Preparation of a Nanoemulsion of Essential Oil of Acroptilon repens Plant and Evaluation of Its Larvicidal Activity against 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 cleevenger 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 LC90 and LC99 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
**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). The use of natural products is an interesting 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 quinquefasciatus* 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 (45.08° E, 37.55° N, elevation ~ 1332 m above sea level) (Fig. 1).
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](http://jad.tums.ac.ir)
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**

| Initial temperature (°C) | 40          |
| Initial time (min)       | 1           |
| Program rate (°C/min)    | 3           |
| Final temperature (°C)   | 270         |
| Final time (min)         | 10          |
| Split ratio (ml/min)     | 100         |
| Septum purge (ml/min)    | ---         |
| Flow rate (ml/min)       | 1           |

Temperate Program

http://jad.tums.ac.ir

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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 dilute 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% (LC50 and LC90) 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-Cubeene (12.054%), 1-Heptadecene (5.181%), delta-Cadinene (3.771%) and β-Cubeene (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 LC50 and LC90 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 nanouemulsion after 200 times dilution, showing instability for the preparation after dilution.
| 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-Cubebeene | 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-Cubebeene | 157435883 | 3.771 | 97     | 204.188         |
| 6   | 31.287   | Caryophyllene | 155313108 | 3.720 | 99     | 204.188         |
| 7   | 28.316   | α-Cubebeene | 148603368 | 3.559 | 98     | 204.188         |
| 8   | 38.858   | 3-Unsocene | 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     | 284.201         |
| 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]Naphthylidine-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 | 45385061 | 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  | 33.945   | δ-Cadinene | 29461311 | 0.706 | 80     | 204.188         |
| 34  | 32.922   | Bicyclo[221]heptane | 27543459 | 0.660 | 98     | 204.188         |
| 35  | 36.641   | Tricosane (CAS) | 25909857 | 0.621 | 98     | 324.376         |
| 36  | 35.212   | δ-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     | 300.438         |
| 45  | 67.226   | Benzenedicarboxylic acid | 18814084 | 0.451 | 91     | 211.012         |
| 46  | 30.925   | Methanazulene | 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         |
### 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   | Cycloisosativeness | 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   | Heneicosene | 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 dihydro 1 16 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   | Germacrane-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   | a-Calacorene | 4672444 | 0.112 | 38 | 200.157 |
| 91  | 51.906   | Eicosane | 4631346 | 0.111 | 96 | 282.329 |
| 92  | 28.997   | Cycloisosativeness | 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-Dihydroxyran | 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 |
### 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[3.1.1]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
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
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 Agriculture 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. dracaunculus, Platycladus orientalis, Tagetes patula, Ferulago carduchorum, Chloroxylon

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
In this study, AEO showed that nanoemulsions would not indicate positive results, 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 oil 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).

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$_{50}$ and LC$_{90}$ 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. quinquefasciatus* were 4 ppm and 39.7 ppm, respectively (22).

With the help of nano-techniques, the stability of essential oils in nature increases. Additionally, nano-products 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. pipiens*. 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 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 oil 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
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. sphaericus, Chlorpyrifos, Chlorpyrifos-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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