Original Article

Preparation of a Nanoemulsion of Essential Oil of *Acroptilon repens* Plant and Evaluation of Its Larvicidal Activity agianst Malaria Vector, *Anopheles stephensi*

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Abstract

Background: Extensive use of chemical larvicides to control larvae, has led to resistance in vectors. More efforts have been conducted the use of natural products such as plant essential oils and their new formulations against disease vectors. Nanoformulation techniques are expected to reduce volatility and increase larvicidal efficacy of essential oils. In this study for the first time, a larvicide nanoemulsion from the essential oil of *Acroptilon repens* was developed and evaluated against *Anopheles stephensi* larvae under laboratory conditions.
Methods: Fresh samples of *A. repens* plant were collected from Urmia, West Azarbaijan Province, Iran. A clevenger type apparatus was used for extracting oil. Components of *A. repens* essential oil (AEO) were identified by gas chromatography–mass spectrometry (GC–MS). All larvicidal bioassay tests were performed according to the method recommended by the World Health Organization under laboratory condition. Particle size and the morphologies of all prepared nanoformulations determined by DLS and TEM analysis.
Results: A total of 111 compounds were identified in plant. The LC_{50} and LC_{90} values of AEO calculated as 7 ppm and 35 ppm respectively. AEO was able to kill 100% of the larvae in 4 days.
Conclusion: The nanoemulsion of AEO showed a weak effect on the larvar mortality. It may therefore be suggested that this kind of nanoemulsion is not appropriate for the formulation as a larvicide. It is important to screen native plant natural products, search for new materials and prepare new formulations to develop alternative interventions with a long-lasting impact.

Key words: *Acroptilon repens;* Nanoemulsion; Larvicidal effect; Vector control; *Anopheles stephensi*

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Introduction

Vector borne diseases are infections which are transmitted by the bite of infected arthropod species and account for 17% of all infectious diseases. Every year more than one billion people are infected and more than one million people die from vector-borne diseases including malaria, dengue, schistosomiasis, leishmaniasis, chagas disease, yellow fever, lymphatic filariasis and onchocerciasis (1). The most important diseases transmitted by mosquitoes are malaria, dengue fever, lymphatic filariasis, yellow fever, chikungunya, Zika virus, as well as viral encephalitis (2). *Anopheles* mosquitoes are widely distributed and found in the tropics temperate regions (3). The most important disease transmitted by *Anopheles* mosquitoes is human malaria, which is the most important parasitic disease in the world (4). The disease is still a major health problem worldwide, including Iran. Most malaria cases were reported from the south and southeastern of Iran. *Plasmodium vivax* was the dominant species (5). *Anopheles stephensi* is an important malaria vector in the Middle East and south Asia (6, 7).

To control the disease, larval control is currently being performed in 55 countries (8, 9). Theuseofnaturalproductsisaninteresting approach in this regards. Today, there are loads of studies and recommendations on plant extracts and essential oils as larvicides, insecticides and repellents (10, 11). Extracts and essential oils (EOs) are biocompatible and have minimum toxic effects on non-target organisms. Along with many novel formulations, nanoemulsions of pesticides have been considered recently due to their greater efficiency, lesser adverse effects on non-target organisms (12, 13). However, extracts and EOs have volatile components that restrict their use in natural environments (14-16). This can be overcome by formulating them in the form of nanoemulsions. There has been a lot of research recently on EOs as natural larvicides, but there are a few available articles on nanoemulsions as larvicides. In a study, the larvicidal activity of eucalyptus essential oil and its nanoemulsion against *Culex quinquefasciatus* was investigated. The result showed that the bioactivity of the nanoemulsion was improved than the bulk EO (17). In a study, nanoemulsion of *Artemisia dracunculus* essential oil showed better larvicidal efficiency on *An. stephensi* larvae in comparison with its essential oil (18). Likewise, encapsulation of *A. dracunculus* essential oil in chitosan nanoparticles presented very good larvicidal activity with 9 days residual effect (19). Volpato et al (2016) investigated the effect of essential oil and its nanoemulsion against *Alphitobius diaperinus*. The nanoemulsion showed a three-fold better effect as compared to the essential oil (20). Balasubramani et al (2017) in their experiments obtained similar results with the nanoformulation of *Vitex negundo* essential oil compared to its essential oil against *Aedes aegypti* (21). In a recent study, larvicidal activity of *Cinnamomum zeylanicum* essential oil was compared with its nanoemulsion. The formulated nanoemulsion showed 32% better larvicidal effect as compared to the essential oil, the residual effect of the formulation was 3 days. These results indicated an increase in larvicidal activity and residual effects of an essential oil nanoemulsion compared to bulk essential oil (11).

As the extract of *A. repens* had very good larvicidal activity against *Anopheles stephensi*, *Culex pipiens* and *Culex quinquefaciatus* in the previous work (22), we decided to extract its essential oil and provide the nanoformulation in order to investigate their larvicidal effect against *An. stephensi* larvae.

Materials and Methods

Collection, identification and extraction of *Acroptilon repens*

Fresh samples of *A. repens* were collected in Jun- Jul 2018 from Urmia, West Azarbaijan province, Iran (45.08º E, 37.55º N, elevation ~ 1332 m above sea level) (Fig.1).

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Collection and identification of plant

Acroptilon repens plants were collected (Fig. 2), rapidly transferred to the laboratory and then was identified by experts in Department of Plant Sciences, Tehran University.

Extraction of essential oil

All collected plants were washed with water, then shad dried. Dried samples were hydrodistilled, using clevenger type apparatus for five hours. The extracted oil dried over anhydrous sodium sulfate. In total, 65 ml extracted oil obtained from 650 kg of dried plant. To prevent degradation and oxidation, the essential oil was stored in dark glass containers, completely away from sun light at 4-8 °C.

Analysis of essential oil by gas chromatography–mass spectrometry (GC-MS)

GC-MS analysis used to identify compounds of the essential oil. The essential oil diluted using hexane with the specifications given in Table 1. The compounds of the essential oil were analyzed using GC-MS and compared with standard mass spectra available in the device library.

Mosquito rearing

Anopheles stephensi larvae were reared in the insectary at 29 ± 1˚C with relative humidity of 70 ± 5% under 12 h light/12 h dark conditions. The cages for keeping mosquitoes were wooden cubes with dimensions of 30 cm × 30 cm × 30 cm, covered with fine mesh. The stock culture of adult *An. stephensi* fed twice a week on sheep blood (artificial feeding). The egg rafts laid transferred to enamel larval trays. The larvae were fed with fish food.

Preparation of nanoemulsion

In this study, surfactant (Tween 80) and co-surfactant (Span 20) were stirred for 6 minutes at 600 rpm. The essential oil was then added at 90% lethal concentration of the bulk essential oil and stirred for 10 minutes at 600 rpm. Water was then added dropwise and stirring was continued at 600 rpm for 38 minutes. Ten different nanoemulsion preparations having a constant amount of essential oil (1.4 %) and different amounts of surfactant (2 to 9.2 %) and co-surfactant (0.8 %). The nanoemulsion stored in a dark place at room temperature for 24 hours, then checked visually for signs of phase

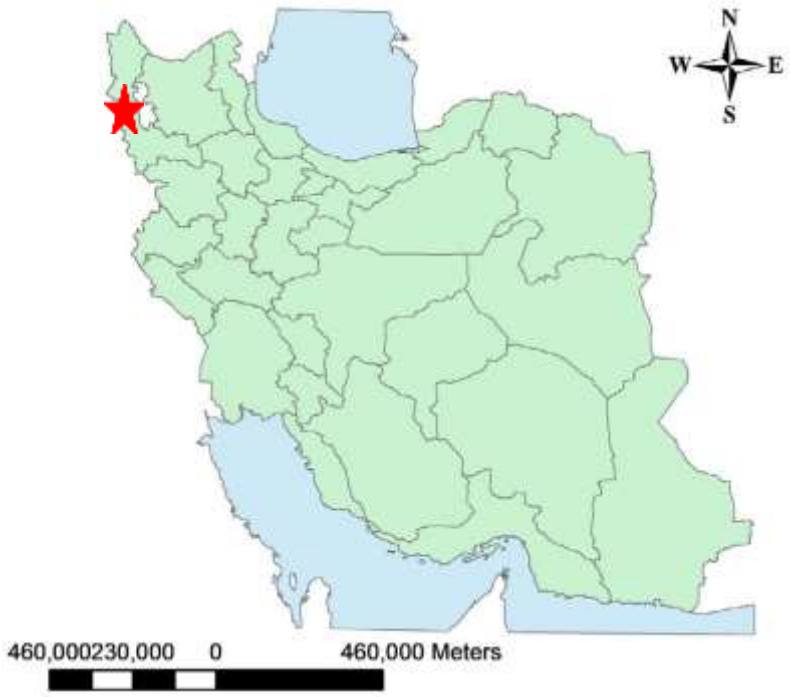

Fig. 1. Collection site of plant *Acroptilon repens* in Urmia, West Azarbaijan Province, Iran

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Fig. 2. *Acroptilon repens*

separation, precipitation or creaming.

Dynamic light scattering (DLS, K-ONE. LTD, Korea) used to determine the particle size (PS) of the prepared nanoformulations. Transmission electron microscopy (TEM) used to confirm the PS and to investigate the morphology of the particles.

Determining the larvicidal efficacy

Larvicidal bioassays performed according to the WHO guideline. Logarithmic dilutions prepared from bulk essential oil by dissolving in ethanol. All the nanoemulsion/ bulk samples diluted 200 times before performing the larvicidal tests (23). Third and early 4th instars larvae were used. One ml of the essential oil was added to 249 ml of chlorine-free (pH=7) water and stirred and 25 healthy larvae added to the containers. Containers were covered and after 24 hours, the number of living and dead larvae counted.

Table 1. Analysis conditions and specifications of GC-MS device

Instrument Specifications	
Manufacturer company	Agilent Technologies
1. GC system	7890A
2. Mass Selective Detector	5975C VL MSD with Triple-Axis Detector
3. Ion source	Electron Impact (EI) 70eV
4. Analyzer	Quadrupole
5. Column	Rtx 5 MS
-Length	30m
-I.D.	0.250 mm
-Film thickness	25 μm
Conditions	
1. Injection port temperature	230˚C
2. Ion source temperature	230˚C
3. Carrier gas	He 99.999%
4. Sample volume	0.2 μL
Temperature Program	
Initial temperature (ºC)	40
Initial time (min)	1
Program rate (ºC/min)	3
Final temperature (ºC)	270
Final time (min)	10
Split ratio (ml/min)	100
Septum purge (ml/min)	---
Flow rate (ml/min)	1

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Determining the duration of action of bulk and nanoemulsions of *A. repens* essential oil

To determine the duration of larvicidal activity, according to the instructions of the WHO, the formulation prepared for the larvicidal test diluted 100 times (23). One ml of specified concentrations of bulk or nanoemulsion samples added to 249 ml of chlorine-free (pH=7) water, then, 25 live larvae were added to the solution. After 24 hours, the number of dead/ live larvae was counted, without changing the solution; the larvae (dead and live) were removed from the containers, followed by the addition of 25 new live larvae in the containers. The larvae were replaced for 8 days. All the larvicidal bioassays repeated 16 times in four different replicates. In each replicate, two control groups containing ethanol were considered.

Statistical analysis

The lethal concentrations of 50% and 90% (LC_{50} and LC_{90}) were calculated using Probit analysis (24). The regression line plotted using Excel 2007 software. If mortality of the control group was less than 5%, the data from the bioassay tests were considered correct. When the control mortality was between 5- 20%, it was corrected using the Abbott formula (24). If the larvae became pupae or the larvae mortality were more than 20% in the control group, the test was repeated.

Results

Determination of chemical composition of *A. repens* essential oil

Components of AEO identified by GC–MS analysis. One hundred and eleven components were determined, with five major components including Caryophyllene oxide (12.055%), α-Cubebene (12.054%), 1-Heptadecene (5.181%), delta-Cadinene (3.771%) and β-Cubebene (3.771%) as listed in supplementary data (Table 2).

Characterization of AEO nanoemulsion

From preliminary studies to find on the optimum nanoemulsion (i.e., highest stability and lowest particle size), a nanoemulsion preparation with 6.8% Tween 80, 0.8% Span 20, 1.4% AEO and water was prepared. Figure 3 shows DLS results of the nanoemulsion with d50= 106 nm.

The morphology of nanoemulsion particles was determined using transition electron microscopy (TEM) (Fig. 4). The results show that the nanoemulsion was well-formed and the particles were almost spherical.

The AEO nanoemulsion did not show any sign of phase separation after 4-month storage (4 °C and room temperature) and centrifugation (25000 rpm, 30 min).

Larvicidal bioassay of *A. repens* essential oil

The results of larvicide activity of six different concentrations of AEO are shown in Figure 5. Mortality rate at 3.125 ppm was 0% and increased to 100% at 50 ppm. There was no mortality in the control groups. In regression line, a positive correlation was observed between essential oil concentrations and the probit mortality (Fig. 6). The LC_{50} and LC_{90} values of AEO against *An. stephensi* larvae calculated as 7 and 34 ppm, respectively.

Figure 7 shows comparison of the residual larvicidal properties. AEO killed 100% of the larvae in the first four days of the experiment. After the 4th day, larval mortality decreased and reached 76%. AEO nanoemulsion showed weaker activity. It had 84% mortality on the first day and the mortality rate decreased to 0% on the 7th day.

To compare larvicidal activity of AEO with AEO nanoemulsion against *An. stephensi* larvae, equal concentrations of AEO used in the short time test (24 hours). The larvicidal effects of AEO were 88%, while the nanoemulsion properties of AEO reduced to 44% (Fig. 8).

Figure 9 shows the particle size of the nanomeulsion after 200 times dilution, showing instability for the preparation after dilution.

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Table 2. Chemical composition of the essential oil of *Acroptilon repens*

No.	RT (min)	Compound	Peak area	%	Quality	Mol Weight (amu)
1	37.993	Caryophyllene oxide	503332611	12.055	96	220.183
2	29.563	α-Cubebene	503308397	12.054	99	204.188
3	41.823	1-Heptadecene	216337204	5.181	99	238.266
4	35.537	delta-Cadinene	188186597	4.507	98	204.188
5	30.091	β-Cubebene	157435883	3.771	97	204.188
6	31.287	Caryophyllene	155313108	3.720	99	204.188
7	28.316	α-Cubebene	148603368	3.559	98	204.188
8	38.858	3-Undecyne	128983727	3.089	55	152.157
9	47.078	2-Pentadecanone	108810045	2.606	96	268.277
10	33.756	Tricyclo[2210(2,6)]heptane-3,5-diol	96224253	2.305	91	204.188
11	40.881	1,3-Cyclooctadiene	93566390	2.241	74	108.094
12	34.201	1-Pentadecene	91513407	2.192	99	210.235
13	38.285	Dihydrotanshinone I	87244445	2.090	91	220.183
14	37.172	[1,5]Naphthyridine-4-carbaldehyde	77809615	1.864	58	220.183
15	38.673	2(1H)-Naphthalenone	64550157	1.546	86	220.183
16	41.231	Azulene	63504092	1.521	97	198.141
17	38.063	β-Copaen	62409995	1.495	70	220.183
18	30.689	4,4-Dimethyl-3	56695349	1.358	47	202.172
19	33.406	delta-Elemene	53675197	1.286	58	204.188
20	41.422	Methyl α-oxo-7-azaindole-3-acetate	50884172	1.219	59	218.167
21	32.655	5,9-Undecadien-2-one	46366238	1.110	90	194.167
22	40.652	Bicyclo	45358016	1.086	89	204.188
23	29.881	β-Damascenone	45288877	1.085	98	190.136
24	36.249	Calacoene	44746403	1.072	78	172.125
25	25.752	1-Tridecene	41473191	0.993	98	182.203
26	36.612	Caryophyllene oxide	39040647	0.935	60	220.183
27	33.972	β-Selinene	37808861	0.906	99	204.188
28	39.526	Naphthalene	36960428	0.885	90	204.188
29	30.212	3,5-Octadiene	33754464	0.808	64	194.203
30	39.869	10,10-Dimethyl-2,6-dimethylenebicyclo	33082573	0.792	99	220.183
31	33.546	β-Selinene	32759075	0.785	96	204.188
32	40.048	Bicyclo	30063246	0.720	84	204.188
33	34.354	delta-Cadinene	29461311	0.706	80	204.188
34	32.922	Bicyclo[221]heptane	27543459	0.660	98	204.188
35	60.641	Tricosane (CAS)	25909857	0.621	98	324.376
36	35.212	delta-Cadinene	25336335	0.607	62	204.188
37	36.803	Bicyclo[310]hexane	24755522	0.593	42	136.125
38	55.424	Phytol	23404833	0.561	90	296.308
39	37.579	Cyclohexane	22932610	0.549	84	192.188
40	75.287	Nonacosane	21848026	0.523	99	408.47
41	51.238	Hexadecanoic acid	20517153	0.491	99	256.24
42	34.557	Naphthalene	19954822	0.478	99	204.188
43	32.77	Trimethylcyclohex	19947825	0.478	80	278.134
44	70.75	Heptacosane	18873599	0.452	95	380.438
45	67.226	Benzenedicarboxylic acid	18814084	0.451	91	211.012
46	30.925	Methanoazulene	18182957	0.435	99	204.188
47	40.474	Isoaromadendrene epoxide	18089104	0.433	43	220.183
48	31.904	Bicyclo[311]hept	17605938	0.422	98	204.188

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Continued Table 2. Chemical composition of the essential oil of *Acroptilon repens*

No.	RT (min)	Compound	Peak area	%	Quality	Mol Weight (amu)
49	30.409	7-Methanoazulene	15474913	0.371	99	204.188
50	35.823	Naphthalene	14843167	0.356	99	204.188
51	32.515	Khusimene	14686454	0.352	91	204.188
52	21.929	Decanal	13680181	0.328	91	156.151
53	33.851	Tricyclo	13035063	0.312	91	204.188
54	39.717	Dimethyl-2	12491034	0.299	83	220.183
55	40.347	4-Methanoazulene	12398829	0.297	55	204.188
56	34.977	Tridecanal	12181378	0.292	94	198.198
57	34.474	Pentadecane	11697698	0.280	93	212.25
58	49.457	13-Pentadecatrien-2-one	11566338	0.277	86	262.23
59	25.161	Vitispirane	11436343	0.274	98	192.151
60	28.914	Cycloisosativene	11154885	0.267	97	204.188
61	40.226	gamma-Selinene	11037619	0.264	64	204.188
62	41.097	Quinoline, 2,6-dimethyl-	10239966	0.245	35	157.089
63	65.877	Heneicosane	9939147	0.238	91	296.344
64	41.988	Heptadecane	9896521	0.237	97	240.282
65	36.491	2-Methyl-6-nitrophenol	9850609	0.236	53	153.043
66	44.463	Alloaromadendrene oxide-(2)	8884878	0.213	83	220.183
67	32.146	4-Dimethylaminopyridin-2-amine	8576066	0.205	52	137.095
68	39.316	6-Methoxy-1-acetonaphthone	8242200	0.197	78	200.084
69	48.751	Nonadecane (CAS)	8087481	0.194	98	268.313
70	26.408	1H-Indene	7844665	0.188	92	174.141
71	30.835	1H-Cycloprop[e]azulene	7747134	0.186	99	204.188
72	54.941	Heneicosane	7588497	0.182	99	296.344
73	37.045	Naphthalene	7339014	0.176	80	172.125
74	42.192	Vulgarol B	7325012	0.175	55	220.183
75	44.164	Ledene oxide-(II)	7233022	0.173	60	220.183
76	39.138	Trimethyl-2'-methylidene-9'-oxabicyclo	7090758	0.170	41	220.146
77	43.025	2-Dodecen-1-yl(-)succinic anhydride	7006122	0.168	30	266.188
78	45.735	Eicosane	6974224	0.167	38	282.329
79	28.443	Naphthalene 12 dihydro 1 1 6 trimethyl	6527008	0.156	97	172.125
80	39.221	Tricyclo	6429488	0.154	38	220.183
81	45.43	Octadecane	6266398	0.150	98	254.297
82	31.618	Germacrene-D	5570492	0.133	98	204.188
83	43.407	Valerenol	5558781	0.133	70	220.183
84	43.559	Isopropylidene	5353049	0.128	42	218.167
85	49.673	Hexadecanoic acid	5318288	0.127	98	270.256
86	22.323	Pentylthiophene	5122791	0.123	83	154.082
87	54.547	1-Heptadecanol	5095930	0.122	95	256.277
88	46.066	Bicyclo[1310]hexadecan-2-one	4816216	0.115	55	236.214
89	13.595	dl-Limonene	4755721	0.114	99	136.125
90	36.129	α-Calacorene	4672444	0.112	38	200.157
91	51.906	Eicosane	4631346	0.111	96	282.329
92	28.997	Cycloisosativene	4487495	0.107	99	204.188
93	42.554	Tetradecanal	4420395	0.106	91	212.214
94	48.178	Cyclotetradecane	4361575	0.104	90	196.219
95	16.483	Benzene	4265136	0.102	96	132.094
96	22.075	Naphthalene, 1,2,3,4-tetrahydro	4132000	0.099	97	174.141
97	44.666	7,8-Dihydropyran	4116843	0.099	50	173.084
98	27.68	Benzene, 1,2,3,4-tetramethyl-	4066183	0.097	46	134.11
99	11.991	Furan, 2-pentyl-	3750856	0.090	91	138.104

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Continued Table 2. Chemical composition of the essential oil of *Acroptilon repens*

No.	RT (min)	Compound	Peak area	%	Quality	Mol Weight (amu)
100	32.019	Aromadendrene	3697814	0.089	99	204.188
101	33.189	Widdrene	3626118	0.087	83	204.188
102	13.423	Benzene, 1-methyl-4-(1-methylethyl)-	3546573	0.085	97	134.11
103	57.836	Docosane	3527160	0.084	94	310.36
104	17.214	Nonanal	3429151	0.082	91	142.136
105	56.277	4,4,6-Trimethyl	3326248	0.080	43	140.12
106	42.923	4,4-Dimethyl-3	3080628	0.074	89	202.172
107	63.294	Tetracosane	2968794	0.071	97	338.391
108	32.324	Bicyclo[311]heptane	2911317	0.070	60	204.188
109	43.311	4-Tetradecene	2856951	0.068	84	196.219
110	23.316	cis-3-Hexenyl isovalerate	2576062	0.062	72	184.146
111	43.19	2-Cyclopenten-1-one	2205731	0.053	90	164.12

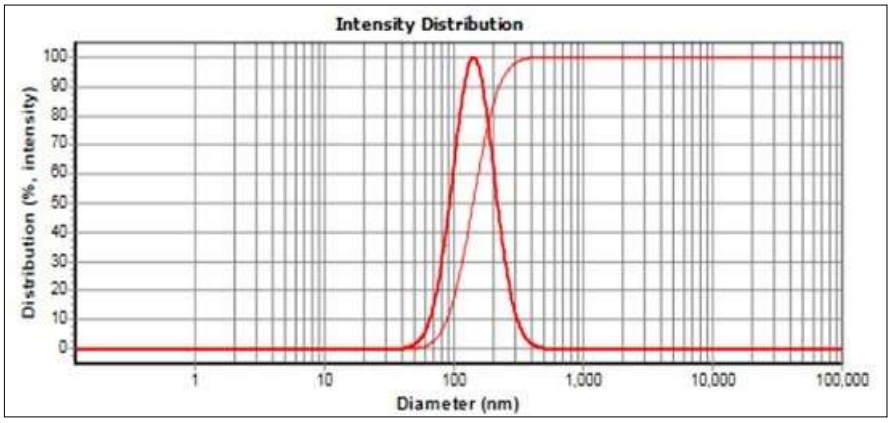

Fig. 3. DLS results of AEO nanoemulsion

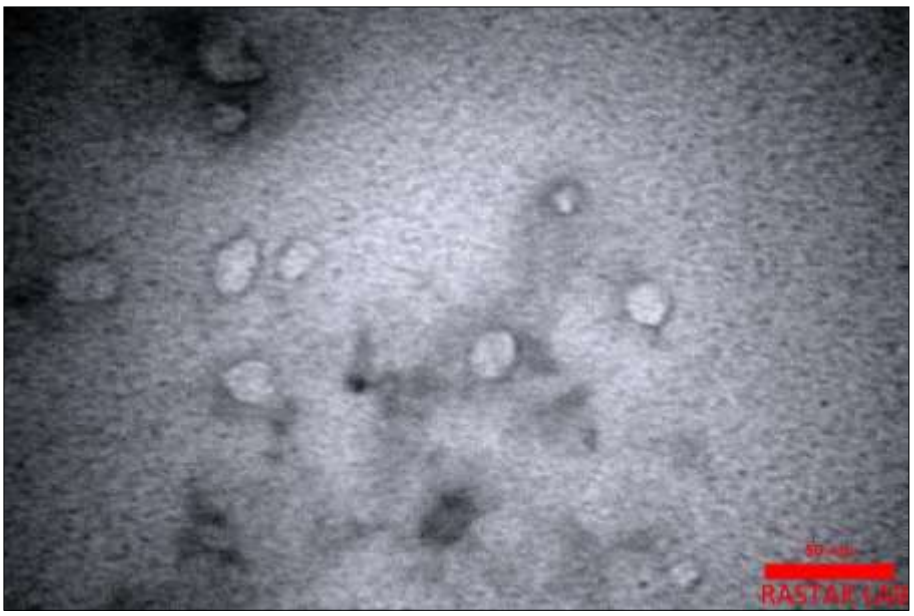

Fig. 4. Transition electron microscopy (TEM) image of AEO nanoemulsion

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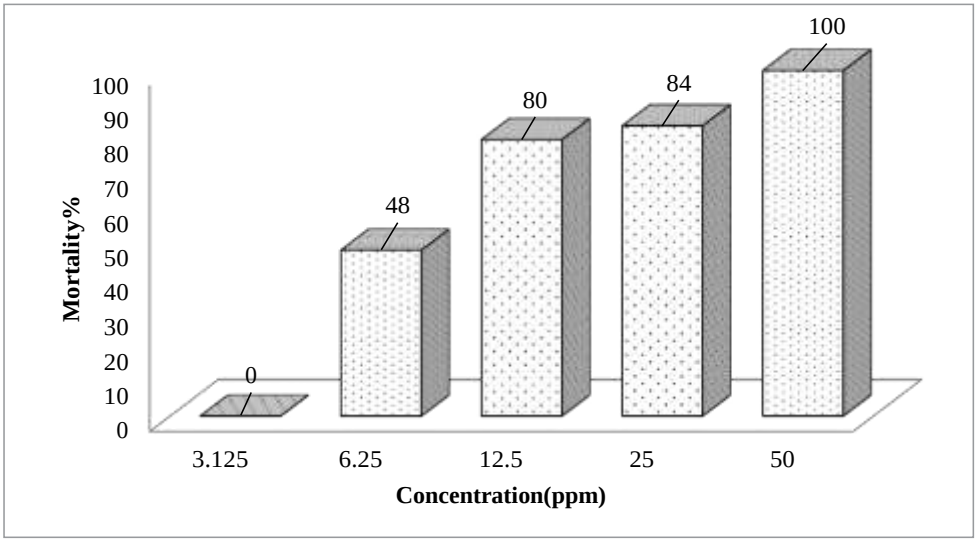

Fig. 5. Larvicidal activity of AEO against *Anopheles stephensi*

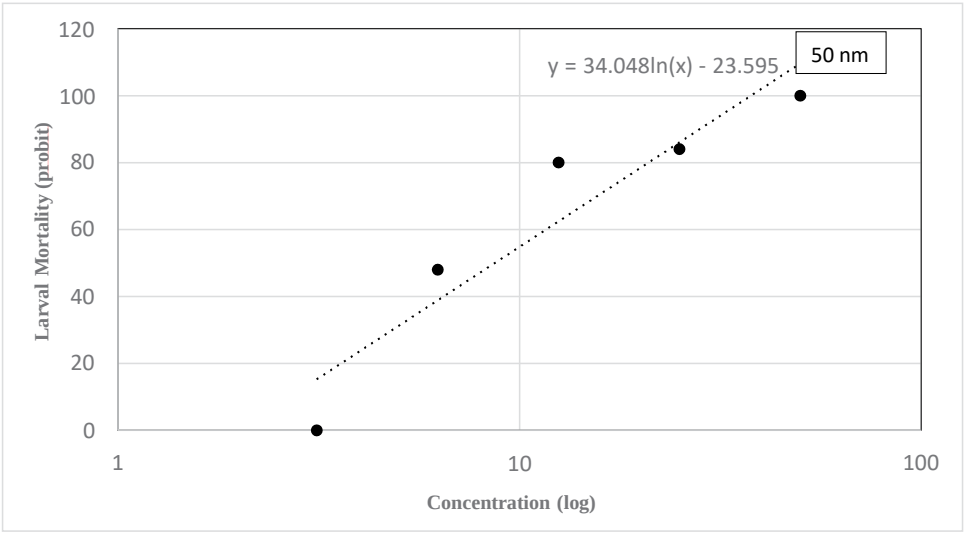

Fig. 6. Probit regression line of AEO against *Anopheles stephensi* larvae

Larvicidal effects of AEO and AEO nanoemulsion

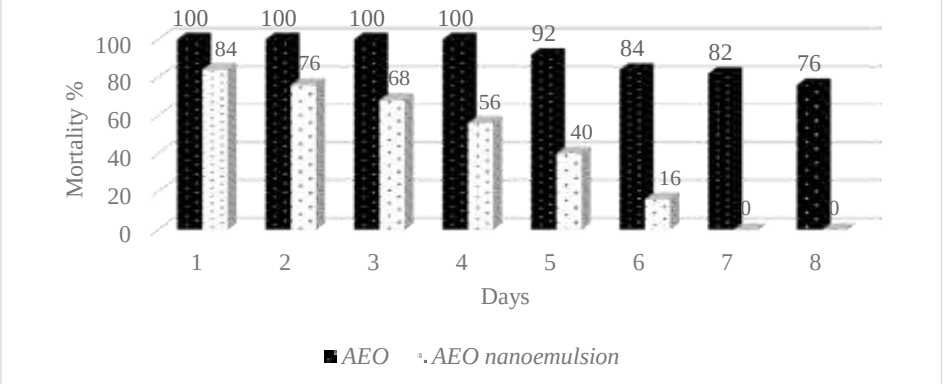

Fig. 7. Comparison of residual larvicidal effect of AEO vs. AEO nanoemulsion (after diluting 100 times) during an 8-day study

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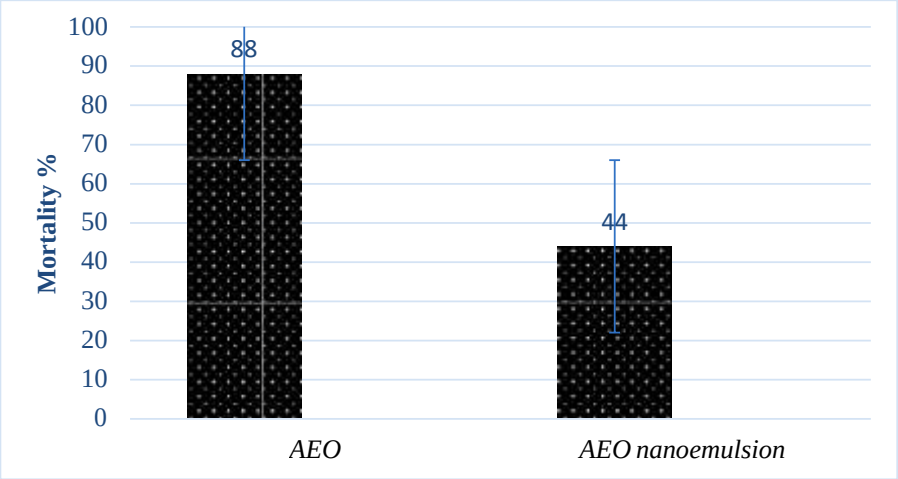

Fig. 8. Comparison of larvicidal activity of AEO vs. AEO nanoemulsion (after diluting 200 times) during an 24

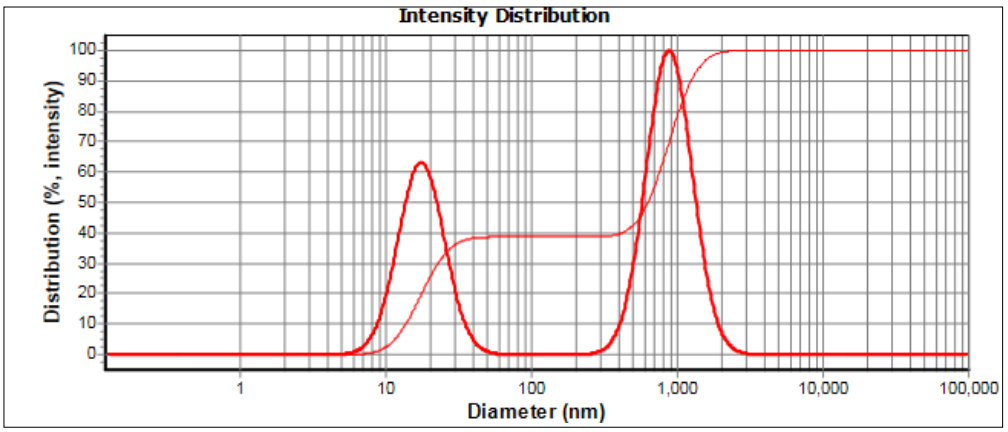

Fig. 9. DLS results of AEO nanoemulsion after dilution

Discussion

Today, associated with extensive use of various chemical pesticides, serious damages have been observed in the environment and non-target organisms which is being carefully considered by international organizations such as United States Environmental Protection Agency (USEPA), World Health Organization (WHO) and Food and Agricultue Organization (FAO) (25). In addition, frequent use of insecticides has led to their resistance for vectors (26). To reduce environmental damages and increase the effectiveness of insecticides on target organisms, the use of novel preparations such as nano-formulations has been suggested (10).

In this study the most components of AEO were identified in comparison with the similar studies. Total number of components in AEO in earlier studies varied from 11 to 77 compounds (27-32) while we were able to identify 111 components in the essential oil due to timely GC analysis. Our research showed LC_{50} and LC_{90} of AEO as 7 and 34 ppm against *An. stephensi*, respectively. Reviewing other reports have shown different values for other essential oils against *An. stephensi*. Depending on the obtained results, LC_{50} values are summarized as follows: LC_{50} < 10 ppm: 1 EO (*Kelussia odoratissima*), 10 ppm < LC_{50} < 50 ppm: 22 EOs (*C. zeylanicum* (11), *Ar. dracunculus*, *Platycladus orientalis*, *Tagetes patula*, *Ferulago carduchorum*, *Chloroxylon*

swietenia, Ipomoea cairica, Feronia limonia, Chloroxylon swietenia, Foeniculum vulgare, Satureja bachtiarica, Bunium persicum, Plectranthus amboinicus, Citrus aurantium, Plectranthus mollis, Achillea kellalensis, Citrus paradisi, Anethum graveolens, Achillea wilhelmsii, Zingiber nimmonii, Zingiber cernuum and *Blumea eriantha*) 50 ppm < LC_{50} < 100 ppm: 14 EOs (*Murraya exotica, Syzigium aromaticum, Zanthoxylum armatum, Zhumeria majdae, Origanum scabrum, Boswellia ovalifoliolata, Lavandula gibsoni, Origanum vulgare, Lawsonia inermis, Cionura erecta, Cupressus arizonica, Trachyspermum ammi, Eucalyptus camaldulensis, Coccinia indica*) and LC_{50} > 100 ppm: 9 EOs (*Kadsura heteroclita, Stachys byzantina, Heracleum persicum, Coriandrum sativum, Stachys setifera, Thymus vulgaris, Stachys inflata, Ajuga chamaecistus tomentella* and *Cedrus deodara*) (10).

According to proposed categories of larvicidal activity of plant essential oils against mosquito larvae, AEO lies in the third category as an active plant (33). In previous work, LC_{90} and LC_{50} of *A. repens* extract against *An. stephensi* were 0.37 ppm and 3.39 ppm, against *Culex pipiens* were 3.5 ppm and 60 ppm and against *Cx. quinquefaciatus* were 4 ppm and 39.7 ppm, respectively (22).

With the help of nano-techniques, the stability of essential oils in nature increases. Additionally, nanoproducts cause faster absorption in the target insect (25, 34). In a report, nanoemulsions of *Azadirachta indica* essential oil with different particle sizes (31, 93 and 251 nm) were prepared and tested against *Cx. quinquefasciatus*. The nanoemulsion with smallest particle size was found to be the most effective larvicidal agent (35). In another study, nanoemulsion of *Ar. dracunculus* essential oil was investigated against *An. stephensi*. The size of the prepared nanoemulsions was 12 to 291 nm. Similar to the above, larvicidal properties of the nanoemulsion increased significantly with decreasing droplet size (18). Previous studies had shown good results of new nanoformulations as larvicides (11), although all prepared nanoformulations had no similar effects. It is possible that different effects can be observed among different plant natural products and their formulations. In this experiment, the comparison has been made between the larvicidal activity of bulk essential oil and its nanoemulsion against one of the main vectors spreading malaria, *An. stephensi*. In this study, AEO showed complete mortality of larvae for up to 4 days, while its corresponding nanoemulsion failed to indicate 100% mortality even on the first day after diluting 100 times. Besides, the bulk preparation showed more larvicidal effect compared with the nanoemulsion after diluting 200 times (i.e. 88% vs. 44%). In a similar study, nanoemulsion of *Artemisia dracunculus* essential was broken or at least showed substantial changes in its nanostructures; it was not able to show a change in larvicidal activity of the essential oil (18). In another study, after dilution, by breaking nanostructure of *Anethum graveolens* essential oil, practically, no difference may be determined between nanoemulsion and bulk essential oil (36).

In total, our nanoemulsion preparation failed to show good efficacy compared with the bulk essential oil. To investigate the possible reason, we measured the particle size after 200 dilutions and found that the nanoemulsion breaks up after dilution. In other studies, nanoemulsions of essential oils have been tested against larvae. The results appear to be promising. For instance, nanoemulsion of *Copaifera duckei* (37), *Rosmarinus officinalis* (32) and *Ocimum basilicum* (38) have shown potential against *Ae. aegypti* however, considering the reports, the nanoemulsions have not been diluted 100 or 200 times (as recommended by WHO). Additionally, in these studies, the results of nanoemulsions have not been compared with the bulk essential oils. It is arguable that by performing the studies similar to ours, the nanoemulsions would not indicate positive results. Based on the result of the current study, difficulty in obtaining AEO and the negative larviciding results, we therefore do

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not recommend considering AEO as a good candidate for the next studies. However, it is suggested that for performing the larvicidal studies, nanoemulsions which are stable after 200 dilutions, should be considered.

Different extractions of the following Iranian native plants were evaluated against main malaria vector, *An. stephensi*, such as *Mentha spicata*, *Cymbopogon olivieri*, *Azadirachta indica*, *Melia azedarach*, *Tagetes minuta*, *Calotropis procera*, *Eucalyptus camaldulensis*, *Cupressus arizonica*, *Thymus vulgaris*, *Lawsonia inermis*, *Cedrus deodara*, *Cionura erecta*, *Bunium persicum*, *Carum carvi*, *Artemisia dracunculus*, *Rosmarinus officinalis.* (39-44). World Health Organization recommended several biological and chemical insecticides for mosquito larval control including: *Bacillus thuringiensis* H-14, *B. spahericus*, Chlorpyrifos, Chlorp-yrifos-methyl, Deltamethrin, Diflubenzuron, Etofenprox, Fenitrothion, Fenthion, Fuel oil, Malathion, Methoprene, Permethrin, Phoxim, Pirimiphos-methyl, Pyriproxyfen, Temephos, and Triflumuron (45). Monitoring and mapping of insecticide resistance is appr-opriate measure for vector control.

Conclusion

The larvicidal effects of AEO compared to its nanoformulation against *An. stephensi* larvae reported. According to the LC_{50} and LC_{90} of AEO, it is considered an active natural product. However, the prepared nanoemulsion did not show even equal efficacy in comparing with AEO, probably due to instability after 200 times dilution. Use of nanoemulsions with better stability profiles or other types of nanoparticles such as polymeric ones may be suggested. Furthermore because of the increasing importance of these alternative larvicides for vector control, the study and screening of native plant natural products should not be neglected.

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Conflict of Interest

The authors declare that there is no conflict of Interest.

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