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Preparative Capillary GC for Characterization of Five *Dracocephalum* Essential Oils from Mongolia, and their Mosquito Larvicidal Activity

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The chemical composition and mosquito larvicidal and adulticidal activity of five essential oils (EOs), *Dracocephalum ruyschiana* L. (**DR**), *D. foetidum* Bunge (**DF**), *D. moldavica* L. (**DM**), *D. fruticulosum* Steph. ex Willd. (**DFr**) and *D. peregrinum* L. (**DP**) were evaluated. Simultaneous GC-FID and GC-MS analyses revealed in the EOs of **DR**, **DF** and **DM** an unidentified compound (**1**) ([M^+122], 5.4%, 57.9% and 74.0%, respectively). Therefore, we aimed to isolate compound **1** using Preparative Capillary GC (PCGC) connected to a Preparative Fraction Collector (PFC) system. Structure determination of **1** was determined by ^{1}H- and ^{13}C-NMR as *p*-mentha-1,8-dien-10-al (**1**, limonen-10-al). Other detected major constituents were thymol (34.0%) and carvacrol (6.1%) in **DR**; limonene (28.8%) in **DF**; *cis*-chrysanthenol acetate (29.1%) and *trans*-verbenol (5.0%) in **DFr**; and linalool (17.9%), *trans*-β-bergamotene (7.9%), (*E*)-nerolidol (7.7%) and eugenol (5.5%) in **DP**. The **DFr**, **DP**, **DR** and **DM** EOs produced 100% mortality to 1st instar larvae of *Aedes aegypti* L. at 250 ppm and **DR** and **DM** EO exhibited the strongest activity and killed 100% 1st instar larvae at 62.5 ppm. No adulticidal activity was observed against female *Ae. aegypti.* In the scope of the present study, for the first time isolation of limonen-10-al (**1**) from **DR**, **DM**, and **DP** EOs and larvicidal activity of five *Dracocephalum* EOs were reported.

Keywords: *Dracocephalum* essential oils, Preparative gas chromatography, NMR, Limonen-10-al, *Aedes aegypti*, Mosquito control.

Dracocephalum L., one of the most important genera of Lamiaceae, encompasses *ca*. 75 species, mainly distributed in alpine and steppe regions of mainly Eurasia, northern America and extending to the alpine steppes of the Pamir Altai and Tian-Shan, Siberia, Kazakhstan, Central Asia and Mediterranean.

Dracocephalum species are used as a food ingredient (*e.g.* in yogurt), as a tea, as a herbal drug for their reputed medicinal properties: the treatment of stomach and liver disorders, headaches, cardioprotection, chronic bronchitis, hepatitis, gastritis, laryngitis, acute respiratory infection, diarrhea, and rheumatoid arthritis [1a]. *D. moldavica* is used in stomach and liver disorders, congestion, for coronary heart disease and hypertension [1b]. In Uygur medicine, *D. moldavica* was reported to be effective against chronic mountain sickness [1c]. A wide range of biological activities of *Dracocephalum* species have been scientifically investigated: antioxidant [2a-b], anticancer [2c], antibacterial [2d] and insecticidal [2e-f] properties.

A comprehensive review of chemical constituents of plants from the genus *Dracocephalum* has recently been reported by Zeng *et al*. [3a]. Previous phytochemical investigations of *Dracocephalum* species resulted in EOs [3b-c], triterpenoids [3d], phenolics [3e] and alkaloids [3f]. The most investigated *Dracocephalum* species are *D. moldavica* (dragonhead) and *D. kotschyi*, which are cultivated in many countries [4a-b].

Our work aimed to elucidate further knowledge on the secondary metabolites of five *Dracocephalum* species from Mongolia and search for novel biological properties of these species. The list of detected compounds with their relative percentages, retention indices and method of identification is given in Table 1 in order of their elution on the HP-Innowax FSC column. GC analysis of **DR**, **DF**, **DM**, **DFr** and **DP** EOs resulted in 98, 31, 53, 47 and 35 constituents representing 89.4%, 95.0%, 93.1%, 87.3% and 98.6% of the oils, respectively. The EOs were characterized by a high diversity of volatile constituents which were classified as mono- and sesquiterpene hydrocarbons and their oxygenated forms, phenylpropanoids, fatty acids and their esters, and aliphatic esters. Oxygenated monoterpenes were the most abundant of the groups in **DR**, **DF**, **DM**, **DFr** and **DP** EOs, representing 59.1%, 63.7%, 85.1%, 77.2% and 34.7% of the oils, respectively. They were followed by oxygenated sesquiterpenes (7.3%, 0.1 %, 2.7%, 7.6% and 27.6%, respectively).

Table 1: The chemical composition of the *Dracocephalum* EOs.

RRI[a)]	RRI[b)]	Compound	%[c)]					ID Method
			DR	DF	DM	DFr	DP	
1032	1032	α-Pinene	0.2	0.6	0.2			d, e, f
1035	1035	α-Thujene	0.2	t				d, e, f
1076	1085	Camphene	0.1	t				d, e, f
1118	1118	β-Pinene	0.5	0.6				d, e, f
1132	1132	Sabinene	t	0.2				d, e, f
1148		Thuja-2,4(10)-diene				t		d, e, f
1159		Hexyl-2-methyl butyrate			t	0.2		f
1174	1156	Myrcene	0.2	t				d, e, f
1183		*p*-Mentha-1,7(8)-diene		0.1				d, e, f
1203	1205	Limonene	0.5	**28.8**	3.5			d, e, f
1213	1216	1,8-Cineole	0.4	0.2	0.2		2.3	d, e, f
1246	1230	(*Z*)-β-Ocimene	0.1	t				d, e, f
1255	1256	γ-Terpinene	0.1		t			d, e, f
1266		3-Octanone	0.3	t	t			d, e, f
1279	1279	*p*-Cymene	1.7		t			d, e, f
1290	1283	Terpinolene	t					d, e, f
1345		3-Octyl acetate	0.8					d, e, f
1391	1368	(*Z*)-3-Hexenol	0.1					d, e, f
1393		3-Octanol	0.4					d, e, f
1400	1381	Nonanal	0.3					d, e, f
1437	1437	α-Thujone		0.1	t			d, e, f
1450		*trans*-Linalool oxide (*Furanoid*)	0.2	0.2	t	0.8		d, e, f
1452		1-Octen-3-ol	0.5	0.3	0.2			d, e, f
1466		Dimethyl tetradecane	0.2		t	0.3		d, e, f
1468		*trans*-1,2-Limonene epoxide			t			d, e, f
1471		(*Z*)-3-Hexenyl butyrate				t		d, e, f
1474	1474	*trans*-Sabinene hydrate	0.2		t	t		d, e, f
1478	1449	*cis*-Linalool oxide (*Furanoid*)	t		t	0.7		d, e, f
1482		(*Z*)-3-Hexenyl-2-methyl butyrate				t		d, e, f
1483	1464	Octyl acetate	0.3					d, e, f
1484		α-Longipinene			t			d, e, f
1497	1497	α-Copaene	0.3		t			d, e, f
1522		Chrysanthenone				t		d, e, f
1532	1532	Camphor	0.2			t	0.3	d, e, f
1535	1519	β-Bourbonene	1.2			0.5	0.2	d, e, f
1536		Pinocamphone				0.1		d, e, f
1538	1508	Benzaldehyde	t					d, e, f
1540	1516	α-Gurjunene	t					d, e, f
1554	1556	Linalool	0.8	0.3		4.3	**17.9**	d, e, f
1554		8,9-Limonene epoxide		0.2				d, e, f
1556	1556	*cis*-Sabinene hydrate	0.1			0.3	0.6	d, e, f
1562		Isopinocamphone			t	3.8		d, e, f
1582		*cis*-Chrysanthenyl acetate			t	**29.1**		d, e, f
1584		Nonyl acetate	t					d, e, f
1586	1585	Pinocarvone	0.1					d, e, f
1590	1571	Bornyl acetate	0.5		t	0.3	0.9	d, e, f
1594	1594	*trans*-β-Bergamotene	0.1				7.9	d, e, f
1596		α-Guaiene					2.2	d, e, f
1600	1594	β-Elemene	0.3		t			d, e, f
1612	1616	Terpinen-4-ol	1.4	t	0.4	0.3	4.2	d, e, f
1613	1604	β-Caryophyllene	0.4	0.1	0.1			d, e, f
1624		*trans*-Dihydrocarvone			t			d, e, f
1637		*p*-Menth-1-en-9-al		t	t			d, e, f
1638		β-Cyclocitral	t					d, e, f
1642		Thuj-3-en-10-al				0.2		d, e, f
1643		*trans*-*p*-Mentha-2,8-dien-1-ol	0.1	0.2	0.4			d, e, f
1645		*cis*-Verbenyl acetate				1.1		d, e, f
1648	1645	Myrtenal				1.6		d, e, f
1661		*trans*-Pinocarvyl acetate				1.3		d, e, f
1668	1705	Safranal	0.1					d, e, f
1670	1646	*trans*-Pinocarveol	0.1			1.7		d, e, f
1672		*p*-Mentha-1,8-dien-10-al (= Limonen-10-al)	**5.4**	**57.9**	**74.0**			g
1678		*cis*-*p*-Mentha-2,8-dien-1-ol	0.2	0.2	0.2			d, e, f
1682		δ-Terpineol				1.8		d, e, f
1683	1680	*trans*-Verbenol	0.2			5.0		d, e, f
1686	1687	α-Humulene	0.1	t				d, e, f
1687		*cis*-Dehydrosesquicineole		0.1				d, e, f
1689		Methyl chavicol					1.6	d, e, f
1690	1687	Cryptone					1.5	d, e, f
1694	1697	Neral	1.0		1.0			d, e, f
1695	1695	(*E*)-β-Farnesene					0.5	d, e, f
1700		*p*-Mentha-1,8-dien-4-ol		t				d, e, f
1702	1700	Heptadecane	0.1					d, e, f
1704		Myrtenyl acetate				1.7		d, e, f
1705	1710	γ-Muurolene	t					d, e, f
1706	1706	α-Terpineol	1.2	0.4	0.5	2.2	1.9	d, e, f
1715		Geranyl formate			0.3			d, e, f
1719	1717	Borneol	0.3					d, e, f
1725	1725	Verbenone				3.7		d, e, f
1726	1716	Germacrene D	2.9	0.5			1.6	d, e, f
1730		δ-Guaiene					1.8	d, e, f
1733	1725	Neryl acetate				0.5		d, e, f
1737	1728	(*Z,E*)-α-Farnesene	0.2					d, e, f
1739	1716	α-Muurolene	0.7					d, e, f
1742	1746	Geranial	1.8		2.3			d, e, f
1743	1779	α-Cadinene	0.2					d, e, f
1751	1751	Carvone	0.1	0.2	0.6	1.7		d, e, f
1755	1742	Bicyclogermacrene						d, e, f
1760		*p*-Mentha-1,8-dien-10-yl acetate		0.6	0.3			d, e, f
1764		*cis*-Chrysanthenol				2.6		d, e, f
1765	1742	Geranyl acetate	1.2		1.1			d, e, f
1770		*trans*-Linalool oxide (*Pyranoid*)				0.7		d, e, f
1769	1764	δ-Cadinene	1.7					d, e, f
1776	1766	γ-Cadinene	0.8				2.1	d, e, f
1800	1800	Octadecane	0.1					d, e, f
1804	1790	Myrtenol	0.2			1.4		d, e, f
1807		Perilla aldehyde				0.6		d, e, f
1808	1807	Nerol	0.2					d, e, f
1827		(*E,E*)-2,4-Decadienal	0.3					d, e, f
1838	1838	(*E*)-β-Damascenone	0.7		t			d, e, f
1845	1845	*trans*-Carveol	0.2		0.4	2.2		d, e, f
1853	1857	*cis*-Calamenene	t					d, e, f
1857	1851	Geraniol	1.5		0.3			d, e, f
1860		4,7-(endo)-Dimethylbicyclo(3.2.1)-oct-3-en-6(exo)-ol			0.7			f
1864	1864	*p*-Cymen-8-ol	t			2.5	0.8	d, e, f
1868		(*E*)-Geranyl acetone	0.2					d, e, f
1875		*trans*-2-Hydroxy-1,8-cineole				0.8		d, e, f
1880		Benzyl 2-methylbutyrate				0.6		d, e, f
1890		Carvacryl acetate	0.7					d, e, f
1900	1879	*epi*-Cubebol	0.1					d, e, f
1900	1900	Nonadecane	0.2					d, e, f
1912		*cis*-Dihydrocarveol			0.1			d, e, f
1941	1916	α-Calacorene	0.2					d, e, f
1945		*neo*-Isodihydrocarveol		0.9				d, e, f
1953	1980	Palustrol					2.1	d, e, f
1976		*trans*-2-Hydroxy pinocamphone				1.7		f
1988		2-Phenylethyl-2-methylbutyrate				0.5		d, e, f
2000		*p*-Menth-1-en-9-al (isomer 1)			0.5			f
2008	2008	Caryophyllene oxide	0.6		0.6	0.5	3.9	d, e, f
2009		*p*-Mentha-1,8-dien-10-ol	0.4	2.3	0.3			d, e, f
2018		4a-β-7α-7a-α-Nepetalactone					t	d, e, f
2026	2023	Methyl eugenol			0.1		2.6	d, e, f
2031	2016	Salvial-4(14)-en-1-one	0.3					d, e, f
2050	2044	(*E*)-Nerolidol	0.7		1.3		7.7	d, e, f
2071	2071	Humulene epoxide-II			0.1			d, e, f
2084	2084	Octanoic acid	0.1					d, e, f
2088	2054	1,10-di-epi-Cubenol	0.1					d, e, f
2093		(*Z*)-γ-Curcumyl 2-methyl butyrate			0.1			f
2096		(*E*)-Methyl cinnamate					2.6	d, e, f
2100		Heneicosane	0.6					d, e, f
2104	2113	Viridiflorol				2.8		d, e, f
2113		Cumin alcohol				t	2.8	d, e, f
2131	2132	Hexahydrofarnesyl acetone	0.9		0.3	2.1	1.7	d, e, f
2142		Salviadienol	0.3					d, e, f
2144	2150	Spathulenol	0.5		0.1	2.2	3.8	d, e, f
2156		α-Bisabolol oxide B					t	d, e, f
2179		3,4-Dimethyl-5-pentyli-dene-2(5H)-furanone	0.2					d, e, f
2186	2136	Eugenol					5.5	d, e, f
2187	2167	T-Cadinol	0.8				5.8	d, e, f
2198	2205	Thymol	**34.0**		1.8	0.9	3.7	d, e, f
2209	2209	T-Muurolol	0.5					d, e, f
2226	2191	Methyl hexadecanoate	0.1					d, e, f
2239	2240	Carvacrol	**6.1**		0.4	1.6	2.1	d, e, f
2255	2231	α-Cadinol	1.4				t	d, e, f
2256		*epi*-α-Bisabolol					2.6	d, e, f
2265		Torilenol	0.6		0.2			d, e, f
2291		Limonene glycol			0.2			d, e, f
2298		Decanoic acid	0.1					d, e, f
2300	2300	Tricosane	0.5					d, e, f
2349		Geranic acid			0.2			d, e, f
2389	2396	Eudesma-4(15),7-dien-1-β-ol	0.5		0.1			d, e, f
2503	2448	Dodecanoic acid	t					d, e, f
2622	2606	Phytol	1.3				1.4	d, e, f
2740		Anthracene	1.1					d, e, f
2931	2862	Hexadecanoic acid	2.0				2.0	d, e, f
		Total	**89.4**	**95.0**	**93.1**	**87.3**	**98.6**	

RRI: Relative retention indices calculated against *n*-alkanes; * Tentatively identified from WileyNIST library; % calculated from FID data; t Trace (< 0.1%); **DR** (*D. ruyschiana*); **DF** (*D. foetidum*); **DM** (*D. moldavica*); **DFr** (*D. fruticulosum*); **DP** (*D. peregrinum*); [a)] Relative Retention Indices calculated against *n*-alkanes (C_9-C_{40}) on HP-Innowax column; [b)] Relative retention indices reported in literature; [c)] Percentage calculated from FID data; ***t***, trace (<0.1%); [d)] Identification based on retention index of genuine compounds on the HP-Innowax column; [e)] Identification on the basis of computer matching of the mass spectra and retention times from Başer Library, [f)] Identification on the basis of computer matching of the mass spectra from Adams, MassFinder, WileyNIST libraries; [g)] Identification on the basis of NMR spectra.

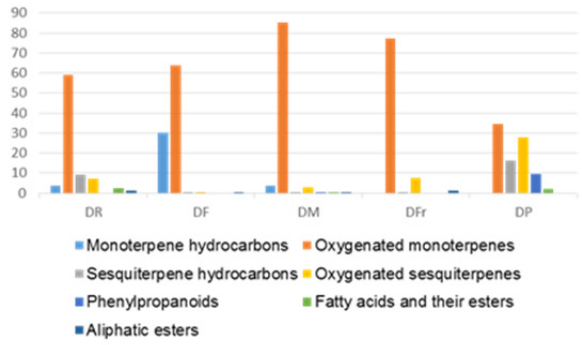

Figure 1: Distribution of major compound groups in *Dracocephalum* EOs. **DR** (*D. ruyschiana*); **DF** (*D. foetidum*); **DM** (*D. moldavica*); **DFr** (*D. fruticulosum*); **DP** (*D. peregrinum*).

Thymol (34.0 %) and carvacrol (6.1%) in **DR**; limonene (28.8%) in **DF**; *cis*-chrysanthenol acetate (29.1%) and *trans*-verbenol (5.0%) in **DFr**; and linalool (17.9%), *trans*-β-bergamotene (7.9%), (*E*)-nerolidol (7.7%) and eugenol (5.5%) in **DP** were the major volatile constituents. The distribution of the major compound classes in *Dracocephalum* EOs is presented in Figure 1.

Preliminary GC and GC-MS analyses of *Dracocephalum* EOs revealed the presence of an unidentified constituent (**1**) with M^+ 122 in **DR**, **DF**, and **DM**, (5.4%, 57.9% and 74.0%, respectively). In this current study, fractionation of the EOs and isolation of **1** was performed using PCGC connected with PFC. PCGC has generated far superior purity: the targeted constituent (**1**) with relative retention index (RRI = 1672) was isolated from the *Dracocephalum* EOs with 97.0% purity. The target peak was trapped into the sample microcollector (U-shaped trap), while the other peaks were trapped in the waste. Trapping of the isolated compound **1** with an external cryotrap collection device during the course of multiple injections produced sufficient quantity to facilitate subsequent NMR spectroscopic analysis, as well as mass spectrometry. The isolated constituent **1** was characterized as *p*-mentha-1,8-dien-10-al (syn. limonene-10-al) by comparison of its ^{1}H- and ^{13}C-NMR spectroscopic data with those published [4c]. Compound **1** had earlier been reported for *D. subcapitatum* (O. Kuntze) Lipsky, *D. foetidum* and *D. kotschyi* [4c-e].

Through the Deployed War-Fighter Protection (DWFP) Research Program, we have expanded our role in exploration and identification of new natural compounds for insecticidal and mosquito deterrent activity. Ultimately, our goal is to find new insecticides with low mammalian and environmental toxicity. These four *Dracocephalum* EOs gave 100% mortality to 1st instar larvae of *Aedes aegypti* L. at 250 ppm, except for the **DF** EO, which showed only 40% mortality, which dropped to 10% at 125 ppm (Table 2). Among the EOs, those of **DR** and **DM** demonstrated 100% mortality at 125 and 62.5 ppm and mortality down to 40% (**DR**) and 60% (**DM**) at 31.25 ppm, respectively.

Table 2: Larvicidal activity of *Dracocephalum* EOs against 1st instar *Ae. Aegypti*.

Dracocephalum species	Mortality %					
	250 ppm	125 ppm	62.5 ppm	31.25 ppm	15.625 ppm	8 ppm
DR	100	100	100	40	0	0
DF	40	0	0	0	0	0
DM	100	100	100	60	0	0
DFr	100	80	60	0	0	0
DP	100	60	0	0	0	0

In our previous study, thymol and carvacrol had LC_{50} values of 13.9 and 20.1 ppm, respectively against 1st instar *Ae. aegypti* larvae [4f], which would explain why **DR** EO had good larvicidal activity in the current study. Limonene-10-al (**1**) was a major component in **DM** EO (74%) and might be responsible for the larvicidal activity. Due to insufficient amounts, compound **1** could only be tested in the larval bioassay. Although limonene-10-al (**1**, 57.9%) and limonene (28.8%) are the major components of **DF** EO, this **DF** oil demonstrated the weakest larvicidal activity. Antagonistic interactions may be responsible for this decrease in activity. Future studies are planned to test these two compounds individually and in combination in mosquito larval bioassays.

The isolation of the target constituent from the oils through the use of automated PCGC connected to PFC was considered to be a valuable approach. This combination allowed separation and recovery of sufficient quantities of individual compounds of high purity quickly from a complex oil matrix with minimal prior fractionation. The present work is the first contribution to the detailed chemical composition and mosquito larvicidal, and adulticidal activities of Mongolian *Dracocephalum* EOs against *Ae. aegypti*.

Experimental

General: All organic solvents and reagents used for PCGC were of analytical or chromatographic grade. An optical rotation measurement of the isolated compound was carried out with an A. Krüss Optronic polarimeter (Germany). NMR spectra were recorded in $CDCl_3$ on a Varian AS 400 spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Plant material: The aerial parts of the *Dracocephalum* species were collected during their flowering period from Mongolia (Table 3). The identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the Mongolian Academy of Sciences (MAS, Ulanbaatar), Mongolia. The plant material was identified by Prof. Dr S. Shatar.

Table 3: *Dracocephalum* species subjected to chemical and larvicidal activity.

Studied species	Voucher Specimen	Collection site	Oil yield, %
DR	S.Shatar No7823	Mongolia, Ulanbaatar, Khairt Kairham Mountain	0.12
DF	S.Shatar No7819	Mongolia, Ulanbaatar, Dund - Gobi Province	0.22
DM	S.Shatar No7820	Mongolia, Ulanbaatar, Dund – Gobi Province	0.44
DFr	S.Shatar No7821	Mongolia, Gobi - Altay Mountains	0.35
DP	S.Shatar No7822	Mongolia, Mongol - Altay Mountains	0.02

Essential oil distillation: The EOs were isolated by steam distillation (3 h) of the dried aerial parts of the *Dracocephalum* species. The oil yields were calculated on a dry weight basis (Table 3).

Gas-chromatographic analysis: All the oils were analyzed by GC-FID and GC/MS techniques in conditions of previously reported methods [4g].

Essential oil fractionation and isolation of target compound 1 with PCGC system: At the second stage of the experiment, the oils with the unknown constituent (target compound) were subjected to fractionation in order to isolate and concentrate the target compound using PCGC connected to PFC.

PCGC system: Briefly, the oils containing the compound of interest were repeatedly injected by an autosampler into the GC equipped with a cooled injection system (CIS) and preparative capillary

column. The end of the column was connected to a zero dead volume effluent splitter which diverted a portion (1.0%) of the effluent to the detector while the majority (99.0%) was transferred to preparative trapping device and selected fraction was trapped using a PFC unit. The PCGC system consisted of an Agilent 7890 GC (Agilent, USA), equipped with FID and 5975 MSD with Triple-Axis detector, Agilent G 4513 autoinjector, integrated with the CIS (Gerstel, Germany).

Conditions of PCGC procedure: The compound of interest was isolated from the oils using a HP Innowax (30 m × 0.53 mm × 1.0 μm film thickness, USA) preparative capillary column with helium as carrier gas (flow rate 6 mL/min, average flow rate 46.84 cm/sec). GC oven temperature was programmed from 90°C to 195°C at a rate of 15°C/min then 60°C/min from 195°C to 230°C. It was kept at 230°C for 2.4167 min. Total time was 10 min. The oils were analyzed in splitless mode. The injector temperature was 250°C. Mass spectra were taken at 70 eV and the mass range was from *m*/*z* 35 to 450. PFC transfer and PFC distribution temperatures were kept at 220°C and 230°C, respectively. The PFC trap cooled with liquid N_2 was kept at -30°C. The retention time selection interval (cut time) was selected as 7.15-7.60 min for the target constituent. Injector volume was 4 μL.

Identification and quantification of compounds: Identification of the individual compounds was performed according to previously reported methods [4g]. 1D and 2D NMR spectra of the isolated compounds were recorded in $CDCl_3$ on a Varian AS 400 spectrometer.

Larvicidal activity: The *Dracocephalum* EOs were evaluated for larvicidal and adulticidal activity against *Ae. aegypti* [4g] using procedures described previously. Permethrin (Chemical Service West Chester, PA) was used as positive control in both bioassays. Permethrin had 100% mortality at 0.55 ppm against 1st instar *Ae. aegypti* larvae. DMSO was used for the larvicidal negative control and acetone for the adulticidal assays.

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