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.
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, Ravassoli 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±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° 39.428'E 50° 46.659'N, 1136 meters above sea level. Satureja hortensis was collected from natural habitat in Sheyda district in Ben at coordinate 32° 37.206'E 50° 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 Anhydrous. 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 4x5x18cm having four rectangular holes 4x3cm. 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 50 1 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 0.25 μm), 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 3 min.

The injector temperature was 280 °C. The flow rate of the carrier gas was 1 ml/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 1849 µl of essential oil of 525 gr of dried A. vermiculata flowers was extracted. Also about 4480 µl of essential oil of 420 gr 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)-β-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-oplopane 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.
Table 1. Chemical constituents of flower essential oil from Achillea vermiculata

| NO | Compound               | Composition % | 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  | α-campholenal          | 2.8           | 1111 |
| 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)-β-damascenone      | 27.4          | 1382 |
| 18 | cis-a-bergamotene      | 5.6           | 1416 |
| 19 | trans-a-bergamotene    | 3             | 1433 |
| 20 | geranyl acetone        | 6             | 1452 |
| 21 | allo-aromadendrene     | 0.8           | 1462 |
| 22 | gamma-gurjunene        | 0.3           | 1473 |
| 23 | a-muuroleone           | 0.6           | 1499 |
| 24 | a-cadinene             | 1.2           | 1537 |
| 25 | Spathulenol            | 0.7           | 1579 |
| 26 | b-oplopenone           | 0.4           | 1601 |
| 27 | humulene epoxide II    | 0.3           | 1609 |
| 28 | silphiperfol-6-en-5-one| 0.6           | 1623 |
| 29 | 1-epi-a-eudesmol       | 2             | 1660 |
| 30 | 8-cedren-13-ol         | 0.3           | 1694 |
| 31 | Xanthorizol            | 0.3           | 1751 |
| 32 | 8-a-acetoxyelemol      | 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 |

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

| NO | Compound                | Composition % | RI   |
|----|-------------------------|---------------|------|
| 1  | isovaleric acid         | 0.06          | 831  |
| 2  | (Z)-3-hexenal           | 2.1           | 856  |
| 3  | trans-sabinene hydrate  | 0.3           | 1095 |
| 4  | Linalool                | 1             | 1109 |
| 5  | α-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 | β-carotyophyllene       | 3             | 1424 |
| 12 | b-oplopenone            | 57            | 1600 |
| 13 | 10-epi-gamma-eudesmol   | 0.3           | 1620 |
| 14 | Hinesol                 | 0.6           | 1637 |
| 15 | 8-cedren-13-ol          | 1             | 1704 |
| 16 | Oplopanone              | 0.2           | 1726 |
| 17 | 8-a-acetoxyelemol       | 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 | Henecicosane            | 0.1           | 2097 |
| 23 | n-docosane              | 0.2           | 2213 |

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) |
|------------------|---------|------------------------|---------------------|
| A. vermiculata   | Lordegan| Range: 2–2.5 Mean: 2.16| Range: 3–3.5 Mean: 3.16 |
| S. hortensis     | Ben     | Range: 4–4.5 Mean: 4.16| Range: 5–5 Mean: 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/cm²) ± 95% C.L. | ED90 (mg/cm²) ± 95% C.L. | $\chi^2$ (heterogeneity) | $\chi^2$ table (df) | p-Value |
|-------------|-------|---------|--------------------------|--------------------------|-------------------------|---------------------|---------|
| A. vermiculata | -0.092 | 1.22±0.27 | 5.67 (2.25–8.68) | 63 (38.21–198.07) | 2.35 | 2 | 0.01 |
| S. hortensis  | -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)-β-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-oplopenone 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 γ-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 hydrate 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 μl/cm² respectively, while for S. hortensis they were 5.63 and 45.75 μl/cm² 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 ED₉₀ 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 has 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. communis 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 ED₉₀ 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. communis 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 Jeanesahn 2007, Mullai et al. 2008, Karunamoorthei 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 trail should be conducted in a malarious area.

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