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Composition and Larvicidal Activity of *Artemisia vulgaris* L. Stem Essential Oil Against *Aedes aegypti*

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Abstract

This study investigates the composition and potential larvicidal activity of the essential oils isolated from the elite plant, *Artemisia vulgaris* L. which is commonly known as mugwort. Essential oils were hydrodistilled from *in vitro* raised stems and analysed by GC-MS. The major components of this oil were camphor, camphene, α-thujone, 1,8-cineole, γ-muurolene and β-caryophyllene. Mosquito larvicidal assays were carried out to test the efficacy of the oil against the dengue vector, *Aedes aegypti*. Laboratory reared third instar larvae were exposed to different concentrations of the oil solution and activity was assessed at different exposure times according to standard WHO procedure. Results showed that 100% larval mortality was achieved when the larvae were treated with 500 ppm oil solution with an exposure time of 8 h. The present study has shown that mugwort oil is a potential larvicide against *Aedes aegypti* even in low doses of the test solution. The results indicate that the natural insecticides could be used in the place of synthetic insecticides and save our environment from chemical hazards.

Keywords*: Artemisia vulgaris*, stem essential oil, larvicidal, *Aedes aegypti*, exposure time.

1. Introduction

The mosquito species, *Aedes aegypti* L. is a vector of major diseases such as dengue haemorrhagic fever, chikungunya and yellow fever. *A. aegypti* is reported to infect more than hundred million people every year in more than 110 countries in the tropics (Halstead, 2000). The present resurgence of these diseases is due to the higher number of breeding places in today's throwaway society (Ravikiran *et al*., 2006). Further, the indiscriminate use of synthetic insecticides is creating multifarious problems like environmental pollution, insecticide resistance, and toxic hazards to humans. Globally, there have been conscientious efforts to overcome these problems, and great emphasis has been placed recently on enviro-friendly and economically viable methodologies for pest control.

Phytochemicals obtained from the huge diversity of plant species are important source for safe and biodegradable chemicals, which can be screened for mosquito repellent, larvicidal, and insecticidal activities; and tested for mammalian toxicity. A large number of plant products have been reported to have mosquito larvicidal and/or repellent activity against adult mosquitoes. The discovery of insecticide activity in Asteraceae species has stimulated interest in this plant family as part of the search for new plant derived insecticides (Rawls, 1986; Prashant *et al*., 2006; Masotti *et al.*, 2012). In recent years, essential oils have received much attention as potent bioactive compounds against various mosquito species (Tripathi *et al.*, 2009).

Artemisia vulgaris L. (mugwort) is a member of the Asteraceae family. It is a tall (0.8-1.4 m), aromatic, threatened perennial herb distributed throughout the northern temperate regions of Africa, Asia, Europe, India, and North America. In traditional medicine, this plant is widely used for the treatment of diabetes and extracts of the whole plant is used for epilepsy and in combination for psychoneurosis, depression, irritability, insomnia and anxiety states (Lewis and Elwin-Lewis, 2003). Numerous medicinally active components of *A. vulgaris* have been identified, including coumarins, essential oils, flavonoids, polyacetylenes, sesquiterpene lactones, and sterols (USDA-ARS-NGRL, 2004). Essential oils make a major contribution to the plant's biological activity (Judzentiene and Buzelyte, 2006).

Mugwort essential oil is used in India for its insecticidal, antimicrobial and antiparasitical properties (Judzentiene and Buzelyte, 2006). It was reported to exhibit 90% repellence against *Aedes aegypti*, a mosquito that transmits yellow fever (Hwang *et al.*, 1985). Repellent and fumigant activity of *A. vulgaris* essential oil against *Musca domestica* L. and the stored-product

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insect pest *Tribolium castaneum* (Herbst) were also reported (Judzentiene and Buzelyte, 2006; Wang *et al*., 2006). The essential oil exhibits many other biological activities such as analgesic, anaesthetic, antidiarrheic, antineuralgic, antiseptic, antispasmodic, antiasthmatic, anti-inflammatory,sedative,CNS-stimulant, decongestant, expectorant, antiacne, larvicidal, nematicide, pesticide, antibacterial, and it is also used in the flavour and perfumery industry (Teixiera da Silva, 2004). To date there are no published reports on larvicidal activity of *in vitro* grown stem essential oils of *A. vulgaris*. Therefore, we herein present a brief report on the composition of *A. vulgaris* stem essential oil and its larvicidal activity against *Aedes aegypti*.

2. Materials and Methods

2.1. Plant material and essential oil extraction

A. vulgaris seeds were collected from National Medicinal Plants Board, India and cultures were raised *in vitro* as previously reported (Sujatha and Ranjitha Kumari, 2007). Stem material was collected from *in vitro* raised plants before the onset of flowering and subjected to essential oil analyses. A 100 g sample of the air-dried stems was mixed with 1000 ml distilled water and subjected to hydrodistillation in a Clevenger-type distilling apparatus for 2 h. The resulting oil was dried over anhydrous sodium sulphate and stored in airtight fuscous glassware in a refrigerator at 4 ºC until analysis.

2.2. Gas chromatography – mass spectrometry (GC-MS)

GC/EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures at 220° and 240° C respectively; oven temperature was programmed from 60° C to 240° C at 3° C/min; carrier gas helium at 1 ml/min; injection of 0.2 µl (10% hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their Linear Retention Indices relative to the series of *n*-hydrocarbons, and by computer matching against commercial (NIST 98 and ADAMS 95) and home-made library mass spectra built up from pure substances and components of known essential oils and MS literature data (Stenhagen *et al*., 1974; Jennings and Shibamoto, 1980; Adams, 1995). Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using MeOH and CI ionizing gas.

2.3. Procurement of eggs and rearing of Aedes Aegypti mosquito larvae

Aedes aegypti eggs (Fig. 1a) were obtained from a colony maintained at the Center for Medical Entomology, Indian Council for Medical Research, Madurai, Tamil Nadu, India. The eggs of *A. aegypti* were obtained as egg rafts on a filter paper. The egg rafts were brought to the laboratory and kept in a tray containing tap water (as culture medium) at laboratory conditions (29 ± 1°C; RH 70–75%; photoperiod 14 : 10 (light : dark)). On the next day, the eggs were observed to hatch out into first instar larvae. Appropriate amounts of nutrients (yeast powder and dog biscuit (1:2 w/w) were added to the culture medium. On the third day after hatching, the first instar larvae moulted into second instar larvae. On the fifth day, third instar larvae were observed. The third instar larvae of *A. aegypti* were used for the larvicidal experiments in the present study (Fig. 1b).

2.4. Preparation of the oil solution

A sufficient amount of target (TR) oil was dissolved in tap water using 2 ml of 100 % acetone to produce a stock solution of 500 ppm. This solution was used to prepare other serial dilutions of target oil in concentrations of 500 – 1 ppm through dilution of stock with tap water. Five replicates of each concentration were made, in addition to five control replicates containing 0 ppm of oil, 2 ml of 100 % acetone and tap water (WHO, 1981).

2.5. Larvicidal bioassay

The tests were conducted at room temperature. The oil dilutions were tested against the third instar larvae of *A. aegypti* mosquitoes (WHO [World Health Organization], 1981; Ansari *et al*., 2000; Rey *et al*., 2001; Amer and Mehlhorn, 2006) to detect their toxicity on mosquito larvae. Five replicates of each oil dilution (1-500 ppm) were prepared. Each replicate containing 200 ml of the described oil solution was placed in a 500 ml glass beaker. Ten third-instar larvae of target mosquito were transferred into each beaker (Mohtar et *al*., 1999). After that, the beakers were left on the laboratory table for 24 h. The number of dead larvae in each beaker was counted after 1, 8, 16, 20 and 24 h.

2.6. Statistical analysis

Experiments were set up in a complete randomized block design and each experiment was repeated five times. Data were recorded on the percentage of larval mortality. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means, and the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5 % level of significance (Gomez and Gomez, 1976). The results were analyzed statistically using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) for windows.

3. Results and Discussion

3.1. Essential oil analyses

The oil was a fluid liquid, slightly greenish in colour and possessed powerful fresh-camphoraceous aroma with sweet bitter taste. The intense odour could be due to the presence of a large number of oxygenated mono- and sesquiterpene components in the stem oil. Fifty one compounds were identified, accounting for 91.0% of the essential oil. The identified constituents and corresponding percentage of the essential oil composition are listed in Table (1).

Table 1. Composition of *in vitro* stem essential oils of *A. vulgaris*

Compound	Rt	% MS	RI[#]	identification[Φ]
cis-salvene	3.39	trace	846	MS, RI
santolinatriene	4.44	0.1	906	MS, RI
tricyclene	4.86	0.2	920	MS, RI
artemisiatriene	5.17	0.8	930	MS, RI
alpha-fenchene	5.57	3.9	943	MS, RI
camphene	5.61	6.0	944	MS, RI, co-GC
sabinene	6.37	2.4	969	MS, RI, co-GC
beta-pinene	6.47	0.8	973	MS, RI , co-GC
2,3-dehydro-1, 8-cineole	6.94	0.1	988	MS, RI
2-carene	7.92	0.1	1012	MS, RI
p-cymene	8.28	0.8	1020	MS, RI, co-GC
1,8-cineole	8.58	5.1	1026	MS, RI, co-GC
arbusculone	10.24	0.4	1061	MS, RI
alpha-thujone	12.52	10.7	1105	MS, RI, co-GC
beta-thujone	13.04	2.8	1114	MS, RI, co-GC
camphor	14.88	17.3	1140	MS, RI, co-GC
isoborneol	15.39	trace	1147	MS, RI, co-GC
borneol	16.16	3.7	1158	MS, RI, co-GC
3-thujanol	16.80	0.6	1167	MS, RI
myrtenal	18.00	0.6	1185	MS, RI
myrtenol	18.10	trace	1186	MS, RI
verbenone	18.85	trace	1197	MS, RI
trans-carveol	19.66	trace	1210	MS, RI
cumin aldehyde	21.05	0.2	1232	MS, RI
perilla aldehyde	23.55	0.2	1272	MS, RI
bornyl acetate	24.58	0.6	1288	MS, RI
iso-3-thujyl acetate	25.38	0.8	1301	MS, RI
delta-elemene	28.35	0.4	1334	MS, RI
alpha-copaene	31.26	0.8	1366	MS, RI
beta-cubebene	32.46	0.6	1379	MS, RI
beta-elemene	32.64	0.3	1381	MS, RI
alpha-gurjunene	33.83	1.3	1394	MS, RI
beta-caryophyllene	34.71	5.8	1405	MS, RI
beta-gurjunene	35.30	0.3	1413	MS, RI
alpha-humulene	37.17	1.9	1438	MS, RI
allo-aromadendrene	37.64	0.3	1444	MS, RI
gamma-muurolene	39.58	9.0	1469	MS, RI
bicyclogermacrene	40.58	1.6	1482	MS, RI
alpha-muurolene	41.00	0.6	1488	MS, RI
gamma-cadinene	41.90	0.6	1499	MS, RI
davana ether isomer [*]	42.42	0.4	1506	MS, RI
delta-cadinene	42.85	2.5	1512	MS, RI
davana ether isomer [*]	43.87	0.2	1525	MS, RI
germacrene D-4-ol	46.60	trace	1560	MS, RI
spathulenol	46.65	1.1	1561	MS, RI
caryophyllene oxide	46.88	1.2	1564	MS, RI
cis-davanone	47.94	2.5	1577	MS, RI, co-GC
cadinol-epi-alpha	51.32	0.4	1623	MS, RI
alpha-cadinol	52.29	0.5	1637	MS, RI
davanone-2-ol-beta	57.50	0.4	1712	MS, RI
cadinene-14-hydroxy-delta	62.67	0.1	1789	MS, RI

Rt = Retention time expressed in minutes; Trace: <0.05; [#] the retention index was calculated using a homologous series of n-alkanes C8-C18; [*] correct isomer not determined; [Φ]Co-GC: co-injection with an authentic sample.

There are no previous reports on the essential oil composition of *in vitro* raised *A. vulgaris* stems. Monoterpenes reached about 70% of the whole essential oil. The main ones were camphene (6.0%) and α-fenchene (3.9%). Oxygenated monoterpenes constituted the main chemical class of the oil (51.3%) and they were represented with camphor (17.3%) and α-thujone (10.7%) as principal chemicals. Sesquiterpenes constituted about 26% of the whole oil. Among them, hydrocarbons (27 compounds) reached 21.5%, whereas oxygenated derivatives (12 compounds) represented 4.4% of the oil. Among sesquiterpene hydrocarbons, β-caryophyllene (5.8%) was the main constituent. In the case of oxygenated sesquiterpenes, the principal one was *cis*-davanone (2.5%). Thus, the isolated essential oil was characterized by a high content of oxygenated monoterpenes [camphor (17.3 %), α-thujone (10.7 %), and 1,8-cineole (5.1%)]; the monoterpene hydrocarbon camphene (6.0%); and by the sesquiterpene derivatives, β-caryophyllene (5.8%) and γ-muurolene (9.0%).

It has been demonstrated that *A. vulgaris* grown in different countries possessed qualitative and quantitative differences in their essential oil composition. The oil from Italy were rich in camphor (47.7%), camphene (9.1%) and verbenone (8.6%) (Mucciarely *et al*., 1995). The oil from the Republic of Bashkortostan was found to contain high amounts of α-pinene (53.7 %), trans-chrysanthenol (13.1%), β-myrcene (8.8%) and β-pinene (7.4%) (Khalilov *et al*., 2001). Whereas in Croatia, the chief components reported were β-thujone (20.8%), α-pinene (15.1%) and 1,8-cineole (11.7%) (Jerkovic *et al*., 2003). The oil isolated from North Lithuania was high in amounts of sabinene, β-pinene, 1,8-cineole, artemisia ketone, cis- and trans- thujone, chrysanthenyl acetate, germacrene D, and β-caryophyllene (Judzentiene and Buzelyte, 2006). The oil isolated from Indian grown plants was characterized with high amount of camphor (38.7%), isoborneol (8.2%) and artemisia alcohol (4.5%) (Haider *et al*., 2003). α-thujone was stated as the main constituents of *A. vulgaris* oil (Misra and Singh, 1986), while we found an average value of 10.7% in this study. Thus the oils isolated from plants native to different countries significantly differ in their composition. This clearly depicts that variations in population genetics, environmental conditions, and the stress factors the plant faces during its survival and growth influence the accumulation of essential oils.

3.2. Larvicidal activity of stem essential oil

Different concentrations (1-500 ppm) of stem essential oil solutions were bioassayed against the third instar larvae of *Aedes aegypti*. The results were recorded after 1, 8, 16, 20 and 24 h of treatment (Table 2). In control treatments, no larvicidal effect was observed; the larvae remained alive, and they moulted into fourth instar larvae. Whereas, when the different oil concentrations were tested, different mortality rates were recorded with respect to exposure time.

At 1 ppm oil solution, the larvae remained immobile after 24 h of treatment. When 10 ppm oil solution was

tested, 5.0 % and 12.3 % larval mortality was recorded after 20 h and 24 h of treatment respectively. When 100 ppm oil solution was tested, 78.2 % mortality was recorded after 24 h (Table 2).

Table 2. Effect of different concentrations of oil solution and exposure time on larvicidal bioassay of third instar larvae of *Aedes aegypti*

Oil Solution (ppm)	Larval Mortality Rate (%) After				
	1 h	8 h	16 h	20 h	24 h
Control (0)	-	-	-	-	-
1	-	-	-	-	-
10	-	-	-	5	12.3 d
100	10.4 e	45.9 e	64.8 d	70.1 c	78.2 c
200	20.1 d	52.7 d	72.9 c	85.3 b	91.5 b
300	59.4 c	71.2 c	89.5 b	100 a	100 a
400	65.1 b	83.2 b	100 a	100 a	100 a
500	89.7 a	100 a	100 a	100 a	100 a

* Treatment means followed by different letters are significantly different from each other (p<0.05) according to Duncan's Multiple Range Test

At 300 ppm, complete mortality was recorded after 20 h of exposure, and at 400 ppm, 100 % mortality was recorded after 16 h (Table 2). The maximum result (100 %) was recorded with 500 ppm oil concentration after 8 h exposure (Fig. 1c). Lethal concentration (50 % larvicidal activity) was observed from 100 ppm oil concentration onwards. The exposure time is very important for 50 % larvicidal activity of the oil solution. Very low concentrations of the oil led to high mortality rates.

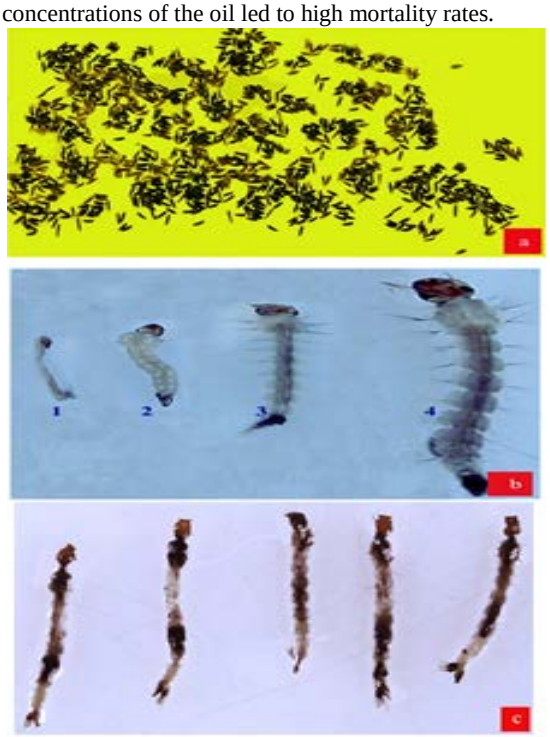

Figure 1. Larvicidal Activity of *Artemisia vulgaris* stems essential oil solution. a – Eggs of *Aedes aegypti* b – Different stages of larval development (1, 2, 3 & 4 instars) c – Treated larvae (at 500 ppm test solution) exhibiting complete mortalit (after 8 h)exposure time on larvicidal activity was also previously reported (Amer and Mehlhorn, 2006).

The present study has shown that *in vitro* produced *A. vulgaris* stem essential oil is a potential larvicide against *Aedes aegypti* in low concentrations (100 ppm) of the oil solution. High doses of the oil solution will be required for large breeding habitats, to be effective. However, the oil might be used as a selective larvicide in small breeding places where water is stagnant, such as in domestic containers and desert coolers. *A. vulgaris* essential oils were previously reported as potent larvicidals against *Aedes aegypti* (Ram and Mehrotra, 1995). But this is the first larvicidal bioassay carried out using essential oil extracted from *in vitro* propagated stems. *In vitro* production of source material helps in conserving the wild resources and thus this protocol can be used for the steady production of *A. vulgaris* plants for essential oil production.

This study indicates that the essential oil of *in vitro* propagated *A. vulgaris* stems has larvicidal properties and its use as a larvicide against mosquitoes should be explored. It is worthwhile to study extensively the larvicidal properties of the plant's essential oil by isolating and identifying the active components responsible for larval mortality, and then test them in field trials in order to assess their potential as an alternative to synthetic chemical larvicides.

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