011; Abdel-Hamid *et al*., 2011; Fortified *et al*., 2019). *Cx. pipiens* strains pose a threat to human life as it is a vector for many arboviruses like; West Nile Virus, St. Louis encephalitis, rift valley fever and sindbis viruses (Turell, 2012). Female *Culex pipiens* is the main vector of lymphatic filariasis in Egypt (Salamah *et al*., 2016).

Chemical insecticides are the main method for controlling mosquitoes for centuries (Hemingway *et al*., 2006), but such chemicals adversely affected the environment and non-target organisms including humans (Aktar *et al*., 2009). Moreover, the prolonged use of a group of insecticides has led to the development of resistance in insect species (Ghorbani *et al*., 2018; Sh *et al*., 2020). In Egypt, mosquito species showed different levels

of resistance against chemical insecticides (Tageldin, R.A. *et al*, 2018; El-Hosainy *et al*., 2018; Abdelbaset B. Zayed *et al*, 2019; Meshrif *et al*., 2021). There are several alternatives to chemicals including botanicals and plant-based insecticides which are eco-friendly and have toxic, growth-reducing and repellent effects against insects (Regnault-roger *et al*., 2012; Zahran *et al*., 2017; Baz *et al*., 2021; Baz *et al*., 2022a, b; Radwan *et al*., 2022).

Several studies illustrated the toxicity levels of different commercial plant oils against *Cx. pipiens* larvae (Khater & Shalaby, 2008; Desouky *et al*., 2019; Nagwa M. Elhawary *et al*., 2020; Baz *et al*., 2022 a, b).

Bee products including bee venom; which is composed of active molecules and propolis; which is a resin composed of more than 300 components have shown a wide range of biological activities (Robertson, 1990; Wehbe *et al*., 2019; Przybyłek & Karpiński, 2019; Anjum *et al*., 2019). For insecticidal activities; Propolis showed toxicity against larval stages of the lesser wax moth (*Achroia grisella*) (Ararso & Legesse, 2016) and the Greater Wax Moth (*Galleria mellonella*) (Fawzy *et al*., 2017). Bee venom showed toxicity against eggs and larvae of *Achroia grisella* (Mahgoub *et al*., 2018) and larvae of the black soldier fly (*Hermetia illucens*) (Nassar *et al*., 2020).

The aim of the present work included studying the efficacy of three essential oils; anise, garlic and marjoram, as well as bee venom and propolis against late 3rd larval instars of *Culex pipiens* field strain. The effect of the applied materials on the metabolic rate of the larvae also has been studied through the estimation of total protein, carbohydrate and lipid profiles within the larval body.

MATERIALS AND METHODS

Mosquito Larvae:

Cx. pipiens larvae were collected from Sinnuris district, Fayoum governorate, Egypt (at latitude 29˚ 24′ 32″ N and longitude 30˚ 51′ 45.5″ E). The larvae were collected from a highly infested long canal located next to houses and farmland and contained shallow polluted water with grasses grown on its margins (Fig. 1). Larvae were collected by dipping method (World Health Organization, 1975) and transported to the laboratory in buckets. At the laboratory, late third larval instars were identified under a microscope according to Harbach (1985) and counted in groups; each group contained 25 larvae.

Fig. 1: Breeding site of the collected larvae.

Tested Materials:

A) Essential Oils:

Three essential oils were licensed for human uses by the Egyptian Ministry of

Health, including; anise (*Pimpinella anisum, Apiales: Apiaceae*), garlic (*Allium sativum, Asparagales: Amaryllidaceae*) and marjoram (*Origanum majorana, Lamiales: Lamiaceae*) were purchased from EL-CAPTAIN Company for cosmetics, extracting natural oils and plants (El-Obor city, Cairo, Egypt).

B- Bee Products (bee venom and propolis):

Both (bee venom and propolise) were purchased from Imtenan Company (El-Nozha, Cairo, Egypt) as powder.

Bioassay Experiments:

A series of concentrations (200, 400, 600, 1500, and 2000 ppm) were prepared by using water with bee venom and ethanol 95% with essential oils and propolis.

Test procedures were carried out according to the world health organization (WHO, 1981). Twenty-five mosquito larvae were put in a cup containing 24 ml of water and left for 10 minutes before adding to a glass beaker containing 225 ml of water and 1 ml of the tested concentration. In the control group, 1 ml of the appropriate solvent (ethanol or water) was added. For each concentration and also the control group, 100 larvae were used as the experiments were replicated four times. After 24 h of post-treatment, mortalities were recorded. The experiment was conducted at room temperature (25 ˚C ± 2), normal relative humidity (62% ± 2) and natural photoperiod.

Biochemical Studies:

Larvae were treated with the LC_{50} of the toxic materials while control group was treated with the solvent. After 24 h, life larvae were filtered (Fig. 2), and frozen at -20 ˚C for further analysis.

Larvae were homogenized in distilled water (50 mg/ml) by using a chilled glass Teflon tissue homogenizer (ST – 2 Mechanic-Preczyina, Poland). The homogenate was centrifuged at 8000 r.p.m. for 15 minutes at 2 ºC in a refrigerated centrifuge. The deposit was discarded and the supernatant was used to determine the total proteins, carbohydrates and lipids. All experiments were conducted in triplicate (Amin, 1998).

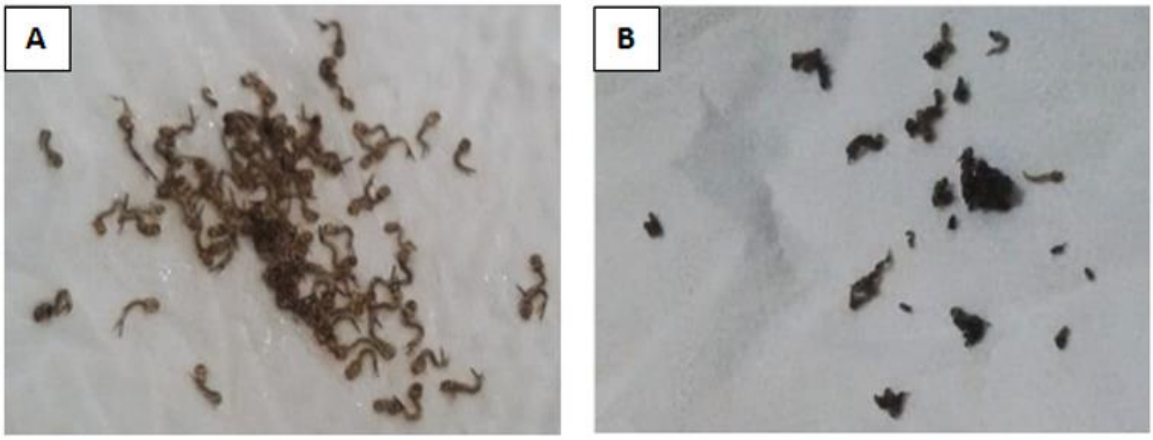

Fig. 2: A) larvae during filteration, B) after complete filteration.

1. Determination of the Total Proteins:

The total Proteins were determined as follows; Protein reagent was prepared from Coomassie Brilliant blue G-250, ethanol (95%) and phosphoric acid (85%). After that, 50 µl containing 10 to 100 µg of bovine serum albumin (serial concentrations) were treated with phosphate buffer solution and protein reagent. The absorbance rate was measured at 595 nm and the standard curve was plotted between protein weight (bovin serum albumin) and its corresponding absorbance. This curve was used in the determination of protein in the unknown sample by the same method of the standard determination, (Bradford, 1976).

2. Determination of the Total Carbohydrates:

Carbohydrates were extracted and prepared for assay (Crompton & Birt, 1967) and estimated in the acid extract by the reaction of the phenol-sulphuric acid (Dubois *et al*., 1956). The absorbance of the characteristic yellow-orange color was measured at 490 nm against blank. The total carbohydrates were expressed as µg glucose/gm fresh weight.

3. Determination of the Total Lipids:

Lipids were estimated as follows; 250 ul of the sample were added to 5 ml of concentrated sulphuric acid in a test tube and heated in a boiling water bath for 10 minutes. After cooling to room temperature, the digest was added to phospho-vanillin reagent. After 45 minutes, the developed color was measured at 525 nm. The optical density was compared to that of a reference standard and results were expressed as mg lipids/ml haemolymph, (Knight *et al*., 1972).

Data Analysis:

Data were analyzed by using IBM SPSS statistics program version 26 for Windows. The total mortalities were subjected to Probit analysis for determining the lethal concentrations. The mortality of each group and also the biochemical results were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey's test (Post-Hoc multiple comparisons). Data were significant if $P < 0.05$.

RESULTS AND DISCUSSION

1. The Two Bee Products:

Bee venom and propolis didn't show any significant effect on larval mortality percentage up to 2000 ppm.

2. Essential Oils:

The larvicidal activity of the essential oils against *Cx. pipiens* larvae (Table 1) indicated that larval mortalities were increased by increasing concentration values. Mortalities weren't corrected by Abbotts's formula because control mortalities were less than 5% (World Health Organization, 2018).

Table (1): Larvicidal activity of essential oils and bee products against late 3rd larval stages of *Cx. pipiens* after 24 h of post-treatments.

	Mean mortality% ± SD				
Concentration (ppm)	***Pimpinella anisum***	***Origanum majorana***	***Allium sativum***	**Bee venom**	**Propolis**
0	3.00 ± 2^{f}	4.00 ± 0^{d}	3 ± 2^{e}	4.00 ± 0^{a}	4.00 ± 0^{a}
200	33.00 ± 2^{e}	32.75 ± 4.27^{c}	9.25 ± 1.50^{e}	7.0 ± 3.83^{a}	5.51 ± 1.91^{a}
600	41.75 ± 2.06^{d}	42.25 ± 3.30^{b}	27.50 ± 2.65^{d}	8.50 ± 3.27^{a}	10.00 ± 5.16^{a}
1000	58.00 ± 5.89^{c}	$43.75\pm2.2.36^{b}$	34.00 ± 1.83^{c}	10.0 ± 4.00^{a}	8.50 ± 5.66^{a}
1500	68.75 ± 6.60^{b}	47.00 ± 1.82^{b}	40.75 ± 5.12^{b}	10.0 ± 5.16^{a}	7.48 ± 3.41^{a}
2000	78.25 ± 2.22^{a}	54.25 ± 2.22^{a}	55.50 ± 1.92^{a}	8.00 ± 3.26^{a}	9.0 ± 5.03^{a}

Values followed by the same letter(s) are not significantly different (Tukey's HSD, $P > 0.05$).

The calculated lethal concentrations for anise, marjoram and garlic oils were displayed in Table 2. Anise oil (*Pimpinella anisum*) was the most effective oil with LC_{50} of 577.53 ppm, followed by marjoram oil (*Origanum majorana*) and then garlic oil (*Allium sativum*) with LC_{50} of 840.71 and 1323.81 ppm, respectively.

Table 2: Lethal concentrations of the essential oils against the late 3rd *Cx. pipiens* larval instars 24 h post-treatments.

	Allium sativum	***Origanum majorana***	***Pimpinella anisum***
LC_{50} (LCL-UCL)	1323.81 (876.36-1954.82)	840.71 (90.01- 2000.04)	577.53 (149.25- 1030.61)
LC_{90} (LCL-UCL)	5303.29 (3151.51- 18433.67)	7948.44 (2813.71- 696381.26)	3827.76 (1829.27- 86590.64)
LC_{95} (LCL-UCL)	7859.74 (4209.02- 37478.31)	15026.61 (4162.48- 4262513.17)	6543.24 (2628.25- 430679.64)
LC_{99} (LCL-UCL)	16440.3 (7128.50-144130.69)	49622.33 (8355.492- 9560603.11)	17888.59 (4987.80- 9078410.43)
X^2 (Sig)	28.65 (0.000)	54.07 (0.000)	25.19 (0.000)
df	5	5	4
Equation	y= -6.69+ 2.17*x	y= -4.51+ 1.56*x	y= -5.44+ 1.98*x
R^2	0.872	0.663	0.716

LC: lethal concentration, LCL: lower Confidence Limit, UCL: Upper Confidence Limit, X^2: Chi-square (significant at $P < 0.05$), df: degree of freedom.

3. Determination of Total Protein, Carbohydrate and Lipid:

Data in Table (3) showed that protein content in larvae treated with anise oil increased from 33.8 to 35.3 mg/g while decreased to 31.8 and 30.4 mg/g with marjoram and garlic oils. The reduction of protein content in the case of garlic oil was statistically significant.

Anise oil treatment increased carbohydrate content from 15.76 to 15.93 mg/g while there was a significant reduction to 11.43 and 11.2 mg/g with marjoram and garlic treatments.

Anise and garlic treatments increased lipid content from 3.11 mg/g in control larvae to 3.57 and 3.36 mg/g in treated larvae while marjoram treatment reduced lipid content of larvae to 2.98 mg/g. The increase in the case of anise treatment was statistically significant.

Table 3: Effect of essential oils on total protein, carbohydrate and lipid contents.

	Protein		**Carbohydrate**		**Lipid**	
	Mean± SD	**Mean difference**	**Mean± SD**	**Mean difference**	**Mean± SD**	**Mean difference**
Control	33.8±0.98	__	15.76±0.7	__	3.11±0.21	__
Anise oil	35.3±1.33	-1.53-	15.93±0.92	-.17-	3.57±0.14	-.46-*
Marjoram oil	31.8±1	1.97	11.43±0.66	4.33*	2.98±0.07	0.13
Garlic oil	30.4±0.4	3.40*	11.2±0.7	4.57*	3.36±0.15	-.25-

Note: All values in units of mg/g. *. Significant value at the 0.05 level.

DISCUSSION

Essential plant oils are an affordable solution for controlling mosquitoes**.** In this work marjoram, garlic and anise oils showed toxicity against *Cx. pipiens* larvae, The LC_{50} values for anise, marjoram and garlic oils were 577.53, 840.71 and 1323.81 ppm, respectively.

Propolis and bee venom didn't show toxicity against the larvae up to 2000 ppm

although these products were toxic against other insects (as previously mentioned), this may be because the larvae used in the experiment were reared in the field and had a higher natural tolerance (Sehgal *et al*., 2014).

For essential oils, the mortality increased by increasing concentration during the experiment, similar findings were recorded by (Iqbal *et al*., 2018; Desouky *et al*., 2019)
The present study indicated that anise oil was the most effective one against the larvae followed by marjoram oil. It was reported from previous studies that anise oil contained anethol (as a major component) and this component has a high toxicity against *Cx. pipiens* larvae (Konstantopoulou, 2012; Oz *et al*., 2018), *Cx. quinquefasiatus* larvae (Benelli *et al*., 2017; Benelli *et al*., 2018; Sergio Andrade-Ochoa *et al*, 2018), the gypsy moth (Kosti´c *et al*., 2021) and mites (El-sayed *et al*., 2022).

O. majorana essential oil was reported to contain monoterpene and sesquiterpenes (Fouad et al., 2014) and Garlic essential oil was reported to contain ally disulfide and diallyl trisulfide as major components (Muturi *et al*., 2018). These components were responsible for the toxicity against the larvae (Kimbaris *et al*., 2009).

The previous analysis of garlic and marjoram oils (purchased from El-Captain company) indicated that the major constituents of garlic were 2,3,3-trimethyl Hexane, Tetradecane and 4 (Prop-2-enoyloxy) pentadecane while marjoram contained 4 (Prop-2-enoyloxy) pentadecane, Sabinene and ç-Terpinene (Meguid *et al*., 2022). Another analysis of garlic oil (from the same company) revealed that the major component was 9-Octadecenamide, (Z)- followed by Trisulfide, di-2-propenyl and isochiapin B (Baz *et al*., 2022b). The insecticidal activity of the plant extracts may be due to the presence of many components like; phenolics, alkaloids and terpenoids (Pavela & Ocimum, 2008; Desouky *et al.,* 2019). Each essential oil is composed of volatile constituents and its insecticidal activity results from a synergistic effect between its all components (Dhifi *et al.,* 2016). The activity of the whole plant oil is more than that of its major components (Burt, 2004; Hategekimana & Erler, 2020). The minor constituents of the oil have an important role in its biological activity as they can strengthen its effect (El-Shemy, 2018).
Plant extracts affected negatively on larval development and metabolic rate (Sharma *et al.,* 2011). In this study, larvae treated with anise oil showed an increase in protein content from 33.8 mg/g to 35.5 mg/g in treated larvae; similar findings recorded an increase in protein content after the treatment of Cx. pipiens larvae by Borago officinalis ethanolic extraction as a result of the increasing detoxification enzymes activity (Draouet *et al.,* 2020).

Treatment by garlic and marjoram oils reduced protein content in this experiment and the reduction caused by garlic oil was significant. This may be because of the larvicidal interference between plant extracts and hormones that regulates protein synthesis (Vijayaraghavan *et al.,* 2010; Sharma *et al.,* 2011). Similar to our findings, treatment with Ocimum basilicum oil decreased protein content; which may be because of the degradation of proteins or the inhibition of its synthesis (Dris *et al.,* 2017), treatment by Origanum glandulosum oil (Bouguerra & Boukoucha, 2021) and also treatment by methoxyfenozide (Hamaidia *et al.,* 2018).

Anise oil increased carbohydrate content to 15.76 mg/g in this experiment and this increase was not significant. It was reported a non-significant increase in carbohydrate content after treatment of Cx. pipiens adults by Tecoma stans ethanolic extracts (Hafsi *et al.,* 2022).
Garlic and marjoram oils significantly decreased carbohydrate contents to 11.2 and 11.43 mg/g, respectively. similar to our results, it was reported a significant decrease in carbohydrate content after larval treatment with Ocimum basilicum oil (Dris *et al.,* 2017) and also a reduction in carbohydrate and lipid contents after larval treatment by ethanolic

extraction of Borago officinalis as a result of the treatment stress (Draouet *et al.*, 2020). Treatment with garlic and anise oils increased lipid content to 3.36 and 3.57 mg/g, respectively. The increase in the case of garlic treatment was significant. Our findings agreed with similar studies indicating that lipid content increased after larval treatment with Azadirachta indica extract (Sharma *et al.*, 2011) and adult treatment with Tecoma stans ethanolic extracts (Hafsi *et al.*, 2022).

Marjoram treatment decreased lipid content to 2.98 mg/g in this experiment, similar to our findings it was recorded that lipid content declined after larval treatment by the extract of *Artemisia annua*; this may be as a result of a shift in metabolism towards lipid catabolism because of the treatment stress (Sharma *et al.*, 2011), after the treatment of Cx. pipiens and Cs. longiareolata larvae by methoxyfenozide (Hamaidia *et al.*, 2018) and also (Draouet *et al.*, 2020) (as mentioned above). It was explained that the physiological stress resulting from the larval treatment was responsible for the decrease in lipid content (Senthilkumar *et al.*, 2009).

In conclusion, Propolis and bee venom didn't show any significant effect on larval mortality while garlic, marjoram and anise oils showed larvicidal activity with LC_{50} values of 1323.81, 840.71 and 577.53 ppm, respectively. Out of the three essential oils, anise oil was the most effective one. Garlic oils significantly reduced protein content from 33.8 to 30.4 mg/g. Marjoram and garlic oils significantly reduced carbohydrate content from 15.76 to 11.43 and 11.2 mg/g respectively. Anise oil significantly increased lipid content from 3.11 to 3.57 mg/g. The study demonstrated the important role of these oils in keeping water free of *Cx. pipiens* larvae and getting rid of an important vector-borne disease like mosquitoes.

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