Chemical Composition and Larvicidal Activity of Essential Oils from Different *Mentha* L. and *Pulegium* Species against *Culex quinquefasciatus* Say (Diptera: Culicidae)

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Abstract

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Twenty samples of essential oils (EO) obtained from *Mentha aquatica*, *M. longifolia*, *M. spicata*, *M. suaveolens*, *M.* × *piperita*, *M.* × *piperita* var. *crispa*, *M.* × *villosa,* and *Pulegium vulgare* were tested for larvicidal activity against *Culex quinquefasciatus*. EO were obtained via hydrodistillation and subsequently analysed by gas chromatography-mass spectrometry (GC-MS). Concentrations causing 50% or 90% larval mortality ranged from 17171 mg/l to 171 mg/l or 28171 mg/l to 577 mg/l, respectively. EO obtained from *M. longifolia* 2 and *M. suaveolens*, which were the only ones containing a majority share of piperitenone oxide, showed the highest effects. LD_{50} was estimated as 17 mg/l for both EO, and LD_{90} was estimated as 28 mg/l.

Keywords: plant extracts; mosquito; terpenoid botanical insecticides

The mosquito species *Culex quinquefasciatus* Say is the most important vector of the filarial parasite *Wuchereria bancrofti* and a variety of arboviruses on the East African coast and the islands of the Indian Ocean, including Zanzibar (Jones *et al*. 2012; Kalaivani *et al*. 2012). Synthetic insecticides used for mosquito control are derived from those developed for agriculture and are very often based on organophosphates, pyrethroids, organochlorines, carbamates or cyclodienes (McAllister & Adams 2010). The use of synthetic pesticides leads to many ecological problems. The most important negative impacts include ecological imbalance, high toxicity, residues in soil and water that affect human and animal health (Casida 2012; Roberts & Karr 2012), and the resistance of *Culex* against insecticides (Gordon & Ottea 2012). It was found that *Culex* populations could develop resistance on account of the widespread use of insecticides (Jones *et al*. 2012). Considering the above, there is an urgent need to develop new insecticides which are biodegradable and environmentally safe (Pavela *et al*. 2009).

Natural products are suitable alternatives to synthetic pesticides (Isman 2006, 2008; Koul *et al*. 2008) as they offer a large number of compounds that exhibit larvicidal, adulticidal, and repellency activities against vectors that transmit disease to humans (Isman 2000; Shaalan *et al*. 2005; Isman 2006). Botanical pesticides have many advantages, such as low mammalian toxicity; they are also biodegradable and there is practically no risk of developing resistance (Isman 2008; Prakash *et al*. 2008).

An important group of plant secondary metabolites are the essential oils (EO). They are the active principles responsible for the odours of plants. Plant EO (or their constituents) have been valued as insecticides owing to their broad spectrum of activity. Direct toxicity, oviposition and feeding deterrence, and repellency and attraction appear

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to result from interaction with the insect nervous system, either by acetylcholinesterase inhibition or antagonism of the octopamine receptors (Rattan 2010). In general, EO consist of chemical mixtures involving from several tens to hundreds of different types of molecules (Almeida *et al*. 2011), most of them complex natural mixtures of terpenes and phenylpropanoids, which are responsible for their biological activities (Astani *et al*. 2010). The insecticidal properties of EO are very well documented (Traboulsi *et al*. 2002; Koul *et al*. 2008; Pavela 2008a,b; Urzúa *et al*. 2010; Rossi *et al*. 2012), and no development of resistance against the botanicals has yet been reported (Sharma *et al*. 1992).

EO have been used since ancient times throughout the world as perfumes, pharmaceuticals, and food flavouring agents (Derwich *et al*. 2010). The *Mentha* species (*Lamiaceae*) is one of the most popular EO crops (Padalia *et al*. 2013). EO obtained from plants of the genus *Mentha* L. are widely used in food, flavours, cosmetics, and for pharmaceutical purposes (Al-Rawashdeh *et al*. 2011). Determination of species in the genus *Mentha* L. is difficult because the genus has a complex taxonomy; in addition, there is phenotypic plasticity and genetic variability because most species are able to produce hybrids (Anca-Raluca *et al*. 2011). Chemotypes have a genetically based ability to produce an EO with a typical composition, and the composition of EO is very important for the required insecticidal effectiveness (Pavela *et al*. 2009). Selection of the most suitable and effective chemotypes is the key factor in ensuring proper plant material, which would be cultivated as a source of active compounds of botanical insecticides. The effects of different EO from the genus *Mentha* L. against the larvae and adults of mosquitoes have been studied (Koul *et al*. 2008; Pavela 2008a,b; Kalaivani *et al*. 2012). However, to our knowledge, there is no previous study focused on selecting the most suitable chemotype among the commercially available taxa. For this reason, the study is focused on selection of the most suitable chemotype, based on larvicidal effectiveness and chemical composition, among seven taxa of *Mentha* spp. and *Pulegium*, which could be cultivated as a source of future botanical larvicides against mosquitoes.

MATERIAL AND METHODS

Essential oils. Samples of plant material were harvested from plants cultivated in the experimental garden of the Faculty of Horticulture in Lednice. Plants were cultivated in experimental beds (area of 10 m^2) enclosed by a plastic barrier against overgrown stolons. The experimental fields are situated at an altitude of 174 m a.s.l. The soil is classified as a modal black soil, loam on loess, with alkaline soil reaction and carbonates throughout the profile. Soil organic matter content is higher than 1%. Fields are irrigated, but no fertilisers or herbicides were used during cultivation, and the field was weeded manually. Plant material was harvested manually in the stage of full bloom. After harvest, plants were dried at a temperature of around 40°C. The EO of the dry herbage were extracted by hydro-distillation using a Clevenger apparatus (FISHER Slovakia, spol. s r.o., Levoča, Slovakia). Samples of dried herbage were milled in a laboratory mill ILABO MF 10 basic (IKA, Staufen, Germany) (maximum grain size 3.15 mm). Plant material (20 g) was distilled in 500 ml dH_2O in a 1000 ml flask for 120 minutes. All samples were analysed twice and averaged (Table 1). The EO content is expressed in per cent. Oil samples were stored at 4.2°C until bioassay.

Chemical analyses. All samples were analysed by a Shimadzu gas chromatograph GC-17A (Shimadzu, Korneuburg, Austria). Preparation of EO samples: 20 µl of EO was diluted in 980 µl of cyclohexane and then

Table 1. List of plant material including status, origin, and essential oil yield

	Status	Locality	Essential oil (%)
Mentha aquatica	wild	Czech Republic	1.812
Mentha longifolia 1	wild	Czech Republic	1.624
Mentha longifolia 2	cultivar	Czech Republic	1.565
Mentha longifolia 3	cultivar	Czech Republic	1.676
Mentha longifolia 4	wild	Czech Republic	1.304
Mentha spicata 1	cultivar	Czech Republic	1.524
Mentha spicata 2	wild	Czech Republic	1.507
Mentha spicata 3	wild	Austria	0.727
Mentha suaveolens	cultivar	Germany	0.966
Mentha × *piperita* 1	cultivar	Czech Republic	1.747
Mentha × *piperita* 2	cultivar	Great Britain	0.690
Mentha × *piperita* 3	cultivar	Czech Republic	1.694
Mentha × *piperita* 4	cultivar	Czech Republic	2.046
Mentha × *piperita* 5	cultivar	Czech Republic	1.777
Mentha × *piperita* 6	cultivar	Czech Republic	2.199
Mentha × *piperita* 7	cultivar	Russia	2.337
Mentha × *piperita* var. *crispa*	wild	Czech Republic	2.123
Mentha × *villosa*	wild	Czech Republic	1.863
Pulegium vulgare	wild	Czech Republic	1.898
Pulegium vulgare 2	cultivar	Czech Republic	0.913

Table 2. Composition of essential oil samples from *Mentha* L. and *Pulegium* L.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
MONOTERPENES																				
Hydrocarbons																				
α-Pinene																				
α-Thujene		0.7																		
β-Pinene																				
(+)-Sabinene		2.4			0.8															
β-Myrcene		1	1					1.2			1.2									
α-Terpinene		6.7			3.3															
R-(+)-Limonene	1.2				0.6	2.9	2.5		1.2	3.7		0.9	1			1.2		5.3	1.4	
β-*trans*-Ocimene		0.9						0.9			1									
p-Cymene		1.7			1															
Terpinolene		2.9			1.3															
Oxygenated hydrocarbons																				
Eucalyptol (1.8-cineole)	3.7	1.8	1.5	2.1	1.5	1.1	1	1.2		1.4	0.6	3	3.6		1.9	3.5	2.1	1.1		0.9
p-Menthone	22.0			2	5.5	1.1	1.1	4.4	0.6	0.7	2.5	12.9	22.1	6.3	12	27	16.8		9.1	7.4
cis-β-Terpineol	0.6	8.2	0.9		5.8						1	1.2		2.2	1	0.6	0.7			
Menthofurane	1.3							0.8				3	0.6		1.8	1.5	0.7			
Isomenthone	4.3				0.7		0.8	0.7			0.7	1.9	4.7	1.9	3.8	4.9	3.8		0.7	14.9
Linalool		2.8			0.7			0.6			3.3	1.6								
Isomenthyl acetate	4.0							1				4.4	3	1.4	2.3	2.6	3.2			0.7
Neomenthol	2.9				1			1.2			0.7	4.5	2.8	1.8	4.4	2.2	3.3			0.7
Dihydrocarvone						11.8	12.7		2.8	10.2								3.3	2	
4-Terpineol	0.6	32.5			16.5						1.7	1	0.7	3.6	1.4	0.6	1.3			
Carvenone								1.6			1.1									
Pulegone	7.9			0.9	2.2	2.3	4	2.4	1.5	1.7	1	4.5	9.7	1.5	16.3	12.3	10.2	1.1	65	55.1
L-(–)-Menthol	36	1.7	0.7	3.4	3.5	1.1	1.5	14.9	1		20.5	46.9	35.6	57.3	39	27.1	39			7.3
Piperitone oxide		2.7	4.9	62.1	21.3			34.1			31.8			4.2	0.6					
Carvone			2.9	0.6	6.4	57.4	59.1	0.7	14	56.8	1.5			1.6	1		2.2	67.9	13.3	
cis-Carvone oxide						0.7	0.8			0.7								0.8		
Menthane-1,2,3-triol				0.6				3.1			2.9									
Verbenone																			0.6	1.3
cis-Jasmone			0.6			1.8	1.6		1.2	1.6										
Piperitenone oxid			65	0.9		1.7			55.5	7						1.4		4.5	0.8	
Thymol				0.7				0.6												
SESQUITERPENES																				
Hydrocarbons																				
Edulan I		0.8		0.6	0.7			0.7			0.6									
β-Bourbonene						2.5	1.8	0.9	0.8	2	0.7		0.6					1.2		
β-Caryophyllene	2.9	5.6	5.3	5.5	5.3	2.9	2.2	3	1.3	2.5	3	2	3.2	3.1	2.3	3	3.3	2.3		0.8
β-Cubebene			0.8			0.7														
β-*cis*-Farnesene								0.6	1.4											
Germacrene D	3	7.1	4.6	7.6	6.6	1.5	1.1	14.6	7	1	12.7	3	3.1	5.4	2.7	2.8	3.6	4.5	0.8	
γ-Elemene	0.8			0.6									0.7			0.7				
Eremophilene					0.6															
Oxygenated hydrocarbons																				
Nerolidol																				
Viridiflorol									1.6			0.8					0.6			
Total (%)	91.4	92.0	88.2	91.6	79.5	89.2	88.5	89.9	89.5	91.2	91.4	87.6	89.3	90.8	90.3	93.7	90.2	89.1	85.3	90.5

1 – *Mentha aquatica*; 2 – *M. longifolia* 1; 3 – *M. longifolia* 2; 4 – *M. longifolia* 3; 5 – *M. longifolia* 4, 6 – *M. spicata* 1; 7 – *M. spicata* 2; 8 – *M. spicata* 3; 9 – *M. suaveolens*; 10 – *M.* × *piperita* 1; 11 – *M.* × *piperita* 2; 12 – *M.* × *piperita* 3; 13 – *M.* × *piperita* 4; 14 – *M.* × *piperita* 5; 15 – *M.* × *piperita* 6; 16 – *M.* × *piperita* 7; 17 – *M.* × *piperita* var. *crispa*; 18 – *M.* × *villosa*; 19 – *Pulegium vulgare*; 20 – *P. vulgare* 2

deprived of water by anhydrous magnesium sulphate. A sample of 1 µl was injected under the following conditions: column: DB-Wax (30 m × 0.25 mm, film thickness 0.25 µm); carrier gas helium (1 ml/min, linear speed 36 cm/s); injection temperature 210°C, column temperature –70 to 250°C, MS 2–60 min, 14–254 *m/z*. The identification of compounds was based on two methods: (a) comparing mass spectra with standards; (b) comparing mass spectra with those described in the literature.

Test organisms. The test organisms, third instar of *Culex quinquefasciatus*, were obtained from a laboratory breed which is maintained at the Crop Research Institute, Prague, Czech Republic. The larvae were fed on dog biscuits and yeast powder (ratio 3:1). Adults were provided with a 10% sucrose solution and a 1-week-old chick for blood feeding for females. Mosquitoes were held in a growing chamber under the following conditions: 28 ± 2°C, 70 ± 5% RH, and a photo regime of 16:8 h (light:dark).

Acute toxicity. The experiment was performed according to the World Health Organisation (1996) standard procedures, with a few modifications (Pavela 2009). Samples of EO were diluted in dimethylsulphoxide (DMSO) to prepare a serial dilution of the test dosage. Third instar larvae were selected and transferred in 224 ml of dH_2O. For experimental treatment, 1 ml of serial dilutions was added to 224 ml of dH_2O in a 500 ml flask and shaken a little bit to produce a homogeneous mixture. The selected larvae were transferred in distilled water into a basin of prepared test solutions with a final surface area of 125 cm^2 (25 larvae/beaker). Four replicates were running simultaneously with at least five dosages (from 2 to 20 mg/l). The larvicidal assay was carried out in a growth chamber under the following conditions: 16 h light: 9 h dark; 26°C. Mortality was determined after 24 h of exposure; no food was given to the larvae during the experiment.

Statistical analysis: Mortality was corrected by Abbott's formula (Abbott 1925) and the LC_{50} and LC_{90} regression equation; the 95% confidence limits of the upper and lower confidence levels were calculated using probit analysis (Finney 2009). The obtained data were analysed using the software STATGRAPHICS Plus 4.0 (STATPOINT TECHNOLOGIES, Inc., Warrenton, Virginia).

RESULTS

The yield of EO obtained from 7 species of the genus *Mentha* L. is presented in Table 1. Although the plants were grown under the same climatic and pedological conditions, the EO yield values ranged (depending on the species and genotype) from 0.69%

Table 3. Larvicidal activity of mint oils against third instar of *C. quinquefasciatus* after 24 hours

	LC_{50} (mg/l)	CI_{95}	LC_{90} (mg/l)	CI_{95}	χ^2
Mentha aquatica	118	(104–141)	298	(221–525)	0.862
Mentha longifolia 1	171	(152–200)	357	(282–544)	2.707
Mentha longifolia 2	17	(15–19)	28	(25–34)	2.329
Mentha longifolia 3	55	(50–59)	78	(71–91)	0.007
Mentha longifolia 4	97	(89–106)	181	(155–232)	4.557
Mentha spicata 1	111	(102–121)	186	(162–231)	3.113
Mentha spicata 2	119	(112–127)	168	(152–194)	0.205
Mentha spicata 3	92	(83–100)	160	(144–185)	4.799
Mentha suaveolens	17	(16–20)	28	(25–35)	2.147
Mentha × *piperita* 1	141	(108–240)	493	(275–1815)	1.099
Mentha×*piperita* 2	110	(100–127)	190	(156–270)	0.038
Mentha × *piperita* 3	104	(95–113)	175	(158–202)	1.122
Mentha × *piperita* 4	79	(70–92)	192	(147–306)	0.815
Mentha × *piperita* 5	54	(46–62)	88	(75–116)	2.369
Mentha × *piperita* 6	88	(75–100)	204	(172–273)	2.406
Mentha × *piperita* 7	83	(73–92)	167	(143–216)	0.144
Mentha × *piperita* var. *crispa*	97	(76–144)	577	(302–2377)	4.913
Mentha × *villosa*	134	(125–143)	195	(179–220)	2.233
Pulegium vulgare	64	(60–68)	86	(80–97)	0.337
Pulegium vulgare 2	86	(77–92)	133	(120–159)	1.606

CI_{95} – 95% confidence intervals; essential oil activity is considered significantly when 95% CI fail to overlap; χ^2-value – significant at $P < 0.05$ level

to 2.33%. The qualitative composition of the EO (Table 2) also showed differences, similarly to the yield values of active substances. The contents of majority substances were determined to be different not only in individual species, but between species as well and genetically conditioned chemotypes were also determined. A majority share of two monoterpenes was found for *M. aquatica* – L-(–)-menthol and *p*-menthone, which together represent a 58% share in the EO. One significant chemotype was found in the *M. longifolia* group, which had a majority content of monoterpene piperitenone oxide (65%), in contrast to the other chemotypes containing two majority monoterpenes, piperitenone oxide, and 4-terpineol, in various mutual ratios. A majority share (55%) of piperitenone oxide was also found in the EO from *M. suaveolens*. A majority share of the monoterpenes carvone and piperitenone oxide was found in the *M. spicata* species group, and the group *M.* × *piperita* showed monoterpenes in various mutual ratios: L-(–)-menthol, whose share in the EO differed depending on genotype from 20.5% to 57.3%, together with carvone (content range 0.0–56.8%) and piperitenone oxide (content range 0.0–31.8%). For EO from *M.* × *villosa*, carvone content (67.9%) was typical, and pulegone content (55.1–65%) was characteristic for both taxa of *Pulegium vulgare*. The majority share of substances in the obtained EO was formed by monoterpenes (69.4–92.9%), most of which belonged to the group of oxygenated hydrocarbons.

The varied composition of the EO also caused significantly different larvicidal efficacy (Table 3). Concentrations causing 50% or 90% larval mortality ranged from 17171 mg/l to 171 mg/l or 28–577 mg/l, respectively. EO obtained from *M. longifolia* 2 and *M. suaveolens*, which were the only one oils containing a majority share of piperitenone oxide, showed the highest effects. LD_{50} was estimated as 17 mg/l for both EO, and LD_{90} was estimated as 28 mg/l. Significantly higher ($P = 0.05$) lethal doses were estimated for the other EO.

DISCUSSION

Based on a comparison of the lethal doses, we were able to select two genotypes of the *Mentha* genus (*M. longifolia* and *M. suaveolens*), which provided the most significantly effective EO. Chemical analysis of these EO indicated that they have a different composition and a majority share of piperitenone oxide; this substance can be considered as the responsible chemical associated with significantly higher larvicidal efficacy.

Piperitenone oxide (1,2-epoxy-4(8)-*p*-menthene-3-one) belongs to the group of epoxyketone monoterpene, characterized by the presence of the epoxy functional group (Reitsema 1957). This monoterpene represents the majority share of EO of some species of the *Mentha* L. genus, such as *M. rotundifolia* (Reitsema 1957), *M. spicata* (Tripathi *et al*. 2004), *M. longifolia* (Saeidi *et al*. 2012), and *M. suaveolens* (Oumzil *et al*. 2002), and is also present in other species of aromatic plants, such as *Micromeria dalmatica* (Karosou *et al*. 2012) and *M. albanica* (Marinković *et al*. 2003); however, these plants show a much lower monoterpene content than the *M. longifolia* genotype that we tested.

The insecticidal efficacy of EO containing piperitenone oxide has been explored by some other authors. For example, Mohamed and Abdegaleil (2008) mentioned that EO obtained from *Mentha microphylla* with a majority share of piperitenone oxide was significantly more effective against *Sitophilus oryzae* and *Tribolium castaneum* than other tested EO. Tripathi *et al*. (2004) tested piperitenone oxide isolated from EO obtained from *M. spicata* var. *virids* for larvicidal, ovicidal, and antiovipositional effects against *Anopheles stephensi*. The results indicated a higher efficacy of piperitenone oxide than the crude EO of the *M. spicata* variety *viridis* in all bioassay experiments. The lethal response of piperitenone oxide and the oil toward fourth instar larvae showed LD_{50} values of 61.64 and 82.95 mg/l, respectively. Female adults of *A. stephensi* exposed to the oil laid approximately 42 times fewer eggs at a dose of 60.0 mg/l compared with the control, whereas exposure of piperitenone oxide at the same dose completely inhibited oviposition. Furthermore, piperitenone oxide also completely inhibited egg hatching at a dose of 75.0 mg/l in the ovicidal assay. The significantly higher efficacy of piperitenone oxide alone confirms our hypothesis that this is the substance responsible for insecticidal effects. Koliopoulos *et al*. (2010) tested piperitenone oxide for larvicidal efficacy against the *Culex pipiens* biotype *molestus* and estimated LC_{50} as 9.95 mg/l. However, as far as we know, our study is the first to focus on the larvicidal efficacy of EO containing piperitenone oxide against *Culex quinquefqsciatus* larvae. Compared to other mosquito species, the larvae of this species are less sensitive to larvicidal substances (Belinato *et al*. 2013; Govindarajan *et al*. 2013), thus the test results can also be generalised for other larvae of the species of the *Culex*, *Anopheles* or *Adres* genus.

The view that the *M. longifolia* species genotype we have selected, the chemotype piperitenone oxide, is a plant material suitable for obtaining a sufficient

amount of active substance for the development of a botanical larvicide is also highlighted by the fact that these plants are perennials with an economic life of 3–5 years, and with a relatively high yield capacity of active substances from a growing area of 112–150 kg/ha (Alsafar & Al-Hassan 2009); some authors provide even higher yield capacities (Lacy *et al*. 1981; Matovic & Lavadinovic 1999). In addition, botanical pesticides based on extracts and EO obtained from *Mentha* spp. are safe for the environment and for human health. (Speiser & Tamm 2006). Moreover, as we ourselves have discovered, this species can also be grown in the climate of Central Europe with very good yields for the EO (1.6%). All papers published until the present have focused on the larvicidal efficacy of EO obtained from *Mentha* spp., and have presented results obtained from materials grown in climates different in terms of both temperature and precipitation (Tripathi *et al*. 2004; Mohamed & Abdegaleil 2008; Koliopoulos *et al*. 2010). As far as we know, this is the first study to demonstrate that good-quality plant materials suitable as a source of active substances for botanical larvicides can be obtained.

In conclusion, we can recommend the *M. longifolia* chemotype piperitenone oxide and *M. suaveolens* as suitable genetic materials for growing plant material to be used to obtain the active substance piperitenone oxide, which is suitable for the production of new botanical larvicides against important vectors.

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