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Chemical composition of the essential oil and *n*-hexane extract of *Stachys tmolea* subsp. *Tmolea* Boiss., an endemic species of Turkey, and their mosquitocidal activity against dengue vector *Aesdes aegypti*

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ABSTRACT

Stachys tmolea subsp. *tmolea* Boiss. is endemic to Turkey and is a species of the genus *Stachys* L. which is one of the largest genera of the family Lamiaceae with about 300 species. The aims of this study were to examine the chemical composition of the essential oil and *n*-hexane extract of *S. tmolea* subsp. *tmolea* as natural sources of insecticidal activity against the dengue vector, *Aedes aegypti*. Analysis of the essential oil by GC-FID and gas chromatography-mass spectrometry (GC-MS) systems identified hexahydrofarnesyl acetone (15%), viridiflorol (10%), hexadecanoic acid (7%) and 9-geranyl-*p*-cymene (6%) as major components. The volatile components of the *n*-hexane extract were extracted using headspace solid-phase microextraction (HS-SPME) and were analyzed using GC-MS. The principal constituents were 3,4-dimethyl decane (16%), 3-methyl-3-pentanol (15%), 2-methyl-2-pentanol (12%), 1,4-bis (1,1-dimethylethyl) benzene (12%), heptanal (10%), acetic acid (6%) and decane (4%). Bioassay of the *n*-hexane extract, at 5 μg/mosquito, produced 90% mortality against adult *Ae. aegypti* while the *S. tmolea* essential oil demonstrated 13% mortality. No larvicidal activity was observed both in essential oil and *n*-hexane extract. Further studies are needed to assess the adulticidal activity of the responsible compounds in the crude extract.

1. Introduction

Aedes aegypti (L) (Diptera: Culicidae) is the primary vector of dengue, Zika, and yellow fever in humans with approximately 50 million cases worldwide every year (WHO, 2011). One factor in the proliferation of these diseases is the increasing resistance of mosquitoes to currently available commercial insecticides, e.g. in pyrethroid-resistant larvae to the oxadiazine indoxacarb and juvenile hormone mimic pyriproxyfen based upon higher detoxification activity of glutathione-S-transferases and general esterases (Wilson et al., 2019). Several different chemical subgroups of Amaryllidaceae isolates including alkaloids, cytochalasans, sterols and terpenes containing sesquiterpenes and diterpenes were assayed for their larvicidal and adulticidal activity against *Ae. aegypti*. Compounds being part of the cytochalsin, diketopiperazine, naphthoquinone and organic acid groups with low molecular weight are active and a good avenue for further structure-activity investigations (Masi et al., 2017b). Larvicidal activity of *Eucalyptus globulus* leaf essential oil discovered for 4th instar larvae of *Anopheles stephensi*, *Ae. aegypti* and *Culex quinquefasciatus* mosquitoes equal to commercial available chemical larvicide Temephos (Vivekanandhan et al., 2019), while lignans and the alkaloid pellitorine extracted from the bark of *Zanthoxylum piperitum* (Rutaceae) were toxic to third-instar larvae from insecticide-susceptible *C. pipiens pallens* and *Ae. aegypti* as well as wild *C. pipiens pallens* resistant to pyrethroids and organophosphates (Soon-Il and Young-Joon, 2017). Also new alkaloids sarniensine and crinsarnine isolated from *Nerine sarniensis*, an indigenous South African Amaryllidaceae, showed promising larvicidal and adulticidal activity against *Ae. aegypti* (Masi et al., 2016; 2017a, b). Recent study

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results performed with *Prosopis juliflora* seed pod hexane extract was indicated as an alternative agricultural pesticide which was effective in producing lepidopteran larval mortality due to the presence of 9-Octadecyne (Dhivya et al., 2018). Adulticidal and smoke toxicity of *Cipadessa baccifera* (Roth) was showed against three important mosquitoes vectors, An. stephensi, *Ae. aegypti*, and *Cx. quinquefasciatus* (Diptera: Culicidae). Leaf extracts of *C. baccifera* have a potential to be used as an ideal eco-friendly approach for the control of mosquitoes (Ramkumar et al., 2015).

The genus *Stachys* L. is one of the largest representative genera of herbs and shrubs of the Lamiaceae (Labiatae) family, and involves about 300 species (Rechinger and Hedge 1982, Greuter et al., 1986), in the subtropical and tropical regions of both hemispheres excluding Australia and New Zealand (Evans, 1996; Salmaki et al., 2012). The species name arises from the Greek and means "an ear of grain" attributing to the inflorescence spike found in many members. The genus is represented in Turkey by 83 species (109 taxa) belonging to 12 subsections, 15 sections and 2 subgenera and 55 of which are endemic (Bhattacharjee, 1982; Davis et al., 1988; Duman, 2000; Özhatay et al., 2009; Akcicek, 2010; Radulovic et al., 2007, Dündar et al., 2013, Baştürk et al., 2015). *Stachys tmolea* Boiss., as being a Labiatae member with basal rosettes of sterile shoots, is an endemic species of Turkey which is named tmolea as being the ancient name of Boz mountain (in Ödemiş, İzmir) by Boissier who collected the species (Avcı, 2004). Flowering stems are simple or with few branches, densely adpressed-tomentose, lanate-villous above. Species are distinguished from the other *Stachys* species with its lower cauline leaves being oblong-lanceolate to oblanceolate, narrowed towards base; base usually attenuate to rounded, rarely subcordate and calyx tube ± regular with distinctly visible veins; stem patently pilose with dense glandular hairs; corolla creamish-white (Davis et al., 1988). The species is a listed endangered species in Turkey (Güner and Akçiçek, 2014).

Stachys species have a history of use as a traditional medicines. Decoctions or infusions of some of *Stachys* species, locally known as "mountain tea", are applied as tonics to heal skin or taken by orally for stomach disorders in Anatolia (Ozturk et al., 2009). The aerial parts of *S. tmolea* are known as "smutty tea" (sürmeli çayçe) in the Ulus mountains (Balıkesir) (Güner and Akçiçek, 2014) and "quester" (kestire) in the city of Bilecik, Turkey where the plant is consumed as a hot tea for the treatment of colds (Koyuncu et al., 2010). Many *Stachys* species are used for the healing of skin, stomach, ulcer, asthma, rheumatic deseases and vaginal tumors (Goren et al., 2011a,b). Some of the species have been reported as having anti-inflammatory, antibacterial, antianxiety, antioxidant or antinephritic properties.

In the current study, we examined the chemical compositions of the essential oil and *n*-hexane extract obtained from aerial parts of *S. tmolea*, an endemic species from Turkey, and evaluated the insecticidal activity against the dengue vector *Ae. aegypti*.

2. Material and methods

2.1. Plant material

S. tmolea subsp. *tmolea* Boiss. was collected in July 2013 in İzmir/Ödemiş, Bozdağ, at an altitude 1500 m (voucher specimen code: Ö. Seçmen 4282) (Fig. 1). Botanical identification was carried out by Prof. Dr. Özcan Seçmen.

2.2. Isolation of the essential oil

The essential oil from air-dried aereal parts of *S. tmolea* was extracted by hydrodistillation for 3 h, using a Clevenger-type device to produce oil in 0.098% yield. The collected oils were dried over anhydrous sodium sulphate and stocked at +4 °C in the dark until examined and tested.

Fig. 1. A voucher specimen of *S. tmolea*. Photo credit: Hatice Demiray.

2.3. Preparation of n-hexane extract

Aerial parts of plants were air dried under shade and were then ground. The powder material was macerated with *n*-hexane 100% in 1:10 (w/v) in a percolator at room temperature for 3 × 48 h. The extracts of plants were filtered through cotton and subsequently with Whatman filter paper (12.5 cm size). The rotary evaporator was used to remove different solvents from the extract at 40 °C (0.14 g). The *n*-hexane extract was collected in small vials and stored at +4 °C until used in mosquito larvicidal and adulticidal activity.

2.4. Chromatographic analysis of volatiles

The GC-FID analysis of *S. tmolea* essential oil was accomplished using an Agilent 6890 N GC system. The temperature of FID detector was 300 °C. Simultaneous auto-injection was done on a duplicate column under the same operational conditions to get the same elution order for GC-MS analysis. Relative percentage amounts of the separated compounds were adjusted from FID chromatograms. The analysis values are given in Table 1.

The volatile compounds were isolated by the manual SPME device (Supelco, Bellefonte, PA, USA) with a fiber-precoated 65 µm thick layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB-blue). The vial involving the plant extract was sealed with parafilm. The fiber was drived through the film layer for exposure to the headspace of the extract for 15 min at 40 °C. The fiber was then embedded immediately into the injection port of the

Table 1
The composition of the essential oil of *Stachys tmolea*.

RRI	Compound	%	Identification method
1280	*p*-Cymene	tr	RRI, MS
1466	α-Cubebene	0.1	MS
1400	Tetradecane	tr	RRI, MS
1497	α-Copaene	1.6	MS
1535	Dihydroedulane I*	0.6	MS
1600	Hexadecane	0.4	RRI, MS
1661	Alloaromadendrene	0.5	MS
1704	γ-Muurolene	1.8	MS
1722	Dodecanal	0.1	RRI, MS
1740	α-Muurolene	1.2	MS
1755	1,2-Dihydro-1,1,6-trimethyl naphthalene	0.3	MS
1773	δ-Cadinene	1.7	MS
1776	γ-Cadinene	0.5	MS
1838	(*E*)-β-Damascenone	1.1	MS
1849	Calamenene	1.7	MS
1868	(*E*)-Geranyl acetone	1.1	MS
1941	α-Calacorene	1.1	MS
1945	1,5-Epoxy-salvial(4)14-ene	2.3	MS
1958	(*E*)-β-Ionone	0.4	MS
2041	Pentadecanal	1.4	MS
2080	Cubenol	0.7	MS
2088	1-*epi*-Cubenol	1.1	MS
2104	Viridiflorol	9.9	MS
2130	Salviadienol	0.9	MS
2131	Hexahydrofarnesyl acetone	15.1	MS
2145	Valeranone	2.3	MS
2192	Nonanoic acid	0.5	RRI, MS
2174	Fokienol	0.6	MS
2209	T-Muurolol	1.3	MS
2256	Cadalene	2.6	MS
2298	Decanoic acid	1.5	RRI, MS
2289	Oxo-α-Ylangene	0.9	MS
2312	9-Geranyl-*p*-cymene	6.1	MS
2384	Farnesyl acetone	0.4	MS
2396	13-*epi*-Manoyl oxide	0.4	MS
2384	1-Hexadecanol	0.6	MS
2503	Dodecanoic acid	1.3	RRI, MS
2617	Tridecanoic acid	1.1	RRI, MS
2622	Phytol	1.7	MS
2670	Tetradecanoic acid	2.8	RRI, MS
2700	Heptacosane	1.3	RRI, MS
2900	Nonacosane	0.9	RRI, MS
2931	Hexadecanoic acid	7.4	RRI, MS
	Monoterpene Hydrocarbons	tr	
	Sesquiterpene Hydrocarbons	10.2	
	Oxygenated Sesquiterpenes	22.6	
	Fatty acids	14.6	
	Diterpenes	8.2	
	Alkans	2.6	
	Others	21.1	
	Total	79.3	

RRI Relative retention indices calculated against *n*-alkanes; % calculated from FID data; tr Trace (<0.1%).
Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.
* : Tentative identification.

GC-MS for desorption of the adsorbed volatile compounds for analysis (Table 2).

2.5. GC-MS analysis

The GC-MS analysis of essential oil and *n*-hexane extract of *S. tmolea* was performed with an Agilent 5975 GC-MSD system. An Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was operated with helium as the carrier gas (0.8 ml/min). GC oven temperature was kept at 60 °C for 10 min and then increased to 220 °C at a rate of 4 °C/min, and maintained at 220 °C for 10 min and then increased to 240 °C at a rate of 1 °C/min. The injector temperature was arranged at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from *m*/*z* 35 to 450.

Table 2
The volatiles identified from the *n*-hexane extract of *Stachys tmolea* by SPME-GC-MS.

RRI	Compound	%	Identification method
1000	Decane	4.1	RRI, MS
1023	3,4-Dimethyl decane*	16.0	MS
1087	2-Methyl-2-pentanol*	12.1	MS
1100	3-Methyl-3-pentanol*	14.5	MS
1194	Heptanal	9.6	RRI, MS
1400	Nonanal	2.7	RRI, MS
1402	1,4-bis(1,1-dimethylethyl)benzene*	11.7	MS
1441	(*E*)-2-Octenal	0.6	MS
1475	Acetic acid	5.9	RRI, MS
1482	2,5-Hexanedione*	0.4	MS
1506	Propanoic acid*	0.4	RRI, MS
1562	Octanol	0.1	RRI, MS
1570	2-Methyl propanoic acid*	0.2	MS
1574	2,2-Dimethyl propanoic acid (=Pivalic acid)*	0.3	MS
1602	6-Methyl-3,5-heptadien-2-one	0.3	MS
1628	4,4-Dimethylbut-2-enolide	0.8	MS
1685	Isovaleric acid	0.1	RRI, MS
1751	3,4-Dimetyl-2,5-furandione	0.4	MS
1762	Pentanoic acid	0.4	RRI, MS
1871	Hexanoic acid	0.9	RRI, MS
1981	Heptanoic acid	0.5	RRI, MS
	Alkane	20.1	
	Aliphatic alcohol	26.7	
	Alkyl aldehyde	12.3	
	Phenylpropane	11.7	
	Aliphatic aldehyde	0.6	
	Carboxylic acid	8.7	
	Aliphatic diketone	0.4	
	Linear ketone	0.3	
	Tetralactone	0.8	
	Furane	0.4	
	Total	82.0	

RRI Relative retention indices calculated against *n*-alkanes; % calculated from TIC data; tr Trace (<0.1%).
Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.
* : Tentative identification.

2.6. Identification of the components

Determination of the volatile and essential oil constituents of the samples was performed by comparison of their relative retention times with those of pure samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer coordinating used commercial (Wiley GC/MS Library, MassFinder Software 4.0) (McLafferty and Stauffer, 1989; Hochmuth, 2008) and in-house "Başer Library of Essential Oil Constituents" built from authentic compounds and components of known oils.

2.7. Mosquito bioassays

The mosquito lineage used for this study was the ORL1952 strain of *Ae. aegypti* which has been cultivated in continuous, laboratory colony after initial colonization in 1952. The rearing and maintenance have been previously described (Pridgeon et al., 2008). Adult and larval mosquito bioassay protocols have been previously described in detail but are described here briefly (Estep et al., 2016; Masi et al., 2017a,b). In the adulticidal assay,

three to seven days post-emergence adult females were cold anesthetized and sorted into groups of 20. The *n*-hexane and the essential oil of *S. tmolea* subsp. *tmolea* samples were applied at 5 μg/mosq in acetone using a Hamiton 7100 series gastight syringe with a PB-600 repeater. Mosquitoes were recovered in TK-35 (Solo Corporation) cups covered in tulle mesh and sealed with a rubber band. Each cup was provided access to a cotton ball saturated with 10% sucrose. Three repetitions of the bioassays were carried out on different days. Mortality was recorded 24 h after application of the extract or permethrin. Negative controls treated with acetone only and positive controls dosed with permethrin were included in all assays.

The larvicidal screening assay method used here is designed for use in microplates in order to produce useful information from mg quantity samples of isolated compounds and allows screening of large numbers of individual compounds and has been well published (Pridgeon et al., 2008; Chang et al., 2014; Tsikolia et al., 2018; Shen et al., 2018) samples were tested at concentrations of 1, 0.5, 0.25 and 0.1 μg/μL against 1st instar *Ae. aegypti* larvae. Assays were performed in a final 200ul volume in 96-well flat bottom cell culture plates with 5 organisms in each well. Each well was provided with 10ul of a 2% (w/v) slurry of alfalfa powder to encourage feeding. After application of the sample, plates were maintained at room temperature (22.5 ± 2 °C) for 24 h. Negative controls were solvent only (DMSO) and positive controls with permethrin were included in all bioassays. Three repetitions of the bioassays were carried out on different days. Mortality in all assays was concluded at 24 h after application of the extract or permethrin.

3. Results and discussion

Essential oils are mixture of volatile and hydrophobic secondary metabolites of plants consist of terpenes and phenylpropanoids. These compounds have a lot of different bioactivities including the antioxidative, cytoprotective, larvicidal, insecticidal, and antiparasitic activities. The hydrophobicity of these compounds combat some diseases like American and African trypanosomiasis, leishmaniasis, and arboviruses, specially dengue (Luna et al., 2019) as crossing the membranes of parasites and the blood-brain barriers. Results obtained from studying the 361 essential oils of 269 plant species against denge virus displayed a larvicidal activity with LCs < 100 mg/L in a review (Dias and Moraes, 2014).

In this study; the chemical composition of essential oil obtained from the aerial parts of *S. tmolea* subsp. *tmolea* was analyzed by GC-FID and GC-MS (Table 1). The composition of the 79.3% total essential oil yielded monoterpene hydrocarbons in trace amounts, sesquiterpene hydrocarbons (10.2%), oxygenated sesquiterpenes (22.6%), fatty acids (14.6%), diterpenes (8.2%), alkanes (2.6%) and others (21.1%). Hexahydrofarnesyl acetone 15.1%, viridiflorol 9.9%, hexadecanoic acid 7.4%, 9-geranyl-*p*-cymene 6.1%, tetradecanoic acid 2.8%, cadalene 2.6%, valeranone 2.3% and 1,5-epoxy-salvial(4)14-ene 2.3% were the majority of compounds in the oil. In contrast, the *n*-hexane extract was characterized mainly by hydrocarbons, aliphatic alcohol, aldehydes, carboxylic acids and phenylpropane. The volatile compounds present in the *n*-hexane were measured by headspace solid phase microextraction (HS-SPME) and followed by GC-MS. The identified components accounted for 82% and major components were identified as 3,4-dimethyl decane (16.0%), 3-methyl-3-pentanol (14.5%), 2-methyl-2-pentanol (12.1%), 1,4-bis(1,1-dimethylethyl)benzene (11.7%), heptanal (9.6%), acetic acid (5.9%), nonanal (2.7%) (Table 2).

Both the essential oil and *n*-hexane extract were screened for the insecticidal activity against *Ae. aegypti*. The n-hexane extract produced 90% ± 10 mortality against adult female *Ae. aegypti* at 5 μg/mosquito, while essential oil demonstrated 12.5% ± 3.6 mortality at the same concentration in the adulticidal bioassays. Neither *n*-hexane extract and nor essential oil showed larvicidal activity at 1, 0.5, 0.25 and 0.1 μg/μL against 1st instar *Ae. aegypti*. Control mortality in these experiments was 0% for solvent only and 100% for the 0.62 ng/mosquito permethrin positive control (Table 3).

Studies on the essential oil composition of *Stachys* species are numerous (Khanavi et al., 2004; Cavar et al., 2010; Giuliani et al., 2009; Hajdari et al., 2011; Ali et al., 2010). In a study on twenty-two *Stachys* species, β-caryophyllene, germacrene D, α-cadinene and caryophyllene oxide were the major constituents (Goren et al., 2011a,b). The essential oil of *S. tmolea*, which 93.2% of the composition was determined, exhibited a high proportion of sesquiterpenes (58.4%) ensued by oxygenated sesquiterpenoids (21.9%). Of the 28 constituents detected, the most amplewere germacrene D (22.2%), β-caryophyllene (19.7%) and valeranone (8.5%), α-cadinene, spathulenol, caryophyllene oxide (Goren et al., 2011a,b). The essential oil content of aerial parts of *S. parviflora* was analyzed by GC-FID and GC-MS systems. Twenty-three constituents, representing 99.9% of the oil, were identified. Epi-α-muurolol (48.4%) and (*Z*)-caryophyllene (11.2%) were the major constituents of the oil. Oxygenated sesquiterpenes (71.4%) was the major fraction of the essential (Bashi et al., 2013). Sesquiterpenes such as spathuleol, T-muurol, elemene, cadinol, α-eudesmol, isoledene, caryophyllene was found in higher percent in an endemic species of *S. rupestris* (Erdoğan, 2014). *Stacyhs iberica* subsp. *iberica* essential oil was characterized by hexadecanoic acid (41.5%), %), phytol (8.2%) and germacrene D (9.7%) (Goger at al., 2016) whereas *S. iberica* subsp. *stenostachya* essential oil was rich in linalyl acetate (42.2%), linalool (18.9%), geranyl acetate (8.2%), and α-terpineol (5.3%) as the main components (Kaya et al., 2001). It clearly showed that the oil compositions of two taxa are different (Goger et al., 2016).

In conclusion, the essential oil and *n*-hexane extract of *S. tmolea* had different chemical composition. In addition, the chemical composition in this analysis of *S. tmolea* was different than a previous study of the essential oil (Goren et al., 2011a,b). It is the fact that different geographic regions, seasons, harvest periods, properties of soils and climatic conditions strongly affect the secondary metabolite content of the plant species, especially essential oil composition. We also observed differences in the insecticidal activity. The *n*-hexane extract produced 90% mortality whereas the essential oil showed only 13% mortality against adult *Ae. aegypti*. Further studies need to plan on the *n*-hexane extract of the aerial parts from *S. tmolea* to identify the constituents responsible for the activity against adult *Ae. aegypti*.

Table 3
The mortalities of essential oil and *n*-hexane extract of *Stachys tmolea* against adult female *Ae. aegypti* and 1st instar *Ae. aegypti* larvae.

	Adult female mosquito *Aedes aegypti*[*]	1st instar *Aedes aegypti* larvae (% mortality)[**]			
Samples	5 μg/mosquito (% mortality)	1 μg/μL	0.5 μg/μL	0.25 μg/μL	0.1 μg/μL
essential oil	12.5 ± 3.6	0	0	0	0
n-hexane extract	90 ± 10	0	0	0	0

[*] Control mortality in adulticidal experiments was 0% for solvent only (acetone) and 100% for the 0.62 ng/mosquito permethrin positive control.
[**] Control mortality in larvicidal experiments was 100% for 41.36 ppb permethrin positive control. Negative control solvent control (DMSO) had 0% mortality.

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