Asian Journal of Chemistry; Vol. 29, No. 11 (2017), 2488-2492

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2017.20813



Chemical Composition and Mosquitocidal Activity of *n*-Hexane and Methanolic Extracts from *Euphorbia anacampseros* var. *tmolea*: An Endemic Species of Turkey against *Aedes aegypti*

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Received: 26 May 2017; *Accepted*: 13 July 2017; *Published online*: 29 September 2017; AJC-18578

Volatile composition of the *n*-hexane and methanol extracts from *E. anacampseros* var. *tmolea* was analyzed by head space-solid phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS). Chemical characterization of the methanolic extract was determined by LC-ESI-MS/MS. Both extracts were bioassayed against 1st instar larvae and adult female *Ae. aegypti*. The main components identified from the *E. anacampseros* var. *tmolea* *n*-hexane fraction were 1,8-cineole (27.5 %), *p*-cymene (25 %), γ-terpinene (12.8 %), limonene (9.9 %). Methyl hexanoate (18.8 %), methyl nonanoate (13.3 %), dimethyl succinate (6.5 %), methyl octanoate (6 %) and methyl phenylacetate (5.3 %) were identified from the methanolic fraction. The *n*-hexane extract showed 100 % mortality at 0.1 µg/µL against 1st instar larvae of *Ae. aegypti* and the methanolic extract exhibited 83.3 % mortality at 5 µg/mosquito against adult female *Ae. aegypti*. The bioassay-guided study demonstrates that *n*-hexane and methanol extracts of *E. anacampseros* var. *tmolea* contain compounds with natural mosquito larvicidal and adulticidal activity.

Keywords: Euphorbiaceae, Mosquito control, Biopesticide, Monoterpenes, Aliphatic esters.

INTRODUCTION

The genus *Euphorbia* is the largest in the spurge family (Euphorbiaceae), comprising about 50 tribes, 300 genera and more than 2000 species; probably the highest species richness with a world-wide distribution [1]. They are widely distributed throughout both tropical and temperate regions and range in morphology from small, annual or perennial herbaceous plants to woody shrubs, lianas, trees and large desert succulents [2]. In Turkey 108 '*Euphorbia*' taxa are known, 14 of which are endemic species. One of the endemics, *Euphorbia anacampseros* var. *tmolea* Boiss., occurs on Bozdag in Ödemis, Izmir, Turkey. It is a glabrous, glaucous, decumbent-ascending perennial herb or subshrub common to rocky slopes (sometimes present in *Pinus brutia* or *Quercus* forest), mountain steppe, phrygana, lake and stream sides, at elevations of 600-1900 m. It has several or simple stems arising from a woody stock reaching 30-45 cm tall and it has cauline leaves suborbicular, ovate, rhombic, obovate or obtrullate [3]. Due to the rich cultural heritage and relatively rich flora in Turkey, some *Euphorbia* species such as *E. amygdaloides* L., have been used medicinally to treat skin diseases and wounds in different provinces [4].

Mosquitoes can be controlled at different development stages including egg, larval and adult stages. During larval stages, mosquitoes are active and aquatic. Their larval habitat is limited to water bodies where both food and air for gaseous exchange are obtained, making them susceptible to any changes that occur in the water body [5]. This limitation can be exploited, by blocking nutrient uptake, breathing systems or both. The use of larvicides is one of the oldest methods of controlling *Anopheles* spp. that vector malarial parasites [6]. Among other advantages, larvicides control mosquitoes before they are able to spread and transmit pathogens that cause diseases [5,7]. While other methods like adult spraying may show quick results, larval control has been successfully applied to bring malaria under control, including in countries such as Brazil, Egypt and Zambia [6].

The primary method for the control of mosquito-borne diseases is the use of insecticides and many synthetic agents have been developed and employed in the field with considerable success. However, major drawbacks to the use of

chemical insecticides are that most are non-selective and can be harmful to other organisms in the environment [8]. This non-target toxicity problem, together with the growing incidence of insect resistance, has called attention to the need for novel insecticides [9] and for more detailed studies of naturally occurring insecticides [10]. Control of the mosquito larvae is largely dependent on continued applications of organophosphates (chlorpyrifos, temephos and fenthion), insect growth regulators (diflubenzuron and methoprene) [11] and *Bacillus thuringiensis israeliensis* [12]. Frequent use of synthetic insecticides has disturbed natural biological systems and led to insecticide resistance and amplified environmental and human health concerns [8]. This warrants the need for the development of new strategies for selective control of mosquito populations. Plants are a good source of alternative agents for control of mosquitoes [13,14] because they are rich in bioactive chemicals which are biodegradable. The *Euphorbia* genus is known to contain a wide variety of terpenoids, ranging from mono-, sesqui- and diterpenes to triterpenoids, flavonoids and steroids known for their toxicity or potential therapeutic activity [15]. Taking this into consideration, we investigated the chemical composition of hexane and methanol extracts from *E. anacampseros* var. *tmolea* and their efficacy against 1st instar larvae and adult female *Aedes aegypti* L.

EXPERIMENTAL

Euphorbia anacampseros var. *tmolea* Boiss., an endemic species of Turkey, was collected from its only natural habitat of Bozdag, Odemis, Izmir (Turkey). A voucher specimen has been deposited at the Herbarium of Ege University, Faculty of Science, Izmir, Turkey (Voucher specimen no: 42191 Leg.). The plant material was identified by Dr. Volkan Eroglu (Ege University, Faculty of Science, Department of Biology, Izmir, Turkey).

Extraction procedure: Aerial parts of *E. anacampseros* var. *tmolea* were powdered and macerated with *n*-hexane (100 g plant material in 1 L solvent) in cold. The solvent was removed and extraction was repeated until the solvent remained colourless. After removal of remaining *n*-hexane, the residue was macerated with aliquots of methanol until the solvent remained clear. Solvents were removed from each extract under vacuum, yielding a dark brown residue. Both extracts were used in mosquito bioassays and also phytochemical analysis.

Headspace-SPME: The manual SPME device (Supelco, Bellafonte, PA, USA) with a fiber-precoated 65 μm thick layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB-blue) was used for extraction of the plant volatiles. The vial containing plant extract was sealed with parafilm. The fiber was pushed through the film layer for exposure to the headspace of extract for 15 min at 40 °C. The fiber was then inserted immediately into the injection port of the GC-MS for desorption of the adsorbed volatile compounds for analysis.

GC-MS analysis: The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and increased to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 min and then increased to 240 °C at a rate of 1 °C/min. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV. Masses ranged from *m/z* 35 to 450.

Identification of the compounds: Identification of the volatile components was carried out by comparison of their relative retention times with those of pure samples or by comparison of the relative retention index (RRI) of a series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) [16,17] and in-house "Baser Library of Essential Oil Constituents" composed of genuine compounds and components of known oils, as well as MS literature data [18,19] was used for the identification.

LC-ESI-MS/MS analysis: LC-MS/MS analysis was carried out using an Absciex 3200 Q trap MS/MS detector. Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC-MS/MS instrument equipped with an ESI source operating in negative ion mode. For the chromatographic separation, a GL Science Intersil ODS 250 × 4.6 mm, i.d., 5 μm particle size, octadecyl silica gel analytical column operating at 40 °C was used. The solvent flow rate was maintained at 0.7 mL/min. Detection was carried out with a PDA detector. The elution gradient consisted of mobile phases (A) acetonitrile:water:formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1, v/v/v). The contribution of B was increased from 10 to 100 % over 40 min. LC-ESI-MS/MS data were collected and processed by Analyst 1.6 software. For enhanced mass scan (EMS), the MS was operated at a mass range of 100-1000 amu.

Mosquito bioassays: The mosquito strain used for this series of bioassays was the ORL1952 strain of *Aedes aegypti* which has been maintained in continuous, unsupplemented laboratory colony after initial colonization in 1952. This strain is broadly susceptible to a number of pesticides [20]. Rearing and maintenance have also been previously described.

Adult and larval mosquito bioassay screening against natural products has been previously described [21,22]. In the larval assay, concentrations of 1, 0.5, 0.25 and 0.1 μg/μL are screened against 1st instar ORL1952 larvae. For the adult assay, the extracts were applied at 5 μg/mosq in acetone. Negative controls containing solvent only with no extract and positive controls containing permethrin were included in all bioassays. Three repetitions of the bioassays were performed on different days. Mortality in all assays was determined at 24 h after application of the extract or permethrin.

RESULTS AND DISCUSSION

The *n*-hexane and methanolic extracts were evaluated for larvicidal and adulticidal activity against *Ae. aegypti* (Table-1). The *n*-hexane extract produced larvicidal activity at each screening concentrations of 1, 0.5, 0.25 and 0.1 μg/μL, while the methanolic extract did not show any larvicidal activity at the same concentrations. However, the methanolic extract showed higher adulticidal mortality than the hexane extract, although both had activity above 70 % at the screening dose.

Subsequently, the volatile composition of *n*-hexane and methanolic extracts from *E. anacampseros* var. *tmolea* was identified using headspace-solid phase microextraction (HS-SPME) and GC-MS systems. Forty-four compounds in total

TABLE-1
MOSQUITOCIDAL ACTIVITY AGAINT 1st INSTAR AND ADULT FELMALE *Ae. aegypti* ORL1952 STRAIN

Samples	Mortality (%)				
	Larvicidal activity*				Adulticidal activity**
	1 µg/µL	0.5 µg/µL	0.25 µg/µL	0.1 µg/µL	5 µg/mosquito
n-Hexane extract	100	100	100	100	76.7 ± 15.3
Methanol extract	0	0	0	0	83.3 ± 11.5

*In larval bioassays, positive control permethrin at 0.04 ng/µl; negative control solvent control (DMSO) had 0 mortality.
**In adult bioassays, two positive control permethrin doses were included at 0.19 ng (60 ± 10 % mortality) and 0.86 ng (100 % mortality) in all assays; negative control solvent control (acetone) had 0 mortality.

were characterized in the *n*-hexane and 36 in the methanolic extracts (Table-2). Monoterpene hydrocarbons, *p*-cymene (25 %), γ-terpinene (12.8 %), limonene (9.9 %), myrcene (3.2 %), α-pinene (2.3 %) and oxygenated monoterpenes, 1,8-cineole (27.5 %), linalool (3.4 %) and camphor (1.7 %) were the main components of *n*-hexane extract, while the methanolic extract was dominated mostly with linear esters, methyl hexanoate (18.8 %), methyl nonanoate (13.3 %), dimethyl succinate (6.5 %), methyl octanoate (6 %), methyl pentanoate (3.7 %), dimethyl malonate (3.1 %), methyl heptanoate (2.9 %) and with an aromatic ester methyl phenylacetate (5.3 %).

TABLE-2
VOLATILE COMPOSTION OF *E. anacampseros* var. *tmolea*

RRI	Compound	*n*-Hexane extract (%)	Methanolic extract (%)	Identification method
1032	α-Pinene	2.28	-	RRI, MS
1090	Methyl pentanoate	-	3.7	RRI, MS
1076	Camphene	0.2	-	RRI, MS
1197	Methyl hexanoate	-	18.8	RRI, MS
1118	β-Pinene	1.3	-	RRI, MS
1132	Sabinene	1.0	-	RRI, MS
1159	δ-3-Carene	1.1	-	MS
1174	Myrcene	3.2	-	RRI, MS
1188	α-Terpinene	1.4	-	RRI, MS
1203	Limonene	9.9	-	RRI, MS
1213	1,8-Cineole	27.5	-	RRI, MS
1255	γ-Terpinene	12.8	-	RRI, MS
1280	*p*-Cymene	25.0	-	RRI, MS
1290	Terpinolene	0.4	-	RRI, MS
1382	*cis*-Alloocimene	0.1	-	MS
1296	Methyl heptanoate	-	2.9	RRI, MS
1360	1-Hexanol	-	0.4	RRI, MS
1398	2-Nonanone	0.3	-	MS
1399	Methyl octanoate	0.1	6.0	RRI, MS
1400	Nonanal	0.3	-	MS
1406	α-Fenchone	0.4	-	MS
1437	α-Thujone	0.2	-	RRI, MS
1443	2,5-Dimethylstyrene	0.1	-	MS
1450	*trans*-Linalool oxide (Furanoid)	0.1	-	MS
1452	1-Octen-3-ol	0.3	0.5	MS
1474	*trans*-Sabinene hydrate	0.1	-	MS
1475	Menthone	1.9	-	RRI, MS
1487	Citronellal	0.2	-	RRI, MS
1475	Acetic acid	-	0.4	RRI, MS
1500	Methyl nonanoate	0.1	13.3	RRI, MS
1503	Isomenthone	0.7	-	MS
1505	Dihydroedulane II*	-	0.4	MS
1510	Dimethyl malonate≠	-	3.1	RRI, MS
1532	Camphor	1.7	-	RRI, MS
1553	Linalool	3.4		RRI, MS
1562	Octanol	-	0.4	RRI, MS
1565	Linalyl acetate	0.1	-	RRI, MS
1573	(*E,E*)-3,5-Octadien-2-one		0.2	MS
1586	Pinocarvone	0.1	-	RRI, MS
1591	Bornyl acetate	0.3	-	RRI, MS
1591	2-Methyl propanoic acid	-	0.3	MS
1601	Methyl decanoate	-	1.3	RRI, MS
1602	Dimethyl succinate≠	-	6.5	RRI, MS
1602	6-Methyl-3,5-heptadien-2-one	-	1.3	MS
1611	Terpinen-4-ol	0.3	-	RRI, MS
1621	2-Octen-1-ol	-	0.1	MS
1625	4,4-Dimethyl but-2-enolide	-	0.4	MS
1638	Menthol	0.1	-	RRI, MS
1631	γ-Pentalactone	-	0.2	RRI, MS
1641	Methyl benzoate	-	0.9	RRI, MS
1645	*cis*-Isodihydrocarvone	tr	-	MS
1648	Myrtenal	0.1	-	MS
1651	γ-Butyrolactone	-	0.2	RRI, MS
1662	Pulegone	0.1	-	RRI, MS
1664	Nonanol	-	0.2	RRI, MS
1670	*trans*-Pinocarveol	0.1	-	RRI, MS
1687	Methyl chavicol	0.3	-	RRI, MS
1706	α-Terpineol	0.1	-	RRI, MS
1719	Borneol	0.1	-	RRI, MS
1726	γ-Hexalactone	-	0.4	RRI, MS
1751	Carvone	0.3	-	RRI, MS
1762	Pentanoic acid	-	0.4	RRI, MS
1779	Methylphenyl acetate	-	5.3	MS
1802	Cumin aldehyde	0.1	-	RRI, MS
1815	Methyl dodecanoate	-	0.7	RRI, MS
1871	Hexanoic acid	-	1.2	RRI, MS
1896	Benzylalcohol	-	0.2	RRI, MS
1937	Phenyl ethyl alcohol	tr	0.4	RRI, MS
1977	Heptanoic acid	-	0.1	RRI, MS
1984	Benzothiazol	0.1	-	MS
1996	2-Acetylpyrrole	-	0.1	MS
2004	*o*-Cresol	-	0.1	RRI, MS
2022	Methyl tetradecanoate	-	0.3	RRI, MS
2192	Nonanoic acid	-	Tr	RRI, MS
2226	Methyl hexadecanoate	-	0.1	RRI, MS

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
≠Purchased from Sigma-Aldrich Co., St. Louis, MO, USA.

The chemical composition of methanolic extract of *E. anacampseros* var. *tmolea* was also investigated with LC-MS/MS. Four compounds (**1**-**4**) were determined according to their molecular ion peaks and MS fragmentation behaviours (Table-3; Fig. 1). Compound **1** showed molecular ion peak at *m/z* 367 which fragmented the base peak ion at *m/z* 193 due to the loss of a feruloyl unit. Other fragment ions at *m/z* 149 and 134, formed after the loss of a methyl group, was led to the identification of this peak as 3-feruloylquinic acid (**1**) [23]. Compounds **2**-**4** were determined as quercetin derivatives which were previously identified in *Euphorbia* species [24]. Compound **2** showed pseudo molecular ion peak at *m/z* 463 and a base peak ion at *m/z* 300 which was formed after the loss of a glucose unit. Other fragments at *m/z* 271, 255 were also observed. The base peak ion and further fragments are characteristic for quercetin. These data led to identifying compound **2** as quercetin glucoside. Similar identifications were done for compound **3** and compound **4** which have the same quercetin aglycone. Compound **3** presented molecular ion peak at *m/z* 477 and showed product ion at *m/z* 301 due to the loss of a glucuronic acid moiety, so compound **3** was identified as quercetin glucuronide. Compound **4** was identified as quercetin rhamnoside due to a loss of a rhamnosyl unit (-147) from molecular ion peak at *m/z* 447 [25].

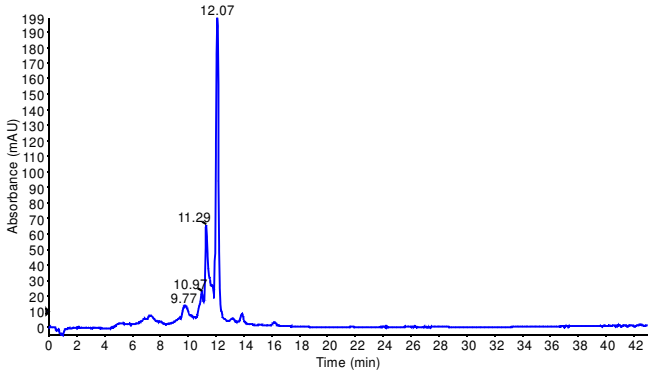

Fig. 1. At 348 nm LC chromatogram of *E. anacampseros* var. *tmolea* methanolic extract

Due to the disadvantages associated with synthetic pesticides, including development of pesticide resistant strains, ecological imbalances and harm to non-target organisms, there is a renewed effort to develop substances of plant origin which are considered to be more environmentally friendly due to their innate biodegradability and lower toxicity to most organisms [26]. Several researchers have investigated the application of plant extracts to fight malaria vectors. For example, *Achyranthus aspera* [27], *Azadirachta indica* [28], *Jatropha curcas*, *Euphorbia tirucalli*, *Euphorbia hirta*, *Phyllanthus amarus* and *Pedilanthus tithymaloides* [29], *Piper nigrum* [30], *Chenopodium album* [31], *Solanum xanthocarpum* [32], *Ajuga remota* [33], *Thymus capitatus* [34], *Tagetes erectes*, *Cleome icosandra*, *Ageratum conyzoides*, *Eichhornia crassipes* [35]. Larvicidal activity of ethyl acetate, butanol and petroleum ether extracts of five species of Euphorbiaceae plants, *Jatropha curcas*, *Pedilanthus tithymaloides*, *Phyllanthus amarus*, *Euphorbia hirta* and *Euphorbia tirucalli* were previously tested against the early fourth instar larvae of *Ae. aegypti* L. and *Culex quinquefasciatus* (Say) [29]. Previous studies reported that *E. tirucalli* have shown larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* [29,36] and *E. lactea* latex had larvicidal activity against three mosquito vectors, *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* [37]. Bioassay-guided fractionation of ethyl acetate extract of *E. lactea* latex resulted in an active fraction and identified the chemical constituents by GC/MS analysis as a tricyclic sesquiterpene and an aliphatic hydrocarbon [37].

Conclusion

The present study showed that components of *n*-hexane extract of *E. anacampseros* var. *tmolea* exhibited mosquitocidal activity against 1st instar larvae and adult female *Ae. aegypti*. In contrast, only adulticidal activity was identified in the MeOH extract from the same source. To the best of our knowledge, this is the first report of the chemical composition of *E. anacampseros* var. *tmolea* hexane and methanol extracts and evaluation of their biological activity against *Ae. aegypti*. Concerning monoterpenes, some of the monoterpenes showed dose-dependent larvicidal activity against *Ae. aegypti*. For example, Santos *et al.* [38] reported that limonene (LC_{50} = 27 ppm for (-)-limonene, LC_{50} = 30 ppm for (+)-limonene) showed the highest larvicidal activity and followed the activity by γ-terpinene (LC_{50} = 56 ppm) against 3rd instar *Ae. aegypti*. Another study reported that α-terpinene, *p*-cymene, (-)-limo-nene, (+)-limonene, γ-terpinene showed 100 % mortality against 3rd instar *Ae. aegypti* at 0.1 mg/mL and mortality decreased for these compounds at lower concentrations [39]. One of our previous studies found that 1,8-cineole was not active at the prescreening dose of 100 ppm against 1st instar *Ae. Aegypti* [40]. Santana *et al.* [41] reported that *p*-cymene rich essential oil from three *Piper* species (*P. grande*, *P. jac-quemontianum* and *P. multiplinervium*) did not show larvicidal activity at $LC_{100} \geq 500$ µg/mL. The moderate adulticidal activity of *n*-hexane extract of *E. anacampseros* var. *tmolea* could be attributed to *n*-hexane extracts being rich in monoterpenes. This hypothesis is supported by reports that monoterpene rich *Hedycium* essential oils had no mortality at the screening dose of 3.125 mg/mL per mosquito against adult *Ae. aegypti* [42]. On the other hand, the methanol extract of *E. anacampseros* var. *tmolea* demonstrated adulticidal activity against adult female *Ae. aegypti*. In the current study, we found that the volatile composition of methanol extract of *E. anacampseros* var. *tmolea* is rich in methyl esters. Chaskopoulou *et al.*

TABLE-3
CHEMICAL COMPOSITION OF *E. anacampseros* var. *tmolea* METHANOL EXTRACT

Compound	Rt	[M-H] *m/z*	Fragments	Compounds	Reference
1	9.7	367	193, 149,134	3-Feruloylquinic acid	[23]
2	11.0	463	300, 271,255,179, 151	Quercetin glucoside	[24]
3	11.3	477	301, 273,179, 151	Quercetin glucuronide	[25]
4	12.1	447	300, 271, 255	Quercetin rhamnoside	[24]

[43] reported that methyl substituted aliphatic esters showed higher mortality (LC_{50} < 0.68 mg/0.5 L) against adult female *Ae. aegypti* than ethyl, propyl, ethyl and hexyl ester. Therefore, methyl esters are highly promising candidates to be evaluated for mortality in adult mosquitoes. The methanol extract is rich in quercetin derivatives and these compounds might be responsible for the adult activity as well. All these findings encourage further research by bioassay-guided fractionation of *E. anacampseros* var. *tmolea n*-hexane and methanol extracts to discover active and safe natural larvicides and adulticides against *Ae. aegypti*.

ACKNOWLEDGEMENTS

This study was partly funded by the Deployed War-Fighter Protection Research Program *via* grants from the U.S. Department of Defense through the Armed Forces Pest Management Board. The authors thank Miss Jessica Louton (USDA-ARS, CMAVE, Gainesville, FL) for the mosquito bioassays.

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