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Larvicidal evaluation of three *Mentha* species essential oils and their isolated major components against the West Nile virus mosquito

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Summary The larvicidal activity of essential oils derived from three different *Mentha* species (Lamiaceae) as well as their major aroma *p*-menthane type components were evaluated against *Culex pipiens* (Diptera: Culicidae). Therefore, pulegone, piperitenone, piperitone, carvone, menthone and menthol were isolated using column chromatography. The LC_{50} values revealed that *M. pulegium* and *M. piperita* oils were the most toxic (46.97 and 40.28 mgl^{-1} respectively) and pulegone was the most effective (27.23 mgl^{-1}) among the major ingredients. The activity of all essential oils is in agreement with the proportion/toxicity rate of their individual major components, apart from the case of *M. piperita* where the LC_{50} values of its major ingredients menthone and menthol stand higher than 100 mgl^{-1}. For the isolated molecules, studies on structure activity relationships revealed that the location of C-C double bond and the presence of the isopropylidene group might be key factors.

Additional keywords: Culex pipiens, essential oil, larvicidal activity, major components isolation, *Mentha* species

Introduction

Control of mosquitoes is crucial due to the fact that they are vectors of many viruses. *Culex pipiens* serves as both an enzootic and an epidemic (i.e. "bridge") vector of West Nile Virus (WNV) to humans, is a wide spread insect pest with medical importance. According to Bakonyi et al. (4) WNV emerged in several European countries within the last 50 years. Outbreaks of WNV encephalitis in humans and horses were reported. Recently, new WNV strains were isolated in Central Europe from mosquito vectors and from encephalitic cases of vertebrate host (18). During the last decade cases are also reported from Russia, Israel, Turkey and other Mediterranean countries (10). During summer of 2010, hundreds of people were seriously affected from WNV and more than 30 died in Northern Greece (18). Currently specific treatment and vaccines are not available for the protection of horses or humans and only mosquito control measures could reduce the risk of the development of serious diseases (6). Therefore the use of improved mosquito control measures is strongly emphasized. According to Floore (11) the success of mosquito control relies on product efficacy and tools that are environmentally friendly. Shaalan et al. (21) stated that ".. the failure to discover a significant new class of insecticides has led many researchers back to biodiscovery studies…". Plant derived pesticides are biodegradable and may be the future arsenal against mosquitoes but there are many topics that need more investigation.

Previous investigations have indicated that various *Mentha* spp. plant extracts displayed larvicidal effect on *Cx. pipiens*, *Cx. quinquefasciatus*, *Aedes aegypti, Anopheles stephensi* and *An. tesselatus* (2, 3, 19, 20, 22, 23). Furthermore, pure substances (thymol, menthone, menthol and pulegone) and menthol derivatives have been tested against mosqui-

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to adults, while menthone, thymol and carvacrol against *Culex* species for larvicidal activity (20, 23). These substances are known active ingredients not only of *Mentha* species essential oils but also of many other plants, in which their concentrations vary (7).

Main objectives of this study were: a) to evaluate the efficacy of essential oils derived from three *Mentha* species, *M. pulegium* (three populations), *M. piperita* and *M. spicata* (spearmint) against *Cx. pipiens* larvae, b) to isolate and evaluate essential oil major components, c) to correlate larvicidal activity of isolated components with their precursor oils and d) to study the structure activity relationships for the isolated molecules.

Materials and Methods

Plant Materials

Three different species of the genus *Mentha* were used: *M. pulegium* (pennyroyal), *M. piperita* (mint) and *M. spicata* (spearmint). All plant materials were collected from various natural habitats in Greece by members of our team, between July and August 2007 in the flowering stage. Specifically, in the case of *M. pulegium* samples were collected from three populations according to their geographical origin, the first one from Orestiada (North-East of Greece, named as PUL1), the second from Karditsa (center of Greece, PUL2) and the third from Heraklion (Island of Crete, PUL3). Samples of *M. spicata* (SP) and *M. piperita* (PIP) were purchased from local farmers from the region of Karditsa. All five plant materials were air dried and stocked for further use.

Chemicals

R-(+)-pulegone,terpinen-4-ol,*β*-farnesene, *α*-pinene, *β*-pinene, mesityl oxide, methyl vinyl ketone, menthol and menthone were purchased from Aldrich (Steinheim, Germany). Eucalyptol, *β*-myrcene, *S*-(-)-limonene, *γ*-terpinene, thymol, carvacrol and *β*-caryophyllene were purchased from Sigma (St. Louis, USA). Carvone was bought from Fluka (Steinheim, Germany). 3-methyl cyclohexanone were bought from Jansen Chimica (Beerse, Belgium). Terpinolene, *iso*-menthone and piperitone were purchased from Extra Synthese (Genay, France). Diethyl ether (BHT free) was purchased from SDS (Cedex, France). Pentane was bought from Lab-Scan (Doublin, Ireland). Piperitenone was prepared according to literature synthetic procedure (5), due to commercial unavailability. Purity of the isolated product (97.3%) was estimated according to GC-MS analytical conditions given bellow. Structural characterization that was accomplished by mass spectral analysis and NMR experiments was in agreement to literature data (5). NMR spectra were recorded on Brucer Avance DRX-500 instrument. Potassium hydroxide, tetrahydrofuran (THF), silica gel 60G and TLC plates (silica gel 60, F254) were purchased from Merck (Darmstadt, Germany).

Isolation of the Essential Oils

Aerial parts from PUL1, PUL2, PUL3, SP and PIP were powdered in an electrical blender and 1 Kg of each sample submitted to hydrodistillation for 4 h in a Clevenger-type apparatus. The obtained essential oils, named as EOpul1, EOpul2, EOpul3, EOsp and EOpip respectively, were dried over anhydrous magnesium sulfate. After filtration their volume were calculated and expressed as ml of essential oil/100 g of dry material (Table 1) and finally stored in labeled sterile screw capped bottles at -22°C until use.

Gas chromatography – Mass spectrometry (GC-MS) analysis

The essential oils were analyzed using a Hewlett Packard II 5890 gas chromatography (GC) system, equipped with a FID detector and HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 220°C and 290°C, respectively. GC oven temperature was programmed from 60°C to 240°C at a rate of 3°C /min and held isothermally for 10 min. Helium was the carrier gas at a flow rate of 1 ml/min. Diluted samples (1/100 in diethyl ether, mgl^{-1}) of 1.0 μl were injected manually and in the splitless mode. Quan-

titative data were obtained electronically from FID area percent data without the use of correction factors. Qualitative analysis of the essential oils was performed using the same conditions with GC, in a Hewlett Packard II 5890 gas chromatograph equipped with Hewlett Packard II 5972 mass selective detector in the electron impact mode (70 eV). Most ingredients of the essential oils were identified on the basis of comparison of GC relative retention times and mass spectra with those of pure standards. As for the rest components, tentative identification was based on the comparison of their mass spectra and elution order with those obtained from the NIST 98 and Wiley 275 library data of the GC-MS system and from Adams CD computer library (1).

Isolation of Essential Oils Major Ingredients

Ten (10) g of the extracted crude essential oils EOpul1, EOpul2, EOpul3, EOpip and EOsp were fractioned by column chromatography on silica gel and eluted with a gradient of solvents of increasing polarity (pentane + diethyl ether: 100 + 0, 97 + 3, 95 + 5, 93 + 7, 90 + 10 and 80 + 20). Column fractions were monitored by thin layer chromatography (TLC) with pentane + diethyl ether (9.5 + 0.5). Fractions with similar R_f values on the TLC plates to authentic co-eluted compounds were pooled. Spots were detected under UV lamp and afterwards by spraying with a mixture of 1% vanillin and 5% sulfuric acid solution (in ethanol) and heating at 120°C and/or PMA solution (phosphomolybdic acid 7.5% mgl^{-1} in ethanol) and charring on a hot plate. The chosen fractions merged to one and elution solvents removed by a rotary flash evaporator. The distillation was stopped when the volume of solvents was reduced to ~10 ml and completed by flushing through nitrogen.

Mosquito Rearing

The colony of the species *Cx. pipiens* biotype *molestus* has been maintained in the laboratory of Benaki Phytopathological Institute, Kifissia, Greece for more than 25 years. Adult mosquitoes are kept in wooden framed cages (33x33x33 cm) with a 32x32 mesh at 25±2°C, 80±2% relative humidity and photoperiod of 14:10 (L:D) h. Cotton wicks saturated with 10% sucrose solution are used as food source. Females lay eggs in round, plastic containers (10 cm diameter x 5 cm depth) filled with 150 ml of tap water. Egg rafts are removed daily and placed in cylindrical enamel pans (with diameter of 35 cm and 10 cm deep), in order to hatch. Larvae are reared under the same conditions of temperature and light and are fed daily with baby fish food (TetraMin, Baby Fish Food) at a concentration of 0.25 gl^{-1} of water until pupation. Pupae are then collected and introduced into the adult rearing cages.

Larvicidal Bioassays

Stock solutions were prepared in ethanol with a concentration of 1% mgl^{-1}. A series of aqueous solutions of the tested material, expressed as mgl^{-1}, were made and tested under laboratory conditions. Preliminary experiments (data not shown) were performed to evaluate toxicity and subsequently different concentrations were employed ranging from 10 to 200 mgl^{-1} for each tested material.

The larval mortality bioassays were carried out according to the test method of larval susceptibility as proposed by the World Health Organization (24). Twenty 3rd to 4th instar larvae of the species *Cx. pipiens* biotype *molestus* were collected from the colony. They were placed in glass beaker with 250 ml of aqueous suspension of tested material at various concentrations and an emulsifier was added in the final test solution (less than 0.05%). Four replicates were made per concentration and a control treatment with tap water and emulsifier was also included. Beakers with larvae were placed at 25±2°C, 80±2% relative humidity and photoperiod of 14:10 (L:D) h.

Data Analysis

Larvicidal effect was recorded 48 h after treatment. Data obtained from each dose–larvicidal bioassay (total mortality, mgl^{-1} concentration in water) were subjected to probit analysis in which probit–transformed

mortality was regressed against log_{10}–transformed dose; LC_{50}, LC_{90} values, and slopes were calculated (SPSS 11.0).

Results

Chemical Composition of the Essential Oils

Yields and chemical composition of the obtained essential oils are shown in Table 1. Literature data suggests that *M. pulegium* is a chemical polymorph species both in qualitative and/or quantitative composition. Recently, it has been demonstrated the link between the oil composition and origin of 38 populations of *M. pulegium* scattered along Greece (12). Thus, the three different samples resulted in different chemical profiles. In detailed, in EOpul1 pulegone (61.1%) was

Table 1. Percentage composition of essential oils isolated from *M. pulegium* (three populations: EOpul1, EOpul2 and EOpul3), *M. spicata* (EOsp) and *M. piperita* (EOpip). Compounds are listed in order of elution from an HP-5 MS column.

Components	Composition (%)				
	EOpul1[c]	EOpul2[c]	EOpul3	EOsp	EOpip[c]
3-methyl cyclohexanone[a]	0.4				
α-pinene[a]			0.2	0.4	0.7
β-pinene[a]			0.2	0.2	1.2
3-octanol[a]	1.4	0.4	0.7	0.2	
limonene[a]	1.2	2.7	1.8		
eucalyptol[a]				9.0	6.9
γ-terpinene[a]				0.4	0.7
menthone[a*]	4.3	1.8	1.6		**39.0**
isomenthone[a*]	13.0	0.3	**24.8**		9.9
menthol[a*]					**25.9**
terpinen-4-ol[a]				0.7	
cis-dihydro carvone[b]				1.0	
isopulegone[b]	2.9				
trans-carveol[b]				0.8	
pulegone[a*]	**61.1**				2.0
carvone[a*]				**71.8**	
piperitone[a*]	1.1	**92.6**	**69.3**		1.3
piperitenone[a*]	4.0	0.1	0.4		
piperitenone oxide[b]	1.8				
β-bourbonene[b]	0.1	0.8	0.2	1.5	0.4
β-elemene[b]				1.1	0.2
β-caryophyllene[a]				2.2	3.5
β-farnesene[a]				0.1	0.6
germacrene D[b]	0.1			1.3	2.9
bicyclogermacrene[b]				0.8	0.5
caryophyllene oxide[a]				0.2	
Total	**91.4**	**98.7**	**99.2**	**91.7**	**95.7**
oil yield (ml/100 dry wt)	2.2	2.1	2.1	1.8	3.2

[a] Comparison with pure standards.
[b] Tentative identification based on data obtained from NIST 98 and Wiley 275 library of the GC-MS system and from Adams CD computer library (16).
[c] For these essential oils the phytochemical analysis for major ingredients (>1%) has already been published (14).
* Components that were isolated and applied in bioassays.

the most abundant ingredient followed by isomenthone (13.0%), menthone (4.3%), piperitenone (4.0%), isopulegone (2.9%) and piperitenone oxide (1.8%). EOpul2 characterized by the dominant occurrence of piperitone (92.6%) and the minor presence of limonene (2.7%) and menthone (1.8%). Finally, EOpul3 consisted of piperitone (69.3%), isomenthone (24.8%), limonene (1.8%) and menthone (1.6%). Concerning the other two plants, the major ingredient of EOsp was carvone (71.8%) followed by eucalyptol (9.0%) and β-caryophyllene (2.2%) among others, while EOpip consisted of menthone (39.0%) and menthol (25.9%) with minor components isomenthone (9.9%), eucalyptol (6.9%) and β-caryophyllene (3.5%) among other less abundant ingredients.

Purity of the isolated major ingredients

The purity of the isolated components was determined by GC-MS analysis and is given as follows: pulegone (5.45 g, 99.2% purity) and piperitenone (0.38 g, 98.6% purity) from EOpul1, piperitone (8.63 g, 99.5% purity) from EOpul2, piperitone (6.21 g, 99.4% purity) and iso-menthone (1.86 g, 98.9% purity) from EOpul3, carvone (6.05 g, 99.6% purity) from EOpip and menthone (3.16 g, 99.2% purity) and menthol (2.17 g, 99.0% purity) from EOsp. Pulegone, piperitenone, piperitone, isomenthone, carvone, menthone and menthol have been isolated in high purities and their chemical structures are given in Figure 1. Although piperitenone does not constitute a major ingredient, it has also been isolated due to the fact that it is structurally similar to pulegone and piperitone.

Larvicidal Assays

Essential oils and their major ingredients were tested for their larvicidal activity against *Cx. pipiens* 3rd-4th instar larvae. EOpul1 was the most active essential oil as well as its major component pulegone among the essential oil major ingredients (based on non-overlapping confidence intervals) (see Table 2 for LC_{50} and LC_{90} values). The essential oils EOpul2 and EOsp and their dominated compounds, piperitone and carvone respectively, presented a rather medium activity (Table 2). EOpul3 was the only inactive essential oil showing no larvicidal effect. The larvicidal pattern of the EOpip was quite different: LC values were high and almost the same with the EOpul1 (LC_{50} value 40.28 and 46.97 mgl^{-1}

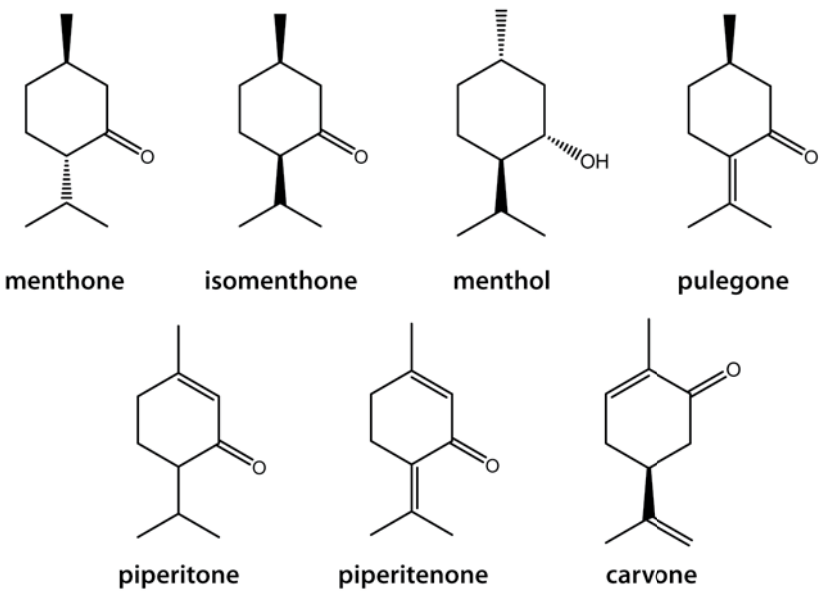

Figure 1. Chemical structures of essential oils' isolated components.

for EOpip and EOpul1 respectively).

Discussion

In this study, essential oils of *Mentha* species and their major ingredients were tested with respect to their larvicidal effect against *Cx. pipiens* mosquitoes. The selection of the three *Mentha* species, *M. pulegium* (three populations), *M. piperita* and *M. spicata*, was based on obtaining essential oils that possessed different chemical profiles. The isolated essential oils can be characterized as pulegone type (EOpul1), piperitone type (EOpul2), piperitone-isomenthone type (EOpul3), menthone-menthol type (EOpip) and carvone type (EOsp). These results of

Table 2. LC_{50} and LC_{90} values for essential oils and their isolated major components against larvae of *Culex pipiens* biotype *molestus*. Major components percentage composition, in relation to their precursor essential oils, is also given in parenthesis.

Essential oil / Major components (% composition in essential oil)	n[c]	Slope (±SE)	LC_{50} (95% CL)[a]	LC_{90} (95% CL)[a]	χ^2	df
EOpul1	400	4.93±0.53	46.97 (43.92-50.73)	85.42 (74.51-104.51)	15.440	18
pulegone (61.1%)	400	4.87±0.52	27.23 (24.39-29.7)	49.91 (45.4-56.61)	12.402	15
piperitenone (4.0%)	320	5.16±0.55	75.91 (72.54-79.2)	97.31 (92.16-104.84)	5.909	10
EOpul2	480	8.84±0.94	168.59 (157.76-182.99)	235.44 (209.67-293.82)	37.275[b]	16
Piperitone (92.6%)	320	8.47±0.9	131.91 (112.92-155.81)	186.84 (157.64-287.79)	47.024[b]	10
EOpul3	320	––	>200	––	––	––
piperitone (69.3%)	400	8.32±0.83	129.51 (118.78-142.17)	178.98 (159.16-223.33)	35.466[b]	13
isomenthone (24.8%)	320	––	> 200	––	––	––
EOsp	320	10.66±1.19	95.9 (80.44-107.13)	126.46 (112.57-162.87)	43.362[b]	10
carvone (71.8%)	400	5.54±0.68	95.31 (84.58-106.77)	162.31 (137.02-228.97)	26.735	13
EOpip	480	22.29±0.22	40.28 (30.9-50.56)	146.17 (105.4-252.54)	44.197[b]	19
menthone (39.0%)	480	9.29±0.92	111.11 (104.54-116.67)	152.63 (145.23-162.51)	18.942	16
menthol (25.9%)	400	8.6±1.31	120.97 (105.35-130.02)	170.45 (157.72-199.31)	20.977	13

[a] LC values are expressed in mgl^{-1} and they are considered significantly different when 95% CL fail to overlap.
[b] Since goodness–of–fit test is significant ($P<0.05$), a heterogeneity factor is used in the calculation of confidence limits (CL).
[c] Total number of larvae tested.

phytochemical analysis are in agreement with literature data (13, 17).

Essential oils are a mixture of different ingredients, mostly terpenes, which in the analysed *Mentha* spp. essential oils included pulegone, piperitone, isomenthone, carvone, menthone and menthol and they are comparable to findings in the literature (8, 13). Besides menthone, the rest isolated ingredients were evaluated for the first time against *Cx. pipiens* larvae. Among them, pulegone demonstrated the strongest larvicidal activity. The activity of all essential oils is in agreement with the proportion/toxicity rate of their individual major components, apart from the case of EOpip. The EOpip consists of menthone and menthol (in a total of 65%) and their LC_{50} values are higher than 100 mgl^{-1}, so it was expected to have the same toxicity pattern with the maternal essential oil. According to our experimental and literature data (10) EOpip did not follow this pattern and found to be one of the two most drastic essential oils (LC_{50} value near 40mgl^{-1}). Two suggestions could be made: either the rest of the ingredients possess independently strong larvicidal ability or some kind of synergistic phenomenon took place. These results are in accordance with those reported by Amer and Mehlhorn (2) where peppermint had moderate larvicidal activity against mosquito larvae (near 53% mortality after 24 h treatment).

Some interesting conclusions can be drawn concerning the relationship between the structure of the isolated *p*-menthane compounds and their larvicidal effect against *Cx. pipiens*. Although, pulegone and piperitone are isomers, the first is 5-fold more active than the later. This differentiation probably stands on account of the location of the C-C double bond that these two molecules contain. We assume that in pulegone the C-C double bond position on the chain group (isopropylidene *versus i*sopropyl group) enhances the toxicity. This hypothesis is strengthened by the case of piperitenone, where toxicity ranges between pulegone and piperitone. Piperitenone is structurally similar to both aforementioned molecules. Particularly, it combines all of the former molecules characteristics to one structure as it bares two double bonds, where the first one is internal at the cyclohexane ring (resembling to piperitone) and the second one external (resembling to pulegone). Therefore, two arguments can be made: either piperitenone's internal double bond reduces the total toxicity or its external double bond increases the toxicity. In any case, toxicity seems to be in direct connection to carbon-carbon double bond's position.

The effectiveness order of the above mentioned molecules, compared to menthone (saturated cetone), is the following: pulegone> piperitenone > menthone > piperitone. This ranking indicates that unsaturation might be a key factor but not necessarily the most determinant; it should be taken into account in combination with the position factor. Our results concerning carvone (a molecule isomer to piperitenone) also support this hypothesis: carvone is less effective than piperitenone while both of them are showing reduced toxicity compared to pulegone. This activity differentiation probably results from the different location of C-C double bond (isopropenyl group) as well as to its endo-exocyclic dienone character. By inference, it seems that in the case of the tested monoterpene cetones, the presence of the isopropylidene group increases the strength of larvicity, comparing to isopropenyl and/or isopropyl group effectiveness.

Finally, the use of the two enantiomers, isomenthone and menthone, revealed that the latter was more toxic, indicating that enantioselectivity may play also an important role for the toxicity of essential oils. This role of enantioselectivity has already been reported in previous projects and is a well-known fact: naphthoquinones (14), linalool (15) and limonene (16).

Essential oils have often proved to be more effective than their ingredients, indicating synergistic phenomena (9). Plants usually produce essential oils as a mixture of many ingredients with strong interactions among them. Results from this study

demonstrated that some *Mentha* species (*M. pulegium* and *M. piperita*) and their *p*-menthane type components (pulegone) could be potential larvicidal agents against *Cx. pipiens*. However, further investigations for their effects on non-target organisms, and their possible toxicity against mammals should be considered to ensure safety of application.

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Τοξική δράση των αιθέριων ελαίων τριών ειδών *Mentha* spp. και των κυριότερων συστατικών τους στο κουνούπι *Culex pipiens*

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Περίληψη Η δράση των αιθέριων ελαίων από τρία διαφορετικά είδη *Mentha* spp. (Lamiaceae), καθώς και τα σημαντικότερα συστατικά τους, αξιολογήθηκαν έναντι προνυμφών του κουνουπιού *Culex pipiens* (Diptera: Culicidae). Ως εκ τούτου οι ουσίες πουλεγόνη, πιπεριτενόνη, πιπεριτόνη, καρβόνη, μενθόνη και μενθόλη απομονώθηκαν με χρωματογραφία στήλης. Τα αποτελέσματα έδειξαν ισχυρή δράση των αιθέριων ελαίων *M. pulegium* και *M. piperita* έναντι των προνυμφών (τιμές LD_{50} 46,97 και 40,28 mgl^{-1} αντίστοιχα) και η πουλεγόνη είχε την πιο ισχυρή δράση ανάμεσα στα κυριότερα συστατικά (27,23 mgl^{-1}). Για τα απομονωμένα μόρια, η διερεύνηση για πιθανή σχέση δομής δράσης αποκάλυψε ότι η θέση του διπλού δεσμού και η παρουσία της ισοπροπυλιδένο ομάδας μπορεί να είναι σημαντικοί παράγοντες δραστικότητας.

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