

Original Article**Chemical Composition and Repellent Activity of *Achillea vermiculata* and *Satureja hortensis* against *Anopheles stephensi***

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Abstract

Background: One of the best ways to control the malaria disease and to be protected human against *Anopheles* mosquito biting is the use of repellents. Throughout repellents, herbal ones may be an appropriate and safe source for protection.

Methods: Chemical constituents of *Achillea vermiculata* and *Satureja hortensis* were determined by using gas chromatography-mass spectrometry. Efficacy and the protection time of these plants were assessed on *Anopheles stephensi* under the laboratory condition.

Results: The mean assessed protection time and efficacy for *A. vermiculata* was 2.16 and 3.16 hours respectively and the obtained ED₅₀ and ED₉₀ for this plant was 5.67 and 63 µl/cm² respectively. The figured for *S. hortensis* was 4.16 and 5 hours respectively. ED₅₀ and ED₉₀ for this plant were 5.63 and 45.75µl/cm² respectively.

Conclusion: Results of investigation showed that *S. hortensis* plant has an acceptable protection time, therefore, this plant could be considered as a good herbal repellent against anopheles mosquitoes.

Keywords: *Achillea vermiculata*, *Satureja hortensis*, *Anopheles stephensi*, Repellency, Protection time

Introduction

Mosquito borne diseases affect human societies by reduction in labor productivity especially in tropical and subtropical countries. The important point is this fact that all countries all over the world have a problem with insect borne diseases (Govindarajan et al. 2011).

Anopheles mosquitoes are bloodsucking insects, responsible for transmission of malaria, filariasis and arboviruses (Service 1980). There are 33 currently recognized *Anopheles* species including sibling, biological forms and

zygotypes, seven of these species have an important role in malaria transmission in Iran. Among these species, *An. stephensi* is considered as a primary vector of malaria in southern parts of the country (Sedaghat et al. 2003, Vatandoost et al. 2004, Sedaghat et al. 2005). According to the WHO report, a total of 627000 people die due to malaria (WHO 2013).

Malaria is caused by *Plasmodium* parasite. Malaria is one of the most important diseases which the parasite is transmitted by female *Anopheles* genus (WHO 2010). There are several methods for malaria vector control.

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Synthetic insecticides which are generally used have side-effects on human, animal health, and the environment. The side-effects of synthetic organophosphorus compounds on fish and other organisms in the environment are being increasingly reported. A lot of attention is being paid to natural products in vector control as they are environmentally safe, degradable and target-specific. Recent studies have demonstrated that use of repellents is one of the effective ways to control the disease and to avoid *Anopheles* bites (Vatandoost et al. 2008).

DEET is slightly yellow oil. It is the most common active ingredient in insect repellents. It is intended to be applied to the skin or to clothing, and provides protection against mosquitoes and many other biting insects. DEET was developed during World War II. The findings bring evidence that, DEET has side effects, so it has proposed to use alternative repellents for protection (Karunamoorthi et al. 2010). Some prefer to use natural insect repellent products. Repellents of plant origin do not pose hazards of toxicity to human and domestic animals and are easily biodegradable. Natural products are safe for human when compared to that of synthetic compounds (Fradin 1998). The effect of some plant origin essential oils have been tested in Iran (Oshaghi et al. 2003, Yaghoobi-Ershadi et al. 2006, Vatandoost and Hanafi-bojd 2008, Tavassoli et al. 2011, Mozaffari et al. 2014).

Achillea vermiculata is a flowering plant in the family Asteraceae with a height of 10–30 cm. It is native to temperate regions of the Northern Hemisphere in Asia, Europe, and North America. It is an erect herbaceous perennial plant. The leaves have varying degrees of hairiness. The leaves are almost feathery (Mozaffarian 2012).

Satureja hortensis has lilac tubular flowers. It grows to around 30 to 60 cm in height and has very slender, bronze-green leaves. This plant belongs to order: Lamiales and family: Lamiaceae. It is used in traditional

medicine as a botanical treatment (Mozaffarian 2012).

This study was conducted to evaluate the repellent properties of two plants *A. vermiculata* and *S. essential oil* against *An. stephensi* in laboratory condition on animal model and also to determine chemical compositions in their essential oils.

Materials and Methods

Mosquitoes rearing

Established colony of susceptible strain of *An. stephensi* obtained from the Insectary of School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Mosquitoes were reared and maintained at 28 ± 2 °C and $65\pm 5\%$ relative humidity (RH) under a 16:8 (L: D) photoperiod. Larvae were fed on a diet of fish food and water lettuce. The adults were maintained in screen cages and fed with 10% aqueous sucrose solution as a source of energy and guinea pigs as blood-feeding female mosquitoes for maturing the eggs. Starved 5 to 8 days old females were used for the repellency tests. The sucrose solution was picking up from the cage, 12 hour before starting the experiments.

Collection, identification and extraction of plants

Fresh flowers and leaves flowers of *A. vermiculata* and *S. hortensis* were collected from Armand and Sheyda district which are located in Chaharmahal and Bakhtiari Province in south-west of Iran in June 2013 (Fig.1,2). They were rapidly transported to the School of Public Health, Tehran University of Medical Sciences. *Achillea vermiculata* was collected from natural habitat in Armand district at coordinate $31^{\circ} 39.428$ E $50^{\circ} 46.659$ N, 1136 meters above sea level. *Satureja hortensis* was collected from natural habitat in Sheyda district in Ben at coordinate $32^{\circ} 37.206$ E $50^{\circ} 42.434$ N, 2219 meters above sea level.

The plant *A. vermiculata* was identified by experts in Department of Plant Sciences, Tehran University. Also *S. hortensis* was identified by experts in Ecotoxicology and herbarium laboratory, School of Public Health, Tehran University of Medical Sciences. The flowers and leaves of *A. vermiculata* and *S. hortensis* were dried at room temperature under good ventilation. About 525 gr of dried *A. vermiculata* and 420 gr of dried *S. hortensis* were chopped into small pieces using a knife mill. The essential oil was extracted from the plants using a Clevenger-type water steam distillation apparatus. It took about 4 hours for extraction of the essential oils. For extraction of essential oil of each plant we used Sulphate Sodium Anhydr. The distilled essential oils were stored in a refrigerator at 4 °C until being used in the experiments. The composition of the volatile constituents was established by gas chromatography-mass spectrometry.

Test method

All series of the experiments of effective dose and protection time were carried out in laboratory condition. We tested these plants on white rabbits. The white rabbits (*O. cuniculus*) (laboratory reared albino male aged 6–8 months) were used to determine both protection time and effective dosage in insectary at the School of Public Health. In this investigation, the 50% concentration of essential oils was used for protection time test. For this purpose essential oils were diluted by absolute ethanol. Then for estimating the protection time of plants essential oils against *An. stephensi* the back of male rabbits by 52cm² were shaved in the next stage, 80µl of 50% essential oils of two plants by sampler on the shaved back of male rabbits were applied. After 5 minutes the rabbits were placed in a box which is designed for the test. Then it is placed in box containing the rabbit in a cage at dimension of 53×53×53 cm containing 150 starved 5–8

days mosquitoes. After 3 minutes of biting and probing records, we brought out the cage and we tested again 30 minutes later. These tests continued until two successive bites. This time is called protection time. We continued these tests until 10 bites. This time is called failure time (Pitasawat et al. 2003).

The procedure for determination of effective dosages of the repellents was adopted by the standard method of American Society for Testing and Material (ASTM 2000).

The testing kit was made of plexiglas cube at dimension of 4×5×18cm having four rectangular holes 4×3cm. Before starting the test for determination of effective dosage, the abdomen skins of rabbits were cleaned with alcohol and the kit was fixed on the abdomen.

Each of 4 adjacent cells of kit was provided with 5 female 5–8 days mosquitoes that randomly selected from a cage containing 150 starved mosquitoes. Circles were drawn on the rabbit's skin. The drawn circles on the abdomen skin's of hold rabbit were treated with 50µl of essential oil diluted with absolute ethanol at 6.6, 13.2, 26.4 and 52.8µl for *A. vermiculata* and 3.3, 6.6, 13.2, 26.4 and 52.8µl for *S. hortensis* microliter with 4 repetitions. The same dilutions were applied on 3 holes because of prevention of contamination as well as the absolute ethanol was applied in remaining control circle. We used 5 mosquitoes for each hole. The treated circles were allowed to dry, and then test apparatus containing starved mosquitoes were fixed on the treated skin. The counts of probing and biting were recorded for 5 minutes. After each test, the mosquitoes were transferred to netted cups and the mortality of mosquitoes was recorded after 24 hours. The ED₅₀ and ED₉₀ values and regression parameters were analyzed using probit 79 programs and the regression lines were plotted in Microsoft Excel 2007.

Plants essential oils analysis

Chemical composition of *A. vermiculata*

and *S. hortensis* was analyzed using an Agilent 7890–5975 gas chromatography-mass spectrometer. With a HP- 5MS (5% Phenyl Methyl Silox) capillary column (30m×0.25mm, film thickness 0.25µm), split ratio, 1: 1, and using a flame ionization detector. The GC was programmed at 50 °C for 0.5 min and then increased at 5 °C/min to 280 °C, and finally held with an isothermal for 3min.

The injector temperature was 280 °C. The flow rate of the carrier gas was 1ml/min. The identification of compounds was performed by comparing their retention times and mass spectra with mass spectra from Wiley library.

Results

Essential oil volumes

By the use of Clevenger-type water steam distillation, about 1849µl of essential oil of 525gr of dried *A. vermiculata* flowers was extracted. Also about 4480µl of essential oil of 420gr of dried *S. hortensis* leaves extracted.

GC-mass analysis

One microliter of each essential oil was injected to GC-mass. A total of 40 compounds were identified in flowers of *A. vermiculata*. (E)- -damascenone with 27.4, (E)-2-hexenal with 8, eugenol with 6 and geranyl acetone with 6 percent were the major components (Table 1). We just found a repellent component “camphene” of all identified components by researchers until now in this plant with 0.7%.

Also we identified 23 components in the leaves of *S. hortensis*. B-oplophenone with 57, trans-carvone oxide with 15.13 and thymol methyl ether with 13 percent were the major components (Table 2).

Protection time

The protection time of *A. vermiculata* essential oil against *An. stephensi* on animal

subject provided 2.0–2.5 hours range with a mean of 2.16 hours protection and a failure time of 3–3.5 hours range with a mean of 3.16 hours.

Also the protection time of *S. hortensis* essential oil provided 4–4.5 hours range with a mean of 4.16 hours protection and a failure time of about 5 hours (Table 3).

Significant differences of protection time and failure time between *A. vermiculata* and *S. hortensis* repellents were observed by ANOVA (Games-Howel), $P < 0.05$.

Effective dose

The ED₅₀ and ED₉₀ values of *A. vermiculata* essential oil were 5.67 and 63 µl/cm² with confidence interval ranged, 2.25-8.68 and 38.21–198.07 µl/cm² respectively (Table 4).

The ED₅₀ and ED₉₀ values of *S. hortensis* essential oil were 5.63 and 45.73 µl/cm² with confidence interval ranged, 3.83-7.43 and 30.92–86.55 µl/cm² respectively (Table 4).

We did not observe any significant differences between ED₅₀ and ED₉₀ of *S. hortensis* and *A. vermiculata* by T-test and $P > 0.001$ analysis.



Fig. 1. The plant *Achillea vermiculata* in its natural habitat, Armand district in Chaharmahal and Bakhtiari Province, south-western of Iran (original)

Table 1. Chemical constituents of flower essential oil from *Achillea vermiculata*

NO	Compound	Composi- tion%	RI
1	isovaleric acid	0.2	833
2	(E)-2-hexenal	8	854
3	Tricyclene	0.1	921
4	Limonene	0.3	1029
5	Methylbenzoate	0.5	1093
6	Linalool	3.1	1108
7	-campholenal	2.8	1131
8	Camphene	0.7	1156
9	Pinocarvone	0.7	1164
10	cis-piperitol	0.7	1194
11	Verbenone	0.8	1208
12	trans-carveol	4	1222
13	cis-carveol	3.7	1233
14	Geraniol	1	1258
15	2E,4E-decadienal	3.1	1313
16	Eugenol	6	1359
17	(E)- -damascenone	27.4	1382
18	cis- α -bergamotene	5.6	1416
19	trans- α -bergamotene	3	1433
20	geranyl acetone	6	1452
21	allo-aromadendrene	0.8	1462
22	gamma-gurjunene	0.3	1473
23	α -muurolene	0.6	1499
24	α -cadinene	1.2	1537
25	Spathulenol	0.7	1579
26	b-oplophenone	0.4	1601
27	humulene epoxide II	0.3	1609
28	silphiperfol-6-en-5-one	0.6	1623
29	1-epi- α -eudesmol	2	1660
30	8-cedren-13-ol	0.3	1694
31	Xanthorizol	0.3	1751
32	8- α -acetoxylemol	2	1788
33	Nootkatone	0.4	1802
34	Flourensiadiol	1.8	1864
35	Hexadecanol	1.7	1881
36	methyl hexadecanoate	1.9	1911
37	methyl hexadecanoate	1.8	1925
38	methyl hexadecanoate	2.2	1941
39	methyl hexadecanoate	2.6	1957
40	Heneicosane	0.25	2057
		99.55	

Table 2. Chemical constituents of leaf essential oil from *Satureja hortensis*

NO	Compound	Composition%	RI
1	isovaleric acid	0.06	831
2	(Z)-3-hexenol	2.1	856
3	trans-sabinene hy- drate	0.3	1095
4	Linalool	1	1109
5	-campholenal	0.6	1132
6	Borneol	1	1171
7	trans-carveol	2	1221
8	thymol methyl ether	13	1237
9	trans-carvone oxide	15.1	1277
10	Undecanal	0.4	1306
11	-caroyophyllene	3	1424
12	b-oplophenone	57	1600
13	10-epi- γ - eudesmol	0.3	1620
14	Hinesol	0.6	1637
15	8-cedren-13-ol	1	1704
16	Oplopanone	0.2	1726
17	8- α -acetoxylemol	0.3	1781
18	Flourensiadiol	1.04	1867
19	methyl hexadecanoate	0.12	1910
20	methyl hexadecanoate	0.2	1923
21	methyl hexadecanoate	0.2	1935
22	Heneicosane	0.1	2097
23	n-docosane	0.2	2213
		99.82	

**Fig. 2.** The plant *Satureja hortensis* in its natural habitat, Sheyda district in Chaharmahal and Bakhtiari Province, south-western of Iran (original)

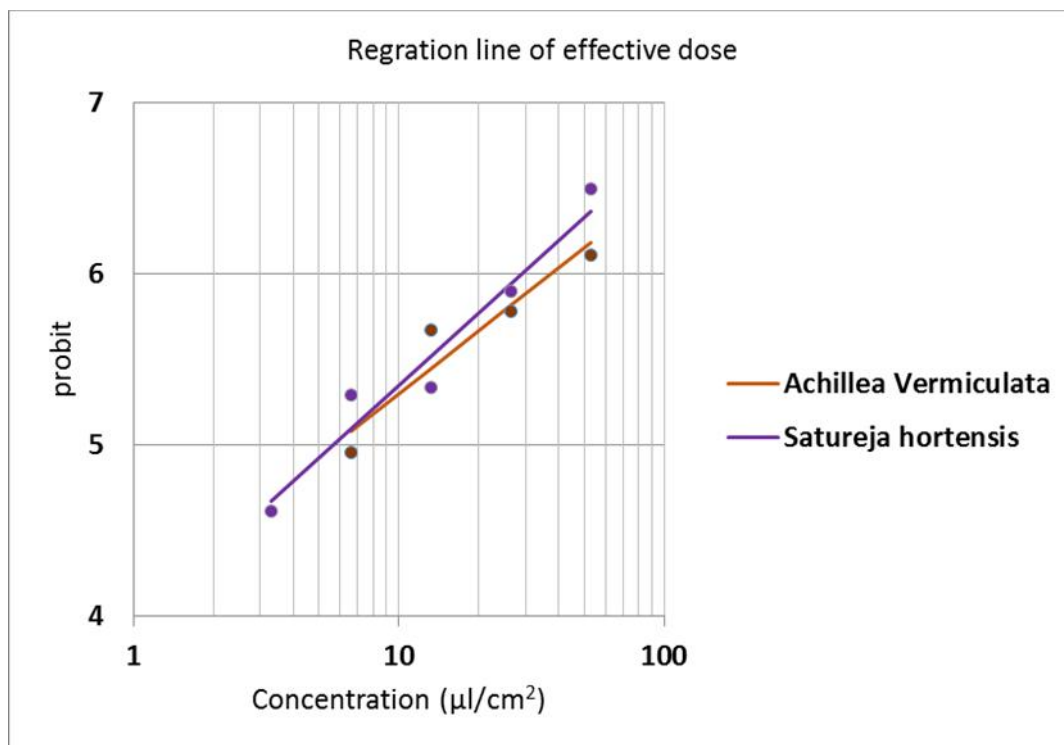


Fig. 3. Dose-response lines for two botanical repellents against *Anopheles stephensi* on animal model

Table 3. Protection time and failure time of *Achillea vermiculata* and *Satureja hortensis* against *An. stephensi* on animal subject in laboratory condition

Species (plants)	District	Protection time (hour)		Failure time (hour)	
		Range	Mean	Range	Mean
<i>A. vermiculata</i>	Lordegan	2–2.5	2.16	3–3.5	3.16
<i>S. hortensis</i>	Ben	4–4.5	4.16	5–5	5

Table 4. Effective dose of *Achillea vermiculata* and *Satureja hortensis* essential oils against *Anopheles stephensi* on animal subject in laboratory condition

plants	a	b ± SE	ED50 (mg/cm ²) ± 95% C.L.	ED90 (mg/cm ²) ± 95% C.L.	2 (heterogeneity)	2 table (df)	p-Value
<i>A. vermiculata</i>	-0.092	1.22±0.27	5.67 (2.25–8.68)	63 (38.21–198.07)	2.35	2	0.01
<i>S. hortensis</i>	-1.05	1.4±0.20	5.63 (3.83–7.43)	45.75 (30.92–86.55)	3.13	3	0.01

Discussion

Application of larvicides and repellents are generally accepted as they play an important role in control of the mosquitoes. The use of botanical essential oils as repellents against

vectors of malaria disease including *An. gambiae* and *A. stephensi* has been tested successfully (Seyoum et al. 2002). In this study the components of *A. vermiculata* essential

oil were identified. (E)- -damascenone with 27.4, (E)-2-hexenal with 8, eugenol with 6 and geranyl acetone with 6 percent were the major components. Totally 40 components were identified from this plant. In an investigation by Ahmadi et al. (2011), on *A. santolina*, 29 components were identified from this plant which the major components were Camphor, Alpha-pinene, Camphene and 1,8 Cineole with 26.27, 10.14, 9.09 and 8.26 percent respectively. We found two same components “Camphene” and “Linalool” of *A. vermiculata* with *A. santolina*.

Also we identified 23 components in the leaves of *S. hortensis*. B-oplophenone with 57, trans-carvone oxide with 15.13 and thymol methyl ether with 13 percent was the major components. In an investigation by Kamkar et al. (2013) (32 components were identified and reported from this plant which the major components belonged to - terpinene with 24.72%, thymol with 29.1% and carvacrol with 26.6%. In this investigation, we identified some components which had been identified in Kamkar et al. (2013) study. These components are: borneol, linalool and thymol. Also cis-sabinene hydrat was identified in Kamkar investigation, but our investigation revealed the presence of trans-sabinene hydrate. In another investigation by Tajalli et al. (2012) on this plant, the major components were thymol, carvacrol, gamma-terpinene with 48.67, 8.96, 9.16 and 9.16 percent respectively. In our investigation, we found thymol and linalool which had been identified in Tajalli et al. (2012) study too. Our investigation revealed that *A. vermiculata* essential oil can have 2.16 hours protection time and also its Failure time is 3.16 hours. Compared to *A. vermiculata*, *S. hortensis* could have 4.16 hours protection time and a Failure time of 5 hours which is about 2 times as much as *A. vermiculata*'s protection time and Failure time. Also we revealed an ED₅₀ and ED₉₀ effective dose of 5.67 and 63 $\mu\text{l}/\text{cm}^2$ respectively, while for *S. hortensis* they were 5.63

and 45.75 $\mu\text{l}/\text{cm}^2$ respectively.

The repellency effect of essential oils of some plants has been studied in Iran. On a laboratory trial by Vatandoost et al. (2008), the repellency of neem tree's essential oil against *An. Stephensi* in animal subject was determined. The ED₅₀ and ED₉₀ values of neem tree's essential oils were calculated 0.159 and 1.388 mg /cm² respectively. Also the protection time and effective dose of this plant calculated 31 minutes and 65 minutes respectively.

The repellency effect of essential oils of both *Myrtus communis* and *Calendula officinalis* had been reported against *An. stephensi* on human subject and the effective dose of these plants was 0.11 and 0.6 mg/cm² respectively. Also the protection time and Failure time for *M. communi* were 4.36 and 4.4 hours respectively. The protection time and Failure time for *C. officinalis* were 2.15 and 3.30 hours respectively (Tavassoli et al. 2011).

In an investigation, the mean protection time of 50% essential oil of *Cionura erecta* (*L*) provided 2.28 hours protection against *An. stephensi*. The figures for ED₅₀ and ED₉₀ values were 10.12 and 23.01ppm respectively (Mozaffari et al. 2014).

We estimate that the most protection time of mentioned investigations and our investigation, belongs to *M. communi* and *S. hortensis* with a protection time of 4.36 and 4.16 hours and failure time of 4.4 and 5 hours respectively. The weakest protection time and failure time belongs to neem plant by 31 minutes and 65 minutes respectively.

There have been so many investigations about repellency effects of plants against mosquitoes all over the world until now (Moore et al. 2002, Rajkumar and Jeanesabn 2007, Mullai et al. 2008, Karunamoorthi et al. 2010, Shahi et al. 2010).

Finally we recommend *S. hortensis* as a candidate for production of insect repellents because of its high protection against mos-

quitoes and also medicinal properties without any side effects. Although this plant did not show any significant differences of effective dose rate with *A. vermiculata*. We recommend doing more investigations on this plant.

Conclusion

According to the results it could be concluded that the plant is appropriate for the repellent formulation for mosquito control, although the field trial should be conducted in a malarious areas.

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