



Study on Larvicidal Effects of Essential Oils of Three Iranian Native Plants against Larvae *Anopheles stephensi* (Liston)

Hanieh Torabi Pour*, Mansoureh Shayeghi, Hassan Vatandoost and Mohammad Reza Abai

Abstract

Objective: To determine the larvicidal activity of three medicinal plants essential oils against larvae *Anopheles stephensi*

Methods: The larvicidal effects of essential oils of seven medicinal plants collected from different parts of Iran including *Carum carvi* (seeds), *Artemisia dracuncululus* (branches and leaves) and *Rosmarinus officinalis* (branches and leaves) were determined. The essential oils were hydro-distilled for 3-5 h using a Clevenger type-apparatus in Ecotoxicology Laboratory, School of Public Health (SPH), Tehran University of Medical Sciences (TUMS). The anti-larvae tests of EOs were carried out according to WHO's guidelines at Bioassay Laboratory at adjacent of Culicidae Insectary of SPH, TUMS.

Results: The 50% and 90% of lethal concentrations of studied plant EOs were 21.59 and 72.44 ppm for *C. carvi*, 1.33 and 4.12 ppm for *A. dracuncululus*, 93.22 and 229.29 ppm for *R. officinalis*, respectively. The chemical constituents of EOs were settled by GC-MS analysis and the main constituents with larvicidal effect were determined.

Conclusion: The results revealed the high larvicidal potential of EOs of *A. dracuncululus* and *C. carvi* and possible cultivation of latter plants, the identification of chemical compounds, trade formulation, semi-field and field evaluations of EOs are strongly recommended at malarious area, southern Iran.

Keywords

Anopheles stephensi; Medicinal plants; Essential oils; Larvicidal effects; malaria; Iran

Introduction

By considering the fact that using chemical larvicidal material in order to control the immature flies' population has destructive consequences on the environment and the process of using these material makes some anopheles' species resistant to these pesticides so the researches are in the direction of extracting larvicidals from the nature specially plants.

Because of this fact, a wide range of researches have been carried

out concerning the effects of larvicidals on anopheles' larvae. In recent years various studies on the poisonous effects of Iranian EOs including *Alliaceae*, *Apiaceae*, *Asteraceae*, *Cupressaceae*, *Graminaceae*, *Lamiaceae*, *Lauraceae*, *Myrtaceae*, *Pedaliaceae*, *Rutaceae*, *Scrophulariaceae*, *Verbenaceae*, *Zingiberaceae* against various types of insects have been reported [1].

The larvicidal effects of three species of Iranian medicinal plants including *Carum carvi*, *Artemisia dracuncululus*, and *Rosmarinus officinalis* against the larva of the main Malaria vector in Iran *Anopheles stephensi* have been studied in this research. *C. carvi* is a plant of *Apiaceae* family. This plant is native to central mountains of Iran especially near Kerman.

a) The most important reported effects of *C. carvi* are: Anti-Worm, Anti-anemia, anti-microbial, anti-histamine, anti-cancer, anti-inflammatory, antispasmodic, carminative, anti-fungal, chloretic, diuretic, digestive, expectorant, larvicide, muscles relaxant, stimulant and stomach tonic [2]. *R. officinalis* is of *Lamiaceae* plants. It is native to Mediterranean Europe and Near East which is being planted in Iran too. Its extract is insect repellent [3].

b) The most important reported effects of *R. officinalis* are: Analgesic, anti-Alzheimer's, anti-arrhythmic, anti-bacterial, anti-atherosclerotic, anti-cancer, anti-vascular fragility, anticonvulsants, anti-edema, anti-swelling, anti-mutagen, anti-oxidants, anti-toxin, anti-prostaglandin, anti-fever, antiseptic, anti-spasmodic, anti-virus, anti-distention, anti-fungus, insecticides, sudorific, stimulant, digestive, diuretic, Hepato protectors and stomach tonic. *R. officinalis* also has a pesticide effect [4]. *A. dracuncululus* of *Asteraceae* family which does not grow naturally in Iran and it can be found in planted and cultivated form [5]. This plant is being cultivated in many areas of Iran, some say that its origin is the North and the West of America.

c) The most important reported effects of *A. dracuncululus* are: Allergen, analgesic, antibacterial, anti-fragility of capillary vessels, anti-inflammatory, anti-fever, anti-spasmodic, anti-distention, diuretic, fungicide, hypnotics, stomach tonic, anti-worm, anti-cancer and stomachic [6]. This research aims to study the larvicidal effects of the EOs of three plant species (*Carum carvi*, *Artemisia dracuncululus*, *Rosmarinus officinalis*) by exposing *Anopheles stephensi* to the prepared logarithmic concentrations of these EOs in laboratory condition and in case of acceptable results these effective, cheap and easy to available larvicides could be used to control Malaria disease.

Methods

Collecting and preparing the plants

This part includes extracting the EOs of the plants in the study including: *Artemisia dracuncululus* which was planted in Iran, *Rosmarinus officinalis* (was collected from Tehran in fresh form then was dried) and *Carum carvi* (which was collected from Jiroft in Kerman province) and also it included identifying the physical and chemical features of these plants and their components and studying the larvicidal effects of them on *Anopheles stephensi*. The said plants were collected in 2013 spring then were dried in shadow based on their main organs and when they were fully dried the final identification of the said species was carried out.

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Rearing of mosquito larvae

Rearing and maintaining mosquito larvae was carried out in the temperature of $29 \pm 2^\circ\text{C}$ and relative humidity of $70 \pm 10\%$ and Light-dark cycle of 16h light and 8h was performed in Culicidae insectarium of the School of Public Health Tehran University of Medical Sciences. The larvae of the late 3rd stage or early 4th stage of *Anopheles stephensi* were used for larvicidal tests.

Extracting the essential oil by distillation with water method

In order to extract the EO of any target plant in this study, first it was taken to Pesticides Chemistry Lab and was identified then it was relented. An amount about 30 gr of the plant was weighted and put in a 1l Laboratory flask then about 600 cc distilled water was added to the flask and after that the Clevenger was mounted on the flask and the heater of the Clevenger was turned on and after 4 hours it was turned off. The collected EO in the calibrated part of the Clevenger was put in small glassy container special for EO collecting then it was dehydrated with Sodium Sulfate and was put in the refrigerator up to the test time.

Biological tests (larvicidal)

The standard WHO method for biological tests was used. The overall temperature of the lab (28°C), test period (24h) and the number of larvae (25 in each 400 cc beaker) has to be constant. The best age range of the larvae for the tests are the larvae of the late 3rd stage or early 4th stage range and preferably dechlorinated water should be used in the tests. At least 5 logarithmic concentrations should be made of the EO. In order to find the suitable concentration first the concentrations should be chosen in a larger domain and based on the results the concentration domain becomes narrower. Usually the concentration in which has the 50% relative mortality and two concentrations lower than it and two concentrations upper than it are used to draw that regression line diagram. In each test 5 concentrations of pesticide and for each concentration 4 repetitions and in general 2 witnesses are considered. In all tests, for all the present material, we had witness but we did not have it for the larvicidal pesticide [7].

Method of EO logarithmic concentration making

The extracted EO of the plants: *C. carvi*, *A. dracunculus*, *R. officinalis* were used to carry out biological tests. For each concentration of the said Eos in the test at least 4 repetitions were considered. In order to evaluate the Larvicidal Effects of each EO the mortality indicator after 24h was used. The tests by five logarithmic concentrations including 80,40,20,10,5 ppm for *C. carvi*, 10,5,2.5,1.25,0.625 ppm for *A. dracunculus* and 20,40,80,160,320 ppm for *R. officinalis* were carried out and for each concentration 4 repetitions were carried out simultaneously. For each series of the tests two series of witnesses (including 1 ml Ethanol in 249 cc dechlorinated water) were also considered. For *C. carvi*, because of the fact that the mortal concentration of 100% of larvae has been 80 ppm, we start the logarithmic concentrations from 5 ppm and with a sequence, make it twice and for *A. dracunculus* the mortal concentration of 100% of larvae has been 10 ppm, we start the logarithmic concentration from 0.625 and make it twice in a sequence and also for *R. officinalis* the mortal concentration of 100% of larvae has been 320 ppm and we start the logarithmic concentration at 20 ppm and in a sequence we make it twice.

Methods of making concentrations are will be discussed below

Larvicidal sensitivity test : The method of the test like the larva test method was WHO method and the tests were carried out in a 400 ml glassy beaker. First the various logarithmic concentrations were produced from the target EO in the before said order then 22 400 cc beakers and 22 50 cc beakers have to be prepared (for each 4 concentration should be done, two repetitions for witness are considered too). Using a graduated cylinder 224 cc dechlorinated water to each 400 cc beaker and 25 cc dechlorinated water to each 50 cc beaker was added. Then 25 larvae of late 3rd stage or early 4th stage which certainly had been fed up with food (fish food) were used.

Then we transferred the larvae to each 50cc beakers and added 1 cc of the EO with the desired logarithmic concentrations to each 400 cc beakers of the same concentration and were mixed what was in the beakers. 1 cc Ethanol was added to the witness beakers instead of the EO. Then we transferred the contents of the 50cc beakers to 400cc beakers and cover the beakers were covered with plate and after 24h the test results were read.

Chemical analysis : The EO of the studied plants after being prepared, were injected to GC-MS apparatus for the components analyses. The used GC was of VARIAN CP-3800 type and its column was 30m in length and inner diameter of 0.25 mm and thickness of 0.25 μm and it was a VF-5MS type too.

The temperature plan of the column was: The initial oven temperature was 50°C and remained in this temperature for 1 min then the temperature increased to 300°C by the rate of 10°C in a minute, the temperature of the injection chamber was 260°C , and Helium was used as the carrier gas with a flow of 1 mm in a minute.

In model of mass set: VARIAN SATURN 2200 the type of column was VF-5MS. The length of GC column was 30 mm, the inner diameter of the column was 0.25 mm, the thickness of the layer was 0.25 μm and the initial temperature of the oven was 50°C . The final column temperature was 300°C , the injection mode was split and the carrier gas was He.

Statistical analysis

The test results after 24h were read as the following way: the number of alive larvae, the number of dead larvae, the number of moribund larvae, number of larvae and the total number and the results were used to draw the mortality tables. The mortal quantities of 50% and 90% of EOs (LC_{50} and LC_{90}) and the level of confidence of 95%, the equation of the regression line will be estimated using a regression probit analysis [8].

When the mortality of the witness group is less than 5% then the resulted data of biometric tests have been correct but if the mortality of the witness group is between 5% to 20% they have to be corrected using the following formul,called Abbot:

$$\text{Formula Abbot} = \frac{\text{The mortality of the witness group} - \% \text{ the mortality of the test}}{\% \text{ the mortality of the witness group} - 100} \times 100$$

Results

The resulted data of the larvicidal activity of the plants in our test showed that:

Rosemarinus officinalis EO in 320 ppm concentration, *Carumcarvi* EO in 80 ppm concentration and *Artemisia dracunculus* EO in 10

ppm concentration show mortality effect of 100%. By decreasing the concentrations into 20 ppm for *Rosemarinus officinalis*, 5 ppm for *Carumcarvi* and 0.625 ppm for *Artemisia dracunculus* mortality became close to zero.

The 50% and 90% of lethal concentrations of studied plant EOs were 21.59 and 72.44 ppm for *C. carvi*, 1.33 and 4.12 ppm for *A. dracunculus*, 93.22 and 229.29 ppm for *R. officinalis*, respectively. Lethal dose of 50% and 90% (LC_{50} and LC_{90}) and other statistical data related to biochemical tests of larvicidal effects of EOs after 24h contact with age 4 larva of *A. stephensi* have been mentioned in the following Table 1.

Also the equation of the regression line of each EO can be seen in the diagrams, the said equation is which is derived from the following Figures 1-3.

GC-MS results of the EO of the tested plants

Types and percentage of the chemical components in the EO of each plant was distinguished by GC-Mass method. Knowing the forming components of EO is necessary for purification of the effective material and correct formulation of the larvicide.

Based on the results of the main chemical components of GC-MS analysis of *Carum carvi* were: 37.2% drima-7,9 (11)-diene,

9% ((E)-2-hexenal), 11.1% trans-carveol, 7.6% AR-curcumene, 6% benzyl-benzoate, 4.4% Cadalene, 4% Geraniol, 3.9% a-cadinene, 5.7% Hexadecanol (Table 2).

The Main chemical components of *Artemisia dracunculus* were: 85.9% (Z)-3-hexenol, 9.4% Isovaleric acid and 2.9% Hexadecanol (Table 2).

The Main chemical components of *Rosemarinus officinalis* were: 46.8% ((E)-2-hexenal), 12.4 (Linalool), 10.3% (a-ylangene), 5.9% Geraniol, 3.4% (Carvacrol), 2.9% (a-campholenal) and 2% (Isovaleric acid) (Table 2).

Discussion

The results shows that each 3 tested EOs have larvicidal effects against *A. stephensi* larvae and *A. dracunculus* in comparison with the other two plants in this test is the most effective one which has the biggest mortality with the lowest concentration LC_{50} . The resulted data of the larvicidal activities of the studied plants says that *R. officinalis* EO with 320 ppm concentration and *C. carvi* with 80 ppm concentration and *A. dracunculus* with 10 ppm concentration show the mortality effect of 100%. By decreasing the concentrations into 20 ppm for *R. officinalis* and 5 ppm for *C. carvi* and 0.625 ppm for *A. dracunculus* respectively, the mortality moved in the direction to LC_{50}

Table 1: Lethal concentration (LC_{50} and LC_{90}) and associated parameters of 24 h bioassay tests of essential oil of (*Carumcarvi*, *Artemisia dracunculus* and *Rosmarinus officinalis*) against 3rd-4th instar larvae of *Anopheles (Cellia) stephensi* (Liston, 1901).

Plant scientific name and family	Used organs of the plant	a	b ± SE	LC_{50} (ppm) ± 95%C.L.	LC_{90} (ppm) ± 95%C.L.	χ^2 (Heterogeneity)	χ^2 table (df)	p-Value
<i>Carumcarvi</i> (Apiaceae)	Seeds	-3.2538	2.4384 ± 0.478	11.2035 21.5974 42.5172	38.2905 72.4433 653.3294	26.010'	16.266 (3)	0.001
<i>Artemisia dracunculus</i> (Asteraceae)	Branches and Leaves	-0.3231	2.6092 ± 0.657	0.2981 1.3300 2.8647	2.1372 4.1213 224.2394	37.518'	16.266 (3)	0.001
<i>Rosmarinus officinalis</i> (Labiatae)	Branches and Leaves	6.4578	3.2788 ± 0.924	85.1680 93.2243 102.1095	200.7901 229.2972 269.4732	59.622'	16.266 (3)	0.001

* Heterogeneity

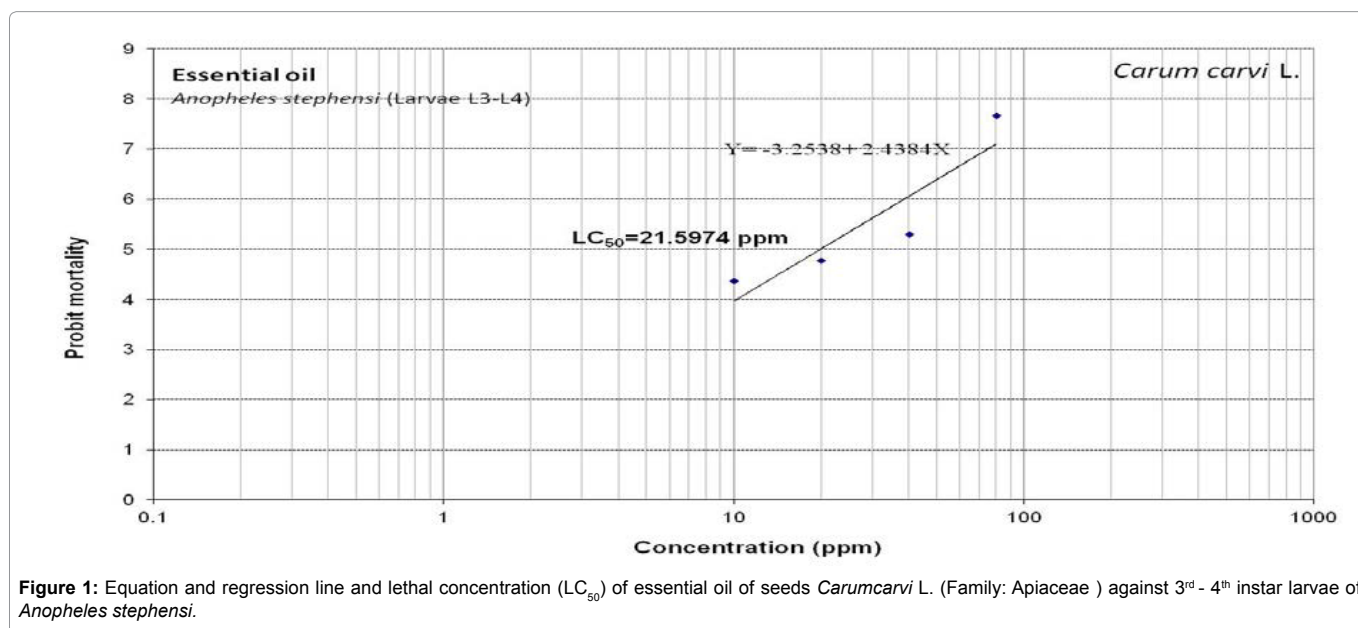


Figure 1: Equation and regression line and lethal concentration (LC_{50}) of essential oil of seeds *Carumcarvi* L. (Family: Apiaceae) against 3rd - 4th instar larvae of *Anopheles stephensi*.

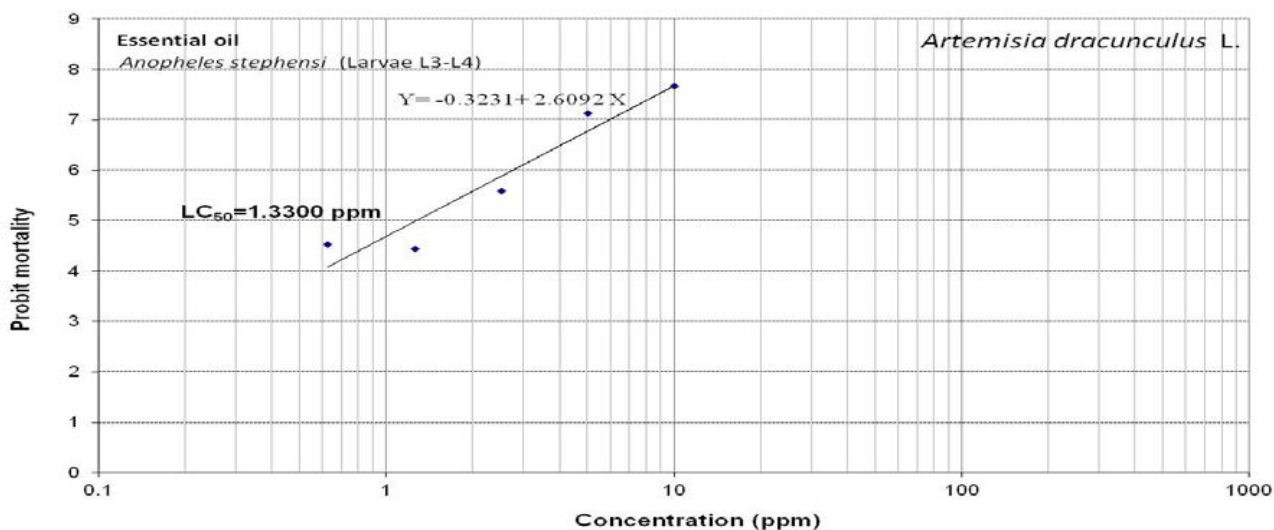


Figure 2: Equation and regression line and lethal concentration (LC_{50}) of essential oil of stems and leaves *Artemisia dracunculus* L. (Family: Asteraceae) against 3rd-4th instar larvae of *Anopheles stephensi*.

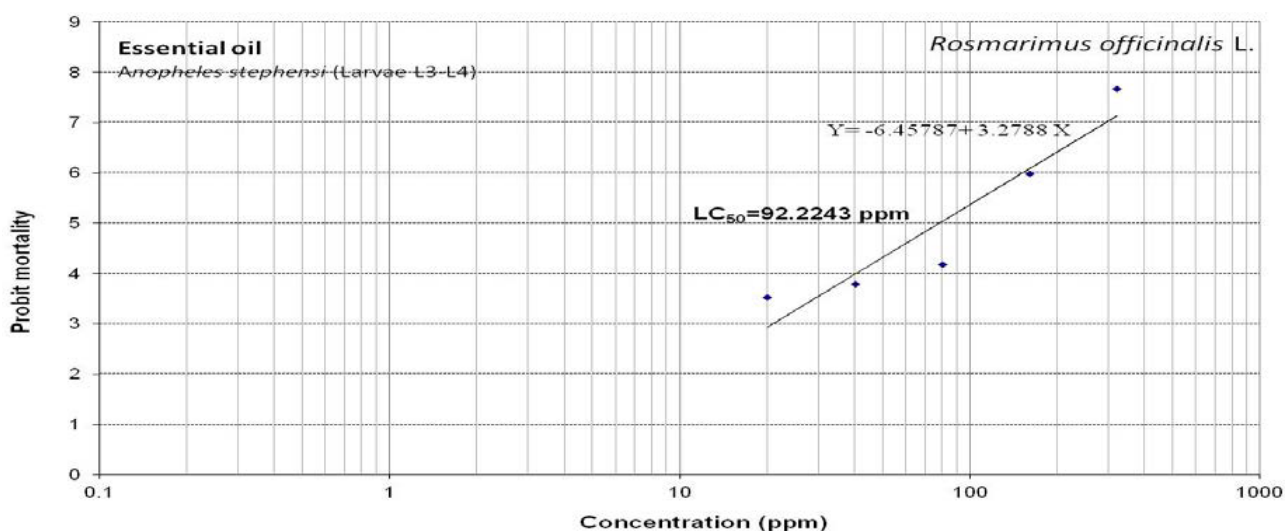


Figure 3: Equation and regression line and lethal concentration (LC_{50}) of essential oil of flowers and leaves *Rosmarinus officinalis* (Family: Lamiaceae) against 3rd-4th instar larvae of *Anopheles stephensi*.

of the studied plants in this biological test were LC_{50} = 21.59 ppm and LC_{90} = 72.44 ppm for *C. carvi* and LC_{50} = 1.33 ppm and LC_{90} = 4.12 ppm for *A. dracunculus* and LC_{50} = 93.22 ppm and LC_{90} = 229.29 ppm for *R. officinalis*.

In this study the larvicidal effect of 3 species of medicinal plants against the larva of the main Malaria vector in Iran *A. stephensi* was studied and based on the results all of the studied plants were effective against *A. stephensi* larva and after 24h showed the larvicidal effects. The most effective plant among these three was *A. dracunculus*.

In a study by Pitasawat et al. [9] the larvicidal effect of *C. carvi* against vector mosquitos of *A. dirus* and *A. aegypti* was studied. In this study *C. carvi* EO in the following concentrations: LC_{50} = 72.28 ppm, LC_{95} = 104.69 ppm and LC_{99} = 128.87 ppm against *A. dirus* and

LC_{50} = 54.62 ppm, LC_{95} = 90.06 ppm and LC_{99} = 119.21 ppm against *A. aegypti* was tested.

In another study which was carried out by Yu et al. [10], about the larvicidal activity of the *R. officinalis* components, the chemical components and their activity against the sensitive species to DDT, resistant to DDT and the *Culex quinquefasciatus* larvae collection were studied. The average lethal dose (LC_{50}) of *R. officinalis* EO against the sensitive species to DDT, resistant species to DDT and *Cu quinquefasciatus* species were 30.6 ppm, 26.4 ppm and 38.3 ppm respectively.

In a study by Govindarajan [11] the lethal effects of *R. officinalis* against two mosquito species: *Culex tritaeniorhynchus* and *Anopheles subpictus* were studied. The results of *R. officinalis* test against *C. tritaeniorhynchus* were LC_{50} = 115.38 (85.27-144.10) ppm and LC_{90} =

Table 2: The GC-MS analysis of the plants EO (*Carumcarvi*, *Artemisia dracunculul*, *Rosemarinus officinalis*).

The name of the plant	The number of distinguished components of the plant	The percentage of the distinguished components	Main components (high percentage)	Components with larvicidal, Antiparasitic and germicidal properties
<i>Rosemarinus officinalis</i>	isovaleric acid	2	Isovaleric acid	linalool
	(E)-2-hexenal	46.8	(E)-2-hexenal	trans-carveol
	linalool	12.4	linalool	carvacrol
	α -campholenal	2.9	α -campholenal	eugenyl acetate
	borneol	1.3	geraniol	
	trans-carveol	0.7	carvacrol	
	cis-carveol	1.8	angelic acid	
	geraniol	5.9		
	p-cymen-7-ol	0.9		
	carvacrol	3.4		
	angelic acid	10.3		
	1,7-di-epi- α -cedrene	2		
	β -isocomene	1.2		
	β -carophyllene	1		
	Z- β -farnesene	0.8		
	germacrene D	1.1		
	eugenyl acetate	1.1		
8-cedren-13-ol	1.7			
1-docosene	1.6			
<i>Carumcarvi</i>	(E)-2-hexenal	9	drimene-7,9 (11)-diene	trans-carveol
	trans-carveol	11.1	(E)-2-hexenal	Hexadecanol
	geraniol	4	AR-curcumene	tetracosane
	(E)- β -farnesene	1.2	benzyl benzoate	
	drimene-7,9 (11)-diene	37.2	Cadalen	
	AR-curcumene	7.6	Geraniol	
	α -cadinene	3.9	α -cadinene	
	cadalen	4.4	Hexadecanol	
	benzyl benzoate	6	tricosane	
	nootkatone	2.1		
	isopropyl tetradecanoate	1.7		
	hexadecanol	5.7		
	n-docosane	2		
	tricosane	3.1		
tetracosane	1			
<i>Artemisia dracunculul</i>	hexanal	0.8	(Z)-3-hexenol	isovaleric acid
	isovaleric acid	9.4	isovaleric acid	hexadecanol
	(Z)-3-hexenol	85.9	hexadecanol	β -carophyllene
	β -carophyllene	1		
	hexadecanol	2.9		

211.53 (176.37-280.69) ppm and against *An. Subpictus* were LC_{50} = 64.50 (49.96-79.16) ppm and LC_{90} = 113.74 (95.58-149.57) ppm.

In another study which was carried by Conti et al. [12] about the lethal activity of *R. officinalis* against *A. albopictus* the highest dose was 300 ppm and the lethal level of *R. officinalis* was 51.7%. The results of the lethality test for *R. officinalis* against *A. albopictus* was $LC_{50} > L_{250}$.

In a study in 2013 in Iran by Valizadeganon *Aphis gossypii* Glove

aphid, the Fumigant toxicity of *A. dracunculul* EO on the adult pest was studied. The results suggested that *A. dracunculul* EO after 24h shows a dramatic mortality on the pest in the study. The LC_{50} in the adult male aphids was 18.63 μ L/L in the air [13].

Recommendations

The results of this study show that the extracted EOs of the medicinal plants could be thoroughly studied as the substitutes for usual pesticides because based on the results which are a proof

for the resistance of Malaria vectors to Pyrethroid insecticides i.e. the last resort of synthetic pesticides to control Malaria vectors. So the need for a conscious and purposeful seek to achieve the rare opportunities for introducing effective EOs in controlling immature flies is an innovation in controlling flies and paves the way for the commercialization of the product and also by considering the obvious effect of the EOs of the studied plants, distinguishing the effective components of these EOs, larvicidal features and formulating them for the field evaluation and use against Malaria vectors is suggested.

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