Original Article

Phytochemical Composition and Bioassay on Iranian *Teucrium Polium* Extracts against *Anopheles Stephensi* (Diptera: Culicidae)

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Abstract

Background: *Anopheles stephensi* is an important malaria vector mosquito in Iran and other western Asian countries. In many human communities, plant products have been used traditionally instead of synthetic pesticides for mosquito control due to their minimal hazardous effects. *Teucrium polium*, known popularly as felty germander, has been introduced in Persian Medicine (PM) as an insect repellent from a long time ago.

Methods: The present study was undertaken to evaluate repellent and larvicidal activity of dichloromethane (DCME-TP) and ethanolic extracts (EE-TP) of *T. polium* against *An. stephensi* under laboratory conditions. The possible chemical components of the extracts were also investigated through gas chromatography/mass spectrometry (GC-MS) technique.

Results: Based on the results, DCME-TP showed better repellent activity than EE-TP with 56.67 and 28.33 % protection, respectively. Larvicidal activity of DCME-TP with 49.41% mortality was also higher than EE-TP (20.24%). The main identified constituents of DCME-TP were long chain alkanes, phenol, aromatic ester, oxaspiro and triterpenoid. While phenolic and aliphatic acid were only the identified components in EE-TP. It is notable that lupeol was detected in DCME of *T. polium* for the first time.

Conclusion: DCME-TP can be considered as a new herbal candidate to control *An. stephensi* mosquitoes. Further studies are required on this extract for the fractionation and identification of the active compounds, and the evaluation of their bioactivity in the laboratory and field.

Keywords: Larvicidal; Repellent; *Teucrium polium*; *Anopheles stephensi*; Phytochemical

Introduction

According to World Health Organization (WHO) report, "no significant gains were made in reducing malaria cases in the period 2015 to 2017. The estimated number of malaria deaths in 2021, at 619000, remained virtually unchanged over the previous year" (1). Malaria is caused by five different species of *Plasmodium*: *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi*, which are transmitted by mosquitoes of the genus *Anopheles. Anopheles* mosquitoes are bloodsucking insects and responsible for the transmission of malaria, filariasis and arboviruses. There are more than 30 species currently recognized as *Anopheles* species, out of which seven of them have important roles in malaria transmission in Iran (2, 3). *Anopheles stephensi* is the

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primary vector of malaria in the southern parts of Iran and other West Asian countries (2).

While the centralized application of malaria control programs has strongly diminished malaria transmission in malarious regions in the past year, malaria elimination remains an unattainable aim in high-endemic areas (1). There are various methods for malaria control, such as, drug chemotherapy, personal protection, and mosquito control using chemical insect repellents and insecticides (4–5).

Control of mosquitoes by repellents is an excellent strategy to avoid biting mosquitoes which can decrease the incidence of mosquito-transmitted diseases. Another control strategy is killing mosquitoes and their larvae by chemical insecticides such as pyrethroids, organophosphates and carbamates (5). Although, control of *Anopheles* mosquitoes with synthetic repellents and insecticides is possible, their environmental effects and the emergence of resistance are the main concerns in the worldwide (5). In this situation, novel control tools can play an important role in the effort to control and eventually eliminate malaria.

Application of herbal preparations is a suitable tool which can be used as an alternative to synthetic repellents and insecticides. Rapid action, minimal side effects on the skin and quick decomposition in the environment are minimum benefits of medicinal plants which encourage the scientists to assess their anti–insect activities (6).

Several studies have shown that some extracts and essential oils of plants presented repellency, ovicidal, larvicidal and pupicidal activities against mosquitoes (7–11). In mosquito control programs, products with botanical origin and traditional medicine may have the potential to be used successfully (12).

Persian Medicine (PM), as one of the oldest types of complementary medicine, has a long history of using plants for treatment and prevention of diseases (13). PM has introduced many plants as repellents or insecticides which could be the suitable alternatives to mosquito control programs (14). One of these plants is *Teucrium polium*, traditionally named "*Joadah*" (15) or "*kalpooreh*" (16), which belongs to the family Lamiaceae. In this regard, PM manuscripts recommended "*Joadah*" could be spread in the environment or smoke to repel insects (15). In a study, the repellency effect and fumigant toxicity of *T. polium* essential oil against *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), as the stored-product insects, have been also evaluated (17). In recent years, the recognition of bioactive chemical agents from plants and other natural sources has been noticed by researchers to control mosquitoes. The aims of this study were to evaluate the repellency and larvicidal activities of dichloromethane extract (DCME) and ethanolic extract (EE) of *T. polium* against *An. stephensi* mosquito and identifying the chemical components of these extracts using gas chromatography- mass spectrophotometry (GC-MS).

Materials and Methods

Plant materials

The aerial parts of *T. poilium* were collected in March 2008 from Sarkouh, one of the parts of Bandar Lengeh City in Hormozgan Province, located on the north coast of Persian Gulf, South of Iran. Originality of the plant was identified by H Moazeni and A Pirani, botanists of Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Iran. The voucher specimen 2095 (TMRC) of the plant has been deposited in the TMRC herbarium.

Extraction

The dried aerial parts of *T. polium* (200g) were powdered and defatted with hexane, and then macerated in dichloromethane (5:1). DCME-TP was separated by filtration and the residue of the plant was dried at room temperature and then macerated in ethanol sol-

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vent (5:1). All extraction operations have been performed for 24 hours at room temperature with constant shaking. Each extraction process was repeated three times and finally three replicate extracts were added to each other. The dichloromethane) and ethanol extracts of *T. polium* were concentrated by a vacuum rotary evaporator under reduced pressure at a maximum temperature of 50 °C to yield 4.1 and 19.88g of residue, respectively and stored at 4 °C.

Sample preparation for repellency test

Formulation of samples composed of DCME-TP and EE-TP were prepared using oil and aqueous phases (18). The oil phase consisted of stearic acid (1.5g), cetyl alcohol (0.4g) and isopropyl myristate (0.3g) separately. The aqueous phase consisted of DCME-TP and EE-TP (2.5g) solved in glycerin (1.5g) and water (7.8mL). Finally, oil and aqueous phases were added to each other under heating about 65–70 °C in a water bath and mixed suitably. The base formulation without extracts was used as control.

Mosquito rearing

The laboratory bred *An. stephensi* strain of Chabahar were used for repellency tests which was reared and maintained at 27±3 °C and 70–80% relative humidity with a photoperiod of 12h light and 12h dark in the Insectarium of the School of public Health, Shiraz University of Medical Sciences (SUM).

Repellency assay

Five to seven days old non-blood fed females *An. stephensi* were used for repellency tests. The repellency study was conducted by a modified 4-celled Klun and Debboun (K and D) module (19) which is used for quantitative measurement of the efficacy of mosquito repellents on 6cm^2 of the forearm skin of healthy male volunteers aged about 45 (Fig. 1). The module was built by Plexiglas to minimize visual error such as: four cells with larger dimensions to hold 20 mosquitoes in each cell. On the test day, the volunteer had no contact with any cream, lotions, perfumes, or perfumed soaps. Before the application of the-formulated samples for repellency test, the arm of the volunteer was cleaned by distilled water. After drying, marked areas on the skin (6cm^2) were covered with 100mg of sample for three cells and a K and D module bioassay system was put on these areas. Only samples foundation served as a control in cell number 4. Twenty mosquitos were then release into each cell by an aspirator. Observations on the number of bites of *An. stephensi* mosquitoes were recorded at 30 minutes post treatment. The percentage of repellency was calculated by the following equation:

$$\% \text{ protection} = [N_m/N_t] \times 100$$

Where N_m is the mean number of unfed females in the treatment group and N_t is the total number of mosquitoes.

Larvicidal bioassay

Based on the preliminary tests, fourth and third instar larvae of *An. stephensi*, Chabahar strain were exposed to serially diluted test concentrations of 62.5, 125, 250, 500, 1000, and 2000ppm of ethanolic and dichloromethane extracts of *T. polium* for 24 hours according to WHO protocol with minor modifications such as: application a surfactant to distribute monotonous of extracts (20). As the extracts do not dissolve in water completely, EE-TP was dissolved in ethanol and DCME-TP was dissolved in acetone. Test solutions were prepared by adding 1mL of appropriate dilution of extracts in ethanol and mixed with 99mL of dechlorinated water containing 0.05% Tween 80 (v/v aq). In the control beaker for EE-TP, 1% ethanol was added into water and Tween 80 (0.05%). For DCME-TP, control beaker was including 1% acetone and 0.05% Tween 80. Also, dechlorinated water served as untreated control. A minimum of 25 healthy larvae per each concentration were used for all the experiments. The dead larvae were counted after 24 hours recovery period, and the percentage of mortality was reported as the average of the four replicates.

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Gas chromatography-mass spectrometry (GC-MS) analysis of the extracts

GC–MS analysis of the extracts was performed using an Agilent 7000, Triple Quad, GC 7890A system operating in EI mode (70ev) equipped with a capillary column (HP-5MS, 30m (length), 0.25mm (diameter), film thickness of 0.25µm) as the stationary phase (7). The column oven temperature was initially held at 50 °C for two minutes, and then increased to 290 °C at the ramp rate of 5 °C/min and held for 10 minutes at the same temperature. The total run time was 60 minutes. The temperatures of the injector and detector were set at 250 and 300 °C, respectively. The flow rate of helium as a carrier gas was 1mL/min. Compounds were identified by comparison of mass spectral fragmentation patterns and retention indices with National Institute of Standards and Technology (NIST) mass spectral library. The relative percentages of the components were obtained according to the peak area in the chromatogram.

Adverse effect of the formulations on the skin of human volunteers

Tests in human volunteers performed to detect allergic contact sensitization following application of extracts formulations. The cutaneous reactions were monitored for any abnormalities including erythema, edema, pruritus and urticaria, skin allergy and irritation after 15-minute, 1 hour and 24 hours.

Results

Repellency assay

The results obtained from repellency test based on the K and D module bioassay with prepared formulations of *T. polium* extracts on *An. stephensi* are presented in Table 1. All formulations showed good results with the 18% concentration used to achieve protection from mosquito bites. Lower blood feeding rate was observed in dichloromethane extract of *T. polium* with the protection percent 56.67% whereas it was 28.33% for ethanolic extract of *T. polium*. The base of formulated sample without extract as a control did not exhibit t repellent activity (Table 1).

Larvicidal bioassay

The consequence of different concentrations of the DCME and EE of *T. polium* at 62.5, 125, 250, 500, 1000 and 2000ppm on the larvicidal activity against *An. stephensi* after 24h exposure is depicted in Table 2. The highest and lowest larval mortality of 49.41% and 0% was observed at 2000 and 62.5ppm concentrations, respectively. The control groups didn't have any mortality. The result indicated the larvicidal activity of DCME-TP was highly dose dependent. Whereas EE-TP with weak larvicidal activity was not completely dose dependent. This study showed medium larvicidal activity of DCME of *T. polium* against fourth- and third-instar larvae of *An. stephensi*.

The cutaneous reactions on the skin of human volunteers

Tests in human volunteers to detect allergic contact sensitization of extracts formulations proved negative. The results did not show any skin irritation, skin sensitization, and skin keratinization.

Chemical composition of the extracts

Different alkanes such as tetradecane, hexadecane, octadecane and eicosane, as well as 2,4-di-tert-butylphenol and di-isooctyl phthalate as the aromatic compounds and 7,9-di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione as a di-keton compound were identified in DCME-TP (Table 3). Lupeol was another chemical component identified by GC-MS in DCME-TP. Chemical components of 2,4-di-tert-butylphenol and 1-methyl-pyrrolidine-2-carboxylic acid were only identified in EE-TP (Table 3).

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Table 1. Repellency assay by the prepared formulation of dichloromethane and ethanolic extracts of *Teucrium polium* (18% concentration) against *Anopheles stephensi*

Blood feeding/non blood feeding					
Sample	**Repeat 1**	**Repeat 2**	**Repeat 3**	**Control**	**%Protection**
DCME-TP	3/17	4/16	4/16	15/5	56.67
EE-TP	8/12	6/14	8/12	13/7	28.33

Abbreviation: DCME; Dichloromethane extract, EE; Ethanolic extract, TP; *Teucrium polium*

Table 2. Larvicidal activity of dichloromethane and ethanolic extracts of *Teucrium polium* against *Anopheles stephensi*

Larvicidal activity of DCME-TP					
Concentration (ppm)	**Repeat 1 Dead/T***	**Repeat 2 Dead/T***	**Repeat 3 Dead/T***	**Repeat 4 Dead/T***	**Mortality%****
2000	12/21	10/21	11/21	9/22	49.41
1000	5/21	6/23	4/20	5/22	25.26
500	4/22	4/24	3/20	2/26	14.13
250	2/23	2/20	1/22	2/24	7.86
125	0/24	0/20	1/20	1/22	2.5
62.5	0/21	0/25	0/24	0/20	0
Larvicidal activity of EE-TP					
Concentration (ppm)	**Repeat 1 Dead/T***	**Repeat 2 Dead/T***	**Repeat 3 Dead/T***	**Repeat 4 Dead/T***	**Mortality%*****
2000	4/21	3/21	6/21	4/21	20.24
1000	6/23	4/22	4/25	4/24	19.15
500	4/22	4/23	5/25	4/22	18.48
250	7/21	2/25	3/20	3/25	16.48
125	3/22	2/21	4/22	4/26	14.23
62.5	3/22	2/21	2/21	1/18	9.5

*T: total number of *Anopheles stephensi larvae*. Abbreviation: DCME; Dichloromethane extract, EE; Ethanolic extract, TP; *Teucrium polium.* **The control of DCME-TP containing 1% acetone, 0.05% Tween 80, and 99ml dechlorinated water did not have any mortality. ***The control of EE-TP containing 1% ethanol and 0.05% Tween 80, and 99ml dechlorinated water did not have any mortality

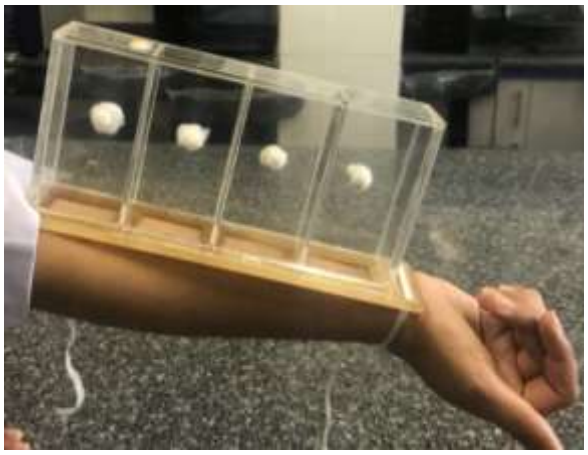

Fig. 1. Modified 4-celled Klun and Debboun module used for the repellency assay of dichloromethane (DCME-TP) and ethanolic extracts (EE-TP) of *T. polium* against *Anopheles stephensi*

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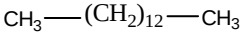
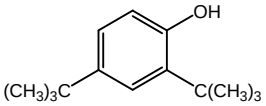
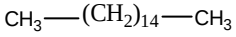
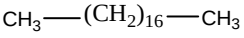
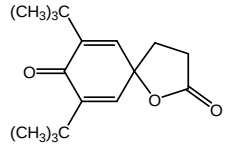
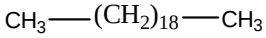
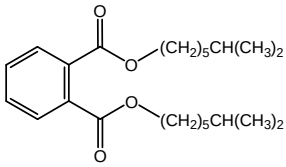
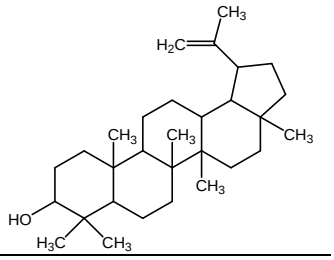
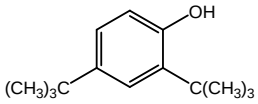
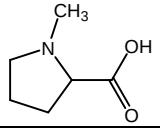
Table 3. The identified compounds in dichloromethane and ethanol extracts of *Teucrium polium* by GC-MS analysis

The identified compounds in DCME-TP					
S. No	**Retention time (min)**	**Area%**	**Name**	**Family**	**Structure**
2	21.864	6.25	Tetradecane	Alkane	
4	24.585	1.49	2,4-Di-tert-butylphenol	Phenol	
5	26.574	21.96	Hexadecane	Alkane	
11	30.826	25.5	Octadecane	Alkane	
14	33.264	10.02	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	Oxaspiro	
20	34.695	21.08	Eicosane (Alkane)	Alkane	
33	43.799	100	Di-isooctyl phtalate	Aromatic ester	
49	57.088	2.78	Lupeol	Triterpenoid	
The identified compounds in EE-TP					
S. No	**Retention time (min)**	**Area%**	**Name**	**Family**	**Structure**
1	16.048	0.27	2,4-Di-tert-butylphenol	Phenol	
2	48.525	100	1-Methyl-pyrrolidine-2-carboxylic acid	Aliphatic acid	

Abbreviation: DCME; Dichloromethane extract, EE; Ethanolic extract, TP; *Teucrium polium*

Discussion

Various species of *Anopheles* mosquitoes showed resistance to synthetic anti-mosquito agents, whilst their environmental risk is worrying due to adverse effects on human, and non-target organisms (7). For these problems, the effectiveness of chemical control agents has been limited in malaria control and eradication programs in recent years.

Herbal anti-mosquito agents are environmentally friendly alternatives for synthetic in-

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secticides with high biodegradability, low residual effect, novelty in mechanism of action, with insignificant effect on the health of humans and pets, and with negligible harm to the non-targeted occupants of the area. Given these facts, mosquitoes control agents from natural sources can be considered as safe and effective alternatives. Several studies introduced mosquito repellent and larvicidal activity of some extracts and essential oils of plants (7–11, 21-22). According to these studies, the products of traditional medicine have the potential to be used successfully in mosquito control programs.

The control of adult malaria vectors with adulticides and repellents is a suitable method to effect on longevity, mosquito densities and other transmission agents. Personal protection is a common approach for preventing mosquito bites which helps indirectly in diminishing the mosquito population by depriving the blood meal which is vital for nourishment of the mosquito eggs in the female *Anopheles* mosquito.

People are mostly being bitten during peak mosquito biting hours, early in the evening, and sometimes throughout the night. Therefore, finding a way to protect people from malaria is essential during these hours which this gap can fill with mosquito repellents including spatial, and topical repellents. The use of repellents protects local people and travelers in endemic areas and consequently reduces the occurrence of mosquito-borne diseases. There are the various repellents, such as N,N-diethyl-mtoluamide (DEET), para-methane-3,8-diol (PMD), icaridin, 3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester (IR3535) (23).

DEET is the most effective insect repellent available for human which has broad-spectrum activity on most mosquitoes, ticks and fleas (24). Today, application of DEET has been restricted due to the adverse effects. Icaridin was also classified as slightly hazardous by the WHO hazard classification category (25). IR3535 and PMD have eye irritation.

Larvicides, pupicides and ovicides treatments can also help to control transmission parameters (7-11, 21-22). A larvicide is a type of insecticide that effects on the larval life stage of an insect and consequently, reduces the adult mosquito population in breeding areas. There are different formulations of larvicides which can be applied directly on water. Therefore, these should not make an unreasonable health risk to humans or other wildlife.

Methoprene, an insect growth regulator agent prevents the normal maturation of insect larvae, has moderate and high toxicity on different aquatic animals and organisms. Temephos, an organophosphate larvicide, affects the central nervous system through inhibition of cholinesterase and results in death before reaching the adult stage. Similarly, there are the concerns about its toxicity on non-targeted aquatic species. In this situation, achievement to new alternatives especially, natural products, is one of the most important approaches of research groups.

Teucrium polium is a traditional plant in Iran belonging to the family Lamiaceae which is recommended in Iranian traditional medicine manuscripts and some papers as an insect repellent (12, 17, 26-27). The present study is the first report of repellent and larvicidal activities of different extracts of *T. polium* against *An. stephensi*.

DCME of *T. polium* showed 49.41% larvicidal activity against *An. stephensi* larvae and 56.67% repellency against mosquitoes. Repellent and larvicidal activity of DCME-TP was greater than EE-TP.

GC-MS analysis of *T. polium* extracts identified eight components in DCME containing alkanes (tetradecane, hexadecane, octadecane, and eicosane); 2,4-di-tert-butylphenol; 7,9-di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione; di-isooctyl phthalate; and lupeol (Table 3). 2,4-Di-tert-butylphenol and 1-methyl-pyrrolidine-2-carboxylic acid were identified in EE-TP(Table 3).

The various studies reported larvicidal and repellent activities of plants containing alkanes (28–30). DCME of *T. polium* was rich in al-

kanes which might be responsible for its anti-mosquito activity. *Citrus hystrix* and *Kaempferia galanga* essential oils presented larvicidal activity against *Aedes aegypti* (28). Both were containing tetradecane. Good larvicidal and adulticidal efficacy of *Acacia nilotica* seed essential oil has been reported against larval mosquitoes; *An. stephensi*, *Ae. aegypti* and *Culex quinquefasciatus* (29). Hexadecane was identified in *A. nilotica* as a major component that probably affects its insecticidal activity.

The acetone leaf extract of *Melia azedarach* was tested by Ranchitha et al. (30) against larvae and pupae of *Ae. aegypti*. Eicosane was determined as one of its components. The various extracts of *Ocimum canum* were evaluated against *Ae. aegypti* and its chloroform extract showed significant larvicidal, pupicidal and adulticidal activity. Eicosan was determined as a major component of this extract (31).

Phthalates are used as plasticizers in the production of plastics which are widely distributed in the environment. Fortunately, their rapid photochemical and biological degradation has led to their low level (32). There are little reports of biological active plants containing phthalates. Di-isooctyl phthalate was identified as a major component of DCME of *T. polium*. Ramamurthy et al. (33) reported moderate larvicidal and pupicidal activity of ethanolic leaf extract of *Mukia maderaspatana* containing di-isooctyl phthalate against *Ae. aegypti*. Based on Babu et al. report (34), di-isooctyl phthalate was identified as a major component in the crude extract of *Pongamia pinnata*. Antifeedant and larvicidal activity of di-isooctyl phthalate against *Spodoptera litura* showed remarkable results. Lupeol was also one of compounds identified in this plant (34).

The nanoparticles of *Acalypha indica* including di-isooctyl phthalate showed the mosquito repellent and larvicidal properties against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* (35).

2,4-Di-tert butylphenol, another identified component of *T. polium* has been found in various species of microorganisms, plants, and animals (36). Chen and Dai (37) reported the ovicidal, larvicidal, and adulticidal activities of 2,4-di-tert butylphenol against *Tetranychus cinnabarinus* in a concentration-dependent manner after treatment.

Lupeol, a pentacyclic triterpene also known as fagarsterol (38), identified in DCME of *T. polium*. According to the literature review, there are no reports about the identification of lupeol in *T. polium*. Duan et al. (39) have claimed the acaricidal activity of lupeol, derived from *Inula japonica*, and its potential as a botanical pest control agent. Díaz et al. (40) isolated lupeol from *Dodonaea viscosa* and reported its anti-insect activity against *Myzus persicae* (green peach aphid) and *Epilachna paenulata* (ladybird beetle).

Lupeol identified in *Vernonia brasiliana* showed a mild inhibition activity against *P. falciparum* growth (41), since the lupeol is a lupane type of triterpenoids, it was considered as an interesting template for derivatization and was led to identify more potent antiplasmodial compounds (42). Ajaiyeoba et al. (43) also reported the antiplasmodial activity lupeol isolated from ethyl acetate fraction of *Cassia siamea* against multi-resistant strain of *P. falciparum* (K1). Dichloromethane extract of *Dendranthema grandiflorum* containing lupeol presented larvicidal activity against *A. aegypti* third instar larvae (44).

Larvicidal activity of chloroform extract of *Carica papaya* latex and silver nanoparticles (CPAgNPs) of aqueous latex extract confirmed better activity of CPAgNPs in lower dose against *Ae. aegypti* and *Cx. quinquefasciatus* (45). Di-isooctyl phthalate, 2,4-di-tert-butylphenol, hexadecane, and 7,9-di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione were some identified compounds in chloroform extract of *C. papaya* similar to *T. polium* in this study (45).

According to the studies mentioned above, each of the main components of *T. polium* identified in the present study has displayed proper anti-insect activity. The larvicidal and

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repellent activity of DCME of *T. polium* can be probably related to the synergistic effect between the mentioned components.

Conclusion

This study introduces an ideal eco-friendly mosquito repellent from the extract of *Teucrium polium* which has a proper potential as a promising candidate for mosquito control. However, further complementary studies such as fractionation extracts to get the molecule(s), mostly responsible for repellent activity, other formulation of extracts, and field trials should be performed to confirm repellent activity.

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Ethical considerations

This study was performed based on ethical considerations and national regulations in animal experiments (no. 1080).

Conflict of interest statement

Authors declare that there is no conflict of interest.

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