Original Article

Evaluating Natural Larvicides: Peppermint and Pepper Extracts versus Temephos on *Aedes aegypti* and *Anopheles stephensi* Larvae Under Laboratory Conditions

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Abstract

Background: *Anopheles stephensi* is an important vector for malaria, while *Aedes aegypti* transmits dengue, chikungunya, Zika, and yellow fever. With the increasing replacement of natural insecticides for conventional ones, it is essential to investigate mosquito resistance to these insecticides and assess the larvicidal potential of new alternatives and their comparison to standard larvicides like temephos.

Methods: The alcoholic extracts of *Mentha piperita* and *Capsicum annuum* were prepared using the maceration method. Mosquitoes were bred at the Bandar Abbas research station in 2024. Biometric tests were performed following the World Health Organization protocol, and the data were analyzed using SPSS version 27 and GraphPad Prism 10.

Results: The lethal concentration 50% (LC_{50}) of *M. piperita* extract was 4.047 ppm against *An. stephensi* larvae and 9.9 ppm against *Ae. aegypti* larvae. Similarly, the LC_{50} of *C. annuum* extract was 5.872 ppm for *An. stephensi* larvae and 11.752 ppm for *Ae. aegypti* larvae. The larvicidal values of temephos were found to be 0.003 ppm against *An. stephensi* larvae and 0.002 ppm against *Ae. aegypti* larvae.

Conclusion: *Mentha piperita* and *C. annuum* extracts possess measurable larvicidal activity against *An. stephensi* and *Ae. aegypti*. However, their effectiveness remains substantially lower than that of temephos. These findings should be considered preliminary evidence rather than an indication of operational readiness. These extracts may represent a promising starting point for future research, but further studies on formulation, environmental persistence, non-target impacts, and field performance are required before use for management programs.

Keywords: *Mentha piperita*; *Capsicum annuum*; Extract; Essential oil; Larvicide

Introduction

Aedes aegypti mosquitoes feed on mammals but show a preference for humans over other hosts (1). Viruses of certain diseases, such as dengue, chikungunya, yellow fever and Zika, are transmitted by this species. Dengue virus (DENV) is the most common arbovirus in the world (2). The above mentioned diseases are endemic in more than a hundred tropical and subtropical regions of the world. Depending on the greenhouse gas emission scenario, it is predicted that by the end of this century, this vector will increase by 10% in the most optimistic case and 30% in the most pessimistic case (3). Studies predict that the development of this vector will accelerate after 2050. It could reach a level where it exceeds 30% of the current value (4). Trade in livestock, goods and human movement between Iran and countries

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where *Ae. aegypti* occurs has increased the risk of transmission and infection of DENV and chikungunya virus (5, 6).

According to reports from the national communicable disease surveillance system in Iran, from March 21, 2025, to January 3, 2026, a total of 1,186 patients with dengue fever were identified in the country. In 2024, a total of 1,126 dengue cases and five cases of chikungunya were reported in Iran and so far, no cases of Zika virus have been detected. Regarding the vectors, *Ae. aegypti* mosquitoes were first detected in Bandar Lengeh, Hormozgan Province, in 2022 and are currently present in the provinces of Hormozgan, Sistan and Baluchestan, Bushehr, Fars and Kerman. *Aedes albopictus* has also been reported in the provinces of Guilan, Mazandaran, Ardebil, Qazvin, Zanjan and East Azerbaijan (7).

Aedes aegypti lives in urban areas and its bite, resting place and egg-laying are inside and outside residential areas. It also lays its eggs mainly in man-made containers in or near residential areas (8). *Aedes aegypti* feeds on blood, mainly in the early morning and at sunset before dark. This vector bites several people for a full-blood meal. This behavior occurs during each gonotrophic cycle, increasing disease transmission potential and can increase the risk of chikungunya, dengue and Zika transmission in areas where *Ae. aegypti* is present (9).

The latest WHO report on malaria shows 263 million cases and 597 000 malaria deaths worldwide in 2023. This represents about 11 million more cases in 2023 compared to 2022, and nearly the same number of deaths (10). According to annual reports, the most important malaria cases are imported to the south and southeast of Iran due to migration from Pakistan and Afghanistan and most positive cases are reported from rural areas.

The southern provinces of Sistan and Baluchestan, Hormozgan and Kerman have long been major malaria hotspots, accounting for the majority of reported cases. In 2018 and 2019, thanks to national malaria control efforts, no locally transmitted cases were reported. However, a cross-sectional study of 8,389 confirmed malaria cases between 2016 and 2022 revealed that approximately 77% occurred in southern regions, with *Plasmodium vivax* being the predominant species among Iranian patients. Since 2019, malaria incidence has been on the rise, peaking in 2022 and highlighting the need for stronger health system preparedness. Alarmingly, Sistan and Baluchestan experienced a more than fivefold increase in cases between 2022 and 2023. Environmental and climatic factors, especially the floods in Pakistan (2022) and Iran (early 2024), have expanded mosquito habitats and accelerated malaria transmission (11).

So far, 30 species of *Anopheles* have been reported from Iran. The main malaria vectors in southeastern Iran are *An. stephensi*, *An. culicifacies* s.l., *An. fluviatilis* s.l., *An. dthali*, while the vectors in the northwest of the country are *An. sacharovi* and *An. maculipennis*, and *An. superpictus* s.l. has been reported as a malaria vector from all malaria foci in Iran (12).

Anopheles stephensi commonly resides near human settlements in livestock areas and feeds indoors (endophilic and endophagous) (13). Studies of *An. stephensi* breeding sites in Africa show that the species thrives in a variety of stagnant-water habitats, including containers, ditches, wells, ponds and both small artificial reservoirs (such as household water tanks and garden cisterns) and larger structures that may also support *Aedes* mosquitoes (14, 15). Factors like temperature (16) and rainfall (17, 18) can change mosquito growth or human and parasite behavior, affecting malaria (19).

There are growing concerns regarding the environmental and health impacts of chemical insecticides. Major chemical groups such as organochlorines, organophosphates, carbamates and pyrethroids are increasingly restricted for these reasons (20). Studies show that pesticides can cause cancer in both direct and indirect users. Some insecticides harm the nervous, kid-

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ney, breathing and reproductive systems in both men and women (21). Pesticides also harm water, soil and air, leaving chemicals in the food chain, reducing biodiversity and nitrogen fixation, damaging marine life and birds, and causing genetic issues in future generations (21, 22). Moreover, the widespread and prolonged use of chemical insecticides has led to the development of resistance in vector populations, particularly in mosquitoes such as *Anopheles*, *Aedes* and *Culex* species. This resistance compromises the effectiveness of vector control programs and contributes to the re-emergence of vector-borne diseases. To address these challenges, alternative approaches are being explored, including ecological methods based on plant-pest-predator interactions (23).

Mentha piperita contains diverse phytochemicals, including terpenoids and phenolic compounds, which contribute to its antimicrobial, anticancer and anti-inflammatory activities (24–30). Due to these properties, *M. piperita* holds potential to create new food, cosmetic and pharmaceutical products (31). *Capsicum annuum*, rich in capsaicinoids, carotenoids and phenolic compounds, exhibits antioxidant, antimicrobial and other pharmacological effects (32).

Temephos (O, O, O′, O′-tetramethyl O, O′-thiodi-p-phenylene bis (phosphorothioate)), a widely used organophosphate larvicide, is effective but poses potential health and environmental risks, including acetylcholinesterase (AChE) inhibition and possible genotoxicity (33).

Consequently, exploring plant-based larvicides offers a potential complementary approach for environmentally sustainable mosquito control strategies.

Materials and Methods

Plant material and extraction

Dried leaves of *M. piperita* were obtained from Isfahan and fruits of *C. annuum* were acquired from Tehran. The extracts of these plants were provided in the pharmacognosy laboratory, Faculty of Pharmacy, Tehran University of Medical Sciences, by the following method. About 167 g of dried *M. piperita* and 200 g of *C. annuum* were transferred separately into the decanter funnel of the percolator device. 2300 mL of pure 70% ethanol was added to the Buchner funnel containing *M. piperita* and 900 mL of 70% ethanol was added to the funnel containing *C. annuum*. The solvent height was about 5 cm above the surface of the crushed plant. After 24 hours, the ethanol was drained and collected in the tray. This operation was repeated 3 times to collect the entire extract. After collecting the material extracted from the decanter funnel in the tray, it is placed under the hood to dry completely. The prepared extract was diluted using water as a solvent, and after making different concentrations, it was used for bioassay.

Rearing of mosquito larvae

Mosquitoes were reared at the Bandar Abbas research station in 2024. *Anopheles stephensi* larvae were reared from a laboratory colony maintained under controlled conditions: 27±2 °C, 60% relative humidity and a 12:12-hour light: dark photoperiod. Adult mosquitoes were kept in metal cages (35×35×35 cm) with a mesh cover and 10% sucrose solution on tissue paper was placed to feed them. Female mosquitoes were fed blood from guinea pigs twice a week. Larvae were reared in trays (35×20×7 cm) filled with tap water and covered with lace and fed with fish food (TetraMin®) until they became pupae. Pupae were collected and moved to rearing cages for emerging as adults. Small cups with 100 mL of water were provided in the cages for egg-laying and the eggs were later moved to trays containing dechlorinated water for hatching.

Aedes aegypti larvae (Bandar Abbas strain) were bred from a lab colony. The breeding conditions were 26±2 °C, 65−67% humidity and a photoperiod of L: D of 12:12 hours. Adult mosquitoes were kept in metal cages (50×50×50 cm)

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covered with a fine mesh and given 10% sucrose solution on paper towels. Female mosquitoes were blood-fed by direct feeding on a rabbit twice a week. An ovitrap was used for egg collection. The ovitrap was a black container with a total volume of approximately 1.5−2 liters. It contained filter paper and two-thirds of the container was filled with water prepared with fermented hay extract as an attractant and placed in the adult cage for 48 hours. The egg paper was subsequently removed, air-dried for four days and stored in large plastic trays (35×20×7 cm) with dechlorinated water, covered with a net. After 48−72 hours, the paper was removed and chopped fish food was used to feed the larvae. The water temperature was 27 °C. Larvae hatched within 6−12 hours and their development time ranged from 7 to 23 days depending on temperature, food and density.

Bioassay tests

The WHO standard method was used for bioassay tests (34). Tests were conducted on late 3rd and 4th instar larvae. All larvae used in the bioassay tests were appropriately fed before testing to prevent starvation bias and had no previous exposure to any insecticides. The lab temperature (28 °C), test period (24 hours) and number of larvae (25 per 400 mL beaker) were kept constant. In this experiment, temephos was used as a positive control and 70% alcohol was used as a negative control.

The plant extract was diluted with ethanol 70% prepare a 1.0% (w/v) test solution. A hydroalcoholic extract uses a mixture of water and alcohol (usually ethanol) as its solvent (35). According to the larval susceptibility assessment guideline, for each beaker, 99 mL of water and 1 mL of plant extract or temephos were used for *Ae. aegypti* (36, 37), while 249 mL of water and 1 mL of plant extract or temephos were used for *An. stephensi* (37). *Mentha piperita* and *C. annuum* extracts were tested at concentrations of 2, 3, 4, 5 and 6 ppm against *An. stephensi* and 7, 8, 9 and 10 ppm against *Ae. aegypti*. Temephos was evaluated at concentrations of 0.0012, 0.0024, 0.0048, 0.0097 and 0.0195 ppm for *An. stephensi* and 0.0025, 0.005, 0.0125 and 0.03 ppm for *Ae. aegypti*. The concentration with 50% mortality, along with two concentrations above and below it, was used to create the regression line. For each test, 4−5 larvicide concentrations were used, with four replications per concentration and two controls included.

Statistical analysis

The test results after 24 hours were recorded, including the number of live, dead and moribund larvae, as well as the total count. These results were used to create mortality rates. The lethal concentrations for 50% (LC_{50}) and 90% (LC_{90}) of the extracts and temephos, along with a 95% confidence interval, were calculated using probit regression analysis based on Finney's method (38). Test data were considered valid when control mortality was below 5%. If control mortality ranged from 5% to 20%, mortality rates were corrected using Abbott's formula. Tests with control mortality exceeding 20% were discarded (39).

Results

The larvicidal effects of *M. piperita* and *C. annuum* hydroalcoholic extracts, along with temephos, were evaluated against late third and early fourth instar larvae of *An. stephensi* and *Ae. aegypti*. Mortality data after 24 hours of exposure were analyzed using probit regression and dose-response curves were created for each treatment (Figs. 1–6). A clear dose-dependent mortality pattern was observed in all treatments, with higher concentrations resulting in more larval mortality. The LC_{50} and LC_{90} values, slope ± SE and R^2 values are summarized in Tables 1 and 2.

For *An. stephensi*, the LC_{50} values were 4.0047 ppm for *M. piperita*, 5.872 ppm for *C. annuum* and 0.003 ppm for temephos. The LC_{90} values followed a similar trend, at 6.280

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ppm, 8.296 ppm and 0.013 ppm, respectively. The regression models showed high goodness-of-fit for *M. piperita* (R^2= 0.984) and temephos (R^2= 0.988), indicating strong dose-response relationships, while *C. annuum* had a lower R^2 of 0.639, suggesting more variability in response.

For *Ae. aegypti*, *M. piperita* and *C. annuum* exhibited LC_{50} values of 9.9 and 11.752 ppm, respectively, while temephos showed a markedly lower LC_{50} of 0.002 ppm. The LC_{90} values for these treatments were 12.777, 21.414 and 0.007 ppm, respectively. Here, the regression lines also demonstrated good fit (R^2= 0.768 for *M. piperita*, 0.916 for *C. annuum* and 0.870 for temephos). Statistical analysis confirmed the suitability of the probit model for all data sets, with p-values from goodness-of-fit tests exceeding 0.15, indicating no significant heterogeneity in the mortality data.

Comparative evaluation showed that temephos was significantly more potent than the plant extracts, requiring much lower concentrations to achieve similar mortality levels. However, among the two botanical extracts, *M. piperita* consistently outperformed *C. annuum* against both mosquito species. The regression lines, along with the slope values, further indicated the steepness of the dose-response curve. Temephos exhibited the shallowest slope, indicating a more gradual increase in mortality across concentrations. In contrast, the steeper slopes observed with *M. piperita* suggest more rapid changes in larval mortality with increasing dose.

Table 1. Lethal concentrations and other associated statistics of bioassay tests of *Mentha piperita*, *Capsicum annuum* and Temephos against *Anopheles stephensi* larvae, 2024

Treatment	Intercept	Slope ± SE	LC_{50} (ppm)	95% CI (ppm)	LC_{90} (ppm)	95% CI (ppm)	R^2
Mentha piperita	0.59	7.252±0.030	4.047	3.864-4.237	6.280	5.845-6.895	0.984
Capsicum annuum	0.8372	4.879±0.055	5.872	4.1038-7.3875	8.296	5.407-12.102	0.654
Temephos	10.349	2.107±0.099	0.003	0.002-0.003	0.013	0.010-0.017	0.988

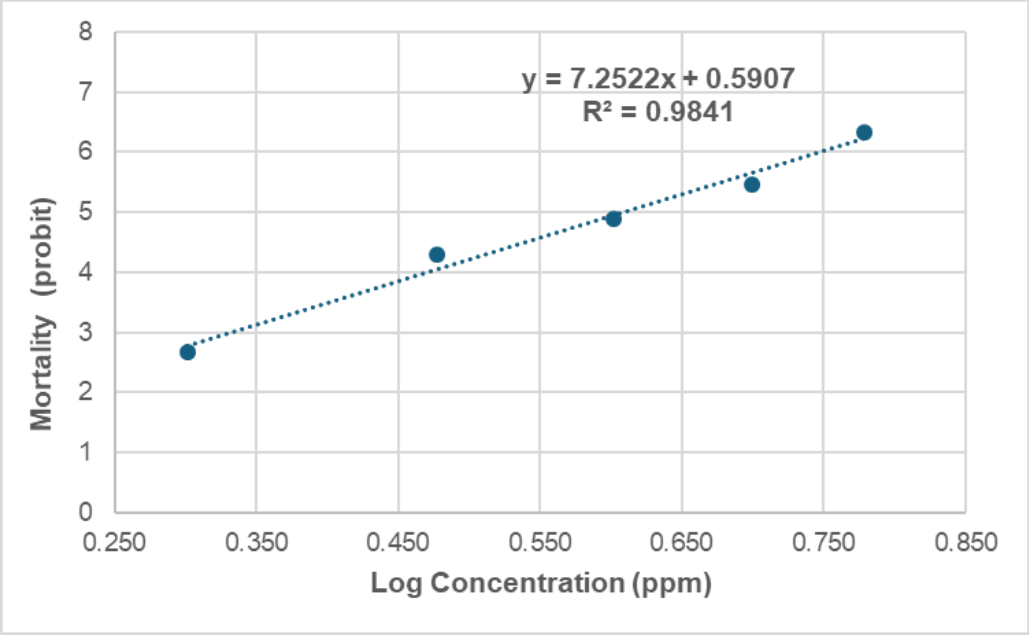

Fig. 1. Diagram, formula, regression line and R^2 for bioassay tests of *Mentha piperita* extract against *Anopheles stephensi* larvae, Bandar Abbas lab strain, 2024

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Published Online: December 30, 2025

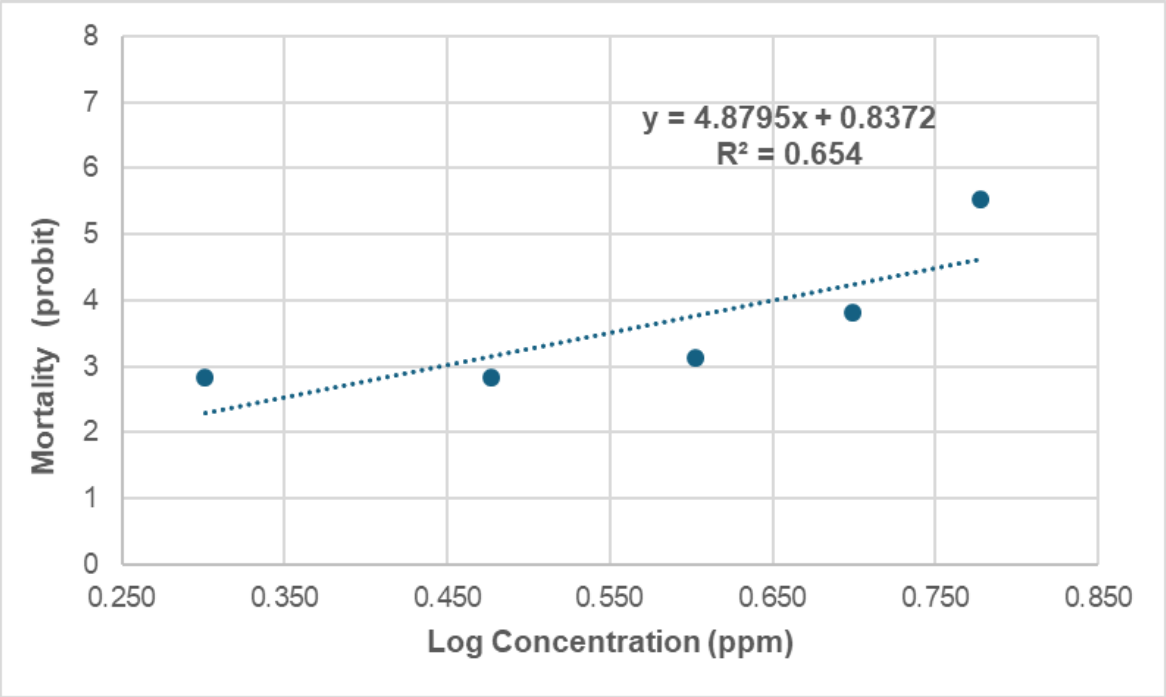

Fig. 2. Diagram, formula, regression line and R^2 for bioassay tests of *Capsicum annuum* extract against *Anopheles stephensi* larvae, Bandar Abbas lab strain, 2024

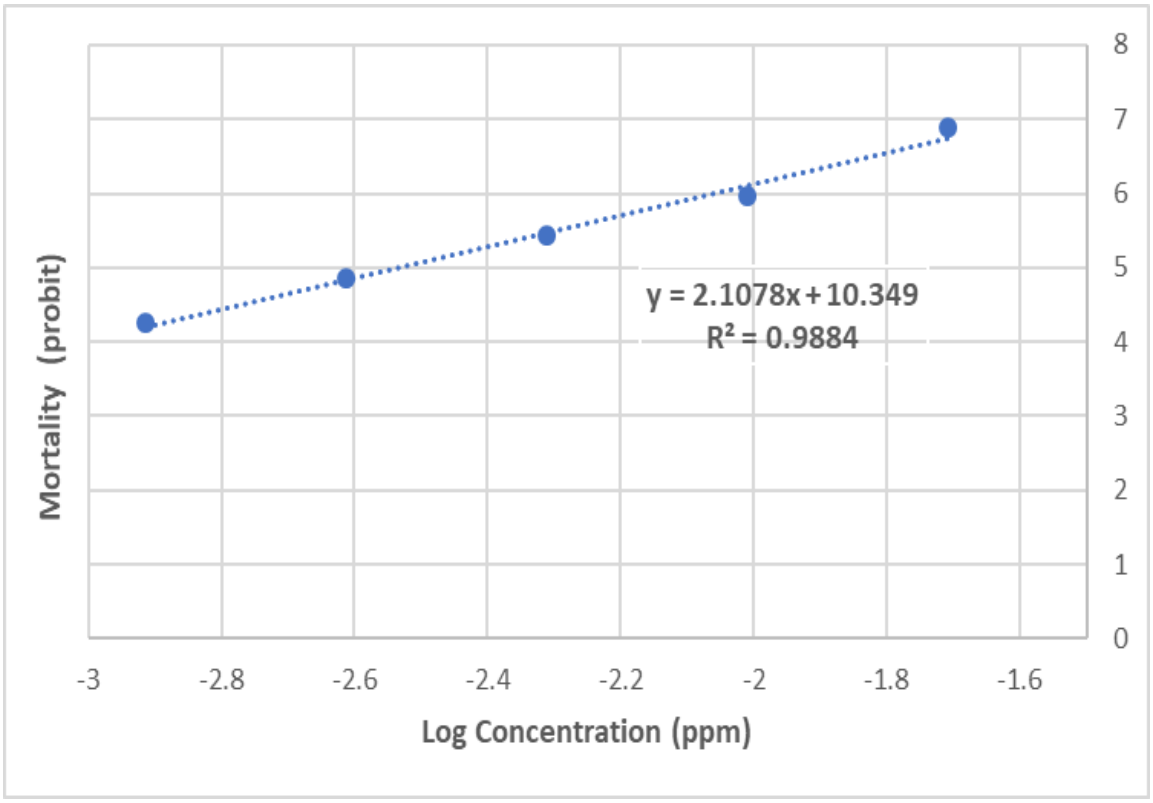

Fig. 3. Diagram, formula, regression line and R^2 for bioassay tests of Temephos against *Anopheles stephensi* larvae, Bandar Abbas lab strain, 2024

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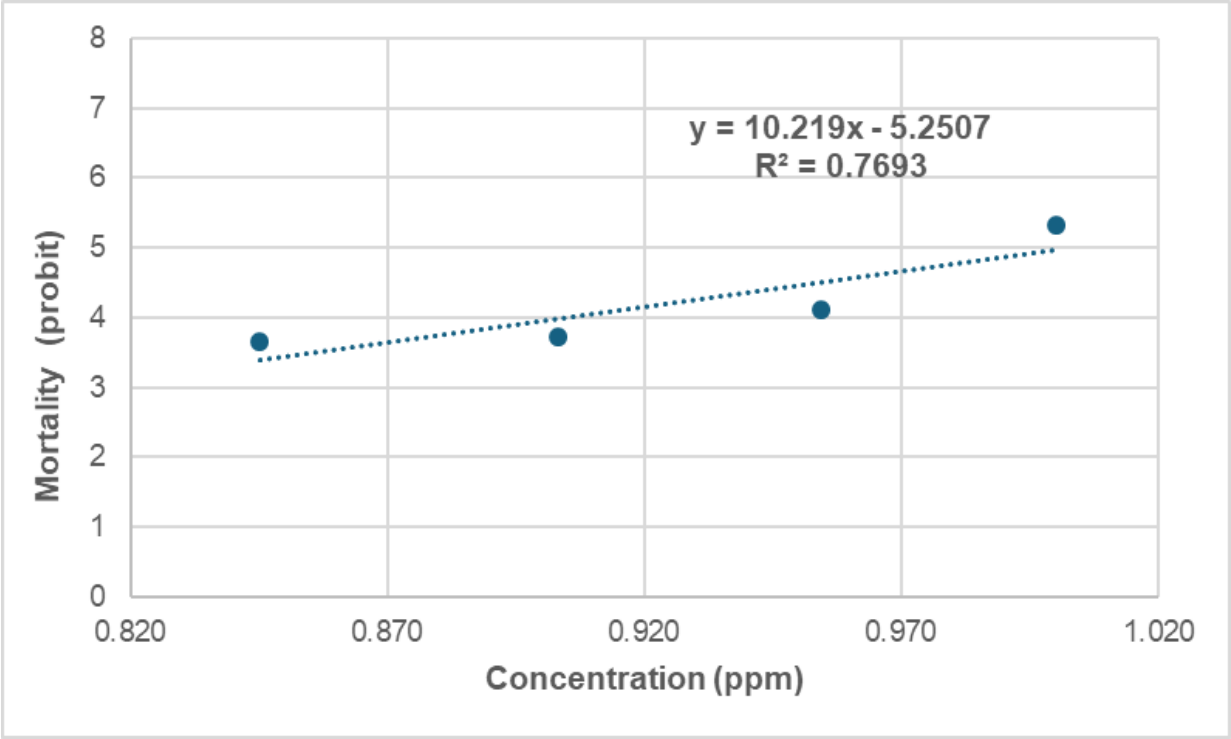

Fig. 4. Diagram, formula, regression line and R^2 for bioassay tests of *Mentha piperita* extract against *Aedes aegypti* larvae, Bandar Abbas lab strain, 2024

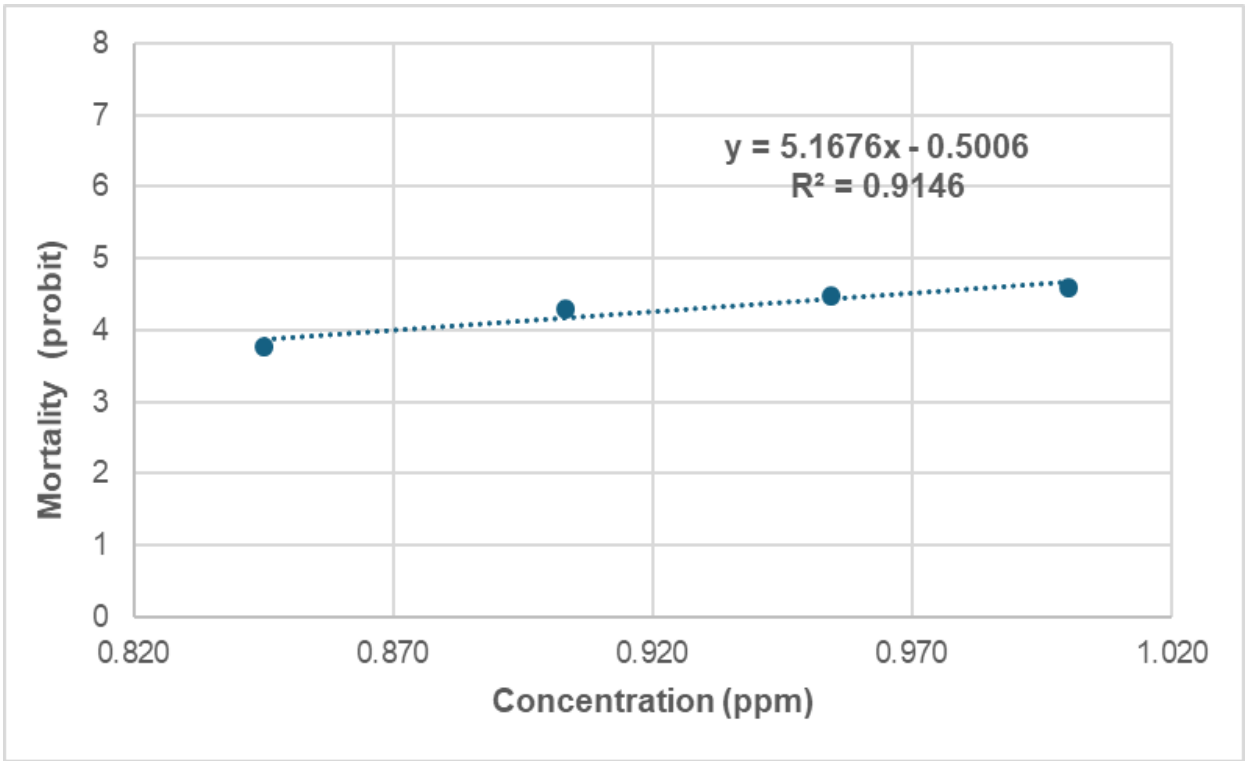

Fig. 5. Diagram, formula, regression line and R^2 for bioassay tests of *Capsicum annuum* extract against *Aedes aegypti* larvae, Bandar Abbas lab strain, 2024

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Published Online: December 30, 2025

Table 2. Lethal concentrations and other associated statistics of bioassay tests of *Mentha piperita*, *Capsicum annuum* and Temephos against *Aedes aegypti* larvae, 2024

Treatment	Intercept	Slope ± SE	LC_{50} (ppm)	95% CI (ppm)	LC_{90} (ppm)	95% CI (ppm)	R^2
Mentha piperita	-5.251	10.219±0.023	9.9	8.090-10.144	12.777	11.277-14.050	0.769
Capsicum annuum	-0.501	5.167±0.043	11.752	10.462-16.191	21.414	15.752-52.854	0.914
Temephos	10.747	2.161±0.144	0.002	0.0012-0.0047	0.007	0.003-0.006	0.879

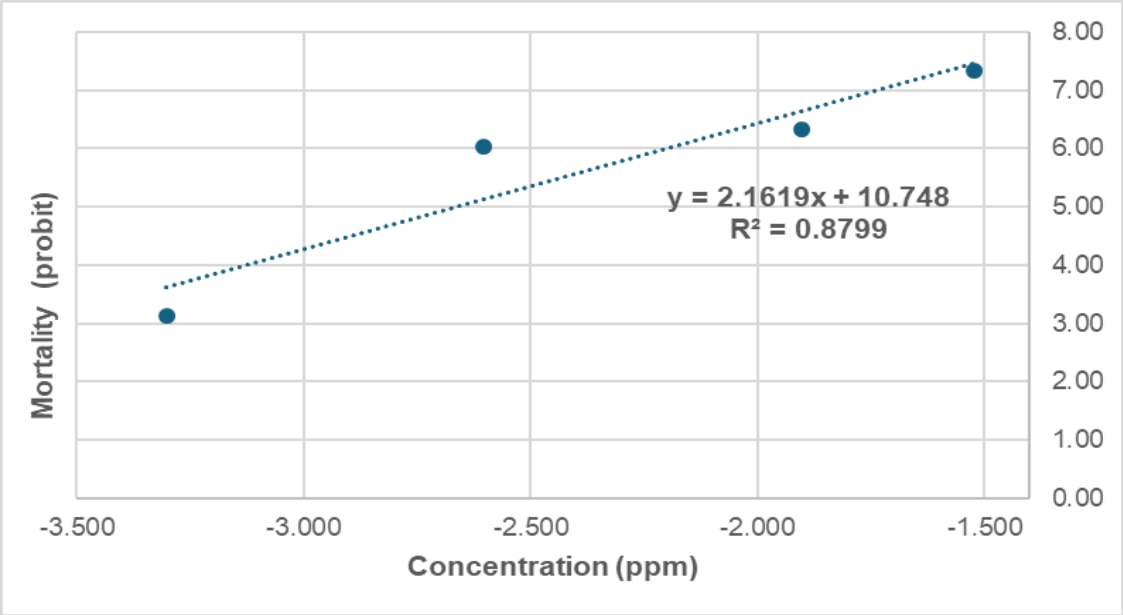

Fig. 6. Diagram, formula, regression line and R^2 for bioassay tests of Temephos extract against *Aedes aegypti* larvae, Bandar Abbas lab strain, 2024

Discussion

In the present study, the larvicidal activity of hydroalcoholic extracts of *M. piperita* and *C. annuum* was evaluated against late third and fourth-instar larvae of *Ae. aegypti* and *An. stephensi*. Temephos was used as the standard larvicide for comparison. The results showed that temephos exhibited the highest toxicity at very low concentrations, with LC_{50} values of 0.002 ppm for *Ae. aegypti* and 0.003 ppm for *An. stephensi*. These values are consistent with previous studies. For instance, Vatandoost and Hanafi-Bojd (40) reported LC_{50} values of 0.00339 mg/L for field strains and 0.00161 mg/L for laboratory strains of *An. stephensi* collected from Bandar Abbas. The LC_{50} obtained in the current study (0.003 ppm) is closely aligned with that of the field strain, with minor differences likely due to population variations, environmental factors, or larval stage. Similarly, Abai et al. (41) reported a significantly higher LC_{50} value of 0.0523 ppm for late instar *An. stephensi*, potentially due to methodological differences or strain susceptibility. Baruah et al. (42) also reported higher LC_{50} and LC_{90} values (0.0148 ppm and 0.0472 ppm, respectively), which is expected given the high susceptibility of the insectary-reared larvae used in the present study, with no prior exposure to insecticides.

The LC_{50} for temephos against *Ae. aegypti* in this study was 0.002 ppm, notably lower than most reported values, indicating high sensitivity

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in the tested population. For example, Singh et al. (43) found LC_{50} values ranging from 0.007 to 0.018 ppm in eight regions of Delhi, while Biber et al. (44) reported values between 0.0017 and 0.0078 ppm in Argentina. Other studies, such as those by Polson et al. (45) in Colombia and Arslan et al. (46) in Pakistan, reported LC_{50} values considerably higher than those observed here. Asgarian et al. (2024) reported LC_{50}, LC_{90} and LC_{99} values of 0.013, 0.065 and 0.238 mg/L for *Ae. aegypti* larvae in Iran. The difference between these values and those obtained in our study may be partly attributed to variations in the timing of bioassays and the specific experimental conditions under which the tests were performed (47). These findings suggest that the *Ae. aegypti* population tested in this study is among the most sensitive to temephos reported to date. While this supports the continued effectiveness of temephos, ongoing resistance monitoring remains essential. Regarding botanical larvicides, the hydroalcoholic extract of *M. piperita* showed an LC_{50} of 9.9 ppm against late instar *Ae. aegypti*. In comparison, Kumar et al. (48) reported LC_{50} and LC_{90} values of 11.9 and 29.18 ppm using essential oil, suggesting that the hydroalcoholic extract may be more effective. Similarly, Aljameeli (49) also evaluated peppermint oil, further supporting its potential as a botanical larvicide. The essential oil of *Mentha piperita* dissolved in n-hexane has previously shown LC_{50} and LC_{90} values of 21.38 ppm and 50.23 ppm, respectively, considerably higher than the values obtained in the present study. Such differences may stem from the type of substance used (essential oil vs. hydroalcoholic extract), extraction method, solvent, plant chemotype, or the ecological background of the mosquito larvae. In this study, the LC_{50} of the hydroalcoholic extract of *C. annuum* against *Ae. aegypti* was determined as 11.752 ppm. To date, no direct study has assessed the larvicidal activity of this specific extract on *Ae. aegypti*. However, Onah et al. (50) in Nigeria reported LC_{50} and LC_{90} values of 567.844 ppm and 2991.191 ppm, respectively, using a methanolic leaf extract prepared via cold maceration. This substantial difference suggests a higher toxicity of the hydroalcoholic extract used in the current study, possibly due to differences in plant parts, solvent polarity, or extraction efficiency. In the same study, various plant extract combinations were tested. For instance, combining extracts of *Melissa officinalis* and *C. annuum* yielded an LC_{50} of 1414.893 ppm. Mixtures of *C. annuum* with *Citrus aurantifolia* or *Cymbopogon citratus* were either much less effective or inactive, indicating that the individual use of *C. annuum* hydroalcoholic extract in the current study resulted in more promising larvicidal activity. This study is also the first to evaluate the hydroalcoholic extract of *M. piperita* against *An. stephensi*, yielding an LC_{50} of 4.047 ppm. While the lack of prior studies limits direct comparison, the result indicates potential for this extract as a natural larvicide against this major vector. The LC_{50} of *C. annuum* extract against *An. stephensi* was found to be 5.872 ppm. Madhumathy et al. (51) reported a significantly higher LC_{50} of 110 ppm using an alcoholic extract of the same plant.

Such discrepancies could be attributed to variations in solvent type, extraction method, plant origin, harvest time and larval susceptibility. These findings demonstrate that although the tested plant extracts exhibit significant larvicidal activity, they generally require higher concentrations than temephos to achieve comparable effects. Nonetheless, their eco-friendliness, lower risk of resistance development, and local availability make them attractive options for integrated vector management (IVM) programs. In both mosquito species, *M. piperita* extract showed greater toxicity compared to *C. annuum*, likely due to differences in active compounds. This can be further elucidated through chemical profiling using GC-MS or HPLC analysis. Overall, the results indicate that hydroalcoholic extracts of *M. piperita* and *C. annuum* have promising larvicidal effects against *Ae. aegypti* and *An. stephensi*. Compared to most

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previous studies using essential oils or other extract types, the extracts in this study demonstrated stronger or more effective toxicity, potentially due to the hydroalcoholic extraction method, harvest time, plant part used, or the local susceptibility of mosquito populations. These findings underscore the need for further research to standardize and operationalize the use of botanical larvicides in vector control strategies. Plant-based larvicides face significant challenges due to their instability under environmental conditions such as light, heat, and oxidation. Nano formulation approaches may help overcome this issue by enhancing stability and efficacy. To better evaluate real-world efficacy, future studies should incorporate wild mosquito strains and be conducted under field conditions.

The present study aimed to evaluate the larvicidal effects of hydroalcoholic extracts of *M. piperita* and *C. annuum* in comparison with temephos against *Ae. aegypti* and *An. stephensi* larvae. The findings can contribute to the development of sustainable and environmentally friendly approaches for vector control. The use of plant-based compounds as potential alternatives to synthetic insecticides such as temephos is particularly valuable in regions where insecticide resistance has emerged, offering a promising strategy for resistance management and environmental safety. Economically, although temephos is currently available at a relatively low cost, its production involves complex chemical processes and dependence on imported raw materials. In contrast, the plant extracts used in this study have the potential for local production, cost reduction through large-scale cultivation and reduced reliance on external sources. Nevertheless, one of the major challenges in the application of plant extracts is their instability when exposed to environmental factors such as light, heat and oxidation, which may limit their efficacy under field conditions. Therefore, it is recommended that future studies explore the use of advanced technologies such as nanoformulations to enhance the stability, bioavailability and effectiveness of these compounds (52).

Conclusion

The results of this study support the potential use of *M. piperita* and *C. annuum* extracts as complementary larvicidal agents in integrated mosquito management programs, particularly where temephos resistance or environmental concerns limit synthetic insecticide use. By the way, since the current experiments were conducted on the insectary strains of mosquitoes, it is advisable to include field strains in subsequent studies to better assess the real-world effectiveness and generalizability of the findings.

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Ethical consideration

This study was approved by the Ethics Committee of the School of Public Health (SPH), Tehran University of Medical Sciences (TUMS) and registered under the code IR.TUMS.SPH.REC.1402.187.

Conflict of interest statement

The authors have declared that no competing interests exist.

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