Anal. Methods Environ. Chem. J. 6 (1) (2023) 69-78

AMECJ

Research Article, Issue 1
Analytical Methods in Environmental Chemistry Journal
Journal home page: www.amecj.com/ir

Chemical analysis of essential oils of Thymus Carmanicus Jalas by gas chromatography-mass spectrometry and toxicity activity against the major Iranian malaria vector, Anopheles Stephensi

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ARTICLE INFO:

Received 13 Nov 2022
Revised form 22 Jan 2023
Accepted 10 Feb 2023
Available online 28 Mar 2023

Keywords:
Chemical analysis,
Gas chromatography-mass spectrometry,
Thymus Carmanicus Jalas,
Essential oil,
Malaria

ABSTRACT

In the last few years, using chemical insecticides to control the malaria vector has caused environmental pollution and resistance to chemical insecticides. This study aimed to investigate the chemical analysis of essential oils of *Thymus carmanicus* Jalas by gas chromatography and mass spectrometry (GC-MS) and toxicity activity against the major Iranian malaria vector, *Anopheles stephensi*. The essential oil of Thymus carmanicus Jalas was prepared from dried leaves using the hydro-distillation method. Gas chromatography mass spectrometer (GC-MS) was used to analyze and identify thyme essential oil compounds. Bioassay was performed using World Health Organization (WHO) standard test. The *T. Carmanicus* Jalas essential oil consisted of 15 compounds, with Carvacrol (61%), Thymol (6%), and β-caryophyllene (5%) being the major components by volume. The LC_{50} and LC_{90} of thyme oil were 20.37 and 41.38 mg L^{-1} at 24h after application, respectively. At 24h after application, significant differences were observed between the toxicity of 5%, 20%, 25%, 40%, 50%, and 80% concentrations of Thyme essential oil ($P<0.05$). The 80% concentration of Thyme essential oil exhibited 100% toxicity against *A.stephensi* larvae at 24h after application. *T. Carmanicus* has a rich source of bioactive compounds for use as a mosquito larvicide.

1. Introduction

Malaria is among the most important parasitic diseases transmitted to humans by Anopheles mosquitoes. This disease has been reported in 91 countries worldwide [1]. Five Plasmodium species are transmitted by the bite of Anopheles mosquitoes [2]. More than 90% of malaria cases were reported in three southeastern provinces of Iran, including Sistan and Baluchistan, Kerman, and Hormozgan [3]. Out of the 476 Anopheles

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https://doi.org/10.24200/amecj.v6.i01.225

species identified in the world, 70 species are capable of transmitting malaria, and 40 species are known as the main vectors. In addition to malaria transmission, Anopheles could transmit filariasis and some arboviruses. In Iran, 7 Anopheles species are disease carriers, the most important of which is Anopheles stephensi, found in the southern regions [4]. Insecticide resistance has become a threat to the effectiveness of chemical vector control methods. This issue is of particular importance considering the malaria elimination program in Iran [5]. Therefore, special attention has been paid to plants as natural reservoirs with fewer side effects to fight against disease vectors. Currently, controlling mosquito larvae using larvicides is a major part of controlling mosquito-borne diseases. The most common larvicides contain organophosphorus compounds such as temephos, fenthion, and chlorpyrifos. However, their toxicity for aquatic organisms and the environment as well as the phenomenon of insecticide resistance and acute and chronic toxicity for humans are increasingly reported. Therefore, it seems necessary to find new larvicides from alternative sources such as plants [6, 7]. Herbal insecticides have been recently used to control vectors due to their relatively high efficiency, degradability, and lack of adverse effects on the environment. Several plant products have been reported as insecticides for mosquito control. Various vegetable oils have shown a wide range of insecticidal activity against pests. Also, these oils have anti-nutritional and repellent properties, reduce oviposition and disrupt the natural growth process of pests [7]. Thyme is among the important genera of the Lamiaceae family and has been widely used in food industry, pharmaceutical and cosmetic due to its various biological activities [8]. According to recent studies, Lamiaceae family consists of 4,000 plant species with 220 genera. Thyme, with 300-400 species, is among the most important species of this family. This genus includes 18 perennial and aromatic species that grow in different regions of the country. *Thymus caramanicus* is among the species of this genus [9]. Thyme branches contain essential oil, tannin, basic bitter substances, saponin and herbal disinfectants. The plant essential oil contains variable amounts of phenolic compounds such as thymol and carvacrol [10]. Various constituents of the oil of *T. carmanicus* were reported in previous studies. Although carvacrol is the most abundant constituent in the oils obtained from all the mentioned studies, percentage of the compound varied, based on the origins of the plant. While it has been reported as 47.55-96.2.7% from *T. carmanicus* cultivated in Kerman, 42.1-93.4 % in Isfahan, 42% in Shahroud, and 19.8-81.1% Semnan [11-15]. Generally, there are two devices including Soxhlet and Clevenger to essential oil extraction [16]. To analysis the composition of an essential oil sample, use Gas chromatography columns with different polarities [17]. The previous studies used several methods to essential oil analysis, such as Gas Chromatography and Mass Spectrometry (GC/MS) and capillary gas chromatography (GC) [18]. Netopilova et al. in 2021 used the GC– flame ionization detector (FID)/MS for analysis the *Origanum vulgare* and *Thymus vulgaris* [19]. In the present study, we aimed to investigate the chemical analysis of essential oils of *Thymus carmanicus* Jalas by GC-MS and also the toxicity activity of this essential oil was evaluated against the major Iranian malaria vector, *Anopheles stephensi* as an environmentally friendly method.

2. Materials and methods

2.1. Plant collection

The fresh leaves of the *Thymus carmanicus* Jalas plant were collected from the Hezar mountain (Kūh-e Hazār) located at 29° 30′ 42″ N and 57° 16′ 18″ E at 120 km from Kerman city in southeast of Iran (Fig. 1). Collected Thyme specimens were identified by the Department of Pharmacognosy in Kerman University of Medical Sciences.

Fig 1. Map of the location of collected Thyme specimens in Kerman province (southeast of Iran)

2.2. Extraction of eessential oil

To extract essential oils, 100 g of dried leaves of *Thymus caramanicus* was poured into a 1L flask [10]. Then, 600 cc of deionized water was added to the flask and then were subjected to hydrodistillation in a clevenger-type apparatus for 3.5 to 4 h. The essential oil was extracted approximately 3.5 to 4 h at 60 °C [20] (Fig. 2). Then, the extracts were exsiccated by anhydrous sodium sulphate and stored in a dark glass vial at 4 °C in a refrigerator for further experiment [11, 12].

2.3. Gas chromatographic-mass spectral analysis

Gas chromatography-mass spectrometer (GC-MS) was used for the analysis and identification of thyme essential oil compounds (Hewlett-Packard 6890, Agilent Technology, Santa Clara, California, USA) (Fig. 2). It is equipped with HP–5MS column (30 m× 0.25 mm× 0.25 µm). The initial temperature was 40 °C for 1 min and later was raised to 220 °C at a rate of 3 °C per min and finally raised to 270 °C for 5 min at a rate of 20 °C per minute. Other parameters of the GC-MC machine included carrier gas Helium (99/999%), injector temperature (260 °C), detector temperature (FID, 270 °C), split-less mode, the ionization potential of 70eV, scan rate of 1 scan per sec, the scan range of m/z 40–48 was used for all analysis. The essential oil constituents were identified by comparing their retention indices, and mass spectra fragmentation with those in a stored Wiley 7n.1 mass computer library and those of National Institute of standards and Technology (NIst) [21-23].

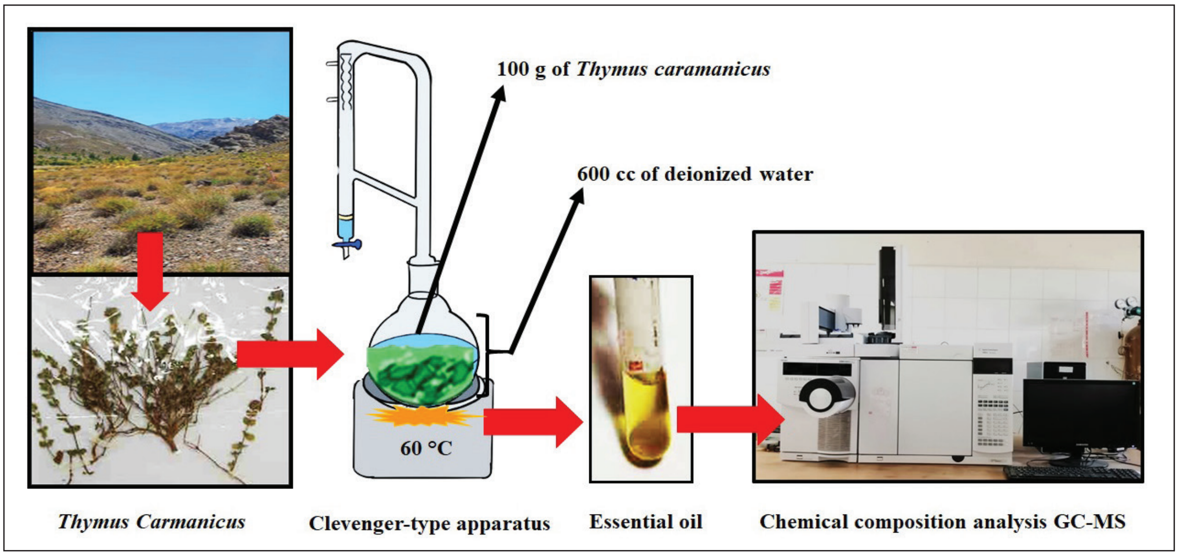

Fig. 2. The procedure of collecting plants, extracting and GC-MS analysis of essential oils.

2.4. Larvae collection and Toxicity assay

Anopheles stephensi larvae were collected from the Paykam area, Bam, south of Kerman Province. Bioassay was performed using World Health Organization (WHO) standard test [24]. Different concentrations (20, 40, 80, 160, and 320 mg L^{-1}) of the essential oils obtained from the studied plant were prepared using ethanol as the solvent. Thus, twenty-five of the 3rd or 4rd instar larvae of *Anopheles stephensi* were exposed to these concentrations in each 400 mL beaker. The experiments were replicated four times for any concentrations of thyme essential oil and ethanol.

2.5. statistical Analysis

Probit analysis was used to calculate the LC_{50} and LC_{90}. Toxicity indices were compared using analysis of (ANOVA) followed by the Dunnett test to distinguish between the treatments. All statistical analyses were performed using the SPSS version. 16. A p-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

Thyme essential oil was found to contain 15 compounds using GC-MS analyses. Carvacrol, thymol, and beta-caryophyllene had the highest frequency with 61.92%, 6.13%, and 5.55%, respectively. The most common compounds are shown in Table 1. In addition, the chemical analysis of the essential oil of *Thymus carmanicus* Jalas is shown in Figure 3.

Table 1. Constituents of *Thymus carmanicus* Jalas essential oil by GC-MS analyses.

Thyme essential oil components	Retention time	Major Constituents (%)
γ-cadinene	10.029	0.58
β-caryophylene	11.099	5.55
Copaene	11.187	1.1
Limonene	12.274	0.99
Isoledene	14.224	2.63
β-burbunene	14.634	1.26
terpinene	15.251	1.92
Sabinene	16.609	0.41
β-pinane	17.932	1.95
Carvacrol	18.541	61.92
β-elemene	24.681	0.87
P-cymene	41.959	1.26
β-phelanderene	45.059	0.32
Thymol	48.209	6.13
Naphthalene	54.345	3.34
Other compounds	-	9.77

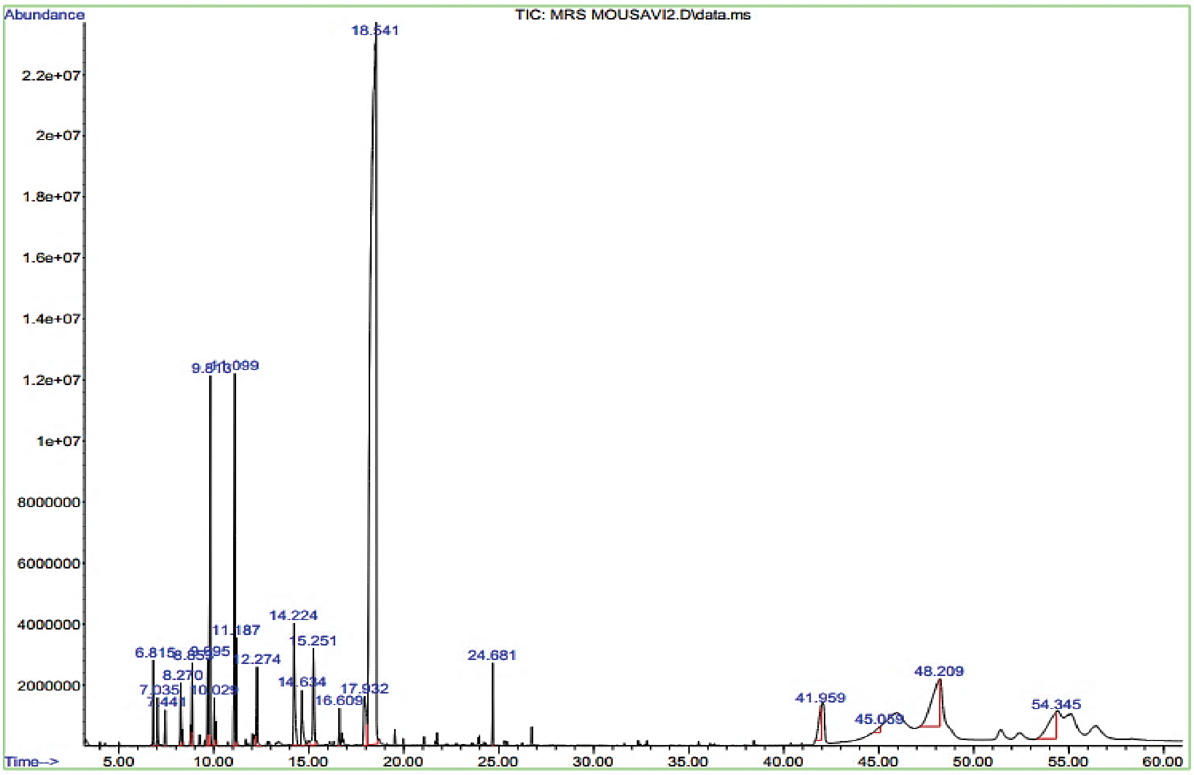

Fig. 3. A typical GC-MS chromatogram showing the chemical analysis of essential oil from *Thymus carmanicus* Jalas.

The results of the dose-response test are shown with the calculation of toxicity lethal concentration as ppm (mg L^{-1}) essential oil (LC_{50} and LC_{99}) in Table 2. They were 20.37 and 41.38 mg L^{-1} for LC_{50} and LC_{90} at 24h after application, respectively. The calculated dose-response curve for Thyme essential oil after 24h is shown in Figure 4.

The Dunnett test showed no significant difference in toxicity between 5%, 20%, and 25% of Thyme essential oil (P>0.05). As well as, there was no significant difference in toxicity between 40%, 50%, and 80% of Thyme essential oil (P>0.05). At 24h after application, significant differences were observed between the toxicity of 5%, 20%, 25%, and 40%,

Table 2. Lethal doses of thyme essential oil against *Anopheles stephensi* larvae.

Time	LC_{50} (CL*) mg L^{-1}	LC_{90} (CL*) mg L^{-1}	Slope (±SE)**	Chi-Square*** (*df*)	*P*
After 24 h	20.37 (18.02-22.52)	41.38 (38.26-45.41)	40.6 (0.005)	1.16 (4)	0.001>

LC_{50} and LC_{90}: Lethal dose necessary to kill 50% and 90% of larvae, respectively.
*Confidence limits.
**standard error.
***Chi-square (degree of freedom).
P: significance level of Probit model.

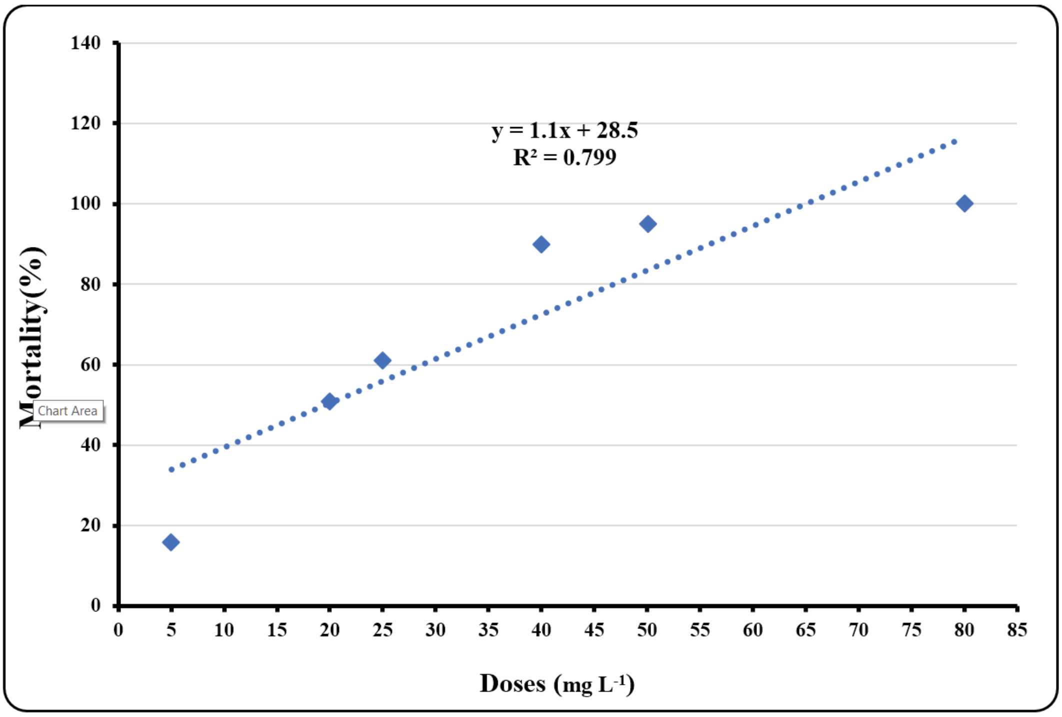

Fig. 4. Dose-response curve for *Thymus carmanicus* Jalas essential oil after 24h

50%, and 80% concentrations of Thyme essential oil ($P<0.05$). The 80% concentration of Thyme essential oil exhibited 100% toxicity against *Anopheles stephensi* larvae at 24h after application (Fig. 5).

3.1. Discussion

Tropical regions are more vulnerable to parasitic diseases and risk contracting diseases due to climate change and increased globalization. Mosquitoes

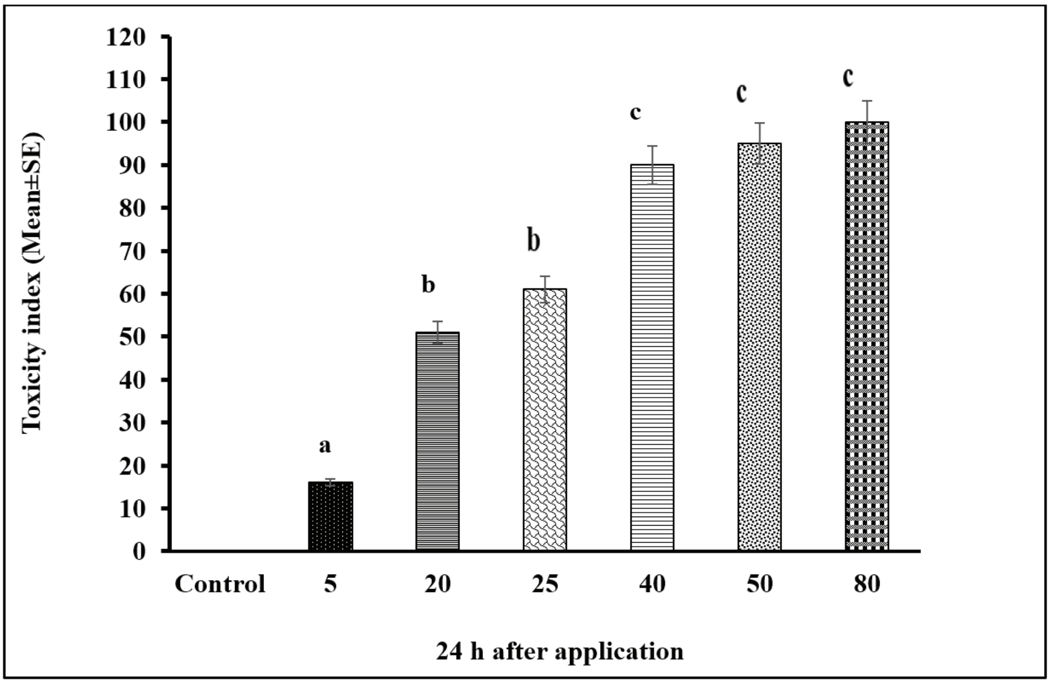

Fig. 5. Toxicity of ethanol, different concentrations of *Thymus carmanicus* Jalas essential oil against *An. stephensi* larvae. Different letters above the bars indicate significant differences at α= 0.05

are the most important public health insects in tropical and subtropical regions because they carry important parasites and pathogens worldwide that cause death, poverty, and social impairment [25]. Makizadeh Tafti et al. (2010) stated that carvacrol constituted the largest part of the essential oil in all thyme ecotypes in Kerman Province, followed by thymol, para cymene, and gamma-terpinene. It is worth noting that the content of each element underwent changes in this research that could be due to geographical differences and reproduction or even in-vitro conditions [10]. Ebrahimi et al. (2009) observed the same compounds with different contents in GC-MS device investigations [26]. The contents observed in the study by Makizadeh Tafti showed that carvacrol constituted about 80% of the composition. However, Ebrahimi's analysis reduced this amount to 60%. Eftekhar et al. (2010) found 68% carvacrol in *Thymus caramanicus*. This study showed that the superiority of carvacrol content compared to other constituents was around 62% [27]. In the research by Mazandarani and Rezaei (2005) on *Thymus caramanicus* grown in Mazandaran Province, it was observed that pulegone (26%) was the most frequent element in the essential oil due to climate change, and carvacrol content decreased to 8% [28]. It was observed that *Thymus caramanicus* desirably affected malaria larvae and significantly increased larval mortality. The lethal property of *Thymus caramanicus* essential oil was extremely high, so lethality reached 50% and 90% at concentrations of 20 and 41, respectively. Damtie and Mekonnen (2021) found that several genera of thyme could effectively prevent Anopheles larvae proliferation and growth at concentrations of 20-50 and showed desirable resistance to adult insects at lower concentrations. This resistance was significantly observed in groups with different doses ($P<0.05$), which was consistent with observations [29]. Dargahi et al. (2014) found that Thymus transcaspicus essential oil exhibited strong insecticidal activity against An. stephensi, which could be due to its constituent compounds, especially carvacrol and thymol phenols [30]. These compounds were present in abundance in *Thymus caramanicus*. *Thymus transcaspicus* could significantly eliminate 50% and 90% of larvae at 154 and 248 µg L^{-1}, respectively ($P<0.05$). There was a significant difference between these two plants in terms of concentration, which could be attributed to the high concentration of carvacrol and thymol in *Thymus caramanicus* compared to *Thymus transcaspicus.*

Gupta et al. (2022) stated that the phenolic compounds present in the thyme could significantly increase the larval population of various disease-carrying mosquitoes ($P<0.05$). The results showed LC_{50} and LC_{90} values of this plant for *An.stephensi*, *Ae. aegypti* and *tritaeniorhynchus* larvae were equal to 56 and 124 µg L^{-1}, 58 and 270 µg L^{-1}, and 22.58 and 193 µg L^{-1} [31]. In the current study, LC_{50} and LC_{90} values were decreased due to changes in the phenolic compounds present in *Thymus caramanicus*, which could be attributed to the greater toxicity of these compounds. Kelidari et al. (2021) investigated the effect of solid-lipid nanoparticles containing *Zataria multiflora* essential oil and found that these particles could significantly prevent the proliferation of *Anopheles stephensi* larvae [32]. Firooziyan et al. (2022) investigated the effect of *Myrtus* nanoemulsion and found that this plant could eliminate 50% and 90% of *An. stephensi* larvae at concentrations of 26 and 46 µg L^{-1} [33]. Zarenzhad et al. (2021) confirmed the promising larvicidal effects of chitosan nanoparticles containing *Laurus nobilis* and *Trachyspermum ammi* essential oils against *An. stephensi* and stated that the essential oil of these plants was a significantly lethal effect [34]. Similarly, in the present study, the Thyme essential oil provided a significant toxic against *An. stephensi* larvae.

4. Conclusion

This study indicated that *T. Carmanicus* has a rich source of eco-friendly bioactive compounds for use as a mosquito larvicide. Its considerable capability might be the high percentage of Carvacrol, which can be used as a larvicidal agent for mosquito control programs. In that way, our findings provide a possible way for further studies to determine the active molecule. Carvacrol with 61.92% was the highest compound of Thyme essential oil. LC_{90} of Thyme essential oil at 24h after the application was 41.38 mg L^{-1}. A concentration of 80% of this essential oil killed 100% of larvae at 24 hours. However, further investigations must be conducted to describe the mode of action of each constituent s independently and also its effects on non-target organisms.

5. Acknowledgements

The authors wish to thank Dr. Fariba sharififar, Zahra Mahdavi and Miss Pakravanan for helping in the essential oil preparation and interpretation of GC-MS results. This article is part of Nazanin Sadat Mousavi MSPH thesis, and it was financially supported by and conducted at Kerman University of Medical Sciences (Project Number 400001144). This study was approved by the Ethics Committee of Kerman University of Medical Sciences (IR. KMU.REC1401.059).

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