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Larvicidal effects and phytochemical evaluation of essential oils of *Trachyspermum ammi* and *Ziziphora clinopodioides* against larvae *Anopheles stephensi*

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ABSTRACT

Introduction: The main purpose of this research was to evaluate larvicidal effects of two native medicinal plants in Iran including seeds of Ajwain (*Trachyspermum ammi*) and leaves and shoots of Blue Mint Bush (*Ziziphora clinopodioides*) against larvae of *Anopheles stephensi*. Their phytochemical compounds of essential oils were also determined using GC-MS method.
Methods: The plants were collected from various regions of the country in the spring of 2013, and the aqueous essential oils were prepared using a Clevenger apparatus. The standard WHO method for anti-larval experiments against third and fourth instar larvae of *A. stephensi* was employed at the Biometric Laboratory next to the Culicidae Insectarium at the School of Public Health of Tehran University of Medical Sciences.
Results: The LC50s (lethal concentrations to kill 50%) and LC90s of the essential oils were 14.26 and 39.54 ppm *T. ammi* and 18.61 and 48.51 ppm for *Z. clinopodioides*. Phytochemical analysis of the essential oils of the tested plants revealed that thymol for *T. ammi* and pulegone for *Z. clinopodioides* had the highest percentage which were 71.989 and 48.609, respectively.
Conclusion: Considering the results of this research, the effective compounds in these essential oils that had larvicidal properties might be used against malaria vectors.

Implication for health policy/practice/research/medical education:
Considering the results of the present research, it seems that the scientific identification of the active ingredients in *Trachyspermum ammi* and *Ziziphora clinopodioides* against larvae *Anopheles stephensi* and utilization of the results of biometric experiments for developing suitable formulations of these active ingredients for controlling pests are necessary.
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Introduction

At present, international organizations have restricted the use of toxic chemicals and have started replacing them with less hazardous ones. This has increased interest in natural products and led to discovery and production of less hazardous toxic chemicals including plant-based pesticides by researchers. Essential oils are the most widely known materials that have been tested against insects. The present research studied the larvicidal effects of two native medicinal plants in Iran (Blue Mint Bush and Ajwain) against the main malaria vector in this country (*Anopheles stephensi*). *Ziziphora clinopodioides* belongs to the mint family (Labiatae), grows wild in mountainous regions of central Iran (1), and is a native plant of western and northwestern Iran (2). Based on previous researches, it has various therapeutic effects including antioxidant (3,4), antibacterial (5), antifungal (6), larvicidal properties (7,8). It is also a protector of the digestive system (9), and lowers blood pressure (10). Ajwain is a member of the Umbelliferae family and grows in eastern and southern parts of Iran (1,11). It is also grown in many regions of Egypt, Afghanistan, and India, especially in Bengal (12). The most important reported therapeutic effects reported for it include being an antioxidant, anti-fever,

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antimicrobial, anti-spasm, anti-flatulence, sudoriferous, expectorant, antifungal, gastric tonic and diuretic (13). The present research studied larvicidal activities of two medicinal plant species (Ajwain and Blue Mint Bush) against the main malaria vector in Iran (*A. stephensi*), and used logarithmic concentrations of the essential oils of these two plant species under laboratory conditions to determine their larvicidal effects so that, if results were desirable, their use could be recommended as effective larvicides against the main malaria vector in Iran. The phytochemical components of these plants were also determined using GC-MS method.

Materials and methods

Blue Mint Bush plant was collected from Bojnord in Khorasan province and Ajwain plant from Kazeroon in Fars province in the spring of 2013. Their organs were separated and air-dried in the shade, and identified. The physicochemical characteristics of the plants were determined, their compounds were identified, and the effects of their extracts against larvae of *A. stephensi* were studied. Features of the applied apparatus for phytochemical analysis of *Trachyspermum ammi* and *Z. clinopodioides* are shown in Table 1.

Rearing *Anopheles stephensi* larvae

Mosquito larvae were reared and kept in the Culicidae Insectarium of the School of Public Health at 29±2ºC, relative humidity of 70±10%, and a photoperiod of 16 hours light, 8 hours dark. Late third instar or early fourth instar larvae of *A. stephensi* were used in antilarval experiments.

Preparing essential oils

A Clevenger apparatus was used for aqueous extraction of the essential oils of the two plant species. The plants were first powdered, about 30 g of the powders were poured in a 1-L flask, about 600 mL of distilled water were added, the Clevenger apparatus was mounted on the flask, and the heater was turned on. After the water boiled and evaporation began, the temperature was kept at a level that the water did not stop boiling (at about 60-80°C) and the heater was turned off. After about 4 hours, the essential oil was collected from the graduated part of the apparatus and poured into a small glass container specifically used for collecting essential oils. The essential oil was dried using anhydrous sodium sulfate and kept in a refrigerator to be used for the experiments.

Table 1. Features of the applied apparatus phytochemical composition of *Trachyspermum ammi* and *Ziziphora clinopodioides*

Apparatus Model GC	Agilent 6890N (USA)
Type of the column	HP-5MS
Column length GC	30 m
Inner diameter of the column	0.25 mm
Thickness of the layer	0.25 µm
Primary oven temperature	40°C
Temperature of the injection location	250°C
The amount of injection	1 µL
Final column temperature	250°C
Injection mode	Split
Type of carrier gas	He
Detector	Mass

Biological (antilarval) tests

The World Health Organization (WHO) method for antilarval biological tests was employed. The mean temperature at the laboratory was kept constant at 28°C, test duration was 24 hours, and 25 larvae were used in each 400 mL beaker. The larvae used for these tests were end of third instar or early fourth instar ones, and chlorine-free water was preferably used. Five concentrations of the insecticide, four replications for each concentration, and two controls are used, and in the control experiments, all materials employed in other experiments were used except the insecticide itself (14).

Preparation of logarithmic concentrations of the essential oils

Essential oils that were obtained from seeds of *T. ammi* and leaves and shoots of *Z. clinopodioides*) were used to conduct biological experiments. Four replications were considered for every concentration of each essential oil. The mortality rate of the larvae after 24 hours of contact with each essential oil was used to evaluate its larvicidal effect. The experiments were performed at the logarithmic concentrations of 5, 10, 20, 40, and 80 ppm for Ajwain and 10, 20, and 40 ppm for Blue Mint Bush with 4 replications for each concentration. The experiments had 2 series of controls (containing 1 mL of ethanol in 249 mL of chlorine-free water). Ajwain essential oil concentration of 80 ppm caused 100% larval mortality and, therefore, the logarithmic concentrations started at 5 ppm and were successively doubled. Blue Mint Bush essential oil concentration of 80 ppm also led to 100% larval mortality and, hence, the logarithmic concentrations started at 10 ppm and were successively doubled. The method for preparing logarithmic concentrations is presented below.

Anti-larval sensitivity test

Similar to the biological tests, the WHO method was employed and the tests were conducted in 400 mL glass beakers. As previously mentioned, logarithmic concentrations of the essential oils were first prepared. Twenty-two 400 mL beakers and twenty-two 50 mL beakers were prepared (4 replications for each concentration, and 2 replications for the control). Using a graduated cylinder, 224 mL of chlorine-free water were added to each 400 mL beaker and 25 mL to each 50 mL

beaker. Following that, 25 late third instar or early fourth instar larvae reared in cuvettes (and fed fish meal four hours earlier) were transferred to each 50 mL beaker and, using an insulin syringe, 1 mL of the essential oil with the desired logarithmic concentrations was added to the 400 mL beaker used for that specific concentration and the contents were mixed. In the control beakers, 1 mL of pure ethanol was added instead of the extract. The contents of each 50 mL beaker was then transferred to the related 400 mL beaker and the beaker was covered with a plate, and results of the test were read after 24 hours.

Chemical analysis

Conditions of the applied apparatus for phytochemical analysis of *T. ammi* and *Z. clinopodioides* are shown in Table 2. After the essential oils of the plant species were prepared, they were injected into the GC-MS instrument to analyze the products in the extracts. The GC instrument was a model VARIAN CP-3800 having a VF-5MS column that was 30 m long, had an inner diameter of 0.25 mm, and a film thickness of 0.25 µm. The thermal regime was as follows: oven temperature was initially maintained at 50°C for 1 minute, it was gradually raised to 300°C at 10°/min, and the injection chamber had the temperature of 260°C. Helium was used as the carrier gas at the flow rate of 1 mL/min. The GC instrument used for Blue Mint Bush and Ajwain was a model Agilent 6890N (USA) having an HP-5MS column that was 30 m long and had an inner diameter of 0.25 and a film thickness of 0.25 µm. The initial oven temperature was 40°C, it was gradually raised to 250°C, the injection chamber had a temperature of 250°C, and helium was used as the carrier gas.

Table 2. Conditions of the applied Apparatus for phytochemical analysis of *Trachyspermum ammi* and *Ziziphora clinopodioides*

Initial temperature (°C)	Step 1
Initial time (min)	40
Program rate (°C/min)	1
Final temperature (°C)	3
Final time (min)	250
Split flow (mL/min)	60
Septum purge (mL/min)	25
Flow rate (mL/min)	6
Initial temperature (°C)	1

Statistical analysis

Results of the tests were read after 24 hours as the number of live, dead, and moribund larvae, the number of pupae, and the total number of larvae and pupae. This information was used to draw up mortality tables. LC50s and LC90s of the essential oils were determined and the linear regression equation was estimated with a 95% confidence interval using the probit-analysis method (15). If mortality rate of the control group was less than 5%, the data obtained from the biometric tests were correct, but if mortality rate of the control group was in the 2%-20% range, the data would be corrected using Abbot's formula as follows:

Abbott's formula = (mortality percentage of the test group – mortality percentage of the control group)/ (mortality percentage of the control group) × 100.

Results

Trachyspermum ammi and *Z. clinopodioides* essential oils at 80 ppm caused 100% larval mortality, but this mortality rate declined and approached zero percent at Ajwain concentrations of 5 and 10 ppm, respectively. The LC50s and LC90s were 14.26 and 39.54 ppm for Ajwain and 18.61 and 48.51 ppm for Blue Mint Bush.

Table 3 presents LC50s and LC90s and other statistical data related to biochemical tests on larvicidal effects of the essential oils after the contact time of 24 hours between them and fourth instar larvae of *A. stephensi*.

Linear regression equations for all essential oils are presented in Figures 1 and 2.

GC-MS results concerning the tested essential oils

GC-MS results concerning the tested essential oils are presented in Table 4. Analysis of the essential oils of the tested plant by using the GC-MS method revealed the

Table 3. Lethal concentration 50% and 90% (LC_{50} and LC_{90}) and associated parameters of 24 hours bioassay tests of essential oil of *Trachyspermum ammi* and *Ziziphora clinopodioides* against 3rd-4th instar larvae of *Anopheles stephensi*

Scientific name and family of the plant	Organs of the plant	A	B ± SE	LC_{50} (ppm) ± 95% CI	LC_{90} (ppm) ± 95% CI	χ^2 (Heterogeneity)	χ^2 table (*df*)	*P* value
Trachyspermum ammi (Apiaceae)	Seeds	-3.9130	3.0812 ± 0.234	16.7725 18.6190 20.4846	42.4791 48.5169 57.3649	8.781*	13.816 (2)	0.001
Ziziphora clinopodioides (Labiatae)	Branches and leaves	-2.3913	2.0503 ± 0.574	1.3240 14.0587 49.6484	26.2726 61.2474 79.2400	45.473*	16.266 (3)	0.001

A = y-intercept; B = The slope of the line; SE = standard error; LC50, 95 % CI = Lethal concentration causing 50% mortality and its 95% confidence interval; LC90, 95 % CI = lethal concentration causing 90% mortality and its 95 % confidence interval; *X* 2 = Heterogeneity about the regression line; *P* value = represent heterogeneity in the population of tested.

* No heterogeneity.

compounds that constituted them and their percentages. This information is necessary for purifying the active ingredient and developing the correct formulation of the larvicide (Figures 3 and 4).

Discussion and Conclusion

This research studied the larvicidal effects of essential oils obtained from two plant species against larvae of *A. stephensi* (the main malaria vector in Iran). Both tested essential oils had larvicidal effects against this vector. The essential oils of *T. ammi* and *Z. clinopodioides* at 80 ppm caused 100% mortality of the vector larvae. When the concentrations of the extracts declined, mortality rates decreased and approached zero percent at 5 ppm for Ajwain and at 10 ppm for Blue Mint Bush extracts.

In the biological experiments, the LC50s and LC90s essential oils were 14.26 and 39.54 ppm for *T. ammi* and 18.61 and 48.51 ppm for *Z. clinopodioides*.

In research conducted by Verdian-Rizi in Iran in 2008 on larvicidal effects of Blue Mint Bush against larvae of *A. stephensi* and *C. pipiens*, the LC50s and LC90s were 14.9 and 22.3 ppm and 16.5 and 28.6 ppm, respectively (7).

Seo et al carried out a study in 2012 to investigate larvicidal effects of Ajwain essential oil against *Aedes aegypti*. This extract caused 100% mortality of the larvae at 0.1 mg/mL (16). In 2007, Chaubey conducted research on insecticidal effects of Ajwain extract and found that its LC50 for the larval stages of *Tribolium castaneum* was 11.62 mg/mL (17).

In 2009, Pandey et al carried out a study on larvicidal activity of Ajwain seed extract, and of its pure constituent thymol, against *A. stephensi*. They found that thymol was 1.65 times more toxic to fourth instar larvae of *A. stephensi* compared to the extract itself, and the LD50s and LD99s were 48.88 and 105.49 ppm for thymol and 80.44 and 172.12 ppm for the extract (18).

Considering the results of the present research, accurate and scientific identification of the active ingredients in these medicinal plants and use of results in biometric tests seem to be necessary in developing an accurate and suitable formulation for controlling pests. Utilization of larvicidal and repellent effects of these plants can, in general, play a substantial role in preventing diseases transmitted by mosquitoes.

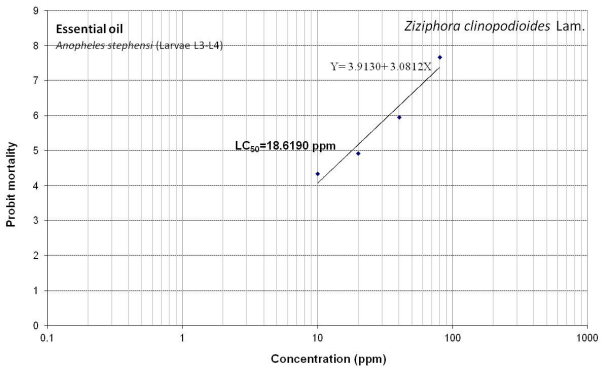

Figure 1. The equation, regression line, and LC_{50} (lethal concentration, 50%) of *Ziziphora clinopodioides* seed essential oil against third- and fourth-instar *Anopheles* stephensi larvae.

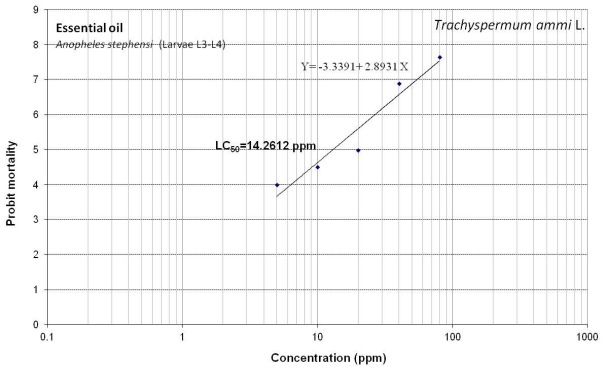

Figure 2. The equation, regression line, and LC50 (lethal concentration, 50%) of *Trachyspermum ammi* seed essential oil against third- and fourth-instar *Anopheles stephensi* larvae.

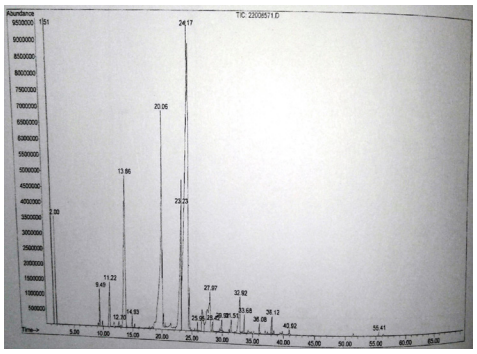

Figure 3. Peaks in the spectrum obtained from GC-Mass of cinnamon plant (*Ziziphora clinopodioides*) essential oil.

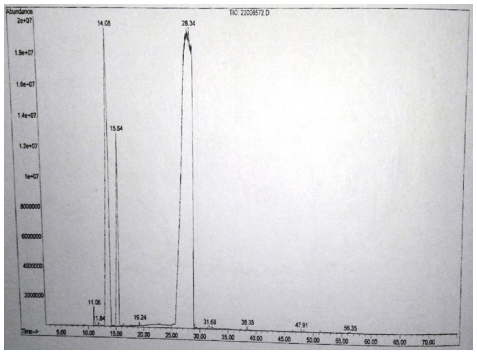

Figure 4. Peaks in the spectrum obtained from GC-Mass of cinnamon plant (*Trachyspermum ammi*) essential oil.

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Table 4. GC-MS results concerning the tested essential oils

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 larv icidal, Antiparasitic and germicidal properties
Ziziphora clinopodioides	Ethanol	9.497	Pulegone	Limonene
	Pentanal	0.036	p-Menthan-3-one	Carvacrol
	alpha-Thujene	0.090	Ethanol	Sabinene
	alpha-Pinene	0.887	1,8-Cineole	3-Octanol
	Camphene	0.097	Piperitenone	
	Sabinene	1.032	Verbenone	
	beta-Pinene	0.732	Ethanethioic acid, S-phenyl ester	
	beta-Myrcene	0.173		
	3-Octanol	0.166		
	alpha-Terpinene	0.026		
	p-Cymene	0.084		
	1,8-Cineole	9.869		
	cis-Ocimene	0.024		
	gamma-Terpinene	0.230		
	3,8-p-Menthadiene	0.056		
	alpha-Fenchene	0.032		
	trans-2-Caren-4-ol	0.074		
	Limonene	0.573		
	p-Menthan-3-one	14.203		
	p-Menthone	1.152		
	trans-Decalone	0.203		
	Neomenthyl acetate	0.138		
	Citronellol	0.062		
	Pulegone	48.609		
	Carvacrol methyl ether	0.045		
	Piperitone	1.128		
	Bornyl acetate	0.051		
	Menthol acetate	0.149		
	Carvacrol	1.512		
	Verbenone	2.129		
	Piperitenone	2.307		
	Isoeugenol	0.272		
	alpha-Cubebene	0.089		
	beta-Bourbonene	0.228		
	trans-Caryophyllene	0.052		
	Artemeisole	0.311		
	gamma-Elemene	0.059		
	Ethanethioic acid, S-phenyl ester	2.109		
	Sinularene	0.394		
	Caryphyllene oxide	0.087		
	1-p-Menthen-8-yl acetate	0.552		
	Vulgarol A	0.126		
	Visopathulenol	0.137		
	n-Hexadecanoic acid	0.119		
	Pterine	0.023		
	Isophytol	0.079		
Trachyspermum ammi	alpha-Thujene	0.013	Thymol	beta-Pinene
	alpha-Pinene	0.010	p-Cymene	Thymol
	beta-Pinene	0.312	gamma-Terpinene	Carvacrol
	beta-Myrcene	0.108		
	p-Cymene	19.536		
	cis-Ocimene	0.107		
	gamma-Terpinene	7.389		
	Dehydro-p-cymene	0.006		
	Turpentine comphor	0.033		
	Thymol	71.989		
	Carvacrol	0.207		
	trans-Caryophyllene	0.069		
	Caryphyllene oxide	0.013		
	Dillapiole	0.061		
	Fenchyl acetate	0.030		
	p-Menth-3-en-ol	0.022		
	cis-9-Octadecenoic acid	0.027		
	trans-9-Octadecenoic acid	0.055		
	Tetracosane	0.013		

their cooperation during this research.

Authors' contributions

HTP was the main researcher and performed the experiments, MS supervised the research, HV and MRA helped the supervision and preparation of the manuscript. All read and confined the final version of the manuscript for publication.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues in research have been completely observed by the authors.

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